FORMATION OF SUBVIRAL PARTICLES BY TRANSLATION OF SUBGENOMIC POLIOVIRUS RNAs IN A CELL-FREE SYSTEM AND IN *SACCHAROMYCES CEREVISIAE*

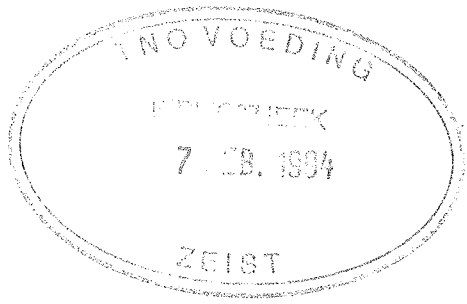
Proefschrift tot het verkrijgen van de graad van doctor in de farmaceutische wetenschappen.

Jan Jore
december 1993

Promotor: Prof. A. Boeyé
Co-promotoren: Prof. B. Rombaut
Prof. P.H. Pouwels

Het onderzoek beschreven in dit proefschrift is uitgevoerd in de afdeling Moleculaire Genetica en Gen-technologie van het Medisch Biologisch Laboratorium van de Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO) onder leiding van Prof. Dr. P.H. Pouwels. Delen van het onderzoek werden uitgevoerd in de afdelingen Biochemie van de Universiteit van Cambridge (hoofd: Dr. R.J. Jackson) en Microbiologie en Hygiëne (faculteit Geneeskunde en Farmacie) van de Vrije Universiteit Brussel (hoofd: Prof. Dr. A. Boeyé).

Niets komt vanzelf
en weinig is blijvend
(Willy Brandt)



Aan mijn vader en moeder
Voor Ans en Matthijs

CONTENTS

GENERAL INTRODUCTION

1. Poliovirus and picornaviruses

1.1 Classification of poliovirus.

Poliovirus, the causative agent of poliomyelitis in man, belongs to the family of picornaviruses (pico, from the Italian piccolo, = small, rna = ribonucleic acid). This family harbours a large variety of human and animal pathogens. Recently, an official division in five genera has been established by the International Committee on Taxonomy of Viruses (Classification and Nomenclature of Viruses, 1991). These genera are:

- Aphthoviruses, comprising the 7 serotypes of foot-and-mouth disease virus (FMDV).
- Enteroviruses, comprising poliovirus (3 serotypes), coxsackieviruses, echoviruses, and others.
- Hepatoviruses (hepatitis A virus (HAV); 4 serotypes).
- Cardioviruses, comprising encephalomyocarditis virus (EMCV), Mengovirus and others.
- Rhinoviruses (common cold viruses; over 100 serotypes).

This division differs from the one suggested by Palmenberg (1989). Based on crystallographic studies and on computer-generated alignments of the P1 genomic region (encoding the capsid proteins, see 1.5) of over 40 picornaviruses, she suggested a division in four genera only, by combining rhinoviruses and enteroviruses in one genus (renteroviruses). Suggestions for a still different division have recently been put forward by Hyypiä *et al.* (1992). They found a molecular base for the unusual biological properties of echovirus 22 and proposed to place it in an additional genus.

Members of the family of picornaviruses are characterized by having:

- a capsid composed of a nonenveloped 60-subunit protein shell (20-30 nm diameter). Each subunit contains four different proteins VP1, VP2, VP3 and VP4. The capsid has intrinsic 5:3:2 icosahedral symmetry.
- a genome consisting of one molecule of single-stranded (positive-sense) RNA. The most remarkable feature of this RNA is the presence of a single translation unit, encoding one giant polypeptide that is co- and posttranslationally cleaved to functional proteins (see 1.5).

1.2 Poliomyelitis and classical vaccines (see also Koch and Koch (1985) and Melnick (1985), and references cited therein).

Until the beginning of this century poliomyelitis (from the Greek polios, =grey and myelos, =marrow, spinal cord) was primarily an occasional disease of infants (hence its Dutch name 'kinderverlamming'= infantile paralysis). This pattern is still seen today in communities with primitive sanitation where the disease is endemic. Spread of virus takes place via the oral-faecal as well as the pharyngeal route.

Vaccination is in principle a very effective way of protection against the effects of a viral infection. Protection is based on the immunological principle that humans and animals are never attacked a second time by the same infectious agent. Vaccination with a less or non-pathogenic form of the infectious agent provides the vaccinated person with defense before infection.

Although the nature of the causative agent for poliomyelitis has been known since 1909, development of a vaccine had to wait for possibilities to grow sufficiently large quantities of the virus in tissue culture, which succeeded in 1949. In the meantime, improvement of sanitation in industrialized countries resulted in a switch in the nature of poliomyelitis from an endemic disease to an epidemic disease, due to the interrupted circulation of the virus and, hence, loss of natural immunity. At the same time adults began to fall victim of the disease as well. The severe paralytic harassment of the central nervous system (=poliomyelitis) must

be regarded as an unpredictable complication of the infection. The vast majority of infections (over 99%), however, is not clinically apparent. Immunization against poliomyelitis became feasible with Salk's formalin-inactivated vaccine (IPV) (Salk *et al.*, 1953) soon followed by Sabin's live attenuated oral vaccine (OPV) (Sabin, 1955). A characteristic difference between the two types of vaccine is that the inactivated vaccine, that is given parenterally, does not induce local (intestinal) immunity, as opposed to the orally given attenuated vaccine (some authors challenge this view, e.g. Selvakumar and John, 1987). Persons who have been immunized with OPV therefore serve as a block to transmission of wild polioviruses. Both vaccines contain the three different serotypes. Partly due to inadequate inactivation, resulting in the notorious Cutter incident (Nathanson and Langmuir, 1963), the United States and the United Kingdom replaced IPV by OPV in their vaccination programmes. In other countries (Canada, Finland, Sweden, The Netherlands) IPV is still used, or IPV in combination with OPV (Israel).

Although both vaccines have proven to be very effective since their introduction, there is an ongoing debate about the immunization policy, to prevent vaccine-associated cases and to eradicate poliomyelitis globally (Beale, 1990; Roberts, 1988; Oostvogel and De Jong, 1991). This debate is fostered by the pros and cons of IPV and OPV, as summarized in Table I (adapted from Oostvogel and De Jong, 1991). The drawbacks of both vaccines are especially prevalent in developing (tropical) countries.

Table 1

Vaccine	Advantages	Disadvantages/dangers
IPV	- Active in tropical countries (temperature stable, no interference by enteric infections) - Can be combined with DPT* - Simplifies virus surveillance	- Expensive to produce - Unclear whether wildtype virus circulation is halted - Incomplete inactivation
OPV	- Cheap - Easy to adminster - Induces gut immunity - Efficient and rapid interruption of virus transmission	- Reversion to wildtype neurovirulence of type 2 and type 3 - No take due to interference in the gut - Inactivation at elevated temperatures - Frustrates virus surveillance

* DPT: diphteria, pertussis, tetanus

1.3 Genomic structure of picornaviruses (Palmenberg (1990), and references cited therein).

Picornaviral RNAs are polyadenylated (50 to 150 A residues) at the 3'-terminus, like most eukaryotic mRNAs. Their 5' ends differ from eukaryotic mRNAs, however: while the latter are capped, i.e. carry a 7-methyl guanylic acid residue that is linked to the primary transcript through a 5'-5' triphosphate bridge, picornavirus genomic RNAs carry a small, virally coded protein VPg. This protein is attached by a tyrosine-O^4-phosphodiester bond to the 5' uridylyl nucleotide of

the RNA. Moreover, unlike the usual cap-structure, VPg is not involved in initiation of protein synthesis, since it is present only in genomic RNA, but is missing in cytoplasmic RNA that is to be translated (Ambros *et al.*, 1978). VPg probably has a role in viral RNA replication, since all newly synthesized RNA molecules, whether they are positive-stranded or negative-stranded, have a 5'-terminal VPg (Petterson *et al.*, 1978). The actual role of VPg in this process is, however, still not clear (Kuhn and Wimmer, 1987). The lengths of the genomic RNAs vary from ~7100 nucleotides (Rhinovirus type 2) to ~8300 nucleotides (FMDV-O1K), not including the poly (A) tail. Each of the picornaviral RNAs encodes a single, large polyprotein. Noncoding regions are present at both 5' and 3' ends (610-1194 nucleotides and 42-126 nucleotides, respectively), of which the 5' noncoding region mediates translational control (see next paragraph).

1.4 Viral protein synthesis.

Eukaryotic ribosomes -more correctly stated eukaryotic 40S ribosomal subunits- make use of a scanning mechanism as part of the initiation of protein synthesis (Kozak, 1983). This scanning is preceded by binding of several initiation factors to the mRNA and formation of a preinitiation complex, as reflected in Fig. 1.

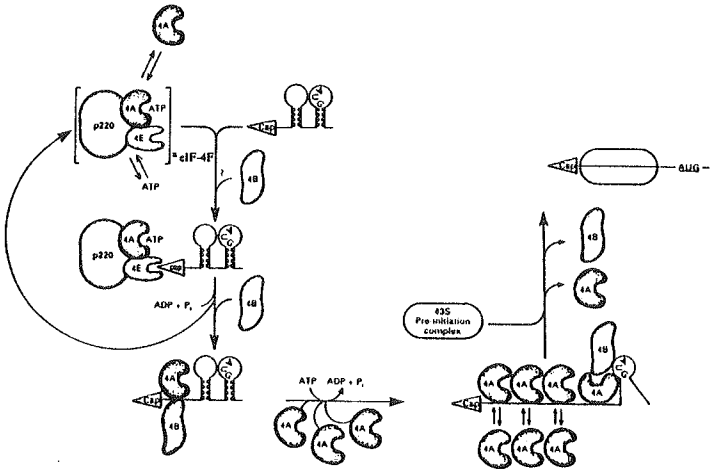


Fig. 1. Cap-dependent ribosome binding to mRNA. See text for details. Adapted from Pelletier and Sonenberg (1989).

Among the initiation factors depicted, the cap binding protein complex (CBPII), also called eIF-4F, is of special interest. It consists of three subunits (Sonenberg and Pelletier, 1989): 1) eIF-4E (eukaryotic initiation factor 4E), which carries the binding site for the cap structure; 2) eIF-4A, that destabilizes mRNA secondary structure in an ATP dependent manner; 3) p220, whose integrity is required for functioning of eIF-4F. The helicase (=destabilizing) activity of 4A is enhanced by 4B, as is its affinity for mRNA. The affinity of 4A for mRNA is further stimulated when it is present as a subunit of eIF-4F. Therefore, it is thought that a key function of eIF-4F is to align eIF-4A on the mRNA, via the cap structure. Once the secondary structure has been melted, 40S ribosomal subunits, supplied with several additional initiation factors, bind to the mRNA. Scanning starts and proceeds until an initiation site is encountered. This initiation site is normally the first AUG codon, but flanking sequences play an important role in the selection (Kozak, 1987; 1990). The actual protein synthesis starts after 60S subunit joining. The general model of initiation of protein synthesis in eukaryotes does not hold for picornavirus protein synthesis, however, as exemplified by poliovirus. As mentioned before, its RNA is not capped. Moreover, it contains a long, nontranslated 5′ region in which -depending on serotype- 8 or 9 AUG codons are present, each followed by a short reading frame, none of which are probably ever used (Pelletier *et al.*, 1988). Finally, poliovirus protein synthesis proceeds even when cap-dependent initiation of protein synthesis is suppressed ["host shut off", occurring shortly after infection of susceptible cells; under these conditions cleavage of p220 can be shown, but whether this cleavage is solely responsible for host shut off is still a matter of debate (Bonneau and Sonenberg, 1987; Pérez and Carrasco, 1992)]. Therefore, an alternative model was developed that does not rely on the presence of a cap structure and a functional eIF-4F (Pelletier and Sonenberg, 1989). In this model (Fig. 2) binding of eIF-4A and 4B results in melting of mRNA secondary structure in the 5′ nontranslated region, followed by ribosome binding at some site, called ribosomal landing pad (RLP). An as yet unidentified factor X, possibly the p52 described by Meerovitch *et al.* (1989), may assist in the binding of 4A and 4B. The concept of internal initiation has been

proven convincingly with the aid of bicistronic mRNAs. Translation of the second cistron (preceded by the poliovirus 5'-nontranslated region) was found to be independent of 5'-end mediated initiation (Pelletier and Sonenberg, 1988; 1989). Analogous experiments, in which the 5'-nontranslated region of EMC virus RNA was employed, yielded similar results (Jang *et al.*, 1988).

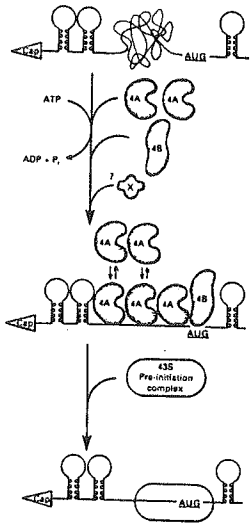


Fig. 2. Cap-independent ribosome binding to mRNA. See text for details. From Pelletier and Sonenberg (1989).

1.5 Co- en posttranslational cleavages.

The cleavages that are responsible for the generation of functional, structural as well as nonstructural proteins, are shown schematically in Fig. 3.

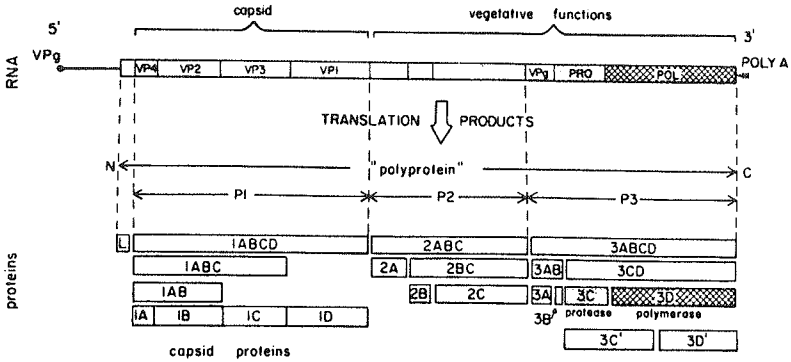


Fig. 3. Structure of the picornaviral genome and cleavage map of the polyprotein. Adapted from Rueckert (1985).

This proteolytic cascade is quite similar among the different members of the family of picornaviruses. There are, however, some deviations from the general scheme as exemplified by poliovirus:

Picornavirus RNA contains a single long open reading frame. The large polyprotein encoded, is cleaved at specific sites and has been divided into the regions P1, P2 and P3. P1 is the capsid precursor, comprising the capsid proteins VP4, VP2, VP3 and VP1, whereas P2 and P3 are precursors of the nonstructural proteins. The primary cleavage event is cotranslational, i.e. takes place as soon as a ribosome has traversed the relevant sequence, resulting in P1 (entero- and rhinoviruses) or L.P1.2A (cardio- and aphthoviruses). While this primary cleavage is catalyzed by 2A in entero- and rhinoviruses (Toyoda *et al.*, 1986; Sommergruber *et al.*, 1989), it is autocatalytic in cardioviruses and probably also in aphthoviruses. Cleavage in the latter genera occurs in a tetrapeptide sequence spanning the 2A/2B junction. All coding regions outside 2A and 2B, as well as large segments of the 2A and 2B coding regions, are dispensable for cotranslational cleavage (Palmenberg, 1990). The activity responsible for generating the precursor L.P1.2A must therefore be either nonvirally coded, or a very short stretch of aminoacids must provide the autocatalytic activity. The latter alternative is supported by the spontaneous cleavage of a synthetic tetrapeptide, containing the viral sequence Asn-Pro-Gly-Pro, to Asn-Pro and Gly-Pro, even upon incubation in buffer only. The identity of the proteolytic agent reponsible for the primary cleavage in hepatitis A virus is still unknown.

Although the P2P3 region is commonly referred to as a region encoding nonstructural proteins involved in processing and replication, functions have not yet been ascribed to all proteins originating from this part of the polyprotein precursor, notably 2B, 2C and 3A. 3B is the genome-linked protein VPg, whereas RNA-dependent RNA synthesis is associated with 3D. 3C is responsible for all defined processing reactions in picornaviruses, with the exception of 1) the primary cleavage event, as described above, and 2) the maturation cleavage (resulting in cleavage of VP0 to VP4 + VP2). A third exception is the cleavage of L.P1.2A to P1.2A in FMDV, which is catalyzed by the leader protein L.

1.6 3-Dimensional structure of picornaviruses

The 3-D structures of poliovirus type 1 (Hogle *et al.*, 1985), rhinovirus type 14 (Rossmann *et al.*, 1985), Mengovirus (Luo *et al.*, 1987) and FMDV type O_1 (Acharya *et al.*, 1989) have been resolved. These viruses represent 4 of the 5 genera in picornaviruses. A type strain of the fifth genus, hepatitis A, has not been subjected to X-ray cristallography yet. Based on aminoacid sequence alignments with the structures of Mengovirus and rhinovirus, Luo *et al.* (1988) predicted 3-D models for both FMDV and hepatitis A virus. Although the FMDV model correctly predicted the basic structural features common to all picornaviruses, it failed to predict the distinctive features of FMDV as described later by Acharya *et al.* (1989), and therefore the predicted 3-D model of hepatitis A virus may be of rather limited value.

From the papers referred to above, features common to all picornaviruses can be summarized as follows:

i. The shell consists of 60 identical protomers, each comprising one copy of VP1, VP2, VP3 and VP4.

ii. The large capsid proteins VP1, VP2 and VP3 form eight-stranded antiparallel wedge shaped ß-barrels flanked by two α-helices (Fig. 4), a structure conserved in the capsid proteins of icosahedral eukaryotic RNA viruses (Rossman and Johnson, 1989). The thin end of the VP1 wedge is directed towards the fivefold axis of symmetry, while the thin ends of the VP2 and VP3 wedge alternate about the particle threefold axis.

The greatest differences between the capsid proteins are in the size and conformation of the loops linking the strands of the ß-barrels and in the N- and C-terminal sequences. This plays a major part in giving the proteins and the viruses their unique structural features (Minor, 1990). Morever, the loops decorate the surface of the virion and mostly determine the antigenic sites of the virus (Hellen and Wimmer, 1992).

iii. The N-termini of the large capsid proteins are internally located, while the C-termini extend from the external surface. The N-termini, including the fully

internally located VP4, form an intertwined network within the virion that contributes significantly to its stability (Hellen and Wimmer, 1992).

iv. The N-terminal aminoacid, glycine, of VP4 is myristoylated via an amide linkage (Chow *et al.*, 1987). From studies on mutant viruses carrying substitutions in the myristoylation consensus sequence, it appears that the myristic acid (C_{14} saturated fatty acid) facilitates the assembly of multimeric protein subunits (Moscufo *et al.*, 1991).

v. No unique structure can be detected in the central cavity of the virion upon X-ray diffraction, indicating a random orientation of the RNA molecule (Rossmann *et al.*, 1985).

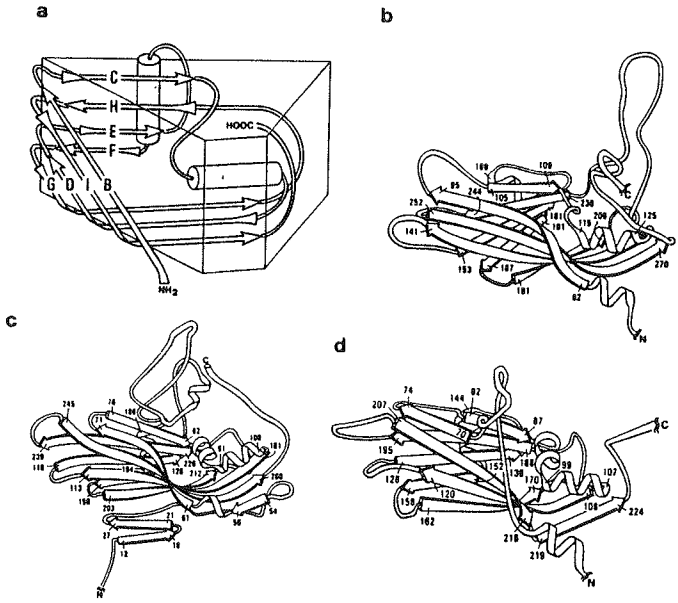


Fig. 4. Schematic representation of poliovirus capsid proteins. (a) Core structure as present in each of the capsid proteins VP1, VP2 and VP3. β-strands are represented as arrows, the flanking helices as cylinders. Ribbon diagrams show VP1 (b), VP2 (c) and VP3 (d). The amino-terminal extensions of VP1 and VP3 and the carboxy-terminal extension of VP1 have been truncated for clarity. From Page *et al.* (1988).

1.7 Poliovirus subviral particles

P1 -the precursor to the capsid proteins- is already cleaved off from the polyprotein P1P2P3 while this protein is still nascent (see 1.5). Processing of P1 results in capsid proteins VP0, VP1 and VP3, that in the so-called L434 nomenclature (Rueckert and Wimmer, 1984) are called 1AB, 1C and 1D, respectively.

While P1 can be found as such in poliovirus-infected cells, the capsid proteins are never found in a free form, but always complexed to one another as subviral or viral particles (Bruneau *et al.*, 1983). The smallest structure in which VP0, VP1 and VP3 are present, is the so-called pentamer, consisting of five copies each of the capsid proteins VP0, VP1 and VP3 (Phillips and Fennell, 1973). Since it sediments in sucrose gradients with an S-value of 14, it is also called the 14S particle. Larger subviral particles have been isolated from infected cells as well, notably 45S particles, consisting of 5 pentamers (Rombaut *et al.*, 1985) and 65S particles (procapsids, consisting of 12 pentamers) (Putnak and Phillips, 1981). [It should be noted here that procapsids are readily converted to 74S particles, even at - 20°C (Rombaut *et al.*, 1989)]. However, since the presence of these larger subviral articles is dependent on the cell type in which the virus is replicating, it is doubtful whether they are true intermediates in poliovirus morphogenesis.

The 14S particle is of special interest, since it is the only particle that is always present in picornavirus infected cells, independent of the cell type (Putnak and Phillips, 1981). Morever, using selective labeling conditions it was shown that polio 14S particles can be converted to virions *in vivo,* be it that an intermediate role of a - supposedly short lived- 65S particle cannot be excluded (Rombaut *et al.*, 1990). This does not detract from the likeliness that the 14S particle is a genuine precursor to virions. Whereas 14S subviral particles can be converted to empty capsids *in vitro* (see next paragraph), their *in vitro* conversion to virions has never been reported. The latter holds for all subviral particles.

1.8 Antigenic structure of poliovirus and subviral particles.

Four neutralizing antigenic sites have been defined, regardless of serotype, by selection of escape mutants with the aid of murine monoclonal antibodies. The location of the four sites is shown in Fig. 5. All epitopes are situated on loops extending from the viral surface. Aminoacid residues involved in the four neutralizing antigenic sites are (Page *et al.*, 1985; Minor, 1990):

i. site 1 : VP1 91-102, 144
ii. site 2 : VP1 221-226; VP2 164-170, 270
iii. site 3A: VP3 58-60, 71,73
iv. site 3B: VP2 72, VP3 76

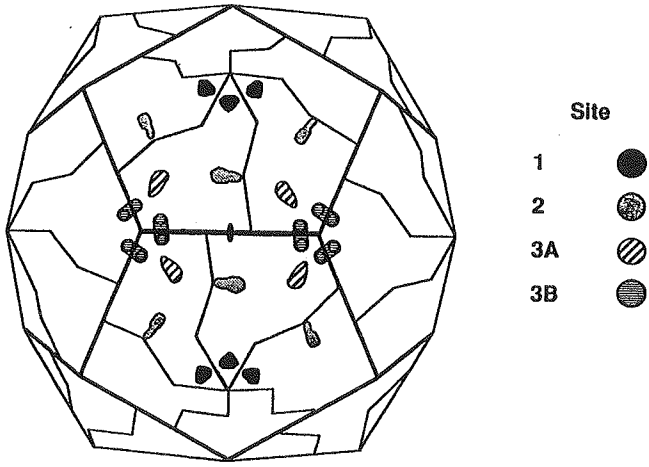


Fig. 5. The surface of a poliovirion, with location of the four neutralization antigenic sites. The limits of the 60 structural units or protomers are shown by thin lines. Pentamers (groups of 5 structural units) are enclosed by bold lines. From Rombaut *et al.* (1990).

Site 3B is a neotope, created by assembly of 14S subunits (see below) into capsids (Rombaut *et al.*, 1990b). The sites 1, 2, 3A and 3B are present on intact poliovirions and are serotype-dependent. In other words, monoclonal antibodies directed against site 1 of type 1 do not react with site 1 of type 2 etc. Native virions are by definition N-antigenic. Denaturation of poliovirus, for instance by gentle heating (56°C), causes release of viral RNA and of capsid protein VP4; the resulting particle ("empty capsid") is characterized as H-antigen. Antibodies directed against such an antigen are not neutralizing.

The antigenic structure of 14S particles has been elucidated by Rombaut *et al.* (1983). They are N1, H-antigenic, meaning that they share antigenic properties of both native and heated virions. In a more extended version 14S particles are now labelled antigenically as [1,2,3A;H], in other words they lack site 3B but carry H-antigen. 14S particles can be converted *in vitro* to empty capsids (74S particles; Putnak and Phillips, 1982). Assembly to 74S particles can only take place if a threshold concentration of 14S subunits is exceeded (Rombaut *et al.*, 1991b). The antigenicity of these 74S particles is, however, dependent on assembly conditions: whereas assembly in the absence of infected cell extracts will yield H-antigenic empty capsids (Rombaut *et al.*, 1984), assembly in the presence of infected cell extracts (Rombaut *et al.*, 1986) or certain chemical agents that are stabilizing against thermal denaturation (Rombaut *et al.*, 1990a; 1991a), will yield empty capsids of antigenic conformation [1,2,3A,3B]. In other words, 74S particles obtained in this manner are antigenically indistinguishable from procapsids and native virions. Since the antigenicity conferring factor is present in infected cell extracts, but not in uninfected cell extracts, it must be either virally encoded or induced; it lacks serotype specificity (Rombaut *et al.*, 1984).

2. Viral subunit vaccines.

It has been known for quite some time that isolated structural components - particularly proteins- of infectious pathogens are often inmunogenic and able to evoke a neutralizing response (see Collett (1989), and references cited therein). The possibility of developing a vaccine based on such isolated structural

components (subunits), is especially attractive in the case of exotic or highly hazardous pathogens and pathogens that can not be propagated in tissue culture or only very poorly so.

The availability of nucleic acid sequencing methods has made it possible to obtain knowledge of virtually any aminoacid sequence rapidly. This, in combination with the use of monoclonal neutralizing antibodies and synthetic peptides, has led to the definition of sequences in viral proteins that are involved in neutralization. For synthesis of the cloned gene product thus defined, a wide variety of biological expression systems have been developed. Yet, in spite of these favourable conditions, progress in the development of viral subunit vaccines has been slower than initially expected, largely due to problems in finding the correct antigenic configuration and presentation to the immune system, as exemplified below:

a) Hepatitis B virus (HBV) (for a review, see Collett, 1989).

HBV poses a major, world-wide public health problem. This virus cannot be propagated in cell culture. Infected individuals contain subviral 22nm particles in their plasma. These particles can be considered as surplus hepatitis B virus surface antigen (HBsAg) and are very immunogenic. They have provided the basis for an effective vaccine. However, being plasma-derived such a vaccine is in limited supply and not without risk. Both yeast and mammalian cell expression systems have been employed for the production of HBsAg. It was found that HBsAg aggregates spontaneously into particles strongly resembling the 22nm particles found in the plasma of infected individuals. The particles are very stable, highly immunogenic and protective and form the basis of the first -and till now only- approved human recombinant vaccine. The spontaneous assembly of HBsAg into 22nm particles is a fortunate feature, since dissociated HBsAgs are 1000-fold less immunogenic.

b) FMDV

The current vaccine against FMDV consists of inactivated virus. Preparation and application of this vaccine is not without problems (see Brown, 1989). Therefore, a recombinant-derived FMDV vaccine has been pursued since long. It was shown that capsid protein VP1 would elicit the formation of low-titered neutralizing antibody (see Brown (1989), and references cited therein). Testing the immunogenic activity of synthetic peptides encompassing the entire sequence of VP1 resulted in attribution of neutralizing activity to peptides representing the aminoacids 141 to 160 and, to a much lesser extend, the aminoacids 194 to 204. Although the 141-160 peptide produced high levels of neutralizing antibody, and even protected guinea pigs against FMDV, it had to be applied in vast molecular excess over the same sequence as present in virus. Clearly, the chief factor influencing the response is the difference in configurations of the aminoacid sequence. Enhancement of the reponse was sought in expressing the peptide as part of a fusion protein with ß-galactosidase (Winther *et al.*, 1986; Broekhuijsen *et al.*, 1987). This did not result in a more immunogenic product then could be obtained by a peptide that was randomly attached to carrier protein by chemical methods. However, 2 or 4 copies of the peptide, expressed head to tail at the amino terminus of ß-galactosidase gave a response in guinea pigs that was several orders of magnitude higher than that obtained with the hybrid containing only one copy of the peptide (Broekhuijsen *et al.*, 1987). An even better reponse, approaching that of inactivated FMDV particles, could be evoked by presenting the peptide sequence as part of a particle-forming hepatitis B core protein (Clarke *et al.*, 1987), be it that this enhancement may be due to immunogenic properties of the carrier protein itself (Brown, 1989). In spite of these promising results, a recombinant-derived subunit FMDV vaccine is not available yet.

All in all it will be clear that even for a relatively simple neutralization antigen like the 141-160 peptide of FMDV, features of antigen configuration and presentation are of prime importance.

c) Poliovirus

Among the four different types of capsid proteins (VP1, VP2, VP3 and VP4), three (VP1, VP2 and VP3) are capable of eliciting a neutralizing reponse, be it inconsistently or very weakly (see Emini *et al.* (1984), and references cited therein). These results were the first indication that multiple neutralization antigenic sites exist on the surface of the poliovirus. Moreover, the influence of the method of isolation on the quality of the response, as observed by several authors (Dernick *et al.*, 1983; van Wezel *et al.*, 1983), already underlined the importance of antigen configuration. The discontinuous character of the majority of neutralization epitopes on poliovirus (see paragraph 1.8) blocks the potential use of separate capsid proteins as a human vaccine.

3. Outline of this thesis.

The aim of the studies described in this thesis was to investigate the possibilities of developing a biosynthetic vaccine, i.e. a non-infectious protective subunit immunogen that is produced in or by a biological system (Collett, 1989). Although primarily oriented at poliomyelitis virus, it was assumed that the results might also be of value for development of biosynthetic vaccines against other picornaviruses. In chapter 2, *in vitro* transcription and translation techniques were used to investigate whether P1, synthesized as such, would serve as an authentic substrate for virally encoded proteases. A second question addressed in this chapter concerned determination of which viral gene products are necessary for processing of P1 to capsid proteins VP0, VP1 and VP3. The answers obtained were used in experiments as described in chapter 3, to determine which sequences are required for formation of 14S and 74S subviral particles *in vitro*. Chapter 4 deals with experiments in which the yeast *Saccharomyces cerevisiae* is used for preparative synthesis of subviral particles. In chapter 5 experiments are described aimed at translation of the whole coding sequence of poliovirus RNA in *S.cerevisiae*, as well as expression studies with both protease encoding sequences in *S.cerevisiae*. Chapter 6 contains final remarks and recommendations for further research. A

summary of the work described in this thesis is presented in chapter 7.

REFERENCES

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D. and Brown, F. (1989). The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. Nature 337, 709-716.

Ambros, V., Petterson, R.F. and Baltimore, D. (1978). An enzymatic activity in uninfected cells that cleaves the linkage between poliovirion RNA and the 5′ terminal protein. Cell 15, 1439-1446.

Beale, A.J. (1990). Poliovaccines: time for a change in immunisation policy? The Lancet 335, 839-842.

Bonneau, A.M. and Sonenberg, N. (1987). Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. Journal of Virology 61, 986-991.

Broekhuijsen, M.P., van Rijn, J.M.M., Blom, A.J.M., Pouwels, P.H., Enger-Valk, B.E., Brown, F. and Francis, M.J. (1987). Fusion proteins with multiple copies of the major antigenic determinant of foot-and-mouth disease virus protect both the natural host and laboratory animals. Journal of General Virology 68, 3137-3143.

Brown, F. (1989). The development of chemically synthesized vaccines. *In:* Vaccine Biotechnology (J.L.Bittle and F.A.Murphy, eds.) pp. 173-193. Academic Press.

Bruneau, P., Blondel, B., Crainic, R., Horodniceanu, F. and Girard, M. (1983). Poliovirus type 1 capsid polypeptides: absence of a free form in the cytoplasm of infected HeLa cells. Annales de Virolologie (Institut Pasteur) 134E, 151-154.

Chow, M., Newman, J.F.E., Filman, D., Hogle, J.M., Rowlands, D.J. and Brown, F. (1987). Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature 327, 482-486.

Classification and Nomenclature of Viruses (1991). Fifth Report of the International Committee on Taxonomy of Viruses (R.I.B. Francki, C.M. Fauquet, D.L. Knudson and F. Brown, eds.). Springer-Verlag, Wien, New York.

Clarke, B.E., Newton, S.E., Carroll, A.R., Francis, M.J., Appleyard, G., Syred, A.D., Highfield, P.E., Rowlands, D.J. and Brown, F. (1987). Improved immunogenicity of a peptide after fusion to hepatitis B core protein. Nature 330, 381-384.

Collett, M.S. (1989). The development of biosynthetic vaccines. *In:* Vaccine Biotechnology (J.L. Bittle and F.A. Murphy, eds.) pp. 109-172. Academic Press.

Dernick, R., Heukeshoven, J. and Hilbrig, M. (1983). Induction of neutralizing antibodies by all three structural poliovirus polypeptides. Virology 130, 243-246.

Emini, E.A., Jameson, B.A. and Wimmer, E.(1984). Identification of multiple neutralization antigenic sites on poliovirus type 1 and the priming of the immune response with synthetic peptides. *In:* Modern approaches to vaccines. Molecular and chemical basis of virus virulence and immunogenicity (R.M. Chanock and R.A. Lerner, eds.) pp. 65-75. Cold Spring Harbor Laboratory.

Hellen, C.U.T. and Wimmer, E.(1992). Maturation of poliovirus capsid proteins. Virology 187, 391-397.

Hogle J.M., Chow M. and Filman D. (1985). Three dimensional structure of poliovirus at 2.9 Å resolution. Science 229, 1358-1365.

Hyypiä, T., Horsnell, C., Maaronen, M., Khan, M., Kakkinen, N., Auvinen, P., Kinnunen, L. and Stanway, G. (1992). A distinct picornavirus group identified by sequence analysis. Proceedings of the National Academy of Sciences U.S.A. 89, 8847-8851.

Jang, S.K., Kräusslich, H-G., Nicklin, M.J.H., Duke, G.M., Palmenberg, A.C. and Wimmer, E. (1988). A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during *in vitro* translation. Journal of Virology 62, 2636-2643.

Kozak, M.(1983) Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiological Reviews 47, 1-45.

Kozak, M.(1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. Journal of Molecular Biology 196, 947-950.

Kozak, M. (1990). Downstream secondary structure facilities recognition of initiator codons by eukaryotic ribosomes. Proceedings of the National Academy of Sciences U.S.A. 87, 8301-8305.

Kuhn, R.J. and Wimmer, E. (1987). The replication of picornaviruses. *In*: The molecular biology of positive strand RNA viruses (D.J. Rowlands, B.W.J. Mahy and M.A. Mayo, eds.) pp. 17-51. Academic Press.

Luo, M., Vriend, G., Kamer, G., Minor I., Arnold E., Rossmann, M.G., Boege, U., Scraba, D.G., Duke, G.M. and Palmenberg, A.C. (1987). The atomic structure of Mengo virus at 3.0Å resolution. Science 235, 182-191.

Luo, M., Rosmann, M.G. and Palmenberg A.C. (1988). Prediction of three-dimensional models for foot-and-mouth disease virus and hepatitis A virus. Virology 166, 503-514.

Meerovitch, K., Pelletier, J. and Sonenberg, N. (1989). A cellular protein that binds to the 5′-noncoding region of poliovirus RNA: Implications for internal translation initiation. Genes and Development 3, 1026-1034.

Minor, P.D. (1990). Antigenic structure of picornaviruses. Current Topics in Microbiology and Immunology, vol. 161, 121-154. Springer-Verlag Berlin Heidelberg.

Moscufo, N., Simons, J. and Chow, M. (1990). Myristoylation is important at multiple stages in poliovirus assembly. Journal of Virology 65, 2372-2380.

Nathanson, N. and Langmuir, A. (1963). The Cutter incident: poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the spring of 1955. I.Background. American Journal of Hygiene 78, 16-18.

Oostvogel, P.M. and de Jong, J.C. (1991). De vaccins voor de mondiale uitroeiing van poliomyelitis. Infectieziekten-bulletin 2(5), 2-7.

Page, G.S., Mosser, A.G., Hogle, J.M., Filman, D., Rueckert, R.R. and Chow, M. (1988). Three-dimensional structure of poliovirus serotype 1 neutralizing determinants. Journal of Virology 62, 1781-1794.

Palmenberg, A. (1990). Proteolytic processing of picornaviral polyprotein. Annual Review of Microbiology 44, 603-623.

Pelletier, J., Flynn, M.E., Kaplan, G., Racaniello, V. and Sonenberg N. (1988). Mutational analysis of upstream AUG codons of poliovirus RNA. Journal of Virology 62, 4486-4492.

Pelletier, J. and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 334, 320-325.

Pelletier, J. and Sonenberg N. (1989). Internal binding of eukaryotic ribosomes on poliovirus RNA: translation in Hela cell extracts. Journal of Virology 63, 441-444.

Petterson, R.F., Ambros V. and Baltimore, D. (1978). Identification of a protein linked to nascent poliovirus RNA and to the polyuridylic acid of negative-strand RNA. Journal of Virology 27, 357-365.

Phillips, B.A. and Fennell, R. (1973). Polypeptide composition of poliovirions, naturally occuring empty capsids, and 14S precursor particles. Journal of Virology 12, 291-299.

Putnak, J.R. and Phillips, B.A. (1981). Picornaviral structure and assembly. Microbiological Reviews 45, 287-315.

Roberts, L. (1988). Change in polio strategy? Science 240, 1145.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1983). Epitope evolution in poliovirus maturation. Archives of Virology 76, 289-298.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1984). *In vitro* assembly of poliovirus empty capsids: antigenic consequences and immunological assay of the morphopoietic factor. Virology 135, 546-550.

Rombaut, B., Vrijsen, R., Delgadillo, R., Vanden Berghe, D. and Boeyé, A. (1985). Characterization and assembly of poliovirus-related 45S particles. Virology 146, 111-119.

Rombaut, B. (1986). Antigeniteit en morfogenese van poliovirus. Thesis doctor Pharmaceutical Sciences. Vrije Universiteit Brussel, Brussels, Belgium.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1986). Assembly factors in poliovirus morphogenesis. Virology 153, 137-144.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1989). Denaturation of poliovirus procapsids. Archives of Virology 106, 213-220.

Rombaut, B., Brioen, P. and Boeyé, A. (1990a). Disoxaril stabilization and immunogenicity of poliovirus procapsids. Journal of General Virology 71, 1081-1086.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1990b). New evidence for the precursor role of 14S subunits in poliovirus morphogenesis. Virology 177, 411-414.

Rombaut, B. and Boeyé, A. (1991a). *In vitro* assembly of poliovirus 14S subunits: disoxaril stabilization as a model for the antigenicity conferring activity of infected cell extracts. Virology 180, 788-792.

Rombaut, B., Foriers, A. and Boeyé, A. (1991b). *In vitro* assembly of poliovirus 14S subunits. Identification of the assembly promoting activity of infected cell extracts. Virology 180, 781-787.

Rossmann, M.G., Arnold, E., Erickson, J.W., Frankenberger, E.A., Griffith, J.P., Hecht, H.J., Johnson, J.E., Kamer, G., Luo, M., Mosser, A.G., Rueckert, R.R., Sherry, B. and Vriend, G. (1985). Structure of a human common cold virus and functional relationship to other picornaviruses. Nature 317, 145-153.

Rossmann, M.G. and Johnson, J.E. (1989). Icosahedral RNA virus structure. Annual Review of Biochemistry 58, 533-573.

Rueckert, R.R. (1985). *In*: Virology (B.N.Fields, ed.), 705-738. Raven Press, New York.

Rueckert, R.R. and Wimmer, E. (1984). Systematic nomenclature of picornavirus proteins. Journal of Virology 50, 957-959.

Sabin, A.B. (1955). Characteristics and genetic potentialities of experimentally produced and naturally occurring variants of poliomyelitis virus. Annuals of the New York Academy of Sciences 61, 924-938.

Salk, J.E., Bennet, B.L., Lewis, L.J., Ward, E.N. and Youngner, J.S.(1953). Studies in human subjects on active immunization against poliomyelitis: I. A preliminary report of experiments in progress. Journal of the American Medical Association 151, 1081-1098.

Sommergruber, W., Zorn, M., Blaas, D., Fessl,F., Volkmann, P., Maurer-Fogy, I., Pallai, P., Merluzzi, V., Matteo, M., Skern, T. and Kuechler, E. (1989). Polypeptide 2A of human rhinovirus type 2: Identification as a protease and characterization by mutational analysis. Virology 169, 68-77.

Sonenberg, N. and Pelletier, J. (1989). Poliovirus translation: A paradigm for a novel initiation mechanism. BioEssays 11, 128-131.

Toyoda,H., Nicklin, M.J.H., Murray, M.G., Anderson, C.W., Dunn, J.J., Studier, F.W. and Wimmer, E. (1986). A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell 45, 761-770.

Van Wezel, A.L., van der Marel, P., Hazendonk, A.G., Boer-Bak, V. and Henneke, M.A.C (1983). Antigenicity and immunogenicity of poliovirus capsid proteins. Developments in Biological Standardization 55, 209-215.

Winther, M.D., Allen, G., Bomford, R.H. and Brown, F. (1986). Bacterially expressed antigenic peptide from foot-and-mouth disease virus capsid elicits variable immunologic responses in animals. Journal of Immunology 136, 1835-1840.

INTRODUCTION TO CHAPTERS 2 AND 3

In chapters 2 and 3 ample use has been made of *in vitro* transcription and translation techniques.

a) *In vitro* transcription.

Using *in vitro* transcription it is possible to obtain very pure RNAs of virtually any desired sequence. These RNAs can be used as probes in various molecular biological techniques. An important application lies in *in vitro* translation (see below) to characterize (c)DNAs and to prepare specific proteins in a biologically active form.

For *in vitro* transcription, vectors were used that carry a promoter from bacteriophage T7. This promoter (like other bacteriophage promoters such as those of SP6, T3 and T5) is highly specific, i.e. is only recognized by its own RNA polymerase (Chamberlin *et al.*, 1970). Moreover, DNAs unrelated to T7 DNA do not contain active T7 promoters (Studier and Moffatt, 1986), nor do they contain termination signals that are efficiently used by T7 RNA polymerases (McAllister *et al.*, 1981). T7 RNA polymerase should therefore be capable of making complete transcripts from almost any DNA that is linked to a T7 promoter. Since *E.coli* polymerases do not recognize T7 promoter motifs, the (c)DNA sequence of interest can be introduced and amplified on plasmids in *E.coli* without concomitant expression of the the (c)DNA-coded protein. Plasmid DNA is then isolated, linearized at a site downstream of the (c)DNA insert to provide for a transcription termination site (and thus for defined 3′ ends) and *in vitro* transcription is carried out. Besides being very selective, transcription by T7 RNA polymerase is very efficient. From 1 μg of template DNA several μg's of RNA can be obtained. After purification of the RNA product from the transcription reaction it is translated *in vitro* (see below).

The gene for T7 RNA polymerase has been cloned and highly active preparations can be obtained commercially.

b) *In vitro* translation.

The *in vitro* translation system that has been used in chapters 2 and 3, and that is based on rabbit reticulocyte lysates, has been described by Jackson and Hunt (1983). Briefly, rabbits are made anaemic by injecting small amounts of acetylphenyl hydrazine. The need for extra oxygen-transporting capacity in the blood is met by a strongly increased number of reticulocytes. Unlike their fully mature form, erythrocytes, these reticulocytes still possess protein synthesizing capacity, be it that this is mainly devoted to the synthesis of globin. Reticulocytes are isolated, lysed and treated with micrococcal nuclease to destroy any globin mRNA. The nuclease is then inactivated and the lysate, complemented with several components, can be used to translate exogenous RNA, for instance RNA from an *in vitro* transcription reaction. Provided that certain precautions are taken, a high fidelity of translation can be obtained.

REFERENCES

Chamberlin, M., McGrath, J. and Waskell, L. (1970). New RNA polymerase from *Escherichia coli* infected with bacteriophage T7. Nature (London) 228, 227-231.

Jackson, R.J. and Hunt, T. (1983). Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. Methods in Enzymology 96, 50-74.

McAllister, W.T., Morris, C., Rosenberg, A.H. and Studier F.W (1981). Utilization of bacteriophage T7 late promoters in recombinant plasmids during infection. Journal of Molecular Biology 153, 527-544.

Studier, F.W. and Moffatt, B.A (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Journal of Molecular Biology 189, 113-130.

POLIOVIRUS PROTEIN 3CD IS THE ACTIVE PROTEASE FOR PROCESSING OF THE PRECURSOR PROTEIN P1 *IN VITRO*.

Jan Jore[1], Bernard de Geus[1], Richard J. Jackson[2], Peter H. Pouwels[1] and Betty E. Enger-Valk[1].

[1]Medical Biological Laboratory TNO, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands
[2]Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

Published in Journal of General Virology (1988), 69, 1627-1636.

SUMMARY

A transcription/translation system for generating poliovirus proteins *in vitro* has been used to assess the proteolytic activity of various polypeptides containing the virus-coded 3C region towards the poliovirus precursor protein P1. Plasmids containing a phage T7 promoter followed by either the complete poliovirus P1 sequence or various sequences containing the 3C region were used for this purpose. We showed that all except one of the 3C-containing polypeptides had a very restricted activity towards P1, generating only a small amount of VP1 and no VP0 or VP3. The only polypeptide capable of fully processing P1 into VP0, VP3 and VP1 *in vitro* was protein 3CD, consisting of the complete 3C and 3D sequences.

INTRODUCTION

Poliovirus is a member of the enterovirus subgroup of the picornavirus family. All picornavirus genomes encode a single, large precursor polyprotein, which is processed in a series of proteolytic steps to yield the virion capsid proteins and nonstructural proteins (reviewed by Nicklin *et al.*, 1986). In the case of poliovirus, evidence has been presented that protease 2A is responsible for cleavage of a tyrosine-glycine (Y-G) bond generating the capsid precursor protein P1 (Toyoda *et al.*, 1986; Nicklin *et al.*, 1987). A second viral protease is involved in cleavage of glutamine-glycine (Q-G) bonds, including processing of P1 into the viral capsid proteins VP1, VP3 and VP0 (Hanecak *et al.*, 1982). The final processing event, cleavage of VP0 into VP4 and VP2, may occur by an autocatalytic mechanism, in which viral RNA serves as a proton acceptor (Nicklin *et al.*, 1986).
The second viral protease is usually referred to as protein 3C, which is the smallest molecule that contains the sequences required for Q-G cleavage (Hanecak *et al.*, 1982). From expression studies in bacterial cells it can be concluded that a protein of the size of mature 3C is generated from a longer precursor by a proteolytic process requiring the 3C protein sequence (Hanecak *et al.*, 1984; Ivanoff *et al.*, 1986). Nevertheless, although data have been presented indicating that 3C alone is sufficient for cleavage at Q-G sites

in the P2 region (Ypma-Wong and Semler, 1987), it has been suggested that processing of P1 may require additional sequences, possibly from the 3B and/or 3D regions (Hanecak *et al.*, 1982; Ypma-Wong and Semler, 1987). However, no direct test has been made to discover which polypeptide containing the 3C region is responsible for efficient processing of P1.

Because of our interest in the possibility of obtaining poliovirus intermediate structures such as 14S particles and/or procapsids (Rombaut *et al.*, 1983 and references mentioned therein) without the use of infectious poliovirus RNA, we wished to know which 3C-containing polypeptide is required to process P1 into the capsid proteins VP0, VP1 and VP3. To address this question, we constructed plasmids containing the phage T7 promoter which encoded either the precursor protein P1 or various 3C-containing proteins. The set of constructed plasmids encoded proteins harbouring either 3C sequences only (protein 3C), 3C plus part of 3A and the complete 3B sequences (protein 3A'BC), 3C plus part of 3D (protein 3CD'), 3C and the complete 3D sequences (protein 3CD) or 3C with both 5' and 3' sequences (protein 3A'BCD'). Some of these 3C-containing proteins possess autocatalytic activity and generate a product that comigrates with 3C. However, this autocatalytic activity appeared to have no correlation with the capacity of these 3C-containing proteins to process P1 into VP0, VP1 and VP3: protein 3A'BC, like mature protein 3C, generated only a small amount of VP1. On the other hand, protein 3CD but not 3CD' was found to be capable of efficiently processing P1 *in vitro* into the separate viral capsid proteins.

METHODS

Restriction endonucleases, enzymes and linkers.

Restriction endonucleases were purchased from New England Biolabs, Boehringer and Bethesda Research Laboratories. T4 DNA ligase, calf intestinal phosphatase and T4 polynucleotide kinase were purchased from Boehringer. RNasin was obtained from Promega Biotec. Cap analog [m^7G(5')ppp(5')G] was purchased from Pharmacia. T7 RNA polymerase was purified from an overproducing clone as has been described by

Tabor and Richardson (1985). Synthetic oligonucleotides were purchased from Dr J. v. Boom (University Leiden, the Netherlands).

Bacterial strains and plasmids.

E. coli JA221 [C600, *trp*E5, *leu*B6, *str*, *lac*Y1, *hsd*R, *hsd*M] (Clarke and Carbon, 1978) was used as the host strain for all plasmids and constructions. *E. coli* GM48 [*dam*-3, *dcm*-6, *thr*-1, *leu*-6, *thi*-1, *lac*Y, *gal*K2, *gal*T22, *ara*-14, *ton*A31, *tsx*-78, *sup*E44] (Marinus, 1973) was used for obtaining plasmid DNA that was digestible with *Sau*96I.

Plasmids pVR104 (Racaniello and Baltimore, 1981b), pT7-1 (Ypma-Wong and Semler, 1987) and pT7-6 were gifts of Drs D. Baltimore, B. Semler and S. Tabor, respectively. Plasmid pVR104 comprises the cDNA sequence of poliovirus type 1 Mahoney, starting at nucleotide 114, cloned into the *Pst*I site of pBR322. Plasmid pT7-1 comprises the entire cDNA sequence of poliovirus type 1 Mahoney inserted into the *Eco*RI site of pGEM-1 (Promega Biotec). Plasmid pT7-6 (Fig. 1a) contains the T7 promoter region followed by a series of unique cloning sites.

Construction of T7 plasmids.

a) The P1-encoding vector pLOP324 (Fig. 1A).

The *Hgi*AI/*Nde*I fragment (poliovirus nucleotides 747-3381; Racaniello and Baltimore, 1981a), isolated from pVR104, was provided at the 5′ end with the synthetic oligonucleotide 5′ AGCTTCCACCATGGGTGCT 3′, restoring the original 5′ sequence up to the
AGGTGGTACCCC
translation initiation codon (ATG) of the P1-coding sequence. The translation initiation codon is preceded by a *Hin*dIII site and nucleotides that conform to the optimal context for efficient initiation (Kozak, 1984). The *Nde*I end of the fragment was provided with the synthetic oligonucleotide 5′ TATTAGTAAGTT 3′, restoring the 3′ end of the P1-coding
AATCATTCAA
sequence. The latter linker also provides translational stop codons at the 3′ end of the P1 sequence. The fragment thus generated was joined to the *Hin*dIII/*Sma*I fragment of pT7-6 resulting in the plasmid pLOP324.

b) The 3C-encoding transcription vectors pLOP311 to 315 (Fig. 1A, B).

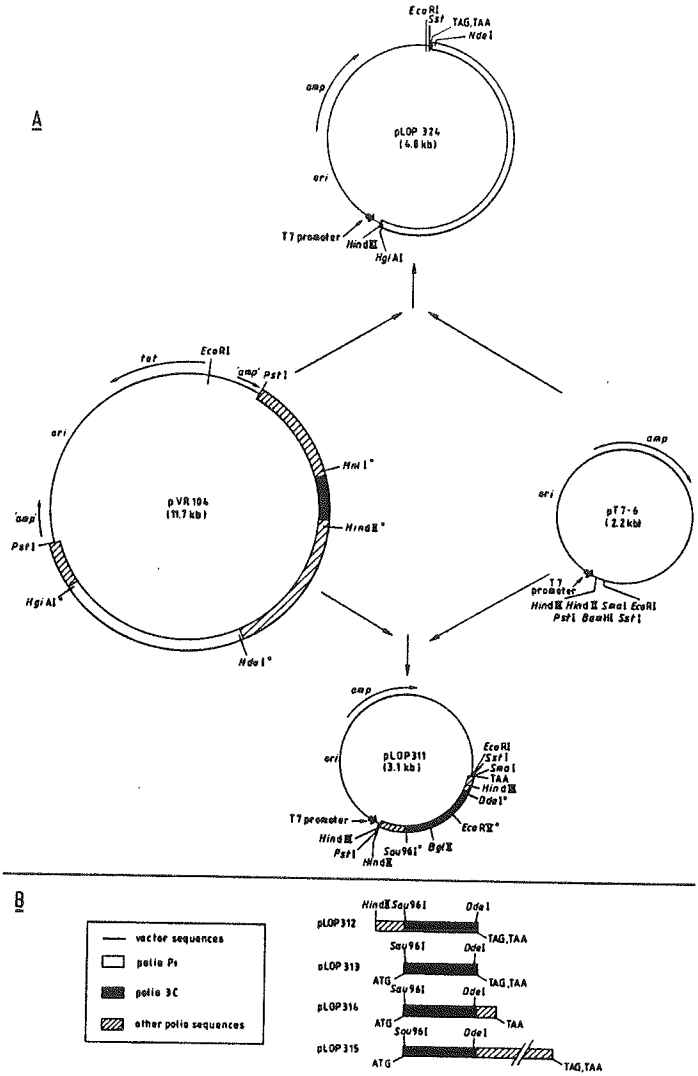


Fig. 1. Schematic presentation of transcription vectors. The plasmid tetracycline and ampicillin resistance genes are marked *tet* and *amp*, the origin of replication *ori*. (A) Parental vectors and strategy for construction of vectors pLOP324 and pLOP311 and (B) diagram of the poliovirus-specific inserts in vectors pLOP312 to -315. Only relevant cleavage sites for restriction enzymes are shown. Non-unique cleavage sites are marked by an asterisk. Open, shaded, and hatched boxes represent poliovirus sequences P1, 3C and other poliovirus sequences respectively.

The parental plasmid of this series, pLOP311, was constructed as follows.

A *Hin*dII/*Mnl*I fragment (poliovirus nucleotides 5240-6112; Racaniello and Baltimore, 1981a) was inserted into plasmid pT7-6 which had been digested with *Hin*dII and *Bam*HI. The *Bam*HI site was ligated to the oligonucleotide 5′ CCAGATAA 3′ providing a
GGTCTATTCTAG
translational stop codon. The translational start codon ATG is present within the poliovirus 3A sequence (ATG at nucleotides 5288 to 5290; Racaniello and Baltimore, 1981a). The plasmid thus obtained, pLOP311, codes for a polypeptide which is denoted 3A′BCD′; a schematic diagram of it is shown in Fig. 1A. This 3C-containing plasmid was used for construction of the various 3C vectors of which the poliovirus-specific parts are schematically depicted in Fig. 1B.

Plasmid pLOP312 comprises the 2.75 kb *Eco*RV/*Sst*I fragment and the 0.17 kb *Eco*RV/*Dde*I fragment from pLOP311; the *Dde*I end was joined to the *Sst*I end using an oligonucleotide linker with the sequence 5′ TCAGAGTCAATAGTAAGAGCT 3′. The polylinker
TCTCAGTTATCATTC
sequence joined the translational stop codons to the C-terminal codon of the 3C sequence. The translation initiation codon is the same as in plasmid pLOP311. Plasmid pLOP312 codes for a polypeptide which is denoted 3A′BC.

Plasmid pLOP313 was obtained by ligation of the *Sau*96I/*Eco*RV fragment (containing the 5′ part of 3C) and the *Eco*RV/*Pst*I fragment (containing the 3′ part of 3C and the pT7-6 vector) of plasmid pLOP312; a *Pst*I/*Sau*96I linker 5′ CCAGCATGG 3′,
CGTCGGTGGTACGCCTG
containing a translation initiation codon ATG in an optimal context (Kozak, 1984), was used for joining the fragments at the *Pst*I- *Sau*96I junction. The resulting vector contained 3C sequences only, preceded by a translational start codon and therefore coded for a protein referred to as 3C.

Plasmid pLOP314 comprises the 2.5 kb *Eco*RV/*Pst*I fragment of pLOP311 (obtained by partial *Eco*RV and complete *Pst*I digestion) and the 0.37 kb *Eco*RV/*Pst*I fragment from plasmid pLOP313. The polypeptide encoded by this plasmid is designated 3CD′.

Plasmid pLOP315 was obtained by ligating the large *Bgl*II/*Sst*I fragment from pLOP313 to the small *Bgl*II/*Sst*I fragment from pT7-1. This plasmid encodes a polypeptide which is denoted 3CD.

***In vitro* transcription with T7 polymerase.**

Prior to transcription, the DNA template was linearized with an appropriate restriction enzyme, extracted with phenol, precipitated with ethanol and dissolved in sterile double-distilled water to a concentration of 0.5 μg DNA/μl. Transcription reactions were carried out as described by Verver *et al.* (1987). Briefly, 2.5 μg DNA was transcribed in a reaction mixture (25 μl) containing 40 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 10 mM DTT, 1mM ATP, CTP and UTP, 0.2 mM GTP and 1 mM $m^7G(5')ppp(5')G$ to which 100 μg/ml BSA, 25 to 50 units T7 RNA polymerase and 20 to 40 units RNasin were added. The GTP concentration was increased to 0.6 mM after 5 min incubation at 37°C and subsequently to 1 mM, again after 5 min incubation. The incubation was stopped after 20 min at 37°C by extraction with phenol and precipitation with ethanol. A sample of the RNA thus obtained was glyoxylated and electrophoresed on an agarose gel to check both the size and the yield. The RNA was used in translation experiments without prior DNAse treatment.

***In vitro* translation of transcripts.**

Messenger RNA-dependent rabbit reticulocyte lysate was prepared according to previously published procedures (Jackson and Hunt, 1983). Conditions for protein synthesis were, excluding the contribution of the lysate, 10 mM creatine phosphate, 0.1 mM aminoacids (no methionine), 80 mM KCl, 0.1 mM $MgCl_2$, 10 mM DTT and, if a labelled product was required, ^{35}S-methionine (10 μCi/25 μl reaction mixture). Optimal concentrations of RNA, defined as the concentrations showing a high yield of the authentic product and minimal relative yield of aberrant products (Dorner *et al.*, 1984), were determined by translation of a range of serial RNA dilutions in reaction mixtures containing ^{35}S-methionine and analysis of proteins patterns on polyacrylamide-SDS gels. If unlabelled proteins were required, translation was checked by separately incubating a small sample of the reaction mixture in the presence of ^{35}S-methionine. In order to ensure faithful initiation of translation, protein synthesis of virus RNA was performed in the presence of uninfected HeLa cell extracts (Dorner et *al.*, 1984). Translation reactions were carried out at 30°C for time periods that were calculated to be sufficient for full-length translation, that is from 20 min for pLOP313 RNA to 75 min for viral RNA. In

order to analyse polypeptide processing, samples from translation reactions were supplemented with cycloheximide and RNase A to final concentrations of 0.5 mM and 10 μg/ml respectively. These preparations were then incubated at 30°C for the indicated periods (see legends to figures). Finally, all samples were diluted 1:1 by addition of 100 μg/ml RNase A in 10 mM EDTA and incubated for 20 minutes at room temperature. Analysis of the products synthesized was performed by subjecting the mixtures to electrophoresis in 15% polyacrylamide-SDS gels. Before electrophoresis, the translation mixtures were diluted with Laemmli sample buffer (Laemmli, 1970) and boiled for 2 min. After electrophoresis the gels were fixed in 7% acetic acid, soaked in Amplify (Amersham), dried and exposed to preflashed Fuji RX film at -70 °C.

RESULTS

Generation of defined RNAs by *in vitro* transcription of various DNAs.

In order to synthesize defined transcripts of poliovirus cDNA that can subsequently be used to program an *in vitro* translation system, we have cloned various DNA fragments downstream of a T7 promoter region. Plasmid pLOP324, the P1 vector, contains the poliovirus coding sequence P1 from nucleotide 743 to 3386 (Racaniello and Baltimore, 1981a), preceded at the 5′ side by nucleotides that conform to the Kozak rule (Kozak, 1984) and which are expected to be required for efficient translation. The ATG codon proximal to the promotor is the authentic poliovirus precursor start codon. At the 3′ end, translation stop codons were introduced allowing the synthesis of precursor P1 without the requirement for proteolytic processing by protease 2A.

The 3C plasmids pLOP311 and pLOP312 both contain 3B and 3C-coding sequences as well as part of the 3A sequences (pLOP312), or parts of both 3A and 3D sequences (pLOP311). In both cases, protein synthesis can start at an in-phase translation initiation codon present in the 3A-coding sequence (Racaniello and Baltimore, 1981a). Experiments with virion RNA *in vitro* showed that initiation of translation occurred efficiently in the P3 region (Dorner *et al.*, 1984). The other 3C plasmids all contain the 3C-coding sequences, with either no additional sequences (pLOP313), part of the 3D

sequences (pLOP314), or the whole 3D sequence together with the complete 3′ non-translated region (pLOP315). In these plasmids, protein synthesis starts at an ATG codon introduced directly upstream of the 3C-coding sequence using a synthetic oligonuleotide that supplies the optimal context for initiation (Kozak, 1984).
On *in vitro* transcription, all plasmids yielded RNAs of the expected size with similar efficiencies (data not shown).

***In vitro* translation of P1 RNA and cleavage by a virion RNA-directed lysate.**
Run-off transcripts of plasmid pLOP324 were translated *in vitro* with rabbit reticulocyte lysate in the presence of ^{35}S-methionine. Optimal concentrations of RNA, determined from a serial dilution range, provided synthesis of a specific protein of the expected size (Fig. 2, lanes 2 and 3). In order to assess whether this plasmid-coded P1 has properties similar to those of the natural capsid precursor protein P1, that is, whether it can be processed to the viral capsid proteins VP0, VP3 and VP1, the protein P1 was subjected to processing by virion RNA-encoded proteases. To this end, a sample of the *in vitro* translated ^{35}S-labeled P1 preparation was mixed with an equal volume of an unlabelled translation mixture which had been incubated with virion RNA. In a separate *in vitro* translation experiment in the presence of ^{35}S-methionine (Fig. 2, lane 1) this virion RNA was shown to direct synthesis of the viral capsid proteins VP0, VP1 and VP3, indicating that the virion-RNA directed lysate contained proteins that were capable of proteolytic processing of P1 synthesized *in vitro*. From the data in Fig. 2 it can be concluded that the plasmid-encoded protein P1 is cleaved quite efficiently into proteins with molecular weights corresponding to those of the viral capsid proteins only upon addition of the virion RNA-directed lysate.

***In vitro* translation and autocatalytic cleavage of various 3C-containing polypeptides.**
Transcripts of plasmids pLOP311, -312, -313, -314 and -315 were translated in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine. The data in Fig. 3 show that all transcripts directed the efficient synthesis of proteins of the expected molecular weights. In the case of pLOP311 (protein 3A′BCD′) and pLOP312 (protein 3A′BC) a smaller product was also observed; on prolonged incubation the yield of the smaller protein

increased and that of full-length product decreased. In the case of pLOP312 (protein 3A'BC) the smaller processed product comigrated with the protein 3C encoded by pLOP313 (Fig. 3). On prolonged incubation (overnight) of the 3A'BCD' preparation some 3C could also be detected (data not shown). However, only very low amounts of 3C were generated, making the data irreproducible. No protein of the size of mature 3C could be detected in pLOP314 (protein 3CD') and pLOP315 (protein 3CD) preparations.

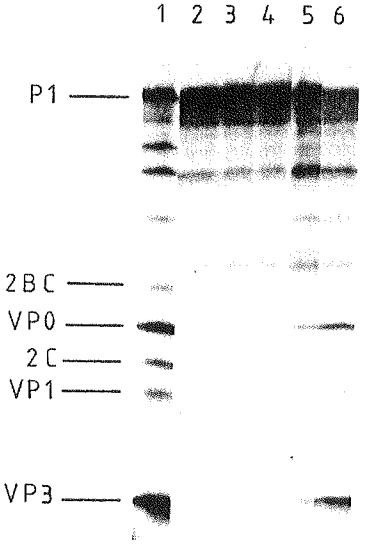


Fig. 2

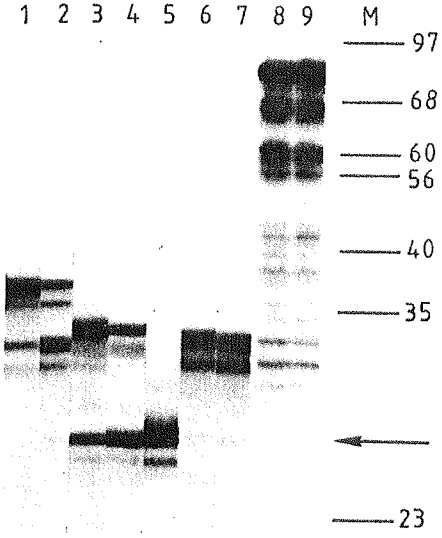


Fig. 3

Fig. 2. Analysis of processing of *in vitro* synthesized pLOP324-P1 protein by virion RNA-encoded proteins. Translation reactions were carried out as described in Methods. After translation cycloheximide and RNase were added. Incubation was resumed and samples, taken at various time points and subsequently incubated with RNase, were added to Laemmli sample buffer. Samples taken at time points 0 min (lanes 2 and 4), 15 min (lane 5) and 80 min (lanes 3 and 6) were subjected to electrophoresis. The reactions were programmed as follows: lane 1, virion RNA; lane 2 to 6, pLOP324 RNA. Lanes 2 and 3 received 10 μl control lysate; lanes 4, 5 and 6 received 10 μl virion RNA-coded lysate. Control lysate contains no added RNAs.

Fig. 3. Analysis of autocatalytic activity of 3C-containing polypeptides. Translation reactions were carried out and stopped as described in Methods and in legend to Fig. 2. Samples taken at time points 15 min (lanes 1, 3, 6 and 8) and 80 min (lanes 2, 4, 5, 7 and 9) were subjected to electrophoresis. The reactions were programmed as follows: lanes 1 and 2, pLOP311 RNA; lanes 3 and 4, pLOP312 RNA; lane 5, pLOP313 RNA; lanes 6 and 7, pLOP314 RNA; lanes 8 and 9, pLOP315 RNA. The M_r values ($x10^{-3}$) on the left side refer to protein markers. The arrowhead indicates the position of mature protein 3C.

Cleavage of precursor protein P1 by the various 3C-containing polypeptides.

To determine the proteolytic activity of the 3C-containing polypeptides towards the P1 molecule, *in vitro* translated ^{35}S-labelled P1 was incubated with *in vitro* prepared unlabelled 3C-containing proteins encoded by the plasmids pLOP312 and pLOP313. The data in Fig. 4 (lanes 4 and 6) show that incubation of P1 with proteins 3A'BC or 3C generated a very low amount of VP1. This processing seemed to be very inefficient and the further cleavage products of P1, that is VP0 and VP3, could not be detected even on prolonged exposure of the fluorogram. In order to check the correctness of the active site (C-terminal portion; Hanecak *et al.*, 1984; Ivanoff *et al.*, 1986) of the 3C encoded by pLOP313 (and the related plasmids pLOP311, -312 and -314), plasmid P1-P2-3ABC was constructed. *In vitro* translation of the RNA from this plasmid (data not shown) indicated a processing activity similar to that of the pT7-1 RNA (Ypma-Wong and Semler, 1987), that is only traces of the processed products VP1 and high yields of the proteins 2A, 2C and 2BC. This demonstrates that the 3C entity from the plasmid pLOP313 and its derivatives harbours the correct poliovirus sequence.

To assess whether the presence of other poliovirus proteins might render these 3C-containing proteins more active, processing of the precursor protein P1 was assayed in the presence of the products from the poliovirus P2 region. Plasmid pT7-1, linearized at a restriction site (*Bgl*II) in the 3C sequence, has been found to be a suitable template for such a study. *In vitro* translation with this truncated template gave proteins from the P1 and the P2 region, but without the appearance of processing at the Q-G bonds in the P1 region (Ypma-Wong and Semler, 1987; Fig. 5, lane 2). This pT7-1-encoded ^{35}S-labelled protein preparation was mixed with the *in vitro* synthesized protein 3A'BC (pLOP312) or 3C (pLOP313). The results in Fig. 5 (lanes 3 to 6) show that similarly to the experiments with P1 encoded by plasmid pLOP324, VP1 could be detected but no VP0 or VP3. These data show that the 3C proteins are equally inefficient at processing P1 generated from a longer precursor protein (P1-P2), and that they are not activated by P2-encoded proteins.

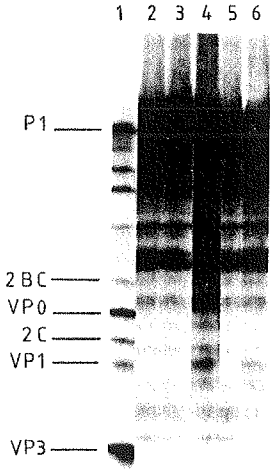


Fig. 4

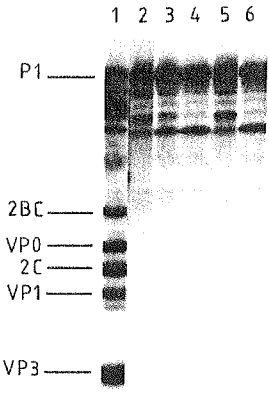


Fig. 5

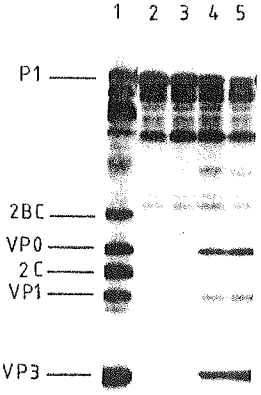


Fig. 6

Fig. 4. Analysis of processing activity of proteins 3A'BC and 3C towards P1 encoded by pLOP324. Translation reactions and subsequent incubations were carried out as described in Methods and in legend to Fig. 2. Samples taken at time points 0 min (lanes 3 and 5) and 80 min (lanes 2, 4 and 6) were subjected to electrophoresis. The reactions were programmed as follows: lane 1, virion RNA; lanes 2, 3, 4, 5 and 6, pLOP324 RNA. Lane 2 received an equal volume of control lysate (no added RNA); lanes 3 and 4 received protein 3A'BC (pLOP312); lanes 5 and 6 received protein 3C (pLOP313). It should be mentioned that the fluorogram (except lane 1) has been exposed for a prolonged period to allow detection of protein VP1.

Fig. 5. Analysis of processing activity of proteins 3A'BC and 3C towards P1 encoded by pT7-1. Translation reactions and subsequent incubations were carried out as described in Methods and in legend to Fig. 2. Samples taken at time points 0 min (lanes 3 and 5) and 80 min (lanes 2, 4 and 6) were subjected to electrophoresis. The reactions were programmed as follows: lane 1, virion RNA; lanes 2, 3, 4, 5 and 6, pT7-1 RNA. Lane 2 received an equal amount of control lysate (no added RNA); lanes 3 and 4 received protein 3A'BC (pLOP312); lanes 5 and 6 received protein 3C (plasmid pLOP313). Plasmid pT7-1 was digested with *Bgl*II before use as template for RNA synthesis.

Fig. 6. Analysis of processing activity of protein 3CD towards P1 encoded by pLOP324. Translation reactions and subsequent incubations were carried out as described in Methods and in legend to Fig. 2. Samples taken at time points 15 min (lane 4) and 80 min incubation (lanes 2, 3 and 5) were subjected to electrophoresis. The reactions were programmed as follows: lane 1, virion RNA; lane 2 received an equal amount of control lysate (no added RNA); lane 3 received protein 3C (pLOP313); lanes 4 and 5 received protein 3CD (pLOP315). It should be noted that, in contrast to results shown in Fig. 4, protein VP1 is barely detectable in lane 3, because of the much shorter exposure time of the fluorogram.

Since the other 3C-containing proteins 3A'BCD' and 3CD' showed essentially similar inefficient processing activities (data not shown), it is likely that poliovirus sequences other than the P2 or 3AB are required for cleavage of the Q-G bonds in the precursor

protein P1. As the 3D protein sequence downstream of nucleotide 6112 was the only sequence not yet included in our study, plasmid pLOP315 was constructed and used for synthesis of the longest form of the 3C protein, that is protein 3CD. The data in Fig. 6 (lanes 4 and 5) clearly show that this form of the 3C protein efficiently generates all three capsid proteins, that is VP0, VP1 and VP3 in proportions similar to those obtained with virion RNA translated *in vitro*.

DISCUSSION

Formation of picornavirus is dependent on proper proteolytic processing of a polyprotein to yield the mature structural and nonstructural proteins. In the case of encephalomyocarditis virus proteolytic activity towards P1 has been unequivocally assigned to the 3C protein (see Parks *et al.*, 1986 and references mentioned therein). In contrast, data for poliovirus suggest that whereas 3C is required for proteolytic processing of P1 (Hanecak *et al.*, 1982) this sequence in itself is not sufficient (Ypma-Wong and Semler, 1987). The present study using *in vitro* transcription and translation of defined poliovirus sequences has been carried out to investigate which 3C-containing protein is necessary for proper processing of P1. To exclude the influence of other poliovirus proteins we constructed a vector, pLOP324, whose run-off transcripts yield only P1 aminoacid sequences. This P1 preparation was used as a substrate for analysing proteolytic activity of the various 3C-containing polypeptides described in this paper.
Our study with the various 3C-encoding templates shows that 3C and 3C-containing polypeptides do have proteolytic activity *in vitro* for a number of Q-G bonds in the poliovirus precursor polyprotein. As deduced from the data in Fig. 3, a protein of the size of mature 3C is formed with template pLOP312 encoding 3A'BC. This indicates that the B-C junction is prone to autocatalytic cleavage. In addition, the appearance of a low amount of mature 3C with template pLOP311, encoding protein 3A'BCD', indicates that some proteolytic cleavage of the C-D junction also takes place, although less efficiently than at the B-C junction. The requirement for two cleavages might render formation of mature 3C from protein 3A'BCD' less efficient. Alternatively, the low

amount of mature 3C obtained with the latter template might indicate that the C-D junction is less susceptible to cleavage than the B-C junction.

Finally, these data might be explained by assuming inhibition or even prevention of C-D cleavage once the B-C junction has been cleaved. The absence of detectable amounts of mature 3C with templates pLOP314 (encoding protein 3CD') and pLOP315 (encoding protein 3CD) favours the latter hypothesis. The high yield of protein 3CD and very low yield of protein 3C on translation of viral RNA both *in vivo* and *in vitro* (Ypma-Wong and Semler, 1987) might also result from this impaired C-D cleavage.

In addition to autocatalytic activity, the 3C-containing polypeptides, except protein 3CD, also possess proteolytic activity towards the precursor protein P1. However, in contrast to the protein patterns of poliovirus RNA translated *in vivo* (Dorner *et al.*, 1984) and *in vitro* (Ypma-Wong and Semler, 1987), and to those of P1 processed *in vitro* with a preparation of virion RNA-encoded protease (Fig. 4), only small amounts of VP1 are formed. Even after prolonged incubations of the protein P1 with the various 3C-containing preparations and after long exposure of the fluorogram no VP0 and/or VP3 can be detected. Taking into account that protein VP1 contains fewer methionine residues than VP0 and VP3 it is to be expected that, under normal processing conditions, VP0 and VP3 would certainly be detectable.

These results may suggest the possibility that the Q-G cleavage sites in the precursor protein P1 are not accessible. However, the results with a protease preparation encoded by virion RNA show that the protein P1 synthesized with template pLOP324 can be processed normally (Fig. 2). Moreover, the results with plasmid pT7-1 indicate that similar processing patterns were obtained with another P1 substrate. Therefore, incorrect folding of the precursor protein P1 encoded by pLOP324 and a consequent inaccessibility of the Q-G bond between VP2 and VP3 cannot explain the incomplete processing.

Instability of protein 3C as suggested by Thomas *et al.* (1983) might possibly be responsible for the observed partial proteolysis. However, co-translation of the various 3C-containing proteins *in vitro* in the presence of protein P1 does not alter processing of P1 (data not shown). In addition, the protease activity in *in vitro* translated virion RNA-encoded preparations appears to be stable enough to be used as a source of the proteolytic activity.

On the other hand, the 3C-containing proteins encoded by the plasmids pLOP311 to -314 might show an abnormal proteolytic activity. However, the low proteolytic activities of our 3C-containing proteins appear to be similar to that of partially purified 3C isolated from bacteria (E. Wimmer, personal communication), indicating that 3C proteins from different origins have similar activities. It therefore seems legitimate to conclude that *in vitro* synthesized mature 3C or the 3C-containing proteins encoded by plasmids pLOP311 to -314 are not capable of efficiently cleaving the Q-G bonds in the P1 sequence. The data obtained using template pT7-1 (Fig. 5) show that P2-encoded poliovirus proteins are not involved in rendering protein 3C more active towards Q-G bonds in the precursor protein P1. Therefore, it should be concluded that another 3C-containing protein is the active protease for cleavage of the precursor protein P1.

Our data clearly show that the only polypeptide capable of fully processing P1 *in vitro* is protein 3CD. The absence of detectable amounts of mature 3C both before (Fig. 3, lanes 8 and 9) and after (data not shown) *in vitro* processing of P1 by protein 3CD suggest that 3CD itself rather than 3C is the active form of the protease for processing P1 into VP0, VP3 and VP1. This argument is supported by the fact that *in vitro* translation of virion RNA gives an activity that processes P1, and a high yield of 3CD but undetectable amounts of 3C (Ypma-Wong and Semler, 1987). Whether the presence of the 3D sequence has an indirect effect, such as conversion of the 3C sequence into a more optimal conformation, or a direct effect by being itself involved in proteolytic processing, cannot be determined from the present study. Further experiments with these or other 3C-containing proteins should reveal clues to the answers of these questions. The *in vitro* approach used in this study is useful for further research on the influence of other genomic sequences on proteolysis in poliovirus.

It remains an open question whether 3CD is also the active protease for cleavage of the Q-G bonds in the precursor protein P1 *in vivo* in infected cells. However, one might argue that formation of capsid proteins can occur quickly if only one processing step (B-C junction) is required to obtain a protease which is active towards the precursor protein P1. Assuming efficient and fast autocatalysis of this bond, as might be indicated by data obtained with protein 3A'BC (Fig. 3), processing of Q-G bonds in the P1 region might take place before any processing in the P2 region.

In conclusion, this study shows that both P1 and P3-CD regions of the poliovirus genome are required for formation of the separate capsid proteins VP0, VP1 and VP3 such as are present in empty viral capsids (Rombaut *et al.*, 1983), opening the possibility of studying the synthesis of these structures without the use of the complete viral genome.

ACKNOWLEDGMENT

We thank J.M.A. Verbakel for helpful discussions, Dr E. Wimmer for providing virion RNA and Drs B. Semler and M. F. Ypma-Wong for the generous gift of plasmid pT7-1. We also thank Dr P. Vos for providing details for *in vitro* transcription before publication. This work was supported by a short-term grant from the Wellcome Foundation in the U.K.

REFERENCES

Clarke, L. and Carbon, J. (1978). Functional expression of cloned yeast DNA in *Escherichia coli*: Specific complementation of argininosuccinate lyase (*arg*H) mutations. Journal of Molecular Biology 120, 517-532.

Dorner, A.J., Semler, B.L., Jackson, R.J., Hanecak, R., Duprey, E. and Wimmer, E. (1984). *In vitro* translation of poliovirus RNA: Utilization of internal initiation sites in reticulocyte lysate. Journal of Virology 50, 507-514.

Hanecak, R., Semler, B.L., Anderson, C.W. and Wimmer, E. (1982). Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. Proceedings of the National Academy of Sciences, U.S.A. 79, 3973-3977.

Hanecak, R., Semler, B.L., Ariga, H., Anderson, C.W. and Wimmer, E. (1984). Expression of a cloned segment of poliovirus in *E. coli*: Evidence for autocatalytic production of the viral proteinase. Cell 37, 1063-1073.

Ivanoff, L.A., Towatari, T., Ray, J., Korant, B.D. and Petteway, S.R., jr (1986). Expression and site-specific mutagenesis of the poliovirus 3C protease in *Escherichia coli*. Proceedings of the National Academy of Sciences, U.S.A. 83, 5392-5396.

Jackson, R.J. and Hunt, T. (1983). Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. Methods in Enzymology 96, 50-74.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNA's. Nucleic Acids Research 12, 857-872.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227, 680-685.

Marinus, M.G. (1973). Location of DNA methylation genes on the *Escherichia coli* K12 genetic map. Molecular and General Genetics 127, 47-55.

Nicklin, M.J.H., Toyoda, H., Murray, M.G. and Wimmer, E., (1986). Proteolytic processing in the replication of polio and related viruses. Bio/Technology 4, 33-42.

Nicklin, M.J.H., Kräusslich, H.G., Toyoda, H., Dunn, J.J. and Wimmer, E. (1987). Poliovirus polypeptide precursors: Expression *in vitro* and processing by exogenous 3C and 2A proteinases. Proceedings of the National Academy of Sciences, U.S.A. 84, 4002-4006.

Parks, G.D., Duke, G.M., and Palmenberg, A.C. (1986). Encephalomyocarditis virus 3C protease: efficient cell-free expression from clones which link viral 5′ noncoding sequences to the P3 region. Journal of Virology 60, 376-384.

Racaniello, V.R. and Baltimore, D. (1981a). Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proceedings of the National Academy of Sciences, U.S. 78, 4887-4891.

Racaniello, V.R. and Baltimore, D. (1981b). Cloned poliovirus complementary DNA is infectious in mammalian cells. Science 214, 916-919.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1983). Epitope evolution in poliovirus maturation. Archives of Virology 76, 289-298.

Tabor, S. and Richardson, C.C., (1985). A bacteriophage T7 RNA polymerase-promotor system for controlled exclusive expression of specific genes. Proceedings of the National Academy of Sciences, U.S.A. 82, 1074-1078.

Thomas, A.A.M., Voorma, H.O. and Boeyé, A. (1983). Relationship between synthesis and cleavage of poliovirus-specific proteins. Journal of Virology 48, 309-313.

Toyoda, H., Nicklin, M.J.H., Murray, M.G., Anderson, C.W., Dunn, J.J., Studier, F.W. and Wimmer, E. (1986). A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell 45, 761-770.

Verver, J., Goldbach, R., Garcia, J. and Vos, P. (1987). *In vitro* expression of a full-length DNA copy of cowpea mosaic virus B RNA: identification of the B RNA encoded 24-kd protein as a viral protease. EMBO Journal 6, 549-554.

Ypma-Wong, M.F. and Semler, B.L. (1987). *In vitro* molecular genetics as a tool for determining the differential cleavage specificities of the poliovirus 3C proteinase. Nucleic Acids Research 15, 2069-2088.

FORMATION OF SUBVIRAL PARTICLES BY *in vitro* TRANSLATION OF SUBGENOMIC POLIOVIRUS RNAs

Jan P.M. Jore[1], Gerrit Veldhuisen[1], Peter H. Pouwels[1], Albert Boeyé[2], Raf Vrijsen[2] and Bart Rombaut[2]

[1]Medical Biological Laboratory TNO, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands

[2]Department of Microbiology and Hygiene, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium

Published in Journal of General Virology (1991), 72, 2721-2726.

SUMMARY

Rabbit reticulocyte lysates were programmed with either RNA extracted from purified poliovirus or a mixture of mRNAs encoding the capsid precursor, P1, and proteinase 3CD. In both cases, 14S subunits were formed at 30°C, and empty capsids at 37°C. Both the 14S subunits and empty capsids had the expected polypeptide composition and neutralization epitopes. It is concluded that the proteinase 3CD gene is the only viral genetic information needed for the correct processing of P1 and the formation of 14S subunits, and their assembly into antigenically correct empty capsids.

INTRODUCTION

The genome of picornaviruses consists of a positive-stranded RNA molecule encoding a single polyprotein. All functional structural, as well as non-structural, proteins are derived from this polyprotein by a cascade of proteolytic cleavages. The common precursor of the structural proteins, P1, is produced by the action of virus-encoded proteinases like 2A in the case of poliovirus (Toyoda *et al.*, 1986). In encephalomyocarditis (EMCV) and foot-and-mouth disease (FMDV) viruses, proteinase 3C is responsible for the cleavage of P1 into the structural proteins VP0, VP1 and VP3 (Parks *et al.*, 1986; Vakharia *et al.*, 1987; Clarke and Sangar, 1988). In poliovirus, however, this function is associated with proteinase 3CD rather than 3C (Jore *et al.*, 1988; Ypma-Wong *et al.*, 1988).

Subviral particles consisting of VP0, VP1 and VP3 are formed in all picornavirus-infected cells (Boege *et al.*, 1986; and references therein). These include 14S subunits (pentamers of the basic structural unit consisting of one molecule of each of the capsid proteins) and procapsids; the latter particles are made of 60 structural units, and differ from mature virions in lacking RNA and containing VP0 instead of VP4 and VP2.

Mature poliovirions express four neutralizing antigenic sites (1, 2, 3A and 3B; Page *et al.*, 1988). Native procapsids express the same four sites whereas 14S subunits possess only three (the fourth site spans the boundary between two 14S subunits and is formed as a

result of capsid assembly) (Rombaut *et al.*, 1990a); in addition, 14S subunits have at least one of the epitopes of the H antigen (i.e. heat-denatured virions). The latter epitope is called H1 to distinguish it from H2 epitopes, which are present on heat-denatured virions only (Rombaut and Boeyé, 1991).

It has recently been shown that poliovirus procapsids, when protected against thermal denaturation by chemical stabilizers such as WIN 51711, elicit the production of high neutralizing antibody titres in mice (Rombaut *et al.*, 1990b). Poliovirus 14S subunits spontaneously assemble into empty capsids at 37°C *in vitro* (Putnak and Phillips, 1981). When assembly occurs in the presence of cellular components (Rombaut *et al.*, 1983) or WIN 51711 (Rombaut and Boeyé, 1991), these empty capsids possess all the antigenic sites present on native procapsids and virions.

Obviously 14S subunits, native procapsids extracted from infected cells and empty capsids assembled *in vitro* qualify as potential immunogens for use in vaccines, although as long as the subviral particles have to be purified from infected cells the cost would be prohibitive. The solution to this problem is to try and produce immunogenic particles by means of recombinant DNA techniques. In the case of poliovirus, the first question to be answered is which genome elements are required in addition to that which encodes P1, the precursor of the capsid proteins. Since two Gln-Gly bonds within P1 (i.e., those at the VP0-VP3 and VP3-VP1 boundaries) must be cleaved, the 3CD proteinase is also required. We therefore investigated whether a cell-free translation system programmed with a mixture of two subgenomic RNAs representing the P1- and proteinase 3CD-encoding regions of the genome could synthesize 14S subunits and empty capsids.

METHODS

Plasmids and *in vitro* transcription.

Plasmid pLOP315 contains the 3CD-coding sequence starting at nucleotide 5438 preceded by a translation start codon and the T7 promoter. Plasmid pLOP324 contains nucleotides 743 to 3386 of the P1-coding region followed by a translation stop codon and preceded by the T7 promotor. Both plasmids as well as the conditions for *in vitro*

transcription of the plasmid DNA have been described (Jore *et al.*, 1988). Briefly, DNA was linearized with an appropriate restriction enzyme and transcribed in a reaction mixture composed of 40 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 10 mM dithiothreitol (DDT), 1 mM each of ATP, CTP and UTP, 0.2 mM GTP, 1 mM $m^7G(5')ppp(5')G$ and 100 μg/ml bovine serum albumin (BSA). For each μg of DNA 10 to 20 units T7 RNA polymerase and RNAsin were added. During the transcription reaction (20 min at 37°C) GTP was added to a final concentration of 1 mM in two steps. The reaction was stopped by extraction with phenol. RNA was purified by gel filtration of the inorganic phase and precipitation with ethanol, and the RNA precipitate was dissolved in sterile water.

Preparation of viral RNA.

The purification of type 1 (Mahoney) poliovirus (Rombaut et al., 1985) and the extraction of vRNA from purified virions (Phillips and Emmert, 1986) were essentially as described.

***In vitro* translation.**

Translation in a rabbit reticulocyte lysate was essentially as decribed (Jore *et al.*, 1988), but with the substitution of potassium acetate for KCl. Briefly, nuclease-treated rabbit reticulocyte lysate (Promega Biotec) was supplemented with 50 μM amino acids (minus methionine), 10 mM DTT, ^{35}S-methionine (10 μCi/25 μl) and RNA as indicated in the legends. Incubation was at 30°C.

Analysis of subviral particles.

(i) Sucrose gradients.

Samples (25 to 50 μl) from *in vitro* translation reactions were diluted fourfold in 20 mM Hepes, pH 6.9, 0.2 M KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and loaded onto 12 ml 5 to 30% or 14 ml 15 to 30% sucrose gradients in the same buffer. The 5 to 30% gradients were centrifuged for 8 h at 197,000 g_{av} and 20°C to isolate particles $\leq$14S and 2 h for larger particles; the 15 to 30% gradients were centrifuged for 17 h at 64,000 g_{av} and 4°C. After centrifugation, the gradients were fractionated and acid precipitable counts determined according to Palmenberg (1982). To prevent loss of 14S, and possibly

larger particles due to stickiness (Onodera and Phillips, 1987), preincubation of tubes with PBS, supplemented with 1% foetal calf serum and 0.05 % tween 20 (2 h at 37°C) proved to be effective.

(ii) Gel electrophoresis.

Samples (100 μl) from gradient fractions were diluted with 300 μl sterile water containing 10 μg of BSA, and proteins were precipitated by addition of 1 ml acetone. After overnight incubation at -20°C, precipitates were collected by centrifugation and dissolved in 15 μl sample buffer (Laemmli, 1970). The samples were run on 12.5%, homogeneous, 0.8 mm thick polyacrylamide gels as described (Jore *et al.*, 1988). When samples from translation mixtures were to be analysed directly, i.e. without prior fractionation on gradients, they were not concentrated by acetone precipitation. Further processing of gels was as described (Jore *et al.*, 1988).

(iii) Immunological characterization.

Eight monoclonal antibodies (MAbs) directed against the three different immunodominant, neutralizing sites of poliovirus type 1 were used (Page *et al.*, 1988; Rombaut *et al.*, 1990a). As the MAbs directed against the same site always yielded identical results we used data from one against each site: MAb 36-5h2 (site 2), 424 (site 3A) and 35-2b6 (site 3B). The non-neutralizing MAbs 39-5d6 and 39-5b4 were also used; both recognize H antigen, but neither native virions or procapsids. The site recognized by MAb 39-5d6 is called H1, which is expressed equally well on 14S subunits and heated virus (identical immunoprecipitation titres for 39-5d6 against both antigens). In contrast, antibody 39-5b4 recognizes 14S subunits poorly, the immunoprecipitation titre against 14S subunits being 1000-fold lower than against heated virus (Rombaut *et al.*, 1983) and, at a 10^{-2} dilution, there is no reaction with 14S subunits. The binding site of 39-5b4 is called H2.

Protein A-aided immunoprecipitation in microtitration plates was as described (Vrijsen *et al.*, 1983). Briefly, 80 μl radiolabeled antigen was mixed with 10 μl antibody. The mixture was allowed to stand for 1 h at 4°C, and 40 μl of a 10% suspension of formalin-fixed *Staphylococcus aureus* (strain Cowan 1) was added. After 30 min, the plate was centrifuged for 15 min at 1100 g and a 50 μl sample of each supernatant was removed for the radioactivity assay.

RESULTS

In vitro translation of full-length vRNA

RNA prepared from purified virions was used to program a messenger-dependent rabbit reticulocyte lysate, which was then incubated at 30°C. Samples were collected at various times up to 24 h and analysed by sucrose gradient ultracentrifugation (Fig. 1); radioactivity was found in a broad peak around 5S. This material, the radioactivity of which failed to increase after 3 h of incubation, consisted of a complex mixture of polypeptides, some of which migrated close to the position of the capsid proteins (Fig. 1). However, these polypeptides did not exhibit the epitopes of capsid proteins because none was precipitated by an antiserum raised to disrupted virus and able to bind all capsid proteins and their precursors (Vrijsen *et al.*, 1980) (results not shown).

A second peak of radioactive material with a sedimentation coefficient of 14S was formed slowly. The protein composition of this material was VP0, VP1 and VP3 as expected, with minor admixtures of proteinase 3CD (Fig. 1).

It has been reported that 14S subunits are formed in the infected cell at 30°C, but only assembled into virions after the temperature is raised to 37°C (Rombaut *et al.*, 1990d). We therefore examined whether the 14S subunits formed at 30°C in a vRNA-programmed reticulocyte lysate would assemble similarly after shift-up to 37°C. One portion of a lysate was shifted up after 3 h, and another portion after 15 h. In both cases, incubation was continued for 1 h at 37°C. In controls, incubation was continued at 30°C for the same period. The results are shown in Fig. 2.

As can be seen, only a trace of empty capsid material (peak fraction 17) was formed after 16 h at 30°C (tracing III), but the amount of empty capsids increased greatly after 1 h at 37°C; simultaneously, the amount of slow sedimenting material decreased (tracing IV). On the other hand, no empty capsids were formed upon incubation at 37°C without a preincubation at 30°C (results not shown), or when the temperature was shifted up after only 3 h at 30°C (tracing II). The results suggest that at 37°C empty capsids are rapidly assembled from 14S subunits, provided these are allowed to accumulate sufficiently. This requirement presumably reflects the need for a threshold concentration of 14S subunits for assembly to occur (Rombaut *et al.*, 1991).

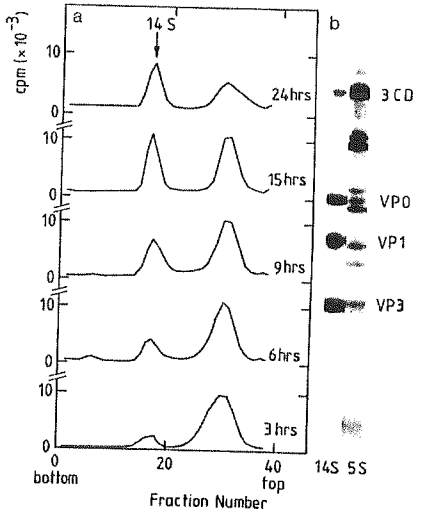


Fig. 1

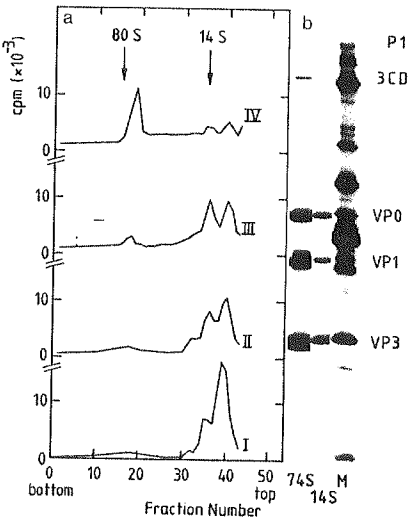


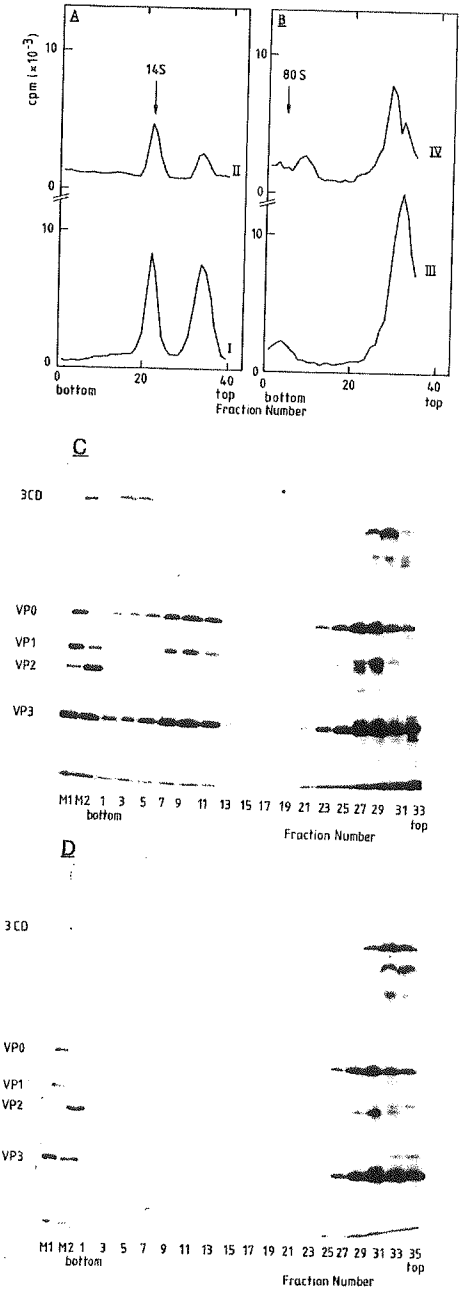
Fig. 2

Fig. 1. Formation of subviral particles in a lysate programmed with full-length poliovirus vRNA. (a) A rabbit reticulocyte lysate programmed with 20 μg/ml poliovirus RNA was incubated at 30°C and 25 μl samples were taken at the times indicated. The samples were centrifuged through 5 to 30% sucrose gradients for 8 h at 197,000 g_{av}. Fractions (250 μl) were collected, of which 20 μl was used for ^{35}S determination as indicated in Methods. Haemoglobin (4.2S) peaked in fractions 31 and 32. The position of the 14S marker was determined by running a preparation of purified, labelled 14S subunits on a separate gradient. Fractions 17 (14S) and 30 (5S) from the gradient marked 15 h were precipitated with acetone. (b) Polypeptide patterns obtained after PAGE and fluorography.

Fig. 2. Formation of subviral particles and the effect of shift-up from 30°C to 37°C. (a) A rabbit reticulocyte lysate was programmed with 20 μg/ml poliovirus vRNA. Samples (25 μl) were taken after 4 h at 30°C (tracing I), 3 h at 30°C plus 1 h at 37°C (II); 16 h at 30°C (III), or 15 h at 30°C plus 1 h at 37°C (IV). The samples were subjected to centrifugation through 5 to 30% sucrose gradients for 2 h at 197,000 g_{av}. Fractions (250 μl) were collected, of which 20 μl was used for ^{35}S determination as described in Methods. Labelled, purified 14S subunits and 80S particles (i.e. virions heated for 20 min at 56°C) served as external markers. Aliquots from gradient fractions (tracing IV) were precipitated with acetone, and separated on a polyacrylamide gel. (b) Peak fractions 14S and 74S after fluorography. Lane M shows the polypeptide composition of the lysate before fractionation.

Fig. 2 also shows the polypeptide composition of peak fractions from the gradient shown in tracing IV (incubation for 15 h at 30°C and 1 h at 37°C). As can be seen, the presumed empty capsids had the expected VP0, VP1 and VP3 composition, with minor admixtures of proteinase 3CD. Before fractionation the lysate contained a considerable amount of non-structural proteins as well as the capsid proteins. Two observations give

some indication as to the fate of these non-structural proteins. First, upon inspection of the complete fluorogram the non-structural proteins appeared to be more or less evenly distributed over he whole gradient, whereas the structural proteins appeared in distinct peaks. Second, a pellet was often present after centrifugation, the polypeptide composition of which was rich in non-structural proteins. However, we cannot explain why non-structural proteins smear or pellet preferentially.



In vitro translation of subgenomic RNAs

Subgenomic RNAs encoding P1 and proteinase 3CD separately were prepared as described in Methods and cotranslated in a reticulocyte lysate. After 15 h at 30°C incubation was continued for 1 h at 37°C (or at 30°C in the control). The lysates were analysed by sucrose density gradient centrifugation. As can be seen in Fig. 3A and B, both 5S and 14S material (tracing I), but no empty capsids (tracing III), accumulated at 30°C.

Fig. 3. Formation of subviral particles in a lysate programmed with subgenomic P1 and proteinase 3CD mRNAs. (A and B) A rabbit reticulocyte lysate was programmed with 150 μg/ml P1 RNA and 10 μg/ml proteinase 3CD RNA. Samples (25 μl) were taken after 16 h at 30°C (tracings I and III), or 15 h at 30°C plus 1 h at 37°C (II and IV). The samples were centrifuged through 5 to 30% sucrose gradients for 8 h at 197,000 g_{av} (tracings I and II), or through 15 to 30% sucrose gradients for 17 h at 64,000 g_{av} (tracings III and IV). Fractions (400 μl) were collected, of which 10 μl was used to measure incorporation of ^{35}S-methionine (see Methods). The position of 14S and 80S particles is indicated. (C and D) Aliquots of odd-numbered fractions were run on polyacrylamide gels. Lane M1 shows the polypeptide composition of an extract from infected HeLa cells, and lane M2 that of purified virions; (c) corresponds to tracing IV (15 h, 30°C and 1 h, 37°C) and (d) to tracing III (16 h, 30°C).

After shift-up to 37°C, the radioactivity in the 5S and 14S regions decreased (compare tracings I and II), and empty capsids were formed (compare tracings III and IV). Moreover, tracings III and IV show counts at the bottom of the gradient which did not appear on the gel; the identity of this material is not clear. The identification of the 14S subunits and empty capsids was confirmed by their VP0, VP1 and VP3 polypeptide composition (Fig. 3C and D).

Antigenicity of subviral particles synthesized in the reticulocyte lysate

The antigenicity of 14S subunits and empty capsids from *in vitro* translation experiments was ascertained by micro-immunoprecipitation using MAbs (Vrijsen *et al.*, 1983). The MAbs used were directed against sites 2, 3A, 3B, H1 and H2 (see Introduction); site 1 was disregarded, as it is not very immunogenic in inbred mice (Page *et al.*, 1988). Table 1 shows the results. The 14S subunits, originating from *in vitro* translation of either poliovirus vRNA or a mixture of P1 and proteinase 3CD RNAs, showed the same (sites 2, 3A and H1) antigenic conformation as native 14S subunits isolated from infected HeLa cells. The empty capsids showed the (2, 3A and 3B) antigenicity of native procapsids (Rombaut *et al.*, 1990a).

Table 1 Antigenicity of subviral particles

Translation conditions in reticulocyte lysate		Fig. showing sedimentation profile	Particle	Input radioactivity precipitated by MAb(%)				
Origin of mRNA	Duration and temperature			2	3A	3B	H1	H2
vRNA	16 h, 30°C	1	14S subunits	100	99	6	98	17
vRNA	15 h, 30°C +1 h, 37°C	2 (tracing IV)	Empty capsids	92	87	94	13	14
Subgenomic, P1+3D	16 h, 30°C	3A (tracing I)	14S subunits	98	100	0	99	6
Subgenomic, P1+3CD	15 h, 30°C +1 h, 37°C	3B (tracing IV)	Empty capsids	98	96	97	22	12

DISCUSSION

The formation of subviral particles in reticulocyte lysates programmed with genomic RNA has been reported for several picornaviruses. 14S subunits are formed with the vRNAs of EMCV, Mengo virus and FMDV (Palmenberg, 1982; Grubman, 1984; Grubman *et al.*, 1985; Boege *et al.*, 1986), and empty capsids with the vRNAs of EMCV and FMDV (Palmenberg, 1982; Grubman, 1984; Grubman *et al.*, 1985). The reason there has not been a similar report for poliovirus is possibly the much lower efficiency with which poliovirus vRNA is translated in a rabbit reticulocyte lysate (Jackson, 1989). However, the high protein synthesis activity of present-day available lysates, like those which have been used here, may, at least partially, have abolished this problem.

In this study we show the formation of both 14S subunits and empty capsids in reticulocyte lysates programmed with poliovirus genomic RNA. Although 14S subunits accumulate at 30°C, empty capsids appear only after shift-up to 37°C, in agreement with the known temperature requirements of assembly (Putnak and Phillips, 1981). The subviral particles synthesized in the lysates possess all known neutralization epitopes of native 14S subunits and procapsids (Table 1).

The presence of antigenic site 3B on the empty capsids shows that the 14S subunits assembled correctly (Rombaut *et al.*, 1990a). It should be emphasized that the acquisition of N antigenicity is not an automatic consequence of assembly; it requires a still unidentified "antigenicity conferring activity", present in poliovirus-infected HeLa cells, that can be mimicked by WIN 51711 (Rombaut and Boeyé, 1991). Our results show that this activity is also present in reticulocyte lysates.

14S subunits and empty capsids were also formed when the reticulocyte lysate was programmed with a mixture of RNA transcripts encoding P1 and proteinase 3CD, and the temperature requirements were the same as when the lysate was programmed with vRNA. Again, the 14S subunits and empty capsids were antigenically correct (Table 1). We conclude that N antigenic particles can be synthesized in a cell-free system programmed with the P1 and proteinase 3CD portions of the genome only. Thus, the system has been reduced to the absolute minimum. Avoiding the simultaneous expression of superfluous poliovirus-encoded proteins is important as they may have adverse effects.

It has indeed been reported that attempts to grow poliovirus-vaccinia recombinants for vaccine purposes were thwarted by expression of proteinase 2A (Turner *et al.*, 1989; Jewell *et al.*, 1990).

Is a subunit vaccine based on 14S subunits and/or empty capsids feasible? Although full purification of these particles is now possible (Rombaut *et al.*, 1990c), their large-scale production in either infected cells or cell-free lysates would be prohibitively expensive. However, three other systems are being explored, i.e. adenovirus recombinants in mammalian cells, baculovirus recombinants in insect cells (Urakawa *et al.*, 1989), and autonomously replicating expression vectors in the yeast *Saccharomyces cerevisiae*. In the yeast expression system, we have achieved relatively high expression of polio VP2 (Verbakel *et al.*, 1987), P1 (J.M.A. Verbakel *et al.*, unpublished results), as well as synthesis of active proteinase 3CD (J.P.M. Jore *et al.*, unpublished results). Recently, we have been able to show that joint expression of P1 and 3CD in *S.cerevisiae* leads to the production of processed P1; the possible formation of subviral particles is being studied.

Note. After submission of this manuscript a paper concerning the assembly of virus particles by recombinant vaccinia virus expressing P1 and P3 was published (Ansardi *et al.*,1991). Although one may presume that the particles described are identical to the particles described here, immunological data to support this assumption are not available.

ACKNOWLEDGMENTS

The authors are grateful to Morag Ferguson for the gift of monoclonal antibody 424. B.R. is a Senior Research Assistant of the National Fund for Scientific Research (Belgium).

REFERENCES

Ansardi, D.C., Porter, D.C. and Morrow, C.D. (1991). Co-infection with recombinant vaccinia viruses expressing poliovirus P1 and P3 proteins results in polyprotein processing and formation of empty capsid structures. Journal of Virology 65, 2088-2092.

Boege, U., Ko, D.S.W. and Scabra, D.G. (1986). Toward an *in vitro* system for picornavirus assembly: Purification of Mengovirus 14S capsid precursor particles. Journal of Virology 57, 275-284.

Clarke, B.E. and Sangar, D.V. (1988). Processing and assembly of foot-and-mouth disease virus proteins using subgenomic RNA. Journal of General Virology 69, 2313-2325.

Grubman, M.J. (1984). *in vitro* morphogenesis of foot-and-mouth disease virus. Journal of Virology 49, 760-765.

Grubman, M.J., Morgan, D.O., Kendall, J. and Baxt, B. (1985). Capsid intermediates assembled in a foot-and-mouth disease genome RNA-programmed cell-free translation system and in infected cells. Journal of Virology 56, 120-126.

Jackson, R.J. (1989). Comparison of encephalomyocarditis virus and poliovirus with respect to translation initiation and processing *in vitro*, p.51-71. *In* B.L.Semler and E.Ehrenfeldt (eds.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington D.C.

Jewell, J.E., Ball, L.A. and Rueckert, R. (1990). Limited expression of poliovirus by vaccinia virus recombinants due to inhibition of the vector by proteinase 2A. Journal of Virology 64, 1388-1393.

Jore, J., De Geus, B., Jackson, R.J., Pouwels, P.H. and Enger-Valk, B.E. (1988). Poliovirus protein 3CD is the active protease for processing of the precursor protein P1 *in vitro*. Journal of General virology, 69, 1627-1636.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227, 680-685.

Onodera, S. and Phillips, B.A. (1987). A novel method for obtaining 14S pentamers from procapsids and their self-assembly into virus-like shells. Virology 159, 278-287.

Page, G.S., Mosser, A.G., Hogle, J.M., Filman, D.J., Rueckert, R.R. and Chow, M. (1988). Three-dimensional structure of poliovirus serotype 1 neutralizing determinants. Journal of Virology 62, 1781-1794.

Palmenberg, A.C. (1982). *In vitro* synthesis and assembly of picornaviral capsid intermediate structures. Journal of Virology 44, 900-906.

Parks, G.D., Duke, G.M. and Palmenberg, A.C. (1986). Encephalomyocarditis virus 3C protease: Efficient cell-free expression from clones which link viral 5′ noncoding sequences to the P3 region. Journal of Virology 60, 376-380.

Phillips, B.A. and Emmert, A. (1986). Modulation of the expression of poliovirus proteins in reticulocyte lysates. Virology 148, 255-267.

Putnak, J.R. and Phillips, B.A. (1981). Picornaviral structure and assembly. Microbiologial Reviews 45, 287-315.

Rombaut, B. and Boeyé, A. (1991). *In vitro* assembly of poliovirus 14S subunits: disoxaril stabilization as a model for the antigenicity conferring activity of infected cell extracts. Virology 180, 788-792.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1983). Epitope evolution in poliovirus maturation. Archives of Virology 76, 289-298.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1985). Stabilization by host cell components and Mg^{2+} of the neutralization epitopes of poliovirus. Journal of General Virology 66, 303-307.

Rombaut, B., Boeyé, A., Ferguson, M., Minor, P.D., Mosser, A. and Rueckert, R.R. (1990a). Creation of an antigenic site in poliovirus type 1 by assembly of 14S subunits. Virology 174, 305-307.

Rombaut, B., Brioen, P. and Boeyé, A. (1990b). Disoxaril stabilization and immunogenicity of poliovirus procapsids. Journal of General Virology 71, 1081-1086.

Rombaut, B., Foriers, A. and Boeyé, A. (1990c). Purification of poliovirus 14S subunits by sucrose gradient ultracentrifugation and high-performance size-exclusion chromatography. Journal of Virological Methods 29, 303-312.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1990d). New evidence for the precursor role of 14S subunits in poliovirus morphogenesis. Virology 177, 411-414.

Rombaut, B., Foriers, A. and Boeyé, A. (1991). *In vitro* assembly of poliovirus 14S subunits. Identification of the assembly promoting activity of infected cell extracts. Virology 180, 781-787.

Toyoda, H., Nicklin, M.J.H., Murray, M.V., Anderson, C.W., Dunn, J.J., Studier, F.W. and Wimmer, E. (1986). A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell 45, 761-770.

Turner, P.C., Young, D.C., Flanegan, J.B. and Moyer, R.W. (1989). Interference with vaccinia virus growth caused by insertion of the coding sequence for poliovirus protease 2A. Virology 173, 509-521.

Urakawa,T., Ferguson, M., Minor, P.D., Cooper, J., Sullivan, M., Almond, J.W. and Bishop, D.H.L. (1989). Synthesis of immunogenic, but non-infectious, poliovirus particles in insect cells by a baculovirus expression vector. Journal of General Virology 70, 1453-1463.

Vakharia, V.N., Devaney, M.A., Moore, D.M., Dunn, J.J.and Grubman, M.J. (1987). Proteolytic processing of Foot-and-Mouth disease virus polyproteins expressed in a cell-free system from clone-derived transcripts. Journal of Virology 61, 3199-3207.

Verbakel, J.M.A., Dekker, K.A., Rutgers, C.A., Pouwels, P.H. and Enger-Valk, B.E. (1987). Construction of expression plasmids for *Saccharomyces cerevisiae*: application for synthesis of poliovirus protein VP2. Gene 61, 207-215.

Vrijsen, R., Brioen, P. and Boeyé, A. (1980). Identification of poliovirus precursor proteins by immunoprecipitation. Virology 107, 567-569.

Vrijsen, R., Rombaut, B. and Boeyé, A. (1983). A simple quantitative protein A micro-immunoprecipitation method: assay of antibodies to the N and H antigens of poliovirus. Journal of Immunological Methods 59, 217-220.

Ypma-Wong, M.F., Dewalt, P.G., Johnson, V.H., Lamb, J.G. and Semler, B.L. (1988). Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor. Virology 166, 265-270.

INTRODUCTION TO CHAPTERS 4 AND 5

The yeast *Saccharomyces cerevisiae* has been used for synthesis of poliovirus proteins and subviral particles on a preparative scale. The plasmids that were constructed for this purpose were derivatives of *Escherichia coli-S.cerevisiae* vectors, i.e., plasmids that can be maintained in *E.coli* as well as in yeast. Maintenance in yeast has been made possible by insertion of sequences that provide for replication in yeast (a) and of a yeast selection marker (b). The expression cassettes for poliovirus proteins that have been inserted in these shuttle vectors comprise efficient promoter sequences that are either inducible or constitutive (c).

(a) Replication in yeast is provided by *ARS CEN* sequences or by 2μm derived sequences. The presence of *ARS* (autonomic replicating sequences) results in a plasmid copy number of 1-20 per cell (Hitzeman *et al.,* 1981). These plasmids are mitotically highly unstable; inclusion of *CEN* (yeast centromeric) sequences leads to a stable, be it reduced number (1-2) of plasmids per cell (Clarke and Carbon, 1980). The presence of an origin of replication in combination with a stabilization locus, both derived from the yeast indigenous 2 μm plasmid, results in 10-20 of such 2μm derived plasmids per cell (Erhart and Hollenberg, 1983). The 2μm plasmid itself provides for *REP1* and *REP2* gene products that are necessary for efficient segregation of the 2μm derived plasmids (Kikuchi, 1983).

(b) For selection in yeast, use has been made of auxotrophic markers, namely *URA3* (orotidine-5'-phosphate decarboxylase, Gerbaud *et al.,* 1979) or *LEU2d* (3-isopropylmalate dehydrogenase, Beggs, 1978). *LEU2d* is a *LEU2* gene with a truncated promoter. The low activity of this gene leads to an increased plasmid copy number when selective pressure is applied. In the case of 2μm derived plasmids carrying *LEU2d* the copy number can increase fourfold (Erhart and Hollenberg, 1983).

(c) For a constitutive promoter, use was made of the *PGK* promoter (phosphoglycerate kinase, Mellor *et al.,* 1983). Especially in the presence of glucose this is a very strong promoter, yielding 5% *PGK* mRNA from a single copy (Romanos *et al.,* 1992).

Inducible promoters were from genes involved in galactose metabolism, notably *GAL1* (galactokinase) and *GAL7* (α-D-galactose-1-phosphate uridyltransferase). *GAL1*, *GAL7*, and *GAL10* (uridine diphosphogalactose-4-epimerase, the promoter of which forms a divergent promoter with the *GAL1* promoter) are induced >1000-fold to approximately 1% of total mRNA on addition of galactose (St.John and Davis, 1981). Galactose regulation in yeast involves many genes, but the central interaction is between the trans-activator encoded by *GAL4*, the repressor encoded by *GAL80* and the *GAL* UAS (upstream activation sequences) (for a review see Johnston, 1987). Binding of *GAL4* protein to the UAS is necessary for induction; *GAL80* protein binds *GAL4* and acts as a repressor unless galactose is added. Since *GAL4* is present in one or two molecules per cell there is a disproportional increase in expression going from a one copy *GAL* regulated expression cassette to a multicopy situation. Although some improvement can be obtained by providing for (preferably *GAL*) regulated *GAL4* expression (Schultz *et al.,* 1987), other factors also become limiting with multi-copy promoters (Baker *et al.,* 1987).

REFERENCES

Baker, S.M., Johnston, S.A., Hopper, J.E. and Jaehning, J.A. (1987). Transcription of multiple copies of the yeast *GAL7* gene is limited by specific factors in addition to *GAL4*. Molecular and General Genetics 208, 127-134.

Beggs, J.D. (1978). Transformation of yeast by a replicating hybrid plasmid. Nature 275, 104-109.

Clarke, L. and Carbon, J. (1980). Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature 287, 504-509.

Erhart, E. and Hollenberg, C.P. (1983). The presence of a defective LEU2 gene in 2μm DNA recombinant plasmids of *Saccharomyces cerevisiae* is responsible for curing and high copy number. Journal of Bacteriology 156, 625-635.

Gerbaud, C., Fournier, P., Blanc, H., Aigle, M., Heslot, H. and Guerineau, M. (1979). High frequency yeast transformation by plasmids carrying part or entire 2μm yeast plasmid. Gene 5, 233-253.

Hitzeman, R.A., Hagie, F.E., Levine, H.L., Goeddel, D.V., Ammerer, G. and Hall, B.D. (1981). Expression of a human gene for interferon in yeast. Nature 293, 717-722.

Johnston, M. (1987). A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. Microbiological Reviews 51, 458-476.

Kikuchi, Y. (1983). Yeast plasmid requires a *cis*-acting locus and two plasmid proteins for its stable maintenance. Cell 35, 487-493.

Mellor, J., Dobson, M.J., Roberts, N.A., Tuite, M.F., Emtage, J.S., White, S., Lowe, P.A., Patel, T., Kingsman, A.J. and Kingsman, S.M. (1983). Efficient synthesis of enzymatically active calf chymosine in *Saccharomyces cerevisiae*. Gene 24, 1-14.

Romanos, M.A., Scorer, C.A. and Clare, J.J. (1992). Foreign gene expression in yeast: A review. Yeast 8, 423-488.

Schultz, L.D., Hofmann, K.J., Mylin, L.M., Montgomery, D.L., Ellis, R.W. and Hopper J.E. (1987). Regulated overproduction of the *GAL4* gene product greatly increases expression from galactose-inducible promoter on multi-copy expression vectors in yeast. Gene 61, 123-133.

St.John, T.P. and Davis, R.W. (1981). The organisation and transcription of the galactose gene cluster of *Saccharomyces*. Journal of Molecular Biology 152, 285-315.

FORMATION OF POLIOMYELITIS SUBVIRAL PARTICLES IN THE YEAST *SACCHAROMYCES CEREVISIAE*

J.P.M. Jore[1], G. Veldhuisen[1], M. Kottenhagen[1], P.H. Pouwels[1], A. Foriers[2], B. Rombaut[3] and A. Boeyé[3]

[1] Medical Biological Laboratory TNO, P.O.Box 5815, 2280 HV Rijswijk, The Netherlands;
[2] Department of Medical and Special Biochemistry and
[3] Department of Microbiology and Hygiene, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium.

Submitted in a condensed form to Yeast.

SUMMARY

The sequence of the poliovirus genome encoding 3CD (a protease) was transferred to the yeast *Saccharomyces cerevisiae* on expression vectors with either a constitutive or an inducible promoter. Transformants could only be obtained with vectors carrying the inducible transcription unit. Extracts of induced cells were able to cleave cell-free synthesized P1, the precursor of the poliovirus capsid proteins, into VP0, VP3 and VP1. In yeast cells constitutively expressing P1, induction of 3CD expression resulted in only trace amounts of processed products. Processing could be improved considerably by simultaneous induction of both P1 and 3CD expression. Analysis of extracts of such induced cells revealed the presence of particles that resembled authentic subviral particles.

INTRODUCTION

Poliovirus is a member of the family of picornaviruses. Its genome encodes a single large precursor polyprotein, which is processed in a series of proteolytic steps to yield the virion capsid proteins and non-structural proteins (reviewed by Nicklin *et al.*, 1986). A capsid precursor protein, P1, is generated by cleavage of a tyrosine-glycine (Y-G) bond. This cleavage is catalyzed by the viral protease 2A (Toyoda *et al.*, 1986; Nicklin *et al.*, 1987). A second viral protease, 3C, is responsible for cleavage of glutamine-glycine (Q-G) bonds outside of P1, thus generating non-structural proteins. From *in vitro* studies it is clear that 3C has a limited activity towards Q-G bonds within P1; it liberates only small amounts of VP0 (Nicklin *et al.*, 1988; Jore *et al.*, 1988). Full processing of P1 to VP0, VP3 and VP1 requires a protein comprising the entire 3C and 3D regions (Jore *et al.*, 1988; Ypma-Wong *et al.*, 1988). Cleavage of VP0 to VP4 and VP2 is considered to be a final step in morphogenesis; it is probably autocatalytic and may involve basic groups from the viral RNA (Arnold *et al.*, 1987).

The present-day vaccines against poliomyelitis have some drawbacks and potential dangers, largely relating to the presence of infectious virions in one or more stages of the

vaccine production process. Recombinant DNA techniques in principle enable production of a subunit vaccine without the need to refer to infectious virions.

Several types of subviral particles can be found in mammalian cells that are infected with poliomyelitis virus. Among these, pentamers (14S) and empty capsids (74S) are predominant. Both consist of a complete set of capsid proteins, i.e., VP0, VP1 and VP3, and lack viral RNA (Putnak and Phillips, 1981). The antigenic properties of the empty capsids are identical to those of the mature virion, while 14S particles have very similar properties (Rombaut *et al.*, 1990b). Both are therefore attractive candidates to serve as an alternative (subunit) vaccine. However, isolation and purification of such particles from infected cell cultures is neither economically attractive, nor completely safe. Therefore an alternative method of production is pursued.

In previous studies it has been shown that the capsid precursor protein P1 and the protease 3CD were necessary and sufficient not only for processing of P1 to VP0, VP1 and VP3 (Jore *et al.*, 1988; Ypma-Wong *et al.*, 1988), but also for generation of 14S and 74S subviral particles *in vitro* (Jore *et al.*, 1991). We wanted to extend these studies to *Saccharomyces cerevisiae* for the synthesis of subviral particles on a preparative scale. To this end, P1 and 3CD-coding sequences, provided with translational start and stop codons, were cloned in yeast vectors using both constitutive (*PGK*) and inducible (*GAL*) promoters. Their expression in transformed yeast cells was studied, and it was shown that both processing of P1 by 3CD and assembly of the resulting capsid proteins into subviral particles occurred.

MATERIALS AND METHODS

Strains

Escherichia coli strain TG1 (*sup*E, *hsd*Δ5, *thi*Δ(*lac-pro*AB), F'[*tra*D36, *pro*AB$^+$, *laq*I^q, *lac*ZΔM15]) (Gibson,1984) was used as the host strain for all plasmids and constructions. *Escherichia coli* JM109 (Yanisch-Peron *et al.*, 1985) was used to synthesize 3CD for immunization purposes. The *Saccharomyces cerevisiae* strains X904 (*α, leu2, ura1, pep4*-3) (MBL) and 334 (*α,leu2*-3 -112, *gal1*, *reg*1-501, *ura3*-52, *pep4*-3, *prb1*-1122) (Hovland *et al.*, 1989) were used for synthesis of proteins.

Media

Bacteria were cultivated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 μg ampicillin per ml. YEPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for cultivation of yeast cells. YNB medium, which was used to select yeast transformants, contained 0.67% yeast nitrogen base and 2% glucose. Further additions were dictated by the strain/ plasmid combination. Induction media used were YEPG (1% yeast extract, 2% peptone, 2% galactose) for X904 transformants, and YEPDG (YEPD + 1% galactose) for strain 334 transformants.

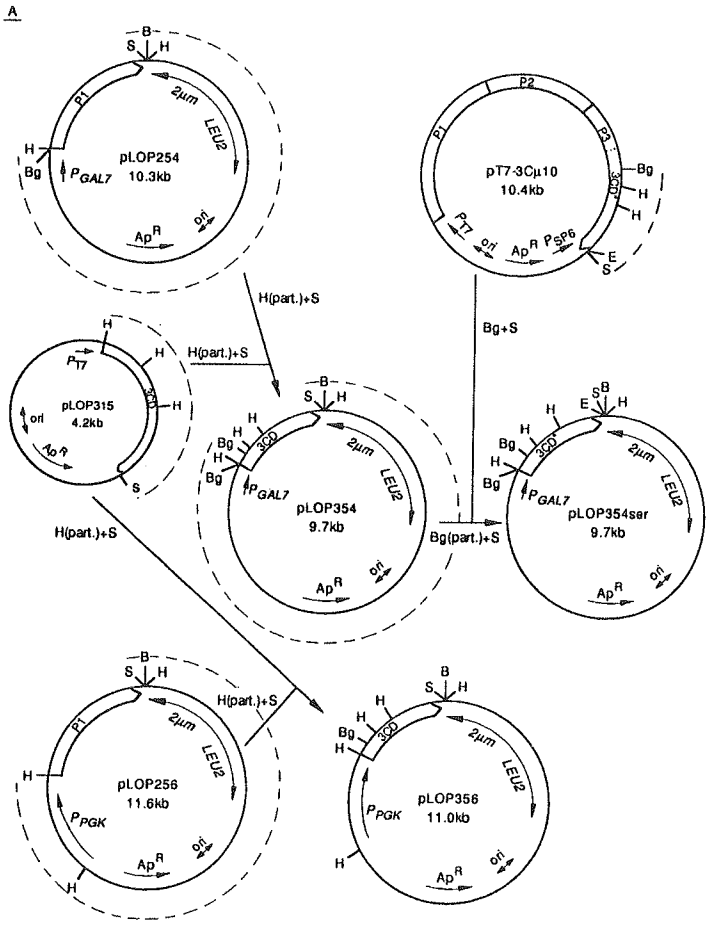
DNA techniques

General recombinant DNA techniques were those compiled by Sambrook *et al.* (1989). Transformation of *E.coli* was carried out according to Hanahan (1983). Transformation of yeast cells was carried out by the protoplast method (Hinnen *et al.*, 1978) using helicase instead of zymolyase for the preparation of protoplasts.

Plasmids

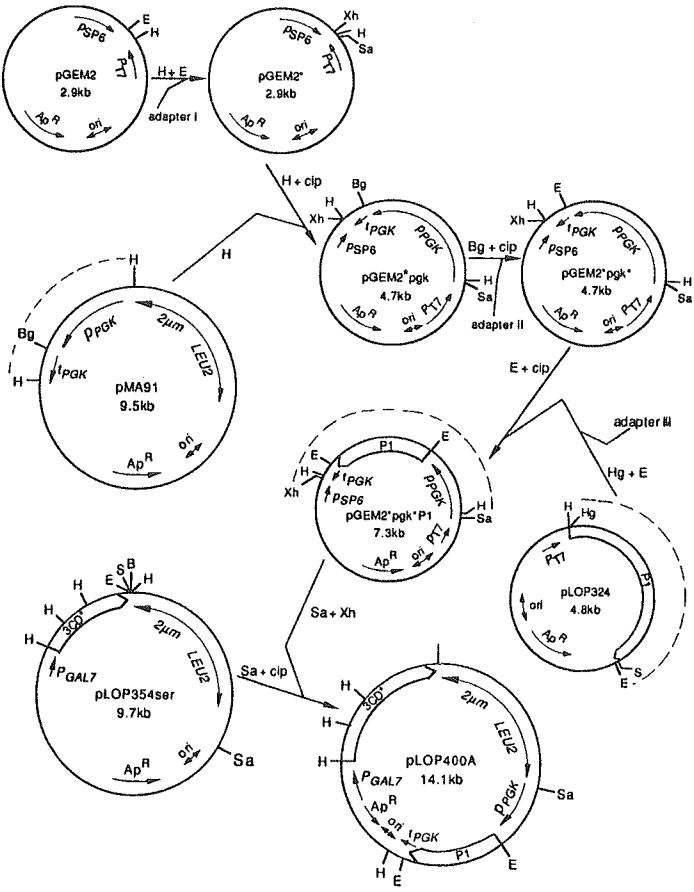
With the exception of the *in vitro* transcription plasmids pGEM2, pLOP315, pLOP324 and pT7-3Cμ10, all plasmids used were yeast-*E.coli* shuttle plasmids. They all contain the *LEU2*d gene as a yeast selection marker, with the exception of BO8-9 which contains the *URA3* gene as a selection marker. Plasmid pMBL204, which contains the *GAL7* promoter followed by a multiple cloning site, has been described (Verbakel *et al.*, 1987). Extracts of yeast cells transformed with this plasmid served as negative controls in expression experiments. Plasmids pLOP254 and pLOP256 carry a transcription unit for P1 with a *GAL7* promoter and a *PGK* promoter respectively and have been described by Verbakel *et al.* (1988), and the *in vitro* transcription plasmids pLOP315 and pLOP324, containing the coding sequences for 3CD and P1 respectively by Jore *et al.* (1988). Plasmid pMA91 (Mellor *et al.*, 1983) was used as source of the *PGK* promoter and terminator. Plasmid pGEM2 (Promega Biotec) was used for subcloning of the *PGK* promoter and terminator. Plasmid BO8-9 was a gift of S.A.Johnston (South Western University, Dallas, Texas) and was used as a source of *ARS* and *CEN* sequences. It contains the coding sequence for *LAC9*, the *Kluyveromyces lactis* analog of *GAL4*. From

plasmid pT7-3Cμ10 (Ypma-Wong *et al.*, 1988) a DNA fragment carrying a serine duplication in the polio 3CD gene was isolated. pEXC3D (Richards *et al.*, 1987) was used to synthesize large amounts of 3CD in *E.coli* for immunization of rabbits.
Construction of the yeast expression plasmids pLOP354, -356, -380 and -400A is outlined in Fig. 1.



-/continued .....

B


pGEM2
2.9kb
pGEM2*
2.9kb
H+E
adapter I
H + cip
pGEM2*pgk
4.7kb
Bg + cip
adapter II
pGEM2*pgk*
4.7kb
E + cip
pMA91
9.5kb
adapter III
Hg + E
pGEM2*pgk*P1
7.3kb
pLOP324
4.8kb
Sa + Xh
pLOP354ser
9.7kb
Sa + cip
pLOP400A
14.1kb

-/continued .....

C

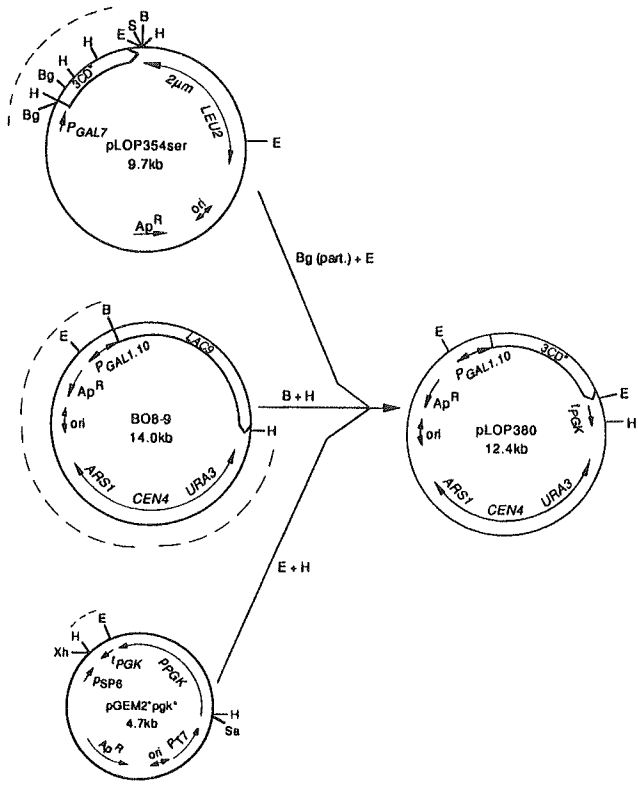


Fig. 1

Fig.1. Strategy for construction of the plasmids used in this study. (A) 3CD-coding plasmids pLOP354, -354ser and -356; (B) P1 and 3CD-coding plasmid pLOP400A and (C) 3CD-coding plasmid pLOP380. In cases where more than one fragment was generated, the fragment of choice is indicated by a dashed arc outside the circle depicting the plasmid. Only relevant sites are indicated. The plasmids are not drawn to scale. Abbreviations, B, *Bam*HI; Bg, *Bgl*II; E, *EcoRI*; H, *Hin*dIII; Hg, *Hgi*AI; S, *Sst*I; Sa, *Sal*I; Xh, *Xho*I; Ap^R, ampicillin-resistance gene; ori, origin of ColE1 DNA replication; 2μm, origin of yeast 2μm plasmid DNA replication. Adapters used were:

5′ AATTGCTCGAGAAGCTTGTCGAC 3′ (I); 5′ GATCGAATTC 3′ (II) and
CGAGCTCTTCGAACAGCTGTCGA CTTAAGCTAG

5′ AATTCATGGGTGCT 3′ (III).
GTACCC

Synthesis of oligonucleotides

Oligonucleotides were synthesized in a Cyclone 8400 DNA synthesizer (Biosearch Inc., San Rafael, California) and purified according to the manufacturer's instructions.

***In vitro* transcription and translation**

The conditions for *in vitro* transcription and translation were as described (Jore *et al.*, 1988).

Cultivation of yeast and preparation of extracts

A fresh X904 transformant was picked from a selective YNB plate and cultivated for 40h in YNB medium at 30°C. Subsequently the cells were counted and diluted in YEPG so as to reach a density of 2-4x10^7/ml after o/n growth. Cells were collected by low speed centrifugation and extracted. In the case of transformants of strain 334, induction was brought about as follows: a fresh transformant was picked from a selective YNB plate and cultivated o/n in YNB. Cells were counted and diluted to 5x10^6/ml in YEPD. After o/n growth cells were counted again, at which time they usually had reached a concentration of 5x10^7/ml. Induction was started by addition of 1% galactose. After the expression period, cells from 10 ml of culture were collected by low speed centrifugation and resuspended in 0.5 ml of ice-cold PBS. Extracts were prepared by vortexing the cells with 0.5-mm glass beads. The clear supernatant fraction, obtained after centrifugation in an Eppendorf centrifuge, was either analyzed immediately or stored at -80°C, as was the resuspended pellet fraction.

Western blot analysis

Extracts and pellet fractions were subjected to electrophoresis in SDS-12.5% polyacrylamide gels (Laemmli, 1970). For analysis by the Western blotting procedure (Towbin *et al.*, 1979) proteins were transferred from gels to a nitrocellulose filter with an electroblotting device (Biorad, Richmond, California), using a buffer with doubly concentrated Tris and glycine (Otter *et al.*, 1987). The filter was treated as described by Hawkes *et al.* (1982), using either polyclonal antisera from rabbits or monoclonal antibodies. Initially, goat antiserum conjugated with horse radish peroxidase was used as

a second antibody. However, in the course of this study this antiserum was replaced by goat antiserum conjugated with alkaline phosphatase.

Antibodies

The preparation of polyclonal VP2-specific rabbit antiserum has been described (Verbakel *et al.*, 1988). Polyclonal 3D-specific antiserum was a gift from O.Richards (University of Utah Medical Center, Salt Lake City, Utah). Polyclonal 3CD-specific antiserum was prepared as follows: *E.coli* JM109 was transformed with pEXC3D. A colony was picked and cultivated o/n in 0.2 l LB medium + ampicillin. Bacteria were harvested by centrifugation and resuspended in 32 ml of 0.1 M Tris, 0.1 M NaCl, 50 mM EDTA, pH7.6, to which 5 mg lysozyme per ml was added. After 3h incubation at 0°C and three passages through a French press the extract was centrifuged at 10k rpm and 5°C in a Beckman JA20 rotor. Since most 3CD proved to be present in the pellet fraction, which was determined on Western blots with 3D-specific antiserum, this pellet fraction was used for preparative purposes. After resuspending in 3 ml of PBS, 300 μl was run on a preparative 7.5% polyacrylamide/SDS gel (dimensions 160x200x3 mm). The gel was stained with copper according to Lee *et al.* (1987), the section with the protein of interest excised, destained and electro-eluted. One of the bands represents 3CD, the other (much more pronounced, but representing a protein with slightly lower molecular weight) represents a breakdown product of 3CD (O.Richards, pers. comm.). A rabbit was injected intradermally at 4 sites on its back with 300 μg of the breakdown product (in CFA) and after 6 weeks boosted with 50 μg of 3CD proper in CFA. A second boost with 50 μg of 3CD proper (in IFA) was given 4 weeks later and the animal bled 9 days thereafter. During the immunization period blood samples were tested for reactivity towards 3CD by Western blotting.

Monoclonal antibodies specific for capsid proteins VP1 (39-5d6a), VP2 (42-1A1a) and VP3 (42-1C9) were prepared as described by Sijens *et al.*(1983). Rabbit polyclonal VP1-, VP2- and VP3-specific antisera were a gift from T.Hazendonk (RIVM, Bilthoven, The Netherlands).

Partial purification of particles from yeast extracts

For preparative purposes yeast transformants were cultivated in 0.5-liter cultures, collected by centrifugation, washed once with PBS, and divided in 3 equal portions. Each portion of packed cells was resuspended in an equal volume of extraction buffer (PBS + 2M NaCl + 5 mM MgCl2 + 2% NP40 + 2 mM PMSF) and vortexed with glass beads until over 90% of the cells were broken, as judged by microscopic examination. To collect the crude extract, tubes were punctured and centrifuged, "piggyback" onto a clean tube, in a MSE tabletop centrifuge at 3000 rpm and 4°C. The supernatant in the recipient tube was saved, the pelleted material washed once with PBS + 1 M NaCl + 2.5 mM $MgCl_2$ + 1% NP40 and centrifuged again. The combined supernatants were subjected to a low speed centrifugation (8000 rpm in a Sorvall SS34 rotor) and the resulting supernatant subjected to a high speed centrifugation step (140,000 x g_{av} during 90 min at 4°C). The pellet thus obtained was resuspended in RSB (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.2) and further purified by high-performance size-exclusion chromatography (HPSEC; Foriers *et al.*, 1990) or by equilibrium sedimentation in 40 to 60% nycodenz in RSB. Western blotting was used to select fractions to be pooled, and pooled material was subjected to sucrose velocity gradient centrifugation (15 to 30% sucrose in RSB). ^{35}S-methionine labelled 14S and 74S particles, obtained from poliovirus-infected HeLa cells, served as external markers.

Determination of protein concentrations

Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, Illinois) with bovine serum albumin as a standard. In samples containing sucrose, known to be a disturbing factor for the BCA reagent, use was made of the BIORAD protein assay (Biorad, Richmond, California) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Synthesis of enzymatically active 3CD in yeast cells.

Previous studies have shown that the capsid precursor protein P1 and the protease 3CD are necessary and sufficient for generation of 14S and 74S subviral particles *in vitro* (Jore *et al.*, 1991). Whereas synthesis of P1 in yeast has already been described (Verbakel *et al.*, 1988), synthesis of the second required component 3CD, has not been studied so far. We introduced the *GAL7* and *PGK* promoters into our first expression plasmids, since these promoters were known to give efficient synthesis of polio-specific proteins in *S.cerevisiae* strain X904 (Verbakel *et al.*, 1987; 1988). Plasmids pLOP354 (*GAL7* promoter) and pLOP356 (*PGK* promoter) were constructed (Fig. 1A) and transferred to strain X904. Even though transferring pLOP354 to strain X904 yielded many transformants, none were obtained with pLOP356. Whereas pLOP354 carries an inducible 3CD transcription unit, the 3CD transcription unit in pLOP356 contains a constitutive promoter; constitutive expression of 3CD is apparently harmful to yeast cells. Yeast cells were transformed with pLOP354 and induced. Their contents were fractionated into a soluble and a pellet fraction. Only the pellet fraction showed two protein bands on a Western blot (results not shown). One band presumably corresponds to 3CD (72 kDa) and the other to 3D, the polio-specific RNA dependent RNA polymerase (52 kDa). The presence of the latter protein may indicate an enzymatic activity of 3CD, since 3C and 3D are connected via a Q-G bond that is a potential cleavage site for 3C/3CD (Nicklin *et al.*, 1986).

Direct proof of the enzymatic activity of 3CD synthesized in yeast was obtained in the following way: ^{35}S-methionine labelled P1 was synthesized in a rabbit reticulocyte lysate and used as a substrate for 3CD activity. Analysis of the enzymatic activity is shown in Fig. 2. Although yeast extracts in which no 3CD was to be expected caused degradation of P1, this was nonspecific, since no VP0, VP1 and VP3 were visible (lanes 3-5 and 6-8 in Fig. 2A). However, extracts of strain X904/pLOP354 transformants generated VP0, VP1 and VP3 (compare lanes 9 and 10, and lanes 12 and 13). These results show that 3CD synthesized in yeast is indeed enzymatically active towards P1. The fact that this activity could only be shown after dilution of the yeast extracts (e.g. compare lanes 10

and 11, and 13 and 14) suggests the presence of an inhibiting factor in the yeast extracts. Moreover, active 3CD is not only present in the pellet fraction, in which 3CD can be observed after Western blotting, but also in the supernatant fraction (e.g. compare lanes 10 and 13).

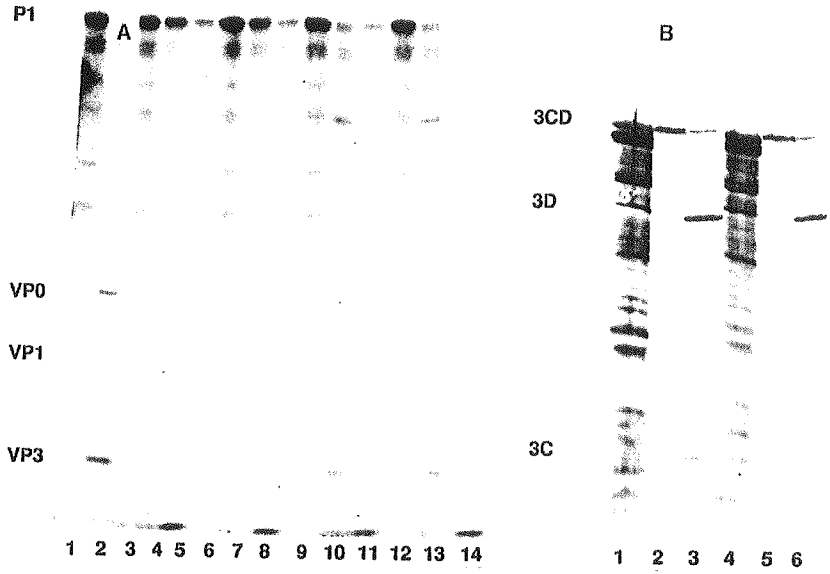


Fig. 2. Analysis of enzymatic activities of 3CD synthesized in yeast (strain X904). (A) Fluorogram obtained after gel electrophoresis of rabbit reticulocyte lysates containing ^{35}S-methionine labelled P1. The lysates were mixed 1:1 with either a lysate in which 3CD had been translated (lanes 1,2), *S.cerevisiae* X904/pMBL204 pellet fraction (3-5), X904/pMBL204 supernatant fraction (6-8), X904/pLOP354 pellet fraction (9-11) or X904/pLOP354 supernatant fraction (12-14). The mixtures applied to slots 1,3,6,9 and 12 served as t=0 controls, the mixtures applied to the other slots were incubated for 3 h at 30°C. Yeast extracts in lanes 3,4,6,7,9,10,12 and 13 were diluted 1:8 in blank lysate before mixing. (B) Western blot showing suppression of autocatalytic activity of 3CD. Lanes 1 and 4 contain *E.coli* JM109/pEXC3D pellet fraction, lane 2 supernatant fraction of *S.cerevisiae* X904/pLOP354ser, lane 5 contains the corresponding pellet fraction, lane 3 the supernatant fraction of X904/pLOP354 and lane 6 the corresponding pellet fraction. Blots were developed using rabbit anti-3CD as a first antibody and goat anti rabbit-IgG conjugated with HRP as a second antibody.

The presence of 3CD in the supernatant fraction may not have been detectable in the former experiment due to the low-titer (anti-3D) antiserum that was used there.

From studies employing *in vitro* transcribed and translated RNAs it has become clear that the whole 3CD polypeptide is required for full processing of P1 to VP0, VP1 and VP3 (Jore *et al.*, 1988; Ypma-Wong *et al.*, 1988). We therefore wanted to know whether the extra polypeptide band observed in the initial experiment indeed represented 3D and hence, whether this autocatalytic activity could be inhibited, since it was conflicting with optimal processing of P1. Therefore, use was made of a mutated 3CD sequence. The actual mutation, a duplication of the serine residue proximal to the C-terminus of 3C, causes a small-plaque phenotype (Ypma-Wong *et al.*, 1988), probably due to a lower autocatalytic activity of 3CD, resulting in a proportional decrease in the amount of 3D generated. The activity of 3CD towards P1 is, however, not influenced by this mutation (Gillis-Dewalt and Semler, 1987).

The polypeptide pattern of a yeast extract containing wild-type 3CD was compared in a Western blot with that of a yeast extract containing mutant 3CD (see Fig. 1A for construction of the corresponding plasmid pLOP354ser). Use was made of a newly prepared, high-titer 3CD-specific antiserum (see M. and M.). The results are shown in Fig. 2B. From this figure the following conclusions can be drawn: (1) The difference in polypeptide pattern between wild-type 3CD and mutant 3CD indeed indicates autocatalytic action of 3CD, since the amount of polypeptides that represent 3D and 3C, is greatly reduced in mutant 3CD (cf. lanes 2 and 3, and lanes 5 and 6). (2) The 3CD-specific serum not only reacts to 3CD in the pellet fractions (lanes 5 and 6), but also reveals the presence of 3CD in the soluble fractions (lanes 2 and 3), as might have been expected from the activity of the latter fractions towards cell-free synthesized P1. The P1 processing activity of such extracts is comparable with that of wild-type 3CD containing extracts (results not shown). The mutation apparently greatly reduced the autocatalytic cleavage of 3CD, thereby providing enough 3CD to process P1. Since it did not adversely influence the processing of P1, we decided to introduce the mutation into all our vectors.

Induced synthesis of 3CD in P1 producing yeast cells.

P1 DNA was inserted in pLOP354ser, carrying the constitutive *PGK* promoter and *PGK* terminator, resulting in the vector pLOP400A (see Fig. 1B). Induced cell extracts were prepared and analyzed by Western blotting (Fig. 3). There was hardly any difference in

P1 content before and after induction of 3CD, and only traces of processed P1 (VP0,

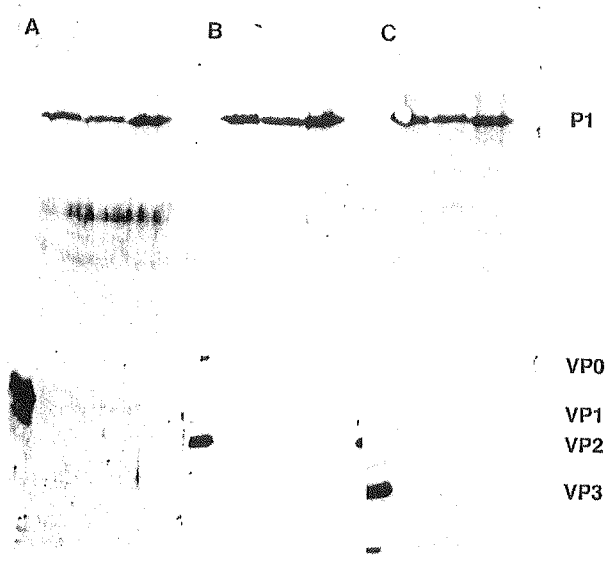


Fig. 3. Western blot analysis of strain X904/pLOP400Aextracts. Each panel presents, from left to right, a virus control, 30 μl of an extract of yeast cells before induction, 30 μl of an extract of yeast cells 6 h after induction, and a crude preparation of unprocessed P1. Panel A has been developed using rabbit anti-VP1, panel B using rabbit anti-VP2 and panel C using rabbit anti-VP3 antibodies. Goat anti rabbit-IgG conjugated with HRP was used as the second antibody.

VP1 and VP3) were present. A possible explanation for this inefficient processing is that transcription of the 3CD-coding region is hindered by transcription of the P1-coding region. However, results obtained with pLOP400B, in which the P1 transcription unit has a reversed orientation, were not different from those obtained with pLOP400A. We did, however, not check the effect of this reversal on transcription. An alternative explanation for the inefficient processing of P1 in pLOP400A, and -400B, transformants, is that only newly synthesized P1 can be cleaved efficiently by 3CD. Furthermore, it can not be excluded that glucose repressed transcription from *GAL* promoters, and thus was frustrating full induction of the 3CD gene. However, use of another yeast strain, 334 (Hovland *et al.*, 1989) in which there is no glucose repression, did not lead to an improved processing. Even so, this strain offers several advantages: *GAL* promoters can be easily induced by simply adding galactose to yeast cells growing in glucose containing medium. Moreover, strain 334 transformed with pLOP354 shows higher levels of 3CD

after induction than its X904 counterpart, and a substantial fraction of P1 synthesized in strain 334 is found as soluble material (results not shown). Further experiments were therefore carried out with this strain 334.

Simultaneous synthesis of P1 and 3CD with the aid of inducible transcription units on two plasmids.

pLOP254, a plasmid carrying an inducible (*GAL7* promoter driven) transcription unit for P1 (Verbakel *et al.*, 1988), was used for expression of P1. In designing an expression plasmid for 3CD, several conditions had to be fulfilled, namely a selection marker different from *LEU2* and an inducible promoter. To avoid complex induction media this promoter should be preferably induced by galactose. Moreover, high-level expression of 3CD should be avoided. So, in plasmid BO8-9 the *LAC9* coding sequence (driven by the *GAL1* promoter) was replaced by the 3CD-coding sequence, resulting in plasmid pLOP380 (see Fig. 1C). This plasmid contains *ARS* and *CEN* sequences and will therefore be present in one copy per cell; moreover, unlike pLOP254, that carries *LEU2* as a selection marker, it carries *URA3* as a selection marker. Plasmids pLOP254 and pLOP380 can therefore be maintained together in the same yeast cell, with the added advantage that selection of transformants can take place in two rounds, first for ura$^+$, then for leu$^+$ (direct selection of strain 334 transformants for leu$^+$ proved cumbersome). Thus strain 334 was transformed with pLOP254 and pLOP380, and extracts prepared at several time points after induction. In Fig. 4 a Western blot analysis of these extracts is shown. The presence of VP0, which became visible after 9 h of induction (lanes 5), indicates that P1 and 3CD were both synthesized during the induction period (since P1 and VP0 contain a complete VP2 polypeptide sequence, anti-VP2 antiserum will recognize P1 as well as VP0). Later time points (lanes 6-8) showed an efficient processing of P1. After 12-16 h of induction there was no further increase in the amount of VP0, of which a considerable part was present in the soluble fraction (presented in panel A). Extracts of pLOP254/380 transformants were further examined for the presence of subviral particles.

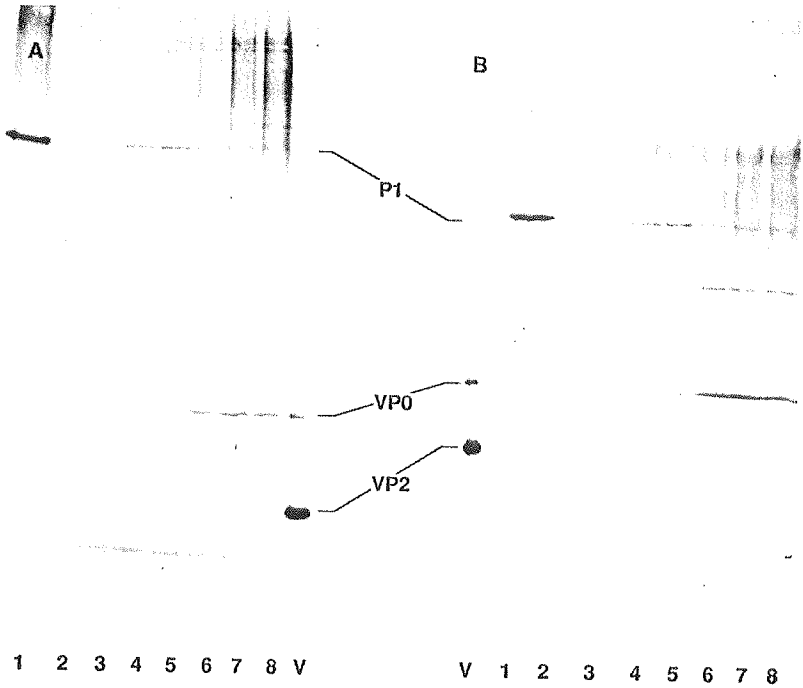


Fig. 4. Western blot analysis of extracts of strain 334 transformed with pLOP254 and PLOP380. Each panel contains as control preparations unprocessed P1 (lanes 1) and poliovirus proteins (lanes V) and samples taken at t=0 (lanes 2) and after 3 h (lanes 3), 6 h (lanes 4), 9 h (lanes 5), 12 h (lanes 6), 16 h (lanes 7) and 25 h of induction (lanes 8). The lanes 2-8 in panel A contain 50 μg of soluble protein, the lanes 2-8 in panel B contain the corresponding pellet fractions. Blots were developed using polyclonal rabbit anti-VP2 as a first antibody and goat anti rabbit IgG conjugated with horse radish peroxidase as the second antibody.

Analysis of subviral particles in extracts of yeast transformants.

As a first analysis total extracts of yeast transformants, induced to express P1 and 3CD, were analyzed on 20 to 60% sucrose gradients. Samples of the gradient fractions were run on a polyacrylamide gel, blotted onto a nitrocellulose filter and probed with VP2-specific antiserum. The results of Western blotting are shown in Fig. 5. Probably due to the large amount of yeast protein that was applied to the gradient, extensive cross reaction with the VP2-specific antiserum was observed (note that this antiserum was generated with the aid of a partially purified VP2 from *E.coli*). Nevertheless, it can be concluded that VP0 is present throughout the whole gradient, with slight enrichment in

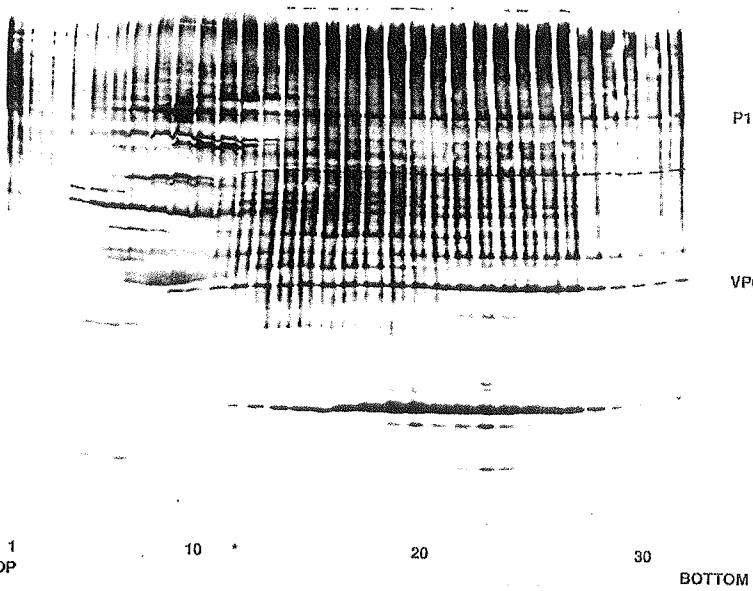


Fig. 5. Western blot analysis of an extract of yeast strain 334 transformant induced to express P1 + 3CD during 16 h. The extract was subjected to centrifugation through a 20 to 60% sucrose gradient (38 h at 5°C and 83,000g_{av}). The gradient was fractionated and 200 μl from each 1-ml fraction was concentrated by precipitation with 1 ml of aceton and 10 μg of BSA, and stored o/n at -20°C. Microfuge pellets were redissolved in sample buffer, electrophoresed and blotted. The blot was developed with polyclonal rabbit anti VP2 as a first antibody and goat anti rabbit-IgG conjugated with alkaline phosphatase as a second antibody. (*) indicates position of an external 14S marker.

the 100-1000S region (fractions 19 to 27). Material from this region was pooled, pelleted, resuspended by sonication and run on a second gradient where it peaked at 90S (a velocity exceeding that of 74S particles, isolated from infected HeLa cells; results not shown). Electron microscopic examination of the pooled material, however, revealed a lot of contaminating structures, so at least some purification was needed to allow visualization of subviral particles. Purification was performed as outlined in M. and M. Briefly, cells were opened by vortexing with glass beads; the 8000g supernatant was pelleted, resuspended and applied to a high-performance size-exclusion chromatography column; pooled fractions were then subjected to sucrose velocity gradient centrifugation. Fractionation was checked with the aid of Western blots, using VP2-specific polyclonal antiserum. Fig. 6A shows a Western blot illustrating this purification process.

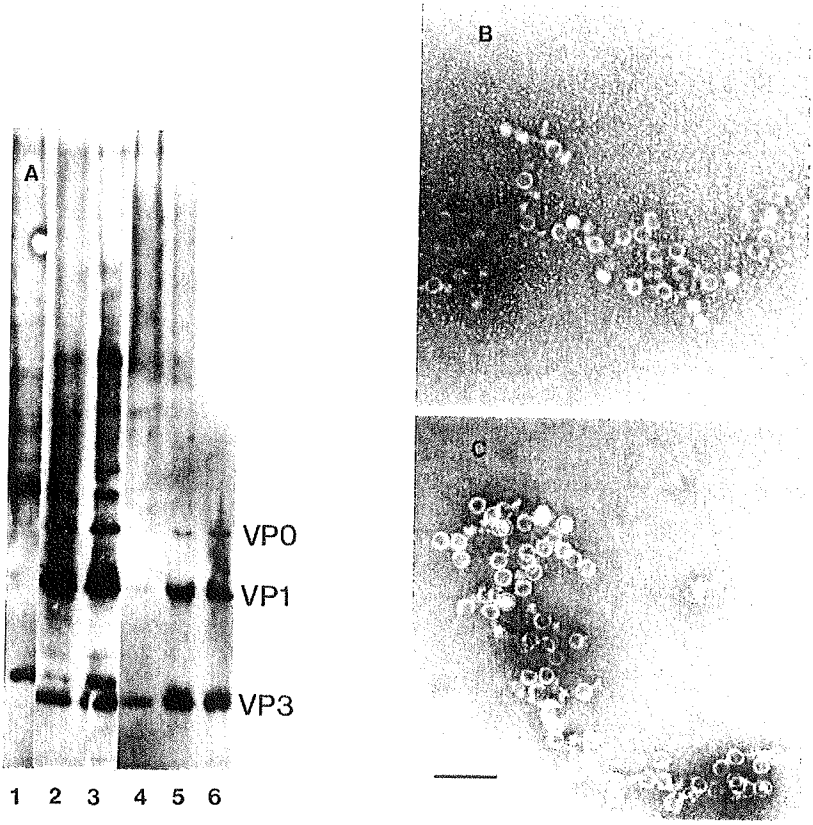


Fig. 6. Analysis of subviral particles synthesized in yeast. (A) Western blot. Lane 1 contains an aliquot of a resuspended high speed pellet of a yeast negative control. Lanes 2-5 contain samples taken at various stages of purification of an extract of yeast strain 334 induced to express P1 + 3CD; lane 2: resuspended high speed pellet; lane 3: material as represented in lane 2 was fractionated on an HPSEC column. Samples from these fractions were then subjected to polyacrylamide gel electrophoresis and Western blotting and those showing the most intense VP0 signal were pooled; lanes 4 and 5: as in lane 3, after velocity sedimentation through 15 to 30% sucrose gradient. Material in lanes 4 and 5 sedimented at the position of external 14S and 74S markers, respectively. Lane 6 contains 100 ng of pure empty capsids isolated from infected HeLa cells. The blot was developed with a mixture of monoclonal anti-VP1, anti-VP2 and anti-VP3 antibodies, and goat anti mouse-IgG conjugated with alkaline phosphatase as the second antibody. (B) and (C) Electron micrographs. Shown in (B) are particles from the same sucrose velocity gradient fraction as present in lane 5 of Fig. 6A and in (C) 74S particles purified from an infected HeLa cell culture. Particles were visualized by negative staining with uranyl acetate. Bar = 100 nm.

The blot was developed with a mixture of monoclonal VP1, VP2 and VP3-specific antibodies. In lane 5, containing material that sedimented at the 74S position, the presence of VP1 and VP3 (besides VP0) was evident. That particles were sedimenting at 74S, as opposed to 90S in the previous experiment, may have been due to the different purification protocol. Comparison with lane 6, that contains purified empty capsids isolated from infected HeLa cells, shows that the capsid proteins VP0, VP1 and VP3 were present in the same ratio as in the empty capsid control. Using Western blots with known amounts of empty capsids as a standard, it can be estimated that 0.1 to 1 mg of particles were obtained from 1 l of yeast culture. An electron micrograph of material from the same fraction as present in lane 5 is shown in Fig. 6B. Comparison with an electron micrograph of 74S empty capsids (Fig. 6C) reveals that the particles observed had the same configuration and size as authentic 74S empty capsids, although the yeast preparation contained a higher proportion of incomplete or damaged particles. Whereas the presumably polio-specific subviral particles were abundantly present in the induced pLOP254/380 transformant (Fig. 6B), only few particles were present in the corresponding fraction of an induced pLOP380 transformant, that served as a control (results not shown). Since the latter were slightly larger than the former, they may represent 40 nm indigenous viral like particles (VLP's).These have a documented sedimentation value of 160S (Herring and Bevan, 1974) and may therefore have been present at a 74S position due to trailing. The origin of the particles observed in the induced pLOP254/380 transformant was further analyzed with a newly developed competition immunoprecipitation assay (Rombaut *et al.*, submitted for publication). This method, that is in fact an improved radio-immunoassay, avoids the use of a solid carrier - and, hence, conformational changes in the antigen- and is very sensitive. Using this method it could be shown that the fraction presented in Fig. 6B was competing with purified empty capsids isolated from infected HeLa cells, whereas the corresponding fraction from the pLOP380 transformant was not. We therefore conclude that the particles observed in the pLOP254/380 transformant were polio-specific. The particles were, however, in the H-antigenic conformation. If this observation holds for other preparations as well, it means that subviral particles can not be assembled properly in *S.cerevisiae*. Rombaut *et al.* (1983) showed that 14S particles can be converted *in vitro*

into 74S particles by addition of an extract of poliovirus-infected HeLa cells. These extracts possess an "antigenicity conferring activity", leading to 74S particles that are antigenically indistinguishable from native virions; extracts of uninfected HeLa cells lack this activity (Rombaut *et al.*, 1984). Our results may indicate that yeast cells are in this respect not different from (uninfected) HeLa cells. Another explanation of our results may be denaturation during extraction and purification of the particles. It will be of interest, then, to investigate the effect of stabilizing agents like disoxaril (Rombaut *et al.*, 1990a; 1991), present either during the extraction and purification of the particles, or from the start of the synthesis.

To our knowledge this is the first example of synthesis in yeast of subviral particles consisting of more than one type of structural proteins. Valenzuela *et al.* (1982) and Miyanohara *et al.* (1986) were among the first to describe synthesis in yeast of heterologous particles consisting of one type of protein only, namely hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) particles, respectively. Yeast derived HBsAg particles are the basis of the first approved recombinant vaccine. Although release of HBsAg and HBcAg particles from the infected liver cell occurs via secretion, expression in yeast does not result in secretion, due to a block in the secretion pathway (Biemans *et al.*, 1992). Neither HBsAg nor HBcAg appears to be toxic to yeast, thus permitting constitutive as well as induced synthesis of these proteins. In the case of HBcAg very high levels of expression, up to 40% of soluble protein, have been obtained (Kniskern *et al.*, 1986).

The approach described in this paper, i.e., use of inducible transcription units on two different plasmids, might lend itself well to the synthesis of other picornavirus subviral particles. Moreover, polio-specific subviral particles can in principle replace attenuated recombinant viral particles as a carrier for heterologous antigenic determinants. The feasibility of antigenic chimeras has been demonstrated firstly by Burke *et al.* (1988), who replaced the antigenic site 1 in poliovirus type 1 by the corresponding site from poliovirus type 3. The resulting recombinant strain induced neutralizing antibodies against poliovirus type 3. Replacement of site 1 of poliovirus type 1 by non polio-specific sequences, for instance by the principal neutralization domain of human immunodeficiency virus (HIV-1), has been reported too (Dedieu *et al.*, 1992). This chimera evoked high levels of HIV-1

specific antibody in rabbits and produced a significant but weak HIV-1 neutralizing response.

Conclusions:

1) The yeast *Saccharomyces cerevisiae* can synthesize the precursor of the poliomyelitis virus capsid proteins in a cleavable form.

2) It can synthesize the poliomyelitis virus specific protease 3CD in an enzymatically active form.

3) Yeast strain 334 supports formation of subviral particles.

ACKNOWLEDGMENTS

The authors are grateful to Dr D. Dekegel and mrs G. De Vuyst for expert electron microscopic analysis.

REFERENCES

Arnold, E., Luo, M., Vriend, G., Rossmann, M.G., Palmenberg, A.C., Parks, G.D., Nicklin, M.J.H. and Wimmer, E. (1987). Implications of the picornavirus capsid protein structure for polyprotein processing. Proceedings of the National Academy of Sciences, U.S.A. 84, 21-25.

Biemans, R., Thines, D., Petre-parent, B., de Wilde, M., Rutgers,T. and Cabezon, T. (1992). Immunoelectron microscopic detection of the hepatitis B virus major surface protein in dilated perinuclear membranes of yeast cells. DNA and Cell Biology 11, 621-626.

Burke, K.L., Dunn, G., Ferguson, M., Minor, P.D. and Almond, J.W. (1988). Antigen chimaeras of poliovirus as potential new vaccines. Nature 332, 81-82.

Dedieu, J.F., Ronco, J., van der Werf, S., Hogle, J.M., Henin, Y. and Girard, M. (1992). Poliovirus chimeras expressing sequences from the principal neutralization domain of human immunodeficiency virus type 1. Journal of Virology 66, 3161-3167.

Foriers, A., Rombaut, B. and Boeyé, A. (1990). Use of high-performance size-exclusion chromatography for the separation of poliovirus and subviral particles. Journal of Chromotography 498, 105-111.

Gillis-Dewalt, B. and Semler, B.L. (1987). Site directed mutagenesis of proteinase 3C results in a poliovirus deficient in synthesis of viral RNA polymerase. Journal of Virology 61, 2162-2170.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology 166, 557-586.

Herring, A.J. and Bevan, E.A. (1974). Virus-like particles associated with the double stranded RNA species found in killer and sensitive strains of the yeast *Saccharomyces cerevisiae*. Journal of General Virology 22, 387-394.

Hawkes, R., Niday, A. and Gordon, J. (1982). A dot immuno binding assay for monoclonal and other antibodies. Analytical Biochemistry 119, 142-147.

Hinnen, A., Hicks, J.B. and Fink, G.R. (1978). Transformation of yeast. Proceedings of the National Academy of Sciences, U.S.A. 75, 1929-1933.

Hovland, P., Flick, J., Johnston, M. and Sclafani, R.A. (1989). Galactose as a gratuitous inducer of gal gene expression in yeasts growing on glucose. Gene 83, 57-64.

Jore, J., de Geus, B., Jackson, R.J., Pouwels, P.H. and Enger-Valk, B.E. (1988). Poliovirus protein 3CD is the active protease for processing of the precursor protein P1 *in vitro*. Journal of General Virology 69, 1627-1633.

Jore, J.P.M., Veldhuisen, G., Pouwels, P.H., Boeyé, A., Vrijsen, R. and Rombaut, B. (1991). Formation of subviral particles by *in vitro* translation of subgenomic poliovirus RNAs. Journal of General Virology 72, 2721-2726.

Kniskern, P.J., Hagopian, A., Montgomery, D.L., Burke, P., Dunn, N.R., Hofmann, K.J., Miller, W.J. and Ellis, R.W. (1986). Unusually high-level expression of a foreign gene (hepatitis B core antigen) in *Saccharomyces cerevisiae*. Gene 46, 135-141.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227, 680-685.

Lee, C., Levin, A. and Branton, D. (1987). Copper staining: a five-minute protein stain for sodium dodecyl sulfate polyacrylamide gels. Analytical Biochemistry 66, 308-312.

Mellor, J., Dobson, M.J., Roberts, N.A., Tuite, M.F., Emtage, J.S., White, S., Lowe, P.A., Patel, T., Kingsman, A.J. and Kingsman, S.M. (1983). Efficient synthesis of enzymatically active calf chymosine in *Saccharomyces cerevisiae*. Gene 24, 1-14.

Miyanohara, A., Imamura, T., Araki, M., Sugawara, K., Ohtomo, N. and Matsubara, K. (1986). Expression of hepatitis B virus core antigen gene in *Saccharomyces cerevisiae*: Synthesis of two polypeptides translated from different initiation codons. Journal of Virology 59, 176-180.

Nicklin, M.J.H., Kräusslich, H.G., Toyoda, H., Dunn, J.J. and Wimmer, E. (1986). Proteolytic processing in the replication of polio and related viruses. Bio/Technology 4, 33-42.

Nicklin, M.J.H., Kräusslich, H.G., Toyoda, H., Dunn, J.J. and Wimmer, E. (1987). Poliovirus polypeptide precursors: expression *in vitro* and processing by exogenous 3C and 2A proteinases. Proceedings of the National Academy of Sciences, U.S.A. 84, 4002-4006.

Nicklin, M.J.H., Harris, K.S., Pallai, P. and Wimmer, E. (1988). Poliovirus proteinase 3C: large-scale expression, purification, and specific cleavage activity on natural and synthetic substrates *in vitro*. Journal of Virology 62, 4586-4593.

Otter, T., King, S.M. and Witman, G.B. (1987). A two-step procedure for efficient electrotransfer of both high-molecular-weight (>400.000) and low-molecular-weight (<20.000) proteins. Analytical Biochemistry 162, 370-377.

Putnak, J.R. and Phillips, B.A. (1981). Picornaviral structure and assembly. Microbiological Reviews 45, 287-315.

Richards, O.C., Ivanoff, L.A., Bienkowska-Szewczyk, K., Butt, B., Petteway, jr., S.R., Rothstein, M.A. and Ehrenfeld, E. (1987). Formation of poliovirus RNA polymerase 3D in *Escherichia coli* by cleavage of fusion proteins expressed from cloned viral cDNA. Virology 161, 348-356.

Rogers Yocum, R., Hanley, S., West, R. and Ptashne, M. (1984). Use of lacZ fusions to delimit regulatory elements of the inducible divergent gal1-gal10 promoter in *Saccharomyces cerevisiae*. Molecular and Cellular Biology 4, 1985-1998.

Rombaut, B. and Boeyé, A. (1991). *In vitro* assembly of poliovirus 14S subunits: disoxaril stabilization as a model for the antigenicity conferring activity of infected cell extracts. Virology 180, 788-792.

Rombaut, B., Brioen, P. and Boeyé, A. (1990a). Disoxaril stabilization and immunogenicity of poliovirus procapsids. Journal of General Virology 71, 1081-1086.

Rombaut, B., Boeyé, A., Ferguson, M., Minor, P.D., Mosser, A. and Rueckert, R. (1990b). Creation of an antigenic site in poliovirus type 1 by assembly of 14S subunits. Virology 174, 305-307.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1983). Epitope evolution in poliovirus maturation. Archives of Virology 76, 289-298.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1984). *In vitro* assembly of poliovirus empty capsids: antigenic consequences and immunological assay of the morphopoietic factor. Virology 135, 546-550.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sijens, R.J., Brioen, P., Vrijsen, R., Rombaut, B., Thomas, A.A.M., Jackers, A. and Boeyé, A. (1983). Monoclonal antibodies to poliovirus and its capsid proteins. *In:* The protides of the biological fluids, vol.30, 513-515. Peeters, ed. Pergamon Press, Oxford.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences, U.S.A. 76, 4350-4354.

Toyoda, H., Nicklin, M.J.H., Murray, M.G., Anderson, C.W., Dunn, J.J., Studier, F.W. and Wimmer, E. (1986). A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell 45,761-770.

Valenzuela, P., Medina, A., Rutter, W.J., Ammerer, G. and Hall, B.D. (1982). Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 298, 347-350.

Verbakel, J.M.A., Dekker, K.A., Rutgers, C.A., Pouwels, P.H. and Enger-Valk, B.E. (1987). Construction of expression plasmids for *Saccharomyces cerevisiae*: application for synthesis of poliovirus protein VP2. Gene 61, 207-215.

Verbakel, J.M.A., Guicherit, O.M.A., van Dijk, M., Kottenhagen, M.J., Pouwels, P.H. and Enger-Valk, B.E. (1988). Synthesis of capsid proteins of poliomyelitis virus in *Saccharomyces cerevisiae.* Molecular Genetics (Life Science Advances) 7, 125-130.

Yanisch-Peron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

Ypma-Wong, M.F., Dewalt, P.G., Johnson, V.H., Lamb, J.G. and Semler, B.L. (1988). Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor. Virology 166, 265-270.

EXPRESSION OF THE POLIOVIRUS PRECURSOR PROTEIN P1P2P3 AND ITS PROTEASES 2A AND 3CD IN THE YEAST *SACCHAROMYCES CEREVISIAE*.

SUMMARY

The coding sequence for the poliovirus precursor protein P1P2P3 was transferred to *Saccharomyces cerevisiae* on a 2μm derived yeast expression plasmid. Transcription of polio-specific sequences was designed to be either constitutive, using the *PGK* promoter (in pLOP336), or inducible, by means of the *GAL7* promoter (in pLOP334). Only transformation with pLOP334 yielded colonies, that -upon induction- showed a relatively low level of expression of polio-specific proteins.

Transformation with 2μm derived yeast expression plasmids containing the coding sequence for the viral proteases 2A or 3CD likewise was only possible when this sequence was part of an inducible transcription unit, as present in pMBL204.2A and pLOP354, respectively. Induction of these transformants proved to be lethal.

In extracts of pMBL204.2A transformants an enzymatic activity was detected towards the same substrates as found for poliovirus polypeptide 2A.

The observed lethality of 2A and of 3CD could not be related to cleavage of identifiable host cell proteins.

INTRODUCTION

Poliovirus genomic RNA encodes a single large precursor polyprotein, P1P2P3, which is processed in a series of proteolytic steps to yield the virion capsid proteins and non-structural proteins (reviewed by Nicklin *et al.*, 1986). In this process two virally encoded proteases are involved, namely 2A and 3C/3CD. Protease 2A is primarily responsible for generating P1, the capsid precursor and 2A, by cleavage of a tyrosine-glycine (Y-G) bond (Toyoda *et al.*, 1986; Nicklin *et al.*, 1987). There are 9 other Y-G bonds present in the precursor polyprotein P1P2P3. Of these, only one -in 3CD- is cleaved by 2A, be it that

cleavage takes place inefficiently (Alvey *et al.*, 1991) and not in all types of strains (Lee and Wimmer, 1988). No functions have been ascribed to 3C′ and 3D′, the polypeptides thus generated. Apart from the enzymatic activity pertaining to the generation of viral structural proteins, 2A is also involved in the inactivation of a host factor (p220) required for cap-dependent initiation of translation (Kräusslich *et al.*, 1987; Lloyd *et al.*, 1988; Sun and Baltimore, 1989). The other virus-specific protease, 3C/3CD, cleaves 6 out of the 10 glutamine-glycine (Q-G) bonds present in P2 and P3, thus generating 3C, 3CD and other non-structural proteins (see Nicklin *et al.* (1986), and references cited therein). Full cleavage of Q-G bonds (2 out of 3) within P1 can be effected by 3CD only and results in formation of capsid proteins VP0, VP1 and VP3 (Jore *et al.*, 1988; Ypma-Wong *et al.*, 1988). No target protein for polio 3C/3CD other than the viral polyprotein has been identified so far, with the exception of the mammalian transcription factor TFIIIC (Clark *et al.*, 1991).

In this chapter experiments are reported, aimed at the expression of the whole poliovirus coding sequence in *Saccharomyces cerevisiae.* Since subviral (14S and 74S) particles can be synthesized in a rabbit reticulocyte lysate programmed with poliovirus RNA (Jore *et al.*, 1991), it was assumed that expression of the viral coding sequence in *S.cerevisiae* might lead to synthesis of 14S and 74S particles. These particles are attractive candidates for an alternative (subunit) vaccine, by virtue of their constitution and antigenic properties (Icenogle *et al.*, 1981). It is shown that *S.cerevisiae* is sensitive to constitutive expression of the viral polyprotein precursor P1P2P3. Experiments will be presented showing that both 2A and 3CD have deleterious effects on yeast cellular metabolism.

MATERIALS and METHODS

Strains

Escherichia coli strain TG1 (*sup*E, *hsd*Δ5, *thi*Δ(*lac-pro*AB), F′[*tra*D36, *pro*AB$^+$, *laq*I^q, *lac*ZΔM15]) (Gibson,1984) was used as the host strain for propagation and construction of all plasmids. The *Saccharomyces cerevisiae* strain 334 (*α,leu2*-3 -112, *gal1*, *reg1*-501, *ura3*-52, *pep4*-3, *prb1*-1122) (Hovland *et al.*, 1989) was used for synthesis of proteins.

Media

Bacteria were cultivated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 μg ampicillin per ml. YEPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for cultivation of yeast cells. YNB medium, which was used to select yeast transformants, contained 0.67% yeast nitrogen base and 2% glucose per ml. Further additions were dictated by the strain/ plasmid combination. Induction was obtained by adding galactose to 1% to cultures growing in YEPD.

DNA techniques

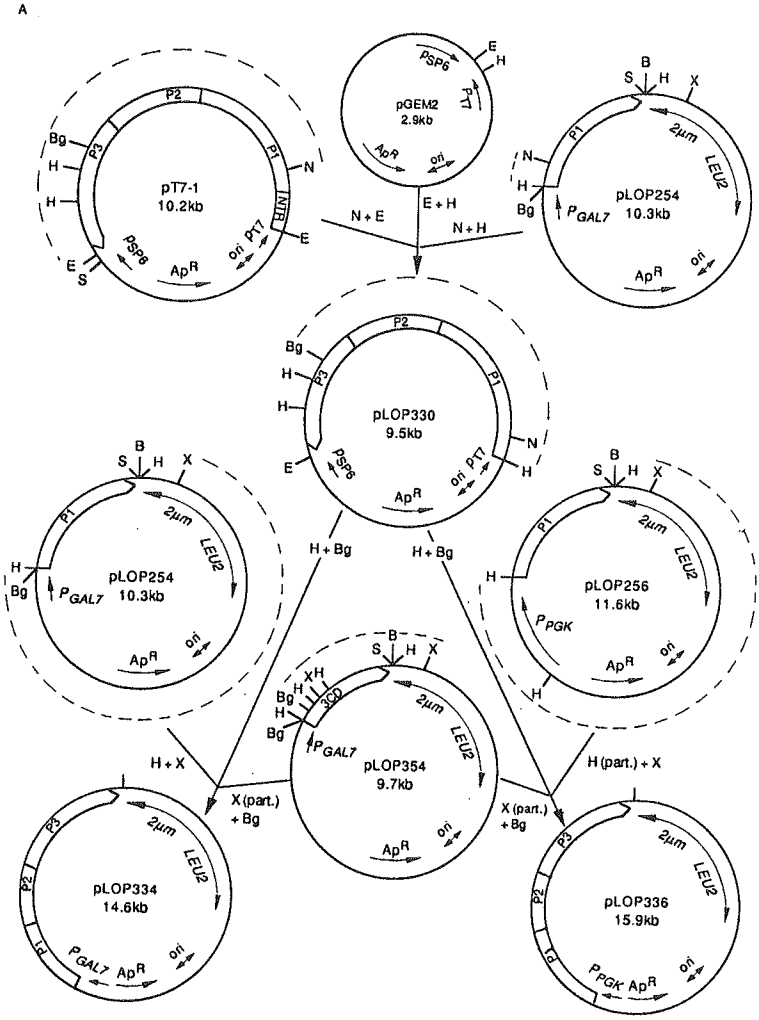
General recombinant DNA techniques were those compiled by Sambrook *et al.*(1989). Transformation of *E.coli* was carried out according to Hanahan (1983). Transformation of yeast cells was carried out by the protoplast method (Hinnen *et al.*, 1978) using helicase instead of zymolyase for the preparation of protoplasts. Since strain 334 does not easily permit direct leu$^+$ selection, protoplasts were, after transformation, covered with 60 μg/ml leucine containing soft agar. Transformants grew as large colonies on a background of nontransformed, small colonies and were subcultured by streaking them on a fresh selective plate without leucine.

Plasmids

The *in vitro* transcription plasmids pT7-1 (Ypma-Wong and Semler, 1987), pCITE-1 (Parks *et al.*, 1986) and pLOP315 (Jore *et al.*, 1988) have been described before. pT7-1 comprises the entire sequence of poliomyelitis virus type 1 Mahoney inserted into the *Eco*RI site of the *in vitro* transcription plasmid pGEM-1 (Promega Biotec, Madison, Wisconsin). pCITE-1 (Novagen, Madison, Wisconsin) was originally published as pELV0. It comprises the 5'cap-independent translation enhancer of encephalomyocarditis (EMC) virus plus the natural translation initiation region of EMC virus. pLOP315 comprises the 3CD-coding region plus a translation start codon inserted into the *in vitro* transcription plasmid pT7-6, a derivative of pT7-1 (Boehringer Mannheim, Mannheim, Germany). pGEM-2 was obtained from Promega Biotec (Madison, Wisconsin).

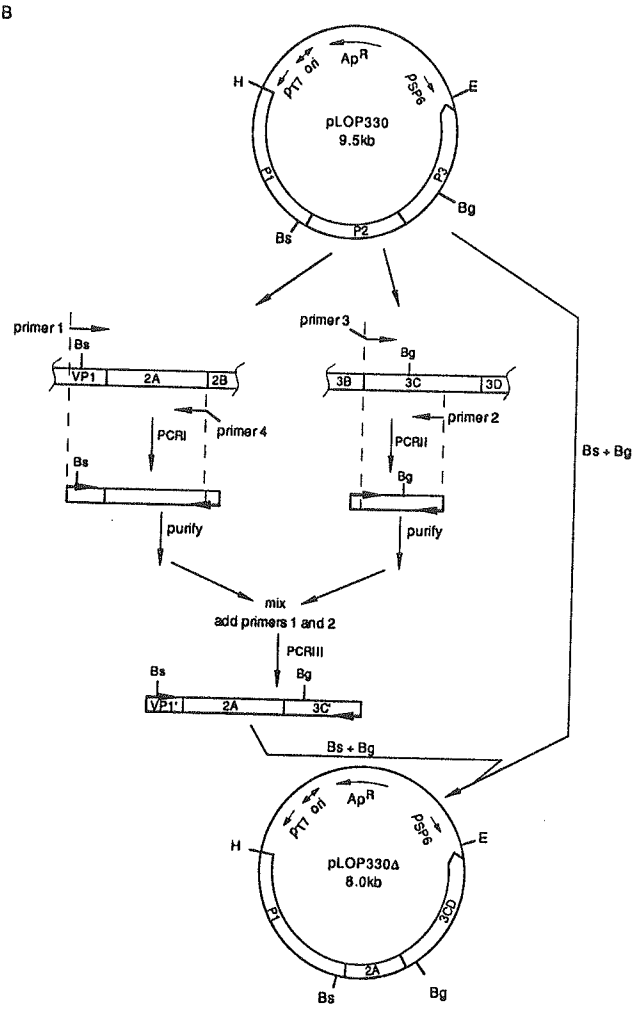
The yeast expression plasmids used in this study are yeast-*E.coli* shuttle plasmids, derived from the 2μm endogenous yeast plasmid and containing *LEU2d* as a yeast selection

marker. pLOP254 and -256, carrying a transcription unit for P1 with the *GAL7* promoter and the *PGK* promoter, respectively, have been described before (Verbakel *et al.*, 1987). Plasmid pMBL204 is a yeast expression plasmid containing the *GAL7* promoter followed by a multiple cloning site (Verbakel *et al.*, 1987). Plasmid pMA91 (Mellor *et al.*, 1983) comprises the *PGK* promoter and terminator. Construction of pLOP354, carrying a transcription unit for 3CD with the *GAL7* promoter, has been described elsewhere (Jore *et al.*, submitted). Construction of the yeast expression plasmids pLOP334, pLOP336, pMA91.2A, pMBL204.2A and of the *in vitro* transcription plasmids pLOP330, pLOP330Δ, pGEM2A and pCITE.VP1′.2A′ is outlined in Fig. 1.


A
pT7-1
10.2kb
pGEM2
2.9kb
pLOP254
10.3kb
pLOP330
9.5kb
pLOP254
10.3kb
pLOP256
11.6kb
pLOP354
9.7kb
pLOP334
14.6kb
pLOP336
15.9kb
N + E
E + H
N + H
H + Bg
H + Bg
H + X
X (part.) + Bg
X (part.) + Bg
H (part.) + X
ApR
ori
LEU2
2µm

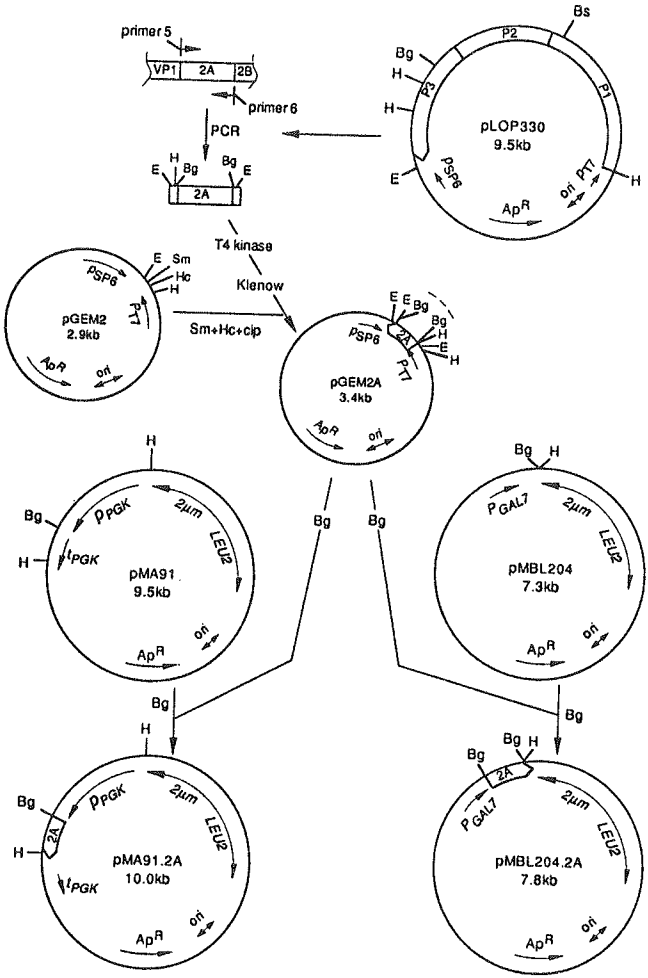
-/continued .....

B


pLOP330
9.5kb
H
E
Bs
Bg
P1
P2
P3
ApR
PT7 on
pSP6
primer 1
primer 3
VP1
2A
2B
3B
3C
3D
PCRI
PCRII
primer 4
primer 2
Bs + Bg
purify
mix
add primers 1 and 2
PCRIII
VP1'
3C'
pLOP330Δ
8.0kb
3CD

-/continued .....

c


primer 5
VP1
2A
2B
primer 6
PCR
E H Bg Bg E
2A
T4 kinase
Klenow
Sm+Hc+cip
pGEM2
2.9kb
pGEM2A
3.4kb
pLOP330
9.5kb
pMA91
9.5kb
pMA91.2A
10.0kb
pMBL204
7.3kb
pMBL204.2A
7.8kb
Ap^R
ori
LEU2
2µm

-/continued .....

D

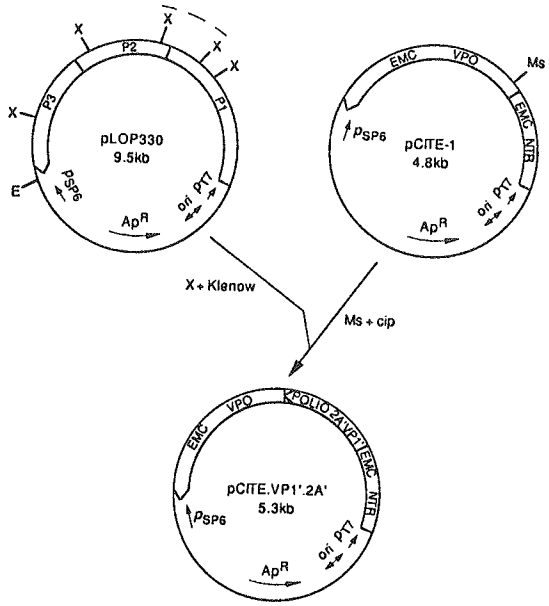


Fig. 1. Strategy for construction of the plasmids used in this study. (A) P1P2P3-coding plasmids pLOP330, -334 and -336; (B) P1.2A.3CD-coding plasmid pLOP330Δ; (C) 2A-coding plasmids pMA91.2A and pMBL204.2A and (D) VP1'.2A'-coding plasmid pCITE.VP1'.2A'. In cases where more than one fragment is generated, the fragment of choice is indicated by a dashed arc outside the circle that depicts the plasmid. Only relevant sites are indicated. The plasmids are not drawn to scale. Abbreviations, B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; Ms, *Msc*I; N, *Nru*I; S, *Sst*I; Sm, *Sma*I; X, *Xba*I; Ap^R, ampicillin-resistance gene; ori, origin of ColE1 DNA replication; 2μm, origin of yeast 2μm plasmid DNA replication; PCR, polymerase chain reaction. Primers used were:
5' ACCAAGGTCACCTCCAAAATCAGAGT 3' (1);
5' ATTTCTCTTTAGAGTGATTATAGTGATTTC 3' (2);
5' CCTACGAAGAAGAAGCCATGGAACAAGGACCAGGGTTCGATTACGCAGTG 3' (3);
5' CACTGCGTAATCGAACCCTGGTCCTTGTTCCATGGCTTCTTCTTCGTAGG 3' (4);
5' GAATTCAAGCTTAGATCTAAAAATGGGATTCGGACACCAAAACAAAGC 3' (5);
5' GAATTCAGATCTATTATTGTTCCATGGCTTCTTCTTCGTA 3' (6).

***In vitro* transcription and translation and analysis of labelled proteins.**
Conditions for *in vitro* transcription and translation as well as the processing of polyacrylamide gels were as described (Jore *et al.*, 1988).

Polymerase chain reaction (PCR).
PCR was performed in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, Connecticut) using *Taq* DNA polymerase (Ampli Taq, Perkin Elmer Cetus, Norwalk, Connecticut). Each of the 25 cycles consisted of a 1 min incubation at 94°C, followed by 1 min at 62°C and 1 min at 72°C, the latter step being the temperature of actual synthesis. Each reaction (100 μl, covered with mineral oil) contained 1 ng DNA, 1.25 units of Ampli Taq, 20 nmole dNTP's, 100 pmole primers and 0.01% (w/v) gelatin in 50 mM KCl, 10 mM Tris (pH 8.3) and 1.5 mM $MgCl_2$.

Synthesis of oligonucleotides.
Oligonucleotides were synthesized in a Cyclone 8400 DNA synthesizer (Biosearch Inc., San Rafael, California) and purified according to the manufacturer's instructions.

Cultivation of yeast and preparation of extracts.
A fresh transformant of strain 334 was picked from a selective YNB plate and cultivated o/n in YNB medium. Subsequently cells were counted and diluted to $5x10^6$/ml in YEPD medium. After o/n growth cells were counted again, at which moment they had normally reached a concentration of $5x10^7$/ml, and induction was started by addition of 1% galactose. After the expression period, cells were collected by low speed centrifugation and resuspended in 0.5 ml of ice-cold PBS. Extracts were prepared by vortexing the cells with 0.5-mm glass beads. The clear supernatant fraction, obtained after centrifugation in an Eppendorf centrifuge, was either analyzed immediately or stored at -80°C, as was the resuspended pellet fraction.

Western blot analysis

Extracts and pellet fractions were subjected to electrophoresis in SDS-12.5% polyacrylamide gels (Laemmli, 1970). For analysis by the Western blotting procedure (Towbin *et al.*, 1979) proteins were transferred from gels to a nitrocellulose filter with an electroblotting device (Biorad, Richmond, California), using a buffer with doubly concentrated Tris and glycine (Otter *et al.*, 1987). The filter was treated as described by Hawkes *et al.* (1982), using either rabbit polyclonal VP2-specific antiserum (Verbakel *et al.*, 1988) or rabbit polyclonal 3CD-specific antiserum (Jore *et al.*, submitted) and goat anti rabbit IgG conjugated with alkaline phosphatase as a second antibody. For analysis of proteolytic activity of 2A towards rabbit p220 the procedure was essentially the same, using rat anti rabbit eIF-4F (=eIF-4A + eIF-4E + p220) as a first antibody (A.Thomas, submitted) and goat anti rat IgG conjugated with alkaline phosphatase as a second antibody. To establish the proteolytic activity of 2A towards yeast protein p150, rabbit anti-p150 antiserum was used as a first antiserum (Altmann *et al.*, 1987) and swine anti rabbit IgG conjugated with alkaline phosphatase as a second antibody.

Determination of protein concentrations.

Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, Illinois) with bovine serum albumin as a standard.

2D-gel analysis of proteins labelled *in vivo*.

Yeast transformants were cultivated in YNB medium untill they reached a concentration of ~3.10^7/ml. For labeling 20 μCi/ml ^{35}S-methionine was added. Induction was started by addition of 1% galactose. 1-ml samples were centrifuged in an Eppendorf microfuge. The pellets were frozen in liquid N_2 and stored at -80°C untill use. 2D-gel electrophoresis was performed essentially as described by Thoraval *et al.* (1990).

RESULTS AND DISCUSSION

Expression of poliovirus proteins from polygenic messengers.

Plasmids pLOP334 and 336 were constructed as depicted in Fig. 1A, and transferred to *S.cerevisiae* strain 334. Whereas transformation of strain 334 with pLOP334 repeatedly gave large numbers of transformants, pLOP336 did not yield a single colony, suggesting that constitutive expression of polio proteins has a lethal effect on yeast. Extracts of 334/pLOP334 (P1P2P3) transformants were prepared after various periods of induction with galactose and compared with extracts of 334/pLOP254 (P1), using Western blotting analysis.

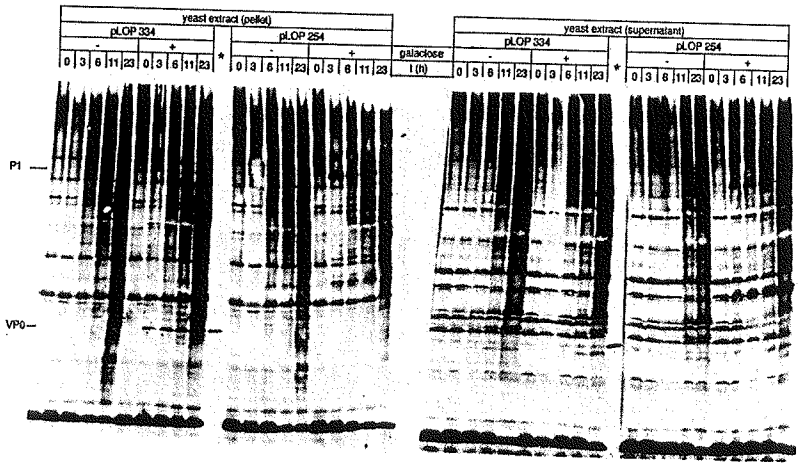


Fig. 2. Western blot of a 10% polyacrylamide gel loaded with extracts of yeast transformants and probed with anti-VP2. In the lanes marked (*) a purified procapsid preparation is present as a marker. Each lane contains 100 μg protein (soluble fraction) or the corresponding amount of insoluble material (pellet). Processing of gel and blot was as described in Materials and Methods.

As can be seen in Fig. 2, induction of 334/pLOP334 transformants results in a relatively low amount of polio-specific anti-VP2 reactive material (= VP0 + P1), that is mainly present in the insoluble fraction. Induction of 334/pLOP254 transformants results in synthesis of considerably more anti-VP2 reactive material (= P1), notably in the soluble fraction. This is remarkable, since no major differences in the transcription pattern are

pattern are to be expected: Plasmid pLOP254 contains a transcription unit that is identical to that in pLOP334 up till the end of the P1-coding sequence. Also, termination of transcription is supposed to take place at the same site in both plasmids (i.e. the 2μm *ABLE* region). The only difference as far as transcription concerns, is a difference in mRNA length, being 2400 nucleotides for P1 RNA (encoded by pLOP254) and 6700 nucleotides for P1P2P3 RNA (encoded by plasmid pLOP334). It cannot be excluded that the latter length causes instability (Brown *et al.*, 1988). Another possible reason for the observed low level of expression in strain 334/pLOP334 may be synthesis of toxic proteins, which prevent a higher overall level of synthesis. Apart from the non-structural proteins 2A and 3CD, both identified as proteinases, 3D (identified as a polymerase) and 3B, a peptide covalently bound to the 5′ end of the viral RNA, are formed. The function of the other products of the P2 and P3 regions, i.e., 2B, 2C and 3A, is essentially unknown. A new plasmid was constructed in which the coding sequence between 2A and 3C was precisely deleted using polymerase chain reactions (see Fig. 1B). This not only reduced the length of the polygenic messenger, but also precluded expression of polypeptides 2B, 2C, 3A and 3B. For this purpose 4 primers were synthesized, two of which were to be used as "outside" primers, each covering a unique restriction site (primers 1 and 4); the other two are fully complementary to each other, so as to connect the last nucleotide of the 2A-coding sequence with the first nucleotide of the 3C-coding sequence (primers 2 and 3). *In vitro* transcription and translation of the plasmid thus obtained, pLOP330Δ, shows a correct pattern of processing (Fig. 3), be it that this processing is not complete. Processing as such is not unexpected, however, since the bond generated between 2A and 3CD is again a Q-G bond in a for 3CD presumed optimal aminoacid context (Blair and Semler, 1991), like in 2A/2B and in 3B/3CD. Deletion of the 2A-coding sequence in pLOP330Δ, yielding pLOP330ΔΔ, creates an Y-G bond that is expected not to be a target for autocatalytic cleavage by 3CD. Indeed, after *in vitro* transcription and translation of pLOP330ΔΔ and analysis of the proteins thus obtained a large protein product, presumably P1.3CD, can be observed, but no VP0, VP1 and VP3 (results not shown), stressing the necessary presence of 2A to liberate P1 from the polygenic messenger encoded precursor protein. A yeast expression vector, pLOP337, was obtained with the same deletion as in pLOP330Δ, by substituting pLOP330Δ for

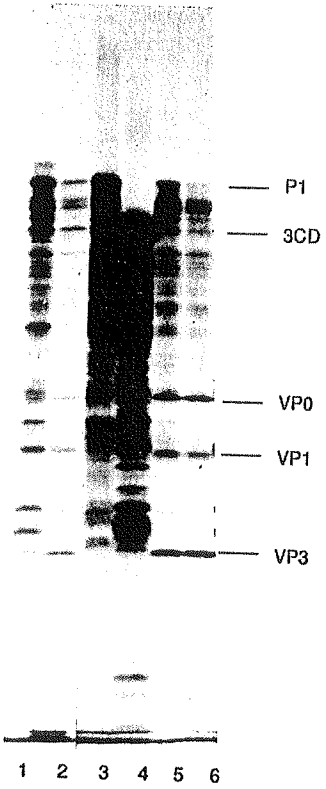


Fig. 3. Fluorogram of a 12.5% polyacrylamide gel showing the polypeptide pattern of a rabbit reticulocyte lysate (RRL) in which pLOP330Δ RNA was translated in the presence of ^{35}S-methionine (lanes 1-2). Lane 1, 1 h incubation; lane 2, 16 h incubation. Lane 3 contains an RRL in which P1 (pLOP324) RNA has been translated for 1 h, lane 4 contains an RRL in which 3CD (pLOP315) RNA has been translated for 1 h. Lanes 5 and 6 were loaded with an RRL containing ^{35}S-methionine labelled P1 after incubation with a nonlabelled 3CD containing RRL for 1 h (lane 5) or 16 h (lane 6). Processing of the gel was as described in Materials and Methods.

construction scheme for pLOP334, as depicted in Fig. 1A. When pLOP337 was transferred to strain 334 and extracts were analyzed for expression after induction, the level of synthesis of polio-specific proteins was at most equal to that in pLOP334 transformants (results not shown). Apparently, a reduction of the length of the transcription unit does not increase the efficiency of expression of the remaining genes. Instability of mRNA as a reason for the low level of expression in pLOP334 and pLOP337 transformants cannot be ruled out on the basis of this experiment, however (a 5.2 kb messenger RNA can still be regarded as long). Nor can it be concluded that polio polypeptides 2B, 2C, 3A and 3B are nontoxic. To study the reason for the low level of expression further experiments were aimed at investigating the possible toxicity of polio polypeptides 2A and 3CD.

Expression of protease 2A (1).

Activity towards virally encoded polypeptides.

To be able to study the effects of 2A on yeast, its coding sequence was subcloned. Correct expression of the cloned gene was demonstrated by testing for the proper enzymatic activities towards virally encoded polypeptides. As a first step towards these

goals, the coding sequence for 2A was supplied with translational start and stop codons with the aid of a polymerase chain reaction and placed in an *in vitro* transcription plasmid (see Fig. 1C). A transcript was made and translated in a rabbit reticulocyte lysate.
Fig. 4 shows the translational products of 2A transcripts, as well as their effects on two different substrates. The main band after *in vitro* translation of 2A RNA is a protein of the expected size (2A has a molecular weight of 16.6 kDa) (lane 1). The supposition that this protein represents 2A is supported by the enzymatic activities that can be detected in rabbit reticulocyte lysates in which it has been expressed (Fig. 4, lanes 2-6). The protein encoded by pCITE.VP1'.2A' (see Fig. 1D) consists of the C-terminal part of VP1 (aminoacids 129-302) connected to the N-terminal part of 2A (aminoacids 1-66). The protein has a molecular weight of 28.8 kDa and contains the main target site for 2A. This protein was labelled with ^{35}S-methionine and incubated together with a rabbit reticulocyte lysate in which 2A had been expressed without any added label. As can be seen in Fig.4 (lane 3), processing of VP1'.2A' took place. (It can be calculated that the proper cleavage products have molecular weights of 19.7 kDa and 9.1 kDa; the apparent absence of the latter on the fluorogram may be due to the presence of 1 methionine residue per molecule only). From these results it can be concluded that the cleavage of the P1.2A bond, that is thought to occur *in cis* under *in vivo* conditions, also takes place *in trans*. Furthermore, if there is a conformational constraint on this bond for cleavage to occur, it is already met by the sequence present in pCITE.VP1'.2A'. Protease 2A, expressed *in vitro*, is also able to cleave ^{35}S-labelled 3CD, resulting in polypeptides 3C' and 3D' (lanes 4-5). Its capability to properly cleave both VP1'.2A' and 3CD polypeptides indicates that our recombinant 2A carries the authentic enzymatic activities.
Having demonstrated that 2A possesses the correct enzymatic activities, and is therefore a *bona fide* 2A, we investigated whether expression of 2A has effects that might explain the nontransformability of yeast by vectors comprising a constitutive transcription unit for -amongst others- 2A. To this end the 2A-coding sequence- including translational start and stop codons- was excised from plasmid pGEM2A and transferred to yeast expression plasmids. The 2A-coding region was placed under the control of the *GAL7* promoter or the *PGK* promoter, resulting in the vectors pMBL204.2A and pMA91.2A respectively (see Fig. 1C). Transformation of yeast with these vectors was only successful in the case

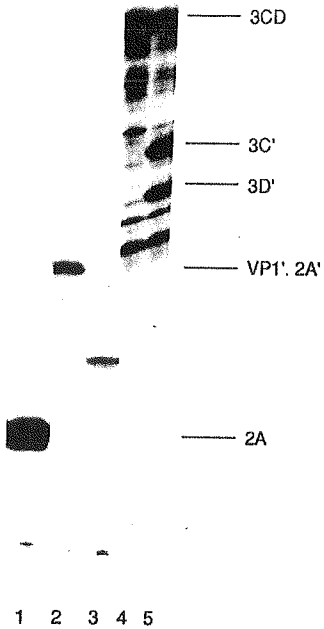


Fig. 4

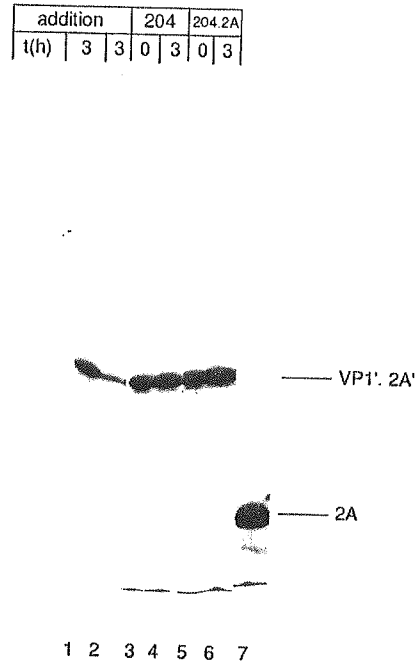


Fig. 5

Fig. 4. Fluorogram of a 15% polyacrylamide gel showing the polypeptide pattern of pGEM2A RNA translated in a rabbit reticulocyte lysate (RRL) in the presence of ^{35}S-methionine (lane 1) and of cleavage products generated by 2A. In lanes 2-3 ^{35}S-methionine labelled VP1'.2A', obtained by *in vitro* transcription and translation, served as a substrate. Lane 2 shows the pattern after 16 h incubation at 30°C with a blank RRL, lane 3 shows the pattern after 16 h incubation with a (nonlabelled) RRL containing 2A. Lanes 4-5 are as lanes 2-3, however with an RRL containing labelled 3CD as a substrate. Processing of the gel was as described in Materials and Methods.

Fig. 5. Fluorogram of a 12.5% polyacrylamide gel showing enzymatic activity of 2A synthesized in yeast. Yeast transformants (referred to as 204 and 204.2A) were cultivated as described in Materials and Methods and induced for 7 h at 28°C by the addition of galactose. Total extracts were tested. pCITE.VP1'.2A' RNA was translated in a rabbit reticulocyte lysate (RRL) in the presence of ^{35}S-methionine and served as a substrate. Lanes 1 and 2 contain substrate mixed with either a blank RRL or with an RRL in which 2A had been translated. Lanes 3-6 contain substrate mixed with either control extracts (204) or with extracts containing 2A (204.2A). Lane 7 contains an RRL in which pGEM2A RNA had been translated in the presence of ^{35}S-methionine. The times indicated on top of the fluorogram refer to the times of incubation after mixing. Incubation took place at 30°C. Processing of the gel was as described in Materials and Methods.

of pMBL204.2A. Plasmid pMA91.2A, containing a constitutive transcription unit for 2A, could not be stably transferred to *S.cerevisiae* strain 334, reminiscent of the problems

encountered with pLOP336 vs. pLOP334 (constitutive vs.induced expression of P1P2P3), and with pLOP356 vs. pLOP354 (constitutive vs. induced expression of 3CD; see chapter 4 of this thesis). Extracts of strain 334 transformed with pMBL204.2A and induced with galactose to express 2A, were mixed with rabbit reticulocyte lysates in which ^{35}S-methionine labelled VP1'.2A' was synthesized. In these extracts enzymatic activity of 2A could be observed with the same specificity as observed in a rabbit reticulocyte lysate expressing 2A: cleavage of the VP1.2A bond, as present in pCITE.VP1'.2A',and cleavage of an Y-G bond in 3CD, resulting in 3C' and 3D' (see Figs. 5 and 6, respectively). The VP1.2A bond could be properly cleaved by 2A -synthesized in yeast-, although very inefficiently, by mixing a total yeast extract with a rabbit reticulocyte lysate containing labelled pCITE.VP1'.2A' (see Fig.5, lane 6). The poor efficiency of cleavage could not be improved by increasing the temperature to 37°C, nor by incubating soluble and insoluble fractions of the yeast extract separately with the substrate. Decreasing the induction period of the pMBL204.2A yeast transformant from 6 to 2 h had no beneficial effect either. Cleavage of 3CD by 2A -synthesized in yeast- could only be demonstrated after separation of 2A-containing yeast extracts in soluble and insoluble fractions. From Fig. 6 it can be concluded that cleavage of 3CD, like that of the VP1.2A bond, is much less efficient with 2A synthesized in yeast, than with 2A synthesized in a rabbit reticulocyte lysate. The activity could be observed in the insoluble fraction of the yeast extract without any dilution. However, the soluble fraction of the yeast extract had to be diluted in order to reveal its 3CD cleaving activity, suggesting the presence of an inhibiting factor in the soluble fraction. From these data it can be concluded that 2A is synthesized in yeast as a polypeptide with proper enzymatic activities towards virally encoded target sequences.

Activity towards mammalian factor p220.

All eukaryotic cellular mRNAs, and most viral mRNAs, carry a 5' cap structure that facilitates ribosome binding to mRNA. The initiation factor that interacts first with the cap structure is eIF-4F. One of the subunits of eIF-4F is a 220 kDa polypeptide (termed p220) (see Sonenberg and Pelletier (1991), and references cited therein). Infection of mammalian cells by poliomyelitis virus results in a complete suppression of cellular protein synthesis ("host shut-of") (for a review see Sonenberg, 1987).

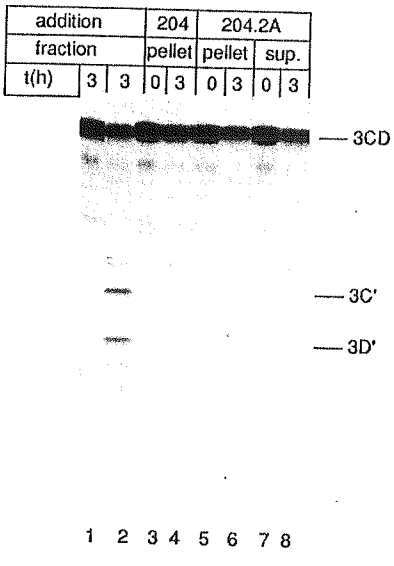


Fig. 6

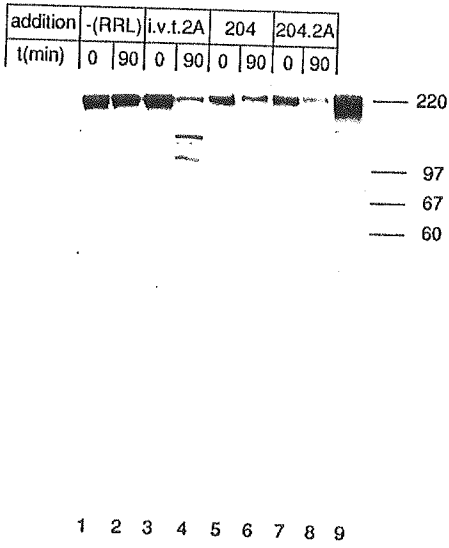


Fig. 7

Fig. 6. Fluorogram of a 12.5% polyacrylamide gel showing enzymatic activity of 2A synthesized in yeast. Yeast transformants were cultivated as described in Materials and Methods and induced for 7 h at 28°C by the addition of galactose. Extracts of yeast transformants (referred to as 204 and 204.2A) were prepared and fractionated in soluble and insoluble material (pellet) by centrifugation in an Eppendorf microfuge (10 min at 12,000 rpm). The pellet was resuspended in the original volume of buffer. pLOP315 (3CD) was translated in a rabbit reticulocyte lysate (RRL) in the presence of ^{35}S-methionine and served as a substrate throughout. Lanes 1 and 2 contain substrate mixed with a blank RRL and with an RRL in which 2A had been translated, respectively. The times indicated refer to the time of incubation after mixing. Incubation took place at 30°C. Prior to incubation the yeast extracts were diluted 1:8 in a blank RRL. Processing of the gel was as described in Materials and Methods.

Fig. 7. Western blot of a 10% polyacrylamide gel showing activity of 2A towards rabbit reticulocyte lysate (RRL) p220. 2A was introduced in the RRL via *in vitro* translation of pGEM2A RNA (i.v.t. 2A, lanes 3-4). Alternatively it was introduced by addition of the resuspended insoluble fraction of a yeast transformant that had been induced for 6 h to synthesize 2A (pMBL204.2A) (lanes 7-8). Lane 9 contains an RRL KCl wash, highly enriched for p220. Incubation took place at 30°C. Processing of gel and blot was as described in Materials and Methods. Molecular weight markers are indicated in kDa on the right.

Although the relationship between cleavage of p220 and (complete) host shut-off is not established yet (Bonneau and Sonenberg, 1987; Buckley and Ehrenfeldt, 1987; O'Neill and Racaniello, 1989; Pérez and Carrasco, 1992), the involvement of poliovirus protease 2A in cleavage of p220 has been proven by several authors (Kräusslich *et al.*, 1987; Lloyd

et al., 1988; Sun and Baltimore, 1989). A further confirmation of the authenticity of 2A as produced *in vitro* and *in vivo* was therefore sought in the analysis of the influence of 2A on p220. From Fig. 7 it can be concluded that 2A, when synthesized in a rabbit reticulocyte lysate *in vitro*, is indeed able to bring about cleavage of (rabbit) p220 (compare lanes 2 and 4). This suggests a similarity in 2A-induced processes in human (HeLa) and rabbit cells. More important, however, is the fact that extracts of yeast in which 2A has been expressed also cause cleavage of (rabbit) p220 when mixed and incubated with a (blank) rabbit reticulocyte lysate (compare lanes 6 and 8). This confirms the authenticity of 2A synthesized in yeast.

Survival of yeast upon expression of 2A and 3CD.

As mentioned before, transformation of yeast strain 334 with a plasmid comprising a constitutive transcription unit for P1P2P3 (pLOP336) or for 2A (pMA91.2A) does not yield any transformants. The same holds for transformation with a plasmid comprising a constitutive transcription unit for 3CD (pLOP356, see chapter 4 of this thesis). To be able to differentiate between arrest in cell growth and cell killing, survival of yeast upon induced expression of 2A or 3CD was determined. The results are presented in Fig. 8. Whereas induction of 3CD and 2A clearly has lethal effects, induction of pMBL204 has not. In other words, proteins 2A and 3CD themselves are toxic to yeast. However, induction of a derivative of BO8-9 encoding 3CD in stead of *LAC9*, pLOP380, is not lethal. This indicates that yeast can survive lower doses of 3CD (see also chapter 4 of this thesis), be it that some retardation in growth can be observed (results not shown). It is not known whether this dose dependency also holds for 2A, since the corresponding derivative of BO8-9 has not been made. Furthermore, it is worth mentioning that codon usage as a reason for lethality (Romanos *et al.*, 1992) is not very likely, since codon usage in P1 -well tolerated by *S.cerevisiae*- is not significantly different from that in 2A and 3CD.

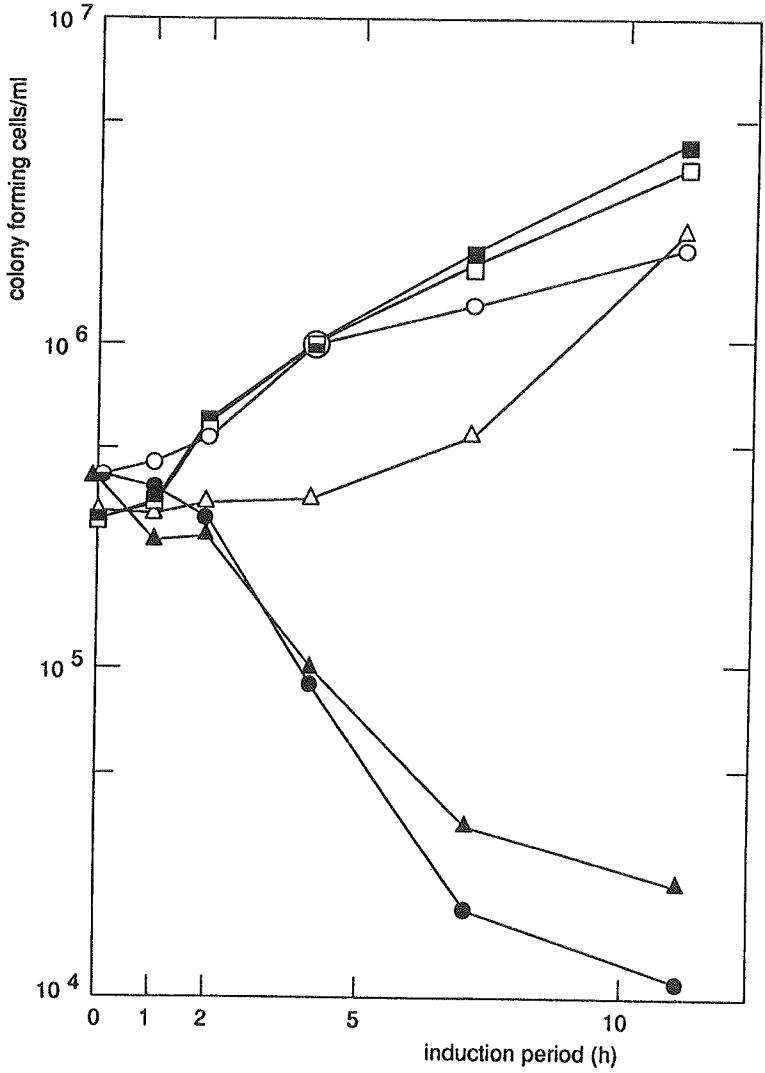


Fig. 8. Survival of yeast transformants upon induction. Transformants (strain 334/pMBL204, strain 334/pMBL204.2A and strain 334/pLOP354) were cultivated overnight in selective medium + glucose and then diluted to 10^6 cells/ml in selective medium + glucose, +/- galactose. Samples were taken at the indicated times after the start of induction and plated in several dilutions on nonselective plates. ■ and □, 334/pMBL204 with and without galactose, respectively; ▲ and △, 334/pMBL204.2A with and without galactose, respectively; ● and ○, 334/pLOP354 with and without galactose, respectively.

Expression of protease 2A (2).

Activity towards yeast factor p150.

Attention has been drawn to a potential functional homology between mammalian p220 and yeast p150 (Goyer *et al.*,1989). It was therefore interesting to know whether 2A, expressed in yeast, brings about cleavage of p150; moreover, we hypothesized that if this does occur it might provide an explanation for the inability to transform yeast with plasmid pMA91.2A, i.e., a plasmid with a constitutive transcription unit for 2A.

Transformants of strain 334 were obtained by transfer of pMBL204.2A or pMBL204 DNA (as described in M.and M.). Two hours and 6 hours after induction samples were taken, electrophoresed, blotted and reacted with anti-p150 antiserum. The results are shown in Fig. 9.

addition	204		204.2A			
galactose	+	+	-	-	+	+
t(h)	2	6	2	6	2	6

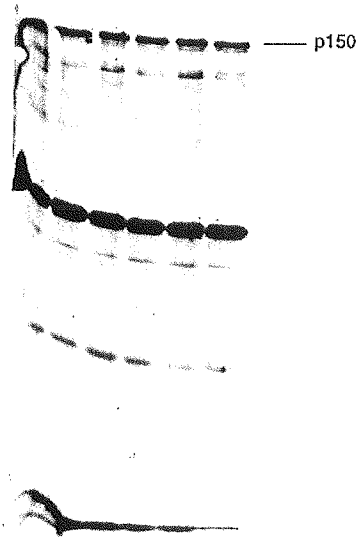


Fig. 9. Western blot of a 10% polyacrylamide gel loaded with the insoluble fractions of extracts of yeast transformants and probed with anti-p150. The times indicated on top of the fluorogram refer to the times of incubation at 30°C after the addition of galactose. Processing of gel and blot was as described in Materials and Methods.

From this Figure it can be concluded that even 6 hours of induction do not result in disappearance of p150. Although some additional bands appear around molecular weight 100 kDa upon expression of 2A, which may well be degradation products of p150, p150 remains largely undegraded. This renders it very unlikely that the deleterious effect of 2A on yeast cellular metabolism is caused by cleavage of p150, unless one assumes that the large majority of p150

molecules is present in an inactive form and that only enzymatically active molecules are prone to 2A-induced cleavage. During the course of these experiments it was shown by others that gene-disruption of the p150 encoding gene in yeast is not lethal, since the protein product of a second gene -only characterized in part yet- can replace the p150 protein in its function (S.Lanker, pers. comm.). Whether the product of this second gene is sensitive to the action of 2A remains to be determined. If so, a further condition for lethality of 2A at this level would be that the p150 gene product cannot replace the "second gene's" product in function.

The fact that 2A does not bring about (full) cleavage of p150 may indicate that there is little homology between p150 and p220. However, since 2A does not by itself cleave p220, but probably activates a latent cellular protease which catalyzes p220 proteolysis (Lloyd *et al.*, 1986), our results may also indicate a lack of this intermediating factor in yeast.

2D-gel analysis of yeast expressing 2A or 3CD.

The fact that 2A cleaves only 2 out of 11 Y-G bonds in the poliovirus polyprotein P1P2P3, whereas 3CD cleaves only 8 out of 13 Q-G bonds, already indicates that additional elements like adjacent aminoacids, tertiary structure etc. are important factors in the cleavage process. Although the poliovirus proteases 2A and 3CD very stringently require the presence of Y-G or Q-G pairs in their respective target sites, this stringency is less adhered to in other picornaviruses (Palmenberg, 1990). From these observations it may be concluded that the site specificity of the proteases from other picornaviruses is primarily determined by the spatial conformation of their target sites. Whatever is decisive for cleavage to occur, one might expect only a low number of mammalian proteins to be a target for 2A and/or 3CD. Indeed, the number of mammalian cellular proteins cleaved by 3CD or 2A is seemingly small (Urzainqui and Carrasco, 1989). With the exception of histone protein H3, cleaved by foot-and-mouth disease virus protease 3C (Falk *et al.*, 1990) and transcription factor TFIIIC, that has recently been shown to be a target for poliovirus protease 3C (Clark *et al.*, 1991), none has been identified so far. This also holds for the protein that is supposed to be directly responsible for cleavage of mammalian p220, and that itself is thought to be a primary target for 2A (Wyckoff *et*

al., 1990; Wyckoff *et al.*, 1992). We have shown that cleavage of p150 does not occur upon synthesis of 2A within our limits of detection. Still we wanted to know whether the lethal effect of 2A and 3CD, when expressed from high copy number plasmids in *Saccharomyces cerevisiae*, results in detectable cleavage of a (discrete) number of host cell proteins. To this end, strain 334/pLOP354, 334/pMBL204.2A and 334/pMBL204 transformants were grown in selective media and induced with galactose. One hour after the start of induction ^{35}S-methionine was added and incubation continued for 10 minutes. Cells from 1-ml samples were extracted and run on 2D-gels. Fluorograms of these 2D-gels are shown in Fig. 10.

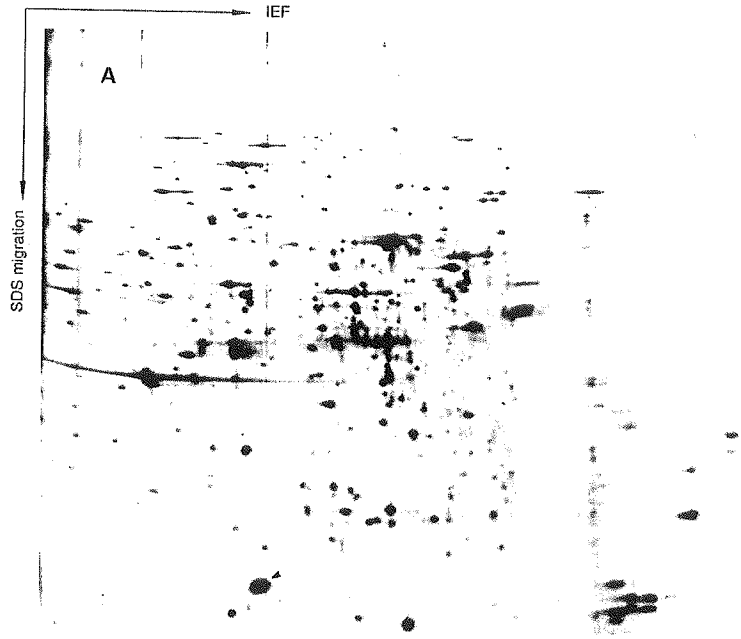
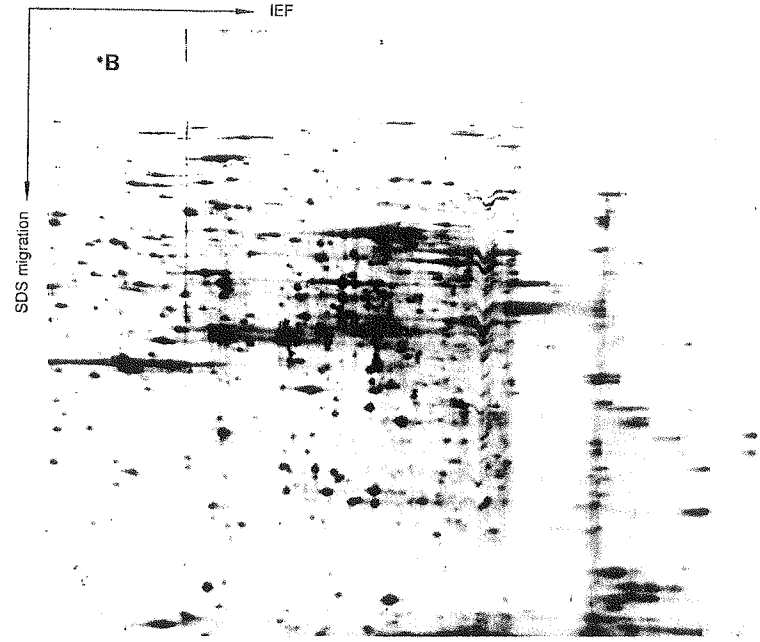
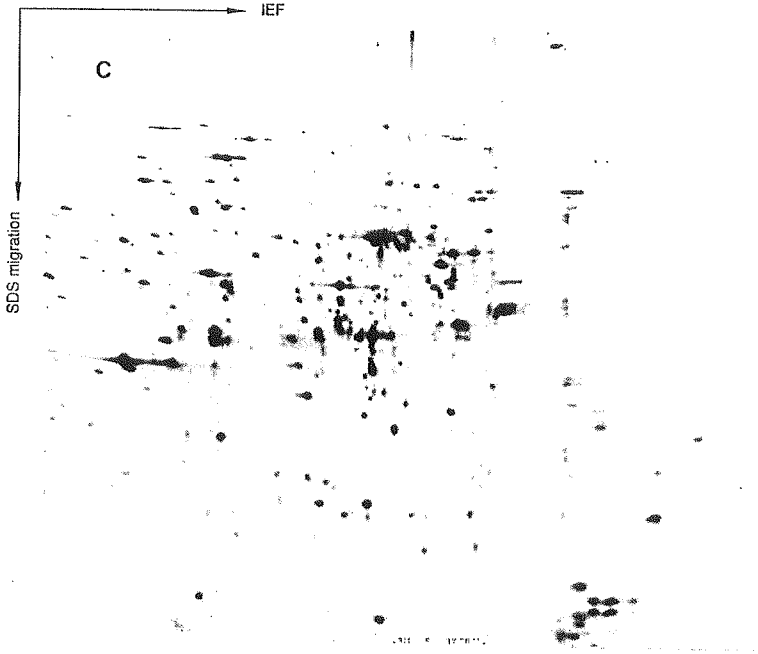


Fig. 10. Fluorogram after 2D-gel electrophoresis of extracts of yeast transformed with either pMBL204.2A (A), pLOP354 (B) or pMBL204 (C). Transformants were cultivated until a concentration of ~3.10^7 was reached. Expression of 2A and 3CD was induced by the addition of galactose. After 60 min at 30°C ^{35}S-methionine was added and incubation continued for 10 min at 30°C. In (A) $9x10^5$ cpm were loaded, in (B) $1{,}1x10^6$ cpm, in (C) $7x10^5$ cpm. Exposure time 35 days.

-/continued .....


IEF
B
SDS migration


IEF
C
SDS migration

In Fig. 10A one extra spot can be observed if compared to Fig. 10C. This extra spot (marked by an arrowhead) represents a protein of low molecular weight. Calculation of the isoelectric point of protease 2A, and taking into consideration its molecular weight (16.6 kDa), makes it very likely that this spot represents 2A. The fact that this spot is not visible after 10 min of combined induction and labeling (results not shown), suggests that the onset of synthesis takes considerably more time than 10 min; this is in agreement with survival studies, in which 10 min of induction of 2A in yeast transformants did not affect viability (experiments not shown). No other extra spots are detectable in Fig. 10A, nor are there any missing in comparison with Fig. 10C.

Induced expression of 3CD results in disappearance of 1 spot (marked ○ in Fig.10B) and the appearance of a cluster of new spots (marked by arrowheads). The new spots may result, in part, from alteration in the isoelectric point of Alcohol Dehydrogenase I (H.Boucherie, pers. comm.). It is remarkable that under these circumstances no 3CD can be observed, although survival of yeast after induction of 3CD is comparable to survival after induction of 2A; however, the 2A protein is detectable, while the 3CD protein is not.

Overall we conclude that more experiments are needed, including other labeling and induction regimes, to get information about the actual number of yeast proteins susceptible to cleavage by polio proteases 2A and 3CD, as well as the identity of these proteins.

In conclusion:

-) 2A can only be expressed in yeast as an inducible protein; transferring plasmids carrying a constitutive transcription unit for 2A does not yield viable transformants.

-) 2A is expressed in yeast in an enzymatically active form.

-) 2A, as well as 3CD, is lethal to yeast when expressed from high copy number plasmids (with inducible transcription units).

-) This lethality can not be attributed to cleavage of identifiable host cell proteins.

REFERENCES

Altmann, M., Handschin, C. and Trachsel, H. (1987). mRNA cap-binding protein: Cloning of the gene encoding protein synthesis initiation factor eIF-4E from *Saccharomyces cerevisiae*. Molecular and Cellular Biology 7, 998-1003.

Alvey, J.C., Wyckoff, E.E., Yu, S.F., Lloyd, R. and Ehrenfeld, E. (1991). *Cis-* and *trans-*cleavage activities of poliovirus 2A protease expressed in *Escherichia coli*. Journal of Virology 65, 6077-6083.

Blair, W.S. and Semler, B.L. (1991). Role for the P4 aminoacid residue in substrate utilization by the poliovirus 3CD proteinase. Journal of Virology 65, 6111-6123.

Bonneau, A.-M. and Sonenberg, N. (1987). Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. Journal of Virology 61, 986-991.

Brown, A.J.P., Purvis, I.J., Santiago, T.C., Bettany, A.J.E., Loughlin, L. and Moore, J. (1988). Messenger RNA degradation in *Saccharomyces cerevisiae*. Gene 72, 151-160.

Buckley, B. and Ehrenfeld, E. (1987). The cap-binding protein complex in uninfected and poliovirus-infected HeLa cells. Journal of Biological Chemistry 262, 13599-13606.

Clark, M.E., Hämmerle, T., Wimmer, E. and Dasgupta, A. (1991). Poliovirus proteinase 3C converts an active form of transcription factor IIIC to an inactive form: a mechanism for inhibition of host cell polymerase III transcription by poliovirus. The EMBO Journal 10, 2941-2947.

Falk, M.M., Grigera, P.R., Bergmann, I.E., Zibert, A., Multhaup, G. and Beck, E. (1990). Foot-and-mouth disease virus protease 3C induces specific proteolytic cleavage of host cell histone H3. Journal of Virology 64, 748-756.

Gibson, T.J. (1984). Studies on the Epstein-Barr virus genome. Ph.D. thesis, Cambridge University, England.

Goyer, C., Altmann, M., Trachsel, H. and Sonenberg, N. (1989). Identification and characterization of cap-binding proteins from yeast. Journal of Biological Chemistry 264, 7603-7610.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology 166, 557-586.

Hawkes, R., Niday, A. and Gordon, J. (1982). A dot immuno binding assay for monoclonal and other antibodies. Analytical Biochemistry 119, 142-147.

Hinnen, A., Hicks, J.B. and Fink, G.R. (1978). Transformation of yeast. Proceedings of the National Academy of Sciences, U.S.A. 75, 1929-1933.

Hovland, P., Flick, J., Johnston, M. and Sclafani, R.A. (1989). Galactose as a gratuitous inducer of gal gene expression in yeasts growing on glucose. Gene 83, 57-64.

Icenogle, J., Gilbert, S.F., Grieves, J., Anderegg, J. and Rueckert, R. (1981). A neutralizing monoclonal antibody against poliovirus and its reaction with related antigens. Virology 115, 211-215.

Jore, J., de Geus, B., Jackson, R.J., Pouwels, P.H. and Enger-Valk, B.E. (1988). Poliovirus protein 3CD is the active protease for processing of the precursor protein P1 *in vitro*. Journal of General Virology 69, 1627-1633.

Jore, J.P.M., Veldhuisen, G., Pouwels, P.H., Boeyé, A., Vrijsen, R. and Rombaut, B. (1991). Formation of subviral particles by *in vitro* translation of subgenomic poliovirus RNAs. Journal of General Virology 72, 2721-2726.

Kräusslich, H.G., Nicklin, M., Toyoda, H., Etchison, D. and Wimmer, E. (1987). Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220. Journal of Virology 61, 2711-2718.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227, 680-685.

Lee, C.-K. and Wimmer, E. (1988). Proteolytic processing of poliovirus polyprotein: elimination of $2A^{pro}$-mediated, alternative cleavage of polypeptide 3CD by *in vitro* mutagenesis. Virology 166, 405-414.

Lloyd, R.E., Toyoda, H., Etchison, D., Wimmer, E. and Ehrenfeld, E. (1986). Cleavage of the cap-binding protein complex polypeptide p220 is not effected by the second poliovirus prorease 2A. Virology 150, 299-303.

Lloyd, R.E., Grubman, M.J. and Ehrenfeld, E. (1988). Relationship of p220 cleavage during picornavirus infection to 2A proteinase sequencing. Journal of Virology 62, 4216-4223.

Mellor, J., Dobson, M.J., Roberts, N.A., Tuite, M.F., Emtage, J.S., White, S., Lowe, P.A., Patel, T., Kingsman, A.J. and Kingsman, S.M. (1983). Efficient synthesis of enzymatically active calf chymosine in *Saccharomyces cerevisiae*. Gene 24, 1-14.

Nicklin, M.J.H., Kräusslich, H.G., Toyoda, H., Dunn, J.J. and Wimmer, E. (1986). Proteolytic processing in the replication of polio and related viruses. Bio/Technology 4, 33-42.

Nicklin, M.J.H., Kräusslich, H.G., Toyoda, H., Dunn, J.J. and Wimmer, E. (1987). Poliovirus polypeptide precursors: expression *in vitro* and processing by exogenous 3C and 2A proteinases. Proceedings of the National Academy of Sciences, U.S.A. 84, 4002-4006.

O'Neill, R.E. and Racaniello, V.R. (1989). Inhibition of translation in cells infected with a poliovirus $2A^{pro}$ mutant correlates with phosphorylation of the alpha subunit of eucaryotic initiation factor 2. Journal of Virology 63, 5069-5075.

Otter, T., King, S.M. and Witman, G.B. (1987). A two-step procedure for efficient electrotransfer of both high-molecular-weight (>400.000) and low-molecular-weight (<20.000) proteins. Analytical Biochemistry 162, 370-377.

Palmenberg, A.C. (1990). Proteolytic processing of picornaviral polyprotein. Annual Review of Micobiology 44, 603-623.

Parks, G.D., Duke, G.M. and Palmenberg, A.C. (1986). Encephalomyocarditis virus 3C protease: efficient cell-free expression from clones which link viral 5′ noncoding sequences to the P3 region. Journal of Virology 60, 376-384.

Pérez, L. and Carrasco, L. (1992). Lack of correlation between p220 cleavage and the shut-off of host translation after poliovirus infection. Virology 189, 178-186.

Romanos, M.A., Scorer, C.A. and Clare, J.J. (1992). Foreign gene expression in yeast: a review. Yeast 8, 423-488.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sonenberg, N. (1987). Regulation of translation by poliovirus. Advances in Virus Research 33, 175-204.

Sonenberg, N. and Pelletier, J. (1989). Poliovirus translation: a paradigm for a novel initiation mechanism. BioEssays 11, 128-132.

Sun, X.-H. and Baltimore, D. (1989). Human immunodeficiency virus tat-activated expression of poliovirus protein 2A inhibits mRNA translation. Proceedings of the National Academy of Sciences USA 86, 2143-2146.

Thoraval, D., Régnacq, M., Neuville, P. and Boucherie, H. (1990). Functional analysis of the yeast genome: use of two-dimensional gel electrophoresis to detect genes in randomly cloned DNA sequences. Current Genetics 18,281-286.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences, U.S.A. 76, 4350-4354.

Toyoda, H., Nicklin, M.J.H., Murray, M.G., Anderson, C.W., Dunn, J.J., Studier, F.W. and Wimmer, E. (1986). A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell 45,761-770.

Urzainqui, A. and Carrasco, L. (1989). Degradation of cellular proteins during poliovirus infection: studies by two-dimensional gel electrophoresis. Journal of Virology 63, 4729-4735.

Verbakel, J.M.A., Dekker, K.A., Rutgers, C.A., Pouwels, P.H. and Enger-Valk, B.E. (1987). Construction of expression plasmids for *Saccharomyces cerevisiae*: application for synthesis of poliovirus protein VP2. Gene 61, 207-215.

Verbakel, J.M.A., Guicherit, O.M.A., van Dijk, M., Kottenhagen, M.J., Pouwels, P.H. and Enger-Valk, B.E. (1988). Synthesis of capsid proteins of poliomyelitis virus in *Saccharomyces cerevisiae.* Molecular Genetics (Life Science Advances) 7, 125-130.

Wyckoff, E.E., Croall, D. and Ehrenfeld, E. (1990). The p220 component of eukaryotic initiation factor 4F is a substrate for multiple calcium-dependent enzymes. Biochemistry 29, 10055-10061.

Wyckoff, E.E., Lloyd, R.E. and Ehrenfeld, E. (1992). Relationship of eukaryotic initiation factor 3 to poliovirus-induced p220 cleavage activity. Journal of Virology 66, 2943-2951.

Ypma-Wong, M.F. and Semler, B.L. (1987). Processing determinants required for in vitro cleavage of the poliovirus P1 precursor to capsid proteins. Journal of Virology 61, 3181-3189.

Ypma-Wong, M.F., Dewalt, P.G., Johnson, V.H., Lamb, J.G. and Semler, B.L. (1988). Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor. Virology 166, 265-270.

FINAL REMARKS AND RECOMMENDATIONS

Vaccination against poliomyelitis has been very successful in the industrialized countries: since the introduction of IPV and OPV the disease has been practically eradicated. Incidental cases are vaccine-associated (i.e. caused by incomplete inactivitation in the case of IPV and by reversion to wild-type neurovirulence in the case of OPV; ever increasing costs by insurance against the latter -still occurring- pose a serious threat to the availability of OPV for a relatively low price). Limited outbreaks occur within groups that refuse vaccination on principle grounds, like -very recently- in the Netherlands. In non-industrialized (tropical) countries poliomyelitis is still an important disease. For these countries IPV is often too expensive and OPV can often not be successfully applied due to lack of necessary facilities (see chapter 1, Table 1 for other drawbacks).

In 1989 the General Assembly of the World Health Organization decided that poliomyelitis should be eradicated by the year 2000. Since this aim has to be achieved using OPV, the possibilities of developing a new poliovaccine, based on subviral particles, were thwarted. It was nevertheless expected that continuation of the research might yield results useful for the development of vaccines against other picornaviruses.

In vitro transcription and translation techniques were already used to elucidate the identity of proteins responsible for some of the proteolytic events in picornavirus precursor proteins at the onset of the research described in this thesis. These techniques have remained valuable since. Thus, Palmenberg *et al.* (1992) showed that the 1D/2A scission in EMCV is carried out by 3C, in other words: the four-step secondary cleavage cascade that converts EMCV L-P1-2A into L, 1AB, 1C, 1D and 2A can entirely be carried out by 3C.

Kusov *et al.* (1992) studied the intermolecular cleavage products of hepatitis A virus (HAV), using the same techniques. They supplied HAV P1-P2 as a substrate for either HAV 3C or 3CD. Since the latter proved to be autoproteolytically very active, their experiments permitted analysis of the activity of 3C only. Protracted incubation of P1-P2 with 3C resulted in several discrete and specific degradation products, whose identity could be confirmed with the aid of immunoprecipitation to be P1, VP1 and VP0-VP3. The authors claimed that one of the other labelled products represented VP3, the

presence of which would indicate cleavage of the VP0-VP3 bond. However, confirmation of the presence of VP3, with the aid of a specific antiserum, was omitted. Therefore, their conclusions about the identity of these products have to be taken with some reserve. Nonetheless, the autoproteolytic activity of HAV 3CD and the processing of the P1-P2 bond by 3C are both reminiscent of the processing events in EMCV. All in all, poliovirus still seems an exception to the rule that secondary cleavages in picornavirus precursor proteins are carried out by virally encoded 3C. Since the picornaviruses that are most closely related to the polioviruses (echo- and coxsackieviruses) have not been studied thoroughly in this respect yet, one still might expect more proteases of the "3CD-type".

Although the experiments described in chapters 2 and 3 clearly indicate that the capsid precursor P1 and the protease 3CD are necessary and sufficient for generation of subviral particles *in vitro*, one can also envisage a system in which the capsid proteins VP0 (VP4+VP2), VP3 and VP1 are synthesized separately, purified and then mixed, in order to obtain subviral particles. However, from X-ray studies it was already apparent that the capsid proteins are not freely moving around during assembly (Hogle *et al.*, 1985). Recent experiments by Bishop's group confirm this: separate synthesis of VP0, VP3 and VP1 in one (insect) cell does yield subviral particles, but the number is low when compared to particle formation from P1 (Bräutigam *et al.* 1993). In other words, synthesis of subviral particles on a preparative scale should be pursued with P1 as one of the constituents.

The successful application of *Saccharomyces cerevisiae* to synthesize subviral particles, as described in chapter 4, was preceded by a comparative study of the levels of expression of VP0 and P1 in adenovirus recombinants, baculovirus recombinants and in *S.cerevisiae*. Adenovirus recombinants designed to express either VP0 or P1, failed to produce any polio-specific protein, as judged by Western blotting (M. Kottenhagen, unpublished results). This may have been an intrinsic property of the system, since high level expression of heterologous proteins in this system has never been reported. A different expression system for mammalian cells was explored by Ansardi *et al.* (1991). Using recombinant vaccinia viruses the authors were able to show the presence of processed capsid proteins (with the aid of immunoprecipitation) and poliovirus empty capsid-like

structures, for which no immunological chacterization was given. The same system has been used successfully for expression of hepatitis A virus (HAV) 14S and 70S subviral particles (Winokur *et al.*, 1991; Stapleton *et al.*,1993). Both types of particles elicited HAV neutralizing antibodies in mice.

Initial results with baculovirus recombinants were encouraging: VP0 was synthesized as fusion product with β-galactosidase in quantities that allowed visualization on stained gels (J.P.M. Jore, unpublished results). However, picking recombinants that expressed P1 failed; plaques without polyhedra contained either a mutated polyhedrin-coding sequence or a scrambled P1 sequence. Since quite acceptable levels of expression of VP0-β-galactosidase and P1 in *S.cerevisiae* had been obtained in the meantime (Verbakel *et al.*, 1987; 1988), the baculovirus recombinant system was no longer employed. Others have since then been able to show the usefulness of the baculovirus recombinant system yet: Urakawa *et al.* (1989) showed the presence of poliovirus empty capsid-like particles in insect cells that were transfected with recombinants containing the whole coding region of the poliovirus genome; moreover, these particles evoked a neutralizing response in mice. Baculovirus recombinants were also employed to synthesize HAV subviral particles (Rosen *et al.*, 1993). Although the immunization schedules employed by these authors obviate a direct comparison, it seems likely that HAV 14S particles have a 20 to 30-fold lower immunopotency than HAV 70S particles.

Introduction in *S.cerevisae* of the 3CD-encoding sequence, the second protein necessary for the formation of subviral particles, was effected by an extension of the P1-encoding sequence to a P1P2P3-encoding sequence. The toxicity of 2A and 3CD, shown by survival experiments and by the lack of transformants if these sequences were comprised on a constitutive transcription unit (chapter 5), may have been a reason for the resulting diminished synthesis of VP2-reactive material. It anyway prevented further use of polygenic messengers comprising -at least- P1 and 3CD-coding sequences. It was expected that 2A, expressed from a 2A-encoding plasmid like pMBL204.2A, would quench its own expression if its mode of action is comparable to that in mammalian cells. Yet, it can be easily detected on 2D-gels, adding to the unlikeliness that it knocks out cap-dependent protein synthesis in yeast. The target for its toxic action remains unclear. The latter also holds for 3CD, for which a dose dependent toxicity could be observed. This dose-

dependency was used to find a compromise between high-level expression of P1 and complete processing with P1 and 3CD encoded on separate transcription units.
Although the results obtained so far are promising, some drawbacks must be noticed as well:

- Due to the intracellular synthesis of poliovirus proteins and particles, the methods of purification are both laborious and time-consuming. Moreover, it can be calculated that the sample depicted in Fig. 6 (chapter 4) still contains 80 to 90 % host cell proteins. A partial solution to this problem may be a further increase in the expression level of P1, for instance by chromosomal integration on the ribosomal DNA locus (see Verbakel, 1991). It remains to be seen whether the level of expression of 3CD is still sufficient under these conditions. Alternatively a 3CD-resistant yeast mutant may be isolated to permit a higher expression level of 3CD, since not all P1 seems to be processed.
- The first results concerning the antigenic configuration of the subviral particles indicate that either yeast is lacking an antigenicity conferring activity or that the antigenicity is lost during extraction and purification of the subviral particles. The antigenicity conferring activity is present in HeLa cells infected by poliovirus (Rombaut *et al.*, 1983), but also in rabbit reticulocyte lysates (chapter 3 of this thesis) and can be mimicked by stabilizing agents like disoxaril (Rombaut *et al.*, 1990). On the other hand, the prolonged cultivation of yeast transformants, required to optimize processing of P1, may have resulted in denaturation of the subviral particles. Whatever the reason, it will be clear that a more intensive research into conditions of assembly and purification is warranted.

Particle formation in infected HeLa cells can be halted, resulting in accumulation of 14S particles, by reducing the temperature of cultivation to 30°C after infection (Rombaut *et al.*, 1990). This temperature effect is not observed in the yeast transformants described in chapter 4, nor does it occur in insect cells infected by baculovirus-polio recombinants (Urakawa *et al.*, 1989), in which the temperature of cultivation is 30°C and 28°C, respectively. In other words, the accumulation of 14S particles is not a direct consequence of the lower temperature. Yet it would be interesting to find a way of accumulating 14S

particles in either system, to study their immunogenic properties and to study morphogenesis of larger subviral particles in a reconstituted system.

A wholly different approach for obtaining particles would be separate synthesis of (preferentially) secreted P1 and 3CD. Both proteins could then be purified, which -for P1 at least, being very hydrophobic- may pose some problems, and cell-free formation of subviral particles studied under well-defined conditions. This approach potentially circumvents the problems mentioned above.

REFERENCES

Ansardi, D.C., Porter, D.C. and Morrow, C.D. (1991). Coinfection with recombinant vaccinia viruses expressing poliovirus P1 and P3 proteins results in polyprotein processing and formation of empty capsid structures. Journal of Virology 65, 2088-2092.

Bräutigam, S., Snezhkov, E. and Bishop, D.L. (1993). Formation of poliovirus-like particles by recombinant baculoviruses expressing the individual VP0, VP3 and VP1 proteins by comparison to particles derived from the expressed poliovirus polyprotein. Virology 192, 512-524.

Hogle, J.M., Chow, M. and Filman, D. (1985). Three dimensional structure of poliovirus at 2.3 Å resolution. Science 229, 1358-1365.

Kusov, Y.Y., Sommergruber, W., Schreiber, M. and Gaus-Müller, V. (1992). Intermolecular cleavage of hepatitis A virus (HAV) precursor protein P1-P2 by recombinant HAV proteinase 3C. Journal of Virology 66, 6794-6786.

Palmenberg, A.C., Parks, G.D., Hall, D.J., Ingraham, R.H., Seng, T.W. and Pallai, P.V. (1992). Proteolytic processing of the cardioviral P2 region: Primary 2A/2B cleavage in clone-derived precursors. Virology 190, 754-762.

Rombaut, B., Brioen, P. and Boeyé, A. (1990). Disoxaril stabilization and immunogenicity of poliovirus procapsids. Journal of General Virology 71, 1081-1086.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1983). Epitope evolution in poliovirus maturation. Archives of Virology 76, 289-298.

Rombaut, B., Vrijsen, R. and Boeyé, A. (1990). New evidence for the precursor role of 14S subunits in poliovirus morphogenesis. Virology 177, 411-414.

Rosen, E., Stapleton, J.T. and McLinden, J.H. (1993). Synthesis of immunogenic hepatitis A virus particles by recombinant baculoviruses. Vaccine 11, 706-712.

Stapleton, J.T., Raina, V., Winokur, P.L., Walters, K., Klinzman, D., Rosen, E. and McLinden, J.H. (1993). Antigenic and immunogenic properties of recombinant hepatitis A virus 14S and 70S subviral particles. Journal of Virology 67, 1080-1085.

Urakawa, T., Ferguson, M., Minor, P.D., Cooper, J.D., Sullivan, M., Almond, J.W. and Bishop, D.H.L. (1989). Synthesis of immunogenic, but non-infectious poliovirus particles in insect cells by a baculovirus expression vector. Journal of General Virology 70, 1453-1463.

Winokur, P.L., McLinden, J.H. and Stapleton, J.T. (1991). The hepatitis A virus polyprotein expressed by a recombinant vaccinia virus undergoes proteolytic processing and assembly into viruslike particles. Journal of Virology 65, 5029-5036.

Verbakel, J.M.A. (1991). Heterologous gene expression in the yeast *Saccharomyces cerevisae*. Ph.D. thesis, Utrecht, the Netherlands.

Verbakel, J.M.A., Dekker, K.A., Rutgers, C.A., Pouwels, P.H. and Enger-Valk, B.E. (1988). Construction of expression plasmids for *Saccharomyces cerevisae*: application for synthesis of poliovirus protein VP2. Gene 61, 207-215.

Verbakel, J.M.A., Guicherit, O.M.A., van Dijk, M., Kottenhagen, M.J., Pouwels, P.H. and Enger-Valk, B.E. (1988). Synthesis of capsid proteins of poliomyelitis virus in *Saccharomyces cerevisae*. Molecular Genetics (Life Science Advances) 7, 125-130.

SUMMARY

The family of picornaviruses harbours a large variety of human and animal pathogens. The causative agent for "infantile paralysis", poliomyelitis virus or poliovirus, is the best known and studied member of this family. Spread of this virus, for which no other host than man is known, takes place via the oral-faecal as well as the pharyngeal route and thrives on primitive sanitation.

Improvement of sanitation changed the nature of poliomyelitis from an endemic disease to a disease with epidemic characteristics, to which adults fell victim as well. This led to an intensive search for vaccines, which ultimately resulted in two types of vaccine: One developed by Salk and one developed by Sabin. The Salk vaccine contains formalin-inactivated virus particles (IPV), whereas the Sabin vaccine contains attenuated virus particles (oral polio vaccine, or OPV). Although poliomyelitis has been practically eradicated with the aid of IPV and OPV in the industrialized countries, it is still an important disease in many non-industrialized countries, partly due to imperfections in both types of vaccine, as explained in chapter 1. These drawbacks largely relate to the presence of infectious virions in one or more stages of the vaccine production process.

It was expected that recombinant DNA techniques would make feasible a biosynthetic vaccine, i.e. a non-infectious protective subunit immunogen, produced in or by a biological system. Initial efforts were aimed at synthesizing capsid proteins, or parts thereoff (see chapter 1); however, emerging insight in the capsid-structure revealed the presence of discontinuous neutralization epitopes. It was therefore obvious to try and mimick this antigenic conformation, that is not only present on mature virions but also on some types of subviral particles that can be observed in infected cells. Some of these particles are rather stable and have been characterized well as to composition and antigenic properties. They are solely composed of capsid proteins VP0 (the precursor of VP4 and VP2), VP3 and VP1 and do not contain RNA.

In chapter 2 experiments are described aimed at elucidating which viral genomic sequences are necessary and sufficient for formation of the separate capsid proteins from the capsid precursor protein P1. A cell-free protein synthesizing system (rabbit reticulocyte lysate) was programmed with genomic and subgenomic polio RNAs. It is

shown that P1 synthesized as such is fully processed to VP0, VP3 and VP1 by virally encoded proteases and that it therefore does not have to be generated by cleavage from a larger precursor protein. Experiments to reveal the identity of the protease responsible for cleavage of P1 showed that it is not 3C -the most likely candidate- but its precursor 3CD that generates the separate capsid proteins by internal cleavage of P1. Further analysis of the products in cell-free extracts in which P1 and 3CD have been translated, is described in chapter 3. The results show that P1 and 3CD are (also) sufficient for formation of subviral particles: 14S and -after a temperature shift- 74S particles are present. Both types of particles have the correct antigenic conformation.

For preparative synthesis of subviral particles use was made of the yeast *Saccharomyces cerevisiae*. Expression of P1 and 3CD was pursued in two ways: (1) For the experiments described in chapter 5, vectors were constructed that comprise a polygenic transcription unit, i.e. contain the whole coding sequence of poliovirus or the coding sequence stripped of superfluous sequences by polymerase chain reactions. (2) For the experiments described in chapter 4 vectors were constructed that comprise separate transcription units for P1 and 3CD. In chapter 5 it is shown that the poliovirus genome contains sequences that cause a lower level of expression of P1 than can be observed if P1 is expressed exclusively. The reason for this suppression may be found in the expression of other poliovirus proteins. The coding sequences for two of these, proteases 2A and 3CD, were subcloned. Both appear to have deleterious effects on yeast, and therefore obstruct the use of a constitutive polygenic transcription unit. A target for these proteases could not be identified. Since the 2A-coding sequence has to be present on the polygenic mRNA to liberate P1, the mRNA has to code for at least P1, 2A and 3CD. Providing yeast with such an (inducible) mRNA, that does not encode poliovirus proteins 2B, 2C, 3A and 3B, does not result in an increased level of synthesis of VP2-reactive material as compared to the level obtained with full length mRNA. Therefore, improvement was sought in the use of separate transcription units for P1 and 3CD, as described in chapter 4. This also allowed manipulation of the expression level of 3CD without interfering with the expression level of P1, to find a compromise between high-level expression of P1 and complete processing of P1 by 3CD. Both promoters had to be inducible, since only newly synthesized P1 could be processed by 3CD. Optimal results were obtained with a

combination of a high copy number plasmid for P1 and a single copy plasmid for 3CD. From a 1-liter culture 0.1 to 1 mg of 74S particles could be obtained. They were composed of VP0, VP3 and VP1. On an electron micrograph the particles showed the same configuration as 74S particles isolated from infected cell cultures. A first analysis, using a competition immunoprecipitation assay, indicated that the 74S particles synthesized in yeast were "antigenically incorrect", i.e. they were H-antigenic instead of N-antigenic. Chapter 6 comprises some final remarks and recommendations for future experiments aimed at obtaining a higher yield of 74S particles, in the "antigenically correct" configuration.

SAMENVATTING

De familie der picornavirussen omvat een grote verscheidenheid aan voor mens en dier pathogene organismen. De veroorzaker van "kinderverlamming", poliomyelitis virus of poliovirus, is het meest bekende en bestudeerde lid van deze familie. Verspreiding van het virus, waarvoor geen andere gastheer dan de mens bekend is, vindt plaats via de oraal-fecale route alsmede via de keelholte en wordt sterk bevorderd door primitieve hygiënische omstandigheden.

Verbetering van de sanitaire voorzieningen veranderde de aard van poliomyelitis van een endemische ziekte in een ziekte met epidemische kenmerken, waaraan ook volwassenen ten prooi vielen. Dit leidde tot een intensieve speurtocht naar een vaccin, die uiteindelijk resulteerde in twee typen vaccin: Eén ontwikkeld door Salk en één ontwikkeld door Sabin. Het Salk vaccin bestaat uit door middel van formaline geïnaktiveerde virusdeeltjes (IPV), terwijl het Sabin vaccin bestaat uit geattenueerde virusdeeltjes (oraal polio vaccin, of OPV). Hoewel poliomyelitis met behulp van IPV en OPV praktisch uitgeroeid is in de geïndustrialiseerde landen, is het in vele niet-geïndustrialiseerde landen nog een belangrijke ziekte, gedeeltelijk ten gevolge van onvolkomenheden in beide typen vaccin, zoals uitgelegd wordt in hoofdstuk 1. Deze nadelen zijn grotendeels terug te voeren op de aanwezigheid van infectieuze virionen in één of meer stadia van het vaccin produktie-proces.

Het was de verwachting dat recombinant DNA technieken het mogelijk zouden maken om een biosynthetisch vaccin te vervaardigen, dit is een niet-infectieus, beschermend immunogeen deeltje, geproduceerd in of door een biologisch systeem. De eerste pogingen waren gericht op de synthese van capside eiwitten, of delen daarvan (zie hoofdstuk 1); mét het groeiend inzicht in de capside struktuur werd echter ook de aanwezigheid van discontinue neutralizatie epitopen duidelijk. Het lag daarom voor de hand om te proberen deze antigene conformatie na te bootsen, welke niet alleen aanwezig is op rijpe virusdeeltjes maar ook op bepaalde typen subvirale deeltjes zoals deze in geïnfekteerde cellen vóórkomen. Sommige van deze deeltjes zijn relatief stabiel en goed gekarakteriseerd wat betreft samenstelling en antigene eigenschappen. Ze bestaan uitsluitend uit de capside eiwitten VP0 (de precursor van VP4 en VP2), VP3 en VP1 en

bevatten geen RNA.

In hoofdstuk 2 worden experimenten beschreven die duidelijk moeten maken welke virale genoom-sekwenties nodig en voldoende zijn voor de vorming van de afzonderlijke capside eiwitten uit het precursor eiwit P1. Een celvrij eiwit synthetiserend systeem (konijne reticulocyten lysaat) werd geprogrammeerd met genomische en subgenomische polio RNAs. Aangetoond wordt dat P1, als zodanig gesynthetiseerd, wordt gesplitst door viraal gecodeerde proteasen in VP0, VP3 en VP1 en derhalve niet behoeft te worden gegenereerd door splitsing van een groter precursor eiwit. Experimenten om de identiteit van het voor de splitsing verantwoordelijke protease te achterhalen toonden aan dat niet 3C -de meest voor de hand liggende kandidaat- maar zijn precursor 3CD de afzonderlijke capside eiwitten genereert door interne klieving van P1. In hoofdstuk 3 wordt de verdere analyse beschreven van de produkten in celvrije extracten waarin P1 en 3CD zijn vertaald. De resultaten tonen aan dat P1 en 3CD (ook) voldoende zijn voor de vorming van subvirale deeltjes, daar 14S en -na een temperatuursverhoging- 74S deeltjes aangetoond kunnen worden. Beide typen deeltjes hebben de correcte antigene conformatie.

Voor preparatieve synthese van subvirale deeltjes werd gebruik gemaakt van de gist *Saccharomyces cerevisiae*. Expressie van P1 en 3CD werd op twee manieren nagestreefd: (1) Voor de experimenten beschreven in hoofdstuk 5 werden vectoren geconstrueerd die een polygene transcriptie-eenheid bevatten, dat wil zeggen de gehele coderende sekwentie van poliovirus of de coderende sekwentie ontdaan van overbodige sekwenties met behulp van polymerase keten reakties. (2) Voor de experimenten beschreven in hoofdstuk 4 werden vectoren geconstrueerd die aparte transcriptie eenheden bevatten voor P1 en 3CD. In hoofdstuk 5 wordt aangetoond dat het poliovirus genoom sekwenties bevat die een lager niveau van expressie van P1 veroorzaken dan wanneer alleen P1 tot expressie wordt gebracht. De reden voor deze onderdrukking zou kunnen liggen in de expressie van andere poliovirus eiwitten. De coderende sekwenties voor twee daarvan, proteases 2A en 3CD, werden in aparte expressie-vectoren gecloneerd. Beide blijken een schadelijk effect op gist te hebben en verhinderen daarmee het gebruik van een constitutieve polygene transcriptie eenheid. Een doelwit voor deze proteasen kon niet worden geïdentificeerd. Aangezien de 2A coderende sekwentie aanwezig moet zijn op

het polygene mRNA teneinde P1 vrij te maken, moet het mRNA tenminste coderen voor P1, 2A en 3CD. Als gist voorzien wordt van zo'n (induceerbaar) mRNA, dat niet codeert voor de poliovirus eiwitten 2B, 2C, 3A en 3B, resulteert dit niet in een verhoogde synthese van VP2-reactief materiaal ten opzichte van het niveau dat behaald wordt met niet-ingekort mRNA. Daarom werd verbetering gezocht in het gebruik van aparte transcriptie-eenheden voor P1 en 3CD, zoals beschreven in hoofdstuk 4. Dit maakte het ook mogelijk het expressie-niveau van 3CD te manipuleren zonder te interfereren met het expressie-niveau van P1, teneinde een compromis te vinden tussen een hoog niveau van expressie van P1 en volledige klieving van P1 door 3CD. Beide promotoren moesten induceerbaar zijn, daar alleen nieuw gesynthetiseerd P1 door 3CD gekliefd kon worden. Optimale resultaten werden verkregen met een combinatie van een multi-kopie plasmide voor P1 en een één-kopie plasmide voor 3CD. Uit een 1-liter kweek konden 0.1 tot 1 mg 74S deeltjes worden verkregen. Ze bestonden uit VP0, VP3 en VP1. Op een electronen-microscopische opname vertoonden ze dezelfde configuratie als 74S deeltjes geïsoleerd uit geïnfecteerde celkweken. Een eerste analyse, gebruik makend van een competitieve immunoprecipitatie test, toonde aan dat de 74S deeltjes die in gist waren gesynthetiseerd, antigeen "onjuist" waren, dat wil zeggen dat zij H-antigeen waren in plaats van N-antigeen. Hoofdstuk 6 bevat enige slotopmerkingen en aanbevelingen voor toekomstig onderzoek gericht op het verkrijgen van méér 74S deeltjes, in de antigeen "juiste" configuratie.

DANKWOORD

Voor mensen uit mijn naaste omgeving zal duidelijk zijn met hoeveel plezier ik aan dit dankwoord begin. Immers, "het ei is gelegd" en daarmee is een eind gekomen aan de bijbehorende geestelijke (maar wel degelijk ook lichamelijke) ongemakken. Het is mij alles bij elkaar niet meegevallen. Dat mijn dagelijks onderzoek al bijna twee jaar niet meer samenvalt met het in dit proefschrift beschreven onderzoek is daaraan niet vreemd. Overigens realiseer ik mij dat dat niet alleen mij extra inspanning heeft gekost, ook vele anderen zullen in de praktijk geconfronteerd zijn met de nadelen van mijn dubbelleven.

Allereerst gaat mijn dank uit naar mijn vader en moeder, die mij aanmoedigden om te gaan studeren en die steeds het vertrouwen in mij behielden, ook toen mijn kennismaking met de academische wereld aanvankelijk op een mislukking dreigde uit te lopen.
Ans en Matthijs zijn de laatste jaren nogal eens in de verdrukking geraakt. Dat ik nog steeds als echtgenoot en vader geaccepteerd word, heb ik misschien wel niet verdiend, maar waardeer ik zeer.
Een speciaal woord van dank ook voor mijn (co-)promotor(s), de professoren Boeyé, Pouwels en Rombaut. Geachte professor Boeyé, uw ruimhartige opstelling om mij de gelegenheid te geven een aantal maanden op uw laboratorium te komen werken en om als mijn promotor op te willen treden is van cruciaal belang geweest. Ik kan u daarvoor niet genoeg bedanken. Beste Peter, in de ruim tien jaar dat jij mijn afdelingshoofd bent, heb ik je leren kennen als een bron van wetenschappelijke kennis en een man met een scherp analyserend vermogen. Vaak heb ik je benijd om je vermogen je op te sluiten in de wetenschap. In díe gevallen dat dit naar mijn mening verkeerd uitpakte, kon ik echter altijd met mijn grieven bij je terecht. Ik hoop dat onze samenwerking nog tot in lengte van dagen zal kunnen voortbestaan. Beste Bart, jouw grenzeloos optimisme en grondige kennis van polio in al zijn facetten, zijn voor mij van onschatbare betekenis geweest. Geen moeite was je teveel. Met jou wil ik ook alle betrokken medewerkers van de VUB bedanken. Ik vind het spijtig dat onze samenwerking naar het zich laat aanzien tot het verleden behoort, maar weet zeker dat er een vriendschap voor jaren is ontstaan.

Speciale woorden van dank ook voor een aantal onderzoekers buiten TNO en de VUB. As the first one of these I would like to thank Dr Richard Jackson, who gave me the opportunity to work in his laboratory for several weeks. Dear Richard, your interest in my work, exemplified by your sudden appearances in the lab late at night when I was trying to force a 25th hour in a space of 24 hours, has been of vital importance. Your wisdom, hospitality and sense of humour won't be forgotten. Hoofdstuk 5 is mede tot stand kunnen komen door de medewerking van Drs Boucherie, Lanker en Thomas: Dr Boucherie, je vous remercie beaucoup pour votre coopération désintéressée apporté au chapitre 5 par l'analyse en gel-2D des extraits de levure. Dr Lanker, ich danke Ihnen recht herzlich für Ihre uneigennützige Mitarbeit am Kapitel 5 durch die anti-p150 Analyse von Hefe-extrakte. Dr Thomas, beste Adri, bedankt voor je belangeloze medewerking aan hoofdstuk 5 middels de anti-p220 analyse.

Het is niet doenlijk om alle (ex-)MBLers, studenten en stagiaires te noemen die, direkt of indirekt, een bijdrage aan dit proefschrift hebben geleverd. Ik wil ze hierbij allemaal bedanken voor de uiterst plezierige en inspirerende sfeer die ze wisten te scheppen. Ik hoop dat ze zich niet beledigd voelen als ik er desondanks toch een paar met name noem: Dat zijn Co van Rotterdam en Christien Lokman voor hun niet te onderschatten invloed op de sociale sfeer in de afdeling MGG; Rob Leer, mijn vaste fietsmaatje, wetenschappelijke toeverlaat en klaagmuur; van mijn (vele) kamergenoten zijn dat John Verbakel en Jan Verdoes, die elk op hun eigen wijze lief en leed met mij hebben willen delen en met wie ik in halve woorden kon communiceren; van "de diensten" zijn dat Frans Wijnants, Jeane Doesburg-Lanooy en Michel Boermans, die leken te kunnen toveren met tijd en steeds prima werk leverden; als laatste, maar zeker niet de minste, wil ik Gerrit Veldhuisen noemen. In Gerrit zijn alle eigenschappen verenigd die het MBL in mijn ogen zo uniek hebben gemaakt: Altijd geïnteresseerd in het hoe en waarom, een grote bereidheid tot meedenken en -doen, en een grote mate van flexibiliteit. Gerrit, je vaderlijk gemopper zal mij altijd als muziek in de oren blijven klinken. Zonder jou was het echt modderen geworden.

Ik wil dit dankwoord afsluiten met de wens dat **voeding** voor alle betrokken MBLers in velerlei opzicht synoniem zal worden met **gezondheid**.

Jan