ISOLATION AND CHARACTERIZATION OF PROTEINS OF THE MOUSE MAMMARY TUMOUR VIRUS

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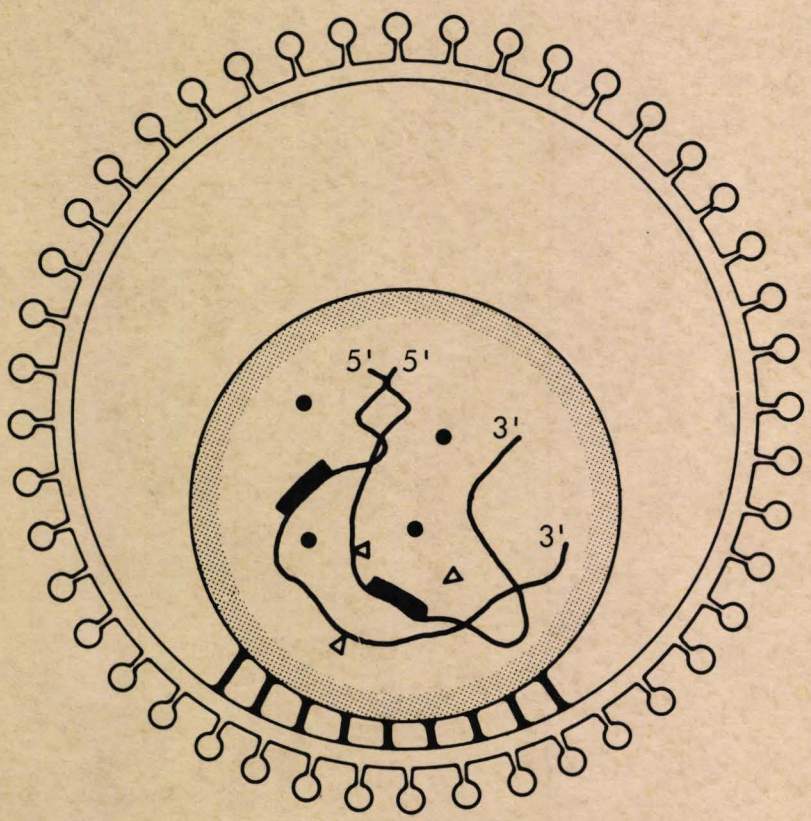

F. WESTENBRINK

Schematic representation of the mouse mammary tumour virus (Fig. 4.7).

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The work described in this thesis has been performed
at the Radiobiological Institute of the
Organization for Health Research TNO

The thesis is available as a publication of the
Radiobiological Institute TNO, 151 Lange Kleiweg,
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ISOLATION AND CHARACTERIZATION OF PROTEINS OF THE MOUSE MAMMARY TUMOUR VIRUS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A.A.H. KASSENAAR, HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN
OP WOENSDAG, 4 JUNI 1980
TE KLOKKE 14.15 UUR

DOOR

FRENS WESTENBRINK

GEBOREN TE BLOKZIJL IN 1950

W.D. MEINEMA B.V. - DELFT

Promotor: Prof.Dr. D.W. van Bekkum

Referenten: Prof.Dr. A.J. van der Eb
Dr. S.O. Warnaar

STELLINGEN

1.

Bij de hypothese dat kanker hoofdzakelijk veroorzaakt wordt door milieufactoren, dient duidelijk onderscheid gemaakt te worden tussen factoren verweven in de persoonlijke levensstijl enerzijds en factoren bepaald door verontreiniging van bodem, water en lucht anderzijds.

2.

De verhoogde tumorincidentie die blijkt op te treden bij ontvangers van transplantaten, wordt veroorzaakt door de invloed die de voor immunosuppressie gebruikte glucocorticoïden hebben op gen-expressie.

3.

Het ontbreken van post-translationele modificatiemechanismen in prokaryoten, houdt een aanzienlijke begrenzing in van de mogelijkheden die de recombinant DNA-techniek biedt op het gebied van de produktie van medisch relevante eiwitten.

4.

Door Biebricher en Druminski is geen evidentie aangedragen voor de hypothese dat de eiwit-elongatie factor Ts betrokken is bij de regulering van de stabiele RNA-synthese.

Biebricher, C.K. and Druminski, M.,
Proc. nat. Acad. Sci. USA 77, 1980, 866-869.

5.

De resultaten behaald in vaccineringsexperimenten met virosomen tonen aan dat liposomen uitermate bruikbaar zijn als adjuvant voor met name membraaneiwitten.

Morein, B. et al.,
Nature 276, 1978, 715-718.

6.

De conclusie van Djeu en medewerkers dat tumorcellen in vivo Type II interferon induceren kan op zijn minst voorbarig worden genoemd.

Djeu, J.Y., Huang,K. and Herberman, R.B.
J. exp. Med. 151, 1980, 781-789.

7.

De veronderstelling van Allan en Michell dat de celfusie-activiteit van Sendai-virus bepaald wordt door de aanwezigheid van diacylglycerol in de virale envelop volgt niet uit hun waarnemingen.

Allan, D. and Michell, R.H.
Biochem. Soc. Trans. 4, 1976, 253.

8.

De stelling dat de "bright" IgM positieve, IgD negatieve lymfocyten, te vinden in de milt van 2-4 weken oude muizen, voorlopers zouden zijn van de volwassen B-lymfocyten ("dull" IgM positief, IgD positief), is niet meer dan een veronderstelling.

Immunological Reviews, vol. 37, 1977.

9.

De toenemende invloed van de overheid op de werkprogramma's van TNO dreigt een wezenlijk facet van met name een researchinstelling als de Gezondheidsorganizatie TNO, zijnde het verrichten van onderbouwende research, aan te tasten.

10.

De ontbossing op grote schaal van de tropische regenwouden in Zuid-Amerika is een adembenemende gebeurtenis.

Stellingen behorende bij het proefschrift
"Isolation and characterization of proteins of the mouse mammary tumor virus"

Frens Westenbrink
Leiden, 4 juni 1980.

THIS PUBLICATION IS PARTLY BASED ON THE FOLLOWING PAPERS:

F. Westenbrink, W. Koornstra and P. Bentvelzen: The major polypeptides of the murine mammary tumor virus isolated by plant-lectin affinity chromatography. Europ. J. Biochem. 76, 1977, 85-90.

F. Westenbrink, W.Koornstra, P.Creemers, J. Brinkhof and P.Bentvelzen: Localization of murine mammary tumor virus polypeptides on the surface of tumor cells. Europ. J. Cancer 15, 1979, 109-121.

F. Westenbrink and W. Koornstra: The purification and characterization of a major glycoprotein of the murine mammary tumor virus. Anal. Biochem. 94, 1979, 40-47.

F. Westenbrink and W. Koornstra: Expression of MuMTV-antigens in rabbits and rats infected with MuMTV. Virology 98, 1979, 493-496.

F. Westenbrink and W. Koornstra: Lack of evidence for nonvirion cell surface antigens on virally induced murine mammary tumor cells. Europ. J. Cancer (in press).

P. Creemers, F. Westenbrink, J. Brinkhof and P. Bentvelzen: Failure to induce protection against transplanted mammary tumors by vaccination with the purified murine mammary tumor virus structural proteins gp52 and p28. Europ. J. Cancer 15, 1979, 679-684.

Aan mijn ouders
Aan Hanneke

CONTENTS

ABBREVIATIONS

MuMTV	- mouse mammary tumour virus
SDS	- sodium dodecyl sulphate
M.W.	- molecular weight
R-MuLV	- Rauscher murine leukaemia virus
MuLV	- murine leukaemia virus
EDTA	- ethylenediaminetetraacetic acid
ASV	- avian sarcoma virus
MuSV	- murine sarcoma virus
ALV	- avian leukaemia virus
HAN	- hyperplastic alveolar nodule
TCSA	- tumour specific cell surface antigen
MCSA	- Moloney virus induced cell surface antigen
FOCMA	- feline oncovirus associated cell membrane antigen
FeLV	- feline leukaemia virus
FeSV	- feline sarcoma virus
PBS	- phosphate buffered saline
DOC	- sodium deoxycholate
Con A	- concanavalin A
MMP	- methyl- α -D-mannopyranoside
BES	- 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid
Vo	- void volume
Vt	- total volume
Gu-HCl	- guanidine-HCl
BSA	- bovine serum albumin
OVA	- ovalbumin
TCA	- trichloro acetic acid
SDS-PAGE	- SDS-polyacrylamide gel electrophoresis
TEMED	- tetramethylenediamine
β -MSH	- β -mercaptoethanol
DMSO	- dimethylsulfoxide
NMS	- normal mouse serum
FCS	- foetal calf serum
PEG	- polyethylene glycol 6000
TEN-buffer	- 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl
cpm	- counts per minute
PMSF	- phenylmethylsulfonylfluoride
DEAE-cellulose	- diethylaminoethyl-cellulose
pI	- isoelectric point

CHAPTER I

GENERAL INTRODUCTION

1.1 HISTORICAL SURVEY

1.1.1 Discovery of exogenous MuMTV

In the beginning of this century, Little started the development of various inbred strains of mice by brother-sister mating. Later, several strains were selectively inbred for either a very high or very low incidence of mammary tumours, resulting in strains such as the DBA, C3H and the A strain versus the C57BL strain. With these inbred strains of mice, it became possible to perform genetically controlled experiments which would eventually lead to the discovery of the mouse mammary tumour virus (MuMTV).

In 1933, experiments showing the involvement of an extrachromosomal influence on the genesis of mammary tumours were reported. In crosses made between a high mammary tumour strain (DBA) and a low mammary tumour strain (C57BL), it became clear that, among the reciprocal hybrids, female mice developed mammary cancers at a high frequency when the female parent came from the DBA-strain (mammary tumour incidence of about 90 per cent), while the frequency was low when the female parent came from the C57BL-strain (incidence of less than 10 per cent). It was concluded that an extrachromosomal factor transmitted through the female to the offspring was responsible for the early development of mammary cancers (Staff of the Roscoe B. Jackson Memorial Laboratory (1933). Bittner (1936, 1937) demonstrated this factor to be transmitted to the offspring by the mother's milk. He discovered that the mammary tumour incidence in females of a high incidence strain (the A strain) was drastically decreased by foster nursing of them by mothers of a low incidence strain (the CBA strain). A few years later, Bittner postulated that, besides this so-called milk factor, genetic and hormonal factors were also involved in the genesis of mammary tumours in the mouse (Korteweg, 1940; Bittner, 1942a), a view which is still accepted.

Establishment of the milk factor as a virus was made when it was shown that infectious material could be passed through a filter which

was known to trap bacteria (Bittner, 1942b; Andervont and Bryan, 1944). In addition, it was found that cell-free extracts of mammary tumours induced similar tumours in mice of low-incidence strains (Visscher et al., 1942; Bryan et al., 1942).

The mouse mammary tumour virus was first visualized in electron micrographs by Dmochowski (1954) and Bernhard and Bauer (1955). In studying thin sections of mammary tumours, they saw intracytoplasmic particles which, after budding, maturated to 105 nm particles with excentrically located nucleoids. The intracytoplasmic particles were later designated as type A and the mature extracellular particles were called type B particles (Bernhard, 1958).

1.1.2 Concept of endogenous MuMTV

As described before, mice of high incidence strains which had been freed of the milk factor through foster nursing by low incidence strain mothers still developed mammary tumours in a low frequency and in old age. It was originally thought, therefore, that B-type particles were not involved in the development of such tumours but that these tumours were the result of an interaction between hormonal and genetic factors only (Heston, 1958). However, Bernhard et al. (1956) detected typical B-type particles in these tumours and such particles were also isolated from C3Hf mammary tumours (Hageman and Calafat, 1973) and from the milk of (C3Hf x BALB/c)F1 hybrids (Hageman et al., 1972). The particles exhibited a considerable oncogenic effect when inoculated in the low cancer strain BALB/c, as reflected in a high incidence of mammary tumours at a late age (Hageman et al., 1972; Bentvelzen et al., 1978). During studies on the presence of virus-like particles in mammary tumours of the C3Hf strain, evidence for the existence of an endogenous virus was discovered. In 1965, Mühlbock described the European mouse strain GR which had a high mammary tumour incidence at an early age and in which males and females transmitted the virus at an equally high efficiency (Mühlbock, 1965). Genetic analysis led to the conclusion that MuMTV in the GR strain was transmitted as a genetic factor of the host. It was hypothesized that a DNA copy of the viral genome was present in the normal cellular DNA. A so-called germinal provirus could thus be transferred to the offspring by either gamete (Bentvelzen, 1968; 1972; Bentvelzen and Daams, 1969).

1.1.3 On the origin of endogenous MuMTV

The germinal provirus hypothesis is confirmed by molecular hybridization studies. With this technique, the rate of formation and the stability of hybrids between labelled DNA complementary to the viral RNA genome (cDNA) and cellular DNA is determined and this gives information on the quantity of viral-related DNA present in the cellular DNA. By this means, multiple copies of proviral MuMTV-DNA have been detected in the normal cellular DNA of all tested inbred strains of mice (Varmus et al., 1972; Michalides and Schlom, 1975; Morris et al., 1977; Michalides et al., 1978a). In addition, proviral MuMTV DNA was detected in female mice from a location in California and in several Asian mouse species (Morris et al., 1977; Drohan and Schlom, 1979a).

There are two hypotheses on the origin of endogenous proviruses in general: 1) they have been established by somatic mutation of cellular genes (protoviruses) (Temin, 1972); 2) they are the result of multiple infections of germline tissues of early vertebrates with retroviruses (Todaro et al., 1975).

Recently, Cohen and Varmus (1979) reported on some results obtained with the so-called blotting technique which they thought to be in favour of the second hypothesis. By studying the MuMTV provirus integration pattern in twelve individual feral mice, Cohen and Varmus detected that varying numbers of proviruses were integrated at various sites in the host DNA. Two wild mice did not contain MuMTV-related sequences. In addition, J.C. Cohen and M.B. Gardner found several wild mice from one location to be free of MuMTV provirus DNA (unpublished results).

For comparison, Cohen and Varmus (1979) studied the MuMTV provirus integration pattern in mice of different sublines of two inbred strains, C3H and BALB/c. Identical patterns were observed for the different sublines of each strain, indicating that MuMTV DNA is stable according to criteria by which a clear heterogeneity in provirus integration patterns of individual feral mice is established.

Cohen and Varmus interpreted their results as offering substantial evidence in favour of the hypothesis on the origin of endogenous proviruses in which these proviruses are considered to be the result of multiple independent infections of germ cells. The authors argued that, if endogenous MuMTV DNA had evolved from genes present in a progenitor of Mus musculus in the same manner as did other cellular genes, it would be expected that individual wild mice should show similar MuMTV provirus integration patterns. As a consequence of this hypothesis, the authors see no functional role for proviral DNA in the

development of normal organisms, although the presence of the provirus itself may have pathological consequences for the animal (Cohen and Varmus, 1979). In this regard, the GR strain in which the presence of endogenous proviruses is responsible for the appearance of early mammary tumours might be an example (Bentvelzen, 1974).

Alternative to the interpretation of Cohen and Varmus, the enormous variation in MuMTV profiles could be explained via the protovirus hypothesis by assuming that endogenous viral genes are original cellular genes which evolve in a different way from other cellular genes. The diversity in integration sites could be explained by supposing the endogenous MuMTV genes to be so-called jumping genes. The absence of proviral sequences in several wild mice would be the result of deletions (Bentvelzen and Hilgers, 1980).

In any event, the two theories have one aspect in common: the presence of proviral MuMTV genomes seems to offer no selective advantage to the host.

1.1.4 In vitro infection with MuMTV

A new chapter was recently added to the history of MuMTV when several authors reported on the successful in vitro infection of different cell types with MuMTV. Lasfargues et al. (1974; 1976) described the infection of a cat kidney cell line with MuMTV derived from the milk of RIII mice. The same group subsequently succeeded in the infection of mink lung cells with the same virus strain (Vaidya et al., 1976). These results were confirmed by Howard et al. (1977) using MuMTV from three mouse strains: C3H, RIII and GR. No difference was observed between virus produced by the infected cells and that produced by murine mammary tumour cells with respect to morphology, buoyant density, sedimentation coefficient, divalent cation requirement of the viral enzyme reverse transcriptase and properties of a major protein constituent of the envelope of the virus when tested in a radioimmunoassay.

In addition, Ringold et al. (1977 a, b) reported on the in vitro infection of a rat hepatoma cell line with MuMTV derived from a GR mammary tumour cell line.

The reported in vitro infections of heterologous cell lines have several interesting features in common: 1) the MuMTV infections involved the synthesis of MuMTV DNA, as was shown by molecular hybridization studies; 2) the viral RNA synthesis and the secretion of virions by the infected cells were stimulated by the synthetic gluco-

corticoid dexamethasone, a phenomenon which was previously observed in MuMTV-producing mouse mammary tumour cells (Fine et al., 1974a; Parks et al., 1974a; Ringold et al., 1975); 3) successful in vitro infections were achieved only when high multiplicities of infection were used (10^5 virions/cell); 4) no morphological alterations or changes in the growth characteristics of the infected cells were observed.

The implication of point 2 is that either the MuMTV DNA itself carries a glucocorticoid receptor binding site or that MuMTV DNA integrates into a region of the cellular DNA that is regulated by the hormone with respect to the transcription process.

With respect to point 3, Howard and Schlom (1979) published results which showed that serial passage of MuMTV in feline kidney cells resulted in the appearance of variants with an increased efficiency to productively replicate in heterologous cells. Just one passage in feline cells decreased the required multiplicity of infection from 3×10^6 to 1. Whether these results were the consequence of a preexisting MuMTV variant in the wild-type MuMTV population or a recombination event between MuMTV DNA and normal feline cellular DNA or with feline proviral DNA, resulting in a more virulent recombinant virus, remains to be established. With these MuMTV variants, Howard and Schlom succeeded in the productive infection of cells of feline, canine, bat, mink, human and murine origin at low multiplicities of infection.

Besides the infection of heterologous cells with MuMTV, Vaidya et al. (1978) reported on the in vitro infection of its original target cell, the mouse mammary epithelial cell, with MuMTV. Transcription of endogenous MuMTV DNA sequences in a C57BL mammary gland cell line could not be detected, even after addition of dexamethasone to the culture medium. Infection with RIII milk derived MuMTV resulted in an increase in the amount of MuMTV DNA and RNA and in the induction of MuMTV protein synthesis. In analogy with the situation in heterologous cells infected with MuMTV, viral RNA and protein synthesis as well as the production of complete virions was stimulated by dexamethasone. Large quantities of virus were required for successful infection. Again, no morphological alterations or changes in growth characteristics of the infected cells were observed. The authors could not give a definite answer to the question of whether the virus released by the infected cells was the progeny of the infecting virus, the endogenous virus or a mixture of both.

1.2 STRUCTURAL FEATURES OF MuMTV

1.2.1 Morphology

The family of Retroviridae to which MuMTV belongs is typified by the possession of a single stranded RNA genome and a RNA-dependent DNA polymerase called reverse transcriptase (Fenner, 1976). The different members of the family are classified largely on the basis of morphological criteria:

The group of type A particles consists of two forms, intracisternal A particles and intracytoplasmic A particles. They are 60-90 nm in diameter, double-shelled and have an electronlucent center. The role of the intracisternal type A particles is unknown. They have been observed in mouse embryos (Calarco and Szollosi, 1973) and a variety of mouse tumours (Bernhard, 1960; Dalton et al., 1961) and are located in the cisternae of the endoplasmic reticulum. Molecular hybridization studies indicated that there are no major sequence homologies between the RNA of these particles and the RNA of known mouse RNA tumour viruses (Lueders and Kuff, 1979). Intracytoplasmic A particles, which can be found in the cytoplasm of mouse mammary tumours (Tanaka et al., 1972) in certain leukaemias (Stuck et al., 1964) and in mouse Leydig cell tumours (Nowinski et al., 1971), are considered as immature core components of MuMTV virions (Bernhard, 1960). These type A particles share common antigenic determinants with mature MuMTV particles (Nowinski et al., 1971; Tanaka et al., 1972; Smith and Wivel, 1973) and it was recently shown that these common determinants are localized in the core of the virion (Smith and Lee, 1975; Tanaka, 1977; Sarkar and Wittington, 1977; Smith, 1978). The RNA genome of intracytoplasmic A particles appeared to be homologous to the RNA of MuMTV according to molecular hybridization data (Michalides et al., 1977).

MuMTV belongs to the genus oncovirus B, generally termed type B virus. The envelope of the virus consists of a lipid bilayer whose exterior surface is covered with stable surface projections (70-90 A in length) called spikes and these consist of two distinguishable components: knobs (45-50 A in diameter) at the ends and thin stalks which connect the knobs to the viral membrane (Sarkar and Moore, 1974). Another characteristic of the B-type virion is the eccentric location of the electron-dense core (or nucleoid). The core of MuMTV is smaller when compared to the core of members of the genus oncoviruses C, more commonly termed type C viruses.

The type C viruses, which comprise the sarcoma and leukaemia viruses, are characterized by an centrally located core. They differ

from type B particles with respect to the maturation process; while type B particles bud at the cell membrane with a complete nucleoid, type C particles bud with a crescent-shaped nucleoid.

In addition to the type B and type C viruses, there is a third oncovirus group, designated as oncovirus D of which the Mason-Pfizer monkey virus originally isolated from a mammary tumour of a rhesus monkey is the prototype. Although this virus was initially considered to be a B type particle, it was later placed in a different group on the basis of small morphological differences (for a review, see Fine and Schochetman, 1978).

1.2.2 Composition of the mature B-type particle

The MuMTV virion has a diameter of 110-130 nm, a buoyant density in potassium citrate or tartrate or a sucrose gradient of 1.16-1.18 $g.cm^{-3}$ and contains 1.9% RNA and 70% protein. Tween 80-ether treatment of the virus results in disruption of the viral membrane and release of the viral nucleoid, which has a buoyant density of 1.24 $g.cm^{-3}$ and contains 4.4% RNA. These nucleoids are not infective upon inoculation into susceptible mice. The nucleoid shows two distinctive structures: a nucleoid capsule and filamentous nucleoprotein strands (Sarkar et al., 1971).

The genome of MuMTV (like that of all nondefective RNA tumour viruses) codes for at least three classes of proteins: the glycoproteins, nonglycoproteins and the enzyme reverse transcriptase. The size of the RNA genome is 70S, which is reduced to 30-40 S on heating or treatment with dimethylsulfoxide (DMSO) (Duesberg and Cardiff, 1968). It may be assumed that the viral genome in its native state is composed of two identical subunit RNA molecules (Dion et al., 1977a), as is also observed for RNA genomes of other oncornaviruses (Bender et al., 1978). The complexity of the viral genome was estimated to be 3×10^6 daltons (Friedrich et al., 1976), which corresponds to a coding capacity of about 250,000-300,000 daltons of polypeptide (Eisenman and Vogt, 1978).

The B-type virion contains several structural proteins; those located in the envelope are glycosylated, a phenomenon observed for all enveloped viruses studied to date (Compans and Kemp, 1978). The carbohydrates of the glycoproteins are specified by host cell transferases. The nonglycosylated viral structural proteins are mainly located within the core, which also contains the enzyme reverse transcriptase (Spiegelman et al., 1970) in association with the viral genome (Schlom

and Spiegelman, 1971).

The polypeptide composition of MuMTV has been analysed by many investigators, usually by electrophoresis of MuMTV preparations on denaturing sodium dodecylsulphate (SDS)-polyacrylamide gels. The number of polypeptides reported to be present in the virion varies from 11-19. In view of the coding capacity of the MuMTV genome, several of those reported polypeptides must represent breakdown products, aggregates or cellular contaminants.

There is general agreement on the presence of two glycosylated proteins, gp52* and gp36, on the envelope of the virion. Tryptic peptide analysis has revealed gp52 and gp36 to be distinct proteins (Schochetman et al., 1978a; Gautsch et al., 1978), both derived from one precursor protein $gPr73^{env}$ (Dickson et al., 1976; Schochetman et al., 1978a; Gautsch et al., 1978). Lactoperoxidase-catalyzed radioiodination of intact particles revealed gp52 to be the only protein which was labelled (Witte et al., 1973; Teramoto et al., 1974). Carbohydrate labelling by means of the galactose oxidase-tritiated borohydride method showed both gp52 and gp36 to become labelled (Sheffield and Daly, 1976). Protease treatment of B-type particles produces spikeless "bald" particles from which gp52 and gp36 are missing (Cardiff et al., 1974). Acid treatment of MuMTV virions results in "bald" particles from which only gp52 is missing (Sarkar et al., 1976). These results indicate that gp52 is located more at the exterior of the virion, while gp36 is an intramembrane component of which mainly the carbohydrate moiety is exposed on the surface of the virion.

Results reported by various laboratories on the internal MuMTV proteins give rise to some uncertainty with respect to number and molecular weights (M.W.s). The reported numbers varied from three (Teramoto et al., 1974; Kimball et al., 1976; Teramoto et al., 1977a;

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*For the nomenclature for the oncoviral proteins, the proposal of August et al. (1974) in which the proteins are designated according to their molecular weight in thousands is adopted. Non-glycosylated proteins are designated by a "p" and glycoproteins by a "gp" placed before the molecular weight number. Therefore, gp52 stands for a glycosylated protein with a molecular weight of 52,000 daltons.

Gautsch et al., 1978; Cardiff et al., 1978; Sarkar and Dion, 1975; Sarkar et al., 1976; Sarkar et al., 1977) to four (Marcus et al., 1979; Dion et al., 1979a; b), five (Schochetman and Schlom, 1976; Schloemer et al., 1976; Schochetman et al., 1977) and six different nonglycosylated internal MuMTV proteins (Dickson and Atterwill, 1979). A wide range of M.W.'s for the various internal proteins has been reported; for instance, the major internal core protein is designated as p28, p27 or p24. There is much confusion with regard to the viral status of proteins with a lower M.W., p7-p18. The protein composition of MuMTV will be further discussed in Chapter 4.

1.3 THE REPLICATION PROCESS OF MuMTV

1.3.1 The infection process

Viral infection generally proceeds by attachment of the virus to the cell, followed by penetration of the host cell membrane. The adsorption of the virus to the cell is mediated through specific receptors present on the cell surface. Cells without such receptors are "resistant" to infection by the virus (Gazdar et al., 1974). The existence of mouse genes coding for specific viral receptors has been demonstrated (Gazdar et al., 1974).

All oncoviruses studied to date express one or more glycoproteins on the viral envelope and those glycoproteins are evidently involved in the cell-virus interaction through a specific interaction with the host cell surface receptors (Bauer, 1974). For the avian leukosis viruses, the envelope glycoproteins define the host range as well as the classification into subgroups (Tooze, 1973): The specific binding of the virus envelope glycoprotein of an avian sarcoma virus to chicken embryo fibroblasts was recently demonstrated (Moldow et al., 1979). It was further shown that the major glycoprotein of R-MuLV gp71, binds specifically to receptor molecules found on murine cells but not on other mammalian cells (Delarco and Todaro, 1976; Fowler et al., 1977). Using a similar experimental approach, we failed to detect receptors for radioiodinated gp52 on MuMTV-susceptible cells. Recently, the receptor for R-MuLV was partly characterized. A lipoprotein structure seemed to be important for the receptor function. The R-MuLV gp70-receptor interaction was not influenced by C-type viruses of other species (Kalyanaraman et al., 1978).

Although not yet proved, it is likely that, in infection with MuMTV (in analogy with the RNA tumour virus systems discussed above)

the host range of the virus is determined by the major envelope protein of MuMTV, gp52. By radioiodination of intact MuMTV via the lactoperoxidase labelling method, it was shown that gp52 is the only protein exposed on the surface of the virus (Witte et al., 1973; Cardiff, 1973).

Although little is known about the processes underlying the penetration of the virus into the host cell, there are apparent differences between MuLV and MuMTV in this respect. After attachment of R-MuLV to embryonic fibroblasts, the host cell membrane dissolves at the attachment sites, probably due to activation of enzymes present on either the viral envelope, on the cell membrane or on both.

The virus subsequently penetrates into the cell. Intracytoplasmic disruption of the viral evelope then results in the release of the viral RNA genome into the cytoplasm (Miyamoto and Gilden, 1971). With respect to the mechanism underlying the uptake of MuMTV by cells, Sarkar et al. (1970) reported on phagocytosis as a means by which MuMTV enters embryonic epitheloid type mouse cells. In that study, electron microscopic examination of negatively stained preparations showed that the attachment of the MuMTV virions to the cell surface was specifically mediated by the viral projections (spikes). It was also shown that the virus particles became uncoated in the cytoplasm of the infected cell. It must be mentioned, however, that the uptake did not result in replication of MuMTV. It remains to be established whether the results of these studies are representative for the in vivo situation, where the epithelial mammary gland cell is the target for MuMTV.

1.3.2 Synthesis and integration of viral DNA into host cell DNA

After release of the viral RNA genome into the cytoplasm of the oncovirus infected cell, the next step in the replicative cycle of the virus is the synthesis of viral duplex DNA and the subsequent integration of this DNA into the host cell DNA. Studies on in vitro infections of heterologous and homologous cells with MuMTV described above yielded data that are compatible with the model proposed by Shank and Varmus (1978) for the avian sarcoma virus system. The predominant form of MuMTV DNA detected after infection of rat hepatoma cells with MuMTV is a linear molecule composed of a genome-length DNA strand complementary to the viral RNA (minus strand) and plus strand pieces of subgenomic length, indicating a discontinuous synthesis of the plus strand (Ringold et al., 1977b).

The process of viral DNA synthesis is mediated through the oncovirusassociated enzyme reverse transcriptase, which is also present in MuMTV (Spiegelman et al., 1970; Dion et al., 1974a; Marcus et al., 1976). The in vivo mechanism of viral DNA synthesis is not yet completely understood. In vitro studies with the enzyme revealed the DNA synthesis to initiate on a tRNA primer [probably $tRNA^{Lys}$ in the case of MuMTV (Waters, 1978)] located near the 5'terminus of the viral genome. The synthesis of the minus strand proceeds from the site of initiation to the 5'terminus of the RNA molecule. Synthesis is then continued at the 3'terminus of the genome, possibly by means of circularization of the RNA strand. This circularization process is thought to proceed via base-pairing of redundant sequences present on the terminal ends of the viral genome. The mechanism of synthesis of the plus DNA strand is unknown. The hydrolysis of template RNA, which is necessarily coupled with the synthesis of the plus strand, is probably mediated through the RNase H activity associated with reverse transcriptase (Bishop, 1978). The association of this RNase activity with MuMTV reverse transcriptase was demonstrated by Marcus et al. (1976). The reverse transcription process is briefly summarized in Fig. 1.1. See for detailed information Collet and Farras (1978); Gilboa et al. (1979).

According to the model of Shank and Varmus, the completed linear viral duplex DNA molecule is transported to the nucleus where it becomes circularized. Covalently closed circles of double-stranded MuMTV DNA (form I DNA) are detectable only in the nucleus of MuMTV-infected heterologous cells (Ringold et al.,1977b; Shank et al., 1978). Nothing is known about the mechanism of integration of form I DNA into the host cell DNA.

In cases of successful in vitro infections with MuMTV, proviral DNA of the virus, integrated into the host cell DNA has been demonstrated (Vaidya et al., 1976; Ringold et al., 1977b; Vaidya et al., 1978). The MuMTV proviral DNA in infected cells is integrated at multiple sites in the cellular genome. The proviral DNA's have not been found in tandem and the orientation of the integrated provirus is probably colinear with the viral RNA genome (Ringold et al., 1979; Cohen et al., 1979).

1.3.3 Production of viral RNA

The next step in the replicative cycle of MuMTV is the transcription of the integrated proviral DNA into viral mRNA. As reported by

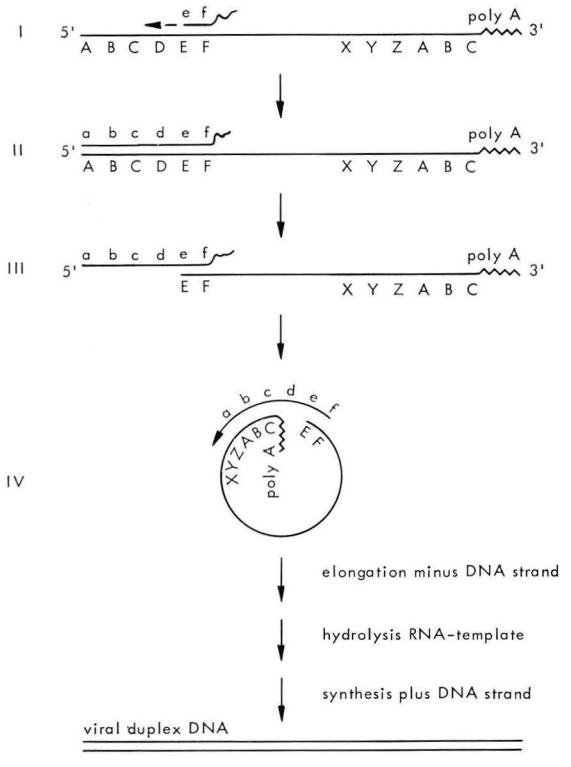


Figure 1.1:

Schematic representation of oncovirus proviral DNA synthesis.
Crucial aspects of the mechanism are: initiation of the synthesis of the minus DNA-strand near the 5' terminal end of the viral RNA genome (I). After completion of the DNA-piece at the 5' end (II) elongation of the minus DNA-strand is continued at the 3' terminus of the viral genome. Possibly this part of the mechanism proceeds via circularization at which the redundant sequences at the ends of the RNA genome are involved (III + IV). Subsequently, the RNA-strand is hydrolysed by means of the RNase-H activity, which is found in association with reverse transcriptase. The duplex proviral DNA molecule is completed by the synthesis of the plus DNA strand.

Ringold et al. (1979), not all proviral DNA copies integrated into the host cell DNA of MuMTV-infected rat hepatoma cells permit the synthesis of viral RNA. This indicates a possible determining role of the cellular site of viral DNA integration in the expression of MuMTV RNA.

Another regulating factor in the transcription process of integrated MuMTV DNA is represented by glucocorticoids (e.g., dexamethason). These hormones stimulate the production of MuMTV in mouse mammary tumour cells as well as in MuMTV-infected heterologous and homologous cells. This stimulation appears to be mediated through an increased rate of MuMTV RNA synthesis (Parks et al., 1974a; Scolnick et al., 1976; Ringold et al., 1977c; Vaidya et al., 1976; 1978). In cells

doubly infected with MuMTV and MuLV, the stimulation of viral RNA synthesis through glucocorticoids appeared to be restricted to MuMTV RNA (Young et al., 1977).

It was recently shown that the transcription of MuMTV-specific proviral DNA in murine and nonmurine cells results in three size classes of MuMTV-specific intracellular RNA (Sen et al., 1979; Robertson and Varmus, 1979; Groner et al., 1979). The first two groups reported on molecules with sedimentation coefficients of respectively 35S and 24S for the two larger RNA-molecules and of 14-18S (Sen et al., 1979) respectively 13S (Robertson and Varmus, 1979) for the smallest RNA molecule. The molecular weights of the RNA molecules as estimated by electrophoresis in denaturing agarose gels appeared to be 3.1×10^6, 1.5×10^6 and 0.37×10^6, which corresponds to 9300, 4500 and 1100 nucleotides per RNA species, respectively.

Groner et al. (1979) identified three different MuMTV-specific mRNA's of 10,000, 8,800 and 4,400 bases, respectively. While, with respect to their base content, the 10,000 and 4,400 RNAs correspond to the 35S and 24S viral RNA's identified by the two other groups, the origin of the 8,800 RNA is not clear. A characteristic of the MuMTV-specific RNA species identified by Robertson and Varmus (1979), the presence of common sequences found at the part of the viral genome adjacent to the 3' terminus, appeared to hold for the 10,000 and 4,400 RNA's pieces, but not for the 8,800 RNA species. Possibly, the 8,800 MuMTV RNA species is related to a deletion occurring in a fraction of the unintegrated double-stranded MuMTV DNA during the circularization process in the nucleus (Groner et al., 1979), a phenomenon which was demonstrated to occur in MuMTV-infected rat hepatoma cells (Shank et al., 1978).

The 35S, 24S and 13S MuMTV RNA species may serve as messengers for the synthesis of proteins, as they display features typical for eukaryotic mRNAs: they are polyadenylated at their 3'ends and are found in association with polyribosomes, from which they are released by treatment with ethylenediamine tetra-acetic acid (EDTA) (Sen et al., 1979; Robertson and Varmus, 1979). The 35S species corresponds to the viral RNA genome with respect to length and coding capacity. *In vitro* translation of the 35S RNA in a cell-free translation system yielded proteins identical to those obtained when genomic MuMTV RNA was translated (Sen et al., 1979).

The 5' ends of the 35S and the 24S MuMTV RNA species, in contrast to the 5' end of the 13S MuMTV RNA species, also contained sequences found at the 5' end of the MuMTV genome (Robertson and Varmus, 1979).

Several features of the MuMTV system are in agreement with the

model of retrovirus gene expression designed for the extensively analysed avian sarcoma virus (ASV) system (see for a review, Bishop, 1978). This model will be briefly discussed in the following paragraphs.

Three virus-specific RNA species with sedimentation coefficients of 38S, 28S and 21S are synthesized in the ASV system. The 38S species is of the same size and genetic composition as the genomic RNA (Hayward, 1977; Weiss et al., 1977). The composition of the different RNA species is shown in Fig. 1.2. Four genes are involved: one coding for the structural proteins of the viral core, gag; one coding for the viral enzyme reverse transcriptase, pol; one coding for the structural glycoproteins of the envelope, env; and one coding for the protein causing cell transformation, src. The order of the genes on the viral genome is as shown in the Figure (Wang et al., 1975; Joho et al., 1975).

The 3' terminus of the genome is polyadenylated (200 residues) (Bender and Davidson, 1976; King and Wells, 1976). A sequence of 1000 nucleotides called the common region "c", is located at the 3' end of the genome; it is present in all ASV's (Tal et al., 1977). The 5' terminus of the genome is "capped" by the structure 7 methylguanosine in 5'-5' linkage with the first encoded nucleotide of the viral genome (Furuichi et al., 1975). Twenty-one nucleotides found at the extreme

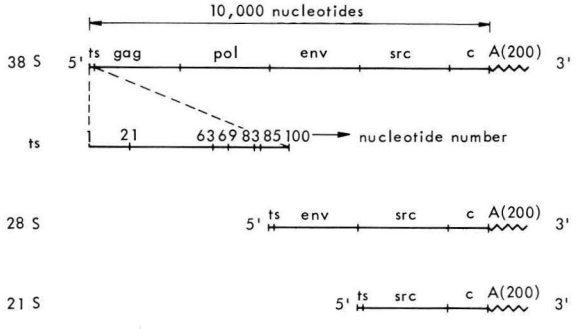


Figure 1.2:

Genetic composition of the ASV-specific RNA molecules present in ASV-producing cells. Four genes are involved: the gag-gene codes for the internal viral proteins; the pol-gene codes for the enzyme reverse transcriptase; the env-gene codes for the envelope proteins and the src-gene codes for the transforming protein. "C" represents the so-called common region, ts stands for transpositive segment. Crucial aspects of the ts-segment are: nucleotide 1 is a methylated cap: m^{7}GpppG.....C ; 1-21 are nucleotides which are repeated at the 3' terminus; 63-69 are nucleotides which are complementary to nucleotides at the 3' end of 18S rRNA; 83-85 represents an AUG initiator codon.

5' end are also present at the 3' end of the genome (terminal redundancy) (Schwartz et al., 1977). The subgenomic 28S and 21S viral RNA species encode env, src, "c" and src,"c", respectively (Hayward, 1977; Weiss et al., 1977).

A striking feature of the three ASV mRNA species is the presence of a capped segment of at least 100 nucleotides at their 5'-ends, which is also found at the 5' end of the viral genome (Mellon and Duesberg, 1977; Cordell et al., 1978). This phenomenon indicates ASV mRNAs to be synthesized by a mechanism which covalently links transcripts from nonadjacent DNA templates (a process termed splicing). A similar phenomenon was demonstrated in Moloney-MuLV (Rothenberg et al., 1978) in adenovirus (Klessig, 1977; Chow et al., 1977) and in simian virus 40-infected cells (Lavi and Groner, 1977). Therefore, splicing seems to be a general feature in the biogenesis of viral mRNAs in eukaryotic cells.

A possible reason for such a phenomenon could be the need to introduce a cap structure at the 5' end of a potential messenger RNA molecule. The presence of a cap moiety at the 5'-terminus has been demonstrated in most eukaryotic (in contrast to prokaryotic) messenger RNAs. Such caps appear to promote efficient translation of the mRNA (Shatkin, 1976; Beemon and Hunter, 1977; Paterson and Rosenberg, 1979). In addition, the splicing mechanism might introduce a ribosome binding site at the 5' ends of the mRNA molecule. The transposed 5' end sequences in the ASV mRNA species do contain several features of a ribosome binding site, such as the presence of AUG initiation codons and the presence of sequences complementary to nucleotides located at the 3' end of avian 18S ribosomal RNA (Haseltine et al., 1977; Shine et al., 1977). Several features of the transpositive segment are depicted in Fig. 1.2. The mechanism underlying the transposition process is unknown at present.

Although the presence of a methylated cap structure at the 5'end of the MuMTV genome has not been reported and detailed information on the structure of the transposed 5' segment is lacking, it is tempting to assume that the transcription process in the MuMTV system proceeds in a way similar to the model emerging from the ASV system. Contrary to this model, the smallest (13S) MuMTV specific intracellular RNA species lacks sequences found at the 5'end of the MuMTV genome (Robertson and Varmus; 1979) a finding which might indicate this RNA-species to be an artefact. The absence of the specific sequences on the other hand does not necessarily imply that it is impossible for this viral RNA species to serve as a messenger for protein synthesis: in vitro translation experiments with 3' ASV genome fragments, which

should not contain genomic 5' end sequences, resulted in the translation of at least a portion of the src-gene (Beemon and Hunter, 1977; Kamine et al., 1978; Purchio et al., 1978). Furthermore, the finding of the 13S MuMTV RNA species in association with polyribosomes suggests its involvement in protein synthesis (Robertson and Varmus, 1979).

1.3.4 Synthesis of MuMTV polyproteins

Schochetman and Schlom (1976) demonstrated that the synthesis of glycosylated MuMTV envelope proteins and nonglycosylated MuMTV core proteins in MuMTV infected cells is differentially affected by employment of the NaCl hypertonic shock technique (with this technique, use is made of the phenomenon that increasing hypertonicity selectively inhibits polypeptide chain initiation on different mRNA's). These results indicated the presence of independent initiation sites for the synthesis of the two classes of MuMTV proteins.

This finding was reflected in the results of the in vitro translation experiments of Robertson and Varmus (1979) and Sen et al. (1979), demonstrating the two larger intracellular MuMTV-specific RNA species to serve as messengers for the synthesis of MuMTV core proteins (35S-RNA) and MuMTV envelope proteins (24S-RNA), respectively.

The observations on the specificity of the MuMTV-specific intracellular RNA species indicate the in vivo translation process in the MuMTV system to proceed in a way similar to the processes identified for other retrovirus systems: Polyadenylated viral genome-sized (38S) RNA (see also Fig. 1.2) isolated from ASV-infected permissive cells directed the synthesis in cell-free translation systems of a precursor to the viral internal core proteins (Pawson et al., 1977; Weiss et al., 1978). An analogous situation was found in the MuLV system (Van Zaane et al., 1977). Polyadenylated RNA of subgenomic length (20-28S) isolated from ASV-infected cells ap-peared to serve as messenger for the synthesis of a precursor to the viral envelope proteins (Pawson et al., 1977). Again, similar findings were reported for the MuLV system (Van Zaane et al., 1977).

A virus-specific RNA with the size and composition expected for a messenger directing the synthesis of viral reverse transcriptase has not been identified (Weiss et al., 1977; Hayward, 1977). The pol-gene (see Fig. 1.2) appeared to be expressed by an occasional "read-through" of the gag and pol-genes in genome-sized RNA resulting in a 180,000 molecular weight precursor protein containing both gag and pol

translational products (Philipson et al., 1978; Murphy et al., 1978; Weiss et al., 1978; Kopchick et al., 1978). This read-through translation is thought to proceed in a way analogous to the prokaryotic system, where read-through translation results from suppression of a termination signal by a normal cellular tRNA (Weiner and Weber, 1971). There is evidence for a termination codon located at the 3' terminus of the mammalian type C viral gag gene (Philipson et al., 1978). The augmentation of the in vitro synthesis of the 180,000 molecular weight gag-pol precursor with the genome of MuLV as a messenger by the use of an amber suppressor tRNA (Philipson et al., 1978) is indicative in this respect. In cell culture as well as in in vitro translation experiments, the gag-precursor protein and the gag-pol precursor protein are synthesized in a ratio of 20 mol to 1 mol (Jamjoom et al., 1977; Murphy et al., 1978).

The observations made on the mechanism of the expression of retrovirus genes in ASV, MuLV and MuMTV-infected cells are summarized in the scheme depicted in Fig. 1.3. In general, the different classes of viral proteins are first synthesized as polyproteins, which, by means of several processing mechanisms such as enzymatic cleavage, glyco-

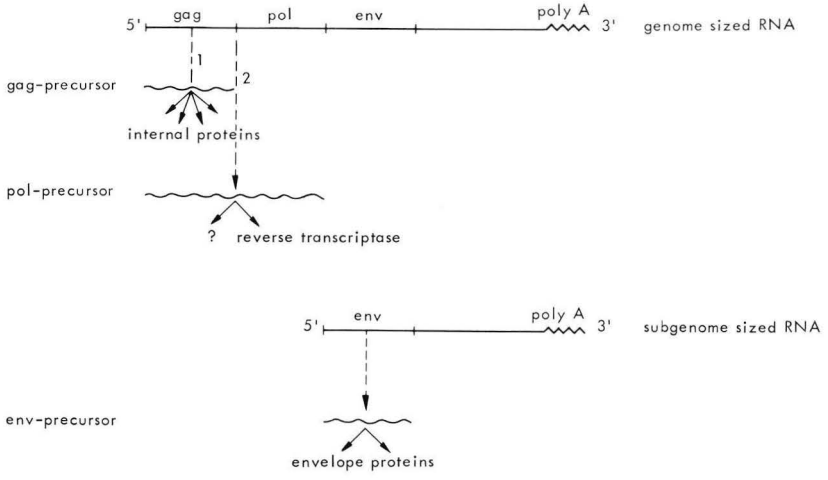


Figure 1.3:

Schematic representation of the expression of oncovirus genes

The process involves translation of messenger RNA (– – – –) and processing of the resulting polyprotein precursors (————). Genome sized RNA serves as messenger for the synthesis of internal viral proteins (1) and for the synthesis of the viral enzyme reverse transcriptase (2). Subgenome sized RNA serves as messenger for the synthesis of the viral envelope proteins.

sylation and phosphorylation, are converted into the ultimate endproducts which are expressed in the mature extracellular virion.

The intracellular synthesis and processing of MuMTV-specific polyprotein precursors has been extensively studied. The major technical approach to this subject was the immunological precipitation of intracellular radiolabelled virus-specific proteins followed by analysis on denaturing SDS-polyacrylamide gels. For specific immunoprecipitation in general, use was made of antisera raised against the major MuMTV envelope protein gp52 and against the major internal protein p28 (for identification of the respective proteins, see Witte et al., 1973; Teramoto et al., 1974; Dickson and Skehel, 1974; Cardiff et al., 1974; Parks et al., 1974b; Sarkar and Dion, 1975; Sarkar et al., 1976).

As the intracellular precursor to the MuMTV envelope proteins, a glycocosylated protein with a molecular weight ranging from 70,000-76,000 daltons was identified (Dickson et al., 1975; 1976; Schochetman et al., 1977; 1978c; Racevskis and Sarkar, 1978). The polyprotein precursor to the internal MuMTV proteins was identified either as a 75,000 molecular weight protein (Racevskis and Sarkar, 1978; Schochetman et al., 1978c) or as a protein migrating as a doublet of 75-76,000 and 77-80,000 daltons on SDS-polyacrylamide gels (Dickson and Atterwill, 1978; 1979; Racevskis and Sarkar, 1979).

By translation of MuMTV virion RNA in Xenopus laevis oöcytes (a translation system in which posttranslational processing mechanisms like glycosylation, phosphorylation and enzymatic cleavage can proceed), Nusse et al. (1978) demonstrated that first a 73,000 molecular weight protein is produced. By phosphorylation this protein is processed into a protein which migrates as a 76,000 dalton protein on SDS-polyacrylamide gels. In addition, the viral core proteins could be identified as final end products.

In addition to the proteins discussed above, two other polyprotein species of 110,000 and 160,000 daltons are immunoprecipitable. With an antiserum directed to the major MuMTV core protein these proteins can be precipitated from both a cell-free system in which genome MuMTV RNA is translated as well as from MuMTV-producing cells. While the role of the 110,000 dalton protein is obscure (it is thought to be a second precursor to particular internal core proteins), the 160,000 dalton protein is considered to be the gag-pol read through product (see Fig. 1.3) which after processing gives rise to the viral enzyme reverse transcriptase (Dahl and Dickson, 1979; Dickson and Atterwill, 1979).

Nothing is known at present about the putative product encoded for by the smallest of the intracellular MuMTV-specific mRNA species. The analogue of this 13S RNA in the ASV system, the 21S mRNA (see Fig.

1.2), codes for pp60^{src}, a transforming protein which will be discussed later in this Chapter.

1.3.5 Processing of MuMTV polyproteins

According to the recently introduced nomenclature for oncoviral polyprotein precursors (Jamjoom et al., 1978), the precursor to the MuMTV envelope proteins is designated as g Pr 73^{env} (in which g stands for glycosylated, Pr for precursor and env directs to the coding gene); the precursor to the internal proteins is designated as Pr 75^{gag}, its phosphorylated form as pPr 78^{gag} (p stands for phosphorylated) and the precursor to the viral enzyme reverse transcriptase is designated as Pr 160$^{gag/pol}$. The processing of gPr73^{env} and of Pr75^{gag} will be discussed below.

1.3.5.1 Processing of the envelope glycoprotein precursor gPr73^{env}

The host cell is responsible for the glycosylation of RNA tumour virus proteins (Witte et al., 1977; Van Eldik et al., 1978; Compans and Kemp, 1978). The processing of the precursors to the envelope proteins in the ASV, the MuLV and the MuMTV systems, appears to proceed in a similar way (Witte et al., 1977; Hayman, 1978; Dickson, personal communication; Eisenman and Vogt, 1978): The env precursor protein is translated and partly glycosylated on ribosomes bound to the endoplasmic reticulum, with the N-terminal end of the protein protruding into the lumen of the endoplasmic reticulum. Posttranslational processes like glycosylation and proteolytic cleavage occur during transport of the protein to the plasma membrane through the endoplasmic reticulum and the Golgi apparatus.

1.3.5.2 Processing of the precursor to the internal non-glycosylated proteins Pr75^{gag}

According to the model constructed for the avian and murine C-type oncoviruses (Eisenman and Vogt, 1978), the oncoviral polyprotein precursor to the internal proteins is translated on free ribosomes present in the cytoplasm. After completion, the precursor protein diffuses to a site on the plasma membrane marked by viral envelope glycoproteins. A small fraction of the ribosomes continue beyond the gag-gene

to synthesize the gag-pol precursor (see also Fig. 1.3). Cleavage of the gag-precursor appears to be connected with viral assembly (Stephenson et al., 1975; Witte and Baltimore, 1978; Hayman, 1978). The detection of gag-precursor molecules in R-MuLV, which upon incubation are converted into the characteristic structural components by precursor-specific proteases present inside the virion, indicates that maturation of precursor-proteins even may take place outside the cell. This appears to be associated with a shift from an immature form of the virus core to a mature one, manifested by its morphological condensation (Yoshinaka and Luftig, 1977a; b).

The first posttranslational step in the processing of the MuMTV $Pr75^{gag}$ is the conversion of this molecule into a phosphorylated form (Racevskis and Sarkar, 1978; 1979; Nusse et al., 1978). In addition to the $pPr78^{gag}$-form, Racevskis and Sarkar (1979) identified a second form of the phosphorylated gag-precursor, $pPr80^{gag}$. The latter form appeared to be more phosphorylated.

No evidence was found for the existence of a dual pathway for the processing of MuMTV-gag related polyproteins in infected cells, as was demonstrated in the murine C-type virus system, where one way leads via phosphorylation and proteolytic cleavage to the mature internal virion components and the other via glycosylation to a stable gag-related polyprotein, expressed on the cell-surface (Schultz et al., 1979; Edwards and Fan, 1979). Nusse et al. (1978) detected in D-[^{3}H]-glucosamine pulse labeled MuMTV-producing cells only labelled $gPr73^{env}$ no evidence for glycosylation of a gag encoded polyprotein was obtained.

Further processing of the phosphorylated gag-gene encoded MuMTV precursor protein proceeds through proteolytic cleavage via a relatively stable phosphorylated intermediate of 30,000-34,000 M.W. (Nusse et al., 1978; Racevskis and Sarkar, 1978; 1979). Evidence has been presented that further phosphorylation takes place at the intermediate p30-p34 level (Racevskis and Sarkar, 1979).

After pulse labelling of MuMTV-producing cells with [^{32}P]orthophosphate for a period of 150 min, Racevskis and Sarkar (1979) could only immunoprecipitate the two phosphorylated gag-gene encoded precursors $pPr78^{gag}$ and $pPr80^{gag}$ and the intermediate pp34. The authors had previously demonstrated that very little of the major mature MuMTV core protein p28 (called p27 by them) was detectable intracellularly after pulse-labelling with [^{35}S]-methionine for a period of 60 minutes and a subsequent chase period of 180 min, while labelled virion associated p28 was readily detectable in extracellular virions at that time (Racevskis and Sarkar, 1978). Similar findings were reported by

Schochetman et al. (1978c). Apparently, the final cleavage step to mature p28 is a very late event in the MuMTV maturation process, occurring at or shortly after budding of the complete virion. As pointed out earlier, there is a precursor product relationship between intracytoplasmic A-type particles and mature extracellular B-type particles. These intracytoplasmic A-type particles contain a major protein of 70,000-75,000 molecular weight, which shows cross-reactivity with antigenic determinants present on internal MuMTV core proteins (Sarkar and Whittington, 1977; Tanaka, 1977; Smith, 1978).

Combining the data on the intracytoplasmic A-type particles, it appears that the processing of the MuMTV gag-gene encoded polyprotein precursor proceeds via a morphologically recognizable structure called the type A-particle. These structures are obviously formed by binding of the gag-precursor and probably the gag-pol precursor, to MuMTV-specific genomic RNA. Such an event is largely in agreement with the model proposed by Bolognesi et al. (1978) for the maturation process of C-type oncoviruses: they suggested that the gag-related precursor molecules possess specific recognition sites for both the viral RNA and the viral envelope proteins, which are inserted into the cellular plasma membrane. After alignment of these respective elements at the cellular budding site, virus maturation proceeds through specific cleavage of the precursor molecules, followed by arrangement of the resulting proteins into the structure typical for the mature extracellular C-type virions. In the MuMTV system the alignment of the different elements composing the A-type particle, is completed inside the cytoplasm (Sarkar et al., 1972). Tanaka (1977) reported that intracytoplasmic A-type particles obtained from several mouse strains contained a major protein of 70,000 molecular weight as shown by analysis on SDS-polyacrylamide gels. On incubation of these particles for 20 h at 37^{o} C, the major protein was converted into proteins identical with respect to molecular weight and antigenicity to proteins present in mature B-type particles. These results suggest the presence of proteolytic activity inside the intracytoplasmic A-type particles.

The envelope of the B-type particle is acquired by budding of the A-type particle through the cellular membrane. The maturation of the virus is completed outside the cell. In the extracellular particle, the spherical nucleoid undergoes a structural transition resulting in a condensed nucleoid. The collapse of the spherical nucleoid is thought to be caused by a change in the microenvironment. At the time that the particle appears in the extracellular space, the interaction between the protein subunits of the nucleoid is altered. The resulting mature B-type particle is typified by an eccentrically located electron dense core (Sarkar et al., 1972).

The results cited on the synthesis and processing of MuMTV proteins are summarized in Table 1.1. The replication process of MuMTV is summarized in Fig. 1.4.

TABLE 1.1

SYNTHESIS AND PROCESSING OF MuMTV PROTEINS

coding gene(s)	polyprotein precursor	Intermediates	(proposed) end product
gag	Pr 75	pPr78, pPr80, pp(30-34)	pp28, pp23, p18, p12, (p10?)
	Pr110		p30
gag-pol	Pr160	?	p100
env	Pr 73	gPr73	gp52, gp36

1.4 VIRUS INDUCED TRANSFORMATION

With respect to their oncogenic capacity, RNA tumour viruses may be divided into two groups: weakly transforming viruses and strongly transforming viruses. Most of the naturally occurring oncogenic RNA-tumour viruses, such as the lymphoid leukosis viruses of mice, cats and chickens and the mouse mammary tumour virus belong to the first group. Such viruses may be present in the target tissue in an actively replicating form for a period of months to years before tumours appear. In contrast, strongly transforming viruses are highly oncogenic and induce tumours a few weeks after infection. Viruses of the latter group usually also induce neoplastic transformation in cell culture.

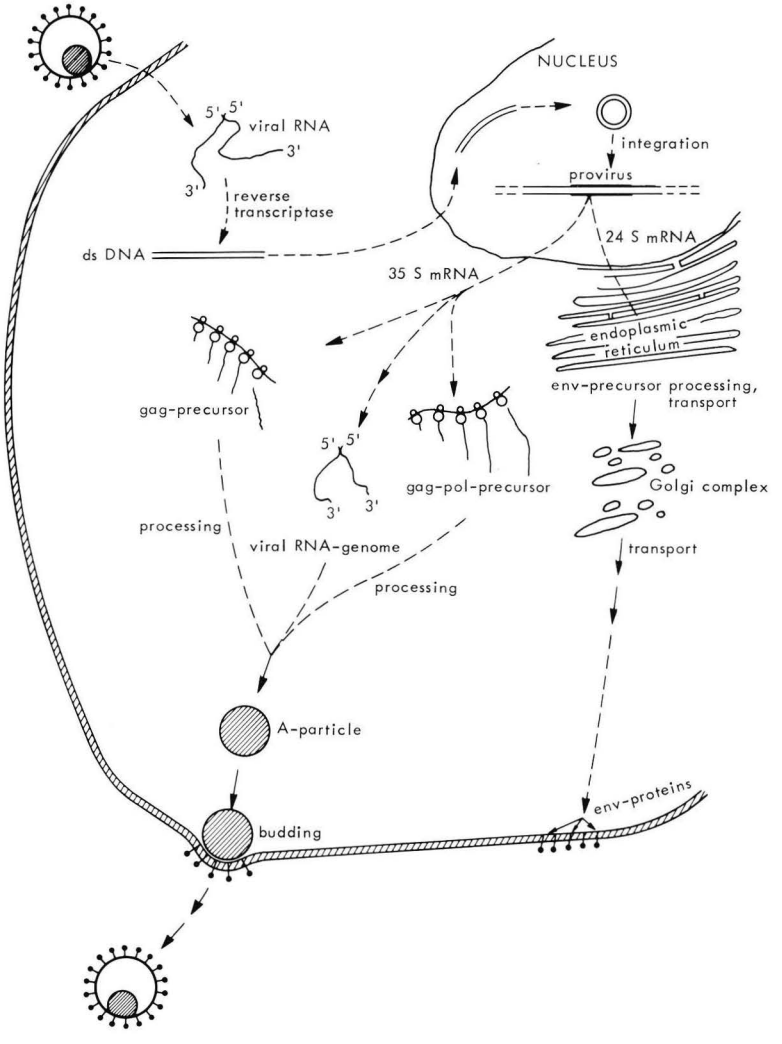


Figure 1.4:

The life cycle of MuMTV
See for detailed information the text of sub-chapter 1.3.

1.4.1 Strongly transforming viruses

Most progress in research on the nature of the oncogenic potential of RNA tumour viruses has been made in the avian system. By studying mutants of ASV, temperature sensitive (ts) for maintaining the transformed state of the cell (Martin, 1970; Kawai and Hanafusa, 1971), it was demonstrated that the transforming capacity of ASV's resides in a single viral gene, the so-called src-gene. By studying transformation defective variants of ASV, which appeared to be deleted for 10-20% of the wild type genome the location of the src-gene was demonstrated to be at the 3'-end of the viral RNA-genome (Lai et al., 1973). The product encoded by this gene was identified as a protein with a M.W. of 60,000 daltons by using antisera of rabbits bearing ASV-induced tumours (Brugge and Erikson, 1977). This 60,000 M.W. protein could be synthesized in a cell free translation system by using either src-gene containing 3' end fragments of the viral genome or virus-specific 21S RNA isolated from ASV-infected cells (see also Figure 1.2) as the messenger (Purchio et al., 1977; 1978; Erikson et al., 1978). The protein is phosphorylated (Brugge et al., 1978a) and displays protein-kinase activity (Collet and Erikson, 1978; Erikson et al., 1978). The src-gene product, designated as $pp60^{src}$, was shown by means of radioimmunoprecipitation of fractionated cells and by means of immunofluorescence studies to be present exclusively in the cytoplasmic fraction of ASV transformed cells (Brugge et al., 1978b). The protein could not be detected on the cell membrane by means of immunofluorescence studies on living cells (Rohrschneider, 1979). Recently, it was demonstrated by means of immunoferritin electron microscopy, that the $pp60^{src}$ protein is concentrated on the cytoplasmic site of the plasma membrane particularly near junctions connecting adjacent cells (Willingham et al., 1979). The protein is eluted from membrane fractions only in the presence of 1% NP40 (a nonionic detergent) (Bishop, personal communication). Purified $pp60^{src}$ which had retained its protein-kinase activity reacted negatively when tested for phosphatase or protease activity (Maness et al., 1979).

Besides the sarcoma viruses, the avian system contains a second class of strongly transforming viruses, the acute lymphomagenic viruses. In contrast to the ASV, these viruses are defective in replication; they require nondefective avian lymphomagenic viruses as helpers for replication.

Based on the type of neoplasms they induce and the haematopoietic target cell they transform *in vitro*, the avian acute lymphomagenic viruses have been assigned to three subgroups named after three typi-

cal representatives: the avian erythroblastosis-type viruses, the avian myelocytomatosis-type viruses and the avian myeloblastosis-type viruses (for a review, see Graf and Beug, 1978). Via hybridization experiments, it was shown that the onc-genes of the acute lymphomagenic viruses are unrelated to src. In addition, the onc-genes of the three subgroups, called erb, mac, and myb, respectively, appeared to be unrelated to each other, as also expressed in the difference in pathogenic spectrum: erythroblastosis, myelocytomatosis and myeloblastosis. Each of the four different viral onc-genes src, erb, mac and myb seem to have their counterpart in normal avian DNA as well as in mammalian DNA (Stehelin et al., 1976; Spector et al., 1978a; Roussel et al., 1979; Sheiness and Bishop, 1979).

The phylogenetic stability of the cellular src- (called endogenous sarc), erb-, mac- and myb sequences suggests these sequences to represent normal cellular genes coding for proteins which are essential for normal cellular metabolism. In agreement with this is the demonstration of a low level of transcription of cellular sarc, erb, mac and myb into RNA in normal uninfected cells (Spector et al., 1978b; Sheiness and Bishop, 1979; Roussel et al., 1979). Sarc-containing RNA has been found in association with polyribosomes (Spector et al., 1978a) and, recently, a phosphoprotein functionally, chemically and antigenically homologous to $pp60^{src}$ was detected in uninfected chicken and vertebrate cells (Collet et al., 1978; Oppermann et al., 1979). The level of expression of $pp60^{sarc}$ in normal chicken cells appeared to be a factor of 30-50 lower than the expression of $pp60^{src}$ in virus transformed cells (Collet et al., 1978).

The accumulating evidence that some virus strains have gained their oncogenic potential by incorporation and possibly modification of host genes is supported by studies on replication defective murine sarcoma viruses (MuSV's) and on Abelson MuLV. Passage of helper independent MuLV's through mice (Moloney-MuLV) and rats (Kirsten and Moloney MuLV) resulted in sarcomagenic virus strains (Moloney, Kirsten, and Harvey MuSV, respectively) in which part of the original viral genome appeared to be replaced by cellular host sequences (Frankel and Fishinger, 1976; 1977; Scolnick et al., 1973; Scolnick and Parks, 1974). Replication-defective Abelson MuLV generated by passage of Moloney MuLV through a BALB/c mouse induces rapid in vivo and in vitro transformation of bone marrow lymphocytes. The genome of Abelson MuLV encodes a 120,000 molecular weight protein which is expressed in Abelson MuLV transformed cells (Witte et al., 1978; Reynolds et al., 1978a) and which can be translated in vitro using the genome of Abelson MuLV as a messenger (Witte et al., 1978; Reynolds et al., 1978b).

This 120,000 M.W. protein contains serological determinants of Moloney MuLV gag-gene products and a 90,000 M.W. region derived from unique Abelson MuLV sequences. Anti-Abelson MuLV syngeneic tumour regressor sera, which are reactive with the 120,000 M.W. protein, immunoprecipitate a 150,000 M.W. protein from extracts of normal mouse thymus and other lymphoid organs. Absorption and competition analysis showed this protein to be closely related to the unique region of the Abelson MuLV p120 protein (Witte et al., 1979). Using a similar serum raised in C57BL mice, Risser et al. (1978) reported the expression of an Abelson MuLV induced tumour specific antigen on the cell surface of normal bone marrow cells and spleen cells but not on thymus cells from BALB/c mice. This suggests that the synthesis of the 150,000 M.W. is not correlated with its expression on the cell surface. Although Witte et al. (1979) detected the 150,000 M.W. protein in bone marrow cells of BALB/c and C57BL/6 mice, Risser et al. (1978) found the Abelson MuLV specific antigenic determinants to be expressed only on BALB/c bone marrow cells.

The results cited indicate the Abelson MuLV to be the result of a recombination process in which a normal host gene is incorporated into the Moloney MuLV genome.

The ability of an oncovirus to incorporate particular host sequences was most elegantly demonstrated by Hanafusa and co-workers. They injected transformation defective mutants of ASV from which the major part of the src-gene was deleted into chickens and this resulted in the development of sarcomas in some chickens. ASV recovered from these tumours appeared to contain a functional src-gene. Apparently, the transformation defective virus had acquired its missing src-gene sequences by incorporation of host-sarc-gene derived sequences (Hanafusa et al., 1977; Halpern et al., 1979).

In addition to the above described isolates of transforming recombinant type C viruses generated by in vivo passage of replication competent helper viruses, the generation of highly leukemogenic variants by passage of a nontransforming type-C virus in cell culture was recently described (Rapp and Todaro, 1978; Rasheed et al., 1978).

Considering the model in which a normal cellular gene turns into an onc-gene upon incorporation into a viral genome, two possibilities concerning the oncogenic potential of the onc-gene product exist. The products of the viral onc-gene and the analogous normal cellular gene are functionally identical; in such a case, inordinate expression of such a normal cellular gene product leads to neoplastic transformation. Alternatively, the viral onc-gene product differs functionally from the analogous cellular gene product. In that case, the expression

of an aberrant functioning protein leads to an altered phenotype of the oncovirus infected cell (see also Oppermann et al., 1979; Collet et al., 1978).

1.4.2 Weakly transforming viruses

In the avian system, the nondefective leukaemia viruses (ALVs) are weakly oncogenic and cause predominantly lymphatic leukaemia only after a period of months to years following infection. In comparison with the ASV's, the nondefective leukaemia viruses do not contain the src-gene (Bishop, 1978; Graff and Beug, 1978). The mechanism of oncogenesis by these types of viruses is not clear. Possibly the "c" region (see Fig. 1.2), present in all nondefective ALV's and the function of which is unknown, is involved in the oncogenic mechanism of the nondefective leukaemia viruses. Alternatively, the weakly-transforming viruses acquire their oncogenic capacity as a consequence of a recombination event in which other viral or cellular genes present in the host organism are involved.

Evidence for the validity of this second hypothesis stems from work in the murine system, in which it was shown that in the AKR mouse strain a recombination event between ecotropic MuLV and a xenotropic virus was associated with spontaneous lymphoma (Hartley et al., 1977). The recombination occurred within the env-gene (Elder et al., 1977) as reflected in the host range of the recombinant virus: while ecotropic viruses reproduce only in mouse cells and xenotropic viruses only in cells of nonmouse origin, the recombinant virus replicates in both mouse and mink cells. This new type, dualtropic, non-defective virus was called mink cell focus-inducing virus (MCF virus).

A possible mechanism by which MCF viruses cause leukaemia in AKR mice is thought to proceed via the expression of the altered glycoprotein on the cell surface of thymocytes. Replacement of gp70, normally expressed on the surface of certain differentiated cells such as thymocytes, by the recombinant gp70 would lead to disturbances in processes such as growth control and differentiation, eventually resulting in oncogenesis (Elder et al., 1978; McGrath and Weissman, 1979).

Further evidence concerning the possibility that RNA tumour viruses gain their oncogenic potential through a recombination event in which the genomes of an ecotropic and a xenotropic virus are involved was given in reports describing dualtropic oncogenic viruses which appeared to be generated through recombination between either

ecotropic Friend- (Troxler et al., 1977a); Rauscher- (Troxler et al., 1977b) or Moloney-(Fischinger et al., 1978; Faller and Hopkins, 1978) MuLV and an xenotropic MuLV. In all cases, the recombination again took place in the env-gene of the viruses. The resulting recombinant viruses, the replication-defective spleen focus forming virus (SFFV) in the cases of the Friend and Rauscher strains and the nondefective HIX virus in the case of the Moloney strain, rapidly induce erythroblastosis (SFFV) or lymphomas (HIX virus) in mice.

Nothing is known about the mechanism underlying the capacity of the nondefective Friend and Rauscher MuLV strains which provide helper functions for the replication-defective SFFVs to induce lymphatic leukaemia several months after inoculation into weanling mice.

Considering the results discussed above, it becomes clear that several mechanisms of RNA tumour virus-induced oncogenesis do exist. The way MuMTV interacts with the cell in inducing malignancy is completely unknown at present.

1.4.3 MuMTV-induced mammary tumorigenesis

There is a long latency period between the neonatal infection with MuMTV by the mother's milk and the appearance of mammary tumours in the adult female mouse. Prior to the development of mammary tumours, preneoplastic lesions called hyperplastic alveolar nodules (HAN) can be observed. According to DeOme et al. (1962), a preneoplastic lesion is a recognizable part of the mammary gland, which lacks morphological resemblance to the mammary tumour, but is the site of the neoplastic transformation.

HAN can be observed in neonatally infected mice after their first pregnancy. The number of nodules increases with age. The appearance of HAN can be accelerated by artificial hormonal stimulation (Nandi, 1963; Nandi and McGrath, 1973). The change of HAN into mammary tumours takes several months.

Besides HAN, which have been observed in all strains of mice with a high mammary tumour incidence (Nandi and McGrath, 1973), another lesion with preneoplastic potential can be observed in certain mouse strains such as DD, RIII and GR. These lesions, which are called plaques (Foulds, 1956), appear during pregnancy, after which they regress. These lesions may reappear during subsequent pregnancies and may develop into tumours.

The growth of HAN is subjected to local growth regulators (Faulkin and DeOme, 1960), a characteristic which distinguishes them from mam-

mary tumours. This dependence on the microenvironment was demonstrated by DeOme et al. (1959), who showed the nodules to grow upon transplantation into mammary gland free fat pads of 3-week-old syngeneic mice. The transplanted nodules produced hyperplastic outgrowths which ultimately changed into mammary tumours.

Although the time of appearance of HAN is well documented, the moment of initiation of transformed cells is not known. DeOme et al. (1978a) showed that transplantation of pieces of morphologically normal mammary gland of virgin BALB/cfC3H mice into gland free fat pads did not lead to nodule outgrowths. However, when they transplanted a cell suspension originating from dissociated equivalent mammary gland, nodules were detectable in 38% of the outgrowths. Apparently, transformed cells were present in the mammary gland several months before the appearance of HAN and tumours. It was hypothesized that organized mammary tissue inhibits the emergence of HAN and tumours. A critical number of transformed cells (already detectable in 2-month-old donor mice) is thought to be needed before HAN emergence takes place (at 8 to 9 months of age) (DeOme et al., 1978a). In a related study, it was demonstrated that pregnancy, probably due to the hormonal milieu associated with it, accelerates the number of MuMTV-transformed cells, the rate of HAN emergence and the rate of appearance of mammary tumours (DeOme et al., 1978b).

1.4.4 Tumour specific cell surface antigens

Several categories of newly expressed cell surface antigens may be detected on RNA tumour virus transformed cells, including viral structural proteins (see Chapter 4), embryonic or fetal antigens and nonvirion, virus-associated tumour specific cell surface antigens (TCSAs). The latter category is of particular interest, as knowledge of the nature of such antigens may give some insight into the transforming mechanism of a particular RNA tumour virus. In addition, there may be a direct relationship between a TCSA and any transforming protein(s) coded for by the virus involved.

Three rather well defined TCSA's have been described in, respectively, the avian, murine and feline RNA tumour virus systems (for a recent review, see Kurth et al., 1979).

In the avian system, a TCSA which is expressed on all cell types transformed *in vivo* or *in vitro* by ASV has been described (Kurth and Bauer, 1972; 1975). Studies with mutants of ASV defective or temperature-sensitive for transformation indicated the expression of avian

TCSA to be under control of the viral src-gene (Comoglio et al., 1978; Kurth and Kitchener, 1978; Wyke and Kurth, 1978). However, as discussed earlier, no evidence for expression of the src-gene product $pp60^{src}$ on the cell surface of transformed cells has been obtained. Kurth et al. (1979) made the suggestion that a processed metabolite of $pp60^{src}$, with altered antigenicity, possibly represents the avian TCSA.

In the murine system, a TCSA is described on Moloney MuLV induced lymphomas. This so-called Moloney virus induced cell surface antigen (MCSA) was characterized originally with sera of syngeneic mice immunized with irradiated lymphoma cells (Klein and Klein, 1964). Evidence indicating the nonrelatedness between MCSA and Moloney-MuLV structural proteins has been reported (Fenyö et al., 1977; Siegert et al., 1977; Troy et al., 1977).

The most thoroughly studied RNA tumour virus induced TCSA is the so-called feline oncovirus associated cell membrane antigen (FOCMA). This tumour-specific cell surface antigen was originally detected on a feline lymphoma cell line by using serum from cats which were resistant to the development of tumours induced by either feline leukaemia virus (FeLV) or feline sarcoma virus (FeSV) (Essex et al., 1971a; 1971b).

It was recently demonstrated that the major antigenic determinants of FOCMA expressed on FeLV induced lymphoid tumour cells and on FeSV transformed fibroblasts, are indistinguishable (Sliski and Essex, 1979). The findings that FeSV-transformed "nonproducer" mink and rat cells do express FOCMA (Sliski et al., 1977) and that specific anti-FOCMA serum does not react with FeLV-structural proteins and the fact that an antiserum directed against FeLV structural proteins shows no reactivity against FOCMA (Snyder et al., 1978) indicate the non-relatedness of FOCMA and the structural proteins of FeLV. The absence of FOCMA on normal lymphoid cells and fibroblasts and its absence from FeLV-producing feline and nonfeline nontransformed cells (Hardy et al., 1977; Sliski and Essex, 1979) indicate FOCMA to be a FeLV- and FeSV-transformation specific protein.

By analysis on SDS-polyacrylamide gels of immunoprecipitates obtained with specific antisera, FOCMA was identified as a 70,000 M.W. protein expressed on FeLV-producing and nonproducing lymphoma cells and on FeSV-transformed nonproducer cells (Snyder et al., 1978). In addition, a 85,000 M.W. protein was characterized specifically on FeSV transformed nonproducer cells (Stephenson et al., 1977; Snyder et al., 1978). Just as the Abelson MuLV induced 120,000 M.W. protein contained MuLV-gag gene coded determinants, the FeSV induced 85,000 M.W. protein

expressed, besides FOCMA, determinants of the FeLV gag-gene coded proteins p15 and p12. Pulse-labelling experiments revealed the 85,000 M.W. protein to be processed in a 65,000 M.W. FOCMA-containing protein and a 25,000 M.W. p15 and p12 containing protein (Stephenson et al., 1977). Probably, the FeLV induced 70,000 M.W. protein and the FeSV induced 65,000 M.W. protein are biochemically similar or even identical (Snyder et al., 1978).

Like the nondefective ALV, FeLV contains a genome expressing the genes gag, pol and env (see Fig. 1.2). The FeSV genome, which shares at least gag-gene sequences coding for p15 and p12 with the FeLV genome (Khan and Stephenson, 1977), contains an additional src-gene which is thought to be responsible for the capacity of FeSV to transform fibroblasts. If FOCMA is encoded by the src-gene, the expression of FOCMA in FeLV induced cat lymphoid cells has to be the result of activation of cellular src-gene related sequences by FeLV. Such a model suggests FeSV to originate by a recombination event between FeLV and cellular sarc. Alternatively, FOCMA could be encoded by a region of the virus genome shared by both FeLV and FeSV. In such a case, there would have to be a mechanism which specifically regulates the expression in FeLV-transformed lymphoid cells of a polyprotein partly coded for by the gag-gene and partly coded for by, for instance, the pol-gene. Such a deletion of the gag-pol read-through precursor polyprotein should not be expressed in FeLV-producing nontransformed cells.

Recently, Van de Ven et al. (1979) mentioned results obtained by tryptic peptide analysis of polyproteins encoded by FeSV, which excluded this alternative possibility for the origin of FOCMA.

The above discussed results clearly indicate that search for and subsequent characterization of RNA tumour virus induced nonvirion TCSAs is a meaningful way to look for virus-coded transformation specific proteins.

1.5 OBJECTIVE OF THE PRESENT STUDY

One of the aims of this study was to develop a vaccination procedure to MuMTV induced mouse mammary tumorigenesis. Whole viruses that have been killed or attenuated may be effective. However, the presence of the viral genome in such vaccins may give rise to undesirable side effects. Therefore, the use of purified viral proteins for vaccination is preferable.

At the time we started our experiments, no suitable method for the isolation of the major MuMTV proteins was available. Therefore, we intended to develop such a procedure, in which special attention had to be paid to the preservation of the immunogenic properties of the proteins.

With antisera developed against the purified proteins we intended to study the expression of MuMTV structural proteins on the mouse mammary tumour cell surface. As events at the cell surface play a central role in the process of communication and regulation of growth of cells, study on newly expressed antigens on the tumour cell surface may give insight into the process of tumorigenesis.

Identification of non-virion virus associated tumour specific cell surface antigens is one approach to the search of the product of the putative MuMTV-onc gene product. In this respect, we characterized hyperimmune sera, raised to MuMTV-induced mouse mammary tumour cells in homologous as well as heterologous animal species. Alternatively, we examined the possibility of infecting non-mouse animal species with MuMTV. The ultimate aim of this experiment was to induce mammary tumours. In analogy with results obtained in the ASV-system the MuMTV-onc-gene product could possibly be identified with sera of MuMTV induced tumour bearing rabbits and rats.

CHAPTER II

MATERIAL AND METHODS

2.1 VIRUS

2.1.1 Purification from mouse mammary tumours

Murine mammary tumour virus (MuMTV) was isolated from mammary tumours by a modification of the procedure developed by Calafat and Hageman (1968). BALB/cFC3H mammary tumours were maintained by subcutaneous passage in BALB/c mice. Fifty grams of tumour were homogenized in 500 ml phosphate buffered saline (PBS: 16 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, pH 7.2, 0.14 M NaCl, 2.7 mM KCl) in a Sorvall omnimixer for 50 seconds at 14,000 rev.min^{-1}. The resulting homogenate was centrifuged for 15 min at 20,000 xg (Sorvall RC2-B, GSA rotor, 12,500 rev. min^{-1}). The pelleted material was resuspended in PBS and subjected to the same centrifugation procedure. The supernatants were combined and ethylene diaminetetraacetic acid (EDTA) was added to a final concentration of 1 mM. After centrifugation for 45 min at 100,000 xg (Beckman 35N rotor, 35,000 rev.min^{-1}), the virus containing pellet was resuspended in 30 ml 1.5 mM Tris-HCl, pH 7.2, 0.025 M sucrose and centrifuged for 20 min at 20,000 xg (Sorvall, SS34-rotor, 12,500 rev. min^{-1}). The pellet was reextracted. The supernatants were combined and 15 ml were layered on a discontinuous sucrose gradient composed of 4 ml 50%, 12 ml 35% and 6 ml 20% sucrose (w/w) in 1.5 mM Tris-HCl, pH 7.2. After centrifugation for 180 min at 140,000 xg (Beckman SW 27 rotor, 27,000 rev.min^{-1}), a sharp light scattering band in the 50% layer was separated with a fraction recovery system (Beckman). The suspension was diluted and layered on a preformed linear sucrose gradient (20-50% sucrose (w/w) in 1.5 mM Tris-HCl, pH 7.2). After centrifugation for 180 min at 100,000 xg, fractions at a density of 1.16-1.18 g.cm^{-3} (determined with a 3 L refractometer, Bausch and Lomb, USA) were separated, diluted with 1.5 mM Tris-HCl buffer and centrifuged for 45 min at 145,000xg (Beckman 50 Ti-rotor, 40,000 rev.min^{-1}). The pellet was resuspended and again subjected to the centrifugation on the linear sucrose gradient. The virus was finally pelleted, resuspended in 1.5 mM Tris-HCl, pH 7.2, 0.025 M sucrose, and stored at -70^{o}C until use.

2.1.2 Tissue culture derived virus

Virus originating from the virus-producing C3H murine mammary tumour cell line Mm5mt/cl (Fine et al., 1974b) was obtained through the Office of Resources and Logistics, Biological Carcinogenisis Branch, National Cancer Institute, Bethesda, Maryland, USA.

2.1.3 Purification from mouse milk

Milk of RIII mice was also obtained through the Office of Resources and Logistics, mentioned above. Virus was purified according to the method of Sarkar and Dion (1975). To 4 ml of milk, 16 ml 0.15 M EDTA, pH 7.4 and 10 ml PBS were added. The mixture was centrifuged for 5 min at 4,500 xg. The cream of the supernatant was discarded and virus was pelleted by centrifugation for 60 min at 140,000 xg (Beckman SW 27-1 rotor, 27,000 rev.min^{-1}). Pelleted material was resuspended in PBS and centrifuged twice on a linear sucrose gradient, as described for the purification of virus from mammary tumours.

2.2 DETERGENT SOLUBILIZATION OF VIRAL PROTEINS

Virus was pelleted from a virus suspension with a protein content of about 40 mg by centrifugation for 45 min at 145,000 xg (50 Ti-rotor, 40,000 rev.min^{-1}) and resuspended in 2.5 ml PBS containing 1% of the nonionic detergent NNP10 (Servo, Delden, the Netherlands) and 0.02% of the preservative sodium azide. In the later experiments on the isolation of viral proteins, this disruption buffer was supplemented with 1% of the detergent sodium deoxycholate (DOC) which resulted in a slight improvement in the yields of viral proteins eventually solubilized.

The virus suspension was subsequently ultrasonicated in ice for four times 15 seconds (MSE, 150-W ultrasonic desintegrator) and incubated for 15 min at 37^{o}C and for 90 min at 4^{o}C. Nondisrupted fragments were collected by centrifugation for 60 min at 164,000x g (Beckman, SW60 rotor, 40,000 rev.min^{-1}). The pelleted material was resuspended and again subjected to the same procedure. The combined supernatants were dialyzed for 16 hr at 4^{o}C against 1 liter of PBS, 0.02% sodium azide, containing 0.02% of the detergent(s), which were present in the disruption buffer used. The protein suspension was usually applied onto a lectin-Sepharose column immediately after the dialyzation step in order to isolate the different viral proteins.

2.3 CHROMATOGRAPHY PROCEDURES

2.3.1 Chromatography on Concanavalin A-Sepharose-4B

Several elution buffers were tested in the development of a procedure for the chromatography of MuMTV proteins on Concanavalin A (Con A)-Sepharose-4B (see Chapter 3). The following procedure was finally chosen. To remove unbound Con A, the Con A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (0.9 x 15 cm) was prewashed extensively with at least 500 ml PBS, 0.02% sodium azide, 0.02% NNP10 and 0.02% DOC (buffer A). The MuMTV protein solution was then applied and, after an incubation period of 2 hr, unbound material was eluted with buffer A.

Bound material was eluted from the column by one of two possible ways: Elution with buffer A containing 0.2 M of the sugar derivative methyl- α-D-mannopyranoside (MMP) resulted in the absorption of all bound material at once; in the other procedure, the column was first equilibrated with PBS, 0.02% DOC and 0.02% sodium azide (buffer B = buffer A minus 0.02% NNP10). Bound material was then partly eluted with buffer B containing 0.2 M MMP. The remaining part of the absorbed material was then eluted with buffer A containing 0.2 M MMP.

2.3.2. Chromatography on phosphocellulose

The flow through material of the chromatography on Con A-Sepharose was chromatographed on a phosphocellulose column (P11, Whatman, Maidstone, England, dimensions: 0.9 x 15 cm). The column was equilibrated with 10 mM 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid (BES), pH 7.0, 1 mM EDTA, 0.02% NNP10 and 0.02% sodium azide (BES buffer) until no UV (280 nm) absorbing material was detectable in the effluent.

The protein fractions eluted from the Con A-Sepharose column were dialyzed against BES buffer and placed onto the phosphocellulose column. Unbound material was eluted and bound proteins were then eluted stepwise with 0.3 M NaCl and 1 M NaCl, or with a linear gradient of 0.3 M - 1 M NaCl.

Note: The phosphocellulose was washed prior to use in order to remove contaminants and to achieve ionic equilibrium with the starting buffer. The material was subsequently washed in the following solutions:

a. 0.5 N NaOH (1 gram phosphocellulose/15 ml, 30 min under continuous stirring;

b. distilled water, until a pH of 8.0 was reached;
c. 0.5 N HCl, 30 min;
d. distilled water, until a pH of 7.0 was reached;
e. BES buffer pH 7.0;
f. the material was finally poured into the column and, after packing, the column was washed with two bed volumes of BES buffer.

2.3.3 Gel filtration on Sephadex-G150

Molecular sieving was performed on Sephadex-G150 (particle size, 10-40 um) (Pharmacia, Sweden) using either a buffer with a high ionic strength, 0.1 M Tris-HCl, pH 7.2, 1 M NaCl and 0.02% sodium azide, or a buffer with a high detergent concentration, 0.05 M Na_2HPO_4-NaH_2PO_4, pH 7.4, 0.5% DOC and 0.02% sodium azide. Column dimensions were 1.6 x 90 cm. The void volume (Vo) of the column was determined with blue dextran and the total volume (Vt) was determined with tryptophan as a marker.

Protein solutions were dialyzed against the elution buffer used and then concentrated by dialysis in vacuo in collodion bags (SM 13200 Sartorius, Germany). Usually, the volume of the applied sample did not exceed 1 ml. Sucrose was added to the sample (10% w/v) and the solution then was layered onto the column bed underneath the eluant. The flow rate of the column was 13 $ml.hr^{-1}$.

2.3.4 Gel filtration in 6 M guanidine-HCl

Gel filtration in 6M guanidine-HCl (Gu-HCl) was performed on Biogel-A5m (Biorad, USA) in 0.02 M Na_2HPO_4-NaH_2PO_4 buffer, pH 6.5, containing 0.001 M dithiothreitol and 6M Gu-HCl (column dimensions were 1.6 x 80 cm). Vo and Vt were determined by using as markers blue dextran and tryptophan, respectively.

The eluted volumes were determined by weight. For the molecular weight determination, the following standards were used: bovine serum albumin (BSA) (MW 69,000 daltons), Ovalbumin (OVA) (MW 43,000 daltons) Carbonic anhydrase (MW 29,000 daltons) and lysozyme (MW 14,500 daltons). Gel filtration in 6M Gu-HCl was performed at room temperature.

Note: Except for the gel filtration in 6M Gu-HCl, all chromatographic procedures were performed at 4°C. Effluent fractions were continuously monitored with an LKB Uvicord (280 nm).

2.4 RADIO-IODINATION OF PROTEIN

Proteins were radioiodinated according to the chloramine-T method of Greenwood et al. (1963), with some modifications. To 25 µl of 0.5 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.5, was added 15 µl of a protein solution, containing 5-10 µg protein, 10 µl sodium [^{125}I] iodide (1 m Ci/10 µl, Amersham, England) and 10 µl chloramine-T solution (1 mg.ml^{-1} chloramine-T in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.5). After an incubation period of 60 sec at room temperature, the reaction was terminated by the addition of 5 µl of 2% sodium meta bisulphite and 50 µl 0.1M potassium iodide. Free iodide was separated from the labeled protein by gel filtration on Sephadex-G25 (0.8 x 15 cm). The percentage of protein bound [^{125}I]iodide was calculated by determining the percentage of trichloro acetic acid (TCA)-precipitable material in the labeled protein sample. For this purpose, a 5 µl sample was diluted to 100 µl and 100 µl 25% TCA was added. After 10 minutes the precipitate was collected by centrifugation and radioactivity was determined by use of a gamma counter. Radioiodinated proteins were regularly analyzed by means of SDS-PAGE.

2.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Weber and Osborn (1969). Cylindrical gels contained 10, 12.5 or 15% acrylamide and either 0.27, 0.33 or 0.41% N'N'-methylen-bis-acrylamide in 0.1 M sodium phosphate buffer containing 0.1% SDS. The gels were chemically polymerized by the addition of 0.15% tetramethylenediamine (TEMED) by volume and 0.05% ammonium persulphate. Gels were preelectrophoresed for 30 min at 1 mA per gel.

The samples were prepared by adding 50 µl 0.005 M sodium phosphate buffer containing 1.5% SDS, 0.05% bromphenol blue, 50% glycerol (by volume) and 0.05% β-mercaptoethanol (β-MSH) to 10-50 µl protein solution. Prior to electrophoresis, samples were heated for 2 min at 100°C in order to increase the solubilization of the proteins. For good detection, 5-10 µg per polypeptide was needed.

When radioiodinated proteins were analyzed, 10^5 cpm per sample was usually applied to the gel. The electrophoresis buffer consisted of a 0.1 M sodium phosphate buffer containing 0.1% SDS. Electrophoresis was performed for approximately 20 h at 2.5 mA per gel.

Gels were stained in an aqueous solution containing 45% methanol (v/v), 9% glacial acetic acid (v/v) and 0.3% Coomassie brilliant blue

for 1 h at room temperature. They were destained in an aqueous solution containing 7.5% glacial acetic acid and 5% methanol (v/v). Destaining usually required 1-2 days. Gels were then analyzed at peak absorbance by means of a recording spectrophotometer (Vernon, France, densitometer model, PH 15).

For electrophoresis of labeled proteins, gels were sliced into 1 mm fractions using a Bio-Rad gel slicer, after which [^{125}I] radioactivity was determined with a gamma counter.

Molecular weights were estimated on the basis of the relative electrophoretic mobility of the polypeptides using BSA, OVA, carbonic anhydrase and lysozyme as standards. The electrophoretic mobility, μ, of a given polypeptide was determined according to the formula:

$$\mu = \frac{\text{length gel before staining}}{\text{length gel after staining}} \times \frac{\text{distance of protein migr.}}{\text{distance of dye-migration}}$$

2.6 ISOELECTRIC FOCUSING

Isoelectric focusing was performed with cylindrical gels (15 x 0.6 cm) containing 5% acrylamide, 0.16% N,N'-methylen-bis-acrylamide, 2% ampholines (LKB, Sweden), pH 3.5-10, in 6.25 M urea. The gels were polymerized by addition of 0.4% TEMED (by volume) and 0.02% ammonium persulphate. The gels were allowed to polymerize for 16 hours at 4° C. Gels were prerun for 30 minutes at 1 mA/gel.

Radioiodinated proteins were applied in a volume of 50 µl containing 6.25 M urea, 0.5 M NaCl, 0.02 M Tris/HCl, pH 7.4, 0.5% NNP10, 1% β MSH and 2% ampholines.

As the upper electrolyte, 0.05 M NaOH was used and, for the lower, 0.025 M H_3PO_4. Electrofocusing was performed overnight at an initial current of 1 mA per gel. When the ampholines and proteins approached their isoelectric points, conductivity in the gels decreased and the applied voltage was increased. When the adjusted maximal voltage of 400 V was reached, the current usually had dropped to 0.2 mA/gel.

Gels were stained for 15 minutes at 60° C in a solution composed of 0.3 gram Coomassie brilliant blue, 9 grams 5-sulfosalicylic acid, 30 grams TCA, 75 ml methanol and 186 ml distilled water. Destaining was performed in a solution composed of 250 ml ethanol, 80 ml acetic acid and 650 ml distilled water. The pH gradient of each gel was determined by sectioning the gels into 1.1 mm slices. Gel fractions were immersed in 2 ml 10 mM KCl for 60 minutes, after which the pH was determined.

2.7 AMINO ACID ANALYSIS

For the quantitative estimation of amino acids, the isolated MuMTV proteins were first subjected to hydrolysis. Samples of 1/2-2 nmol of each protein were heated in 6 N HCl in thick-walled sealed glass tubes under nitrogen for 18 hours at 110°C and then dried in a rotary evaporator. The resulting residue was resuspended in 0.2 M sodium citrate buffer, pH 2.2, and the amino acid composition determined quantitatively on a Beckman amino acid analyzer (for a general description, see Knight, 1975).

Some amino acids are completely or partly destroyed during the hydrolysis procedure. Tryptophane is completely destroyed, cysteine partly. On increasing the hydrolysis time, serine and threonine tend to be proportionately more destroyed (see Knight, 1975). In a standard experiment, it was estimated that under the chosen conditions, the values observed for serine and threonine respectively were usually 10% and 5% lower than the actual value.

2.8 ASSAYING FOR PROTEOLYTIC ACTIVITY

The purified MuMTV proteins were tested for proteolytic activity according to the method of Dittmar and Moelling (1978), who demonstrated the association of proteolytic activity with p15 purified from avian myeloblastosis virus. SDS-denatured BSA or OVA was used as the substrate in the assay. In control experiments, trypsin (trypsin 1-300 from hog pancreas, ICN, USA) and protease (from Streptomyces griseus, type VI, Sigma, USA) were used as proteolytic enzymes. After incubation, the reaction mixture was analyzed for degradation products by SDS-PAGE.

Stock solutions of 1 $mg.ml^{-1}$ BSA or OVA in distilled water were denatured by addition of 0.6% SDS and incubation for 2 minutes at 100°C. They were then stored at -20°C until use.

The assay conditions were as follows: to 30 µl of a buffer containing 0.1 M Tris-HCl, pH 6.0 or pH 7.0, 0.5 mM EDTA, 5% glycerol, 20% DMSO and 0.05% SDS was added 10 µl of a substrate solution. To this mixture, 5-10 µl of test solution containing either a proteolytic enzyme (of a 0.1 $mg.ml^{-1}$ stock solution in sodium phosphate buffer) or a purified MuMTV protein was added. Incubation was performed at 37°C for either 30 minutes, 1 h, 3 h or 16 h. After incubation, the reaction mixture was analyzed by SDS-PAGE as described.

Purified MuMTV proteins were tested under different assay conditions, such as varying the pH of the reaction mixture (either pH 6.0 or pH 7.0), varying the incubation time (which was either 30 minutes, 1 h, 3 h or 16 h) and varying the amount of test protein added (either 0.5, 5 or 10 µg; corresponding to an "enzyme"-substrate ratio of 1:20, 1:2 or 1:1, respectively).

2.9 PROTEIN DETERMINATION

Protein was determined according to the method of Lowry modified in such a way that the assay was possible in the presence of interfering substances (Bensadoun and Weinstein, 1976). After addition of 25 µl 2% DOC, samples to be assayed were diluted with distilled water to 3 ml and incubated for 15 min at room temperature. Protein was precipitated by adding 1 ml 24% TCA. The precipitate was collected by centrifugation for 30 min at 3300 xg and redissolved in 1.5 ml of a solution containing 100 volumes of a solution of 20 g Na_2CO_3 and 200 mg KNa tartrate in 1 liter, 20 volumes 1 N NaOH and 1 volume 1% $CuSO_4$. In addition, 0.15 ml Folin-Ciocalteau reagens was added. After incubation for 45 min at room temperature, samples were read spectrophotometrically at 660 nm. BSA was used as a standard.

2.10 PREPARATION OF ANTISERA

Rabbits were injected with 100-150 µg of protein in 500 µl PBS emulsified with an equal volume of Freund complete adjuvant. One half of the suspension was injected intramuscularly and the other half subcutaneously at multiple sites. Booster injections with the same amount of protein mixed with an equal volume of Freund incomplete adjuvant were given at 2 and 4 weeks. Rabbits were bled from the ear veins 2 weeks after the last injection; 30-40 ml of blood were usually collected. Bleeding was continued every 2 weeks until reactivity strongly decreased.

For the preparation of a polyvalent antiserum to MuMTV proteins, a detergent-solubilized MuMTV protein solution was used as the inoculum.

Antisera were routinely absorbed for activity directed against normal mouse serum (NMS) and fetal calf serum (FCS) in order to remove aspecific reactivites which would interfere with assays such as the immunofluorescence and the humoral cytotoxicity assay. Therefore, pro-

teins were covalently coupled to CNBr-activated Sepharose-4B beads by the method of March et al. (1974). Briefly: to one volume of Sepharose beads (Pharmacia, Sweden) in 1 M sodium bicarbonate, 0.05 volume of a cyanogen bromide solution (2 g CNBr/l ml acetonitrile) was added during continuous stirring. Beads were then washed on a coarse sintered glass funnel with 10 volumes 0.1 M sodium bicarbonate pH 9.5, with distilled water and with 0.2 M sodium bicarbonate, pH 9.5, successively. The activated beads were suspended in a solution containing one volume of 0.2 M sodium bicarbonate and one volume of a solution containing the antigens to be coupled. Usually 2 mg of protein per ml of activated beads was used. The coupling was achieved by incubation at room temperature for 8 h on a roller bank. Residual active groups were deactivated by treatment of the beads with 0.5 M ethanolamine, pH 9.5, for 6 h. Finally, beads were washed with the following solutions: 0.1 M sodium acetate, pH 4.0, 2 M urea and 0.1 M sodium bicarbonate, pH 9.5. Each of these solutions contained 0.65 M NaCl.

For absorption, 1 ml of antiserum was usually chromatographed on FCS-Sepharose and on NMS-Sepharose columns (column dimensions were 1.6 x 30 cm). The chromatography was carried out at 4 °C with PBS as the elution buffer at a velocity of 10 $ml.h^{-1}$. Absorbed material was washed from the columns with 4 M urea in PBS until no UV-absorbing material was eluted. After subsequent equilibration with PBS, columns were ready for use again. Following this procedure, columns could be repeatedly used for a period of at least one year. After absorption, antisera were stored at -20 °C.

2.11 IMMUNOLOGICAL ASSAYS

2.11.1 Immunodiffusion assay

Double immunodiffusion was carried out on microscope slides, employing 0.6% agarose in PBS containing 0.02% sodium azide. Agarose was solubilized by heating. Wells were punched into the solidified agarose with the microimmunodiffusion set of LKB. The wells contained 15 µl of reagent. Slides were incubated for 24-48 h in a humidified box at room temperature, after which they were washed in PBS overnight. Staining was performed in a solution containing 0.5% Coomassie brilliant blue, 45% ethanol (v/v) and 10% glacial acetic acid (v/v). The destaining solution was composed of 10% glacial acetic acid (v/v) and 50% methanol.

2.11.2 Immunoelectrophoresis

This assay was performed in 1.5% agarose in Veronal buffer, pH 8.6, containing 0.02% sodium azide. The plates contained either 1% NNP10 and 1% DOC, or 1% NNP10, 1% DOC and 1% polyethylene glycol 6000 (PEG) or 1% DOC and 1% PEG.

Usually, 1-5 µg of protein was electrophoresed at 2.5 mA/slide for 2-3 h. Immunodiffusion against the appropriate antiserum subsequently took place for 24 h in a humidified atmosphere at room temperature. Staining and destaining were carried out as described for the double immunodiffusion assay.

2.11.3 Immunoprecipitation

For the immunoprecipitation procedure, proteins were radioiodinated as described above. An amount of protein containing 10^6 cpm of radioactivity was usually solved in 100 µl TEN buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA and 100 mM NaCl) containing 20 mg.ml^{-1} BSA, an appropriate amount of antiserum and either 1% NNP10, 0.5% DOC and 0.1% SDS or 0.02% NNP10 and 1 M NaCl.

To determine the amount of antiserum giving optimal precipitation, quantities of 0.5, 1, 2, 5 and 20 µl of the antiserum were tested in the assay. The immunoprecipitation mixture was incubated for 60 min at 37°C; a second antiserum directed to the IgG of the test serum was then added in a quantity giving optimal precipitation. For testing rabbit antisera, pig antirabbit IgG (Nordic, the Netherlands) was used as the second antiserum in a quantity of 50 µl. When mouse sera were tested, a goat antimouse-IgG serum (kindly provided by Dr. J. Radl, Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands) was added in a quantity of 20 µl.

After addition of the second antiserum, there was an incubation period of 2 h at 37°C followed by an additional period of 16 h at 4°C. Then, 0.5 ml ice-cold TEN buffer containing either 1% NNP10, 0.5% DOC and 0.1% SDS or 0.02% NNP10 and 1 M NaCl was added; the immunoprecipitate was collected by centrifugation for 30 min at 3300 x g. The precipitate was washed twice and then the radioactivity was determined by use of a gamma counter. All steps in the collection of the precipitate were carried out at 2°C.

The precipitate containing the maximum amount of radioactivity (usually obtained with 2 µl of antiserum) was then analyzed by SDS-polyacrylamide gel electrophoresis as described earlier.

2.11.4 Radioimmunoassays

With the radioimmunoprecipitation method, in which a test antigen competes with the antigen-antibody reaction of a purified radioiodinated antigen and a specific antiserum, microdetermination of specific proteins in unfractionated mixtures can be accomplished (for a comprehensive description of the assay, see Hunter, 1979). In addition, the qualitative similarities or differences between proteins can be analyzed precisely with this assay. Reactivities among immunologically related proteins have been categorized by Hunter as follows: 1) reactions of identity; 2) complete cross-reactions; and 3) incomplete cross-reactions. At reactions of identity, proteins compete in the assay in a way indistinguishable from that of the antigen assayed. The slopes of the resulting competition curves for proteins are exactly the same as that of the antigen being assayed. In reactions of complete cross-reactivity, proteins compete for all antibodies binding to the antigen; however, because of a reduced affinity, more of the cross-reacting protein than of the antigen is needed for an equal displacement. The result is a shallower slope of the resulting competition curve. In reactions of incomplete cross-reactivity, proteins will compete for only some of the antibodies binding to the antigen. The resulting competition curve will plateau at some level above complete displacement. Fig. 2.1 shows the hypothetical curves which can be obtained upon testing of a protein in a competition radioimmunoassay.

The assays were performed as described by Strand and August (1974) with some modifications.

Proteins were radioiodinated as described and diluted in TEN buffer containing 20 mg.ml^{-1} BSA to yield a 40 ng of labelled protein. ml^{-1} solution. This solution was diluted 1:4 with normal rabbit serum (diluted 1:9 in 2 mg.ml^{-1} BSA in TEN buffer) which should act as a carrier in the immunoprecipitation. The resulting solution was used as a stock solution.

To 50 µl of TEN buffer containing 2 mg.ml^{-1} BSA and 0.2% Triton-X100, 40 µl of the ^{125}I-protein containing stock solution and 10 µl of antiserum at serial twofold dilutions were added (antiserum dilutions were made in TEN buffer containing 20 mg.ml^{-1} BSA). The reaction mixture was incubated for 4 h at 37°C, after which 30 µl of swine anti-rabbit-IgG serum was added (it was previously determined that this amount of the second antiserum gave optimal results). Incubation was continued for 1 h at 37°C and for 16 h at 4°C. Then, 500 µl of ice-cold TEN buffer were added and the precipitate collected by centrifugation for 30 min at 3700 x g. The pelleted material was washed and

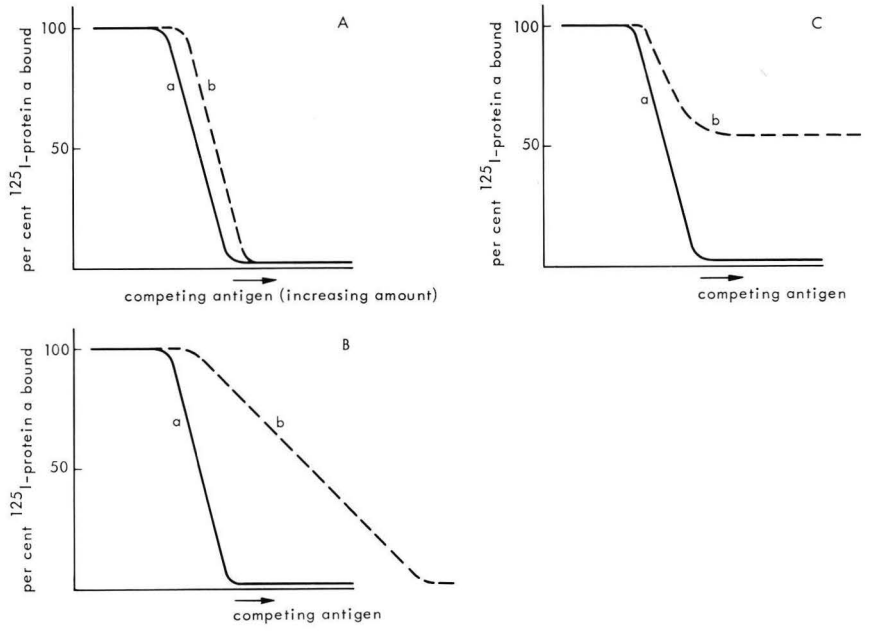


Figure 2.1:

Competition radioimmunoassay
In an assay developed for protein a, three types of reactions can be obtained upon competition with an immunologically to protein a related protein b: Reaction of identity (A); Reaction of complete cross-reactivity (B); Reaction of incomplete cross reactivity (C); —— competition with protein a; – – – competition with protein b.

radioactivity determined with a gamma counter.

By plotting the data, the serum dilution capable of precipitating 50% of the maximal precipitable amount of ^{125}I-labeled protein was calculated. This antiserum dilution was then used in the competition radioimmunoassay.

For the competition assay a binding site occupation assay was used in which the test antigen is incubated with the antiserum for some time prior to the addition of the labeled antigen. Such a preincubation step increases the sensitivity of the assay (Sheffield et al., 1977).

The conditions of the competition assay were as follows: Twofold serial dilutions of test antigen were made in TEN buffer containing 2 mg.ml^{-1} BSA and 0.2% Triton X-100. To 40 µl of this same buffer, 10 µl of antigen dilution and 10 µl of antiserum at a dilution determined as described above were added. After incubation for 2 h at 37°C under continuous agitation, 40 µl of the ^{125}I-labeled protein solution (described above) was added and incubation was continued for 2 h at 37° C.

Then, 30 µl of swine anti-rabbit-IgG serum were added (all assays were performed with rabbit antisera). The procedure was then continued by incubating for 1 hr at 37° C and for 16 hr at 4° C. After addition of 500 µl ice-cold TEN buffer, precipitates were collected, washed once more, after which radioactivity was determined with a gamma counter. In each competition assay, the precipitation reaction was repeated in order to verify that antibody was limiting.

The conditions described for the competition assay were suitable when purified MuMTV proteins were tested; however, in testing crude samples such as tumour extracts and milk samples, the addition of a protease inhibitor to the system is needed in order to prevent the occurrence of false positive results due to the presence of contaminating proteolytic activity in the test sample. Such an activity would degrade the ^{125}I-labeled protein. This would consequently result in a decrease in the amount of precipitated radioactivity in the assay and would erroneously suggest the presence of a particular antigen in the tested sample. Therefore, when such samples were tested in a competition assay, 300 µg.ml^{-1} of the protease-inhibitor phenylmethylsulfonylfluoride (PMSF) was added to the buffers used (including the buffer used for serial dilution of the test antigen).

Note: It was recently shown (James, 1978) that the serine-protease inhibitor PMSF is very labile in aqueous solution. Inactivation increased with pH and temperature. For instance, half-lives of the inhibitor at 25° C appeared to be 110 and 35 min at pH 7.0 and 8.0, respectively. Solutions in isopropanol proved to be stable for months. Therefore, when PMSF was used, it was added to the buffers from a solution made in isopropanol just before use.

In cases where we demonstrated MuMTV-antigens in milk samples of MuMTV-infected rabbits and rats (Chapter 7), in addition to the use of PMSF in the assay, a control experiment which ruled out the possibility of false positive results due to contaminating proteolytic activity was included. For this purpose supernatants of some dilutions in those assays giving significant displacement of precipitated radioactivity were subjected to immunoprecipitation with the corresponding antiserum (either rabbit anti-gp52 or rabbit anti-p28 serum). The resulting precipitates were then analyzed by SDS-polyacrylamide gel electrophoresis. The results were considered to be positive only when the control experiment showed the tested supernatants to contain intact ^{125}I-labeled input protein.

2.12 CELLS

The following cell lines were used during the experiments:

Mm5mt/cl (Fine et al., 1974b) and C3HMT/clll (Parks et al., 1975) are both cell lines derived from the C3H mouse mammary tumour cell line originally isolated and described by Owens and Hackett (1972). Both cell lines produce large quantities of MuMTV.

EMT-6 is a mouse mammary tumour cell line derived from a BALB/c tumour arising after implantation of a HAN. This line produces only C-type viruses (Rockwell et al., 1972).

BALB/3T3 cells chronically infected with Rauscher murine leukaemia virus. The BALB/3T3 cell line is a fibroblast line.

GRSL18 is a GR mouse ascites leukaemia cell line which produces intracytoplasmic A particles and some B particles, but no C type particles (Hilgers et al., 1973).

L1210 is a DBA/2 mouse leukaemia cell line (Himmelfarb et al., 1967). This line produces some MuMTV-particles.

The GRSL18 cell line was grown in RPMI 1640 medium containing 10% FCS, the L1210 cell line in Fischer's medium containing 3% FCS, 7% horse serum and 60 μM β-MSH. The other cell lines were cultured in Dulbecco's modified minimal essential medium supplemented with 10% FCS. When the cells were grown to confluency, they were detached by trypsinization: cultures were incubated for a few minutes in medium containing 0.25% trypsin (wt/wt) until all cells were in suspension. Suspensions were then centrifuged for 10 minutes at 200 x g. Pellets were resuspended in medium and seeded into new flasks (75 cm^2 plastic tissue culture flasks (Costar, England)).

All cultures were supplemented with penicillin (100 $IU.ml^{-1}$) and streptomycin (100 $\mu g.ml^{-1}$). Stimulation of MuMTV antigen expression was mediated by growing cells in the presence of 10^{-5} M of the synthetic glucocorticoid dexamethasone and 10 $\mu g.ml^{-1}$ insulin.

Mammary epithelial cell and mammary tumour cell cultures were prepared in the following way: mammary glands of lactating female mice or mammary tumours were finely minced and placed in Hanks' Balanced Salt Solution (Ca and Mg free) containing 0.1% collagenase. The suspension was incubated for 1 h under continuous agitation. The remaining cell suspension was then forced through nylon gauze. Lipid material and erythrocytes were subsequently removed by low speed centrifugation. Cells were grown in Dulbecco's 300 medium containing 15% FCS and cis hydroxyproline (50 $\mu g.ml^{-1}$). The latter substance served to suppress the growth of fibroblasts in the culture (Kao and Prockop, 1977).

Embryonic fibroblasts were prepared from 10-day-old embryos taken from uniparous mice. Tissue was forced through nylon gauze and cells were cultured in Dulbecco's modified minimal essential medium supplemented with 15% FCS. Again, culture media contained 100 IU.ml^{-1} penicillin and 100 µg.ml^{-1} streptomycin.

2.13 CYTOPLASMIC IMMUNOFLUORESCENCE

After trypsinization, cells were deposited on microscope slides and incubated for 16-20 h in a humidified incubator (5% CO_2) at 37°C. The cells were then washed 3 x 10 minutes with PBS and fixed for 3 x 5 minutes in acetone at 28°C. Fixed cells were washed 2 x 10 minutes in PBS.

The GRSL 18 and L1210 cells, which grow in suspension, were deposited on microscope slides by means of a cytocentrifuge (Vossen et al., 1976) and fixed immediately in cold acetone. Thereafter, slides were processed in the same manner as described above.

Immunofluorescence was performed by incubating the fixed cells with serial twofold dilutions of the appropriate antiserum, starting with a dilution of 1:20 (dilutions were made in PBS). After incubation for 45 minutes at 37°C in the humidified incubator, cells were washed 2 x 10 minutes with PBS. Subsequently, cells were incubated for 45 minutes with a goat antiserum directed to the immunoglobulins of the test antiserum (usually rabbit serum). The goat antiserum was conjugated with fluoresceine isothiocyanate (Nordic, the Netherlands), diluted 1:20 in PBS. After incubation, the cells were washed 2 x 10 minutes with PBS and embedded in Elvanol.

For the negative control, in three separate experiments, cells were incubated successively with normal rabbit serum (absorbed with NMS and FCS) with the conjugated goat anti-rabbit-Ig serum and PBS.

The embedded cells were covered with glass and examined for cytoplasmic fluorescence with a Leitz Orthoplan microscope.

2.14 MEMBRANE IMMUNOFLUORESCENCE

After trypsinization, target cells were seeded in plastic microtiter plates and incubated for 16-20 h in a humidified incubator (5% CO_2) at 37°C. Serial two-log dilutions of test serum were then added and incubation was continued for 45 minutes. After three washings with medium, cells were incubated with goat antiserum directed to the Ig of

the species used for the production of the test antiserum. The goat antiserum was conjugated with fluoresceine isothiocyanate (dilution 1:20 in PBS).

After three washings in medium, cells were subsequently examined with a Leitz Orthoplan microscope for membrane immunofluorescence.

BALB/3T3 cells infected with R-MuLV were used as control cells. Goat anti-MuMTV serum and normal rabbit serum absorbed with NMS and FCS served as positive and negative control sera, respectively.

2.15 HUMORAL CYTOTOXICITY ASSAY

In the humoral complement dependent cytotoxicity assay, use is made of the capacity of antibodies directed to cell surface antigens to lyse target cells in the presence of complement. The test was originally developed by Gorer and O'Gorman (1956). We have used a ^{51}Cr release assay: lysis of ^{51}Cr-containing cells results in release of radioactivity into the medium. By determining the amount of ^{51}Cr in the medium the percentage of cell lysis can be determined.

Antisera were routinely incubated for 30 minutes at 56^{o}C in order to inactivate the complement present in the test serum.

Cells were seeded in plastic microtiter plates at a density of 1-10 x 10^4 cells per well (the number of cells applied depended on the type of cell used; for C3HMT/clll cells, 3 x 10^4 cells/well were seeded; with fibroblasts, 1 x 10^5 cells/well gave optimal results). After incubation for 16-20 h in a humidified incubator at 37^{o}C (5% CO_2) cells were washed three times with 100 µl medium. Then, 20 µl medium containing 2 µCi ^{51}Cr was added per well and incubation continued for 2 h. Cells were washed three times with medium and plates plates were then placed on ice, after which serial twofold dilutions of test serum in 20 µl medium containing 10% FCS were added to the wells. In addition, 20 µl of rabbit complement were added.

Note: Normal rabbit serum was used as the source of complement. To remove natural anti-mouse reactivity, serum was absorbed with agarose. For this purpose, agarose (80 mg.ml^{-1} serum) was preswollen in PBS for 60 minutes at 4^{o}C. It was then centrifuged for 30 minutes at 2000 x g and serum diluted 1:3 in PBS was added. After incubation for 1 h at 4^{o}C, the agarose was centrifuged and the supernatant was stored at -70^{o}C until use.

After addition of complement to the wells there was an incubation period of 60 minutes at 37°C; after this the reaction was terminated by placing the plates on ice and adding 100 µl of medium per well. Radioactivity present in the medium was then determined in a gamma counter. Maximum ^{51}Cr release was determined by disrupting cells with saponin. Blanks were obtained by incubating cells with test serum without complement or with complement without test serum. All experiments were carried out in duplicate.

The percentage of specific ^{51}Cr-release was calculated as follows:

$$\%\ ^{51}Cr\ \text{release} = \frac{\text{cpm (experimental)} - \text{cpm (blank)}}{\text{cpm (maximum)} - \text{cpm (blank)}} \times 100$$

CHAPTER III

ISOLATION OF STRUCTURAL MuMTV PROTEINS

3.1 INTRODUCTION

Analysis of the number of MuMTV proteins, mainly done by means of electrophoresis of purified virus on denaturing SDS-polyacrylamide gels, has resulted in the identification of 11-19 different proteins (Nowinski et al., 1971; Teramoto et al., 1974; Cardiff et al., 1974; Dickson and Skehel, 1974; Sarkar and Dion, 1975). The differences in reported numbers of MuMTV proteins may be due to contaminating cellular proteins and to the source of virus, i.e. tumours or milk or medium of virus producing cell cultures. In addition, some of the demonstrated polypeptide bands may represent aggregates or cleavage products of original viral proteins.

As discussed in Chapter 1, mature MuMTV virions appear to contain two virus coded glycosylated envelope proteins: gp52 and gp36. About the number and M.W.'s of the internal MuMTV proteins much discord does exist, however. In addition to the major core protein, p28, proteins such as p30, p18, p16, p14, p12, p10 and p8 have been reported as constituents of the virion (see Chapter 1). A phosphorylated minor protein, p23, was recently identified as a distinct MuMTV constituent (Sarkar et al., 1978). In view of the limited coding capacity of the viral genome (see Chapter 1), several of the reported proteins probably represent contaminating cellular proteins, intermediate precursor proteins, or degradation products. In addition to the envelope and the internal proteins, the presence of a 100,000 M.W. enzyme, reverse transcriptase, in the core of the virion was reported (Spiegelman et al., 1970; Dion et al., 1974a; Marcus et al., 1976).

With respect to the isolation of structural MuMTV proteins several reports have appeared in the last few years. Nowinski et al. (1971) obtained a partial separation of MuMTV protein constituents by gel filtration of a Tween-80-ether treated MuMTV solution on Sephadex G200.

Parks et al. (1974b) reported on the purification of gp52 and p28. A preparation of MuMTV solubilized with the nonionic detergent Triton-X100 was subjected to a chromatography procedure involving ion

exchange chromatography on diethylaminoethyl (DEAE)-cellulose and gel filtration on Sephadex G100. With this purified gp52, a radioimmunoassay was developed. However, testing of samples in this assay gave satisfactory results only after heating the test sample for 20 min at 57° C. Apparently, only partly denatured antigen was capable of competing satisfactorily with radioiodinated gp52 in this assay.

Sarkar and Dion (1975) also reported on the purification of gp52 and p28 from MuMTV, solubilized with the nonionic detergent Nonidet, by chromatography on DEAE-cellulose. Gel filtration of unbound material yielded purified gp52, while bound material after elution from the DEAE-cellulose column with 0.4 M KCl yielded purified p28 on chromatography on phosphocellulose. In our experience a major drawback of this procedure is the poor quality of the purified p28. No satisfactory reaction with rabbit anti-MuMTV serum could be obtained when the protein was tested in a double immunodiffusion assay. This indicated partial denaturation of the protein.

A report on the purification of a 14,000 M.W. MuMTV protein concerned the chromatography of MuMTV on Biogel-A5m agarose under denaturing conditions (the gel filtration procedure was performed in 6M-Gu-HCl). Analysis of the purified p14 protein on SDS-PAGE revealed the purified p14 preparation to be contaminated with about 25% of a 16,000 M.W. protein (Noon et al., 1975).

In developing an isolation procedure for the major MuMTV structural proteins, we wanted to achieve several purposes. We intended to examine the usefulness of the purified proteins for vaccination against MuMTV-induced mammary tumorigenesis. Furthermore, with antisera raised against the purified proteins we wanted to examine the expression of individual MuMTV proteins on the mammary tumour cell surface.

In view of these purposes several requirements needed to be made to the isolation procedure to be developed, including a high degree of purity, a high yield and no change in the antigenic nature of the viral proteins during the isolation procedure. With respect to these purposes the isolation procedures developed by Parks et al.(1974b) and by Sarkar and Dion (1975) are not suitable. A consequence of partial denaturation of proteins will be the loss of antigenic determinants, which would be detrimental to the planned vaccination experiments. Furthermore, in studying the cell surface expression of proteins with antisera raised against partially denatured proteins, expressed determinants might be missed. Experiments would then lead to false negative results.

Consequently we decided to develop an alternative isolation procedure for the major MuMTV structural proteins.

For the purification of the envelope proteins, use was made of the affinity which Concanavalin A (Con A) shows for glycoproteins containing α-glucopyranosyl- and α-mannopyranosyl residues (Goldstein et al., 1965; Poretz and Goldstein, 1970). This Mn^{2+} containing plant lectin exists in the form of a dimer at pH 7.0 (MW 55,000) and contains two identical binding sites (Becker et al., 1971).

It was previously demonstrated that MuMTV virions can be agglutinated by Con A (Calafat and Hageman, 1972). Successful applications of affinity chromatography with plant lectin columns in the isolation of viral glycoproteins have been reported (Smart et al., 1974; Moennig et al., 1974; Stohlman et al., 1976).

For the separation of the nonglycosylated viral proteins, use was made of ion-exchange chromatography on phosphocellulose. For elution, a so-called BES-buffer was used. This type of buffer appears to be especially suitable for preserving the native conformation of proteins during the chromatography procedure (Strand and August, 1976).

3.2 RESULTS

3.2.1 Solubilization of the virus

Initially virus purified from mouse mammary tumours was used as starting material. Later on we changed to the use of tissue culture derived MuMTV. For purpose of comparison occasionally virus purified from RIII mouse milk was used.

By application of the solubilization procedure described in Chapter 2, 80% of the total amount of protein present in the original virion suspension was usually recovered in the combined supernatants which were obtained by centrifugation of the detergent-treated virus suspension.

Analysis of the nondisrupted virus suspension and the detergent-solubilized viral protein solution by SDS-PAGE showed that the treatment did not lead to preferential solubilization of a particular viral protein (Fig. 3.1).

3.2.2 Affinity chromatography on Concanavalin A-Sepharose 4B

Addition of the solubilized MuMTV proteins to the Con A column

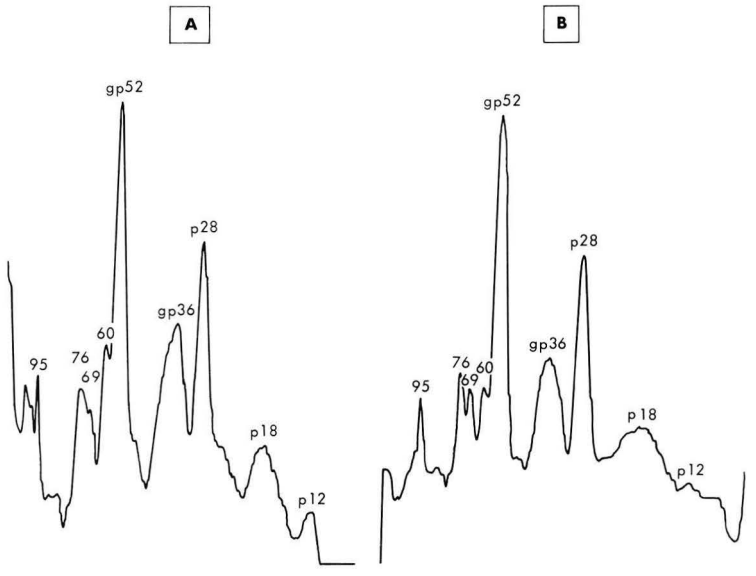


Figure 3.1:

Analysis by means of SDS-PAGE.
Untreated MuMTV (A) and detergent-solubilized MuMTV (B) were analysed by SDS-PAGE. Scans were made of gels stained with Coomassie brillant blue.

usually resulted in specific absorption of the MuMTV glycoproteins gp52 and gp36. Subsequent elution of the bound glycoproteins initially presented some problems. Elution with PBS containing a sugar- derivative (MMP) as a potent competitor of the glycoprotein-Con A interaction did not result in the recovery of either of the glycoproteins. Even the use of borate buffer, which is known to be a competent eluent of glycoproteins tightly bound to Con A-Sepharose (Kennedy and Rosevear, 1973), did not result in the elution of the bound MuMTV glycoproteins.

This difficulty was overcome by the addition of a low concentration of 0.02% of the nonionic detergent NNP10 to the elution buffer used (NNP10, which was also used for the solubilization of the virus, is an analogue of the more commonly used nonionic detergent Nonidet). Satisfactory results were obtained when PBS containing 0.02% NNP10 and 0.02% sodium azide was used as the elution buffer after application of the MuMTV protein solution on a Con A-Sepharose column. Under those conditions, the flow through peak of unbound material consisted mainly

of the core protein p28. When 0.2 M MMP was subsequently added to this buffer, a tailed peak eluted from the column, which, on analysis by SDS-PAGE, appeared to consist of gp52 and gp36. Analysis of the different fractions constituting this peak revealed a heterogeneous distribution of gp52 and gp36, indicating a differential affinity of Con A for the two proteins. A slightly improved recovery of the bound glycoproteins was obtained when, in addition to NNP10, 0.02% DOC was added to the elution buffer.

The chromatography procedure we finally developed is illustrated in Fig. 3.2. The effect which different detergent combinations in the elution buffer exert on the elution is shown.

In the figure, the SDS-PAGE analysis of the starting material, the MuMTV protein solution, is presented. In this case, the virus originated from tissue culture medium (see Chapter 2). In contrast with the scans shown in Fig. 3.1, where the virus was isolated from mammary tumours, almost no contaminating high molecular weight material (MW >52,000) was present. This confirmed the suggestion made in the introduction of this Chapter that analysis of the number of proteins present in a purified MuMTV-preparation by SDS-PAGE, is clearly influenced by the source of virus. Tissue culture derived virus appears to be fairly "clean" in this respect.

After application of the MuMTV protein solution to the Con A-Sepharose column, unbound material is eluted with PBS containing 0.02% NNP10, 0.02% DOC and 0.02% sodium azide. The column is subsequently equilibrated with PBS containing either 0.02% NNP10 plus 0.02% DOC or 0.02% NNP10 or 0.02% DOC as detergents. Addition of 0.2M MMP to the equilibration buffer resulted in the elution of gp52 + gp36 in the first two cases (see Fig. 3.2), while only gp52 was eluted in the presence of 0.02% DOC. Subsequent addition of 0.02% NNP10 to this buffer resulted in the further elution of gp52 + gp36. The latter result suggested two glycoprotein entities to be bound to the Con A-Sepharose column: gp52 and a gp52-gp36 complex and that the complex is only elutable in the presence of NNP10 under the chosen circumstances. On the average, the recovery by the chromatography on Con A-Sepharose appeared to be 70% of the applied MuMTV proteins.

3.2.3 Chromatography on phosphocellulose

In developing a purification procedure for the nonglycosylated MuMTV proteins, we took the material eluting in the flow through peak of the chromatography on the Con A-Sepharose column as starting mate-

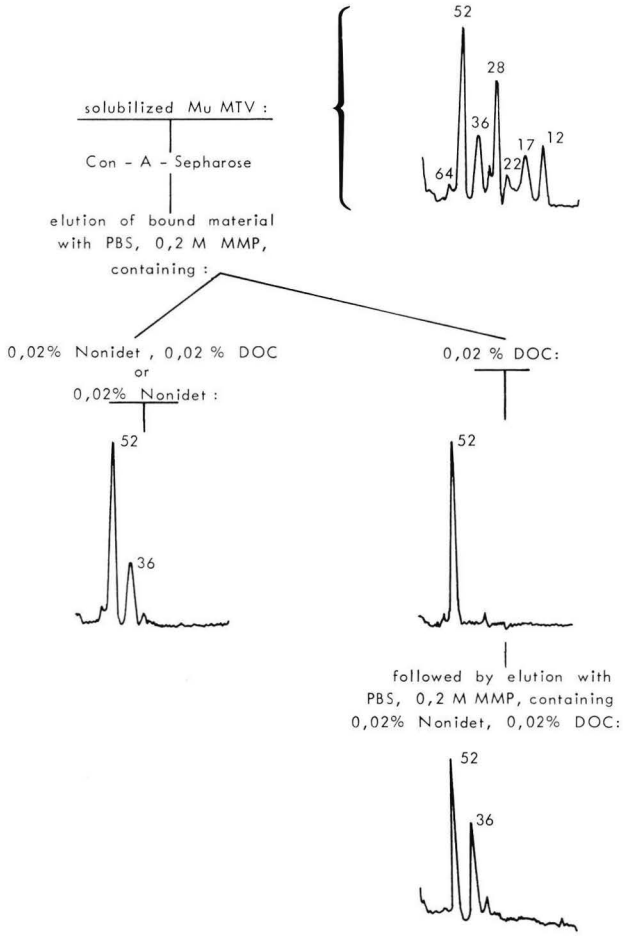


Figure 3.2:

Chromatography of detergent-solubilized MuMTV on Con A-Sepharose.
Bound material was eluted with PBS, 0.2 M MMP, containing different detergents as indicated in the figure. Eluted fractions were analyzed by SDS-PAGE, the results of which are depicted. The numbers given in the scans correspond to the molecular weight x 10^{-3}.

rial. We first experimented with the procedure described by Sarkar and Dion (1975) which was briefly discussed in the introduction to this Chapter. The authors reported on the purification of p28 by chromatography on phosphocellulose of a MuMTV-protein fraction which was enriched for p28 by chromatography on DEAE-cellulose.

Using their buffer system (i.e., 0.01 M sodiumphosphate buffer, pH 6.8, 0.001 M EDTA, 0.2% β-MSH, 0.2% Nonidet and 30% glycerol), chromatography of the nonglycosylated MuMTV protein fraction on phosphocellulose was not very successful. The column regularly showed the tendency to collapse and the elution pattern obtained revealed no clearly separated protein fractions (which in fact was in accord with the results shown by Sarkar and Dion). Furthermore, the p28 fraction ultimately obtained showed no distinct reaction with polyvalent anti-MuMTV sera when tested in a double immunodiffusion assay.

A considerable improvement in the results was obtained after we used the BES-buffer advocated by Strand and August (1976) as an elution buffer. Fig. 3.3 shows the elution pattern obtained by application of the chromatography procedure as described in Chapter 2.

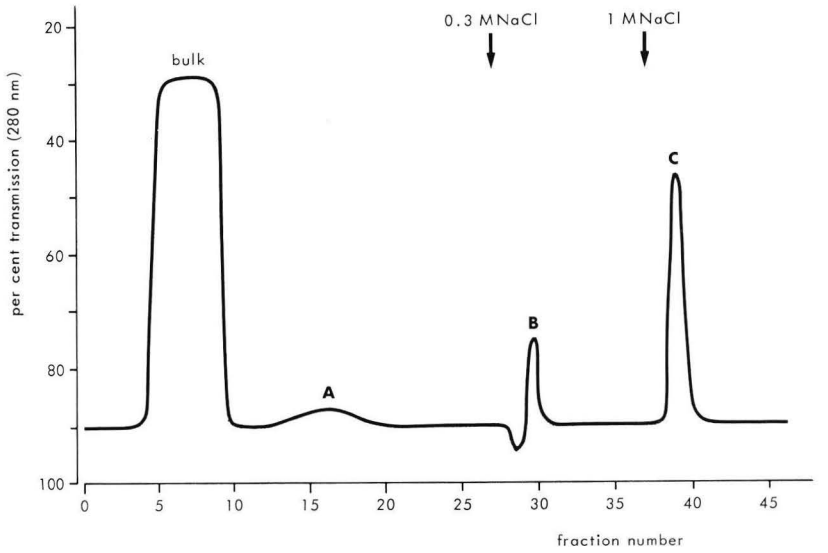


Figure 3.3:

Chromatography on phosphocellulose of the nonbinding material of the chromatography on Con A-Sepharose.
After elution of the unbound material (bulk + peak A), bound material was eluted with 0.3 M NaCl and 1 M NaCl, respectively.

After application of the proteins, most of the U.V.-absorbing material eluted in the flow through peak from the column. Gel filtration of this material on Sephadex-G150 did not result in the purification of any particular protein. All U.V.-absorbing material eluted in the Vo of the column in that case. This material probably consisted mainly of lipids.

A small peak, designated A in the figure, occasionally eluted slightly after the flow through peak. Analysis by SDS-PAGE revealed this peak to contain pure p28, as was also the case for the material which was eluted with 0.3 M NaCl from the column (peak B) (see Fig. 3.4). Subsequent elution with either 1 M NaCl or a 0.3 M - 1 M NaCl gradient resulted in the elution of pure p12 from the column (peak C) in both cases, as was shown by analysis on SDS-PAGE (Fig. 3.4).

Analysis of the p12-containing fraction sometimes revealed the presence of a 18,000 M.W. protein in addition to p12 (Fig. 3.5). Reanalysis of such a sample often showed only the presence of p12. Possibly, the initially demonstrated p18 band represented aggregated proteins. Alternatively, the presence of proteolytic activity in the 1 M NaCl-fraction might be responsible for the disappearance of the initially demonstrated p18 band (see Chapter 4).

As far as the recovery by the chromatography on phosphocellulose is concerned, usually 75% of the proteins placed on the column were collected in the subsequently eluted fractions.

3.2.4 Gel filtration on Sephadex G150

For further purification and characterization the glycoprotein fractions obtained by chromatography on Con A-Sepharose were subjected to gel filtration on Sephadex G150. When the total amount of glycoproteins eluting from the Con A-Sepharose column with NNP10 and MMP with or without DOC (see Fig. 3.2), was subjected to gel filtration in Tris buffer containing 1 M NaCl, an elution pattern consisting of two peaks was obtained: one is recovered in the Vo of the column, the second slightly thereafter (Fig. 3.6). Analysis of those peaks showed them to contain gp52 + gp36 and gp52, respectively (Fig. 3.6). When the material eluting in the Vo of the column was again subjected to the same filtration procedure, all material again eluted in the Vo. Analysis by SDS-PAGE again revealed the presence of gp52 + gp36 in that peak.

As described, it was possible to elute the Con A-Sepharose bound MuMTV glycoproteins from the column in two separate fractions containing pure gp52 and gp52 + gp36, respectively (Fig. 3.2). When the

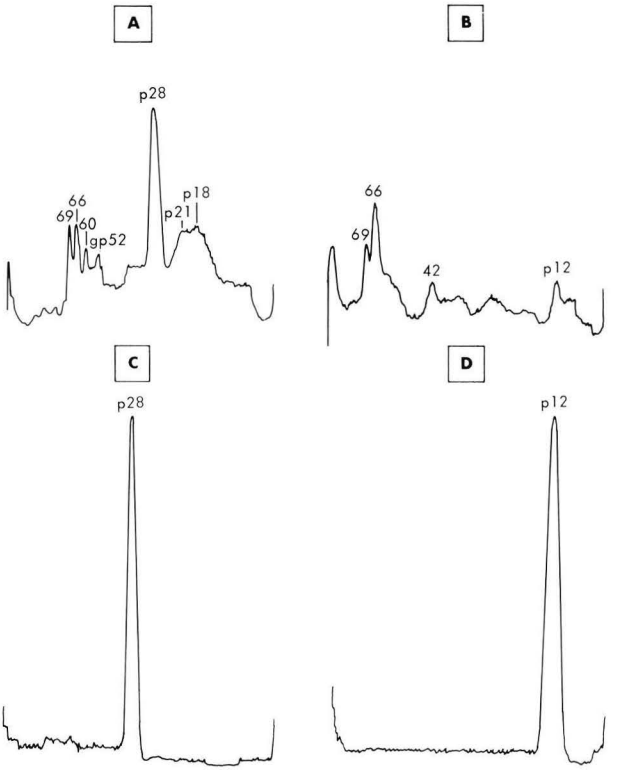


Figure 3.4:

Analysis by SDS-PAGE of the successive fractions eluted from the phosphocellulose column.
A. The protein solution as it was applied to the column. B. The "bulk" of the non-binding material. C. p28 as it elutes in peak A and peak B of the chromatography depicted in Fig. 3.3. D. p12 as it elutes with 1 M NaCl from the phosphocellulose column (peak C, Fig. 3.3).

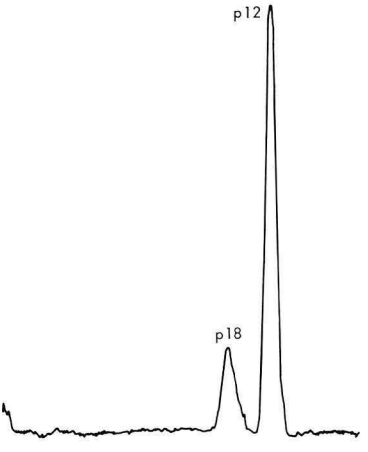


Figure 3.5:

Analysis of a purified protein fraction eluted from the phosphocellulose column. Material as it eluted occasionally with 1 M NaCl from the phosphocellulose column (Fig. 3.3) was analyzed by SDS-PAGE. Upon reanalysis of such a fraction, only p12 was normally detectable.

latter fraction was subjected to the gel filtration procedure, all material eluted in the Vo of the column. No free gp52 eluted from the column in that case. On the basis of the results obtained, we conclude that the solubilized MuMTV protein solution contained two glycoprotein entities: free gp52 and a tightly bound gp52-gp36 complex. Apparently, this complex remains intact under the circumstances of chromatography on Con A-Sepharose (in the presence of low detergent concentrations) and the gel filtration procedure applied, where a high ionic strength (1 M NaCl) buffer was used.

Splitting of the complex was achieved only in the presence of 0.5% DOC. Gel filtration of the complex in sodium phosphate buffer containing 0.5% DOC resulted in an elution pattern similar to the one depicted in Fig. 3.6; however, analysis of the material eluting in the Vo of the column demonstrated this peak to contain only gp36 this time (Fig. 3.7), while the second peak again appeared to contain pure gp52. These results proved to be consistently reproducible; gp36 never eluted at a position after the gp52 peak, as one would normally expect in view of the difference in molecular weight observed for the two proteins on analysis by SDS-PAGE.

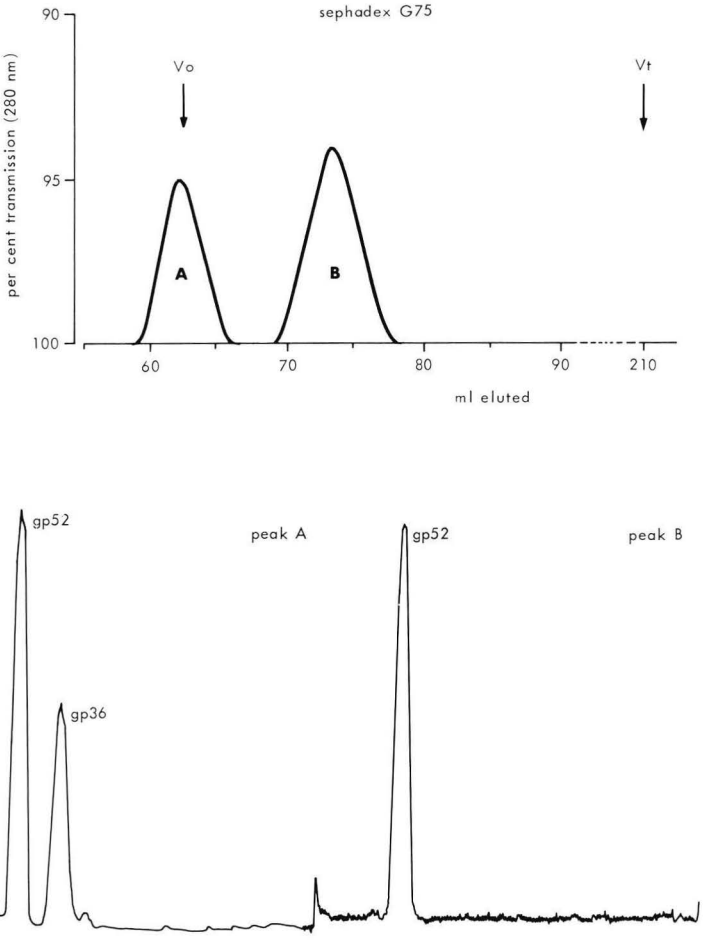
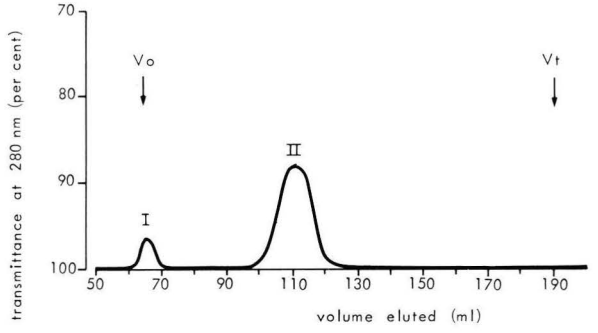


Figure 3.6:

Gel filtration of the MuMTV glycoprotein fraction on Sephadex G150.
In the elution pattern, the volume corresponding to the Vo and Vt is indicated. The two eluted fractions were analyzed by SDS-PAGE. The corresponding scans are shown in the Figure.



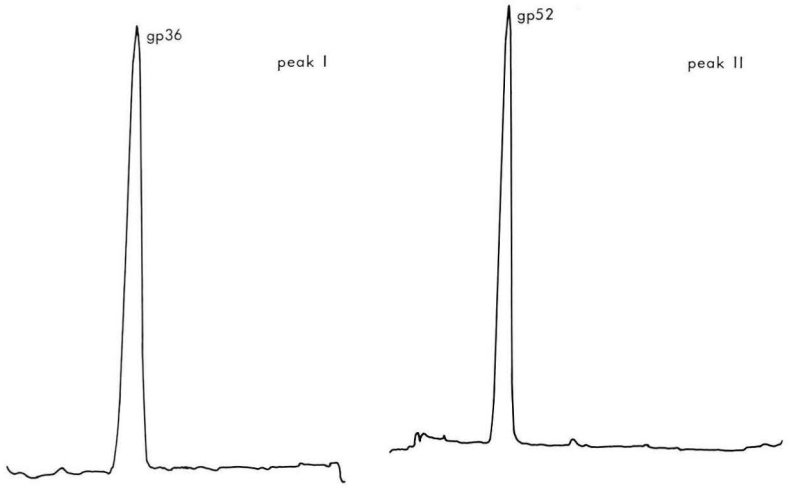


Figure 3.7:

Gel filtration of the gp52-gp36 complex on Sephadex G150.
Gel filtration was made in the presence of 0.5% DOC. The two scans represent the result of the analysis of the two eluted fractions by SDS-PAGE.

As mentioned earlier, gel filtration of the flow through peak of the chromatography of nonglycosylated MuMTV proteins on phosphocellulose produced no significant results. Using Tris-buffer containing 1 M NaCl, as the elution buffer, all U.V. absorbing material eluted in the Vo. This material probably consisted mostly of lipids.

Table 3.1 summarizes the results obtained by application of the overall isolation procedure as described in this Chapter, expressed in the final yields of the respective MuMTV proteins. To estimate the amount of a particular protein present per total amount of MuMTV protein, it was assumed that the optical density of Coomassie blue stained gels is proportional to the mass of each protein.

Table 3.1

PRESENTATION OF THE YIELD OF PURIFIED MuMTV-PROTEINS OBTAINED ON APPLICATION OF THE ISOLATION PROCEDURE DESCRIBED IN THIS CHAPTER

MuMTV protein	mg present in 40 mg MuMTV[1,2]	yield of purified protein (mg)[3]	average percentage recovery
gp52	10	3.0 - 3.4	32
gp36	5	1.5 - 1.7	33
p28	8	3.0 - 3.8	43
p12	4	1.3 - 1.7	38

[1] A purification procedure was usually started with 40 mg solubilized MuMTV protein.
[2] The amount of protein present per total amount of MuMTV protein was calculated on basis of the surface area of the peak representing the corresponding protein in a scan obtained by analysis of MuMTV on SDS-PAGE. For comparison, see Teramoto et al.(1974) and Sarkar et al. (1976).
[3] The yield of 5 isolations is given.

3.3 DISCUSSION

Nonionic detergents, such as NNP10 and bile salts, such as sodium-deoxycholate, in contrast to ionic detergents such as sodium dodecyl-sulphate, do not usually denature proteins (Helenius and Simons, 1975; Tanford and Reynolds, 1976). Therefore, these types of detergents are most suitable for solubilization of integral membrane proteins and stabilization of these proteins in solution, especially when maintenance of the immunogenic integrity of the proteins is essential.

Treatment of a MuMTV suspension with 1% NNP10 and 1% DOC resulted in the solubilization of about 80% of the total amount of viral proteins present.

In the development of the isolation procedure for the glycoproteins of MuMTV, it became clear that the type(s) of detergent(s) added to the elution buffer markedly influenced the elution pattern during chromatography on Con A-Sepharose. Lotan et al. (1977) reported that nonionic detergents had hardly any effect on the binding capacity of immobilized plant lectins; on the contrary, several reports mentioned a decreased binding in the presence of DOC at a concentration of 1% (Allan et al., 1972; Winquist et al., 1976). In our experiments, the binding of gp52 and gp36 by the immobilized lectins was not affected by the addition of low concentrations of NNP10 and/or DOC to the elution buffer; however, effective elution of these proteins by addition of a sugar derivative as competitor to the glycoprotein lectin interaction to the buffer was achieved only in the presence of 0.02% DOC and/or 0.02% NNP10.

When NNP10 was added, all bound material eluted from the column in a heterogeneous peak, whereas, in the presence of DOC, the sugar derivative MMP effected the elution of only gp52 from the column. Under these conditions a gp52-gp36 complex eluted next from the column when NNP10 was also added to the elution buffer. We conclude that fractionation of glycoproteins slightly differing in their affinity for the immobilized lectin can be obtained by using different detergent-combinations in the elution buffer in addition to a competitor of the lectin glycoprotein interaction.

The gp52-gp36 complex remains intact during the chromatography on Con A-Sepharose and during gel filtration in a buffer of high ionic strength (1 M NaCl). Only in the presence of a high concentration of 0.5% DOC does this complex disintegrate; even under such circumstances, gp36 elutes in the form of a multimeric aggregate in the Vo of a Sephadex column. Analysis of such a purified gp36 fraction on SDS-PAGE occasionally showed gp36 to appear partly as a dimer on the gel.

Considering the result obtained with the final isolation procedure developed for the MuMTV structural proteins, we conclude this procedure to meet the requirements we previously stated. The method results in the purification of both glycosylated proteins at an reasonable high yield (see Table 3.1). Furthermore, as was demonstrated by analysis of the purified protein fractions on SDS-PAGE, a high degree of purity was obtained (see also Fig. 4.1). Anticipating the results shown in Chapter 5, the requirement of the preservation of the immuno-

genic nature of the purified proteins was also met. This was demonstrated by the results obtained when the proteins were tested in several immunological assays. No occurrence of partial denaturation as a consequence of the applied isolation procedure was detected.

Since the time we reported on our isolation procedures, several other papers on the isolation of structural MuMTV proteins have appeared. Ritzi et al. (1976) reported on the purification of gp52 and p28, using a procedure involving chromatography on Con A-Sepharose and DEAE cellulose. Dion et al. (1977b) discussed several methods for the isolation of MuMTV proteins. In the first method, Nonidet solubilized MuMTV proteins are subjected to chromatography on DEAE-cellulose and phosphocellulose. This procedure resulted in the purification of gp52, p28, p12 and a protein designated as gp68. This latter protein is identifiable only in RIII milk-purified MuMTV. The second purification method of Dion and co-workers involved gel filtration of MuMTV proteins in the presence of 1% SDS. Solubilization of the viral proteins was achieved by incubation of the virus for 30 min at 56 ^{o}C, a procedure which probably leads to partial denaturation of the proteins (Helenius and Simons, 1975). Using this method, the purification of gp52, gp36, p28 and p12 was achieved. A third method involving gel filtration in 6 M Gu-HCl proved to be unsuccessful; dialysis of separated fractions to remove the guanidine resulted in precipitation of denatured proteins.

Arthur et al. (1978a) reported on the purification of a 14,000 molecular weight MuMTV protein by chromatography on DNA-Sepharose. Probably this p14 molecule is the same as the 18,000 molecular weight protein we occasionally detected in our p12 fraction (see Chapter 4).

The use of hydrophobic chromatography was introduced recently as a very useful method for the isolation of MuMTV proteins (Marcus et al., 1979). Using chromatography of nonionic detergent-solubilized MuMTV on columns of alkyl-agarose derivatives as the initial step, the purification of gp52, gp36, p28, p23, p16 and p12 was described.

CHAPTER IV

SOME CHARACTERISTICS OF THE PURIFIED MuMTV PROTEINS

4.1 INTRODUCTION

The proteins which form part of RNA tumour virions can be distinguished according to several characteristics. For the estimation of the M.W.'s of proteins below 30,000 daltons, it has been generally agreed to use gel filtration in 6M Gu-HCl. SDS-PAGE is recommended for those with a M.W. above 30,000 daltons (August et al., 1974). However, the M.W. estimates of glycoproteins are often incorrect when either of the two methods are used. On gel filtration in 6M Gu-HCl, the virion glycoproteins frequently appear as aggregates in the Vo of the column (Fleissner, 1971; Green and Bolognesi, 1974), while electrophoresis on SDS-polyacrylamide gels often yields aberrant M.W. values. This is because reduced binding of detergent to the carbohydrate component results in a shorter migration distance and, consequently, a higher apparent M.W. value. Yet, due to the excellent resolution of proteins in SDS-PAGE, it was agreed to use these apparent M.W.'s to designate the viral glycoproteins (August et al., 1974).

According to studies on viral proteins in the various RNA tumour viruses, it appears that the localization of the different protein constituents within the virus particle is determined by their chemical characteristics. The glycosylated viral proteins are always associated with the envelope of the virus, from which they protrude as spike-like structures. The nonglycosylated proteins are usually located inside the virion, although the MuLV system represents an exception on this rule. In addition to the glycoprotein gp70, the envelope of MuLV contains a nonglycosylated protein designated as p15E. This p15E component may be cleaved into a slightly smaller form, p12E, which remains associated with the viral membrane (Ikeda et al., 1975; Naso et al., 1976).

As far as the internal proteins are concerned, on the basis of studies made on the proteins of avian leukemia and sarcoma viruses and MuLV's, Eisenman and Vogt (1978) distinguished four types of proteins: 1) an arginine- and lysine-rich basic protein, which is found in association with the RNA of detergent lysed particles; 2) a distinct phos-

phoprotein which binds specifically to homologous RNA; 3) a protein present in a relatively large amount and which probably constitutes the core shell; 4) a rather hydrophobic protein which, in the murine system, is associated with lipid.

In the classification of the internal viral proteins, it is of importance to determine the amino acid composition. By taking the sum of the percentages of methionine, phenylalanine, cysteine, leucine, isoleucine, valine, tryptophan and histidine as an index of hydrophobicity, proteins can be distinguished on the basis of this property (Rackovsky and Sheraga, 1977; Brouwer et al., 1979). In addition, the content of arginine plus lysine will give information on the acidic or basic nature of the proteins. Isoelectric focusing will give additional information in this respect.

The association of some types of enzymatic activities with particular viral protein constituents in the RNA tumour virus system has been reported. As mentioned in Chapter 1 a major characteristic of the RNA tumour viruses is the enzyme reverse transcriptase, with which a RNA-directed DNA polymerase activity and a RNAse H-activity are associated. As described, this enzyme is involved in the process of viral DNA synthesis.

Recently, the association of protease activity with the gag-gene coded ASV-structural protein p15, has been described (Dittmar and Moelling, 1978; Khan and Stephenson, 1979; Vogt et al., 1979). This proteolytic activity is thought to be involved in the processing of the viral gag-precursor proteins. The association of proteolytic activity with R-MuLV was also described (Yoskinaka and Luftig, 1977a, b); however, the protein carrying this activity has not been identified yet.

In this Chapter, we describe the characterization of the two isolated MuMTV envelope proteins gp52 and gp36 and the two nonglycosylated MuMTV proteins p28 and p12 (see Fig. 4.1). The proteins were analysed by gel filtration in 6M Gu-HCl and by SDS-PAGE with respect to their M.W.'s. Furthermore, the amino acid composition of the different proteins has been determined. A search for proteolytic activity possibly associated with one of the purified proteins was made by the method, used by Dittmar and Moelling (1978).

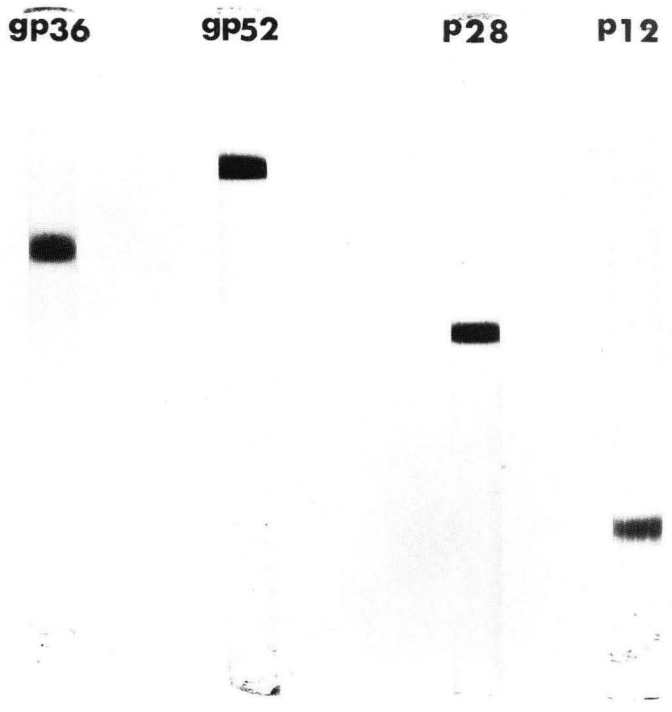


Figure 4.1: Analysis of the four isolated MuMTV proteins by SDS-PAGE. Bands were stained with Coomassie brillant blue.

4.2 RESULTS

4.2.1 Analysis of MuMTV proteins by SDS-PAGE

As was established by Weber and Osborn (1969), polypeptide M.W. estimates may be determined by SDS-PAGE, with an inaccuracy of about 10%. Based on analysis by SDS-PAGE, a great variety of MuMTV-associated polypeptide numbers and M.W.'s has been reported (see Chapter 1). It is uncertain which of the proteins are coded for by the MuMTV genome.

In Fig. 4.2, the results which we obtained on analysis of MuMTV derived from different sources by SDS-PAGE are shown. Analysis of tissue culture derived virus usually gave reproducible results with respect to the number of polypeptides, with the exception of the lowest M.W. protein designated as p10 in Fig. 4.2A, which was seen only occasionally. The source of virus clearly influences the results obtained; RIII milk derived MuMTV demonstrated an extra protein with a M.W. of 66,000 daltons as compared with the other two analyses (Fig. 4.2C). This protein probably corresponds to the MuMTV-associated protein gp68 reported by some investigators (see Moore et al., 1979). In view of the coding capacity of the MuMTV genome (see Chapter 1) and in view of

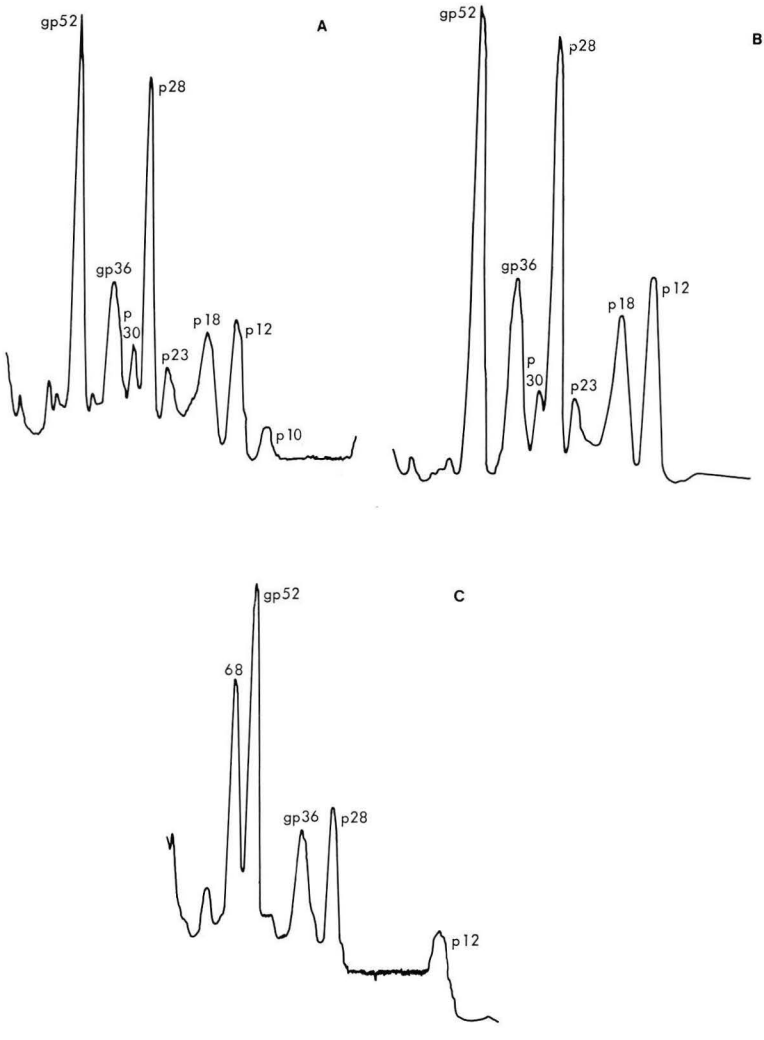


Figure 4.2:

Analysis of MuMTV derived from different sources by SDS-PAGE.
(A) represents MuMTV purified from C3HMT/cl11 culture medium. (B) represents MuMTV purified from Mm5mt/cl culture medium as it was obtained through the Office of Resources and Logistics (see Chapter II). (C) represents a detergent solubilized RIII milk derived MuMTV protein solution centrifuged for 60 min at 164,000 x g (see Chapter II). See text for the designations of the different polypeptides.

the polypeptide composition of MuMTV derived from other sources, it is reasonable to assume gp68 to represent either a host-cell-derived protein or an intermediate precursor of some of the MuMTV-structural proteins.

Fig. 4.1 shows the results of the analysis by SDS-PAGE of the MuMTV proteins which we have isolated. Analysis of purified gp36 occasionally revealed this protein to be migrating as a dimer and/or trimer on SDS-polyacrylamide gels, indicating aggregates of this protein difficult to disperse in the buffer used. Similar results were obtained when the purified MuMTV proteins were analysed on gels containing either 10, 12.5 or 15% acrylamide.

4.2.2 Gel filtration in 6 M Guanidine-HCl

As referred to in the introduction of this Chapter, an alternative to the estimation of the M.W. of a protein as a function of the electrophoretic mobility on SDS-PAGE is gel filtration in 6 M Gu-HCl.

Gel filtration of solubilized total MuMTV proteins in 6 M Gu-HCl did not lead to the isolation of any individual MuMTV-protein; material eluted from the column in one broad peak. For the M.W. estimations of the MuMTV proteins, they were radioiodinated and subjected to the gel filtration procedure individually.

Fig. 4.3 shows the results obtained when the agarose column was calibrated with the M.W. marker proteins (see Chapter 2); the logarithm of the M.W. was plotted against Ve/Vo, where Ve is the elution volume of the protein and Vo the void volume of the column determined by chromatography of a sample of dextran blue.

Of the MuMTV proteins, gp52 and p28 eluted as single homogeneous peaks, gp36 eluted in the Vo and radioiodinated p12 eluted in two different peaks from the agarose column. The corresponding M.W. values for the different proteins were estimated to be 42,500 daltons for gp52 and 27,800 daltons for p28 (see Fig. 4.3).

The M.W. values corresponding to the two peaks eluting from the column after application of radioiodinated p12 were calculated to be 10,400 and 7,400 daltons, respectively. When both fractions were dialysed and subsequently analysed by SDS-PAGE, both proteins displayed the same electrophoretic mobility. A similar finding was reported by Brouwer et al. (1979), who demonstrated the same electrophoretic mobility for the R-MuLV proteins p12 and p10 on analysis by SDS-PAGE, while gel filtration in 6 M Gu-HCl resulted in a clear separation of the two proteins. Gel filtration of unlabelled p12 resulted in the

elution of one peak with a corresponding M.W. of 12,500 daltons, suggesting the ^{125}I-labelled p12 preparation had suffered radiochemical breakdown.

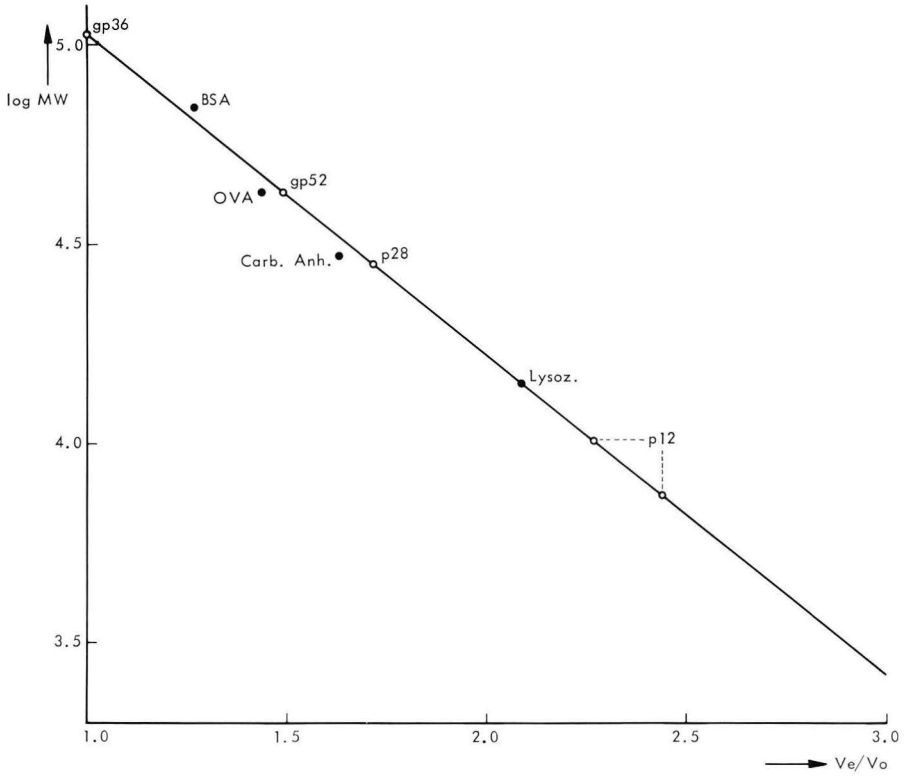


Figure 4.3:

Gel filtration of MuMTV proteins in 6 M guanidine-HCl.
The biogel A .5m column was calibrated with BSA (M.W. 69,000), OVA (M.W. 43,000), Carbonic anhydrase (M.W. 29,000) and lysozyme (M.W. 14,500). In the figure, the log MW was plotted against Ve/Vo, where Ve is the elution volume of the protein and Vo represents the void volume of the column. The corresponding positions of the MuMTV proteins in the curve are shown.

4.2.3 Amino acid analysis

Another approach in characterizing the MuMTV proteins was amino acid analysis, the results of which are shown in Table 4.1. As pointed out in Chapter 2, the hydrolysis procedure used has some limitations:

TABLE 4.1

AMINO ACID ANALYSIS OF MuMTV PROTEINS

Amino acid residue	Number of residues/100 residues of amino acid gp52	gp36	p28	p12
Asp.	10.1	9.9	8.0	8.2
Thr.*	5.7	6.3	5.9	5.2
Ser.**	8.5	6.1	7.0	9.4
Glu.	5.3	10.3	11.7	6.9
Pro.	11.3	3.4	7.1	3.9
Gly.	7.2	4.7	7.3	8.9
Ala.	6.0	11.5	8.2	6.6
Val.	5.7	8.6	6.7	7.1
Cys.***	0.6	0.3	0.2	0.02
Meth.	0.2	-	4.5	1.0
Ile.	5.0	5.6	3.2	4.8
Leu.	9.4	14.3	11.1	11.4
Tyr.	4.5	2.3	3.2	4.2
Phe.	7.3	5.3	1.3	3.5
His.	2.7	1.8	0.6	0.5
Lys.	6.6	5.4	8.7	12.8
Arg.	4.0	3.3	5.4	5.7

Protein	% hydrophobic amino acids	% lysine + arginine
gp52	30.9	10.6
gp36	35.9	8.7
p28	27.6	14.1
p12	28.3	18.5

* The threonine value is usually 5% lower than the actual value (see text).
** The serine value is usually 10% lower than the real value (see text).
***Cysteine is largely destroyed during hydrolysis.

tryptophan and cysteine are largely destroyed; furthermore, the values estimated for serine and threonine, tend to be 10% and 5% lower respectively than the actual value.

For gp52 and p28 our results are in general agreement with those of Parks et al. (1974b). The claim of Sarkar and Dion (1975) that p12 is extremely rich in arginine (a result which led them to suggest p12 to be a ribonucleoprotein) is not confirmed by our results. Two recent reports on amino acid analysis of MuMTV proteins also failed to confirm that statement (Yagi et al., 1978; Dion et al., 1979b).

The amino acid composition reported by Dion et al. (1979b) for purified gp36 and p12, differs considerably from our results, the reason of which is not clear.

The order of hydrophobicity of the MuMTV proteins is calculated by taking the sum of the percentages of the amino acids methionine, phenylalanine, cysteine, leucine, isoleucine, valine and histidine as the index of this property. The order of hydrophobicity was estimated to be gp36 > gp52 > p12 > p28 (see also Table 4.1).

None of the analysed proteins showed an extremely high content of arginine and lysine; therefore, none of them seems to be a candidate for a ribonucleoprotein. For comparison, the ribonucleoprotein of R-MuLV, p10, appeared to contain 23% arginine and lysine (Brouwer et al., 1979).

4.2.4 Isoelectric focusing

The results obtained with isoelectric focusing of the MuMTV proteins, are compiled in Table 4.2. Several isomers of gp52, gp36 and p28 were identified with isoelectric points (pI) at or near pH 7.0. These results are reminiscent of those obtained by Forchhammer and Turnock (1978) for the comparable proteins of Moloney MuLV.

TABLE 4.2

ISOELECTRIC POINTS OF THE MuMTV PROTEINS

MuMTV protein	pI
gp52	7.3, 7.0, 6.6, 6.4, 6.1
gp36	6.9, 6.5, 6.3, 5.7, 5.4
p28	7.6, 7.4, 7.0, 6.4
p12	7.0 - 6.8

By two-dimensional gel electrophoresis, Forchhammer and Turnock (1978) demonstrated the structural proteins of Moloney MuLV to have pI's varying from pH 4.2 to pH 7.8; an exception was the arginine and lysine rich basic ribonucleoprotein, p10, which exhibited a pI of 9.6 (Pfeffer et al., 1976). Forchhammer and Turnock (1978) showed the viral proteins to be present in multiple forms differing in charge but not in size. Especially the major envelope protein gp70 was shown to have a great charge heterogeneity, as about 17 different spots could be identified within one virus preparation, while for the major core protein, p30, 5 different isomers could be identified. The authors postulated the existence of different isomers of particular viral proteins to be due to differences in posttranslational modifications. For glycoproteins, much of the heterogeneity may be attributed to variations in carbohydrate composition (Righetti and Drysdale, 1974). Artificial results may be obtained upon storage of virus for longer periods at -20° C, as deamination of glutamine and/or asparagine may lead to proteins focusing at a different pH (Hamann et al., 1977).

In agreement with the results of the amino acid analysis, p12 appeared not to be a particular basic protein (Sarkar and Dion, 1975); it electrofocused at pH7.0 - 6.8, indicating that is is not a candidate for the major ribonucleoprotein of MuMTV.

4.2.5 Assaying the purified MuMTV proteins for proteolytic activity

To investigate the possibility of proteolytic activity being associated with one of the purified MuMTV proteins or with detergent disrupted MuMTV, the method used by Dittmar and Moelling (1978) for the identification of the proteolytic activity associated with p15 of avian myeloblastosis virus was employed. SDS-denatured BSA or OVA was incubated with the test protein under varying conditions (see Chapter 2). Incubation mixtures were then analysed by SDS-PAGE for degradation products. As a positive control, substrates were incubated with trypsin or protease. Fig. 4.4 shows some results which demonstrate the usefulness of the test system.

No proteolytic activity was detected in association with any of the four MuMTV proteins tested. Incubation for 16 hr at an "enzyme"-substrate ratio of 1:1 did not reveal any degradation of the substrate used. Fig. 4.5 shows some of the results obtained. When a p12-fraction, which initially appeared to be contaminated with an 18,000 M.W. protein (see Fig. 3.5) was incubated with denatured BSA no degradation of BSA was detected. Furthermore only p12 appeared to be present in

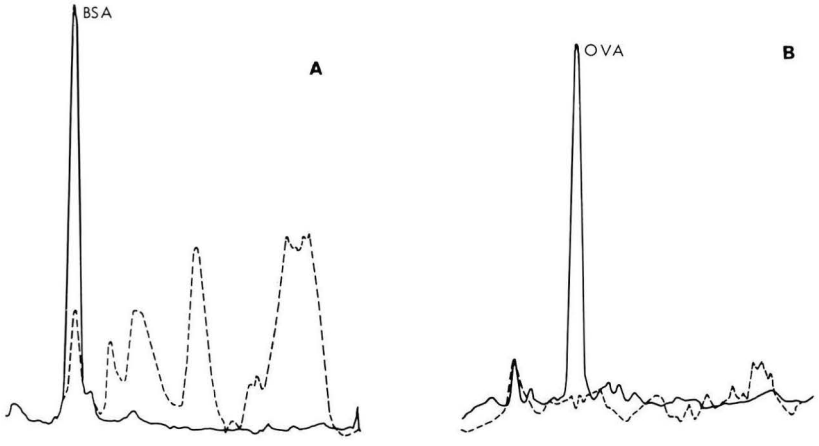


Figure 4.4:

SDS-PAGE analysis of incubation mixtures for the presence of proteolytic activity.
A:——represents BSA;– – – represents 5 µg BSA, incubated with 0.5 µg trypsin for 30 min at 37° C, pH 6.0.
B:—— represents OVA;– – –represents 10 µg OVA, incubated with 0.1 µg protease for 3 hr at 37°, pH 6.0.

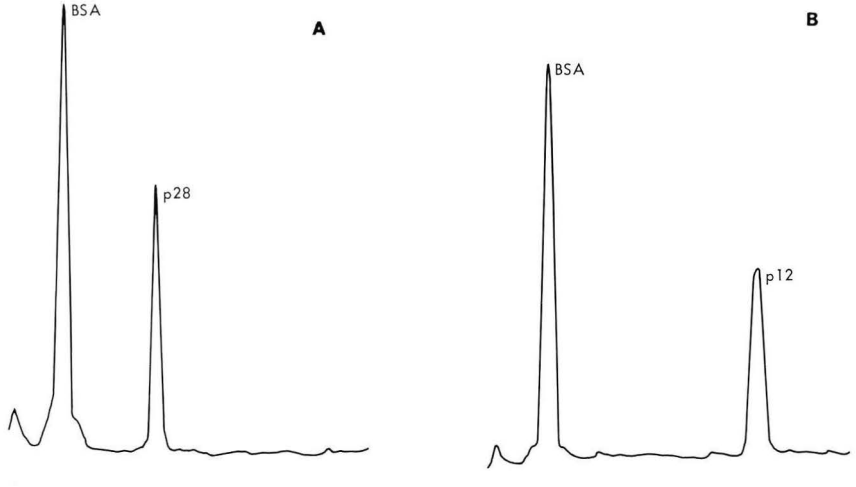


Figure 4.5:

SDS-PAGE analysis of incubation mixtures for the presence of proteolytic activity.
A: represents 10 µg BSA incubated with 10 µg p28 for 16 hrs at 37° C, pH 6.0;
B: represents 10 µg BSA incubated with 10 µg p12 for 16 hr at 37° C, pH 7.0.

the mixture. Although the disappearance of p18 suggested the presence of proteolytic activity in the p12-fraction, no such activity was detected by the test method used.

When denatured OVA was used as a substrate in addition to BSA, there was also no proteolytic activity detected in any of the MuMTV protein fractions tested under any conditions. Some of the results are shown in Fig. 4.6.

Completely disrupted MuMTV was finally tested for the presence of proteolytic activity. It was disrupted in 1% NNP10 and solubilized viral proteins in an amount of 50 ug were incubated with radioiodinated, SDS-denatured BSA (10^6 cpm) for 16 hr at 37°C. No degradation of BSA was detected on analysis by SDS-PAGE.

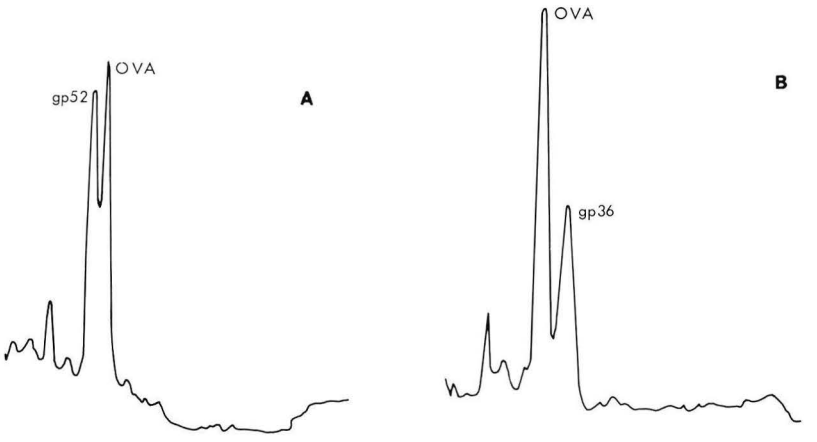


Figure 4.6:

SDS-PAGE analysis of incubation mixtures for the presence of proteolytic activity. A: represents 10 µg OVA incubated with 10 µg gp52 for 16 hr at 37° C, pH 7.0. B: represents 10 µg OVA incubated with 10 µg gp36 for 16 hr at 37° C, pH 7.0.

4.3 DISCUSSION

Analysis of MuMTV preparations by SDS-PAGE for the determination of the number of distinct structural viral proteins is hampered by contamination with host-cell derived proteins such as actin (Racevskis and Sarkar, 1978; Yagi et al., 1978). In addition, some protein bands may represent breakdown products of original viral proteins, such as gp33, which appears to be a breakdown product of gp36 (Yagi et al., 1978), while apparently homogeneous protein bands may be composed of

different proteins, as was demonstrated by analysis of the different p12-related protein fractions on SDS-PAGE. Furthermore, strongly hydrophobic proteins may appear in dimeric and/or trimeric form on SDS-polyacrylamide-gels, which we repeatedly found to be the case for gp36.

Based on data reported in the literature (see Chapter 1) and our own results, we postulate the following proteins to represent MuMTV-coded structural viral proteins: the glycosylated envelope proteins gp52 (47-55,000) and gp36 (34-36,000) and the internal proteins p28 (24-28,000), p23 (20-23,000), p18 (14-18,000) and p12 (10-12,000). The M.W. values for the different proteins are according to our results obtained by SDS-PAGE (see Fig. 4.2), the numbers in parentheses indicate the M.W. values given in the literature (see also Chapter 1). There is uncertainty with respect to the proteins p30 and p10. P30, which contains p18 sequences (Gautsch et al., 1978; Dickson and Atterwill, 1979, who designated this protein as p14), either represents an intermediate precursor of p18 (Gautsch et al., 1978) or it is an end-product of the processing of a minor gag-gene coded precursor protein p110 (Dickson and Atterwill, 1979; see also Chapter 1). Furthermore, it is not clear whether p10 is an MuMTV-coded protein; peptide mapping experiments did not give a definite answer to the question of whether p10 represents a gag-gene coded MuMTV structural protein (Dickson and Atterwill, 1979).

The arrangement of the viral proteins in their respective precursor molecules was estimated to be NH_2-gp52-gp36-COOH (Schochetman et al., 1977) and NH_2-p12-p23-p28-p18-COOH (Dickson and Atterwill, 1979; Massey and Schochetman, 1979).

As part of their characterization the MuMTV proteins gp52, gp36, p28 and p12 were subjected to gel filtration in 6 M Gu-HCl. A M.W. of 42,500 daltons was estimated for gp52, a value which agrees with the M.W. of 41,650, calculated by Parks et al. (1974b) from amino acid composition analysis.

The elution of gp36 in the Vo of the column indicates strong hydrophobic interactions to be present within this molecule. Similar findings were reported for the smaller envelope glycoproteins of avian myeloblastosis virus and R-MuLV. In these cases, the larger envelope glycoprotein also eluted behind the Vo (Fleissner et al., 1975). The hydrophobic nature of gp36 was further indicated by the results of the amino acid composition analysis of the protein; 35.9% of the amino acids appeared to consist of nonpolar amino acids. By using an alternative hydrolysis method, Dion et al. (1979b) were able to estimate the amount of cysteine present. This is one of the amino acids used

for the calculation of the degree of hydrophobicity. They estimated that 46.7% of the amino acids of gp36 were nonpolar.

By means of hydrophobic chromatography Marcus et al. (1978) determined the order of hydrophobicity for different MuMTV proteins to be: gp36 > gp52, p12 > p28 > p18,p23. Their results are in agreement with our observations (Table 4.1).

A M.W. of 27,800 dalton was estimated for p28 by gel filtration in 6 M Gu-HCl, a value corresponding to the M.W. estimated by SDS-PAGE. For p12 a M.W. of 12,500 was estimated. The elution of radioiodinated p12 in separate fractions from the agarose column was possibly a consequence of radiation damage. Massey and Schochetman (1979) reported on the existence of two closely migrating protein bands in the p12 region (p10 in their nomenclature) by analysis of MuMTV by SDS-PAGE on 10-20% gradient slab gels.

Isoelectric focusing revealed the isolated MuMTV proteins to consist of multiple isomers differing in charge. The existence of such isomers could be due to differences in posttranslational modifications, such as glycosylation and phosphorylation. In this respect, the finding of Anderson et al. (1979) that, in addition to the major phosphoprotein pp23 most, if not all, of the MuMTV proteins are phosphorylated is of importance. In contrast to the suggestion of Sarkar and Dion (1975), it was shown, by both isoelectric focusing and amino acid composition analysis that p12 is not a basic protein, and therefore is not a candidate for a ribonucleoprotein.

No evidence for the association of proteolytic activity with any of the four purified MuMTV proteins nor with detergent disrupted MuMTV could be obtained using the method of Dittmar and Moelling (1978). However, despite these results, there is evidence for the association of proteolytic activity with p12. On analysis of purified p12 fractions by SDS-PAGE, the presence of p18 was occasionally demonstrated (see Fig. 3.5). Reanalysis of such fractions revealed the presence of only a homogeneous p12 band on SDS-polyacrylamide gels. It is tempting to assume that proteolytic activity which under certain circumstances degrades p18 is associated with the purified p12 fraction. The failure of the method of Dittmar and Moelling to identify this proteolytic activity, is similar to the results they obtained with R-MuLV (Dittmar, personal communication).

In summary, MuMTV contains at least six distinct structural proteins which are coded for by the viral genome. Two glycosylated proteins are associated with the envelope of the virion. Gp36 is embedded within the lipid bilayer of the envelope with mainly sugar residues exposed to the exterior. The larger envelope protein gp52 is anchored

by gp36 to the envelope surface by hydrophobic interactions. This strong hydrophobic interaction is evidenced by the purification of a tightly bound gp52-gp36 complex from detergent disrupted MuMTV preparations (see Chapter 3).

The major internal protein, p28, is present in relatively large amounts in the virion (see Fig. 4.2) and probably constitutes the capsid shell, in analogy to p27 of the avian tumour viruses and p30 of MuLV (see Eisenman and Vogt, 1978).

The major phosphoprotein of MuMTV is p23. In contrast to the general situation in RNA tumour viruses, a subpopulation of p28 also appears to be phosphorylated (Sarkar et al., 1978; Nusse et al., 1978; Racevskis and Sarkar, 1979). In analogy with the murine C-type viruses, p23 possibly exhibits type-specific RNA binding properties and consequently plays a role in the process of reverse transcription of the viral RNA genome (Sen and Todaro, 1977) or in the integration of the viral genome on infection of a cell (Todaro, 1978).

P18, which is identified by most investigators as p14, is found in association with the core of the mature virion and can be distinguished from the other structural proteins by its DNA-binding property (Long et al., 1977; Arthur et al., 1978a; Massey and Schochetman, 1979). However, the arginine plus lysine content of this protein (9-10%) (Yagi et al., 1978; Dion et al., 1979b) appears to be far less than that which was estimated for the basic R-MuLV protein p10 (about 23%) (Brouwer et al., 1979), a protein which was typified as the viral ribonucleoprotein (Eisenman and Vogt, 1978).

P12 which is not an integral part of the mature MuMTV core (Teramoto et al., 1977a), probably represents a membrane associated protein located on the innerside of the viral envelope linking the nucleocapsid to the envelope (Cardiff et al., 1978).

Whether p30, clearly demonstrated in association with cores of mature MuMTV virions (Teramoto et al., 1977a; Cardiff et al., 1978) and p10 (see Fig. 4.2), represents distinct, MuMTV-genome coded, viral structural proteins, remains to be established (Dickson and Atterwill, 1979). The protein composition of MuMTV discussed above is summarized in Fig. 4.7 and Table 4.3.

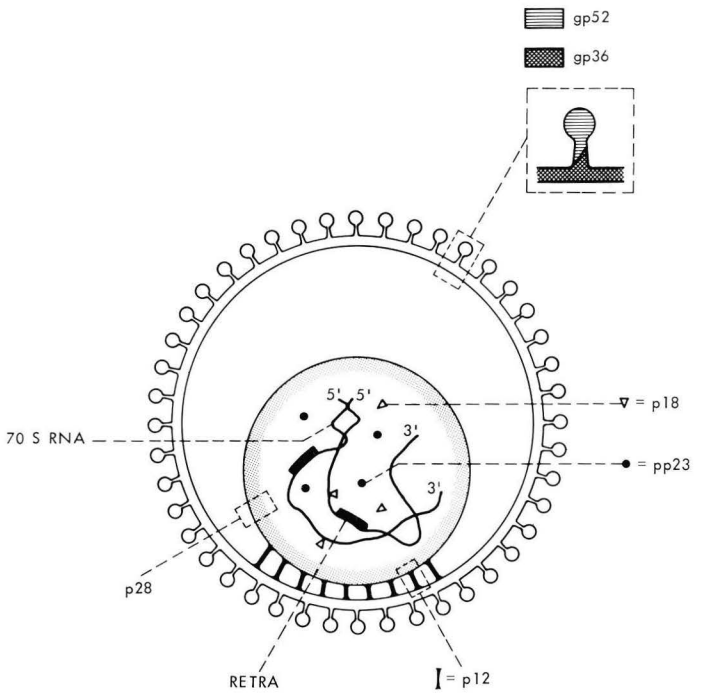


Figure 4.7:

Schematic representation of the location of the different MuMTV structural proteins within the virion (see Table 4.3 for their functions).

TABLE 4.3

LOCATION AND FUNCTION OF THE STRUCTURAL PROTEINS OF MuMTV

protein	location	function (probably)
gp52	on the exterior of the virion	involved in the cell-virus interaction via a host cell surface receptor. Consitutes the greater part of the spikes of the viral envelope
gp36	embedded within the lipid bilayer of the envelope	anchors gp52 to the envelope of the virus
p28	located in the core	constitutes the capsid shell
p23	located in the core	major phosphoprotein involved in processes such as reverse transcription of the viral RNA genome; integration of proviral DNA into the host genome.
p18	located in the core	possibly represents the RNA associated protein of the virus
p12	located on the innerside of the viral envelope	connects the core with the envelope and is therefore responsible for the typical eccentric location of the core in the MuMTV virion

CHAPTER V

CHARACTERIZATION OF ANTISERA RAISED AGAINST THE PURIFIED MuMTV PROTEINS. STUDY ON THE EXPRESSION OF MuMTV ANTIGENS ON TUMOUR CELLS

5.1 INTRODUCTION

Events at the cell surface play a central role in the communication between and regulation of growth of normal and neoplastic cells. Therefore, by studying newly expressed antigens on the tumour cell surface possibly insight will be attained in the processes underlying the altered growth properties and metastatic behaviour of malignant cells. As was discussed in Chapter 1, TCSA's expressed on cells transformed by RNA tumour viruses may represent the transforming protein coded for by the viral genome. In addition, several other types of tumour-specific antigens may be identified, like structural viral antigens and embryonic or foetal antigens.

In this Chapter, our study on the expression of MuMTV antigens on MuMTV-induced mouse mammary tumour cells will be described, whereas in Chapter 6 a search for the expression of non-virion TCSA's will be described.

Expression of MuMTV structural proteins on the surface of murine mammary tumour cells and lymphoid cells was initially studied with polyvalent antisera to MuMTV in immunofluorescence, immunoelectronmicroscopy and humoral cytotoxicity assays (Tanaka and Moore, 1967; Shigematsu et al., 1971; Hilgers et al., 1972; Hoshino and Dmochowski, 1973; Gillette et al., 1974). With such an approach, it is not possible to define the identity of the expressed antigen(s). In addition, antisera raised to MuMTV may contain antibodies directed to normal cellular antigens: As part of their maturation process, B type particles are released from cells by budding. As a consequence, host proteins may be enclosed within the viral envelope. For instance, Calafat et al. (1976) demonstrated the presence of the normal surface antigens Thy 1.2 and H-2.8 on the envelope of B particles produced by GR mouse ascites leukaemia (GRSL) cells. The cellular protein actin was also identified in purified MuMTV preparations (Damsky et al., 1977; Racevskis and Sarkar, 1978). Therefore, in using heterologous polyvalent antisera to MuMTV in techniques like immunofluorescence and im-

munoelectronmicroscopy, there is the danger of misinterpretation of results obtained. Consequently, clearly characterized antisera are required for the study of the cell surface expression of MuMTV-antigens.

In this Chapter, we describe the characterization of rabbit antisera raised against purified MuMTV-proteins. With these antisera, the expression of MuMTV antigens on different types of tumour cells was studied by means of the membrane immunofluorescence assay and the complement dependent humoral cytotoxicity assay.

In addition, the development of competition radioimmunoassays for the different viral proteins is described. Recently, radioimmunoassays for intact MuMTV virions (Cardiff, 1973; Ihle et al., 1976) for disrupted MuMTV virions (Verstraeten et al., 1973; 1975) and for purified MuMTV proteins (Parks et al., 1974b; Ritzi et al., 1976; Sheffield et al., 1977) have been described. In general, it appears that radioimmunoassays developed for purified viral proteins are 10-15 times as sensitive as assays developed for intact virions.

The suitability of the competition radioimmunoassay developed for gp52 for testing of crude samples such as milk and tumour cell extracts was investigated.

5.2 RESULTS

5.2.1 Preparation of antisera

Rabbits were used for the generation of the different antisera. Following the immunization schedule as outlined in Chapter 2, sera usually reacted positive in immunological assays when tested after three immunizations. Antisera were stored at -20° C and remained useful for a period of at least one year.

About 40 ml of blood was collected from the ear vein every two weeks until reactivity declined. When this occurred the titer of the antiserum was raised again by one additional immunization, after which the blood collection was continued. In this way, several hundred ml of antiserum could be obtained from a single animal.

A polyvalent anti-MuMTV-serum was raised by inoculation with the detergent solubilized viral protein solution, which also was used for chromatography on Con A-Sepharose (Chapter 3). Antisera were also raised against the purified viral proteins gp52, gp36, p28 and p12.

The production of antisera against gp52 and p28 presented no problems, in contrast to the production of an anti-gp36 serum. Three antisera were raised against gp36 fractions obtained by three isola-

tion procedures. When tested in different immunological assays (described below), two of the antisera reacted mainly with gp52. A third antiserum reacted only with gp36 when tested against solubilized MuMTV. The results obtained with the latter antiserum are described in this Chapter. The diversity in quality of the three anti-gp36 sera was due to the degree of contamination of the different gp36 fractions with gp52. With a competition radioimmunoassay developed for gp52 (as described below), it was shown that the three gp36 preparations contained 2%, 1% and 0.1% gp52, respectively. Only the antiserum raised against the latter preparation gave satisfactory results: Apparently gp52 is far more immunogenic in rabbits than gp36, even a 1% contamination of the gp36 preparation with gp52 results in an antiserum which reacts mainly with gp52.

In addition to antisera raised against the proteins gp52, gp36 and p28, an antiserum was raised against p12. As will be discussed later, this antiserum reacted somewhat differently from the other anti-MuMTV protein antisera in the immunoprecipitation assay. Different conditions had to be established in order to obtain satisfactory results.

5.2.2 Characterization of the antisera

5.2.2.1 Double immunodiffusion

The anti-MuMTV serum produced three precipitation lines against NNP10-disrupted MuMTV in the double immunodiffusion test (Fig. 5.1). The antisera to gp52, p28 and p12 produced single lines which did not fuse with each other. The line produced by the anti-p12 serum is only weakly discernible (Fig. 5.1). The three lines obtained with the polyvalent antiserum did fuse with the single lines obtained with the monovalent antisera. No reaction was observed when the different antisera were tested against the serum of a normal C57BL mouse. The results obtained with the anti-gp36 serum in this assay were similar to those obtained with the other monovalent antisera: no cross-reaction with the other antisera raised against single proteins was observed. With the anti-gp36 serum, only weakly discernable precipitation lines were obtained in the immunodiffusion assay (not shown).

5.2.2.2 Immunoelectrophoresis

In the immunoelectrophoresis assay, the anti-gp52 serum and the anti-p12 serum each produced a single line at different sites when

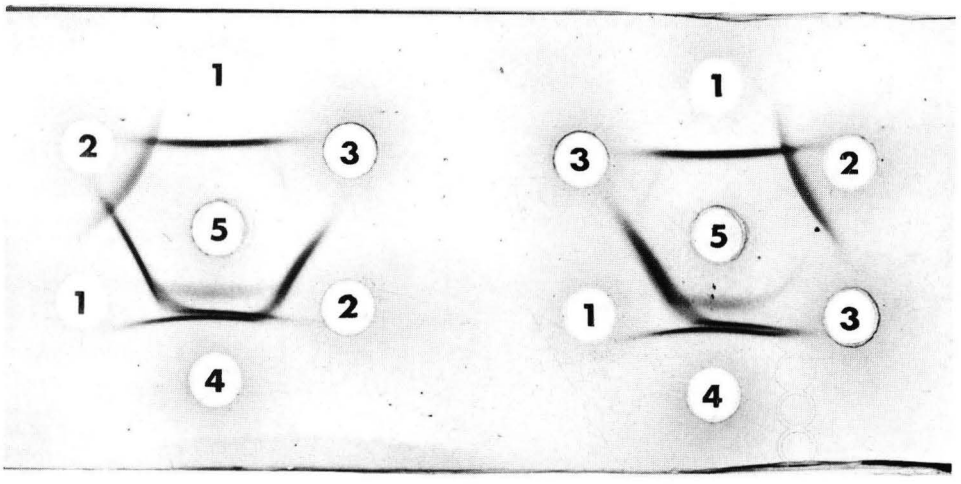


Figure 5.1:
Double immunodiffusion of antisera to the purified MuMTV proteins, against NNP10 disrupted MuMTV.
The wells contained rabbit anti-gp52 (1); rabbit anti-p28 (2); rabbit anti-p12 (3); rabbit anti-MuMTV (4); NNP10-disrupted MuMTV (5).

reacted with detergent disrupted MuMTV, while anti-p28 serum produced two fusing lines, one of which was situated at the application well (Fig. 5.2). This latter line, however, was observed only occasionally. Fig. 5.3 shows another test in which anti-gp52, anti-gp36, anti-p28 and anti-MuMTV were tested in one assay. In this case, the antiserum to p28 produced only one line, like the other two monovalent antisera, which indicates that the line at the application well produced by anti-p28 serum in Fig. 5.1 is probably derived from a p28-containing aggregate which had no electrophoretic mobility in this assay. This was probably due to incomplete disruption of part of the virus preparation.

The presence of detergents in the agarose plates was required in order to obtain satisfactory separation of the different MuMTV proteins in electrophoresis. Without detergents precipitation lines were situated only at or near the application well, indicating a bad solubility of the different proteins under such circumstances. The influence of two different detergent combinations in the agarose on the mobility of glycoproteins gp52 and gp36 is shown in Fig. 5.4. Purified gp52-gp36-complex (see Chapter 3) was tested in the immunoelectrophoresis assay against anti-gp52 and anti-MuMTV sera in the presence of either 1% NNP10, 1% DOC and 1% PEG or 1% DOC and 1% PEG. While the

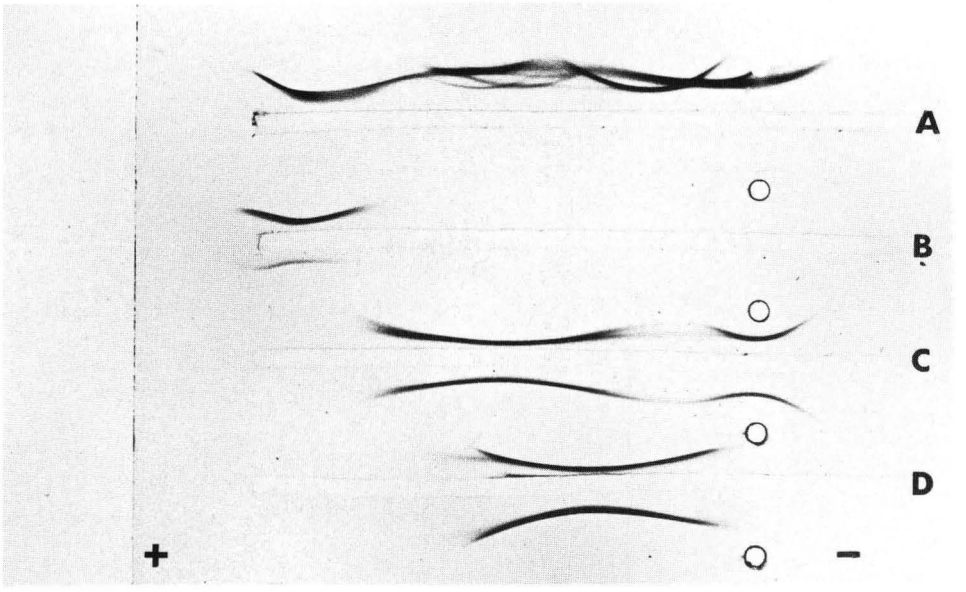


Figure 5.2:
Immunoelectrophoretic characterization of antisera to purified MuMTV proteins.
The trench contained respectively rabbit-anti-normal mouse serum (Nordic, The Netherlands) (A); rabbit anti-p12 serum (B); rabbit anti-p28 serum (C); rabbit anti-gp52 serum (D). The upper well contained normal mouse serum, the other wells NNP10 disrupted MuMTV. The agarose plates contained the detergents NNP10 and DOC (each at 1%).

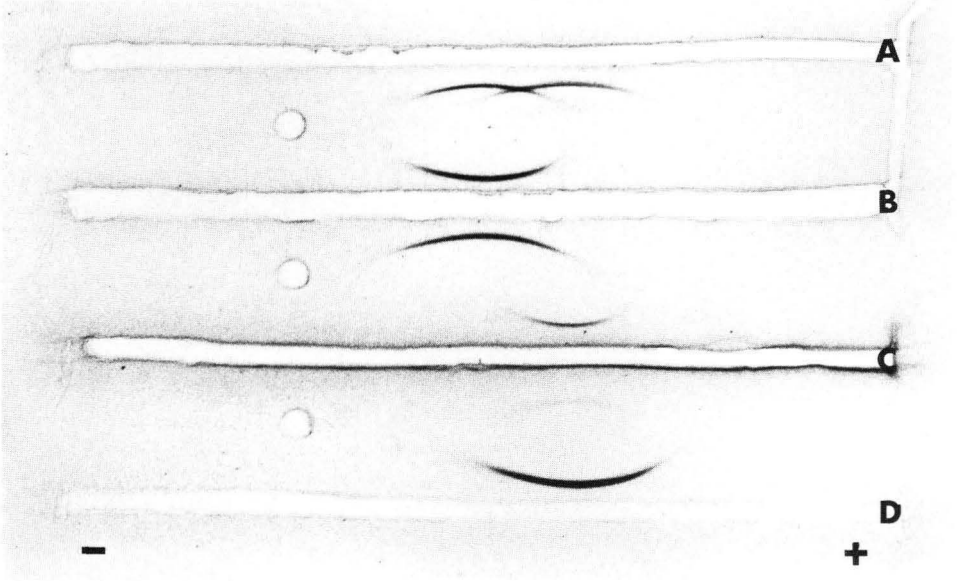


Figure 5.3:
Immunoelectrophoretic characterization of different antisera.
Anti-MuMTV serum (A); anti-gp52 serum (B); anti-gp36 serum (C) and anti-p28 serum (D) were tested against NNP10 disrupted MuMTV. The plates contained 1% NNP10, 1% DOC and 1% PEG.

site of the gp52-derived precipitation line is clearly influenced by the detergent combination used, the position of the gp36-derived line is the same in both systems. This difference in detergent-protein interaction between gp52 and gp36 had already been disclosed in the chromatography of MuMTV proteins on Con A-Sepharose (Chapter 3).

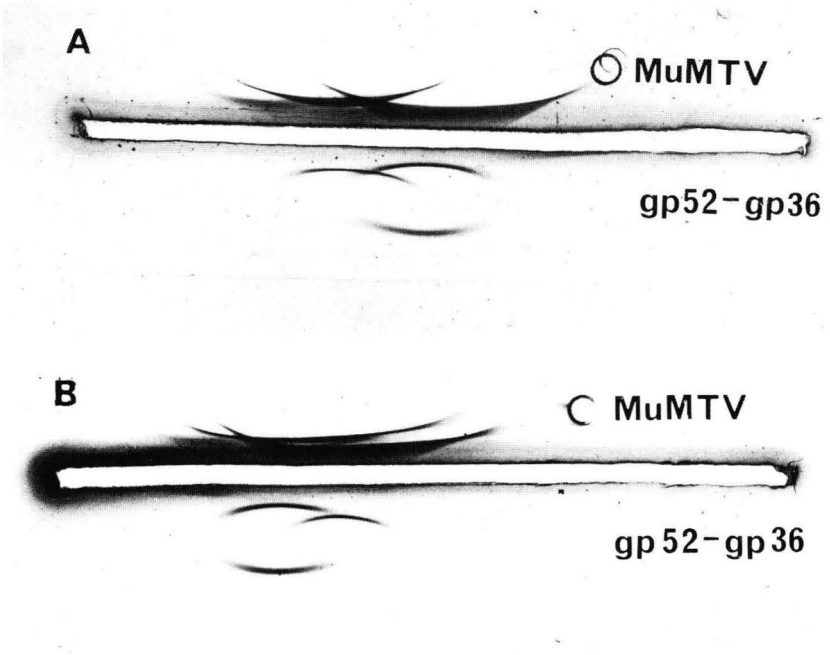


Figure 5.4:

Immunoelectrophoresis in the presence of different detergent-combinations.
The plates contained either 1% NNP10, 1% DOC and 1% PEG (A) or 1% DOC and 1% PEG (B). In both cases, the upper trench contained anti-MuMTV serum and the lower trench anti-gp52 serum. The wells contained NNP10 disrupted MuMTV and purified gp52-gp36 complex (see Chapter 3) as shown in the figure.

5.2.2.3 Immunoprecipitation

In an immunoprecipitation assay a labelled antigen-preparation is reacted with an antiserum. After an incubation period, the reaction mixture is centrifuged after which the precipitate is collected. Analysis of this precipitate on SDS-PAGE subsequently reveals with which antigens the antiserum tested reacts.

For an immunoprecipitation test, detergent solubilized MuMTV was radioiodinated at a specific activity of 1.5-6.4 x 10^4 cpm.ng^{-1} protein. Usually, 75-80% radioiodinated material was TCA precipitable. Fig. 5.5A represents the analysis of a radioiodinated MuMTV sample by means of SDS-PAGE. The results of the analysis of the immunoprecipi-

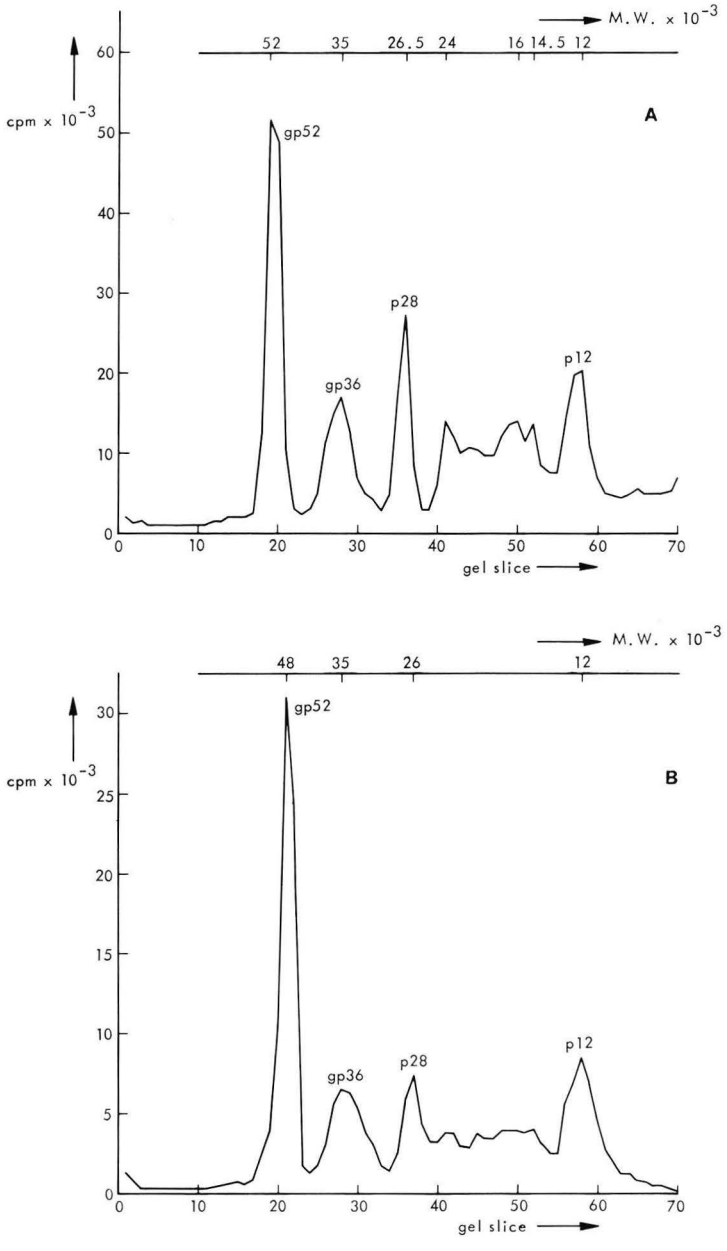


Figure 5.5:

Analysis of immunoprecipitates by SDS-PAGE.
A. Analysis of radioiodinated NNP10 disrupted MuMTV. B. Analysis of the immunoprecipitate obtained by reacting rabbit-anti-MuMTV serum with the radioiodinated MuMTV preparation. After electrophoresis the gels were sectioned in 1.1 mm pieces. Gel fractions were counted in a gamma counter.

tate obtained with polyvalent anti-MuMTV serum is also shown (Fig. 5.5B).

Two different buffers were used in the immunoprecipitation procedure. With anti-MuMTV, anti-gp52, anti-gp36 and anti-p28 sera optimal results were obtained using Tris-buffer containing 1% NNP10, 0.5% DOC and 0.1% SDS (the detergent combination was derived from the procedure used by Van Zaane et al., 1977) as the immunoprecipitation buffer (Fig. 5.6). The presence of detergents in the buffer appeared to be essential; otherwise, the immunoprecipitates obtained always consisted of aggregates of MuMTV proteins, no matter which antiserum was used.

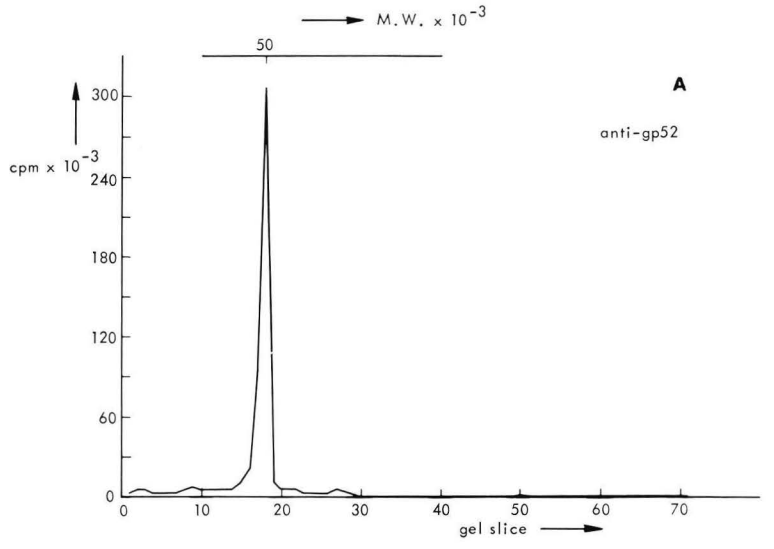
In contrast to anti-gp52, anti-gp36 and anti-p28 sera, which precipitated only those proteins from a radioiodinated MuMTV solution against which they were raised, the anti-p12 serum, in addition to p12, precipitated some other proteins in the presence of the detergents. Considerably better results were obtained when a Tris-buffer containing 0.02% NNP10 and 1 M NaCl was used as the immunoprecipitation buffer. The result which is than obtained is shown in Fig. 5.7. Apparently, in circumstances of high ionic strength, aggregation of p12 with other proteins is circumvented.

Considering the results obtained with anti-gp52, anti-gp36, anti-p28 and anti-p12 sera in the three different immunological assays (see Figs. 5.1-5.7), we conclude that the antisera are monospecific, reacting with the appropriate protein when tested against detergent solubilized MuMTV.

5.2.3 Competition radioimmunoassays

With the availability of purified MuMTV proteins, it was possible to develop specific, highly sensitive radioimmunoassays. Radioiodination of gp52, gp36, p28 and p12 resulted in specific activities ranging from $0.8 - 1.5 \times 10^5$ cpm.ng^{-1} protein. More than 90% of the radioiodinated material was usually TCA-precipitable. Labelling of detergent-disrupted MuMTV (as mentioned above) resulted in specific activities of $1.5 - 6.4 \times 10^4$ cpm.ng^{-1} protein and only 75-80% proved to be TCA-precipitable in such cases.

The stability on storage of the radioiodinated proteins varied: ^{125}I-labelled gp52 was still useful in competition assays for up to 2 weeks after labelling, while gp36 and p28 rapidly decayed. Titers of the different antisera, defined as the input antiserum-dilution which precipitates 50% of the labelled antigen in the precipitation assay varied markedly from 1:200 (anti-p12 serum) to 1:4000 (anti-MuMTV serum, anti-gp52 serum). The titer of the same antiserum varied from



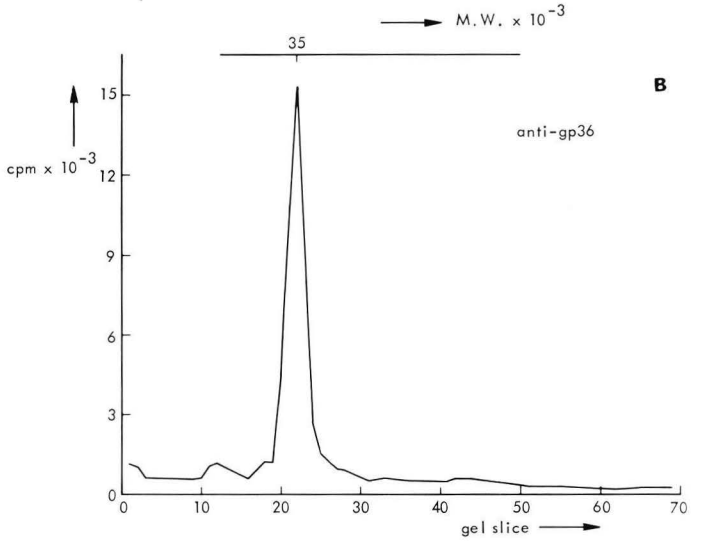


Figure 5.6:

Analysis of immunoprecipitates by SDS-PAGE.
Analysis of the immunoprecipitates obtained by reacting anti-gp52 (A), anti-gp36 (B) and anti-p28 (C), with radioiodinated NNP10-disrupted MuMTV.

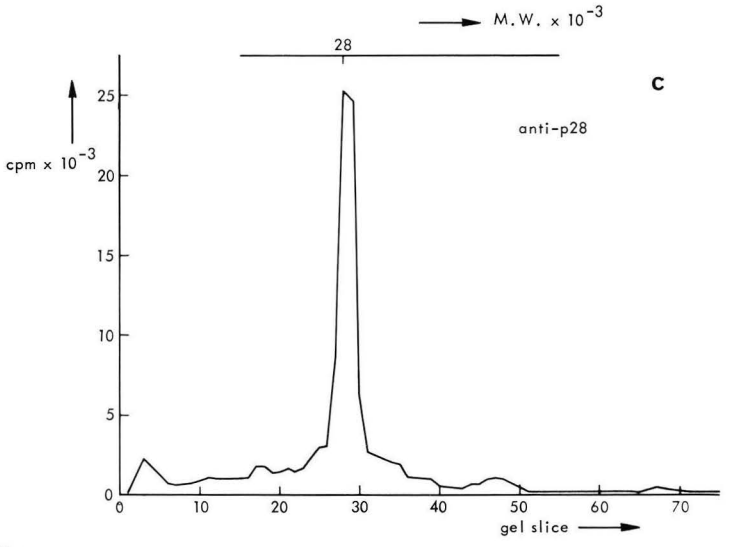


Figure 5.6C:

For legends, see Fig. 5.6.

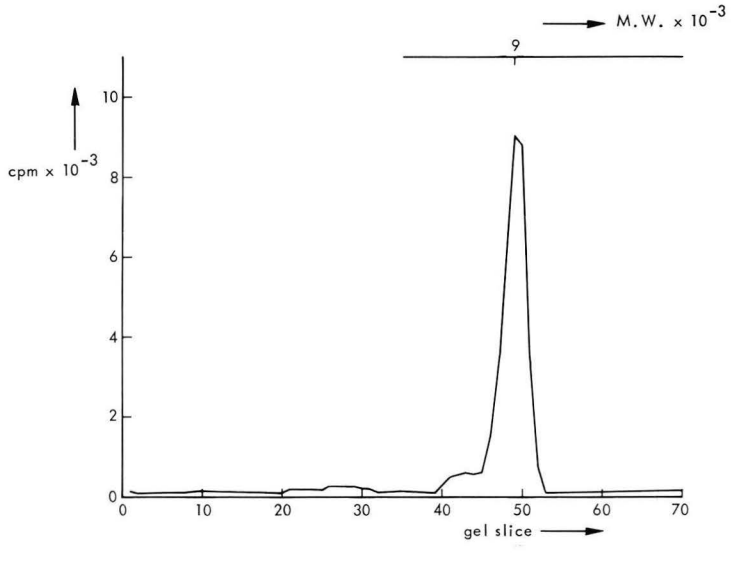


Figure 5.7:

Analysis of immunoprecipitates by SDS-PAGE.
Analysis of the immunoprecipitate obtained by reacting anti-p12 serum with NNP10-disrupted MuMTV in circumstances of high ionic strength (0.02% NNP10, 1 M NaCl).

test to test; this was due to differences in the specific activity of the labelled antigen.

Competition assays were developed for gp52, gp36, p28, p12 and disrupted MuMTV. Each viral protein was tested in the assays developed for the individual MuMTV proteins. Some results are shown in Fig. 5.8. All proteins showed a 90 (p12) to 100% competition when tested in their homologous assays. A blocking of 50% was usually obtained with 0.1-1 ng protein in each assay.

Competition in each assay with the other viral proteins showed that only a 100-fold excess or more resulted in some displacement of the labelled input counts. Testing of C-type oncoviral proteins in competition assays showed similar minor displacements at excess of unrelated protein (Barbacid et al., 1976a). Whether this phenomenon is

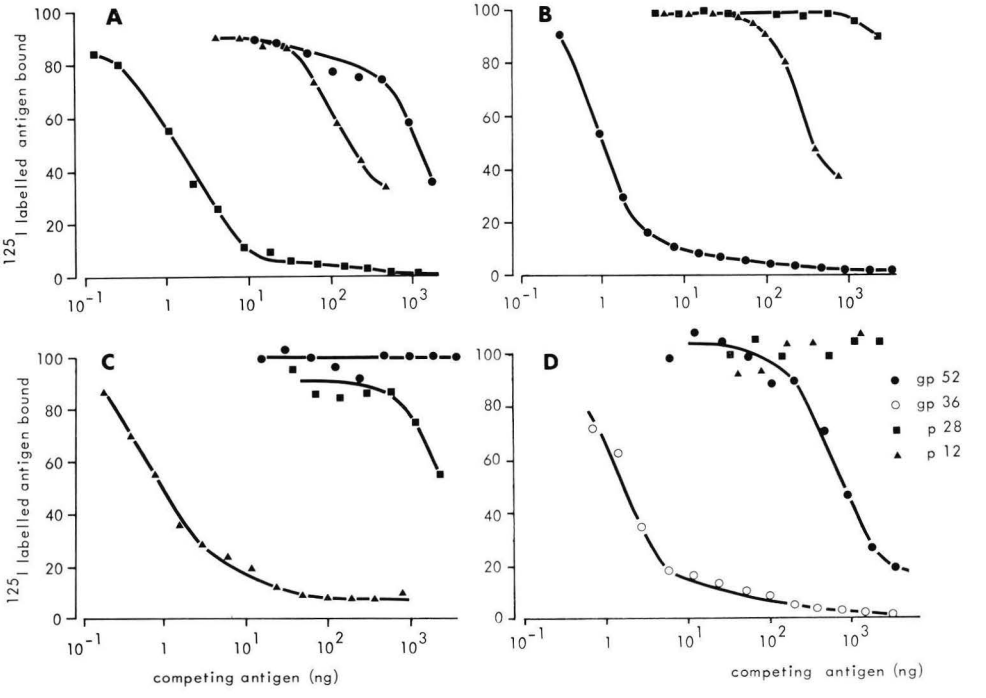


Figure 5.8:

Competition radioimmunoassay.

The capacity of the unlabelled purified MuMTV proteins, to compete with the radioiodinated MuMTV proteins in the respective assays was determined: ^{125}I-p28 vs anti-p28 (A); ^{125}I-gp52 vs anti-gp52 (B); ^{125}I-p12 vs anti-p12 (C); ^{125}I-gp36 vs anti-MuMTV (D). Test antigens were used in the assays at serial twofold dilutions.

due to very minor contaminations of the purified proteins with the other viral proteins to an extent undetectable with other, less sensitive immunological assays or whether the minor displacements are non-specific and due to the presence of excess protein is not clear at present.

Nevertheless, taking also into account the results obtained with the other immunological assays described in this Chapter, the conclusion at this point that the four MuMTV protein fractions gp52, gp36, p28 and p12 are immunologically unrelated to each other is justifiable.

The reliability and applicability of the competition assay for gp52 was tested in a number of experiments. At first, the reliability of the test in assaying crude extracts for the presence of gp52 was determined. An assay in which ^{125}I-labelled, detergent disrupted MuMTV served as antigen and a monovalent anti-gp52 serum as antiserum was used.

Extracts were made from lactating mammary gland tissue from a GR mouse and a WAG/Rij rat in the following way: Tissue was forced through a fine steel mesh and subsequently through nylon gauze. The cells were collected in PBS containing 300 $\mu g.ml^{-1}$ of the protease inhibitor PMSF and washed three times by low speed centrifugation, after which 1% of the nonionic detergent NNP10 was added. The suspension was incubated overnight at 4^{o} C and then subjected to ultrasonication four times at 15 sec. Nondisrupted fragments were removed by centrifugation for 60 min at 160,000 x g (Beckman SW60 rotor, 40,000 $rev.min^{-1}$).

To one half of each extract, a known amount of disrupted MuMTV was added as a control for proteolysis. Five types of samples were tested in the competition assay: 1) disrupted MuMTV (tissue culture derived); 2) GR mouse mammary gland extract; 3) the GR mouse mammary gland extract + MuMTV; 4) WAG-Rij rat mammary gland extract; 5) rat mammary gland extract + MuMTV. All extracts and buffers used contained 300 $\mu g.ml^{-1}$ PMSF. The results are depicted in Fig. 5.9.

Except for the rat mammary gland extract, all samples showed a 100% competition, indicating the presence of gp52 in these extracts, as was expected. From the Figure it can be concluded that the GR mouse mammary gland extract contained about 1.2 μg gp52.mg^{-1} protein, a value approaching those reported for other mouse strains with a high expression of MuMTV in their milk: 2.6 $\mu g.mg^{-1}$ protein for C3H (Noon et al., 1975) and 2.8 $\mu g.mg^{-1}$ protein for RIII (Zangerle et al.,1977). Addition of extra MuMTV proteins to the mouse mammary gland extract results in a shift of the competition curve to the position expected (corresponding to the position of the curve obtained when the MuMTV solution is used as the competing antigen).

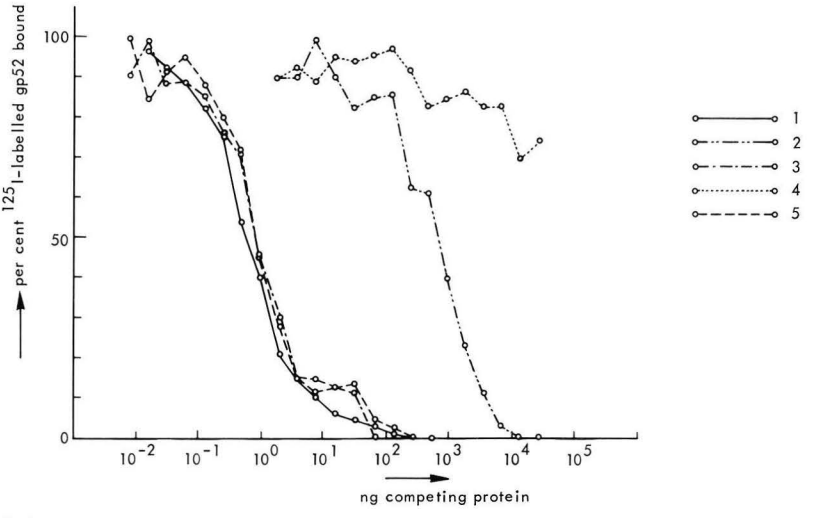


Figure 5.9:

Competition radioimmunoassay.
Anti-gp52 serum was used to precipitate ^{125}I-gp52 from a radioiodinated NNP10-disrupted MuMTV preparation. As competitors were used: NNP10 disrupted MuMTV (1); GR mouse mammary gland extract (2); GR mouse mammary gland extract (as in 2) to which NNP10 disrupted MuMTV was added at a quantity equal to the concentration used in 1 (3); WAG/Rij rat mammary gland extract (4); the rat mammary gland extract used for (4) to which MuMTV was added (5).

Competition with the rat mammary gland extract showed a slight reduction of the percentage ^{125}I-labelled gp52 precipitated at excess protein. This effect is considered to be nonspecific. A similar result was obtained when normal rat milk samples were tested in a competition assay for gp52: competition with the first two samples of the serial twofold dilution series also showed a nonspecific reduction (see Chapter 6). Therefore, when testing protein-rich materials like milk samples and tissue extracts, results obtained with the first two samples of the serial twofold dilution series were not used in plotting the data of the competition assay (see Fig. 6.6).

In a second experiment radioiodinated detergent disrupted tissue culture derived MuMTV (C3H) was used as the antigen and a polyvalent antiserum raised against this same MuMTV (C3H) virus as the antiserum.

Disrupted MuMTV (C3H) and disrupted RIII milk derived MuMTV [MuMTV(RIII)] were compared in a competition experiment. It appeared (Fig. 5.10) that a 100% competition for the binding of ^{125}I-MuMTV(C3H) to anti-MuMTV(C3H) serum was obtained in both cases, but that the slope of the curve resulting from increasing amounts of MuMTV(RIII) was less steep than that of the curve obtained with MuMTV(C3H). These

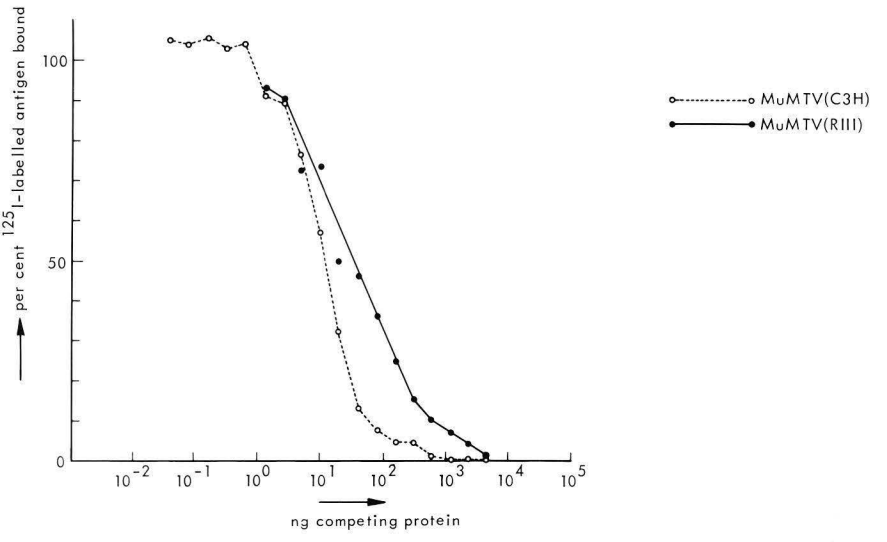


Figure 5.10:

Competition radioimmunoassay.
Radioiodinated detergent disrupted MuMTV(C3H) was precipitated with rabbit anti-MuMTV (C3H) serum. As competitors were used detergent disrupted MuMTV(C3H) and MuMTV(RIII).

results indicate that, although MuMTV proteins from both sources can compete for all antibodies binding to the ^{125}I-labelled antigens (reflected in the 100% displacement), the affinity of the antibodies is reduced in the case of the MuMTV(RIII) proteins, an effect which is due to type-specific differences between the respective proteins (Hunter, 1979). These results confirm the findings of Teramoto et al., who demonstrated in an analogous way type-specific determinants to be present on the viral proteins gp52, gp36 and p28 of MuMTV(C3H) and MuMTV(RIII) (Teramoto et al., 1977b; Teramoto and Schlom, 1978, 1979). These results demonstrate the suitability of the competition radioimmunoassay in distinguishing closely related viral proteins on the basis of type-specific differences in antigenic determinants.

As described in Chapter 3, gp52 can be isolated from detergent solubilized MuMTV in two different forms: as "free" gp52 and as a tightly bound gp52-gp36 complex. These glycoprotein entities show a different affinity for Con A-Sepharose. The question arose as to whether free gp52 was identical to complex bound gp52. A way to answer this question is to test both gp52 forms in a competition radioimmuno-

assay for gp52. Therefore, a homologous assay in which free MuMTV(C3H)-gp52 was used as ^{125}I-labelled antigen and anti-MuMTV(C3H)-gp52 serum as the precipitating antiserum was carried out. In one assay, the following protein solutions were tested as competing antigen: 1) free gp52, isolated from detergent-disrupted MuMTV(C3H); 2) gp52-gp36 complex isolated from detergent-disrupted MuMTV(C3H); 3) gp52 isolated from the gp52-gp36 complex by gel filtration in 0.5% DOC (Chapter 3); 4) gp52 isolated from detergent disrupted RIII milk derived MuMTV. The results obtained are shown in Fig. 5.11. Competition with the protein samples 1-3 resulted in so-called reactions of identity: All three samples showed competition up to 100% and the slopes of the resulting curves are identical. Competition with MuMTV(RIII)-gp52 suggested type-specific differences between gp52 isolated from MuMTV(RIII) and MuMTV(C3H), as reflected in a shallower slope of the competition curve (see also Fig. 5.10).

Apparently, in terms of competition radioimmunoassays, no differences are detectable between free gp52 and complex-bound gp52 of the same virus.

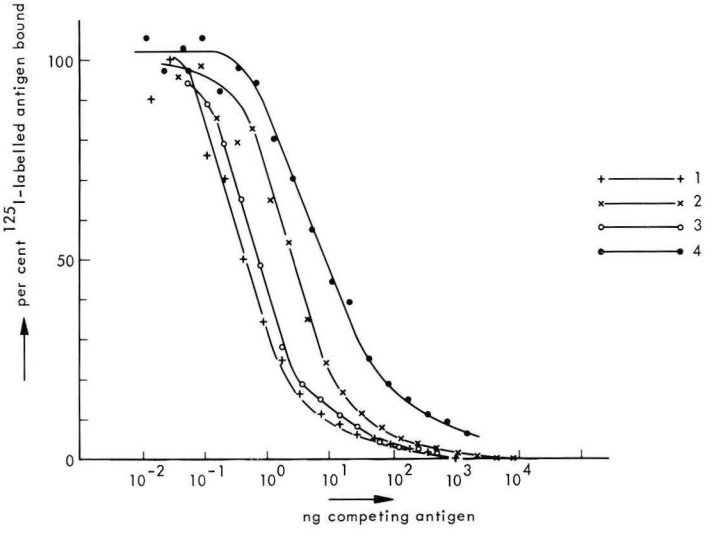


Figure 5.11:

Competition radioimmunoassay.
Radioiodinated gp52, purified from a MuMTV(C3H) preparation was precipitated with rabbit-anti-gp52 from MuMTV(C3H) serum. As competitors were used: gp52 from MuMTV (C3H) (1); gp52-gp36 complex purified from MuMTV(C3H) (2); gp52 purified from the complex used for 2 (3); gp52 from MuMTV(RIII) (4).

5.2.4 Examination on the cell surface expression of MuMTV-proteins

5.2.4.1 Immunofluorescence studies

After we had demonstrated both the immunogenic uniqueness of the isolated viral proteins with respect to each other and the monospecificity of the different antisera raised against these proteins, we investigated the expression of MuMTV proteins on the cell surface of murine mammary tumour cells.

Before use in the cytoplasmic immunofluorescence assay or the membrane immunofluorescence assay, antisera were routinely absorbed with FCS and NMS. Unabsorbed antisera usually reacted in both assays with every cell type tested, irrespective of the virological status of the cell. The applied absorption procedure removed these nonspecific reactivities.

Virus-specific cytoplasmic immunofluorescence was consistently found with all four antisera in two MuMTV-producing C3H mouse mammary tumour cell lines: Mm5mt/cl and C3HMT/clll (Fig. 5.12). No reaction was observed with the mouse mammary tumour cell line EMT-6 (which does not produce MuMTV) or with BALB/3T3 cells infected with R-MuLV. Positive reactions were also obtained when using the leukaemia lines L1210 and GRSL18 as target cells. These lines are known to produce MuMTV antigens. Remarkably, in these cell lines, immunofluorescence with anti-p28 serum was mainly confined to a few large bright dots inside the cell (Fig. 5.12).

In the membrane immunofluorescence tests on all four MuMTV antigen producing cell lines, a very faint reaction was found with the anti-p28 serum in less than 20% of the cells. A completely negative reaction was found with anti-p12 serum and with anti-gp36 serum. A strong positive reaction was found only with anti-gp52 serum in all cases tested (Fig. 5.12). The latter antiserum reacted with more than 90% of the cells when the MuMTV antigen production was stimulated by addition of the synthetic glucocorticoid dexamethason to the culture medium. No reaction was found with any of these antisera with the MuMTV-free cell lines EMT-6 and BALB/3T3 infected with R-MuLV.

5.2.4.2 Humoral cytotoxicity assay

In the complement dependent humoral cytotoxicity assay the antisera to gp36, p28 and p12 showed no reactivity against the mouse mammary tumour cell lines Mm5mt/cl and C3HMT/clll. Only anti-gp52 serum

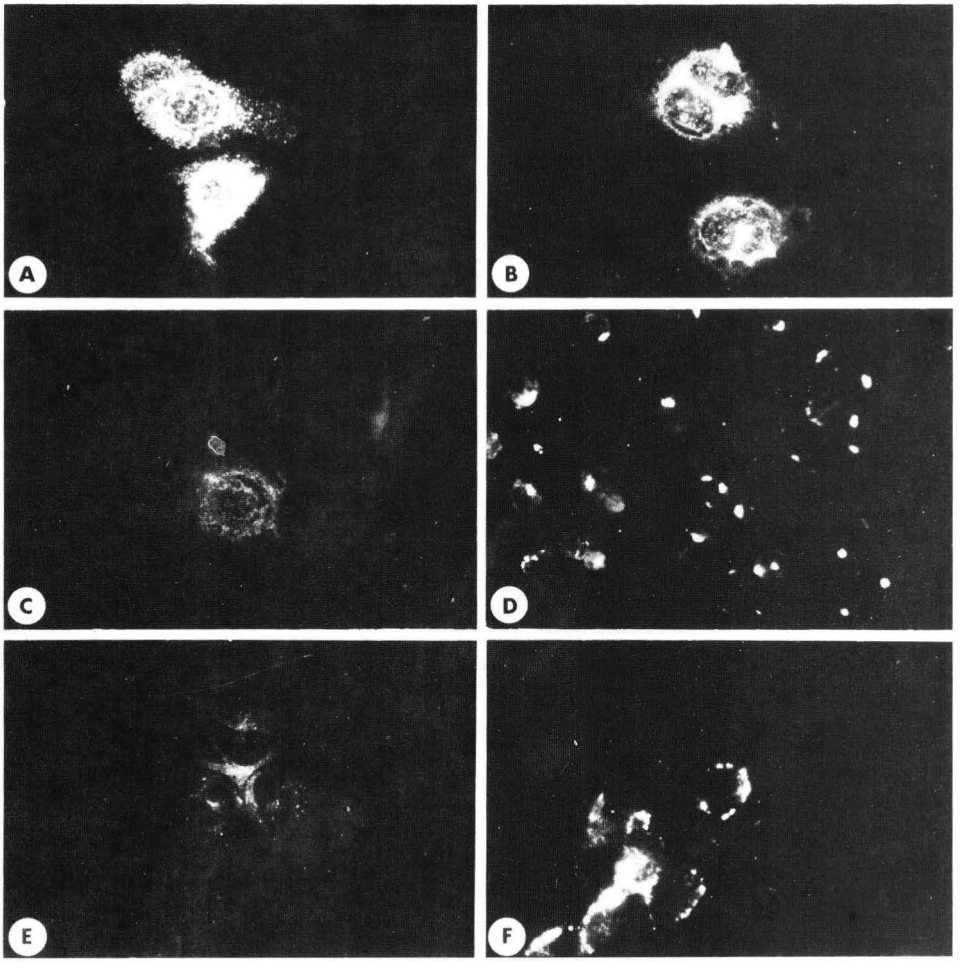


Figure 5.12:

Immunofluorescence with different rabbit antisera to purified MuMTV proteins.
A-E: cytoplasmic immunofluorescence, F: membrane immuno-fluorescence; (A) anti-p12 serum to C3HMT/c111 cells; (B) anti-p28 serum to C3HMT/c111 cells; (C) anti-gp52 serum to C3HMT/c111 cells; (D) anti-p28 serum to L1210 cells; (E) anti-gp52 serum to GRSL18 cells; (F) anti-gp52 to Mm5mt/cl cells.

and polyvalent anti-MuMTV serum showed strong reactions with these cells, during which 80-100% of the cells were usually lysed at a dilution of 1:2 or 1:4. Fig. 5.13 shows the results of an experiment in which anti-MuMTV serum, anti-gp52 serum and anti-p28 serum were tested against C3HMT/c111 cells. Both the anti-MuMTV and anti-gp52 sera failed to show reactions with BALB/3T3 cells infected with R-MuLV.

In addition, anti-gp52, anti-p28 and anti-p12 sera were tested on L1210 and on GRSL18 cells. Only anti-gp52 serum showed a strong reactivity towards these cells. Our results are in disagreement with those Schochetman et al. (1978a) obtained with their anti-gp36 serum. They did find a positive reaction with Mm5mt/cl cells in both membrane immunofluorescence and in ^{51}Cr-release humoral cytotoxicity assays. As

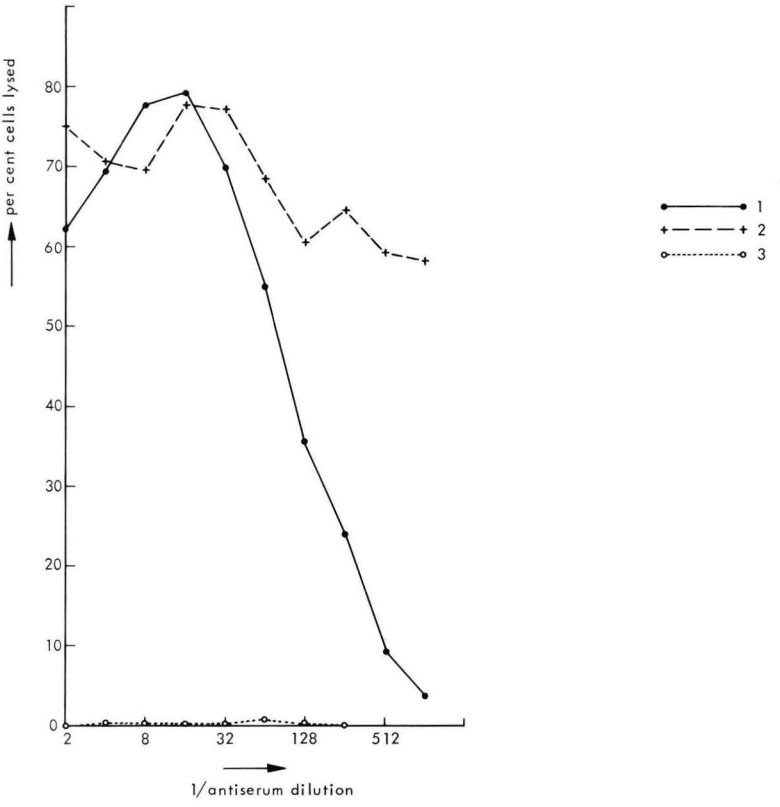


Figure 5.13:

Humoral complement-dependent cytotoxicity assay.
Anti-MuMTV serum (1), anti-gp52 serum (2) and anti-p28 serum (3) were tested against C3HMT/clll cells.

we did, they trypsinized their cells and the assays were performed after one night of incubation (16 hr) at 37° C. When cells were allowed to regenerate for 48 hr instead of the usual 16 hr incubation, they found reactivity only with anti-gp52 serum in both assays. They argued that the gp36 molecule was located within the cellular membrane. Trypsinization should affect the cellular membrane structure in such a way that complete regeneration of the membrane is achieved only after 36-48 hr incubation at 37° C.

In one experiment, we tested the C3HMT/clll cells in the cytotoxicity assay against the anti-gp36 serum at different times after trypsinization. No lysis was detected 1.5, 3 or 18 hr after trypsinization. Anti-gp52 serum, which was used as a positive control in the same experiment, gave maximum percentages of cytolysis of 18, 29 and 92% at the different times after trypsinization.

In addition, we tested our gp36 serum on Mm5mt/cl cells, the same target cell Schochetman and co-workers used in their experiments. Again, no reaction was found 16 hr after trypsinization.

The difference in results obtained by Schochetman et al. and us is probably due to differences between the anti-gp36 sera used. Schochetman et al. (1978a) demonstrated a reaction of their anti-gp36 serum with a protein of 65,000 daltons in an immunoprecipitation assay. This uncharacterized protein appeared to be present on numerous cells of different origin. The positive reaction they obtained with their anti-gp36 serum to Mm5mt/cl cells was probably due to a reaction with this 65,000 dalton protein.

5.3 DISCUSSION

Sera raised in rabbits against the MuMTV structural proteins gp52, gp36, p28 and the p12 fraction showed no cross-reactivity when tested in either the double immunodiffusion test, the immunoelectrophoresis assay or the immunoprecipitation test.

With the competition radioimmunoassays developed for each isolated viral protein, it was shown that the proteins are immunogenically unrelated. The competition assay developed for gp52 appeared to be suitable for testing crude mammary tumour cell extracts and milk samples for the presence of gp52; no interference of possible contaminating proteolytic activity with the results obtained was observed. The slight displacement of the competition curve, observed when the first two samples of twofold dilution series of the protein rich samples were tested was considered to be unreliable.

It was shown that the competition radioimmunoassay is suitable for distinguishing MuMTV's originating from different mouse strains. In an analogous way, Teramoto et al. could not only distinguish MuMTV's from different mouse strains (GR, RIII and C3H) on the basis of type specific differences among the viral proteins gp52, gp36 and p28 (Teramoto et al., 1977a; Teramoto and Schlom, 1978; 1979) but they also succeeded in making distinctions between the gp52 molecules of the high oncogenic milk-transmitted exogenous MuMTV and the low oncogenic endogenous MuMTV of the same C3H mouse strain (Teramoto et al., 1977c).

A reaction of immunological identity was obtained when so-called free gp52 and gp52-gp36 complex derived gp52 were compared with respect to their competing capacity in a competition assay developed for free gp52.

All antisera raised against the purified MuMTV antigens gave positive reactions in the cytoplasmic immunofluorescence assay with four

MuMTV producing cell lines. The reactivity proved to be virus-specific. No reaction was observed with BALB/3T3 cells chronically infected with R-MuLV virus or with the MuMTV-free mouse mammary tumour cell line EMT-6.

The large fluorescence dots produced in the mouse leukaemia cell lines GRSL18 and L1210, by the anti-p28 serum are probably due to the large clusters of A-type particles which were previously observed in these cell lines (Radzikowski et al., 1972; Calafat et al., 1974). As pointed out in Chapter 1, these intracytoplasmic A-type particles share antigenic determinants with the core of mature B-type particles.

The results obtained with the membrane immunofluorescence assay and the humoral complement dependent cytotoxicity assay proved only gp52 to be clearly present on the cell surface of MuMTV producing mouse mammary tumour cells. No reaction with either the anti-gp36 or the anti-p12 serum was observed. Although a faint reaction was observed with anti-p28 serum in the membrane immunofluorescence assay, a completely negative result was obtained with this antiserum in the humoral cytotoxicity assay.

The expression of gp52 on various mouse mammary tumour cells was also demonstrated by Holder et al. (1976) by means of immunoelectronmicroscopy and by Yang et al. (1977) using lactoperoxidase mediated cell surface labelling of cultured mammary tumour cells. Subsequent analysis of solubilized mammary tumour cells immunoprecipitated with a polyvalent anti-MuMTV serum by SDS-PAGE, revealed only gp52 to be present in the immunoprecipitate, indicating the expression of only gp52 on the cell surface. With an experimental set-up similar to that used by the latter group, Schochetman et al. (1978b) also found only gp52 to be expressed on cultured mouse mammary tumour cells, using antisera to the MuMTV proteins gp52, gp36, p28 (called p27 by them) and p12 (p10 in their nomenclature) respectively. A limitation of the experimental procedure in which lactoperoxidase radioiodinated cell surface proteins are immunoprecipitated and subsequently analysed by SDS-PAGE is the need for tyrosine residues of those particular membrane proteins to be exposed on the cell surface. Otherwise, membrane proteins will not become radioiodinated and consequently will not be detected as such. Additional assays such as the humoral cytotoxicity assay and the membrane immunofluorescence assay are needed to complete studies on cell surface expression of particular proteins. For instance, Schneider and Hunsmann (1978), studying the expression of viral proteins on the surface of MuLV producing cells, detected the expression of the C-type viral protein p12 by means of the humoral cytotoxicity assay. By application of the lactoperoxidase mediated cell surface

labelling technique, however, this protein appeared not to become radioiodinated.

The faint reaction obtained with anti-p28 serum in the membrane immunofluorescence assay could be interpreted as meaning that p28 or its precursor protein is an integral part of the cell surface. However, this is contradicted by the negative results repeatedly obtained with the anti-p28 serum in the humoral cytotoxicity assay. In addition we applied the method used by Yang et al. and Schochetman et al. as discussed above and, in this case, we were also unable to obtain any evidence for a surface location of p28 related protein(s) (results not shown). Therefore, the expression of p28 (or its precursor) on the cell surface of mouse mammary tumour cells is unlikely.

CHAPTER VI

SEARCH FOR THE MAM-GENE PRODUCT

6.1 INTRODUCTION

One way to explain the capacity of MuMTV to induce neoplastic transformation in the mammary gland is to assume the existence of a MuMTV-specific onc-gene, the so-called "mam-gene" (Hilgers and Bentvelzen, 1978). As pointed out in Chapter 1, one approach to the search of the product of a putative onc-gene of RNA-tumour viruses in general is to look for nonvirion virus associated tumour specific cell surface antigens (TCSAs).

Research on the MuMTV transforming mechanism is hampered by the lack of an in vitro transformation assay for MuMTV. Consequently, studies on temperature sensitive MuMTV mutants or mutants with deletions in the viral onc-gene (the type of studies by which the viral src-gene was defined in the ASV system) are not possible. Furthermore, tumour induction by MuMTV in heterologous animals has never been reported. This means that searching for a mam-gene product with antisera obtained from nonmouse species bearing MuMTV-induced tumours is not yet possible at the moment [with such a method Erikson and co-workers (see Chapter 1) identified the src-gene product in the ASV-system].

The possibility remains to investigate the expression of TCSAs on MuMTV induced mouse mammary tumour cells. Characterization of such newly expressed antigens will increase insight into MuMTV-induced mammary carcinogenesis and may eventually lead to the identification of the putative MuMTV-onc-gene product.

Transplantation studies have revealed the expression of tumour-specific antigens on MuMTV-induced mouse mammary tumours. Studies on C3Hf-mice which are freed from exogenous MuMTV by foster nursing demonstrated the involvement of viral antigens in transplantation resistance against tumours induced by exogenous MuMTV. As a consequence of neonatal infection with MuMTV, C3H mice are thought to be relatively tolerant to MuMTV antigens; this explains the tolerance of these mice to transplanted syngeneic MuMTV-induced mammary tumours (Lavrin et al., 1966; Morton, 1969; Blair, 1971). Transplantation resistance in C3H mice was developed only against mammary tumours bearing unique,

non-cross-reacting antigens (Vaage, 1968; 1978; Vaage and Medina, 1974). In contrast, mice of the subline C3Hf develop transplantation resistance against all MuMTV-induced C3H mammary tumours after immunization, indicating strong reactivity against viral antigens (Vaage, 1968; 1978; Vaage and Medina, 1974). The expression of cross-reacting and non-cross-reacting (unique) tumour specific antigens on C3H mammary tumours was confirmed by *in vitro* studies in which the response of C3H and C3Hf mice to syngeneic transplantable mammary tumours was characterized by means of cellular cytotoxicity assays (Stutman, 1976; Tagliabue et al., 1979). The latter group suggested the unique antigens to be identical to embryonic antigens, as lymphocytes of mammary tumour bearing C3H and C3Hf mice were reactive to 3 M KCl extracts of various transplantable C3H mammary tumours as well as to an extract of MuMTV negative C3H embryonic tissue.

Cross-reacting antigens were not only demonstrated on C3H mammary tumours but also on MuMTV induced BALB/cfC3H tumours (Dezfulian et al., 1967) and on spontaneous A.SW tumours (Kuzumaki and Klein, 1979).

Blair (1970) found no evidence for the expression of embryonic antigens on BALB/cfC3H mammary tumours: immunization with embryonic extracts had no effect on the development of mammary tumours in the test females.

Slovin et al. (1977) tested the specificity of antibodies, present in the glomerular deposits of mammary tumour bearing RIII mice, in a cytoplasmic immunofluorescence assay against autologous mammary tumour cells and against normal MuMTV-antigen positive mammary gland cells. The antibodies reacted only with the mammary tumour cells. Whether the relevant antigen represented a cross-reacting or a unique tumour specific antigen and whether the antigen was expressed on the tumour cell surface was not made clear in this report.

A study in which we investigated whether, in addition to the viral protein gp52 (see Chapter 5) nonvirion TCSA's are expressed on MuMTV-induced mammary tumour cells, is described in this Chapter. Sera of BALB/c and C3Hf mice hyperimmunized with irradiated syngeneic mammary tumour cells, BALB/c mice bearing transplanted BALB/cfC3H mammary tumours and rabbits immunized with mouse mammary tumour cells were tested on mouse mammary tumour cells in both a membrane immunofluorescence assay and a complement dependent humoral cytotoxicity assay. Reactivity was characterized by means of the radioimmunoprecipitation assay. The ultimate aim of the experiments was to determine whether any tumour specific reactivity was left after absorption of positive sera with a purified MuMTV preparation.

In addition, we investigated whether MuMTV has the capacity to induce mammary tumours in animals other than the mouse. Sera of MuMTV-

induced tumour bearing rabbits or rats would offer us an alternative approach in the search for nonvirion, cross-reactive mouse mammary tumour specific antigens, the type of antigen which is a candidate for the putative mam-gene product.

6.2 RESULTS

The different heterologous and homologous sera raised against MuMTV-induced mouse mammary tumour cells were all tested according to the same principle. Sera were tested first in the complement dependent humoral cytotoxicity assay for reactivity against the mouse mammary tumour cell line, C3HMT/c111. If there was a positive reaction, sera were occasionally tested in the membrane immunofluorescence assay against the same target cell. Reactivity against MuMTV structural proteins was characterized by means of the radioimmunoprecipitation assay. Positive sera were subsequently incubated with a purified MuMTV preparation in order to absorb the reactivity directed against the viral proteins. Absorbed antisera were then tested in both the humoral cytotoxicity assay and the membrane immunofluorescence assay to determine whether any residual reactivity against non-virion, tumour specific cell surface antigens was left.

Antisera directed against mouse mammary tumour cells were obtained in three ways. In method one, a group of 39 ten-week-old female BALB/c mice were inoculated intraperitoneally with 10^7 irradiated (20 Gy) mouse mammary tumour cells 7 times at two week intervals. Fresh cells for each inoculum were prepared from transplanted BALB/cfC3H mammary tumours. In addition a group of 48 ten-week-old C3Hf female mice received 6 immunizations with irradiated cells from the mammary tumour cell culture C3HMT/c111 following the same immunization schedule as used for the group of BALB/c mice. Ten days after the last immunization, mice were bled from the retro-orbital plexus. Sera from both groups of mice were pooled and stored at -20^{o}C.

In method two, sera were collected from 24 BALB/c mice carrying transplanted BALB/cfC3H mammary tumours. In this case, all sera were tested individually.

In method three, rabbits were immunized with mouse mammary tumour cells. Of 10^8 tumour cells in 0.5-1.0 ml PBS emulsified with 0.5 ml Freunds complete adjuvant, one-half was inoculated intradermally and the other half subcutaneously at multiple sites. Inoculations were repeated at 3 to 4 week intervals. After the second inoculation, Freunds complete adjuvant was omitted from the inoculum. Blood was collected

from an ear vein after 3 to 4 inoculations. Sera were then tested in the humoral cytotoxicity assay against the inoculated cell. Usually, a 90-100% cell lysis was obtained. When the percentage of killed cells decreased as a consequence of a decrease in the titer of the antiserum, the entire immunization schedule was repeated.

6.2.1 Sera of mice hyperimmunized with mouse mammary tumour cells

The pooled BALB/c and C3Hf sera were tested in a complement dependent humoral cytotoxicity assay on different cell types. The results obtained are summarized in Table 6.1. Both pools reacted strongly with the C3HMT/clll cells and to a lesser extent with the Mm5mt/cl mouse mammary tumour cell line. The C3Hf antiserum also showed a clear reaction with cultured BALB/cfC3H mammary tumour cells. A sharp decrease in reactivity was observed upon storage of both serum pools at -20° C; for this reason, we were not able to test the BALB/c antiserum against BALB/cfC3H tumour cells.

Values for maximum percentage ^{51}Cr-release, as given in Table 6.1, were obtained only when using antisera at dilutions of 1:32 and 1:64. Antisera at lower or higher dilutions were less cytotoxic or not cytotoxic at all. For instance, when either the C3Hf or the BALB/c antiserum was tested at a 1:2 dilution in PBS, no reaction was detected in the assay.

Reactivities of the C3Hf and BALB/c antisera to embryonic fibroblasts and normal mammary epithelial cells of both strains was negligible, as was the reactivity against BALB/3T3 cells infected with R-MuLV. Apparently, the established immune reactivity of the two sera to the mouse mammary tumour cell lines was tumour specific.

In the membrane immunofluorescence assay, both sera also showed a clear reaction with C3HMT/clll cells, as depicted in Fig. 6.1, in which the reaction of the C3Hf antiserum and the BALB/c antiserum with this cell line is demonstrated.

To characterize the specificity of the two antiserum pools, a radioiodinated NNP10 extract of transplanted BALB/cfC3H mammary tumour cells was immunoprecipitated with both sera. This tumour cell extract was obtained by incubating 10^7 cells per ml PBS containing 1% NPP10, 0.02% sodium azide and 0.1 mM PMSF for 16 hr at 4° C. After centrifugation for 60 min at 100,000 x g, the supernatant was dialyzed against PBS, 0.02% NNP10, 0.02% sodium azide and 0.1 mM PMSF at 4° C for 16 hr. Radioiodination of the resulting supernatant was performed as described in Chapter 2. The results of the radioimmuno precipitation are

Table 6.1

HUMORAL CYTOTOXICITY ASSAY

Immune serum from:	Cell type: Mammary tumour			Fibroblast		Mammary gland		RLV-infected
	C3HMT/c111	BALB/cfC3H	Mm5mt/cl	BALB/c	C3Hf	BALB/c	C3Hf	BALB/3T3
BALB/c	85*	NT**	12	1	3	4	6	0
C3Hf	92	41	24	3	6	1	6	0

* Maximum per cent ^{51}Cr-release
**Not tested
BALB/c and C3Hf hyperimmune sera raised against syngeneic mammary tumour cells were tested in a ^{51}Cr-release assay on different cell types. Rabbit serum was used as a source of complement. All tests were done in duplicate.

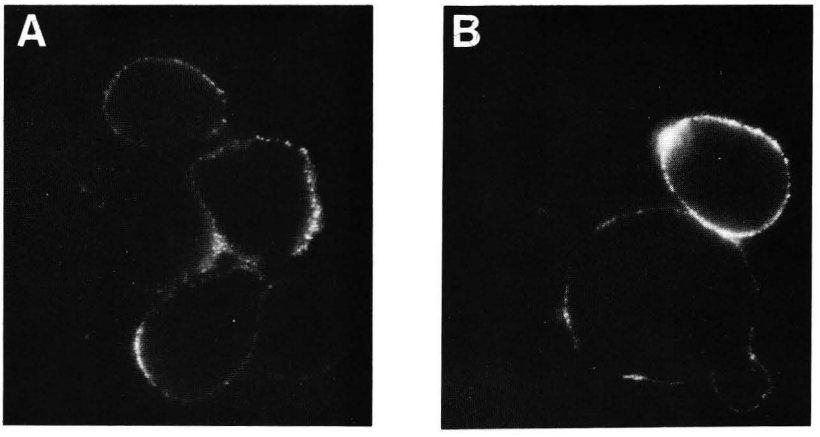


Figure 6.1:
Membrane immunofluorescence assay.
C3HMT/clll cells were used as target cells and the C3Hf (A) and BALB/c (B) hyper-immune sera as test sera, respectively.

shown in Fig. 6.2. The BALB/c antiserum reacted mainly with two proteins migrating at the positions of the two major MuMTV proteins gp52 and p28 (Fig. 6.2.A). The C3Hf antiserum reacted mainly with a protein migrating at the position of MuMTV gp52 (Fig. 6.2.B).

The results of the immunoprecipitation of radioiodinated detergent-disrupted MuMTV with the two antisera are shown in Fig. 6.3. The BALB/c antiserum precipitated only gp52 and p28 (Fig. 6.3.A), while gp36 as well as gp52 and p28 were precipitated by the C3Hf antiserum (Fig. 6.3.B). The ^{125}I MuMTV preparation used for the immunoprecipitation with the BALB/c serum contained, in contrast to the normal situation, far less gp52 than p28 as appeared by analysis by SDS-PAGE (not shown); this probably explains why the BALB/c serum precipitates a relative excess of p28.

The results obtained indicated that the tumour specific reactivity of both sera was directed mainly against MuMTV structural proteins. This was confirmed by the results obtained in the humoral cytotoxicity assay and in the membrane immunofluorescence assay with both sera after absorption with purified tissue culture derived MuMTV. Fig. 6.4 shows the effect of absorption of each antiserum with increasing amounts of MuMTV on the reactivity in the humoral cytotoxicity assay to C3HMT/clll cells. The absorption was performed as follows: Pelleted virus was resuspended in Dulbecco's modified minimal essential medium supplemented with 10% FCS. Serial twofold dilutions of this virus suspension were made. Equal volumes of an antiserum dilution were then added, giving a final antiserum dilution at which optimum cytotoxic activity against C3HMT/clll cells was previously obtained. After an

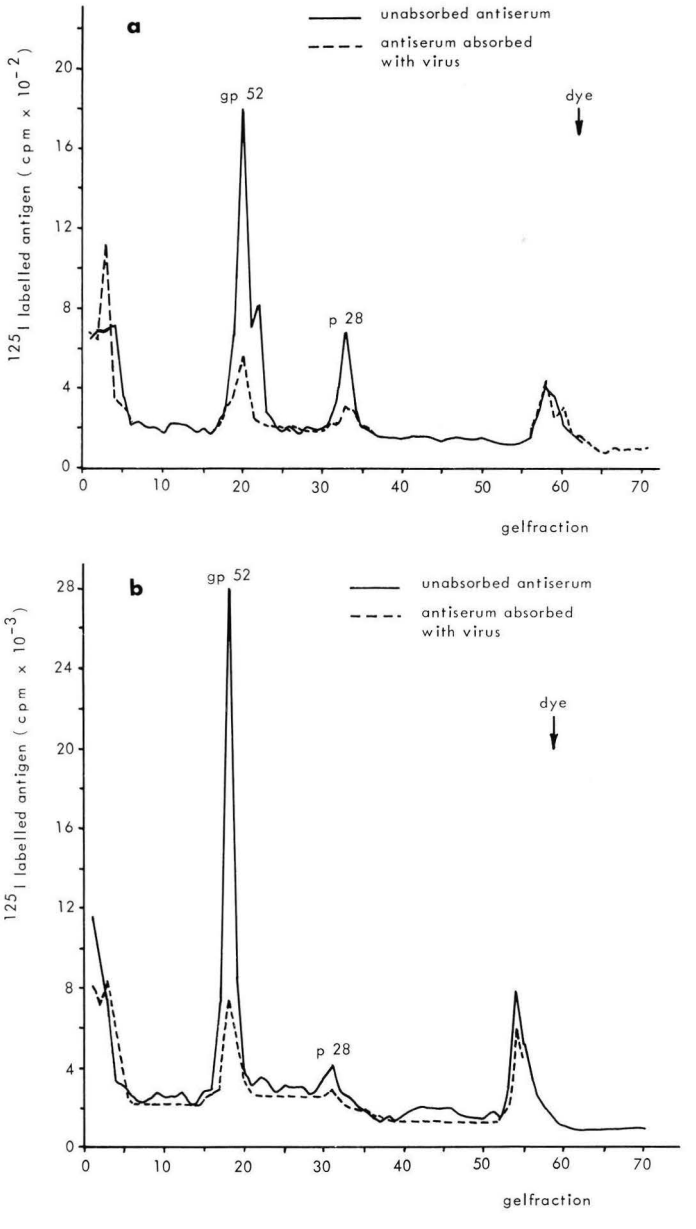


Figure 6.2:

Immunoprecipitation of a radioiodinated mammary tumour cell extract.
A radioiodinated detergent extract of a transplanted BALB/cfC3H mammary tumour was immunoprecipitated with the BALB/c hyperimmune serum (A) and the C3Hf hyperimmune serum (B). Precipitates were analyzed on SDS-PAGE. After electrophoresis, gels were sliced and fractions were counted in a gamma counter. Dotted lines represent the results which were obtained with the same sera absorbed with MuMTV.

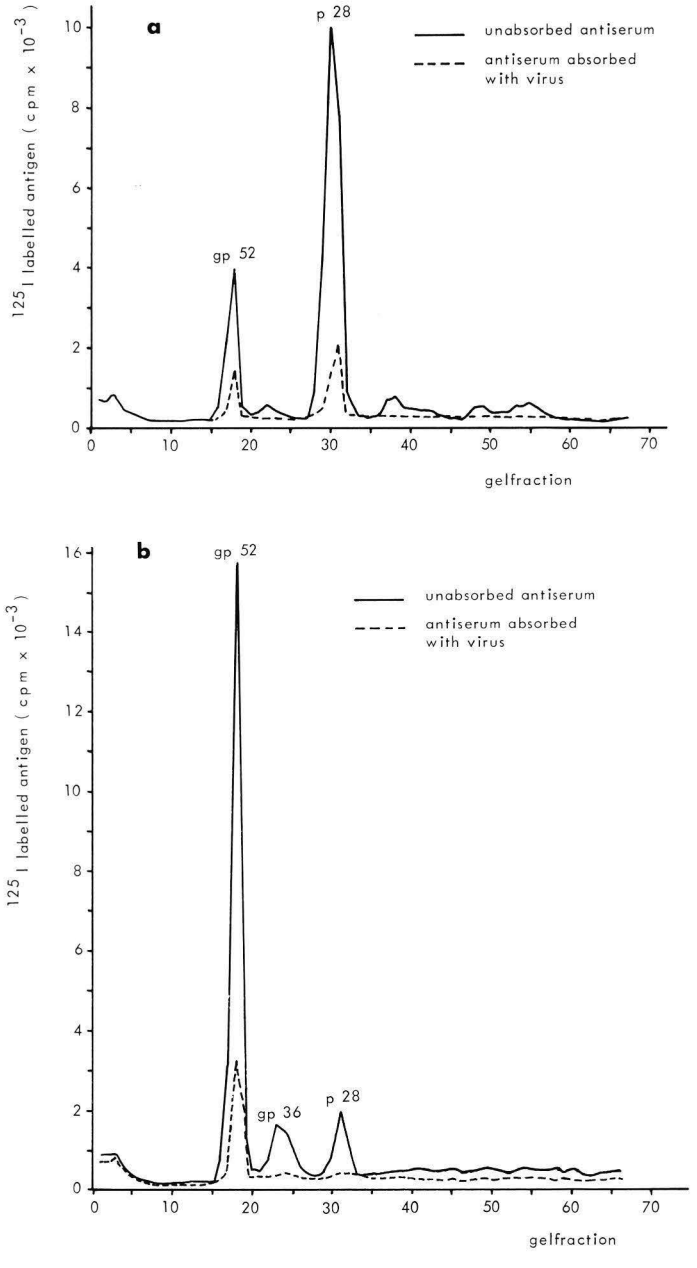


Figure 6.3:

Assaying of mouse hyperimmune sera by immunoprecipitation.
A radioiodinated, detergent-disrupted preparation of MuMTV was immunoprecipitated with the BALB/c hyperimmune serum (A) and the C3Hf hyperimmune serum (B), respectively. For further details, see legend to Fig. 6.2.

incubation period of 30 min at room temperature, sera were either tested directly or stored at -20° C. In both cases, it appeared that the anti-C3HMT/clll reactivity of the sera could be completely abolished through the absorption procedure. Although the group of BALB/c mice received one more inoculum than did the C3Hf strain, the serum pool of the latter group had a considerably higher titre in the cytotoxicity assay (Fig. 6.4). Samples of both sera (which no longer reacted in the humoral cytotoxicity assay against C3HMT/clll cells as a consequence of the absorption with MuMTV) also gave completely negative results when tested in the membrane immunofluorescence assay against the same target cells. The absorption procedure also resulted in a drastic reduction in the amount of proteins immunoprecipitated from the tumour cell extracts as well as from the disrupted virus preparation (Figs. 6.2 and 6.3). This indicates that the proteins which are immunoprecipitated by the respective antisera from the radioiodinated tumour cell extract indeed represent the major MuMTV structural proteins gp52 and p28.

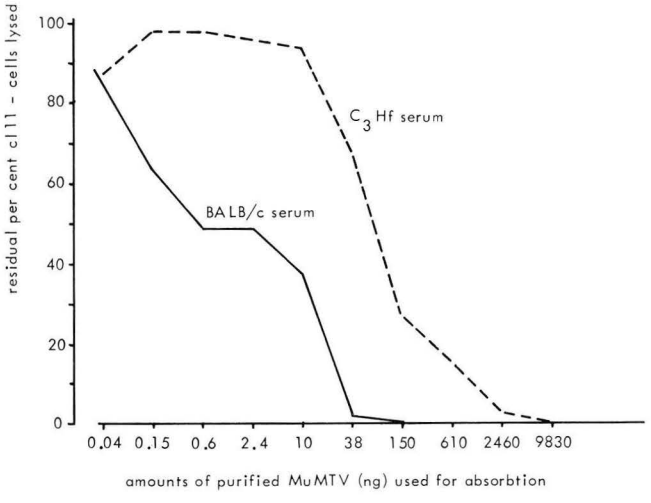


Figure 6.4:

Complement dependent humoral cytotoxicity assays.
The BALB/c and C3Hf hyperimmune sera, absorbed with increasing amounts of purified MuMTV were tested on C3HMT/clll cells. [The MuMTV preparation used for absorption, showed a distinct reaction with rabbit antiMuMTV-p28 serum and rabbit anti-MuMTV-gp52 serum when tested in the double immunodiffusion test. This indicated that the virus was at least partly disrupted]. In both cases, the antiserum dilution at which the maximum percentage of ^{51}Cr-release was previously determined was used in the absorption test.

6.2.2 Sera of mice bearing transplanted mammary tumours

Sera of 25 BALB/c mice carrying transplanted BALB/cfC3H mammary tumours varying in weight between 1-3 grams were tested individually in both the humoral cytotoxicity assay and the membrane immunofluorescence assay in the same way as the above-discussed hyperimmune sera. The results obtained were in agreement with those obtained with the hyperimmune sera. All sera tested reacted with C3HMT/clll cells in the humoral cytotoxicity assay. In no case was cell lysis obtained with a 1:2 serum dilution. The sera exhibited maximum cytotoxic activity at 1:8 - 1:32 dilutions. Values for maximum percentage cell lysis varied from 30-100 per cent among the different sera. In addition, 8 sera were tested on cultured normal BALB/c mammary gland cells. No cytotoxic reaction was observed.

Eight sera were also tested in the membrane immunofluorescence assay. Positive reactions against C3HMT/clll cells were obtained with all sera. The same sera reacted negatively when tested against normal BALB/c mammary gland cells and BALB/3T3 cells chronically infected with R-MuLV.

The mammary tumour specific reactivity could be absorbed with MuMTV in all 25 cases. Absorbed sera no longer showed reaction with C3HMT/clll cells in either the cytotoxicity assay or the membrane immunofluorescence assay.

6.2.3 Sera of rabbits immunized with mouse mammary tumour cells

Rabbit antisera were raised against transplanted BALB/cfC3H mammary tumour cells and against C3HMT/clll cells. The first antiserum was tested in the humoral cytotoxicity assay against C3HMT/clll cells, BALB/3T3 cells chronically infected with R-MuLV(BALB/3T3-RLV) and normal BALB/c mammary gland cells. The serum was subjected to different absorption procedures and the effect of these absorptions on the cytotoxic reactivity of the serum against the different cell types is given in Table 6.2.

The absorptions were performed as follows: For spleen and kidney cells, 20 BALB/c kidneys and 10 BALB/c spleens were finely minced and forced through nylon gauze. Cells were then washed three times in PBS, after which one-half of the cell pellet was added to 4 ml of serum. Absorption was performed by incubation for 30 min at room temperature with constant swirling. Absorption with MuMTV was performed with MuMTV coupled to Sepharose-beads (for the coupling procedure, see Chapter

Table 6.2

HUMORAL CYTOTOXICITY ASSAY

Target cell	Test serum absorbed with:	Test serum dilution 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
C3HMT/clll		82*	95	114	102	104	58	19	- 2
BALB/c mammary gland	-	21	23	37	38	20	8	- 1	- 2
BALB/3T3-RLV		79	80	102	95	89	62	14	-15
C3HMT/clll		- 4	6	90	89	87	24	- 4	- 2
BALB/c mammary gland	BALB/c kidney, spleen	- 2	- 1	2	3	0	- 2	2	- 2
BALB/3T3-RLV		- 9	-15	-10	-10	-13	-11	- 6	1
C3HMT/clll		7	- 7	4	6	- 7	5	3	- 4
BALB/c mammary gland	BALB/c kidney, spleen + MuMTV	3	0	2	2	1	5	4	- 1
BALB/3T3-RLV	MuMTV	-12	- 4	- 8	- 7	- 1	- 5	8	8

Rabbit antiserum raised against BALB/cfC3H mammary tumour cells was tested in a ^{51}Cr-release assay on different cell types. The effect of different *in vitro* absorptions on the reactivity of the serum was determined.
*The numbers indicate the percentage ^{51}Cr-release.

2). Beads were suspended in an equal volume of serum and absorption was performed for 30 min. The beads were then collected by centrifugation. The different absorptions were carried out twice.

The results in Table 6.2 show that, after absorption with spleen and kidney cells, a mammary tumour-specific reactivity is left. There is no longer any reaction with normal BALB/c mammary gland cells or with BALB/3T3-RLV. The tumour specific reactivity can be completely absorbed with MuMTV.

The anti-MuMTV reactivity of the rabbit antiserum was characterized by means of immunoprecipitation of detergent-disrupted radioiodinated MuMTV. The result (Fig. 6.5) demonstrated that the antiserum precipitated mainly gp52.

In addition to the experiments with the rabbit anti-BALB/cfC3H mammary tumour cell serum, similar experiments were performed with the antiserum raised to the C3HMT/clll cells. This serum was tested in the humoral cytotoxicity assay against C3HMT/clll cells and against BALB/3T3-RLV cells. Again, the effect of absorption with spleen and kidney cells and also with MuMTV on the cytotoxic reactivity of the serum was determined. The absorptions were performed in the same way as described for the anti-BALB/cfC3H mammary tumour cell serum.

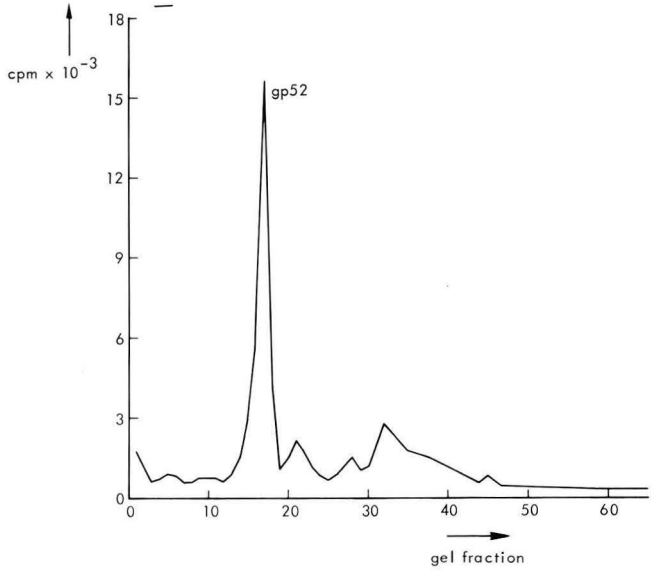


Figure 6.5:

Immunoprecipitation of a radioiodinated detergent-disrupted MuMTV preparation. A rabbit anti-BALB/cfC3H mammary tumour cell serum was used for precipitation. The resulting precipitate was analyzed on SDS-PAGE.

The results shown in Table 6.3 indicate the same tendency as shown by the other rabbit antimouse mammary tumour cell serum: the unabsorbed antiserum showed a strong humoral immune reactivity directed against different types of cells; after absorption with normal kidney and spleen cells, a specific antimouse mammary tumour cell reactivity is left which can be completely absorbed with purified MuMTV. With the immunoprecipitation procedure, the anti-MuMTV reactivity again appeared to be directed mainly to gp52.

When absorbed serum was tested on BALB/3T3-RLV cells, negative values for the percentage of cells lysed were obtained. Apparently in that case serum factors exerted a stabilizing influence on the target cells, resulting in a smaller amount of ^{51}Cr-release in comparison to that occuring when medium is added to the cells for determining the amount of ^{51}Cr-release corresponding to the background level of the assay. At higher serum dilutions, this effect decreases, as would be expected. This phenomenon was observed with both rabbit antisera (see Tables 6.2 and 6.3).

In addition to the above described rabbit antisera, a rabbit antiserum was raised against cells derived from spontaneous GR mouse mammary tumours. The resulting serum was tested in a humoral cytotoxicity assay against C3HMT/clll and BALB/3T3-RLV cells. In contrast to the *in vitro* absorption procedure described above, this serum was absorbed *in vivo*. For that purpose, GR mice were injected intraperitoneally with 1.5 ml of rabbit antiserum. After 16 hr, the animals were bled from the retro-orbital plexus. Absorption with MuMTV was performed with MuMTV coupled to Sepharose beads, as described earlier. The effects of the different absorptions on the cytotoxic reactivity of the serum with C3HMT/clll and BALB/3T3-RLV cells are shown in Table 6.4. After the *in vivo* absorption, a mouse mammary tumour specific reactivity is left which, as was the case with the other two rabbit antisera, is absorbable with MuMTV. Also in this case the anti-MuMTV reactivity appeared to be mainly directed to gp52, as evidenced by immunoprecipitation of detergent disrupted radioiodinated MuMTV with the serum.

Considering the results obtained with the experiments described in this Chapter so far, it is concluded that, in the homologous host, the mouse, as well as in a heterologous host, the rabbit, a strong tumour specific humoral immune response against mouse mammary tumour cells can be evoked. The mammary tumour specific reactivity is directed mainly to the MuMTV-envelope protein gp52, which is expressed on MuMTV-induced mouse mammary tumour cells, as was already demonstrated by experiments described in Chapter 5. No evidence for the expression of an additional, nonvirion, TCSA could be obtained in the chosen experimental systems.

Table 6.3

HUMORAL CYTOTOXICITY ASSAY

Target cell	Test serum absorbed with:	Test serum dilution									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
C3HMT/clll	-	73*	85	73	66	61	46	30	14	10	12
BALB/3T3-RLV		95	107	93	100	94	73	38	5	-12	- 4
C3HMT/clll	C3Hf kidney, spleen	-	3	10	24	32	16	11	2	- 1	- 1
BALB/3T3-RLV		-	-25	-21	-22	-22	-13	- 4	- 2	- 5	- 4
C3HMT/clll	C3Hf kidney, spleen + MuMTV	-	2	- 3	1	- 1	1	0	0	- 1	- 3
BALB/3T3-RLV		-	-27	-23	-23	-11	-13	-10	-14	- 6	- 9

Rabbit antiserum, raised against C3HMT/clll cells was tested in a ^{51}Cr-release assay on different target cells. The effect of different *in vitro* absorptions on the reactivity of the serum was determined.
*The numbers indicate the percentage ^{51}Cr-release.

Table 6.4

HUMORAL CYTOTOXICITY ASSAY

Target cell	Test serum absorbed with:	Test serum dilution 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
C3HMT/cl11	-	35*	46	45	38	28	15	10	6	4	3
BALB/3T3-RLV		46	56	55	26	0	- 4	- 1	- 1	3	0
C3HMT/cl11	in vivo	- 2	3	14	8	4	0	0	1	1	0
BALB/3T3-RLV		- 4	5	3	1	0	- 2	0	- 2	- 2	- 2
C3HMT/cl11	in vivo + MuMTV	0	- 1	0	1	1	1	3	0	0	0
BALB/3T3-RLV		- 4	3	0	- 3	2	1	3	1	0	0

Rabbit antiserum raised against spontaneous GR-mammary tumours was tested in the ^{51}Cr-release assay against different target cells. The effect of in vivo absorption and an additional absorption with MuMTV on the reactivity of the serum was determined.
*The numbers indicate the percentage ^{51}Cr-release.

6.2.4 Infection of rabbits and rats with MuMTV

As discussed in detail in Chapter 1, the most thoroughly characterized onc-gene product in the RNA-tumour virus area is $pp60^{src}$, coded for by the so-called src-gene of ASV. This protein was identified with sera of rabbits bearing ASV-induced tumours.

Although several authors have reported on the successful in vitro infection of nonmouse cells with MuMTV (see Chapter 1), no report on the in vivo infection with MuMTV of animals other than the mouse has appeared.

We have attempted to infect rabbits and rats with MuMTV, with the ultimate aim of obtaining a serum with which, in analogy with the results obtained in the ASV system, we would be able to identify the putative mam-gene coded product.

A group of 10 female rabbits and 8 female rats were injected intraperitoneally at 1 day of age with 0.5 and 0.1 ml, respectively, of the supernatant of the MuMTV-producing C3HMT/clll cell line. MuMTV was routinely isolated from culture medium by centrifugation on a linear sucrose-gradient. Material banding at a density of 1.16-1.18 $g.cm^{-3}$ was then analyzed by means of the double immunodiffusion technique and by SDS-PAGE.

It was estimated that the C3HMT/clll cell line usually produced 1 mg MuMTV protein per litre culture medium, which corresponds to 1.6×10^9 virus particles per ml culture medium (Vaidya et al., 1976). Therefore, the rabbits and rats received about 10^9 and 10^8 MuMTV particles, respectively, on inoculation. To test the virulence of the inoculated MuMTV preparation, 10 female BALB/c mice at 10 weeks of age were inoculated intraperitoneally with 0.5 ml of C3HMT/clll culture medium which was concentrated 3 times by use of an Amicon CH3-concentrator. The mice were force-bred and after 7 months 80% of the test animals developed mammary tumours.

After weaning, the infected rats and rabbits were subjected to breeding and milk samples were collected. Prior to sampling, the milk production was stimulated by subcutaneous inoculation with 0.1 ml (rats) or 0.2 ml (rabbits) of oxytocin-s (10 $IU.ml^{-1}$). Samples were stored at $-20^{o}C$ until they were tested in competition radioimmunoassays for the presence of MuMTV proteins.

Initially, only one milk sample per litter was taken. This sample was usually taken from rats at day 10 postpartum and from rabbits as soon as possible. In the course of the experiments, we changed our strategy and took milk samples daily from each litter for a number of days. Samples were normally tested only in the radioimmunoassay developed for gp52 (see Chapter 5). In most cases, those samples giving

a positive result in the assay were tested several times. These were also tested in the competition assay developed for the MuMTV-core protein p28. As described in Chapter 2, the possibility of false positive results due to contaminating proteolytic activity was excluded. Supernatants of some dilutions in those assays giving significant displacement of input radioactivity were immunoprecipitated with the corresponding monospecific antiserum (either rabbit anti-gp52 serum or rabbit anti-p28 serum). The resulting precipitates were analyzed by SDS-PAGE. In none of the assays reported hereafter in which milk samples were shown to contain MuMTV antigens was degradation of labelled antigen detected.

Fig. 6.6 shows the results obtained with a competition assay for gp52 using the following samples as the competing antigen: MuMTV-containing culture medium of the C3HMT/clll cell line, a milk sample of a rat infected with MuMTV, purified gp52 and nine individual milk samples of noninfected control rats. Using 10 µl of undiluted milk of any control rat as competing antigen, an average displacement of 45% was obtained (see Fig. 6.6). A similar result was obtained in the assay developed for p28. These displacements were considered to be nonspeci-

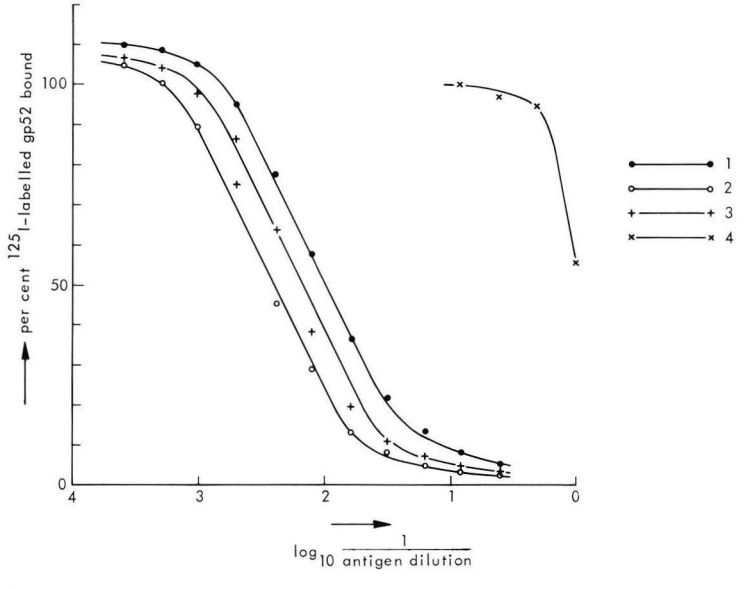


Figure 6.6:

Competition radioimmunoassay for gp52.
As competing antigens in the assay were used: MuMTV-containing culture medium from the C3HMT/clll cell line, diluted 1:10 with TEN-buffer (1); milk of a MuMTV infected rat (the sample was calculated to obtain 17,400 ng gp52/ml milk; see Table 6.5) (2); a solution containing 100 µg purified gp52/ml (3); (4) represents the average of the results obtained using milk samples of nine noninjected control rats.

fic; therefore, 10 µl of milk sample at a dilution of 1:4 was taken as the first point in each assay (see also Chapter 2).

As far as the sensitivity of the assay was concerned, 50% displacement of the amount of ^{125}I-gp52 precipitated was obtained using 0.6 ng purified gp52 as the competing antigen in the assay depicted in Fig. 6.6. Therefore, for this particular experiment, about 200 ng gp52.ml^{-1} milk sample was detectable. An assay using a known amount of purified gp52 as competing antigen, was included in each test, in order to estimate the sensitivity of the respective tests. The sensitivity of the different assays fluctuated between 60-300 ng gp52.ml^{-1} milk sample.

With the particular milk sample of the MuMTV infected rat tested in the assay for which the results are depicted in Fig. 6.6, a 100% displacement of the precipitated radioiodinated gp52 was observed. Furthermore, the slopes of the three competition curves obtained by using the rat milk sample, purified gp52 and MuMTV-containing culture medium as competing antigens are identical. Therefore, by definition, the reactions shown by the three samples tested in the competition assay for gp52 may be classified as reactions of identity (Hunter, 1979). This indicates that all three samples tested contained the same MuMTV gp52. Similar results were obtained with the other milk samples which were positive for either gp52 or p28 (see Table 6.5).

The rat milk sample tested in Fig. 6.6 was calculated to contain 17,400 ng gp52 per ml of milk, which would be a moderate amount for a MuMTV-positive mouse (Altrock and Cardiff, 1979).

Table 6.5 summarizes the results obtained thus far. Rats have produced 3-9 litters, rabbits 0-3. Forty-six litters were born in the group of rats; for the group of rabbits, this number was 15. A total of 146 different milk samples of MuMTV-infected rats and 28 different rabbit milk samples were tested in the competition assay for MuMTV gp52. Of these, 4 different rat milk samples and 1 rabbit milk sample appeared to be positive for gp52; only two of those gp52-containing milk samples appeared to be also positive for p28. Three of eight rats were positive for gp52 at some time, while only one rabbit contained this viral antigen in its milk in one out of two litters.

As mentioned above we changed our strategy for taking milk samples during the course of the experiments. We originally took only one sample per litter; later, milk samples were taken daily on a number of successive days. The gp52-positive milk samples obtained from two of the rats (see Table 6.5) were collected during the period in which we took only one milk sample per litter; however, in the case of the rat positive for gp52 in milk samples of the 4th and 5th litter, milk sam-

Table 6.5

TESTING OF MILK SAMPLES FOR gp52 AND p28 IN COMPETITION RADIOIMMUNOASSAYS

Animal	No. of animals positive for MuMTV antigens in milk per total no. of animals tested	Serial number of litters with milk positive for MuMTV antigens	ng gp52 per ml milk	ng p28 per ml milk
Rat (+ MuMTV)	3/8	4th of 5 litters	480*	-
		4th and 5th of 7 litters	3650**; 17,400***	925; -
		5th of 9 litters	100*	-
Rat (control)	0/9	-	-	-
Rabbit (+ MuMTV)	1/10	1st of 2 litters	380*	60
Rabbit (control)	0/10	-	-	NT

* Tested in three different assays.
** Tested in two different assays.
***Milk sample taken on day 5 postpartum.
N.T.: Not tested.

ples of the 5th litter were taken daily, starting 4 days postpartum and continuing for a period of 20 days thereafter. In that period, samples were taken on five successive days; sampling was omitted in the weekend, after which sampling was continued for the next five days, etc. A positive result was obtained only with the milk sample taken five days post-partum. This sample appeared to contain 17,400 ng gp52 per ml milk. It showed no competition in the assay developed for MuMTV p28 (see Table 6.5). Although milk from this particular rat was positive for gp52 at two successive litters, different milk samples taken during the 6th and 7th litter were all negative in the assay for gp52 (a total of 13 and 9 milk samples taken during the 6th and 7th litter, respectively, were tested).

To determine whether in rats positive for gp52 in the milk a humoral response to this antigen and to other putative TCSA's was developed, sera of these rats were tested in the complement dependent humoral cytotoxicity assay on gp52-expressing C3HMT/c111 cells. In none of the cases was a positive reaction obtained.

The rat milk sample containing 17,400 ng gp52 per ml of milk was examined electron microscopically for the presence of B-type particles. For this purpose, 400 µl of milk was subjected to centrifugation on a linear sucrose gradient (see Chapter 2). Material banding at a density of 1.16-1.18 $g.cm^{-3}$ was collected. In the search for the viral particles, the method of Miller et al. (1973) was used. Briefly, Millipore membrane filter discs (pore size 25 nm) were positioned on top of supports made of Epon embedding medium in cellulose nitrate tubes (tubes for the Beckmann 50 Ti rotor were used). The 1.16-1.18 $g.cm^{-3}$ material was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer and was placed in the pretreated cellulose nitrate tubes. After centrifugation for 45 min at 150,000 x g at 4°C, the supernatant fluid was discarded. The sediment containing discs were cut into strips and immersed in phosphate buffered 1% osmium tetroxide solution (OsO_4) for 60 min at 4°C. Strips were dehydrated in a graded series of isopropanol, treated with toluene and flat embedded in Epon. After polymerization, strips were orientated in the ultramicrotome so that the section could be cut perpendicular to the plane of the millipore membrane. Ultrathin sections were collected on uncoated grids and their contrast was enhanced with an aqueous solution of uranyl acetate and lead citrate. No evidence for the presence of B-type particles in the rat-milk was obtained.

In addition, biopsies of mammary gland tissue of the rat which once had 17,400 ng $gp52.ml^{-1}$ milk were taken. These biopsies were taken three days after the gp52-positive milk sample was collected.

The tissue was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer and subsequently postfixated in 1% OsO_4 in 0.1 M sodium cacodylate buffer. Dehydration was performed in graded series of ethanol; the material was then embedded in Epon (see above). No A-or B-type particles were found in ultrathin sections of the material.

The absence of virus-like particles in the milk and mammary gland tissue in the case discussed above, agrees with the lack of p28 in the 17,400 ng gp52.ml^{-1} containing milk sample. Apparently, a case of partial MuMTV-gene expression was encountered. This, of course, can not lead to the production of complete MuMTV virions.

Up to now, no mammary tumours have been detected in either the group of infected rats (up to 22 months of age) or the infected rabbits (up to 21-24 months of age). Two rats have died of an overdosage of anaesthetics, and one rat as the result of a pituitary tumour. Unfortunately, six of the group of 10 infected rabbits died as the consequence of severe gangrenous mastitis. In none of the deads could involvement of the mammary gland be established.

6.3 DISCUSSION

In discussing the involvement of MuMTV in mouse mammary tumorigenesis, a distinction has to be made between exogenous and endogenous MuMTV.

Exogenous, milk transmitted MuMTV is responsible for the early appearance of mammary tumours in susceptible mice, which is evidenced by the increase in MuMTV RNA and integrated MuMTV DNA levels in mammary tumour cells as compared to normal mammary gland cells and cells of other organs (Michalides et al., 1976; 1978b; McGrath et al., 1978).

Evidence for the involvement of endogenous MuMTV in mammary tumorigenesis in nonexpressor, low mammary tumour incidence strains is less clear. Mammary tumours appearing either spontaneously or after hormonal stimulation in strains like C3Hf, BALB/c and 020 do not contain increased levels of proviral DNA (Michalides et al., 1978b; McGrath et al., 1978). However, in the GR-mouse, in which the role of endogenous MuMTV in mammary tumorigenesis is evident, early appearing mammary tumours do not contain an increased level of proviral copies (Michalides et al., 1976; Morris et al., 1977). Therefore, an increase in proviral copy number is not absolutely required in MuMTV induced mammary tumour cell transformation. As far as the MuMTV RNA level is concerned, some studies have shown an elevated level in mammary tumours arising in hormone-stimulated mice from low-incidence strains (Dudley et al.,

1978b; McGrath et al., 1978). However, Michalides et al. (1978b) found an elevated level of MuMTV RNA only in the mammary glands of hormonal stimulated low-incidence mouse strains, while considerably lower levels were found in the mammary tumours of these hormonal-stimulated mice. These results led the latter group to conclude that continuous expression of MuMTV genes is not required for the maintenance of the transformed state.

In spontaneously arising mammary tumours in nonexpressor low-incidence mouse strains, the MuMTV-RNA level, like the MuMTV DNA level, is similar to those found in liver cells of these animals (Dudley et al., 1978b; Michalides et al., 1978b).

Because of the uncertain involvement of endogenous MuMTV in mammary tumorigenesis, a search for the putative mam-gene product should be made preferably with mouse mammary tumour cells originating from tumours induced by exogenous MuMTV.

The search for a nonvirion mammary tumour-specific TCSA, an antigen which would be a candidate for a putative mam-gene product, is described in this Chapter. Studies were made with tumours, originating in strains with a high mammary tumour incidence: the C3HMT/clll cell line which was originally derived from a spontaneous C3H tumour, and with transplanted BALB/cfC3H mammary tumours. In addition, spontaneous mammary tumours of the GR mouse strain, of which the involvement of endogenous MuMTV in tumorigenesis is evident, were examined for the expression of TCSAs.

As discussed in Chapter 1, the RNA tumour virus associated TCSAs identified up to now, were originally discovered with sera of either mice immunized with irradiated syngeneic tumour cells (MCSA) or animals resistant to virus-induced tumours (FOCMA). The experiments described in this Chapter have shown that antisera raised in mice against irradiated, syngeneic mammary tumour cells and sera of mice bearing transplanted syngeneic mammary tumour cells both show a strong specific antimouse mammary tumour reactivity in the humoral cytotoxicity and the membrane immunofluorescence assays. Absorption with a MuMTV preparation removed this reactivity completely, indicating the humoral response to be directed only to MuMTV structural proteins. As was demonstrated with the immunoprecipitation assay the sera reacted mainly with the MuMTV-envelope protein gp52, the expression of which on MuMTV-induced mouse mammary tumour cells was previously demonstrated (Chapter 5). In addition to gp52, most of the sera tested precipitated low amounts of gp36 and p28 from detergent-disrupted radioiodinated MuMTV preparations. This could indicate that these proteins are expressed in low quantities on mouse mammary tumour cells. Alternative-

ly, the antibodies to gp36 and p28 might be induced by virus particles released by the tumour cells or by viral proteins released from degraded tumour cells in the inoculum. In the results presented in Chapter 5, no evidence for the expression of gp36 on the mammary tumour cell surface was obtained.

The considerable reactivity of the BALB/c antiserum raised against irradiated BALB/cfC3H mammary tumour cells against p28 in the immunoprecipitation of detergent-disrupted radioiodinated MuMTV (see Fig. 6.3.A) can be explained by the quality of the radioiodinated virus preparation used in that particular case: by way of exception an excess of p28 over gp52 was present in the sample.

The results obtained with the rabbit antisera raised against different mouse mammary tumour cells confirm those obtained with the mouse sera: MuMTV structural proteins, and primarily gp52, are the only TCSAs detectable on MuMTV-induced mouse mammary tumour cells. Also no evidence for the expression of unique antigens on the mammary tumour cells tested was found.

Our results are in agreement with those recently reported by Kuzumaki and Klein (1979), who demonstrated a varying degree of MuMTV-antigen expression on spontaneous and MuMTV-induced mouse mammary tumour cells derived from different mouse strains. No evidence was found for the expression of a cross-reactive nonvirion TCSA on mouse mammary tumour cells. With a serum raised in A.SW mice against a spontaneous syngeneic mammary tumour, Kuzumaki and Klein (1979) demonstrated the presence of a unique cell surface antigen on that particular tumour, confirming the findings of Vaage (1968), who showed individually distinct antigens to be expressed on some mouse mammary tumours.

Based on the results obtained, several hypotheses on the putative mam-gene and its product, can be made:

1) the putative mam-gene product is not expressed on MuMTV-transformed cells but is located inside the cell, a situation similar to that demonstrated for $pp60^{src}$ in the ASV system;
2) the mam-gene product is part of MuMTV virions and, consequently, reactivity induced against the mam-gene product is removed from sera by absorption with MuMTV. In the FeSV system, the presence of the FOCMA antigen in pseudotypes of FeSV has been demonstrated (Sherr et al., 1978);
3) the mam-gene product is weakly antigenic as compared to the expressed MuMTV envelope protein gp52 and was therefore not detected in the chosen experimental system;
4) the MuMTV-genome does not contain a mam-gene at all. In that case, the oncogenic capacity of MuMTV must be based on factors other than the production of a specific "transforming" protein.

In an attempt to develop an alternative system for the study of MuMTV-induced mammary tumorigenesis, rabbits and rats were infected with MuMTV. As a result, differential expression of MuMTV antigens was demonstrated in the milk of some infected animals; some animals expressed only gp52, while others also expressed p28. A possible explanation for this phenomenon was given in a report of Dudley et al. (1978a): With two DNA probes representative for the entire MuMTV genome and for the poly(A)-adjacent sequences at the 3'-end of the genome, respectively, they demonstrated in lactating mammary gland tissue of BALB/c mice a 20-fold excess of poly(A)-adjacent RNA sequences over sequences representing the entire MuMTV genome. In BALB/c mammary tumour cells, the ratio decreased to 4:1. In MuMTV-producing C3H-mammary tumour cells, a ratio of 2:1 was determined. The authors suggested these effects to be due to a differential expression of subgenomic MuMTV mRNA species, the existence of which was recently demonstrated (Sen et al., 1979; Robertson and Varmus, 1979; Groner et al., 1979).

In addition, the expression of gp52 in the absence of p28 can be explained with another model, in which the MuMTV DNA is only partly integrated into the host genome of the infected cells. Integration of partial proviral copies is a well-known phenomenon in the oncovirus system (Drohan et al., 1977; Wong-Staal et al., 1979).

A possible explanation for the detection of MuMTV-related antigens in rat milk samples, would be the expression of the MuMTV-related sequences as they were recently demonstrated in rat-DNA by molecular hybridization experiments (Drohan and Schlom, 1979b). However, these sequences appeared to be related to only 20% of the MuMTV genome. Expression of such sequences would result in antigens which, on testing in competition radioimmunoassays developed for MuMTV proteins, would produce curves clearly deviating from those obtained by testing original MuMTV-proteins (see Chapter 2, Fig. 2.1). This appeared not to be the case (Fig. 6.6).

In view of the models described above, the absence of complete MuMTV particles in the MuMTV antigen positive milk sample and in the mammary gland tissue of the rat examined is not unexpected.

Another striking feature emerging from the results obtained, is the transient nature of the MuMTV gene expression in the heterologous host: those rats containing viral antigens in the milk at the fourth and/or fifth litter gave negative results with the successive litters (see Table 6.5). In one case, this transient nature of gene expression could even be demonstrated within one litter: a milk sample taken from a particular MuMTV-infected rat on day 5 postpartum demonstrated a

considerable amount of gp52 (about 17 ug gp52 per ml milk); samples taken on subsequent days were completely negative.

A possible explanation for the limited duration of the viral gene expression could be the immunological elimination of those cells containing MuMTV antigens. With respect to the humoral immune system, sera of rats positive for MuMTV antigen showed no reactivity with gp52-expressing C3HMT/c111 cells when tested in the humoral complement dependent cytotoxicity assay.

An alternative explanation for the transient nature of the MuMTV antigen expression in MuMTV-infected rabbits and rats could be the influence which biological variables might have on the viral antigen expression. In the mouse system, a differential expression of MuMTV-RNA at different stages of mammary gland development and lactogenesis has been demonstrated. Pauley et al. (1978) detected no MuMTV RNA in mammary glands of virgin and early pregnant BALB/cfC3H mice, low levels of MuMTV RNA in midpregnant mice and a considerable increase in the MuMTV RNA level in the later stage of pregnancy. After parturition, an additional twofold increase in the MuMTV RNA level was detected. Later in lactation, the MuMTV RNA level decreased considerably.

McGrath and co-workers demonstrated the concentration of MuMTV RNA in normal mammary cells from the BALB/c strain to vary as a function of the physiological state of the mammary gland and of the age of the animal. Lactating mammary glands contained the highest MuMTV RNA level. A somewhat lower level was detected in glands of midpregnancy animals. No MuMTV RNA was detected at all in later lactation periods. The MuMTV RNA demonstrated in the normal mammary gland cells, saturated the MuMTV cDNA only at submaximum levels in the molecular hybridization experiments. This led the authors to conclude that, in normal BALB/c cells, the expressed MuMTV RNA represents an incomplete or highly disproportionate transcript of the MuMTV genome (McGrath et al., 1978; McGrath and Jones, 1978), a phenomenon compatible with the results we obtained on the expression of MuMTV antigens in the infected rats and rabbits.

In analogy with the mouse system, the expression of MuMTV in the infected animals other than the mouse is possibly influenced by the physiological state of the gland.

With respect to the amount of MuMTV protein detected in the positive milk samples (which varied from 60-17,400 ng protein per ml of milk), it is of interest to consider the experience with the in vitro infection of nonmouse cells with MuMTV. As stated earlier (see Chapter 1) such infections needed a very high multiplicity of infection (about

10^5 virions/cell). When the *in vivo* situation is compared to that *in vitro* in this respect, this would suggest that, on infection with 10^8 (rats) or 10^9 (rabbits) virus particles per inoculum, only a very small percentage of the target cells can become infected. In such cases only low amounts of MuMTV proteins can be expected in the milk of the animals. Furthermore, in the process of postlactation regression of the mammary gland, infected cell populations might get lost. During the next lactation only uninfected cells may be involved in milk production.

CHAPTER VII

GENERAL DISCUSSION

At the moment we started our experiments, no suitable isolation procedure for the major MuMTV proteins was available. For the purposes we wanted to achieve the antigenic integrity of the proteins needed to be conserved during the isolation procedure. The methods which Parks et al. (1974b) and Sarkar and Dion (1975) developed for the isolation of the major MuMTV proteins gp52 and p28 did not meet this requirement. As no suitable alternative did exist at that moment, it was decided to develop a new isolation procedure.

In comparison with the murine C-type viruses, such as R-MuLV, MuMTV is a hardly solubilizable virus. While freeze-thawing of a R-MuLV preparation is sufficient for solubilizing the major portion of the viral protein constituents (Strand and August, 1976), MuMTV must be incubated in the presence of a rather high concentration of detergents in order to obtain satisfactory results.

The presence of low concentrations of detergents in the buffers used in the different chromatographic procedures also appeared to be of utmost importance for successful isolation of both the envelope and core proteins. In the absence of detergents, the elution of the absorbed MuMTV glycoproteins from the Con A-Sepharose column by a sugar derivative acting as a competitor of the lectin-glycoprotein interaction was impossible (see Chapter 3). In addition, low detergent concentrations were of importance for the stabilization of the solubilized viral proteins; dialysis of purified MuMTV protein fractions against buffers without detergents resulted in the precipitation of a considerable amount of the protein.

The isolation procedure finally developed appeared to be applicable not only to MuMTV; subjection of a R-MuLV preparation to this procedure yielded highly purified gp70 and p30-fractions.

Indications of a strong hydrophobic nature of gp36 previously obtained by the elution of this protein in the Vo of the Sephadex column and of a Biogel-A.5 m column (upon gel filtration in 6 M Gu-HCl) were confirmed by results obtained with the amino acid composition analysis. The expected order of hydrophobicity for the different proteins was estimated. The envelope proteins, associated in vivo with the

lipid bilayer surrounding the viral core (see Fig. 4.7), appeared to be most hydrophobic. The existence of a tightly bound gp52-gp36 complex in detergent solubilized MuMTV preparations (a complex which stays intact during chromatography on Con A-Sepharose and gel filtration at high ionic strength) suggests the in vivo association of these proteins, an association most probably mediated through hydrophobic interactions. Possibly the association of such a firm complex with the lipid bilayer surrounding the virus, explains the tenacity of the MuMTV-virion. The easily disruptable C-type viruses contain one major glycoprotein in their envelope. This protein, gp70, is easily released from the virus.

The quality of the purified MuMTV proteins seemed to meet a requirement we previously made: preservation of the antigenicity of the viral proteins during the isolation. Whereas Parks et al. (1974b) needed to denature their samples by heating prior to testing them in the radioimmunoassay developed for their purified gp52, we were able to assay different kinds of test samples in competition radioimmunoassays developed with antisera raised against the respective purified MuMTV-proteins without a need for partial denaturation of the test antigen.

In spite of the preservation of antigenicity, vaccination of different mice strains with either a purified gp52 or a purified p28 preparation failed to protect them against transplanted gp52 expressing mouse mammary tumours (Creemers et al., 1979). In the latter study, gp52 was found to induce a substantial cellular immune response, whereas antibody titers as determined by an enzyme linked immune assay were low or negative. The simultaneous induction of factors blocking the cellular immune reactivity, factors which were possibly represented by antigen-antibody complexes (Creemers and Brinkhof, 1977) probably explains the failure of these vaccination experiments.

The results obtained with the vaccination experiments confirm those of related studies: in general the efficacy of viral subunits in vaccination experiments is low (Wright et al., 1976). A way to increase the immunogenicity of spike proteins of enveloped viruses is to reconstitute such a protein into vesicles of egg lecithin [structures which are called virosomes (Almeida et al., 1975)]. Morein et al. (1978) demonstrated that a lethal infection of mice with Semliki Forest virus could be prevented by a single vaccination with the virus spike protein presented in the form of virosomes. Vaccination with the monomeric form of this protein appeared to be very much less effective. Possibly application of this method for the MuMTV spike protein gp52 would be a useful approach to achieve successful vaccination

against MuMTV-induced mammary tumorigenesis.

Analysis of the four purified MuMTV proteins by isoelectric focusing revealed for the proteins gp52, gp36 and p28 the existence of multiple forms of each protein, differing only in charge. This phenomenon was previously demonstrated for the structural proteins of Moloney MuLV (Forchhammer and Turnock, 1978). Assuming this heterogeneity of MuMTV proteins to be due to posttranslational processes, such as glycosylation and phosphorylation (besides the major phosphoprotein p23, p28 also appeared to be phosphorylated), these results suggest an accurate completion of posttranslational processes not to be essential for the assemblage of the mature virion.

The existence of isomers, differing only in charge possibly explains the observation which Racevskis and Sarkar (1978) made on the analysis of MuMTV on high resolution SDS-polyacrylamide gradient gels. They noted a glycoprotein heterogeneity, especially for gp36, which they found to run as a heterogeneous population of three or four discrete bands. The influence of differences in charge of a particular protein on the results obtained by analysis on SDS-PAGE was demonstrated in case of the polyprotein precursor to the internal MuMTV proteins. Here it was demonstrated that increased phosphorylation resulted in a slower migration on SDS-polyacrylamide gels (Racevskis and Sarkar, 1979; see also Chapter 1).

The functions and properties of those proteins which are thought to be coded for by the MuMTV genome and which form part of the mature MuMTV virion are summarized in Table 4.3 and Fig. 4.7.

Some striking differences between the MuMTV proteins and the proteins which form part of the murine type C viruses (see Bishop, 1978; Eisenman and Vogt, 1978, for reviews) are, apart from the reported differences in the properties of the respective DNA polymerases (Dion et al., 1974b; Marcus et al., 1976): 1) the existence of two distinct MuMTV envelope proteins; the MuLV's contain a major glycosylated envelope protein, gp70, plus a less glycosylated form of this protein, gp45, furthermore the MuLV-envelope contains two nonglycosylated proteins p15 (E) and p12 (E) (Montelaro et al., 1978); 2) the demonstration that the major MuMTV core protein, p28, is phosphorylated, which is in contrast to the situation for the major core protein of the murine C-type viruses, p30.

There seems to be a marked resemblance between the arrangement of the internal proteins of MuMTV and MuLV in the gag-gene coded polyprotein precursor with respect to their characteristics and functions. The order of the internal proteins of the MuLV's within the primary translational product of the gag-gene has been established as NH_2-p15-

p12-p30-p10-COOH (Barbacid et al., 1976b). According to their properties and probable functions, the proteins were characterized respectively as a lipid associated hydrophobic protein (p15), the major phosphoprotein probably constituting the core shell (p30), and a basic protein, found in association with the RNA of detergent lysed particles (p10) (see Eisenman and Vogt, 1978; Bishop, 1978, for reviews). In this respect, the arrangement of the internal MuMTV proteins within the gag-gene, i.e., NH_2-p12-p23-p28-p18-COOH (Chapter 4) correlates well with the MuLV situation (see Table 4.3 and Fig. 4.7 for the properties and proposed functions of the respective MuMTV proteins).

The correlation found between the gag-gene order of the MuMTV and MuLV internal proteins does not hold for the arrangement of the internal avian type C RNA tumour viral proteins, the gag-gene order of which was established as NH_2-p19-p12-p27-p15-COOH (Vogt et al., 1975; 1979); these proteins were characterized as the major phosphoprotein (p19), the viral RNA associated basic protein (p12) and the core shell constituting major internal protein (p27); while p15 was characterized as being associated with proteolytic activity (see Eisenman and Vogt, 1978 for a review).

It was discussed in Chapter 5 that gp52 is most probably the only MuMTV structural protein which is expressed on MuMTV transformed mouse mammary tumour cells, a finding which was in agreement with the results reported by Yang et al. (1977), Schochetman et al. (1978b) and Racevskis and Sarkar (1978). The precursor to the MuMTV envelope proteins, $gPr73^{env}$ (see Chapter 1), is not expressed on the mouse mammary tumor cell surface (Schochetman et al., 1978b). This implies that $gPr73^{env}$ is cleaved prior to the expression of gp52 on the cell surface. The envelope protein precursor which Racevskis and Sarkar (1978) detected in the culture medium of MuMTV-producing cells is obviously not involved in the maturation process of MuMTV. This extracellular envelope precursor protein possibly represented the result of a partial block in the processing of the MuMTV envelope proteins.

No evidence for the expression of the second envelope protein, gp36, on the mouse mammary tumour cell surface could be obtained with either the membrane immunofluorescence assay or the humoral cytotoxicity assay, results which were recently confirmed by Massey and Schochetman (1979). The cell surface location of gp52 is reflected in the humoral immune response as it develops in mammary tumour bearing mice (Arthur et al., 1978b) and in low mammary tumour incidence mice (Arthur and Fine, 1978). In both cases, the response appeared to be directed to MuMTV gp52. Michalides et al. (1979) demonstrated the development of a natural humoral immune response to MuMTV to be related

to the expression of MuMTV in the animals.

Although Massey and Schochetman (1979) were unable to label the polypeptide portion of gp36 by lactoperoxidase catalysed radioiodination of mouse mammary tumour cells, even after EDTA treatment of the cells, they succeeded in labelling the carbohydrate part of gp36 by labelling intact mouse mammary tumour cells with galactose oxidase and sodium [^{3}H]-borohydride. This result lead the authors to conclude the envelope proteins of MuMTV to be present on the surface of MuMTV producing cells in an orientation similar to the one in mature MuMTV-virions. Probably such structures represent the budding site of the virus.

The faint reaction, obtained with anti-p28 serum against mouse mammary tumour cells in the membrane fluorescence assay suggested p28, or its polyprotein precursor, to be expressed on the tumour cell surface. This could not be confirmed by results obtained with the humoral cytotoxicity assay.

Upon reacting anti-p28 serum with GRSL18 and L1210 cells in the cytoplasmic immunofluorescence assay, large fluorescence dots were observed (see Fig. 5.12). Recently, Nusse et al. (1979) compared the processing of the MuMTV gag-gene encoded polyprotein precursor in GR-thymic lymphoma (GRSL-)cells (these cells are deficient in the production of B-type particles) with the processing of this precursor in a GR-mammary tumour cell line (GRMT). The processing of the gag-precursor in GRMT cells appeared to follow the normal pathway as described for MuMTV-producing cells (see Chapter 1), while the processing in the GRSL cells got stuck at the level of the gag-precursor protein $Pr73^{gag}$. It was shown that this $Pr73^{gag}$ molecule was associated with the intracytoplasmic A-type particles. The precursor molecule appeared to be poorly phosphorylated in comparison to the $pPr76^{gag}$ intermediate detectable in the GRMT-cells. The authors concluded that the processing of the $Pr73^{gag}$ molecule in the GRSL-cell was blocked at the phosphorylation level (Nusse et al., 1979). The accumulation of large clusters of intracytoplasmic type A-particles in the leukaemia cells explains the observation we made with the anti-p28 serum in the cytoplasmic immunofluorescence assay.

By way of comparison, the manner of expression of viral proteins in the murine C-type virus system will be briefly discussed. Two serologically distinct antigenic systems were originally distinguished: the G antigens or Gross cell surface antigens (GCSA) (Old et al., 1965) and the FMR antigens (Old et al., 1964). GCSA are expressed on cells infected with Gross MuLV, on spontaneous mouse leukaemias and on normal lymphoid tissues of both high- and low-leukaemia incidence

strains of mice. In the latter two cases, the expression of GCSA is thought to be associated with the expression of endogenous MuLV sequences. The FMR antigens are expressed only on cells infected with Friend, Moloney, or Rauscher MuLV (see Old and Stockert, 1977 for a review).

GCSA was recently identified as a complex of two closely related glycosylated polyproteins containing antigenic determinants related to the internal MuLV core proteins p30, p15, p12 and p10 (Tung et al., 1976; Snyder et al., 1977; Ledbetter and Nowinski, 1977). In addition to these antigens (which are found on the surface of normal lymphoid tissues as well as on spontaneous leukaemias in mice) also the expression of several type-specific determinants of the major envelope glycoprotein gp70 of certain endogenous MuLV's on the cell surface of both normal thymocytes and on spontaneous or X-ray induced leukaemias was described recently. The expression of those latter determinants is not inevitably correlated with the expression of the GCSA; while GCSA is never found in the absence of virus replication, gp70-related antigenic determinants [like G1X, X.1, G(RADA$_1$), G(ERLD)] can be expressed independently of other MuLV genes (Old and Stockert, 1977).

For the FMR antigens it is a matter of controversy as to whether the FMR antigen represents a determinant of the major envelope protein gp70 (Nowinski et al., 1978) or a determinant of the internal core protein p15 (Lejneva et al., 1976).

A major conclusion of the studies on the cell surface expression of viral proteins in the MuLV system, is that core proteins internally located in the intact virion can also be expressed on the cell surface membrane, a phenomenon not yet demonstrated for the MuMTV system. In both the MuMTV and the MuLV system the viral envelope protein is present abundantly on the cell surface. Another similarity is that in none of the two systems the envelope protein can be considered to be transformation related; in both cases, the expression of these antigens can also be demonstrated in virus infected cells which are not malignant. Electron microscopical, biological and immunological studies have revealed the presence of MuMTV in various tissues besides the mammary gland (Nandi and McGrath, 1973; Bentvelzen and Brinkhof, 1977, for reviews). In spite of this, the mammary gland seems to be the only target for the oncogenic capacity of exogenous MuMTV. There is no clear concensus concerning the factors defining this target specificity.

As was presented in discussing the replication process of MuMTV (see Chapter 1), the MuMTV genome contains three genes, gag, pol and env, which code for the proteins which form part of the mature MuMTV

virion. The gag-pol region of the genome codes for 160,000 daltons and the env-gene for 73,000 daltons of polypeptide. Therefore, as the complexity of the total viral genome gives a coding capacity for about 250,000-300,000 daltons of polypeptide (see Chapter 1), a part of the genome, with a capacity for about 20,000-70,000 daltons of polypeptide, is left. It is tempting to assume this part of the genome to code for a transforming protein, in analogy with the ASV system in which the src-gene codes for the transforming protein designated as $pp60^{src}$.

No evidence was obtained for the expression of a tumour specific nonvirion antigen on MuMTV transformed mouse mammary tumour cells (Chapter 6). With sera directed against syngeneic mouse mammary tumour cells, only the expression of structural viral antigens was demonstrated with immunological techniques such as the membrane immunofluorescence assay and the complement-dependent humoral cytotoxicity assay. The results obtained with the mouse antisera were confirmed by the studies made with rabbit sera raised against mouse mammary tumour cells; again, no evidence for the expression of a nonvirion mouse mammary tumour specific cell surface antigen was obtained (Chapter 6). Therefore, if a part of the MuMTV genome codes for a transforming protein, this protein apparently is not expressed on the tumour cell surface. This situation would be analogous to the situation in the ASV system, in which $pp60^{src}$ was reported to be located on the inner side of the cell-membrane of the ASV-transformed cell (see Chapter 1).

As reviewed in Chapter 1, $pp60^{src}$ appeared to be a phosphorylated protein associated with protein-kinase activity. Therefore, the ASV-induced process of transformation possibly proceeds via phosphorylation of particular cellular proteins. Such an event may lead to an altered pattern of polypeptide synthesis. This pattern may lead ultimately to the appearance of abnormal quantities of proteins resulting in the properties which are characteristic for the ASV-transformed fibroblastic cell (Hanafusa, 1977).

Evidence concerning the site of action of the src-gene product was presented by McClain et al. (1978), who microinjected cellular extracts of ASV-transformed fibroblasts into normal cells. As a result, actin containing microfilament bundles which form part of the cytoskeleton, appeared to dissolve within one half hour after microinjection. The specificity of the assay was demonstrated by the finding that the activity of extracts obtained from cells transformed by mutants temperature sensitive for transformation appeared also to be temperature sensitive in the assay. This observed effect on the cytoskeletal elements of the cell was thought to be either directly or in-

directly responsible for the cellular changes occurring upon transformation induced by ASV.

Sen and Todaro (1979) reported on the isolation of a 15,000 M.W. protein kinase-activity from MuSV-particles. The activity could not be detected in the pure murine leukaemia helper virus particles. The identified protein kinase activity was demonstrated to possess a significant binding affinity for actin, a major component of the cytoskeletal system. The authors suggested this protein-kinase activity to be specifically associated with the transforming activity in the virus stock. The mechanism underlying the MuSV induced transformation process is possibly reflected in an inhibitory effect of the protein-kinase activity on the polymerization of microtubules.

The results obtained in the ASV and MuSV systems are compatible with the hypothesis that transformation of certain kinds of cells is due to changes introduced in the structure and organization of cytoskeletal filaments, as was proposed by Edelman (1976) and Nicolson (1976). The demonstrated association of protein-kinase activity with MuMTV-particles (Hatanaka et al., 1972) might suggest that similar activity is associated with the MuMTV transforming system. However, with the indirect immunofluorescence technique using antisera directed to tubulin and actin and by electron microscopy, Asch et al. (1979) found no difference to exist between the cytoskeletal organization of normal, preneoplastic and neoplastic mouse mammary tumour cells. Apparently, MuMTV-induced transformation is mediated through a process different from that by which ASV and MuSV induced transformation occurs.

SUMMARY

The mouse mammary tumour virus (MuMTV) causes tumours of the mammary gland in the mouse. The virus can be transmitted by means of the mothers milk, a way of infection which is called horizontal transmission. In that case the virus is present as a complete virus particle and is called exogenous virus.

All examined laboratory mice strains and almost all examined wild mice appear to contain information for MuMTV in their cellular DNA. Virus, present in a species in this way is called endogenous virus; transmission of endogenous virus to the off-spring (via the gametes) is called vertical transmission.

The existence of a causal relation between infection of mice with MuMTV and the appearance of mammary gland tumours, is without doubt; however, the involvement of endogenous MuMTV in the appearance of spontaneous mammary tumours, is obscure.

In Chapter 1 the replication process of RNA tumour viruses, the group of viruses to which MuMTV belongs, is discussed. By studying transformation defective variants, it became clear that the genome of RNA tumour viruses in addition to genes coding for the protein components of the virus also may contain a so-called onc-gene. Such a gene is thought to code for a protein which finally is responsible for the malignant transformation of the infected cell. In some RNA tumour virus systems such a protein seems to be expressed on the surface of the transformed cell.

In Chapter 2 the materials and methods are discussed.

Chapter 3 is concerned with the procedure we developed for the purification of the structural proteins of MuMTV. In developing the procedure it was attempted to preserve the immunogenic qualities of the proteins during their isolation. The isolation was performed under mild conditions and therefore the use of denaturing agents, like guanidine-HCl and sodium dodecylsulphate was avoided.

The presence of nonionic detergents in the buffers used during the different chromatographic procedures were found to be of decisive importance for the success of the isolation procedure developed. By making use of different chromatographical principles, like affinity chromatography, ion-exchange chromatography and molecular sieving, we succeeded in the isolation of four viral proteins. Two of those proteins normally form part of the envelope of the mature virus particle, whereas the other two proteins are located inside the virion.

In Chapter 4 results are discussed on the characterization of the proteins by molecular weight determinations, amino acid analysis and by defining the isoelectric points. The results obtained, together with data from the literature, are summarized in a model concerning the structure and composition of the virus (see Fig. 4.7).

In Chapter 5 it is demonstrated that the purified proteins had retained their immunogenic qualities. This appeared from the results of competition radioimmunoassays which were developed for the respective proteins. Antisera raised against the proteins did not show any cross reactivity when tested against disrupted MuMTV in immunological assays like the double immunodiffusion assay, the immunofluorescence assay and the radioimmuno precipitation assay. With these antisera it was shown that only one MuMTV protein is expressed on the surface of mouse mammary tumour cells. This conclusion was made with results obtained in the membrane immunofluorescence assay and the humoral cytotoxicity assay.

In Chapter 6 it was examined whether, in addition to the viral protein gp52, other tumour specific antigens are expressed on MuMTV-transformed mammary tumour cells. This appeared not to be the case. This result implies that the putative MuMTV onc-gene product is not expressed on the surface of the MuMTV transformed cell. An analogous situation is encountered in the ASV system where the onc-gene product is localized at the inner side of the cellular membrane.

The ASV onc-gene product was identified by means of sera of rabbits bearing ASV induced tumours. In Chapter 6 MuMTV proteins are demonstrated to be present in milk samples of MuMTV-infected rabbits and rats. So far, no evidence was obtained concerning the oncogenic capacity of MuMTV in these animal species. Possibly the onc-gene product of MuMTV will in the future be identified with sera of rabbits or rats bearing MuMTV induced tumours.

Chapter 7 contains a general discussion on the work presented. It is discussed that the mechanism of ASV induced transformation probably differs from MuMTV induced transformation.

SAMENVATTING

Het muizemammatumor virus (MuMTV) veroorzaakt melkkliertumoren in de muis. Het virus kan worden overgebracht door de moedermelk, een vorm van besmetting die men horizontale transmissie noemt. Het virus is in dat geval als een compleet virusdeeltje aanwezig, en wordt dan aangeduid als exogeen virus.

In alle onderzochte laboratorium muizenstammen en in nagenoeg alle onderzochte wilde muizen heeft men informatie voor MuMTV in het cellulaire DNA aangetoond. Virus dat in die vorm in een diersoort aanwezig is, wordt endogeen virus genoemd; overdracht van endogeen virus op de nakomelingen (via de gameten) wordt verticale transmissie genoemd.

Dat er een oorzakelijk verband bestaat tussen besmetting van muizen met MuMTV en het ontstaan van melkkliertumoren, staat vast; de betrokkenheid van endogeen MuMTV bij het ontstaan van spontane melkkliertumoren, is echter onduidelijk.

In hoofdstuk 1 wordt het replicatie proces van RNA tumorvirussen, de groep van virussen waartoe het MuMTV behoort, besproken. Door transformatie-defectieve varianten te bestuderen werd het duidelijk dat het genoom van RNA-tumorvirussen naast genen, die voor de eiwitcomponenten van het virus coderen, ook nog een zogenaamd onc-gen zou kunnen bevatten. Dit gen wordt gedacht te coderen voor een eiwit, dat uiteindelijk verantwoordelijk is voor de maligne transformatie van de geinfecteerde cel. In sommige RNA-tumorvirus systemen lijkt een dergelijk eiwit tot expressie te komen op het oppervlak van de getransformeerde cel.

In hoofdstuk 2 worden de bij het onderzoek gebruikte materialen en methoden besproken.

Hoofdstuk 3 behandelt de door ons ontwikkelde procedure voor de zuivering van structurele eiwitcomponenten van het MuMTV. Bij het ontwikkelen van de methode werd er naar gestreefd de immunogene eigenschappen van de eiwitten gedurende de isolatie te handhaven. De isolatie werd onder zo mild mogelijke omstandigheden uitgevoerd en daarom werd het gebruik van denaturerende agentia, zoals guanidine-HCl en natrium dodecylsulfaat vermeden.

De aanwezigheid van niet-ionische detergentia in de bij de verschillende chromatografische stappen gebruikte buffers, bleek van beslissend belang voor het doen welslagen van de ontwikkelde isolatiemethode. Door gebruik te maken van diverse chromatografische principes, zoals affiniteitschromatografie, chromatografie over ionenwisse-

laars en gelfiltratie, konden vier virale eiwitten worden gezuiverd. Twee van die eiwitten maken deel uit van de envelop van het rijpe virusdeeltje, terwijl de andere twee eiwitten binnen het virusdeeltje zijn gelocaliseerd.

In het werk beschreven in hoofdstuk 4 worden de eiwitten nader gekarakteriseerd door middel van molekuulgewichtsbepaling, analyse van de aminozuursamenstelling en bepaling van de isoëlektrische punten. De verkregen resultaten werden, samen met gegevens uit de literatuur, samengevat in een model voor de opbouw en samenstelling van het virus (zie Fig. 4.7).

In hoofdstuk 5 wordt aangetoond dat de gezuiverde eiwitten hun immunogene kwaliteiten hebben behouden. Dit bleek uit resultaten verkregen met de competitieve radioimmunoassays, zoals die voor de verschillende eiwitten werden opgezet. Antisera opgewekt tegen de gezuiverde eiwitten, vertoonden geen kruisreactiviteit wanneer ze tegen kapotgemaakt MuMTV werden getest in immunologische testen zoals de dubbele immunodiffusie-test, de immunoelektroforese methode en de radioimmunoprecipitatie-test. Met behulp van deze antisera werd vastgesteld, dat slechts één van de MuMTV-eiwitten tot expressie komt op het oppervlak van de MuMTV-geinduceerde muizemelkkliertumorcellen. Deze conclusie werd getrokken uit resultaten verkregen met de membraan immunofluorescentietechniek en de humorale cytotoxiciteitstest.

In hoofdstuk 6 werd onderzocht of er, naast het virale structurele eiwit gp52 nog andere tumorspecifieke antigenen tot expressie komen op MuMTV-getransformeerde cellen. Dit bleek niet het geval te zijn. Blijkbaar komt het vermeende MuMTV onc-gen produkt niet tot expressie op het oppervlak van de virus-getransformeerde cel. Een analoge situatie wordt aangetroffen in het vogelsarcomavirus (ASV) systeem, waar het onc-gen produkt gelokaliseerd blijkt te zijn aan de binnenzijde van de cel-membraan.

Het ASV onc-gen produkt werd geidentificeerd door middel van sera afkomstig van konijnen die ASV-geinduceerde tumoren droegen.

In hoofdstuk 6 wordt de aanwezigheid van MuMTV-eiwitten in de melk van met MuMTV-geinfecteerde konijnen en ratten aangetoond. Tot nu toe werd geen aanwijzing verkregen omtrent het oncogene vermogen van MuMTV in deze diersoorten. In de toekomst zal blijken, of het onc-gen produkt van MuMTV kan worden geidentificeerd met sera van konijnen of ratten die door MuMTV geinduceerde tumoren dragen.

Hoofdstuk 7 bevat een algemene bespreking van het gepresenteerde werk.

Er wordt aangegeven dat ASV-geinduceerde transformatie volgens een mechanisme verloopt dat waarschijnlijk verschillend is van MuMTV geïnduceerde transformatie.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to:

- Mr. W. Koornstra, Mr. M.A. Dubbeld and Mr. D.S. Luyt for their expert technical assistance.
- Dr. P. Bentvelzen for introducing me into the field of tumour virology, for discussions and for critical reading the manuscript.
- Dr. P. Creemers for her cooperation in several projects and for learning me the humoral cytotoxicity assay.
- Mr. W.C.M. Zwennis of the Medical Biological Laboratory TNO for performing the amino acid analysis.
- Dr. J. Radl of the Institute for Experimental Gerontology TNO for providing several antisera.
- Dr. W.P. Parks for providing the mouse mammary tumour cell line C3HMT/clll.
- Mrs. M.van der Sman for typing and Dr.A.C. Ford and Drs. J.P.M. Jore for aid in the preparation of the manuscript.
- Many other technical and scientific members of the REPGO-TNO Institutes, who made this thesis possible.
- The Netherlands Organization for Health Research TNO for providing facilities and equipment for the performance of this investigation and for financial support.

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