REGULATIE VAN TRANSCRIPTIE IN VITRO VAN HET TRYPTOFAAN OPERON VAN ESCHERICHIA COLI

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REGULATIE VAN TRANSCRIPTIE IN VITRO VAN HET TRYPTOFAAN OPERON VAN ESCHERICHIA COLI

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A. E. COHEN, HOOGLERAAR IN BE FACULTEIT DER LETTEREN, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP WOENSDAG 8 OKTOBER 1975 TE KLOKKE 15.15 UUR.

DOOR

HANS PANNEKOEK

GEBOREN TE HAARLEMMERMEER IN 1944

Promotor: Prof.Dr.Ir. A. Rörsch

Het in dit proefschrift beschreven onderzoek werd uitgevoerd op het Centre d'Etudes Nucléaires de Saclay (Service de Biochimie) te Gif ^{S}/Yvette (Frankrijk) en op het Medisch Biologisch Laboratorium TNO te Rijswijk. Het onderzoek stond onder leiding van Dr. P.H. Pouwels.

STELLINGEN

I

De oriëntatie van de *E. coli* histidine genen op Φ80imm$^{\lambda}$*his* DNA t.o.v. de p_L^{λ} promotor is bepalend voor de waarde die toegekend moet worden aan de resultaten van *in vitro* transcriptie experimenten.

F. Blasi *et al.* Proc. natl. Acad. Sci. (Wash.) *70*, (1973) 2692-26996

II

De conclusie van Shimizu en Hayashi, dat in aanwezigheid van Rho factor de transcriptie *in vitro* van het tryptofaan operon, gelegen op λ *trp* DNA, onafhankelijk is van λ promotoren, is onjuist.

N. Shimizu and M. Hayashi J. molec. Biol. *84*, (1974) 315-355

III

Bij gebruikmaking van het anti-bioticum Rifampicine om *de novo* RNA synthese te verhinderen dient bij de interpretatie van de resultaten rekening te worden gehouden met stabilisatie van aspecifieke transcriptiecomplexen.

E.K.F. Bautz and F.A. Bautz Nature (Lond.) *226*, (1970) 1219-1222

IV

Een systematisch onderzoek naar de invloed van verschillende zuiveringsmethoden voor het enzym RNA-polymerase uit *E. coli* op de eigenschappen van dit enzym zou een nuttige bijdrage kunnen betekenen voor studies over regulatie van gen-expressie.

V

De regulatie van de synthese van regulatie-eiwitten wordt mogelijk autogeen gereguleerd.

VI

De bewering van Klein *et al.*, dat het anti-rheumaticum d-2'-(6'-methoxy-2'-naphtyl)-propionzuur (naproxen) drie herstelenzymen in menselijke lymphocyten en muizemiltcellen remt, is ongegrond.

G. Klein, A. Wottawa und H. Altmann Arzneim.-Forsch. *25*, (1975) 288-290

VII

Het streven om sociaal verschillende groepen gelijke kansen te geven in het vergaren van kennis wordt ondermeer ondergraven door het prijsverschil tussen aluminium- en roestvrij stalen kookpannen.

D.R. Crapper and A.J. Dalton Physiol. and Behaviour *10*, (1973) 925-945

VIII

De relatieve toename van het aantal onderzoekers, dat experimenteert met polyploïde systemen, vertraagt de progressie van de moleculaire biologie.

Leiden, 8 Oktober 1975 H. Pannekoek

Aan mijn ouders

Aan Nelly

INHOUD

VOORWOORD

De experimenten, die in dit proefschrift worden beschreven, zijn uitgevoerd met het doel een bijdrage te leveren aan de opheldering van de fundamentele vraag: "Hoe wordt de expressie van genen gereguleerd?" Daar als model voor deze studie een verzameling van genen van de bacterie *Escherichia coli* is gekozen, zal de Inleiding zich beperken tot algemene informatie over regulatie van gen-expressie bij *E. coli*. De artikelen, waarin de experimenten worden beschreven, worden gevolgd door een Samenvatting van de uitkomsten en een Discussie, waarin de resultaten worden besproken.

In vergelijking met de intensief bestudeerde regulatie van genen, die betrokken zijn bij de afbraak van stoffen (suikers), is de studie van de expressie van genen, die betrokken zijn bij de synthese van bouwstenen voor de cel (b.v. aminozuren) minder ver gevorderd. Dit gegeven is de voornaamste drijfveer voor deze studie naar de regulatie van expressie van genen, die de genetische informatie bevatten voor enzymen die de biosynthese van het aminozuur L-tryptofaan (*trp*) katalyseren. Daarnaast kan een praktisch motief worden aangevoerd voor de bestudering van de genen betrokken bij de biosynthese van L-tryptofaan. De beschikbaarheid van bacteriofagen, die de bacteriële *trp* genen dragen, brengt met zich mede, dat door verrijking van het te bestuderen materiaal t.o.v. het genoom van *E. coli* de detectie van gesynthetiseerde genproducten vereenvoudigd is.

De publicaties, die in dit proefschrift zijn opgenomen, vermelden experimenten die een deelproces (i.e. transcriptie) voor de expressie van de *trp* genen van *E. coli* beschrijven. Voor de bepaling van de verbindingen, die een functie vervullen bij de regulatie van transcriptie, alsmede voor het ophelderen van het werkingsmechanisme van die verbindingen, hebben wij gekozen voor een *in vitro* systeem, dat gebruik maakt van gezuiverde componenten. Dankzij de veelheid aan genetische en biochemische gegevens, die beschikbaar zijn over de regulatie van het *trp* operon *in vivo*, is het mogelijk om de uitkomsten van *in vitro* en *in vivo* experimenten met elkaar te vergelijken.

In het kader van de studie van de expressie van genen is een bijdrage geleverd om de componenten, die essentiëel zijn voor het adequaat functioneren van de transcriptie *in vitro* van de *trp* genen te bepalen. Hiermee is wellicht de basis gelegd voor studies, die inzicht kunnen verschaffen in de moleculaire interacties tussen die componenten en de regulatie-elementen, die gelegen zijn in het gebied van de *trp* genen van *E. coli*.

De informatie van alle genen van een bacterie komt nooit tegelijkertijd tot expressie. Het hangt ondermeer af van de kweekomstandigheden of de informatie van een bepaald gen al dan niet tot expressie wordt gebracht. Men heeft dit verschijnsel van aanpassing aan de omstandigheden "genetische adaptatie" genoemd. In 1961 hebben Jacob en Monod (1,2), in een poging de mechanismen voor genetische adaptatie te beschrijven, het "operon-model" voorgesteld, dat de aanzet betekende voor onderzoek ter bestudering van de regulatie van de expressie van genen. Dit model beschrijft twee aspecten: a) op welke wijze genetische informatie, gecodeerd door de structurele genen, ten dienste komt aan de cel middels biologisch actieve eiwitten en b) op welke wijze de expressie van genetische informatie wordt gereguleerd. Het operon-model, zoals voorgesteld door Jacob en Monod (1,2) en later uitgebreid door Scaife en Beckwith (3), kan als volgt schematisch worden weergegeven (*Fig. 1*)

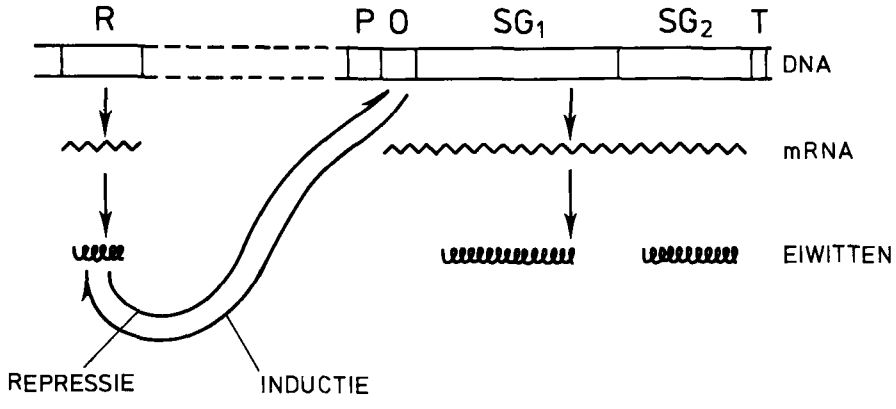


Fig. 1 Verklaring: SG_1, SG_2 = structurele genen; P = promotor; O = operator; T = terminator; R = regulator-gen (codeert voor repressor-eiwit).

Overeenkomstig dit model bestaat een operon uit één of meer structurele genen die naast elkaar zijn gelegen en aan één kant geflankeerd worden door regulatie-elementen. De structurele genen dienen als matrijs voor de synthese van een"boodschapper-molecuul" (mRNA), dat op zijn beurt fungeert als matrijs voor de synthese van een eiwit. De regulatie-elementen bepalen of de genen al

dan niet tot expressie worden gebracht. De synthese van mRNA, de transcriptie, die wordt uitgevoerd door het enzym RNA-polymerase, begint op een specifiek startpunt (de promotor; P) en eindigt eveneens op een specifieke stopplaats (de terminator; T). Een dergelijk mechanisme houdt in, dat de tussen de start- en stopplaats gelegen structurele genen, gezamenlijk tot expressie zullen komen. Wanneer het gesynthetiseerde mRNA-molecuul de informatie bevat voor meerdere eiwitten spreekt men van een polycistronisch mRNA.

Er bestaat voor ieder operon, dat aan regulatie onderworpen is, een specifiek eiwit (apo-repressor), dat het overschrijven van de genetische informatie kan verhinderen. Het gen, dat voor de apo-repressor codeert, het regulator-gen (R), is veelal op een geheel andere plaats op het genoom gelegen dan het betreffende operon zelf. De repressor oefent zijn werking uit door zich te binden met een gedeelte van het DNA dat onmiddellijk naast de promotor is gelegen (de operator; O) en verhindert aldus de synthese van mRNA.

Dickson *et al.* (4) hebben in een studie, waarin de nucleotidevolgorde werd opgehelderd van de regulatie-elementen van het lactose (*lac*) operon van *E. coli*, dat codeert voor enzymen die de afbraak van lactose katalyseren, het bewijs geleverd, dat voor dit operon de promotor niet slechts beschouwd dient te worden als het startpunt voor de mRNA synthese. Niet alleen het RNA-polymerase, maar ook andere eiwitten die een rol spelen bij het tot stand komen van de start van de mRNA synthese, blijken in het promotor-gebied fysisch zowel als functioneel gescheiden aangrijpingspunten te bezitten. Derhalve dient het gehele gebied tussen het einde van het voorafgaande gen en het begin van het eerste structurele gen beschouwd te worden als een verzameling van aangrijpingspunten voor verschillende regulatie-eiwitten. Elk regulatie-eiwit herkent een "eigen" nucleotidevolgorde in dit gebied en kan hiermee interactie aangaan teneinde invloed op de synthese van mRNA uit te oefenen.

TRANSCRIPTIE

In *E. coli* wordt de transcriptie van alle genetische informatie - zowel genen, coderend voor eiwitten, als ook die, welke coderen voor ribosomaal RNA en transfer RNA - uitgevoerd door één enzym, het RNA-polymerase. Dit enzym bestaat uit 5 sub-eenheden ($\beta\beta'\alpha_2\sigma$) en heeft een molecuulgewicht van bijna 500,000 dalton. In deze vorm is het enzym in staat *in vitro* een serie verschillende reacties accuraat uit te voeren (5-10). Ook RNA-polymerase dat de σ-sub-eenheid mist kan zorg dragen voor de synthese van RNA, echter de start van transcriptie vindt in dit geval plaats op willekeurige plaatsen op

het DNA.

Op grond van *in vitro* studies met gezuiverd RNA-polymerase heeft Travers (11) een model opgesteld voor de synthese van RNA. De verschillende reacties, die leiden tot de synthese van RNA, worden in Fig. 2 schematisch weergegeven.

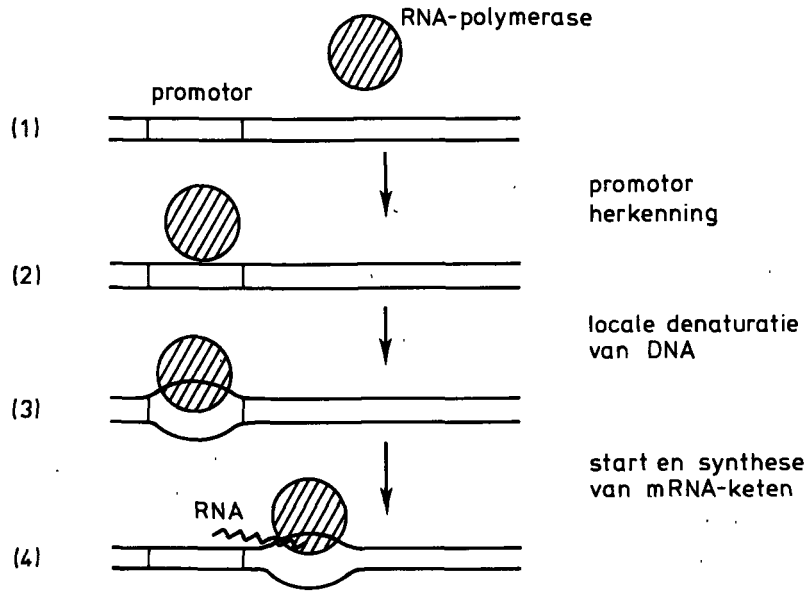


Fig. 2

(1) RNA-polymerase, gecomplexeerd met zijn sub-eenheid σ,"herkent" een specifieke structuur of een specifieke nucleotidevolgorde in of nabij de promotor.

(2) Hierna bindt het enzym zich op of nabij deze plaats aan het DNA.

(3) Door deze binding ondergaat het DNA een conformatie-verandering (11), die zich manifesteert door een locale ontwinding van de dubbele helix over een gebied van 4-8 baseparen (12).

(4) De conformatie-verandering van promotor-DNA is noodzakelijk om RNA-polymerase in staat te stellen de transcriptie te starten.

De energie, die benodigd is voor de conformatie-verandering, wordt "voorgeschreven" door de structuur en/of de nucleotidevolgorde van de betreffende

promotor. De energie nodig voor de conformatie-verandering van het DNA kan beinvloed worden door de aanwezigheid van bepaalde celcomponenten, met name eiwitten. De aanwezigheid van eiwitten, die deze energie verhogen en als zodanig de start van de RNA-synthese kunnen verhinderen, zou ertoe leiden dat de expressie van genetische informatie wordt geremd, terwijl eiwitten, die de energie verlagen daarentegen de start van transcriptie mogelijk zouden maken en dus zorgen voor een verhoging van het niveau van expressie van informatie. Dit concept voor het mechanisme van de transcriptie benadrukt dat regulatie van gen-expressie voornamelijk plaatsvindt op het niveau van de start van de transcriptie (zie tevens paragraaf "Toelichting bij Publicaties I t/m V").

REGULATIE VAN GEN-EXPRESSIE

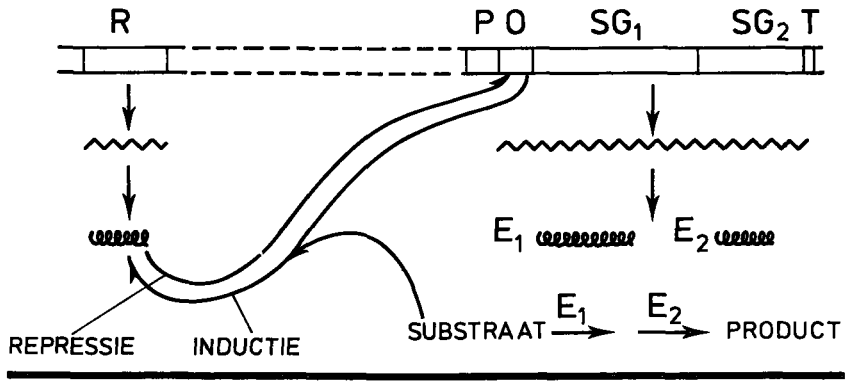
De hoeksteen van het operon-model (1-3) is de hypothese, dat de beslissing of een gen tot expressie komt genomen wordt op het niveau van de transcriptie. De hypothese komt voornamelijk voort uit economische overwegingen, daar energie verspild zou worden door de synthese van onbenutte mRNA-moleculen. Hoewel regulatie van translatie voor de expressie van enkele bacteriële operons gesuggereerd is (13), moet deze vorm van regulatie waarschijnlijk beschouwd worden als een "bijsturen" van de gen-expressie. In hetzelfde licht moet de regulatie van gen-expressie middels een preferentiële degradatie van gedeelte(n) van polycistronisch mRNA gezien worden (14).

Blijkens het voorafgaande is het voornaamste aangrijpingspunt voor regulatie van gen-expressie de start van de mRNA-synthese. Derhalve kan de volgende conclusie getrokken worden: aanpassing aan veranderingen in groeiomstandigheden door de cel komt op het niveau van de synthese van enzymen voornamelijk tot stand door beïnvloeding van de start van transcriptie van de betreffende structurele genen.

DIFFERENTIATIE VAN REGULATIE-MECHANISMEN

De cel beschikt over een aantal principiëel verschillende mechanismen om de expressie van zijn genen te reguleren. In de eerste plaats kan een indeling gemaakt worden in operons, die gereguleerd worden volgens het model van Jacob en Monod (1,2) en operons, die autogeen gereguleerd worden. De eerste groep kan verder onderverdeeld worden in negatief- en positief gereguleerde operons. In de groep van negatief-gereguleerde operons kan tevens onderscheid gemaakt worden in de wijze waarop regulatie gebeurt bij biodegradatieve- en biosynthetische operons. Het verschil in mechanisme van regulatie van deze beide typen negatief-gereguleerde operons wordt geïllustreerd in Fig. 3.

BIODEGRADATIEF OPERON



BIOSYNTHETISCH OPERON

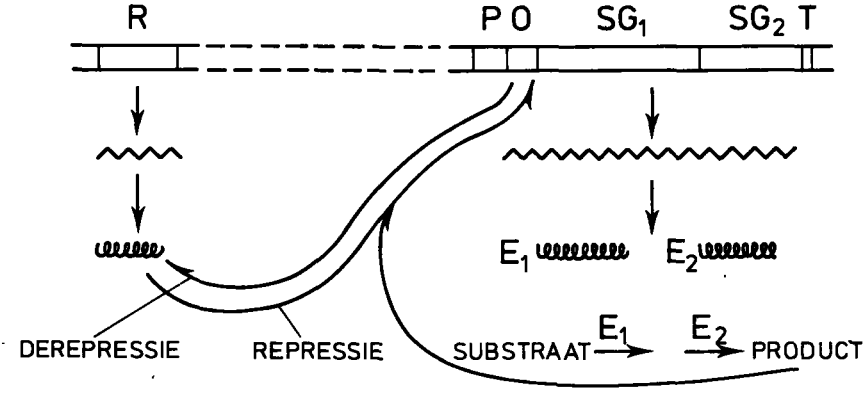


Fig. 3 Voor een verklaring van de gebruikte symbolen zie Fig. 1.

De expressie van zowel biodegradatieve- als biosynthetische operons wordt beïnvloed door een laag-moleculaire verbinding (effector), die de bindingsaffiniteit van de repressor voor de operator op reversibele wijze modificeert. Als effector voor biodegradatieve operons fungeert het substraat, of een vergelijkbare verbinding, voor de katabole enzymen, die door het desbetreffende operon gecodeerd worden. Zolang het substraat aanwezig is kan het zich binden

aan de apo-repressor en daardoor de bindingsaffiniteit voor de operator verlagen (inductie). Zodra het substraat volledig is omgezet en er dus geen behoefte meer bestaat aan de katabole enzymen, neemt de complex-vorming tussen apo-repressor en co-repressor af en daardoor neemt de affiniteit van de apo-repressor voor de operator toe. De expressie van het *lac* operon van *E. coli* wordt via dit principe gereguleerd (voor overzicht ref. 15).

De effector voor biosynthetische operons is, daarentegen, het eindproduct van de anabole enzymreacties. Dit product heeft een tegenovergesteld effect op de binding tussen apo-repressor en operator als het effector-molecuul voor de regulatie van de biodegradatieve operons, t.w. een bevordering van de associatie tussen de apo-repressor en de operator (repressie). Een voorbeeld van een operon, waarvan de gen-expressie op deze wijze gereguleerd wordt, is het tryptofaan (*trp*) operon van *E. coli*. Het eindproduct van de biosynthetische reactieketen is het aminozuur L-tryptofaan. Dit effector-molecuul (co-repressor) fungeert als activator voor de complex-vorming van de *trp*-repressor (het product van het *trpR* gen) en de *trp* operator (zie tevens paragraaf "Bestudering van regulatie van het *trp* operon *in vitro*").

Concluderend kan opgemerkt worden, dat de genetische organisatie van biodegradatieve- en biosynthetische operons niet essentiëel verschilt, maar de aard en de werking van de effector accentueren de principiële verschillen t.a.v. de regulatie van gen-expressie van beide negatief-gereguleerde operons.

Positieve regulatie van gen-expressie is aanmerkelijk minder uitvoerig onderzocht dan negatieve regulatie. Bij bepaalde operons, die aan positieve regulatie onderworpen zijn, is binding van een regulatie-eiwit op een specifieke plaats op het regulatie-gebied van het betreffende operon, een absolute vereiste voor de start van de mRNA-synthese. Kataboliet-gevoelige operons, zoals de lactose, galactose en arabinose operons van *E. coli*, behoren tot de groep van positief-gereguleerde operons. Dergelijke kataboliet-gevoelige operons kunnen uitsluitend tot expressie komen in afwezigheid van glucose (of een metaboliet van glucose). Men veronderstelt, dat positieve regulatie plaatsvindt door tussenkomst van een eiwit, de z.g. CAP-factor (16), dat zich bindt in de nabijheid van de startplaats voor de mRNA-synthese. De CAP-factor wordt geactiveerd door de laag-moleculaire verbinding cyclisch AMP. De concentratie van cyclisch AMP blijkt reciprook gecorreleerd te zijn met de concentratie van glucose in de cel. Derhalve leidt een hoge concentratie glucose tot een verlaging van de cyclisch AMP-concentratie, hetgeen uiteindelijk tot gevolg heeft dat dergelijke operons niet tot expressie zullen komen. In ana-

logie met negatief-gereguleerde operons bepaalt ook bij positief-gereguleerde operons een laag-moleculaire verbinding (cyclisch AMP) de aanzet tot de synthese van mRNA.

Een geheel ander type mechanisme voor regulatie van gen-expressie is autogene regulatie. Operons waarvoor is vastgesteld dat ze autogeen gereguleerd worden ressorteren onder de groep waarvan de gen-expressie negatief gereguleerd wordt. De essentie van dit regulatie-mechanisme is dat een eiwit, gecodeerd door één van de structurele genen, zelf een regulatie-element is in de expressie van dat operon (17). De genetische organisatie van een autogeen gereguleerd operon is derhalve verschillend van het "klassieke" operon-model; er is geen onafhankelijk regulator-gen, dat codeert voor de apo-repressor. De rol van het regulator-gen wordt ingenomen door één der structurele genen, waarvan het genproduct bifunctioneel is, d.w.z. het heeft zowel een katalytische functie in een enzymatische reactie, als een regulerende functie in de repressie van hetzelfde operon. Er zijn aanwijzingen, dat de regulatie tot stand komt door binding van dit eiwit met de operator (18).

Het vóórkomen van autogene regulatie stelt de cel in staat op strikt gecontroleerde wijze zich aan veranderingen in kweekomstandigheden aan te passen. Doordat de beide activiteiten van het eiwit antagonistisch werken wordt vermeden, dat er zich extreme veranderingen voordoen in de expressie van genen.

Autogene regulatie is een mechanisme, dat zowel bij biodegradatieve- als bij biosynthetische operons voorkomt. Bijvoorbeeld het *E. coli* D-serinedeaminase (*dSda*) operon (19), dat betrokken is bij de afbraak van D-serine en het histidine (*his*) operon van *E. coli* (17), dat codeert voor enzymen die de biosynthese van het aminozuur histidine katalyseren, worden beide op autogene wijze gereguleerd.

REGULATIE VAN GEN-EXPRESSIE IN VITRO

Bij studies die gericht zijn op het opheldéren van de regulatie-mechanismen van de expressie van bacteriële genen wordt als matrijs voor mRNA- en enzymsynthese veelal gebruik gemaakt van bacteriofaag-DNA, waarin de betreffende genen zijn ingebouwd. Deze, z.g. transducerende fagen, behoren tot een type waarvan het DNA op reversibele wijze op een bepaalde plaats in een bacteriëel chromosoom kan integreren. Transducerende fagen ontstaan doordat bij het uittreden van het DNA uit het chromosoom ("excisie") de breuken in het DNA niet op de juiste plaats worden aangebracht. Hierbij wordt behalve faag-DNA ook een stukje bacterie-DNA "uitgesneden", dat met het faag-DNA wordt"ingepakt" in

een faagdeeltje. De integratieplaats van de bacteriofaag Φ80 op het *E. coli* chromosoom is gelegen in de onmiddellijke omgeving van het tryptofaan (*trp*) operon. Hierdoor is het mogelijk om transducerende fagen van Φ80 te isoleren die een gedeelte of het gehele *trp* operon hebben ingebouwd.

De systemen, waarmee de regulatie van de expressie van bacteriële genen *in vitro* wordt bestudeerd, kan men onderscheiden in twee typen:

a) het gekoppelde transcriptie-translatie systeem, waarmee *in vitro* biologisch actieve enzymen worden gesynthetiseerd en

b) het *in vitro* transcriptie-systeem, waarmee met gezuiverde componenten functioneel mRNA wordt gemaakt.

Het gekoppelde *in vitro* transcriptie-translatie systeem, waarvoor de grondslag is gelegd door Zubay en medewerkers (20) en Gold en Schweiger (21), bevat alle componenten die nodig zijn voor de synthese van enzymatisch actieve eiwitten. Door een zuivering van het eiwitsynthetiserende systeem kan men bepalen welke componenten essentiëel zijn in hetzij de transcriptie, hetzij de translatie. De opzet van een *in vitro* transcriptie-systeem daarentegen is, uitgaande van een beperkt aantal gezuiverde componenten, de synthese van functioneel mRNA te bewerkstelligen. Wanneer de aanwezige bestanddelen niet toereikend zijn voor dit doel, dienen andere gezuiverde enzymen en/of factoren toegevoegd te worden, teneinde een specifieke transcriptie te verzorgen. Op grond van deze overwegingen kunnen beide typen *in vitro* studies elkaar aanvullen.

De detectie van *in vitro* gesynthetiseerd bacteriëel mRNA kan geschieden m.b.v. DNA-RNA hybridisatie-technieken. Wanneer men beschikt over Φ80 en λ fagen, die beide dezelfde bacteriële genen dragen, kan RNA gesynthetiseerd op het DNA van b.v. de Φ80 transducerende faag gehybridiseerd worden met het DNA van de λ transducerende faag. De hoeveelheid gehybridiseerd materiaal is een maat voor de synthese van bacteriëel mRNA.

BESTUDERING VAN REGULATIE VAN EXPRESSIE VAN HET trp OPERON IN VITRO

Alvorens een toelichting te geven op de in dit proefschrift opgenomen publicaties worden in deze paragraaf enige studies besproken die betrekking hebben op de regulatie van expressie van het *trp* operon van *E. coli*. Dit operon bestaat uit 5 naast elkaar gelegen structurele genen - *trpE, D, C, B* en *A* - die coderen voor 5 verschillende polypeptide-ketens. De start van de transcriptie vindt plaats op of nabij de *trp* promotor, die deel uitmaakt van de *trp* regulatie-elementen. Tot deze elementen behoren tevens de *trp* operator en de *trp* "leader-sequence", welke laatste gelegen is tussen de opera-

tor en het *trpE* gen (22). Op een mogelijke functie van de "leader-sequence" zal in de paragraaf "Toelichting bij publicaties I t/m V" en in de Samenvatting en Discussie nader worden ingegaan. Uit de ligging van de *trp* promotor t.o.v. de structurele genen volgt, dat de genen in de richting van *trpE* naar *trpA* worden overgeschreven. Ook de *trp* enzymen worden *in vivo* in deze volgorde gesynthetiseerd (23).

In een cel-vrij eiwitsynthetiserend systeem geprogrammeerd met Φ80 *trp* DNA voltrekt de synthese van *trp* enzymen zich op een wijze vergelijkbaar met die in de intacte cel (24,25). Zubay en medewerkers (26) hebben de gevoeligheid en de specificiteit van het cel-vrije eiwitsynthetiserende systeem benut om de apo-repressor (product van *trpR*) van het *trp* operon te detecteren en deels te zuiveren. Uit deze studie blijkt, dat de repressor een eiwit is.

Voor een nadere karakterisering van de gedeeltelijk gezuiverde *trp* apo-repressor hebben Yanofsky en medewerkers (27,28) gebruik gemaakt van het *in vitro* transcriptie-systeem, bestaande uit Φ80 *trp* DNA, *E. coli* RNA-polymerase, apo-repressor en L-tryptofaan (co-repressor). Dit systeem blijkt adequaat te zijn om enig inzicht te verkrijgen in het werkingsmechanisme van de apo-repressor. Dit onderzoek heeft, samengevat, de volgende gegevens opgeleverd:

a) de aangrijpingsplaats voor de apo-repressor, geactiveerd door L-tryptofaan, is de *trp* operator,
b) de *trp* apo-repressor gebonden aan de operator verhindert de binding van RNA-polymerase aan of nabij de *trp* promotor en voorkomt op deze wijze de de synthese van mRNA en
c) de co-repressor is L-tryptofaan.

De resultaten van deze studies vormen een bevestiging van het oorspronkelijke operon-model voor de regulatie van de expressie van genen, zoals dat is voorgesteld door Jacob en Monod (1,2).

TOELICHTING BIJ PUBLICATIES I t/m V

Publicatie I: Hans Pannekoek and Peter H. Pouwels: "The specificity of transcription *in vitro* of the *trp* operon of *Escherichia coli*". Molec. gen. Genet. *123*, 159-172 (1973).

In deze publicatie is een onderzoek beschreven, dat ten doel had na te gaan onder welke omstandigheden *in vitro* specifieke transcriptie van het *trp* operon, dat is transcriptie zoals die *in vivo* plaatsvindt, kon worden verkregen. Als criteria voor specifieke transcriptie werden gebruikt:

a) de asymmetrie van transcriptie d.w.z. transcriptie waarbij uitsluitend de

codogene streng van het *trp* DNA fungeert als matrijs voor de synthese van *trp* mRNA en

b) zowel de start als het stoppen van de transcriptie vindt plaats op specifieke plaatsen op het DNA.

Als matrijs voor de transcriptie werd gebruik gemaakt van DNA van een *trp* transducerende hybride-faag λ-Φ80, terwijl de synthese van *trp* mRNA werd gemeten d.m.v. DNA-RNA hybridisatie.

Uit het onderzoek is gebleken, dat specifieke transcriptie van het *trp* operon verkregen kan worden met gezuiverde RNA-polymerase, zonder toevoeging van additionele factoren. Hiermee onderscheidt het *trp* operon zich van biodegradatieve operons, waarbij transcriptie *in vitro* alleen kan plaatsvinden in aanwezigheid van een extra eiwit, de CAP-factor. Om na te gaan of RNA-polymerase in staat is om ook andere biosynthetische operons op specifieke wijze over te schrijven, is de transcriptie *in vitro* onderzocht van het arginine (*argECBH*) operon van *E. coli* (Publicatie IV).

Uit het onderzoek is tevens gebleken, dat de transcriptie terminatiefactor Rho de synthese van *trp* mRNA stimuleert, zowel gemeten naar de absolute hoeveelheid als naar het percentage van het totale RNA. Dit verschijnsel is nader geanalyseerd: in publicatie II is het effect van Rho bestudeerd op de transcriptie van DNA van de hybride-faag λ-Φ80, terwijl in publicatie III het effect van Rho op de transcriptie van het *trp* operon is onderzocht.

Publicatie II: Hans Pannekoek and Peter H. Pouwels: "The influence of ρ factor on the transcription *in vitro* of DNA from phage Φ80imm$^{\lambda}$ at high ionic strength". Biochim. Biophys. Acta *366*, 264-269 (1974).

Het effect van Rho-factor op de transcriptie, zoals dat door Roberts en anderen (29-31) is beschreven, is vrijwel zonder uitzondering bestudeerd in een transcriptie-systeem met lage ionsterkte. Echter bij lage ionsterkte verloopt de transcriptie beduidend minder specifiek dan bij hoge ionsterkte (32-34). Omdat uit ons onderzoek (Publicatie I) bleek, dat Rho-factor ook werkzaam was bij hogere ionsterkte, was het nodig opnieuw het effect van Rho op de transcriptie te bestuderen, zij het nu bij relatief hoge ionsterkte. Wij hebben onderzocht wat het effect van Rho is op het aantal en de lengte van de RNA-ketens, die onder deze omstandigheden gemaakt worden. Tevens hebben we onderzocht het effect van Rho op de relatieve frequentie waarmee bepaalde gedeelten van het DNA van de hybride-faag worden overgeschreven. Uit dit onderzoek is gebleken, dat in aanwezigheid van Rho het transcriptie-patroon meer

overeenkomst vertoont met het transcriptie-patroon *in vivo* dan in afwezigheid van Rho.

Publicatie III: Hans Pannekoek, Bernard Perbal and Peter Pouwels: "The specificity of transcription *in vitro* of the tryptophan operon of *Escherichia coli*. II The effect of Rho factor". Molec. gen. Genet. *132*, 291-306 (1974).

Voor het onderzoek, dat in deze en de volgende publicaties is beschreven, is een nieuwe detectie-methode ontwikkeld voor de specifieke synthese van *trp* mRNA, die ons in staat heeft gesteld op eenvoudiger en nauwkeuriger wijze dan voorheen de synthese van *trp* mRNA kwantitatief te bepalen. Hierbij werd als matrijs DNA gebruikt, afkomstig van een *trp* transducerende faag die behalve de *trp* genen uitsluitend Φ80 genen bevat, terwijl voor de hybridisatie DNA gebruikt werd van een transducerende faag die behalve de *trp* genen uitsluitend genen van faag λ bevat. De fagen van het laatste type werden voor dit doel geconstrueerd.

In de, in deze publicatie beschreven studie is getracht, ter verklaring van de toename van de synthese van *trp* mRNA door Rho, de vraag te beantwoorden of Rho invloed uitoefent op de snelheid van dissociatie van het transcriptie-complex bij het bereiken van een stopsignaal. Uit het onderzoek is gebleken, dat Rho inderdaad de snelheid van dissociatie van het transcriptie-complex verhoogt. Tevens is onderzocht of de lengte van de gevormde *trp* mRNA moleculen beïnvloed wordt door de aanwezigheid van Rho tijdens de synthese. Hierbij is gebleken, dat het *trp* operon wordt afgesloten door een transcriptie-stopsignaal, dat door Rho wordt herkend. Deze uitkomsten vormen een bevestiging van de in Publicatie I en II opgestelde hypothese, dat Rho vereist is voor de specifieke transcriptie van bacteriële en faag genen.

Publicatie IV: Hans Pannekoek, Raymond Cunin, Anna Boyen and Nicolas Glansdorff:"*In vitro* transcription of the bipolar arginine *ECBH* operon of *Escherichia coli* K 12". FEBS Letters *51*, 143-145 (1975).

In het onderzoek, dat in deze publicatie is beschreven, is de transcriptie bestudeerd van een ander biosynthetisch operon, t.w. het *E. coli* arginine (*argECBH*) operon. Onderzocht werd of voor de transcriptie van dit operon, behalve RNA-polymerase, andere factoren nodig zijn. Hierbij werd gebruik gemaakt van een transcriptie-systeem dat vergelijkbaar was met het in Publicatie III beschreven systeem, met dien verstande dat DNA van een *arg* transducerende faag van Φ80 werd gebruikt als matrijs, terwijl DNA van een *arg* transducerende faag

van λ werd gebruikt om de hoeveelheid gesynthetiseerd *arg* mRNA te bepalen. De uitkomsten wijzen er op, dat ook het *argECBH* operon, zonder toevoeging van additionele factoren, op specifieke wijze kan worden overgeschreven door RNA-polymerase.

Publicatie V: Hans Pannekoek, William J. Brammar and Peter H. Pouwels: "Punctuation of transcription *in vitro* of the tryptophan operon of *Escherichia coli*. A novel type of control of transcription". Molec. gen. Genet. *136*, 199-214 (1975).

De experimenten, die in deze publicatie worden beschreven, betreffen een nadere analyse van de regulatie van transcriptie van het *trp* operon. In het bijzonder is aandacht besteed aan de vraag of en hoe de regulatie-elementen van het operon (de promotor, operator en de "leader-sequence") worden overgeschreven. Op grond van de uitkomsten van deze experimenten is een hypothese opgesteld voor een nieuw type regulatie van transcriptie. Deze nieuwe vorm van regulatie zal in de Samenvatting en Discussie nader worden besproken.

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The Specificity of Transcription *in vitro* of the *trp* Operon of *Escherichia coli*

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Summary. The transcription in vitro of the tryptophan (*trp*) operon of *Escherichia coli* was studied with a preparation consisting of purified RNA polymerase from *E. coli* and purified DNA from a *trp* transducing strain of ϕ80. The synthesis of *trp* mRNA was determined a) in a two-step RNA-DNA filter hybridization test (measurement of the amount of RNA which remains free after hybridization with ϕ80 DNA and hybridizes with ϕ80 *trp* DNA)

b) by competition-hybridization between radioactive *trp* mRNA synthesized in vivo and RNA prepared in vitro on ϕ80 *trp* DNA.

Specific transcription of the *trp* genes on ϕ80 *trp* DNA could be demonstrated when RNA synthesis was carried out in a buffer of high ionic strength (0.15 M KCl) with RNA polymerase saturated with σ factor in the presence of the termination factor ρ. In the absence of σ- or ρ factor and/or in buffers of low ionic strength (0.05 M KCl) synthesis of *trp* mRNA was less specific.

Introduction

In the past few years great progress has been made in the understanding of the regulation of gene expression in bacteria (Vogel, 1971). The development of preparations for the synthesis in vitro of mRNA corresponding to specific DNA segments and for the synthesis in vitro of active enzymes has allowed the identification and characterization of components which control the transcription of inducible operons such as the lactose-, the galactose- and the arabinose operon (Zubay *et al.*, 1970; de Crombrugghe *et al.*, 1971; Eron *et al.*, 1971; Greenblatt and Schleif, 1971; Wetekam *et al.*, 1971). A study of the control of synthesis of bio-synthetic enzymes, which may very well be different from that of the synthesis of enzymes involved in degradative pathways has only recently begun. Using a preparation for the synthesis in vitro of enzymes of the tryptophan (*trp*) operon of *Escherichia coli*, we have shown that control mechanisms which are operative in vivo also function in vitro (Pouwels and Van Rotterdam, 1972). Yura *et al.* (1968), who have studied RNA synthesis in vitro with RNA polymerase and DNA from *E. coli*, found that RNA is formed which specifically hybridizes with DNA from phages containing the genes of the *trp* operon and therefore presumably represents *trp* mRNA. In order to gain insight into these control mechanisms, we have started a study of the transcription in vitro of the *trp* operon, using a preparation consisting of purified DNA from a *trp* transducing strain of phage ϕ80 and purified RNA polymerase from *E. coli*. Synthesis of *trp* mRNA was assayed by the technique of RNA—DNA

hybridization. In the present paper experiments are described showing that RNA polymerase (E^{σ}; holo-enzyme) can catalyse the transcription of the *trp* operon in vitro. The specificity of the transcription of the *trp* genes in vitro is strictly dependent on the presence of the initiation factor σ and the termination factor ϱ and also on the ionic strength.

Materials and Methods[1]

Bacteriophages. Bacteriophage ϕ80imm$^{\lambda}$ptAEd62 (Gratia, 1971) is a *trp* transducing strain of phage ϕ80 containing the entire *trp* operon including *trp* promotor and operator (Gratia, 1971; Pouwels and Stevens, 1973). The only difference with the parent phage, ϕ80imm$^{\lambda}$, is the replacement of some non essential phage genes in ϕ80imm$^{\lambda}$ by the bacterial segment. The phages will be indicated in the text as ϕ80 *trp* and ϕ80 respectively. The propagation and purification of bacteriophages and the isolation of DNA have been described previously (Pouwels and Van Rotterdam, 1972).

Enzymes and Factors. E. coli RNA polymerase was prepared from strain Q13 as described by Burgess (1969) or from strain MRE 600 according to Darlix *et al.* (1969). When RNA polymerase was purified according to the latter method an additional purification step was included which consists of a chromatographic separation on hydroxyapatite (Richardson, 1966). Enzyme preparation were more than 95% pure as judged by electrophoresis patterns in polyacrylamide gels in the presence of SDS (Burgess *et al.*, 1969). Holo-enzyme was separated into core-enzyme (E) and σ factor by chromatography on phosphocellulose (Burgess, 1969). Reconstitution of holo-enzyme from core-enzyme and σ factor was carried out as described by Dausse *et al.* (1972). Rho factor was isolated from *E. coli* MRE 600 and purified according to Roberts (1969). The concentrations of purified RNA polymerase, ϱ- and σ factor were estimated from the absorbance at 280 nm (A_{280}) with the assumption that an A_{280} of 0.7 corresponds to 1 mg/ml.

DNA polymerase I was isolated from E. coli KMBL 1068 F'*thyA*, *bio87*, *endA101* (constructed by B. Glickman, Rijswijk) and purified as described by Jovin *et al.* (1969).

Isolation of ^{3}H-Labelled RNA from E. coli. E. coli, Hfr, thi, trpR was labelled with [^{3}H]-uracil during two min and the radioactive RNA was isolated as described by Summers (1970). In addition the isolated RNA was filtered twice through a Millipore HAWP 025 filter to lower the aspecific hybridization values. The presence of *trp* mRNA in this preparation was determined by hybridization with the l-DNA strand of ϕ80 *trp* DNA (see below). The percentage of total radioactivity which hybridized to the l-DNA strand of ϕ80 *trp* DNA reached a plateau (0.75%) at a ratio l-DNA/[^{3}H]-RNA = 2.5. Under these conditions very little, if anything, hybridized with the r-strand from ϕ80 *trp* DNA (0.04%) or with the l-DNA strand of ϕ80 DNA (0.02%) (Pouwels and Stevens, 1973). It thus appears that 0.75% of the radioactive RNA in our preparation represents *trp* mRNA.

Synthesis of ^{3}H-Labelled RNA in vitro. Incubations were carried out during 7.5 min at 37° C unless otherwise specified. The reaction mixture (0.15–0.30 ml) contained: 25 mM TRIS-HCl (pH 7.9), 8 mM $MgCl_2$, 0.05 or 0.15 M KCl as indicated in the text, 0.1 mM DTT, 40 μg phage DNA per ml, 0.5 mM of each of the four ribonucleoside triphosphates (UTP labelled with ^{3}H or GTP labelled with ^{32}P in the α-position), 15 μg/ml RNA polymerase (hydroxy-apatite fraction; 60–80% saturated with σ factor), 6 μg/ml σ factor and 1.5 μg/ml ϱ factor. The reaction was stopped by addition of cold trichloroacetic acid to a final concentration of 5%. The amount of RNA which was synthesized was determined by measuring the radioactivity present in the acid precipitable material. When the RNA had to be used for hybridization studies the reaction was stopped by the addition of SDS and the RNA was precipitated with ethanol and redissolved in 2 × SSC.

Separation of the Complementary Strands of Phage DNA. Separation of the complementary strands of ϕ80 *trp* DNA and ϕ80 DNA was performed in a CsCl density gradient containing poly (U, G) (2:1) as described by Hradecna *et al.* (1967), after denaturation of the DNA with alkali as described by Shapiro *et al.* (1969). After centrifugation the separated strands (l- and r-strand) were incubated in 0.3 N NaOH for 18 hours at 37° C to bring about hydrolysis of

1 *Abbreviations.* SDS = sodium dodecylsulphate; SSC = 0.15 M NaCl, 0.015 M tri-sodium citrate; nxSSC = n fold concentrated SSC buffer; DTT = dithiotreitol.

poly (U, G). After extensive dialysis, to remove the degradation products, the separated strands were incubated for 4 hours at 67° C in 20 mM TRIS-HCl (pH 8.0) containing 0.3 M NaCl, 0.03 M trisodium citrate (2 × SSC) to reanneal any complementary strands that might be present.

DNA-RNA Hybridization. a) DNA-RNA hybridization was carried out in solution as described by Goff and Minkley (1970). To an aqueous solution of ^{3}H-labelled RNA 10 × SSC (saturated with phenol) was added to give a final concentration of 2 × SSC. ^{3}H-labelled RNA (10–20 ng) was mixed with excess (0.30 μg) l or r strand from ϕ80 *trp* DNA or ϕ80 DNA in 0.3 ml 2 × SSC (20% saturated with phenol) and incubated at 67° C during 6 h in a closed vial. Then 0.7 ml 2 × SSC, containing 10 μg pancreatic RNAase and 1 μg T1 RNAase was added to digest non hybridized and partially hybridized RNA segments and the samples were incubated at 37° C during 30 min. DNA-RNA hybrids were collected on Millipore HAWP 025 filters and the filters were washed with 50 ml 10 mM TRIS-HCl (pH 7.4), 0.5 M KCl. After drying of the filters the radioactivity was determined in a liquid scintillation counter. The hybridization efficiency was 80–90%.

b) DNA-RNA hybridization on filters was carried out for 20–30 h at 36° C in 0.4 ml 3 × SSC, 50% formamide according to Gillespie and Gillespie (1971). After hybridization the filters were incubated with pancreatic RNAase and T1 RNAase and further processed as under a).

c) Competition-hybridization experiments were carried out in solution: 1.1 μg ^{3}H-labelled RNA (isolated from *E. coli HfrH, thi, trpR*; 4.6 × 10^5 counts/min/μg)was hybridized in 0.26 ml 2 × SSC,10 mM TRIS-HCl (pH 7.4) (20% saturated with phenol), with 0.11 μg of the l strand of ϕ80 *trp* DNA together with increasing amounts (0–25 μg) of partially purified, in vitro synthesized ϕ80- or ϕ80 *trp* mRNA as competitor. Conditions of hybridization and determination of the amount of RNA which hybridized to DNA have been given above under a).

Chemicals. Ribonucleoside triphosphates were obtained from Sigma (St. Louis, U.S.A.) or from P. L. Biochemical Company (Milwaukee, U.S.A.). [^{3}H]-UTP (7 Ci/mmole), [^{3}H]-uracil (28 Ci/mmole) and [α-^{32}P]-GTP (2 Ci)/mmole) were products of C.E.N. (Saclay, France). Poly (U, G) (2:1) was a product of General Biochemicals (Chagrin Falls, U.S.A.).

Results

Influence of Reaction Conditions on the Synthesis of RNA on ϕ80 trp DNA. DNA dependent RNA synthesis is influenced by the concentration of DNA and triphosphates as well as by the ionic environment. To yield optimal RNA synthesis each of these parameters was varied for ϕ80 *trp* DNA, with a molecular ratio of enzyme to DNA between 10–25. At a fixed RNA polymerase concentration of 15 μg per ml a maximal rate and extent of RNA synthesis was found at a DNA concentration of 40–50 μg per ml, in the presence of 8 mM $MgCl_2$, 0.05 M KCl and 0.5 mM of each of the triphosphates.

These results are essentially the same as those reported by Okamoto *et al.* (1970) for the RNA synthesis on ϕ80 DNA and those of Matsukage (1972a) for RNA synthesis on ϕ80 *plac* DNA. The time-course of RNA synthesis (in the absence of ϱ factor) at high and at low ionic strength is presented in Fig. 1. The initial rate of RNA synthesis was the same at low (0.05 M) and at high (0.15 M) KCl concentration. At high ionic strength RNA synthesis was linear for a short period (10 min) only and reached a plateau value after 50 min while at the lower ionic strength RNA synthesis continued linearly for at least 150 min. These results are different from those found with T4 DNA, where synthesis generally reaches a plateau at 0.05 M KCl and continues linearly at a higher ionic strength (Fuchs *et al.*, 1967; So *et al.*, 1967). Although RNA synthesis continues for a longer period at the lower ionic strength the synthesis of *trp* mRNA is not optimal at 0.05 M KCl but requires a higher ionic strength as will be shown below.

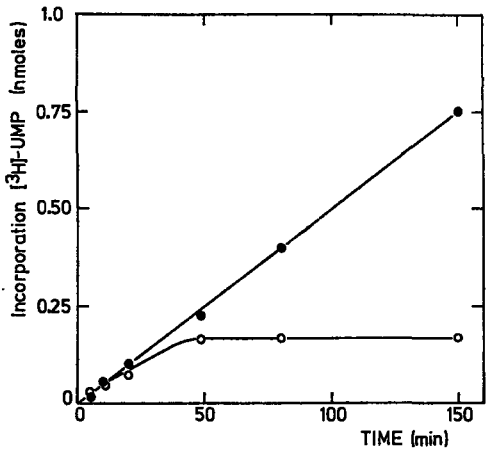

Fig. 1. Influence of the KCl concentration on the time dependency of the transcription of ϕ80 *trp* DNA. RNA was synthesized in the absence of ϱ factor as described in Materials and Methods. The molecular ratio of RNA polymerase (E^σ) to DNA was 10 and the specific radioactivity of [^{3}H]-UTP in the reaction mixture (0.15 ml) was 3×10^4 counts/min/nmole. KCl concentration ○ 0.15 M; • 0.05 M. The results are given for ϕ80 *trp* DNA; those for ϕ80 DNA were essentially the same

RNA Synthesis at Varying Ratios of RNA Polymerase to ϕ80 trp DNA. When the initial rate of RNA synthesis was measured in either 0.05 M KCl or 0.15 M KCl at varying ratios of E^σ to ϕ80 *trp* DNA (in the absence of ϱ factor) a non-linear dependency was observed (Fig. 2; open circles; results show RNA synthesis in 0.05 M KCl). A similar observation was made by Naono and Tokuyama (1970) who used phage λ DNA.

The explanation for this non-linearity may be sought in the presence of single stranded regions at the ends of DNA of lambdoid phages (Hershey, Burgi, and Ingraham, 1963). Since RNA polymerase has a high affinity also for single stranded DNA, the single stranded regions will bind a proportion of the enzyme molecules. These enzyme molecules contribute significantly less to the synthesis of RNA than those that are bound to double stranded DNA (Wood and Berg, 1964; Maitra *et al.* 1967). The following experiment shows that this hypothesis probably is correct. The cohesive ends of ϕ80 *trp* DNA were made double stranded with DNA polymerase I (Kornberg, 1969) and this "repaired" DNA was then used as a template for RNA synthesis. The rate of RNA synthesis now increased linearly as a function of the molecular ratio E^σ to DNA (Fig. 2; closed circles). Even at very high ratios (E^σ/DNA = 100) the rate of synthesis was found to be proportional to this ratio; a plateau was reached at a ratio E^σ to DNA = 250 (results not shown).

The Effect of the Termination Factor ϱ on RNA Synthesis. In 1969 Roberts reported the isolation and purification of a protein, called ϱ factor, which depresses the RNA synthesis on phage λ DNA in vitro (Roberts, 1969). Factor ϱ is believed to act on termination of transcription. We have determined the effect of purified ϱ factor on RNA synthesis on ϕ80 *trp* DNA at low or high ionic strength. The results are presented in Fig. 3. When the reaction was carried out at 0.05 M KCl the maxi-

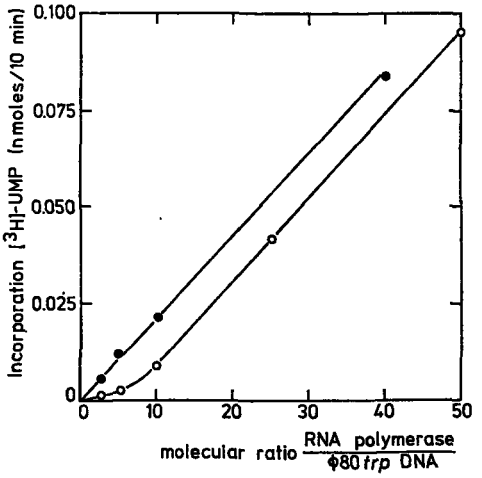

Fig. 2. Dependency of the rate of RNA synthesis on the ratio RNA polymerase to ϕ80 *trp* DNA; the effect of treatment of the DNA with DNA polymerase I. The amount of RNA synthesized during the first 10 min of incubation at varying ratios of RNA polymerase to ϕ80 *trp* DNA, was determined before and after treatment of the DNA with DNA polymerase I. Conditions for RNA synthesis (0.05 M KCl) have been described in Materials and Methods. The specific radioactivity of [^{3}H]-UTP in the reaction mixture (0.15 ml) was 2×10^5 counts/min/nmole. The reaction mixture for the "repair" of the cohesive ends of ϕ80 *trp* DNA contained per ml: 25 μmole TRIS-HCl (pH 7.9), 310 μg ϕ80 *trp* DNA, 0.17 μmole of each of the four deoxyribonucleoside triphosphates, 8.5 μmole $MgCl_2$, 0.1 μmole DTT and 15 μg DNA polymerase I (Amicon XM-50 fraction). The reaction mixture was incubated at 4° C during 16 h. Subsequently the DNA polymerase I activity was destroyed by heat treatment (10 min at 65° C) in the dark. ○ control; ● DNA incubated with DNA polymerase I. Similar results were obtained with ϕ80 DNA instead of ϕ80 *trp* DNA

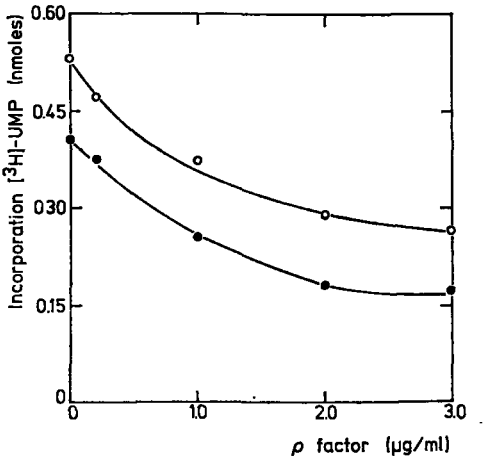

Fig. 3. Influence of ρ factor on the transcription of ϕ80 *trp* DNA. Conditions for RNA synthesis have been described in Materials and Methods. The ratio of RNA polymerase (E^o) to DNA was 10 and the specific radioactivity of [^{3}H]-UTP in the reaction mixture (0.15 ml) was 8×10^4 counts/min/nmole. The duration of incubation was 10 min at 37° C. KCl concentration: ○ 0.05 M; ● 0.15 M. The results which are given for ϕ80 *trp* DNA, were essentially the same as those for ϕ80 DNA

mal depression of RNA synthesis amounted to 50%. Similar values were reported by Roberts for phage λ DNA. In contrast to the results obtained with λ DNA (Goldberg, 1970), with ϕ80 *trp* DNA a depression of total RNA synthesis was also observed at 0.15 M KCl, again of approximately 50% (Fig. 3). Essentially the same results were obtained if ϕ80 DNA was used instead of ϕ80 *trp* DNA (results not shown).

Detection of trp mRNA Made in vitro on ϕ80 trp DNA. To detect the presence of mRNA corresponding to the *trp* genes among the RNA made *in vitro* we have used the technique of DNA-RNA hybridization (two-step hybridization). In this procedure the RNA which is synthesized in vitro on ϕ80 *trp* DNA is first hybridized to ϕ80 DNA to remove RNA segments homologous to the ϕ80 genes. In a second hybridization-step it is determined whether the material which did not hybridize with ϕ80 DNA contains RNA which will specifically hybridize to ϕ80 *trp* DNA. This RNA then would represent *trp* mRNA.

Equal amounts of ^{3}H-labelled RNA, made in vitro on ϕ80 *trp* DNA and ϕ80 DNA were incubated with excess ϕ80 DNA on filters under conditions favouring hybridization (Fig. 4A). After hybridization the filters were removed and the remaining material was subjected to the second hybridization step with excess ϕ80 *trp* DNA on filters (Fig. 4B).

As can be seen from Fig. 4B more radioactivity from the preparation made with ϕ80 *trp* DNA than from the preparation made with ϕ80 DNA hybridized with ϕ80 *trp* DNA. The results of this experiment indicate that the RNA preparation made on ϕ80 *trp* DNA contains RNA segments which hybridize specifically with ϕ80 *trp* DNA. This RNA presumably represents *trp* mRNA.

Comparison of the Transcription of ϕ80 trp DNA and ϕ80 DNA; Determination of the Relative Hybridization Ratios. A further indication for the presence of *trp* mRNA in RNA preparations synthesized in vitro may also be obtained from the relative hybridization ratios. Roberts (1969) and Ikeda (1972) have used this technique to demonstrate the synthesis in vitro of RNA corresponding to early genes of lambda and of $tRNA^{tyr}$, respectively. To determine the relative hybridization ratios ^{3}H-labelled RNA, made in vitro on ϕ80 *trp* DNA, was hybridized with the l strand and the r strand of ϕ80 *trp* DNA.

Likewise ^{3}H-labelled RNA, made on ϕ80 DNA, was hybridized with the l strand and the r strand of ϕ80 DNA. In the following the symbols $l_{\phi 80\ trp}$ and $r_{\phi 80\ trp}$ represent the amounts of RNA, synthesized on ϕ80 *trp* DNA, which hybridize with l- and r strand of ϕ80 *trp* DNA. Likewise $l_{\phi 80}$ and $r_{\phi 80}$ represent the amounts of RNA, made on ϕ80 DNA, which hybridize with the l- and r strand of ϕ80 DNA. The presence of *trp* mRNA, which will hybridize with the l strand of ϕ80 *trp* DNA (Pouwels and Stevens, 1973), may then be determined by comparison of the ratios $l_{\phi 80\ trp}/r_{\phi 80\ trp}$ and $l_{\phi 80}/r_{\phi 80}$ respectively. If these ratios are the same, specific transcription of genes on the λ strand of ϕ80 *trp* DNA (a measure for the synthesis of *trp* mRNA) probably does not occur. If, however, the ratio $l_{\phi 80\ trp}/r_{\phi 80\ trp}$ is greater than the ratio $l_{\phi 80}/r_{\phi 80}$ very likely specific transcription of genes on the l strand of ϕ80 *trp* DNA has taken place.

The ratios $l_{\phi 80 trp}/r_{\phi 80\ trp}$ and $l_{\phi 80}/r_{\phi 80}$ obtained with RNA synthesized under various conditions are given in Table 1. At 0.05 M KCl, both in the absence or presence of ϱ factor, the ratios were not significantly different. Also at 0.15 M KCl, without ϱ factor the ratios were the same. However, at 0.15 M KCl and in the

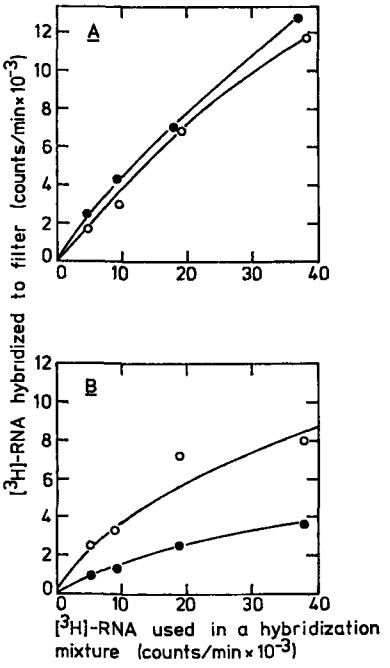

Fig. 4. Two-step hybridization on filters of [^{3}H]-RNA synthesized in vitro on ϕ80 *trp* DNA or ϕ80 DNA. RNA synthesis was carried out as described under Materials and Methods in 0.15 M KCl in the presence of ϱ factor. The ratio of RNA polymerase (E^σ) to DNA was 25. In the first hybridization-step (panel A) [^{3}H]- ϕ80 *trp* RNA (2.1 μg/ml; 4.6×10^5 counts/min/μg) or ϕ80 RNA (2.0 μg/ml; 4.6×10^5 counts/min/μg) was hybridized with denatured ϕ80 DNA on filters (5 μg DNA per filter). Hybridization was carried out in 50% formamide, 3 × SSC during 20 h at 36° C in a volume of 0.4 ml. After hybridization the filters were removed from the solution. In the second hybridization step (panel B) the solution remaining after the first step was incubated with denatured ϕ80 *trp* DNA on filters (5 μg DNA per filter) during 30 h at 36° C. After hybridization the filters were processed as described under Materials and Methods and the radioactivity which was present on the filters was determined. ○ ϕ80 *trp* RNA; • ϕ80 RNA

presence of ϱ factor, a significant difference between the ratio $l_{\phi80\ trp}/r_{\phi80\ trp}$ and $l_{\phi80}/r_{\phi80}$ was observed, indicating that only in the latter case specific synthesis of *trp* mRNA can be detected .

RNA polymerase holo-enzyme (E^σ) consists of a core-enzyme (E) and σ factor (Burgess, 1969). The σ factor presumably mediates the recognition of specific sites (promotors) on DNA templates (Bautz *et al.*, 1969; Goff and Minkley, 1970). In the following experiment we have determined the effect of σ factor on the synthesis of *trp* mRNA with ϕ80 DNA and ϕ80 *trp* DNA, using either core-enzyme or enzyme saturated with σ factor, at 0.15 M KCl and in the presence of ϱ factor (Table 2). The amount of RNA synthesized with core-enzyme was 25% of that synthesized with holo-enzyme. A similar result was obtained by Vogt (1969) for ϕ80 DNA. A small, but significant difference of the hybridization ratios was observed only when transcription was carried out with holo-enzyme, which suggests a dependency of *trp* mRNA synthesis on the presence of σ factor.

Table 1. Influence of ϱ factor and the ionic strength on the relative hybridization ratios: comparison of the transcription of $\phi 80$ *trp* DNA and $\phi 80$ DNA

Exp.	KCl (M)	ϱ factor (3 μg/ml)	RNA synthesized (nmoles) on		Ratio of hybridization with the separated strands of	
			$\phi 80$ *trp* DNA	$\phi 80$ DNA	$\phi 80$ *trp* DNA ($l_{\phi 80\ trp}/r_{\phi 80\ trp}$)	$\phi 80$ DNA ($l_{\phi 80}/r_{\phi 80}$)
1	0.05	−	0.210	0.191	1.02; 1.03	1.04; 1.05
2	0.05	+	0.106	0.094	1.19; 1.16	1.16; 1.15
3	0.15	−	0.162	0.162	0.56; 0.53	0.52; 0.52
4	0.15	+	0.073	0.083	0.97; 0.94	0.79; 0.79

The synthesis of RNA was as given in Materials and Methods. The concentrations of KCl and of ϱ factor were as indicated in the Table. RNA polymerase was a hydroxyapatite fraction (± 70% saturated with σ). The ratio E^σ to DNA was 25. Hybridizations were carried out in duplicate in solution with excess DNA as described in Materials and Methods. The same amount of radioactive RNA was used for the hybridization with $\phi 80$ *trp* DNA and $\phi 80$ DNA. Hybridization efficiencies ranged from 80–90%. $l_{\phi 80\ trp}$ and $r_{\phi 80\ trp}$ represents the radioactivity of the preparation of [^{3}H]-$\phi 80$ *trp* RNA which hybridized with the l strand and r strand of $\phi 80$ *trp* DNA respectively. Likewise $l_{\phi 80}$ and $r_{\phi 80}$ represents the radioactivity of the preparation of [^{3}H]-$\phi 80$ RNA which hybridized with the l strand and r strand of $\phi 80$ DNA.

Table 2. Influence of σ factor on the relative hybridization ratios: comparison of the transcription of $\phi 80$ *trp* DNA and $\phi 80$ DNA

Saturation of core-enzyme with σ factor (%)	RNA synthesized (nmoles) on		Ratio of hybridization with the separated strands of	
	$\phi 80$ *trp* DNA	$\phi 80$ DNA	$\phi 80$ *trp* DNA ($l_{\phi 80\ trp}/r_{\phi 80\ trp}$)	$\phi 80$ DNA ($l_{\phi 80}/r_{\phi 80}$)
0	0.11	0.13	1.04; 1.04	1.05; 1.03
100	0.46	0.49	0.70; 0.71	0.64; 0.65

RNA synthesis was carried out in 0.15 M KCl in the presence of 3 μg ϱ factor per ml, with a molecular ratio E^σ to DNA is 10. RNA polymerase was separated into core-enzyme and σ factor (Burgess, 1969). Reconstitution of holo-enzyme from core-enzyme and σ factor was performed as described by Dausse *et al.* (1972). Conditions of hybridization (duplicate experiment) were the same as those given in Table 1.

Competition-Hybridization. In order to prove that the radioactivity which hybridizes specifically with $\phi 80$ *trp* DNA represents *trp* mRNA, we have carried out competition-hybridization experiments. The hybridization was studied with l-DNA from $\phi 80$ *trp*, which codes for *trp* mRNA in vivo (Pouwels and Stevens, 1973), with radioactive RNA, isolated from bacteria which constitutively synthesize *trp* mRNA, while RNA made in vitro on $\phi 80$ *trp* DNA or $\phi 80$ DNA was used as competitor.

In a competition-hybridization experiment 1.1 μg ^{3}H-labelled RNA, made in vivo, was hybridized to 0.11 μg l-DNA from $\phi 80$ *trp* in the presence of increasing amounts (0–25 μg) of [^{32}P]-RNA synthesized in vitro on $\phi 80$ *trp* DNA or on $\phi 80$ DNA. The results (Fig. 5) show that the RNA synthesized in vitro on $\phi 80$ *trp* DNA can fully

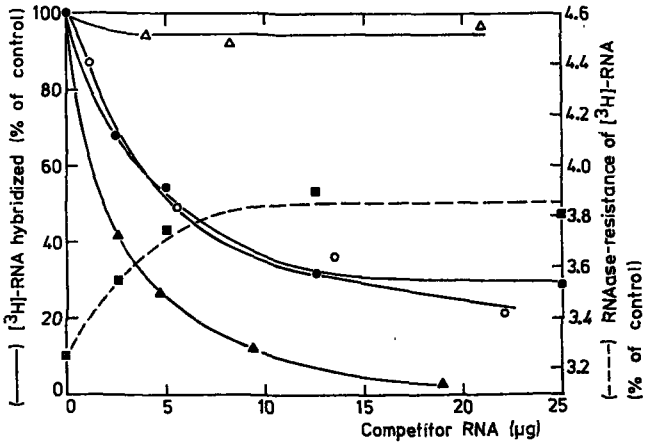

Fig. 5. Competition-hybridization between [^{3}H]-trp mRNA synthesized in vivo and RNA made in vitro on ϕ80 *trp* DNA or ϕ80 DNA, with l-DNA from ϕ80 *trp*. *E. coli trp R* was pulse-labelled with [^{3}H]-uracil and the RNA was isolated as described under Materials and Methods. 1.1 µg [^{3}H]-labelled *E. coli trp R*-RNA was hybridized to 0.11 µg l-DNA from ϕ80 *trp*. Increasing amounts of [^{32}P]-RNA, synthesized in vitro in 0.15 M KCl, were added as a competitor. The efficiency of hybridization in the absence of competitor RNA was 50% of that obtained with large excess of ϕ80 *trp* DNA. The amount of [^{3}H]-RNA, which hybridizes to ϕ80 *trp* DNA in the absence of competitor RNA represents the 100% value of the control. ▲ ϕ80 RNA made with holo-enzyme + ϱ factor (2.5 µg/ml). ▲ ϕ80 *trp* RNA made with holo-enzyme + ϱ factor (2.5 µg/ml). ○ ϕ80 *trp* RNA made with holo-enzyme. ● ϕ80 *trp* RNA made with core-enzyme + ϱ factor (2.5 µg/ml). ■ RNAase-resistance of [^{3}H]-*E. coli trp R*-RNA with increasing amounts of ϕ80 *trp* RNA, made in vitro with core-enzyme + ϱ factor (2.5 µg/ml)

compete with *trp* mRNA made in vivo, but RNA synthesized in vitro on ϕ80 DNA cannot. At increasing concentrations of competitor RNA an increasing amount of ^{32}P-labelled material was bound to the filters (results not shown) indicating that the RNA synthesized in vitro truly competes with the [^{3}H]-RNA made in vivo. These results indicate that RNA synthesized in vitro on ϕ80 *trp* DNA contains RNA segments which are homologous to *trp* genes.

Competition between [^{3}H]-*trp* mRNA made in vivo and [^{32}P]-RNA synthesized with core-enzyme was also observed although it was less efficient than with RNA synthesized with holo-enzyme (Fig. 5). In this case, however, ^{32}P-labelled material was not found on the filters, even at very high concentrations of competitor RNA. Therefore it seems likely that the reduction of the percentage of [^{3}H]-mRNA which hybridized with ϕ80 *trp* DNA was not due to true competition between [^{32}P]-RNA made in vitro and [^{3}H]-*trp* mRNA made in vivo but rather was caused by the presence in our in vitro preparation of RNA segments which are complementary to [^{3}H] *trp*-mRNA. This complementary RNA might form RNA-RNA duplex molecules with [^{3}H]-*trp* mRNA, thus preventing the formation of DNA-RNA hybrids between ϕ80 *trp* DNA and [^{3}H]-*trp* mRNA. This conclusion is supported by the finding that the percentage of RNA (^{3}H-label) forming RNAase resistant complexes increased from 3.2% to 3.8% at increasing concentrations of competitor RNA, made with core-enzyme (Fig. 5). This increase may seem small

but is close to that expected for a RNA preparation containing approximately 0.75% *trp* mRNA. Our results, therefore, suggest that RNA synthesized with core-enzyme contains segments which are complementary to in vivo made *trp* mRNA. The conclusion that RNA synthesis with core-enzyme is less specific than with holo-enzyme is supported by the observation that 37 percent of the in vitro synthesized RNA, made with core-enzyme, formed RNAase resistant complexes; this value is significantly higher than that found for the material synthesized with holo-enzyme (5–8%).

In analogous experiments we have determined the effect of the termination factor ϱ on the synthesis of *trp* mRNA. The results of these experiments (Fig. 5) show that RNA synthesized in vitro at 0.15 M KCl in the presence of ϱ factor can compete more efficiently with in vivo made *trp* mRNA, than RNA which is synthesized in the absence of ϱ factor. RNA synthesized at 0.05 M KCl (in the presence or absence of ϱ factor) could compete less efficiently than RNA synthesized at 0.15 M KCl (results not shown).

Discussion

Under appropriate conditions of synthesis specific transcription in vitro of the *trp* operon of *E. coli* will take place. This conclusion is based upon the following observations. RNA synthesized in vitro on ϕ80 *trp* DNA very efficiently competes with radioactive *trp* mRNA synthesized in vivo, while RNA made on ϕ80 DNA does not (Fig. 5). This result indicates that RNA synthesized on ϕ80 *trp* DNA contains *trp* mRNA. The observation that the efficiency of competition between this in vitro synthesized RNA and in vivo made *trp* mRNA is over 95% suggests that all *trp* mRNA regions which are present in vivo are also found in the in vitro preparations. A comparison of the rate of migration in polyacrylamide gels of the products of transcription in vitro with that of RNA molecules of known length (Pannekoek & Pouwels, in preparation) shows that the fraction of the RNA which specifically hybridizes with ϕ80 *trp* DNA, is approximately 6900 nucleotides long, a value close to that of *trp* mRNA found in vivo (Imamoto and Yanofsky, 1967) indicating that the *trp* mRNA synthesized under these conditions is not composed of short fragments. Apparently the *trp* mRNA is present as a polycistronic messenger, suggesting that initiation and termination do not take place at random, but are limited to specific sites. In our in vitro system *trp* mRNA is transcribed from the same strand from which it is transcribed in vivo, i.e. the l-strand, as could be shown by competition-hybridization experiments.

The specificity of the transcription of the *trp* genes on ϕ80 *trp* DNA depends on several parameters as is indicated by the present studies.

a) Influence of the ionic strength on the synthesis of *trp* mRNA in the presence of ϱ factor: Determination of the relative hybridization ratios shows that specific synthesis of *trp* mRNA can be detected at 0.15 M KCl but not at 0.05 M KCl. Competition-hybridization experiments also indicate that RNA which is synthesized at 0.15 M KCl contains a larger fraction of *trp* mRNA than RNA which is synthesized at 0.05 M KCl. From these results we conclude that transcription of ϕ80 *trp* DNA in buffer of low ionic strength is mainly non-specific and starts randomly at both DNA strands, while a more specific transcription is obtained at the higher salt concentrations. This conclusion is in keeping with the results of studies

with phage T7 DNA. Dausse *et al.* (1972) and Matsukage (1972b) have shown that a higher specificity of transcription is obtained at 0.15 M than at 0.05 M KCl.

b) The initiation factor σ: By determination of the relative hybridization ratios we have shown that a small but significant difference of these ratios only occurs with RNA synthesized in the presence of σ factor (Table 2). Moreover, we have shown by competition-hybridization experiments that RNA synthesized with holo-enzyme can compete efficiently with in vivo made *trp* mRNA while RNA synthesized with core-enzyme cannot (Fig. 5).

These results suggest that only the holo-enzyme initiates *trp* mRNA synthesis specifically. Eron *et al.* (1971) and de Crombrugghe *et al.* (1971) have shown that correct initiation of transcription of another bacterial operon, the *lac* operon, is also dependent on the presence of σ factor. Our results, therefore, lend additional support to the conclusion, originally based on studies with phage DNA, that σ factor is a specificity determinant, which functions in the selection of sites of initiation of transcription (Bautz *et al.*, 1969; Goff and Minkley, 1970; Travers, 1971; Hinkle and Chamberlin, 1972).

c) The termination factor ρ: Our results (Fig. 3) show that ρ factor depresses RNA synthesis on ϕ80 *trp* DNA to the same extent at high and at low ionic strength. Analysis of the products on polyacrylamide gels, moreover, shows that the average RNA chain length is reduced, and that also short RNA chains are synthesized when RNA synthesis is performed in 0.15 M KCl in the presence of ρ factor (Pannekoek & Pouwels, in preparation). These results, although at variance with those of Goldberg (1970) who observed no effect of ρ factor on total RNA synthesis with λ DNA, T4 DNA or T7 DNA at high salt concentration, are in agreement with the results of Takanami (1971) who found a depression of RNA synthesis by ρ factor in buffers of high ionic strength with fd DNA.

RNA synthesized on ϕ80 *trp* DNA in 0.15 M KCl in the absence of ρ factor contains significantly less *trp* mRNA than RNA which is made in its presence, as is revealed by competition-hybridization experiments (Fig. 5). Comparison of the competition efficiencies of RNA made in the presence of ρ factor with equivalent amounts of RNA made in its absence (after correction for the depression of RNA synthesis caused by ρ factor, see Fig. 3) shows that at all RNA concentrations, the former RNA contains more *trp* mRNA than the latter, not only relatively, but also absolutely. The relative hybridization ratios of RNA preparations made in the presence or absence of ρ factor suggest that only in its presence specific synthesis of *trp* mRNA takes place (Table 1). Moreover, the results of two-step hybridization experiments (Fig. 4) suggest that in the presence of ρ factor no read-through transcription of phage genes which has started at a bacterial promoter or transcription of *trp* genes which has started at a phage promotor takes place.

From the arguments presented so far we conclude that in our in vitro system the *trp* operon is fully transcribed from the correct strand, provided that σ- and ρ factor are present and that the reaction is carried out in 0.15 M KCl. The synthesis of *trp* mRNA is at least partly initiated and terminated within the bacterial segment of the phage genome since RNA made on ϕ80 *trp* DNA contains segments which hybridize with ϕ80 *trp* DNA but not with ϕ80 DNA. This argument might not be valid if the RNA had been extensively degraded during the hybridizaton

experiment. To minimize RNA degradation, however, we have carried out these hybridization experiments at a relatively low temperature (36° C).

de Crombrugghe *et al.* (1971) and Eron *et al.* (1971) have shown that under conditions of RNA synthesis, similar to ours, transcription of the *lac* operon is correctly initiated and terminated, provided that σ factor is present. Similarly Arditti *et al.* (1970) have shown that the synthesis of specific *lac* mRNA can only be detected if ρ factor is present during RNA synthesis. Our results indicate that specific *trp* mRNA can be synthesized which is composed of a full-size messenger. Since this reaction also requires the presence of σ- and ρ factor, it seems not unlikely that in our system under appropriate conditions of synthesis, correct initiation and termination of *trp* mRNA synthesis occur.

In any case it appears that other transcription-initiation factors, like CAP factor, which is required for the initiation of transcription of some bio-degradative operons (de Crombrugghe *et al.*, 1971; Eron *et al.*, 1971) are not necessary for the transcription of the *trp* operon, a bio-synthetic operon.

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THE INFLUENCE OF ρ FACTOR ON THE TRANSCRIPTION IN VITRO OF DNA FROM PHAGE φ80imm$^{\lambda}$ AT HIGH IONIC STRENGTH

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Summary

The effects of ρ factor, which is involved in the termination of transcription, were studied in vitro with an RNA synthesizing system consisting of purified RNA polymerase from *Escherichia coli* and φ80imm$^{\lambda}$ DNA. At high ionic strength (0.15 M KCl) several aspects of the transcription process are affected by ρ factor when used at saturating amounts.

1. The number of RNA chains starting with ATP increases 2- to 3-fold and those starting with GTP 2-fold.

2. For both DNA strands the transcription of the "late" genes of φ80imm$^{\lambda}$ DNA is strongly reduced, as is the transcription of the "early" region on the *r*-strand, whereas transcription of the "early" region on the *l*-strand is almost unaffected.

Our results suggest that ρ factor is required for correct transcription at high ionic strength of φ80imm$^{\lambda}$ DNA by *E. coli* RNA polymerase.

Introduction

A protein factor, isolated and purified by Roberts [1], was found to cause specific termination during transcription of λ DNA in vitro. RNA chains made in the presence of ρ factor are shorter than those made in its absence [1—6]. Furthermore, it was reported that ρ factor improves the accuracy of transcription of T3 and T7 DNA in vitro [5].

Conflicting conclusions have been drawn with regard to the effect of ρ factor on the number of RNA chains formed [1,6—8]. According to several investigators ρ factor does not affect the number of λ RNA chains initiated [1,7,8]. Other authors, however, describe that ρ factor does enhance the number of RNA chains during synthesis of φ80 and fd RNA [2] and of T7 RNA

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[6]. Most of these studies were carried out in buffers of relatively low ionic strength. It appeared advisable, however, to study RNA synthesis in vitro at higher ionic strength, since it has been shown that under these conditions the transcription in vitro of T7 and T3 DNA more closely resembles the process in vivo than at low ionic strength [5,9]. Moreover, we have shown recently [10] that ρ factor improves the specificity of synthesis of tryptophan (*trp*) mRNA on $\phi 80imm^{\lambda}$ *trp* DNA at high ionic strength (0.15 M) and not at 0.05 M KCl.

We, therefore, decided to investigate in detail the various effects of ρ factor on RNA synthesis at high ionic strength, using DNA of phage $\phi 80imm^{\lambda}$. In particular we have studied the effect of ρ factor on the number of RNA chains formed and its influence on the asymmetry of transcription.

Materials and Methods

Preparation of RNA polymerase and ρ factor

E. coli RNA polymerase holo-enzyme was prepared as described before [11]. Termination factor ρ was isolated according to the procedure of Roberts [1] as modified by Darlix et al. [12]. The RNA polymerase and ρ factor preparations were shown to be free from contaminating nucleases, in particular RNAase III, as judged by the resistence of in vitro synthesized T7 RNA towards attack by these preparations [13].

In vitro synthesis of RNA

Reactions with RNA polymerase were carried out for 10 min at 37° C in a mixture containing: 25 mM Tris—HCl (pH 7.9), 8 mM $MgCl_2$, 0.15 M KCl, 0.1 mM dithiothreitol, 50 μg/ml $\phi 80imm^{\lambda}$ DNA, 13 μg/ml RNA polymerase holo-enzyme and 0.4 mM ATP, GTP, CTP and [^{3}H]UTP ($1.6 \cdot 10^5$—$9.5 \cdot 10^5$ cpm/nmole). In some experiments γ-^{32}P-labelled ATP and GTP were used at different concentrations as indicated in the next section. ρ factor was used at 1.5 μg/ml, a concentration which caused maximal depression of RNA synthesis.

Determination of the number of RNA chains synthesized

For the determination of the number of RNA chains synthesized, the polymerization was carried out in the presence of either 0.12 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP and the amount of incorporated radioactivity was measured. In order to reduce the background, the RNA synthesized was separated from the lower molecular weight material by chromatography on Sephadex G-50.

Strand separations and hybridization

Separation of complementary strands of $\phi 80imm^{\lambda}$, $\phi 80$ and λ DNA and DNA—RNA hybridization were carried out as described previously [10].

Results

The influence of ρ factor on the number of RNA chains formed

In the first experiment we have investigated the effect of ρ factor on the number of RNA chains initiated. It is known that with a large variety of DNA templates ATP and GTP are the usual starting nucleoside triphosphates for

TABLE I

EFFECT OF ρ FACTOR ON THE NUMBER OF RNA CHAINS, ON THE AVERAGE CHAIN LENGTH AND ON THE AMOUNT OF RNA SYNTHESIZED

ϕ80imm$^{\lambda}$ RNA was synthesized (in a volume of 0.6 ml) and partially purified by Sephadex G-50 gel filtration as described in Materials and Methods. The RNA was labelled with [^{3}H]UTP (spec. radioact. $1.6 \cdot 10^5$ cpm/nmole) and either [γ-^{32}P]ATP ($1.54 \cdot 10^3$ cpm/pmole) or [γ-^{32}P]GTP ($1.12 \cdot 10^3$ cpm/pmole). UMP"$_{ATP}$" represents UMP incorporation in the reaction in which 5′ termini were labelled with [γ-^{32}P]ATP and UMP"$_{GTP}$" the UMP incorporation in the reaction in which 5′ termini were labelled with [γ-^{32}P]GTP. A background of ^{32}P (165 cpm) was substracted from all data.

ρ factor	RNA chains starting with		Amount of RNA synthesized		Average chain length (nucleotides)	Inhibition of RNA synthesis (%)
	ATP (pmoles)	GTP (pmoles)	UMP"$_{ATP}$" (pmoles)	UMP"$_{GTP}$" (pmoles)		
–	2.2	1.1	1911	1902	2350	0
+	5.0	2.3	1090	1090	600	45

RNA synthesis [14]. Therefore, we used reaction mixtures containing either [γ-^{32}P]ATP or [γ-^{32}P]GTP and determined the amount of ^{32}P incorporated in the RNA, from which the number of chains starting with these nucleosides could be calculated. Our data (Table I) show that in an experiment where total RNA synthesis was depressed by 45% due to the presence of ρ factor, the number of 5′ termini starting with either ATP or GTP was approximately doubled. The average chain length was reduced from 2350 nucleotides to 600 nucleotides by the action of ρ factor. This result was confirmed by an analysis of the chain length distribution of in vitro made ϕ80imm$^{\lambda}$ RNA with the use of polyacrylamide gelelectrophoresis: the number of RNA chains of small length increased, whereas the number of long RNA chains decreased when ρ factor was present during RNA synthesis (Pannekoek, H. and Pouwels, P.H., unpublished).

The specificity of transcription

ρ factor has been shown to affect the specificity of transcription in buffers containing 0.05—0.10 M KCl of both phage DNA [1,3,5,6] and bacterial DNA [15—17]. The reports by Goff and Minkley [3] and by Darlix [6] indicate that the termination factor ρ improves the accuracy of transcription of T7 DNA in vitro. Essentially the same results were obtained with T3 DNA as a template [5]. In this section experiments are described which indicate that also at a higher ionic strength the specificity of transcription is improved by the presence of ρ factor.

In the starting experiment we determined the effect of ρ factor on the proportion of the RNA hybridizing with either one of the separated strands of ϕ80imm$^{\lambda}$ DNA. This was done for total RNA and for the RNA starting with either ATP or GTP. The results show that the ratio of RNA hybridizing with the *l*- and the *r*-strand, respectively, increased, both for RNA labelled with [^{3}H]UMP (see also last two lines of Table II) and for RNA labelled with [γ-^{32}P]ATP or [γ-^{32}P]GTP. Furthermore, it was observed that under these conditions of synthesis the formation of complementary RNA is reduced from

TABLE II

RESTRICTION OF TRANSCRIPTION OF THE "LATE" GENES OF $\phi 80imm^{\lambda}$ DNA BY ρ FACTOR

[^{3}H]RNA was synthesized on $\phi 80imm^{\lambda}$ DNA in the presence or absence of ρ factor and hybridized with the separated strands of $\phi 80$, λ and $\phi 80imm^{\lambda}$ DNA. Conditions of RNA synthesis (volume 0.1 ml without ρ factor; 0.2 ml with ρfactor) were as described in Materials and Methods, except for the incubation time which was 15 min. RNA was labelled with [^{3}H]UTP (spec. radioact. $9.5 \cdot 10^5$ cpm/nmole). DNA—RNA hybridizations were done with a 50 fold excess of *l*- or *r*-strand of $\phi 80$, λ or $\phi 80imm^{\lambda}$ DNA. Radioactive input in the hybridization mixture was $1.3 \cdot 10^4$ cpm of $\phi 80imm^{\lambda}$ [^{3}H]RNA made without ρ factor and 31% of this amount ($4.0 \cdot 10^3$ cpm) of RNA made with ρ RNA synthesis was reduced by ρ factor to 31% of the control). The hybridization efficiences of $\phi 80imm^{\lambda}$ RNA, made either without or with ρ factor, with the separated strands of $\phi 80imm^{\lambda}$ DNA were comparable (89 and 93.3%, respectively) to those found by adding the values for the separated strands of $\phi 80$ to those of λ DNA (without ρ factor 81.4%; with ρ factor 94.7%).

DNA strand	Hybridization of $\phi 80imm^2$ RNA (— ρ factor) (cpm)	Hybridization of $\phi 80imm^2$ RNA (+ ρ factor) (cpm)	Reduction of RNA synthesis (%)
l — $\phi 80$	1599	593	63
r — $\phi 80$	1180	473	60
l — λ	1446	1067	26
r — λ	6354	1686	74
l — $\phi 80imm^2$	3764	1604	57
r — $\phi 80imm^2$	7806	2156	72

12.7 to 3.6% (Pannekoek, H. and Pouwels, P.H., unpublished). These results suggest that ρ factor affects the asymmetry of transcription of $\phi 80imm^{\lambda}$ DNA.

In further experiments we have investigated whether the presence of ρ factor during RNA synthesis affects the proportion of RNA transcribed from the immunity region and neighbouring genes (i.e. the λ part) relative to that transcribed outside this region. For this purpose $\phi 80imm^{\lambda}$ RNA, synthesized either with or without ρ factor, was hybridized to the separated strands of $\phi 80$ to determine the fraction of RNA made outside the immunity region, and to the separated strands of λ DNA to determine the fraction of RNA complementary to this region. Our results (Table II; Column 4) show that ρ factor reduces RNA synthesis on the *l*- and *r*-strand of the $\phi 80$ genes by 63 and 60%, respectively. The presence of ρ factor also causes a considerable decrease of the transcription of the λ region on the *r*-strand (74%), but with the corresponding region of the *l*-strand the reduction only amounts to 26%. Consequently, our results indicate that at a high ionic strength factor ρ reduces transcription from "late" genes and from the immunity region on the *r*-strand, but has little effect on the amount of RNA made from the immunity region on the *l*-strand.

Discussion

The results presented in this paper show that at high ionic strength (0.15 M) several aspects of the transcription process are affected by factor ρ: (i) factor ρ causes a pronounced effect on the number of RNA chains formed. In the presence of ρ factor a considerable larger number of RNA chains starting with either ATP or GTP was synthesized than without ρ factor. Roberts [1] and Goldberg [7] have reported that the number of RNA chains formed in

0.10 M KCl with λ DNA as a template is not affected by ρ factor. The difference between their results and ours may be explained by assuming that the lower ionic strength interferes with the recycling of RNA polymerase, irrespective whether ρ factor is present or absent. Results of experiments presented elsewhere [18] show that at high ionic strength (0.15 M KCl), both in the presence and absence of ρ factor, RNA polymerase is released from the DNA template and reinitiation of RNA synthesis takes place. Moreover, we found that at low ionic strength (0.05 M KCl) reinitiation of RNA synthesis does not occur, irrespective of the presence of ρ factor. (ii) The results of hybridization experiments (Table II) with $\phi 80imm^{\lambda}$ RNA and the separated strands of $\phi 80imm^{\lambda}$, φ80 and λ DNA, carried out in order to differentiate between RNA homologous to the "late" and the "early" genes, respectively, indicate that ρ factor strongly reduces RNA synthesis outside the immunity region and neighbouring genes on both strands and on the *r*-strand also within this region, while it affects the amount of RNA transcribed from the immunity region of the *l*-strand to a much smaller extent. In vivo "early" transcription of λ DNA or $\phi 80imm^{\lambda}$ DNA is limited to the region corresponding to the λ portion of $\phi 80imm^{\lambda}$ and takes place predominantly from the *l*-strand [19]. In vitro also the major species of the RNA transcribed from λ DNA are initiated within this region [1,20], but a considerable amount of aspecific transcription takes place outside this region [1,21]. We have shown that ρ factor preferentially reduces RNA synthesis on the "late" genes of $\phi 80imm^{\lambda}$ and as a consequence the transcription pattern in the presence of ρ factor is more reminiscent of that in vivo than the pattern found in the absence of ρ factor.

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Publicatie III

The Specificity of Transcription *in vitro* of the Tryptophan Operon of *Escherichia coli*

II. The Effect of Rho Factor*

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Summary. We have studied the effect of purified Rho factor on the synthesis *in vitro* of tryptophan (*trp*) mRNA. The system used for transcription consists of purified RNA polymerase from *Escherichia coli*, DNA isolated from a *trp* transducing strain of ϕ80 and termination factor Rho. A general characterization of this system showed that in buffers of high ionic strength (0.15 M KCl) Rho did not interfere with the dissociation of the transcription complex after a transcription cycle. This indicates that reinitiation of *trp* mRNA synthesis occurs, a conclusion which is supported by the kinetics of the synthesis of this RNA.

Results of DNA-RNA hybridization experiments with the *in vitro* synthesized *trp* mRNA and λ *trp* DNA containing different sections of the *trp* operon showed that at low concentrations of Rho (below 0.6 μg/ml) the promotor-proximal and the promotor-distal *trp* genes are transcribed with nearly equal efficiency. At high concentrations of Rho (6 μg/ml), however, more than 90% of the *trp* mRNA originates from the promotor-proximal genes. Analysis by electrophoresis on polyacrylamide gels revealed that *trp* mRNA synthesized in the presence of high concentrations of Rho was homogeneous in size and that the length of the molecules was less than that of a complete polycistronic transcript. RNA synthesized in the presence of low concentrations of Rho was significantly longer and reached the size of a full-length *trp* mRNA. When RNA was synthesized without Rho most of the *trp* mRNA was much longer than the length of the *trp* operon.

From these results we conclude that under our conditions of synthesis Rho recognizes a termination signal at the end of the *trp* operon. At high concentrations Rho also causes termination of *trp* mRNA synthesis at a specific site within the operon.

Introduction

We have recently begun a study on the transcription *in vitro* of the tryptophan (*trp*) operon of *Escherichia coli* (Pannekoek and Pouwels, 1973). Our RNA synthesizing system consists of purified RNA polymerase from *E.coli* and DNA from *trp* transducing ϕ80 phages which carry all the structural genes *trp E*, *D*, *C*, *B* and *A* including the *trp* promotor and operator. We showed that with this system *trp* mRNA can be synthesized *in vitro*. Transcription of the *trp* genes proceeds only from the correct strand of ϕ80 *trp* DNA, provided that RNA polymerase is saturated with σ-factor. The synthesis of *trp* mRNA does not require accessory factors, such as CAP (catabolite activating protein) which are indispensable for the transcription *in vitro* of some bio-degradative operons (de Crombrugghe *et al.*, 1971). The addition of the transcription termination factor Rho to the RNA synthesizing mixture led to an increase of the amount of *trp* mRNA made on ϕ80 *trp*

* The first paper in this series is: Pannekoek, H., Pouwels, P. H. Molec. gen. Genet. 123, 159–172 (1973).

DNA. This result might be explained by assuming that Rho somehow stimulates the release of RNA polymerase from the DNA template, allowing reinitiation of RNA synthesis to occur.

Roberts (1969), Goldberg and Hurwitz (1972) and Schäfer and Zillig (1973), who studied the effect of Rho on transcription of phage DNA, have reported that in the presence of Rho factor only one cycle of transcription takes place. An important difference, however, between the experimental conditions of those investigators and ours is that they have used buffers of an ionic strength of 0.05–0.10 M, while we used a higher ionic strength (0.15 M). Therefore, we have investigated whether under our conditions of RNA synthesis Rho factor affects the release of RNA polymerase from DNA and the reinitiation of RNA synthesis.

A major part of this paper is devoted to experiments in which it was investigated to what extent the *trp* mRNA made *in vitro* is homogeneous in size and corresponds to the complete *trp* operon. Furthermore the effect of Rho on the specificity of the termination during the transcription of this operon *in vitro* was studied. De Crombrugghe *et al.* (1973) have found recently that Rho factor affects the transcription of two bacterial operons for fermentation of galactose (*gal*) and lactose (*lac*). When they used DNA with an insertion mutation in the first gene of the *gal* operon, which causes a strong polar effect on the expression of promotor-distal *gal* genes *in vivo*, it was observed that in the presence of low concentrations of Rho RNA synthesis *in vitro* was terminated at a site within the insertion. They found that in the presence of high concentrations of Rho also the transcription of wild-type *gal* operon was terminated at a site within the operon. These observations led them to conclude that Rho might be involved in the phenomenon of polarity *in vivo*.

In our experiments we also observe an effect of Rho on the efficiency of the transcription of the promotor-distal *trp* genes, but in our opinion this is not necessarily related to polarity.

Materials and Methods

Bacteriophages

Bacteriophage φ80ptEA190 (Deeb *et al.*, 1967) and φ80imm$^{\lambda}$ptEAd62 (Gratia, 1971) are *trp* transducing strains of φ80 containing the entire *trp* operon, including the *trp* promotor and operator (Pouwels and Stevens, 1973). φ80ptED (Matsushiro, 1963) and λptEDd3 (isolated by J. P. Gratia) contain the *trp* promotor and operator and the promotor-proximal genes *trp E* and *trp D*. φ80imm$^{\lambda}$ptBA5–2 (isolated by N. Franklin) carries the promotor-distal genes *trp B* and *trp A*. The propagation and purification of bacteriophages and the isolation of DNA have been described previously (Pouwels and van Rotterdam, 1972).

List of Bacterial Strains

Name	Characteristics	Origin
C600	F$^-$ *thi, thr, leu, lac*Y_1, *tonA*, su_{II}, φ80^r	M. Hofnung
LG106	HfrH, *lys*(P2), *su*$^-$	G. Lindahl
$HP_2\phi$	HfrH, *lys*(P2), *su*$^-$, φ80^r	this work
CA275	F$^-$ trp_{am}, lac_{am}, su^+_{III}	M. Hofnung
YMC (λ$^+$)	YMC *lys*(λ)	P. Kourilsky
C600 (φ, λ)	C600 *lys*(φ80), *lys*(λ)	P. Kourilsky
C600 (φ)	C600 *lys*(φ80)	P. Kourilsky

Isolation of λptEA and λptBA Phages

Trp transducing strains of phage λ were obtained by crossing φ80imm$^{\lambda}$ptEAd62 or φ80imm$^{\lambda}$ptBA5–2 with an appropriate λ strain. The selection was based on the property of strains of *E. coli* which are lysogenic for phage P2, to allow the development of λ with an altered *redβγ* region while restricting wild-type λ. In our *trp* transducing φ80imm$^{\lambda}$ phages the *redβγ* region probably is partly or entirely deleted since these phages grow normally on strain LG106. Therefore, we have used this property to select for recombinants carrying the *trp* genes. To counterselect for mutants of λ with spi$^-$ phenotype, which arise spontaneously at a relatively high frequency, phage λcI857N7N53 was used as donor for the host range of λ. Counterselection of parental phages with φ80 host range was achieved by plating at a non-permissive temperature (42° C).

The crossing was performed on strain CA275 which permits growth of the two parental phage strains. Progeny phages, appropriately diluted, were spread on strain $HP_2\phi$ and incubated overnight at 42° C. Single plaques were picked and the phages were purified by restreaking on the same host bacteria. The resulting phages were tested for immunity by spotting on C600(φ), C600(φ, λ) and YMC(λ^+) while the presence of the *trp* genes on these phages was verified by transduction tests. All the candidates tested were *trp* transducing phages which had the λ immunity and the λ host range. The frequency at which the recombinants were formed was 1.3×10^{-7} for λptEA6 and 4×10^{-8} for λptBA24.

Enzymes and Factors

RNA polymerase was isolated from *E.coli MRE600* (*RNAaseI*) according to Burgess (1969), but after chromatography on DEAE-cellulose the enzyme was further purified by native DNA-cellulose chromatography according to Burgess *et al.* (1969). Rho factor was purified according to Roberts (1969) as modified by Darlix *et al.* (1971). RNA polymerase and Rho preparations were more than 95% pure as judged by the patterns obtained after electrophoresis in polyacrylamide gels in the presence of SDS[1]. The preparations were free from DNAase and RNAase activity since they did not degrade [^{32}P]T7 DNA (measured by sedimentation analysis) nor [^{3}H]T7 RNA (measured by the release of acid-soluble material). Other enzymes were from Worthington (Freehold, U.S.A.).

In vitro Synthesis of RNA

Incubations were carried out for 30 min at 37° C unless otherwise specified. The reaction mixture contained: 25 mM Tris-HCl (pH 7.9), 8 mM $MgCl_2$, 0.15 M KCl, 0.1 mM dithiothreitol, 45 μg/ml DNA, 0.2 mM of each of the four ribonucleoside triphosphates, RNA polymerase and Rho as indicated in the Legends to the Figures and Tables. The specific radioactivity of [^{3}H]UTP or that of [α-^{32}P]ATP is given in the Legends. After incubation the reaction was stopped by addition of cold TCA[1] (5% final concentration) and the incorporation of radioactive UMP or AMP was measured. In an alternative procedure, when the RNA had to be used for hybridization experiments, the reaction was stopped by shaking with phenol (saturated with 2 × SSC[1], 10 mM Tris-HCl (pH 7.5), 1 mM EDTA).

Separation of the Complementary Strands of Phage DNA

Separation of the complementary strands of λptEA6, λptEDd3, λptBA24 and λ DNA was performed by centrifugation in a CsCl density gradient containing poly (U,G)[1] according to Hradecna and Szybalski (1967). After centrifugation, hydrolysis of poly (U,G), removal of the degradation products and reannealing of any complementary strands contaminating the separated strands, were performed as described previously (Pannekoek and Pouwels, 1973).

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1 *Abbreviations.* SDS = sodium dodecylsulphate; TCA = trichloroacetic acid; SSC = 0.15 M NaCl, 0.015 M tri-sodium citrate; n × SSC = n fold concentrated SSC buffer; poly (U, G) = copolymer of uridylic- and guanylic acid; poly d(A-T) = copolymer of deoxyadenylic- and deoxythymidylic acid.

DNA-RNA Hybridization

DNA-RNA hybridizations were performed according to Goff and Minkley (1970). ^{3}H-labelled RNA (2–3 ng) was mixed with excess (100 ng) *l*-strand λptEA6, λptEDd3, λptBA24 or λ DNA in 0.1–0.2 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (20% saturated with distilled phenol) and incubated for 16 h at 67° C. Under these conditions maximal hybridization was reached after 12 h. In competition hybridization experiments 6.6 ng [^{3}H]RNA, synthesized *in vitro* on ϕ80ptEA190 DNA, was hybridized in 0.2 ml hybridization buffer with 0.12 μg *l*-λptEA6 or *l*-λptEDd3 DNA in the presence of increasing amounts of non-radioactive RNA isolated from an *E. coli* wild-type strain or an *E. coli trpR* strain. All hybridizations were done in duplicate with results which agreed within 10%.

Polyacrylamide Gelelectrophoresis

Polyacrylamide gelelectrophoresis was carried out as described elswhere (Pannekoek and Pouwels, Biochim. biophys. Acta in the press). Elution of the RNA from gel slices was performed according to the procedure described by Hyman and Summers (1972), which was slightly modified. The slices were crushed in 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA by forcing through a hypodermic needle (19-gauge) and the resulting fragments were left at roomtemperature for several days and washed several times with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The supernatant solutions were pooled and filtered through Whatman n° 1 paper. Each solution was then diluted to give a final concentration of 0.2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and applied to DEAE-cellulose columns (Whatman DE-52: 2 × 0.5 cm). The DEAE-cellulose was washed with 20 ml of the same buffer and the RNA was eluted with 20 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

To the eluates were added, successively, EDTA (final concentration 8 mM, pH 6.0), carrier RNA isolated from *E.coli* wild-type (final concentration 20 μg/ml) and Cetyl-trimethyl ammoniumbromide (final concentration 0.2%) according to the procedure of Sibatani (1970). The resulting solutions were frozen and thawed and the precipitates were collected by centrifugation, washed with 80% ethanol, 0.3 M sodium-acetate (pH 5.2) and left overnight at −20° C. Precipitates were spun down and lyophilized. After solubilization in distilled water the RNA was used for hybridization studies. The efficiency of hybridization of RNA after electrophoresis on polyacrylamide gels was rather low (about 10%) due to some contaminant extracted from the gel slices (Hyman and Summers, 1972).

Chemicals

Ribonucleoside triphosphates were obtained from Sigma (St. Louis, U.S.A.). [^{3}H]UTP (50 Ci/mmole) and [α-^{32}P]ATP (2 Ci/mmole) were products of the Radiochemical Centre (Amersham, England). Cetyl-trimethyl ammoniumbromide was purchased from the British Drug Houses (Poole, England).

Poly (U,G) (2:1) was a product of General Biochemicals (Chagrin Falls, U.S.A). Poly d(A-T)[1] was obtained from Miles Laboratories Inc. (Kanakee, U.S.A.).

Results

Determination of trp mRNA Synthesized in vitro

In order to measure quantitatively the synthesis of *trp* mRNA *in vitro* we have used a one-step DNA-RNA hybridization test. This test takes advantage of the low cross-hybridization between ϕ80 RNA synthesized *in vitro* and λ DNA (Eron *et al.*, 1971). So, DNA of *trp* transducing λ phages was used for the hybridization with RNA synthesized on ϕ80 *trp* DNA.

It was shown (Pouwels and Stevens, 1973) that the strand on which *trp* mRNA is synthesized in *E.coli trpR* corresponds with the *l*-strand of all *trp*

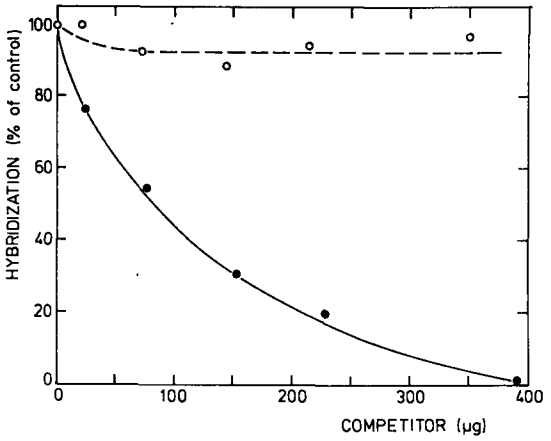

Fig. 1. Competition hybridization between [^{3}H]mRNA synthesized *in vitro* and *trp* mRNA made *in vivo*. RNA was synthesized *in vitro* on ϕ80ptEA190 DNA (45 µg/ml) in a volume of 1.0 ml with 13 µg/ml RNA polymerase (holo-enzyme) and 1.1 µg/ml Rho as described in Materials and Methods. The specific radioactivity of [^{3}H]UTP was 7.0×10^2 cpm/pmole. 6.6 ng of the synthesized RNA was hybridized with 120 ng *l*-λptEA6 DNA in the presence of increasing amounts of RNA isolated either from a strain of *E.coli* which is genetically derepressed for the *trp* operon (*E.coli trpR*) (•—•), or from a wild-type strain of *E.coli* which was grown under conditions of repression of the *trp* operon (o—o). The amount of [^{3}H]RNA which hybridizes with *l*-λptEA6 DNA in the absence of competitor RNA represents the 100% value. This 100% value corresponds with an efficiency of hybridization which is approximately 50% of that obtained with large excess of *l*-λptEA6 DNA

transducing ϕ80, ϕ80imm$^\lambda$ and λ phages tested. Therefore, the *l*-strands were used for the hybridization experiments.

A significant proportion (9–10%) of the RNA synthesized *in vitro* on ϕ80pt-EA190 DNA hybridized with *l*-λptEA6 DNA while the RNA did not hybridize with *l*-λ DNA. In order to determine whether the RNA which hybridized with *l*-λptEA6 DNA represents *trp* mRNA only, or also comprises RNA transcribed from DNA segments immediately adjacent to the *trp* genes and common to the ϕ80ptEA190 and λ ptEA6 DNA's, we have carried out a competition hybridization experiment. ^{3}H-labelled RNA synthesized *in vitro* on ϕ80ptEA190 DNA was hybridized with *l*-λptEA6 DNA together with increasing amounts of RNA isolated from strains of *E.coli* which contain high levels of *trp* mRNA (*E.coli trpR*) or very low levels of *trp* mRNA (wild-type bacteria grown under conditions of repression of the *trp* operon). The results, which are given in Fig. 1, show that the competition reaches nearly 100% when RNA is taken from a strain which constitutively synthesizes *trp* mRNA while no competition was observed with RNA taken from the repressed strain. This result shows that virtually all RNA which hybridized with *l*-λptEA6 DNA represents *trp* mRNA.

Reinitiation of trp m RNA Synthesis

When the progress with time of RNA synthesis on ϕ80ptEA190 DNA in the presence of Rho was studied, the results shown in Fig. 2 were obtained: the syn-

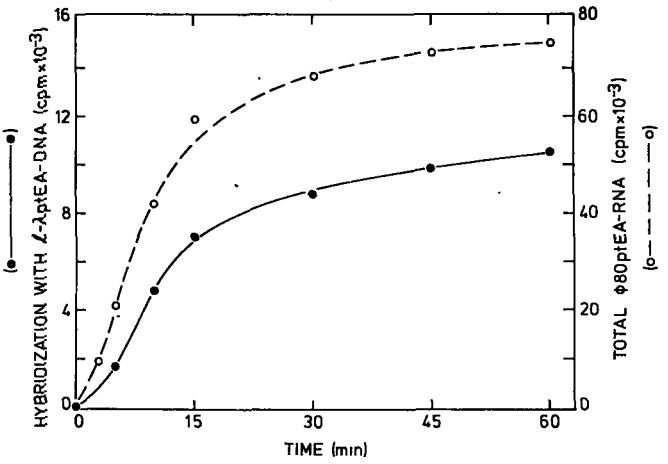

Fig. 2. Kinetics of *trp* mRNA synthesis. RNA was synthesized *in vitro* as described in the Legend to Fig. 1. The volume of the reaction mixture was 0.6 ml. All components were mixed at 0° C and the reaction was started by raising the temperature to 37° C. The specific radioactivity of [^{3}H]UTP was 1.2×10^3 cpm/pmole. At the times indicated aliquots (80 µl) were taken to determine both total RNA synthesis (measuring the radioactivity which was precipitable with 5% TCA) and *trp* mRNA synthesis (measuring hybridization with *l*-λptEA6 DNA). The values have been corrected for background (hybridization with *l*-λ DNA; less than 0.5%)

thesis proceeded at an approximately constant rate for 10–15 min and reached a plateau after 40–60 min. The time-course of synthesis of *trp* mRNA showed a similar profile. The proportion of total RNA which hybridized with *l*-λptEA6 DNA remained constant (approximately 10%) during the entire period of synthesis.

Under the conditions used the amount of RNA polymerase must be considered limiting[2]. Taking 20–30 nucleotides per sec as the rate of φ80ptEA190 RNA synthesis *in vitro* (Pannekoek and Pouwels, unpublished results) and the length of the *trp* operon as 6700 nucleotides (Imamoto and Yanofsky, 1967) the time required to synthesize a full-length *trp* mRNA would be 4–5 min. Consequently, our results indicate that several rounds of *trp* mRNA synthesis have taken place during the 60 min period of synthesis. Since the amount of RNA polymerase is limiting, reinitiation of RNA synthesis must have occurred and probably both RNA and the enzyme must have dissociated from the template.

We verified this hypothesis by studying whether release from the template occurred under our conditions. The release of RNA was measured by determining the proportion of radioactive RNA which passed through a nitrocellulose filter.

2 The molar ratio of RNA polymerase to DNA in the experiments on reinitiation of transcription was 15, assuming a 100% active enzyme preparation. Taking into account that 4–5 enzyme molecules are bound to the cohesive ends of lambdoid DNA's without participating in the synthesis of RNA (Pannekoek and Pouwels, 1973) approximately 10 molecules of RNA polymerase are available for RNA synthesis. Our investigations with φ80imm$^\lambda$DNA and those of others on λ DNA (Blattner *et al.*, 1972) indicate that φ80ptEA190 DNA contains 5–8 promotors. Consequently, our experiments were carried out with 1 or 2 RNA polymerase molecules per promotor, a ratio which probably corresponds to limiting amounts of RNA polymerase.

Table 1. Release of RNA from ϕ80ptEA190 DNA, as measured by filtration through nitrocellulose filters

Reaction stopped with	Radioactivity (cpm × 10^{-3})			
	on filter		in filtrate	
	^{32}P (=DNA)	^{3}H (=RNA)	^{32}P (=DNA)	^{3}H (=RNA)
EDTA	7.5 (12.5%)	9.4 (34%)	53 (87.5%)	17.7 (66%)
SDS	1.3 (2.1%)	1.0 (3.8%)	59 (98%)	24.0 (96%)

RNA was synthesized on [^{32}P] ϕ80ptEA190 DNA (45 μg/ml; specific radioactivity 1.3×10^3 cpm/μg) with 13 μg/ml RNA polymerase (holo-enzyme) and 1.1 μg/ml Rho. The specific radioactivity of [^{3}H] UTP was 5×10^2 cpm/pmole. The reaction volumes were 0.1 ml. Other details of the synthesis of RNA are given in Materials and Methods. The reaction was stopped either by the addition of EDTA (final concentration 80 mM) or by the addition of SDS (final concentration 1%). The reaction mixture was diluted 20 fold in ice-cold 25 mM Tris-HCl (pH 7.9), 0.15 M KCl and filtered through a nitrocellulose filter (Schleicher and Schüll, BA85 0.45 μ) and the radioactivity on the filter and in the filtrate was determined.

Free DNA or RNA will pass through a nitrocellulose filter, but ternary complexes of DNA, RNA and RNA polymerase are retained by the filter (Jones and Berg, 1966). When RNA (labelled with ^{3}H) was synthesized on ϕ80ptEA190 DNA (labelled with ^{32}P) in the presence of Rho and the reaction was terminated by the addition of EDTA, which does not dissociate ternary complexes of DNA, RNA and RNA polymerase, and the mixture was filtered through a nitrocellulose filter, the major part of the RNA and of the DNA was found in the filtrate (Table 1). The remaining part of the RNA which was trapped on the filter probably was present as a ternary complex since virtually all RNA could be released from the template by termination of the reaction with SDS. From these results we conclude that under our conditions release of RNA from the template takes place.

The release of RNA polymerase from the DNA template was studied by determining the effect of poly d(A-T) on RNA synthesis on ϕ80ptEA190 DNA. RNA polymerase efficiently binds and transcribes poly d(A-T) (Stevens, 1961) and thus is expected to limit RNA synthesis on phage DNA by competing with it for free RNA polymerase. We have carried out RNA synthesis in 0.15 M KCl on ϕ80ptEA190 DNA in the presence or absence of Rho with a limiting quantity of RNA polymerase and we have followed the synthesis of RNA by measuring the incorporation of [^{3}H]CMP and [^{3}H]UMP, respectively. Since [^{3}H]UMP is polymerized on poly d(A-T), while [^{3}H]CMP is not, we only expected to observe a reduction of the incorporation of [^{3}H]CMP upon addition of poly d(A-T). When a four-fold excess of poly d(A-T) (compared to ϕ80ptEA190 DNA) was added at the beginning of the reaction, the incorporation of [^{3}H]CMP was reduced three to four-fold, in the presence or absence of Rho, indicating that poly d(A-T) indeed efficiently competes with phage DNA (Table 2; lines 1 and 3 or lines 2 and 4). That the binding of RNA polymerase to poly d(A-T) is responsible for this reduction of incorporation of [^{3}H]CMP is concluded from the fact that the incorporation of [^{3}H]UMP is stimulated three- to four-fold by addition of poly d(A-T) (lines 1 and 3). When poly d(A-T) was added after

Table 2. Release of RNA polymerase from ϕ80ptEA190 DNA during RNA synthesis. RNA synthesis was carried out as described in Materials and Methods, except for the concentration of ϕ80ptEA190 DNA which was 15 μg/ml. The concentration of RNA polymerase (holo-enzyme) was 2.5 μg/ml and that of Rho factor, if present, was 0.5 μg/ml. The specific radioactivity of [^{3}H] CTP and of [^{3}H] UTP was 2×10^3 cpm/pmole. Poly d(A-T) was added either at $t = 0$ min or at $t = 2$ min at a final concentration of 60 μg/ml. n.d. = not determined

Starting system	Time of addition of poly d(A-T)	Incorporation (pmoles/30 min) of	
		[^{3}H] CMP	[^{3}H] UMP
1. ϕ80ptEA190 DNA	not added	24.5	34
2. ϕ80ptEA190 DNA + Rho	not added	14	n.d.
3. ϕ80ptEA190 DNA	0 min	6	140
4. ϕ80ptEA190 DNA + Rho	0 min	5.5	n.d.
5. ϕ80ptEA190 DNA	2 min	10.5	74
6. ϕ80ptEA190 DNA + Rho	2 min	7	n.d.

the start of the reaction (at $t = 2$min) and the incorporation of [^{3}H]CMP was measured after 30 min of synthesis, we also observed a strong reduction of RNA synthesis on ϕ80ptEA190 DNA (line 1 and 5). This result suggests that RNA polymerase indeed is released from the template during the transcription process. In the parallel experiment where Rho was present in the incubation mixture, we also observed a strong reduction of the [^{3}H]CMP incorporation upon addition of poly d(A-T) (lines 2 and 6). This result indicates that Rho factor does not interfere with the release of RNA polymerase from a ϕ80 template. Such an interference was suggested by results of experiments with T4 DNA (Goldberg and Hurwitz, 1972) and with T7 DNA (Schäfer and Zillig, 1973).

In similar experiments, where poly d(A-T) was added 8 min after the start of the reaction, we also observed a reduction of [^{3}H]CMP incorporation when RNA synthesis was carried out in 0.15 M KCl, although less pronounced than in the experiments described above. A reduction was not observed when the reaction was carried out in 0.05 M KCl (results not shown). From these experiments it is clear that in buffers of high ionic strength in the absence, but definitely also in the presence of Rho factor, RNA polymerase and RNA are released from ϕ80ptEA190 DNA allowing reinitiation of RNA synthesis to occur.

The Length of trp mRNA Synthesized in vitro

In a previous paper (Pannekoek and Pouwels, 1973) it was shown by means of competition hybridization that the *trp* mRNA synthesized *in vitro* contained all the regions which are present in *trp* mRNA made *in vivo*. From these results it could not be decided, however, whether the *trp* mRNA made *in vitro* consisted of a homogeneous population of full-length transcripts, specifically initiated and terminated, or was composed of a mixture of partial transcripts. This was determined by measuring the length of the RNA by electrophoresis on polyacrylamide gels.

For these experiments ϕ80ptEA190 RNA was synthesized under the same conditions as used previously (Pannekoek and Pouwels, 1973), i.e. in the presence

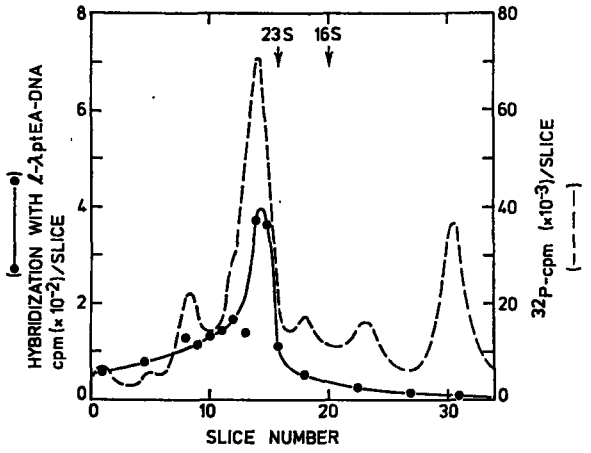

Fig. 3. Polyacrylamide gelelectrophoresis of [^{3}H, ^{32}P]ϕ80ptEA190 RNA. RNA was synthesized on ϕ80ptEA190 DNA (45 μg/ml) with 22 μg/ml RNA polymerase (holo-enzyme) in the presence of 6 μg/ml Rho. RNA was labelled with [^{3}H]UTP (specific radioactivity 1.0×10^4 cpm/pmole) and [α-^{32}P]ATP (specific radioactivity 1.4×10^3 cpm/pmole). The concentration of ATP, GTP and CTP was 0.2 mM and that of [^{3}H]UTP 0.1 mM. The KCl in the reaction mixture was replaced by NaCl in order to prevent precipitation of SDS during processing of the RNA. The reaction volume was 0.15 ml. RNA synthesis was stopped by addition of 15 μl 4% SDS, 0.125 M EDTA, 50% glycerol (v/v). The mixture was applied to polyacrylamide gels (1×12.5 cm; 30 μl/gel) and subjected to electrophoresis. Subsequently the gels were frozen and sliced and the radioactivity in each slice was measured by Cerenkov counting. For the determination of the position of *trp* mRNA, RNA was eluted from the slices following the procedure described in Materials and Methods and the amount of [^{3}H]RNA hybridizing with excess *l*-λptEA6 DNA was measured (*trp* mRNA: •—•). Total RNA (----). The arrows indicate the positions of [^{3}H] *E.coli B* ribosomal RNA which served as markers, run in separate gels

of high concentration of Rho (6 μg/ml). The RNA was radioactively labelled with [^{3}H]UTP and [α-^{32}P]ATP. The ^{32}P-label was used to determine the distribution of total RNA in the gels by Cerenkov-counting of slices, while the ^{3}H-label was employed to assess the distribution of *trp* mRNA. For the latter purpose the RNA was eluted from the gel slices and hybridized to *l*-λptEA6 DNA. The length of the ϕ80ptEA190 RNA species was estimated from the relative position of 23 S and 16 S ribosomal RNA, present in [^{3}H]RNA isolated from *E.coli* which was run in separate gels under identical conditions (Peacock and Dingman, 1968). The results are shown in Fig. 3.

While total RNA is distributed over a number of peaks with a great variation in size, the greater part of the RNA which hybridized with *l*-λptEA6 DNA migrated as a single, narrow peak, next to some more slowly migrating, larger material. This result indicates that the major fraction of *trp* mRNA is homogeneous in size and, consequently, that in general the initiation and termination of the transcription of this RNA take place at specific sites on the genome. From the position of the peak we have calculated that the size of this *trp* mRNA made on ϕ80ptEA190 DNA is between 4000 and 5000 nucleotides which is significantly shorter than the length of the *trp* operon, being 6700 nucleotides (Imamoto and

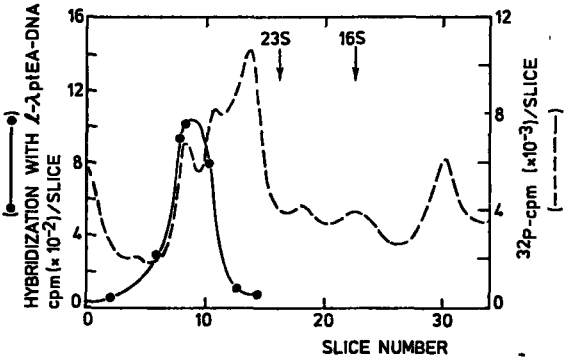

Fig. 4. Polyacrylamide gelelectrophoresis of [^{3}H, ^{32}P] ϕ80ptED RNA. The conditions of RNA synthesis on ϕ80ptED DNA were as outlined in the Legend to Fig. 3. Electrophoresis and processing of the RNA were as described in Materials and Methods and as described in the Legend to Fig. 3 (trp mRNA: •—•, total RNA: ----)

Yanofski, 1967). This striking result suggests that the transcription does not proceed till the end of the *trp* operon but is terminated before this site is reached, unless it is assumed that the RNA synthesis is initiated at a considerable distance from the *trp* promotor.

When the experiment was repeated with DNA of phage ϕ80ptED as a template which contains the *trp* promotor/operator and the adjacent genes *trp E* and *D* only, an electrohoresis pattern as shown in Fig. 4 was obtained. The profile for total ϕ80ptED RNA is very similar to that of ϕ80ptEA190 RNA, indicating that the insertion of *trp EA* DNA or *trp ED* DNA in the ϕ80 genome does not significantly change the transcription *in vitro* of ϕ80 genes. The *trp* mRNA (the material specifically hybridizing to *l*-λptEA6 DNA) again ran as a single peak, the position of which corresponds to a length of 7000–8000 nucleotides. The length of the part of the *trp* operon present on ϕ80ptED DNA amounts to approximately 3300 nucleotides; consequently, this result indicates that ϕ80 genes adjacent to the *trp* genes have been transcribed together with these genes to form a greatly extended *trp ED* mRNA molecule. Since the only difference between ϕ80ptEA190 DNA and ϕ80ptED DNA is the absence of the promotor-distal genes *trp C*, *B* and *A* in the latter DNA, it appears likely that transcription on ϕ80ptED DNA continues beyond the end of the *trp D* gene and is arrested in the ϕ80 part of the genome, while on ϕ80ptEA190 DNA termination occurs in general within the *trp* operon at a site which is deleted in ϕ80ptED DNA.

Disproportionality of trp mRNA Synthesis

If indeed RNA synthesis on *trp EA* DNA starts near the *trp* promotor and is mainly terminated before the end of the operon is reached, one would expect that relatively more RNA is transcribed from the promotor-proximal genes than from the promotor-distal ones. To test whether a disproportional *trp* mRNA synthesis takes place *in vitro* we hybridized [^{3}H]ϕ80ptEA190 RNA, made in the presence of Rho (6 μg/ml), to the *l*-strands of λptEDd3, λptBA24 and λptEA6

Table 3. Hybridization of [^{3}H]ϕ80ptEA190 RNA with *l*-strands of λ DNA containing different sections of the *trp* operon. RNA was synthesized on ϕ80ptEA190 DNA (45 μg/ml) with 22 μg/ml RNA polymerase (holo-enzyme) and 6 μg/ml Rho as described in Materials and Methods. The reaction volume was 0.3 ml. The specific radioactivity of [^{3}H] UTP was 2.0×10^3 cpm/pmole. Radioactive input in the hybridization mixtures was 1.3×10^5 cpm of [^{3}H] ϕ80ptEA190 RNA. The values have been corrected for background (hybridization with *l*-λ DNA; less than 0.5%)

DNA	Hybridizing RNA (% of total RNA)	Ratio[a] (found)	Ratio[b] (expected)
l-λptEDd3	5.4	1.0	1.0
l-λptBA24	0.8	0.15	0.62
l-λptEA6	9.2	1.7	2.0

[a] % of RNA hybridizing with *l*-λptEDd3 DNA taken as unity.
[b] Ratio of the length of the segment of the *trp* operon present in the DNA used for hybridization in relation to the segment present in λptEDd3 DNA.

DNA (Table 3). The λptEDd3 DNA and λptBA24 DNA contain 50% and 31%, respectively, of the complete *trp* operon present on λptEA6 DNA (Rose and Yanofsky, 1971). Therefore, if *trp* mRNA synthesis were to yield equimolar amounts of the promotor-proximal and promotor-distal transcripts one would expect hybridization values with the different *l*-λ*trp* DNA's which are proportional to the size of the *trp* segment present on the DNA. These theoretical hybridization ratios are given in column 4 of Table 3. When we compare the transcription on ϕ80ptEA190 DNA of the promotor-proximal genes *trp E* and *D* (i.e. hybridization with *l*-λptEDd3 DNA) with that of the promotor-distal genes *trp B* and *A* (i.e. hybridization with *l*-λptBA24 DNA) it is obvious that ϕ80ptEA190 RNA does not contain equimolar amounts of *trp ED* mRNA and *trp BA* mRNA. The number of *trp BA* copies is much lower than could be accounted for when the *trp* operon was transcribed as a polycistronic messenger, as has been observed in *in vivo* experiments (Imamoto and Yanofsky, 1967). Our results indicate that for the greater part transcription is terminated before the *trp B* and *A* genes. This conclusion is further supported by comparing the data on the hybridization of ϕ80ptEA190 RNA with *l*-λptEA6 DNA and with *l*-λptEDd3 DNA, respectively, given in Table 3. While the size of the *trp* DNA segments on these λ *trp* DNA's differs by a factor of 2, the hybridization values differ significantly less, supporting the notion that transcription of the promotor-proximal and the promotor-distal *trp* genes on ϕ80ptEA190 DNA proceeds in a disproportional manner, at least under the *in vitro* conditions used.

Effect of Rho on the Size of trp mRNA

The most plausible component in our reaction mixture which might cause this disproportional transcription of the *trp* operon is the termination factor Rho. To verify this assumption we synthesized [^{3}H,^{32}P]ϕ80ptEA190 RNA at various concentrations of Rho and subjected the RNA preparations obtained to gelelectrophoresis on polyacrylamide in order to determine the size-distribution of the *trp* mRNA.

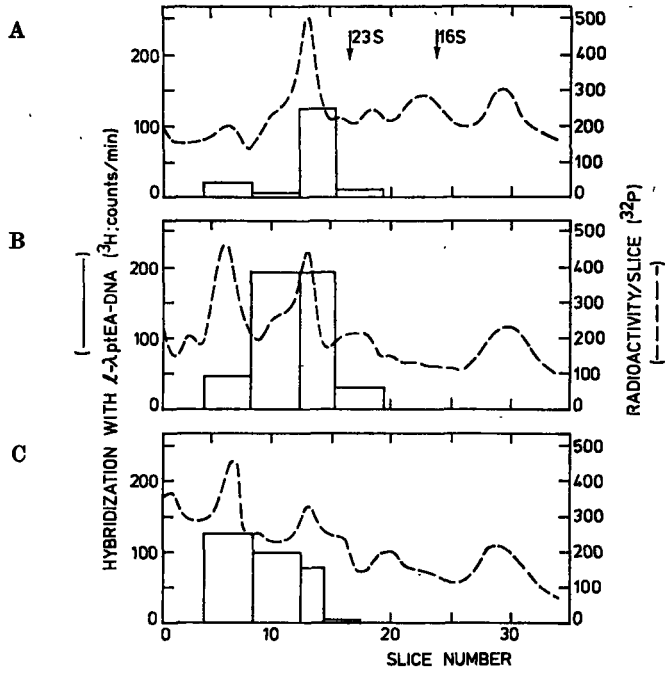

Fig. 5A–C. Polyacrylamide gelelectrophoresis of [³H, ³²P] ϕ80ptEA190 RNA synthesized in the presence of various concentrations of Rho and in the absence of Rho. RNA was synthesized on ϕ80ptEA190 DNA as described in the Legend to Fig. 3 with the following modifications. The synthesis of RNA was carried out either in the presence of 4.5 μg/ml Rho (A), in the presence of 0.6 μg/ml Rho (B) or without Rho (C). Other details concerning the conditions of electrophoresis, processing of the RNA and the determination of the position of *trp* mRNA in the gels were as described in Materials and Methods and in the Legend to Fig. 3. (*trp* mRNA: —, total RNA: ----)

The synthesis of RNA was carried out with a high and a low concentration of Rho (4.5 and 0.6 μg/ml, respectively) and without Rho; the first concentration of Rho caused maximal inhibition of RNA synthesis (50%), the lower reduced synthesis by 10%. The results of this experiment (Fig. 5) show that there is an obvious influence of the concentration of Rho in the reaction mixture on the size of the RNA synthesized. In general, the presence of Rho results in the synthesis of less very long molecules and more medium sized RNA chains. The effect on specific *trp* mRNA follows this general pattern: *trp* mRNA synthesized in the presence of a low concentration of Rho (Fig. 5 B) migrates more slowly than *trp* mRNA made in the presence of saturating amounts of Rho (Fig. 5 A).

From the position of the peak fractions relative to that of marker RNA, we estimate that the length of *trp* mRNA, made in the presence of a low concentration of Rho, is 5000–7000 nucleotides as compared to 4000–5000 found for *trp* mRNA obtained with 6 μg Rho per ml.

An analysis of *trp* mRNA synthesized in the absence of Rho (Fig. 5 C) revealed the presence of *trp* mRNA molecules ranging from 5000 to 12500 nucleotides and very little RNA with a length of 4000 to 5000 nucleotides.

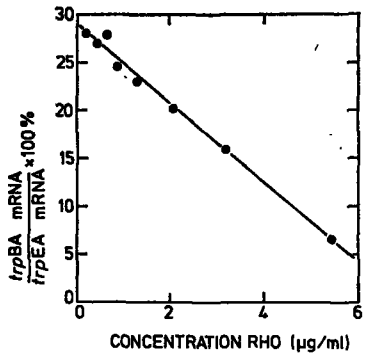

Fig. 6. The effect of Rho on the synthesis of promotor-distal *trp* mRNA. RNA was synthesized on ϕ80ptEA190 DNA (45 µg/ml) with 16 µg/ml RNA polymerase (holo-enzyme). The concentration of Rho was varied between 0.2–6.4 µg/ml. Reaction volumes were 0.08 ml. Other conditions of RNA synthesis are given in Materials and Methods. The formation of *trp BA* mRNA was determined by measuring the hybridization of [^{3}H]ϕ80ptEA190 RNA with excess of *l*-λptBA24 DNA while the formation of total *trp* mRNA was determined by measuring the hybridization of [^{3}H]ϕ80ptEA190 RNA with excess of *l*-λptEA6 DNA

Effect of Rho on the Synthesis of Promotor-distal trp mRNA

If, as is suggested by the experiments in the previous paragraph, Rho factor is responsible for intra-operon termination of *trp* mRNA synthesis, one would predict that the degree of disproportionality found in the synthesis of *trp* mRNA should be influenced by the concentration of Rho. In order to determine the effect of Rho on the synthesis of promotor-distal *trp* mRNA we have determined the ratio of promotor-distal *trp* mRNA to total *trp* mRNA as a function of the concentration of Rho, by comparing the hybridization of [^{3}H]ϕ80ptEA190 RNA with *l*-λptBA24 DNA and *l*-λptEA6 DNA.

The results of this experiment (Fig. 6) show that of the *trp* mRNA synthesized at high concentration of Rho a considerably smaller fraction hybridized with *l*-λptBA24 DNA than of the material formed at low concentration of Rho, ranging from 6.5% at 6.4 µg Rho per ml to 28% at 0.2 µg/ml. If promotor-proximal and promotor-distal *trp* mRNA were present in equimolar amounts, the RNA hybridizing with *l*-λptBA24 DNA would constitute 31% of total *trp* mRNA since λptBA24 DNA contains only that portion of the *trp* operon (Rose and Yanofsky, 1971).

From our results it is clear that at low concentrations of Rho this percentage is approximated, and under these conditions nearly equimolar amounts of promotor-proximal and promotor-distal *trp* mRNA are made; at higher concentrations, however, Rho causes a relative abundance of promotor-proximal *trp* mRNA which increases linearly with the concentration of Rho.

Discussion

The results presented in this paper indicate that the transcription *in vitro* of the *trp* operon is affected by Rho. At low concentrations of Rho equimolar amounts

of promotor-proximal and promotor-distal *trp* mRNA are made and the length of *trp* mRNA synthesized under these conditions corresponds rather well to the size of the *trp* mRNA made *in vivo* (6700 nucleotides; Imamoto and Yanofsky, 1967). When the synthesis is performed in the absence of Rho, however, a large fraction of the mRNA formed consists of molecules which are much longer than the *trp* operon (Fig. 5 C) and which are hardly synthesized when Rho is present. Evidently a specific termination signal at the end of the *trp* operon is recognized in the presence of Rho which is neglected in its absence.

A similar conclusion was reached for the *gal* and the *lac* operon by de Crombrugghe *et al.* (1973). The Rho mediated termination of *trp* mRNA synthesis at the end of the operon might also explain why Rho enhances the specific formation of *trp* mRNA (Pannekoek and Pouwels, 1973). In these experiments limiting amounts of RNA polymerase were used; since in the presence of Rho synthesis is terminated at the proper sites, RNA polymerase is released from the template and becomes available more frequently to start a new round of transcription as is indicated by the results of Table 2 and Fig. 2.

More striking is the influence of Rho when present at high concentrations; under these conditions a strongly polar effect on the transcription of promotor-distal *trp* genes is found (Table 3 and Fig. 6). Since the *trp* mRNA synthesized at these concentrations of Rho is fairly homogeneous in size (Fig. 3), but has a length of approximately two-third of that of a transcript of the entire *trp* operon, this result suggests that a specific, Rho sensitive termination site exists within the *trp* operon. The length of this *trp* mRNA (the transcript of the *trp* operon synthesized at saturating concentration of Rho) places the site of Rho-sensitive termination in the promotor-distal part of the third gene, *trp C*.

On the basis of their experiments de Crombrugghe *et al.* (1973) reached the conclusion that also within the *gal* and *lac* operon of *E.coli* a Rho-sensitive termination site is present which becomes operative at higher concentrations of Rho. These authors suggest that the polar effect of Rho on transcription *in vitro* bears a relationship to the phenomenon of polarity which is observed *in vivo*. Our studies on transcription of the *trp* genes, however, do not necessarily lead to this conclusion, although we also observe a polar effect of Rho factor on transcription *in vitro*. First, phage ϕ80ptEA190, which has been used in our present studies, does not contain polar mutations and makes comparable amounts of promotor-proximal and promotor-distal *trp* enzymes *in vivo* (Pouwels and Stevens, 1973). Secondly, in experiments with a system for DNA-dependent protein synthesis *in vitro* we have shown that equimolar amounts of promotor-proximal and promotor-distal *trp* enzymes are made on the DNA of this phage (Pouwels and van Rotterdam, 1972). The difference between results obtained with the DNA-dependent preparation for protein synthesis and those of the present studies may simply be related to the high concentration of Rho factor used in the present studies. The polar effect of Rho factor on transcription of the *trp* operon and also of the *gal* operon, in particular of the *gal* operon containing an insertion of 1400 base pairs (Jordan *et al.*, 1967; Fiandt *et al.*, 1972), may be explained by the presence of a nucleotide sequence in the *trp C* gene or in the inserted piece of DNA in the *gal* operon which mimics a normal Rho-sensitive termination site, but requires a higher concentration of Rho factor.

Our conclusion that polarity is not necessarily related to the action of Rho is supported by the finding of Wetekam and Ehring (1973) that polarity in a DNA-dependent preparation for protein synthesis *in vitro* is dependent on the presence of the *suA* product, which is believed to be different from Rho factor.

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Publicatie IV

IN VITRO TRANSCRIPTION OF THE BIPOLAR ARGININE *ECBH* OPERON OF *ESCHERICHIA COLI* K 12

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1. Introduction

The arginine (*arg*) regulon of *E. coli* K12 comprises 9 structural genes, four of which are clustered in the order *argECBH*. Genetic data [1,2] and *arg* messenger (m-) RNA determinations [3,4] have shown that the four genes constitute a bipolar operon, divergently transcribed from an internal control region which is situated at or near the *argE–argC* boundary and contains a repressor binding site common to the two wings of the cluster.

The present report describes a system for the in vitro synthesis of *arg* m-RNA, with a ratio of leftwards (*argE*) over rightwards (*argCBH*) transcription similar to that observed in vivo. As in the case of the in vitro transcription of another biosynthetic operon, the tryptophan operon [5], the specific synthesis of m-RNA requires only the presence of RNA polymerase holoenzyme.

2. Materials and methods

Transducing phages: ϕ80d *arg*, also carrying the neighbouring *ppc* gene, 95% cotransducible with *argE* and situated on the *argE* gene side of the *argECBH* cluster, has been isolated by B. Konrad. The λd *arg* phages (λ14, carrying *argECBH* and *ppc*, λ23, carrying only *argECBH*) have been isolated by the method of Shimada et al. [6], starting from λ199 (a gift of Dr Weisberg) which is thermoinducible (c I 857) and lysis defective (S7). They carry the *argECBH* genes in the reverse orientation with respect to ϕ80d *arg* and appear to differ only by the amount of bacterial DNA situated on the side of *argE*, i.e. the *ppc* region (Cunin, Boyen and Glansdorff, in preparation; Palchaudhuri, Mazaitis, Glansdorff and Maas, in preparation). Large amounts of the ϕ80d *arg* and the two λd *arg* phages have been prepared by inducing lysogens with mitomycin C (1 μg/ml) at 37°C or by raising the temperature of the cultures for 35 min from 32°C up to 41.5°C, respectively, under conditions of vigorous aeration. In both cases, incubation was continued for 3 hr at 37°C after induction was over. ϕ80 phages were collected after precipitation with polyethylene glycol [7] ; the λ-phages were liberated by chloroform treatment after concentrating the induced lysogen about 50 times. The transducing phages were purified and separated from the helper phage by differential centrifugation and 3 or 4 isopycnic centrifugations in CsCl. ϕ80 DNA was extracted with phenol and strand separation of the λd *arg* DNA was achieved in the presence of poly U, G (2:1) as described previously [3].

In vitro transcription system: RNA polymerase (Holo-enzyme) was purified from *E. coli MRE 600* (RNase I$^-$) by the method of Burgess [8] up to the phosphocellulose step and was further purified by native DNA-cellulose chromatography [9]. Rho factor was purified from the same strain according to the procedure of Roberts [10] as modified by Darlix et al. [11]. Both proteins were shown to be free from detectable amounts of RNAase. In vitro RNA synthesis was carried out for 1 hr at 37°C. The reaction mixture was as follows : 25 mM Tris–HCl pH 7.9,

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Abbreviations: ppc: genetic determinant of phosphoenol pyruvate carboxylase.

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8 mM $MgCl_2$, 0.13 M KCl, 0.1 mM dithiothreitol, 0.2 mM of ATP, GTP, CTP, 0.1 mM [^{3}H]UTP (specific radioactivity as indicated in the legends), 50 μg/ml φ80d *argECBH ppc* DNA, 25 μg/ml RNA polymerase (holo-enzyme) and 1 μg/ml Rho factor. The reaction was stopped by the addition of Pancreatic DNAase I at a final concentration of 20 μg/ml and the mixture was incubated for 10 min at 37°C. Then sodium dodecyl-sulfate was added up to a concentration of 0.2% and the mixture was chilled for 10 min at 0°C. The precipitate which was formed was removed by centrifugation and the supernatant solution was passed through a Sephadex G-50 column in order to remove unincorporated [^{3}H]UTP.

One step hybridizations and competition hybridization assays were performed as described before [12]. Competitor *E. coli* RNA was extracted from *E. coli* K 12 *argR* P4XB2 and from *E. coli* K12 deletion [*argECBH ppc*](MN42) by the procedure of Summers [13], followed by filtration on Schleicher and Schüll filters BA-85 (0.45 μ).

3. Results

To detect *arg* m-RNA we have used a one-step hybridization assay, taking advantage of the low cross-hybridization between φ80 RNA and λ-DNA [14], especially when φ80 RNA is synthesized in the presence of Rho (Pannekoek and Pouwels, in preparation). RNA was synthesized in vitro on φ80d *argECBH ppc* DNA as template and hybridized with the separated strands of λ23 (d *argECBH*) and λ14 (d *argECBH ppc*) and λ199 (helper). *argE* m-RNA hybridizes with the light strand, *argCBH* m-RNA with the heavy strand of both λd *arg* phages (Cunin et al., in preparation). The results are presented in table 1. Our data reveal an asymmetric transcription pattern with a substantially lower percentage of RNA hybridizing with the light strand of λ23 (lacking *ppc*) than that of λ14, while the values of the heavy strand are not significantly different between the two d *arg* phages. The ratio of leftward over rightward transcription falls in the range of values determined in vivo under conditions of maximal repression of the *ppc* gene. Indeed, in the case of λ23, leftward transcription represents 26% of the total *argECBH* m-RNA, while estimates of the fraction of *arg* m-RNA transcribed in vivo as *argE* m-RNA vary between 15 and 30% [3,15]. The difference observed between the hybridization percentage obtained with λ14 and λ23, plus the fact that the two phages seem to differ only by the *ppc* region, suggests that *ppc* DNA is transcribed in vitro from the same DNA strand as *argE* (see also below).

Table 1
Percentage of RNA synthesized in vitro on φ80 d*argECBH ppc* DNA hybridizing with the separated strands of λd *arg* DNA's

Separated DNA Strands of	% Hybridizable counts retained with	
	Light strand (*argE*)	Heavy strand (*argCBH*)
λ14 (d *arg ppc*)	1.9	3.4
λ23 (d *arg*)	1.3	3.7
λ199 (helper)	0.1	0.4

[^{3}H] φ80d *argECBH ppc* RNA was synthesized as described in Materials and methods. The specific radioactivity of [^{3}H]UTP was 2000 counts/min per μmol. The input of [^{3}H]RNA in the hybridization mixture was 3×10^4 counts/min of acid-precipitable material. A 10-fold excess of separated strands over RNA was used during hybridization.

The asymmetry of the transcription pattern observed is per se an indication that we are dealing with specific in vitro transcription of *argE* and *argCBH* m-RNA. The correctness of this assumption was tested by a competition hybridization experiment. The hybridization percentage of a fixed quantity of radioactive in vitro synthesized RNA with the separated strands of λ14 (d *arg ppc*) was determined in the presence of increasing amounts of cold competitor RNA extracted from an *arg ppc* deletion mutant (MN42) or from a genetically derepressed (*argR*), but otherwise isogenic strain (P4XB2). The results are presented in fig.1. When the heavy strand of λ14 DNA was used competition was not observed with RNA from the deletion mutant, but RNA from P4XB2 (*argR*) could efficiently compete the radioactive in vitro synthesized RNA. Approximately 25% of the hybridized radioactive material can not be competed by P4XB2 RNA. This residual hybridization is likely to be caused, at least in part, by hybridization between φ80 RNA and λ-DNA (see also table 1).

With the light strand of λ14 DNA competition was not observed with RNA from the deletion mutant (results not shown), while competition was observed

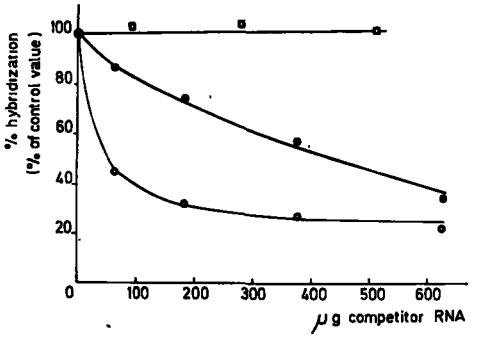

Fig.1. Competition hybridization between RNA synthesized in vitro on φ80d *argECBH ppc* DNA and RNA isolated from *E. coli* K 12 P4XB2 and *E. coli* K12 MN42. 0.08 μg of ^{3}H-labelled RNA synthesized in vitro (1.25×10^5 cpm) was hybridized with 0.11 μg of either light or heavy strands of λ14 DNA, in the presence of increasing amounts of unlabelled competitor RNA (circles = P4XB2 ; squares = MN42). Hybridization efficiency in the absence of competitor RNA, which represents the 100% value of the control is about 50% of that observed with a large excess of separated strands of λ14 DNA. Background, mock hybridizations performed at 0°C, were substracted from all values. (○–○–○) hybridization with the heavy strand of λ14 DNA; (●–●–●) hybridization with the light strand of λ14 DNA; (□–□–□) hybridization with heavy strand of λ14 DNA.

with RNA from P4XB2 although less efficient than for the heavy DNA strand : only 60% competition is achieved for 600 μg of competitor RNA and a plateau value has not been reached yet. Our results therefore clearly show that a large fraction of the radioactivity which hybridizes with the light and the heavy strand of λ14 represents *arg* m-RNA.

Our finding that the competing effect of P4XB2 RNA with the light DNA strand is less efficient than with the heavy strand may be explained by assuming that *ppc* and *argE* are transcribed from the same (= light) strand. Since P4XB2 has been grown under conditions of maximal repression of the *ppc* gene expression, RNA isolated from such a strain is expected to contain only small amounts of *ppc* m-RNA and therefore very large amounts of competitor RNA would be required to obtain complete competition of all the in vitro synthesized RNA.

4. Conclusions

Our results show that it is possible to achieve specific in vitro transcription of the bipolar, divergently transcribed *argECBH* operon. In its present state, the system may be used for the accurate determination of *argCBH* m-RNA levels which constitute the largest fraction (about 3/4) of the RNA transcribed from the whole cluster. No accessory factors appear to be required for the in vitro transcription of the *argECBH* cluster, a result which parallels the conclusions drawn from in vitro transcription of another biosynthetic operon, the tryptophan operon [5].

The exact nature of the corepressor of arginine biosynthesis is one of the pending questions that the system described here may contribute to resolve.

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Publicatie V

Punctuation of Transcription *in vitro* of the Tryptophan Operon of *Escherichia coli*

A Novel Type of Control of Transcription

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Summary. RNA transcribed *in vitro* from DNA of a tryptophan (*trp*) transducing strain of bacteriophage ϕ80 which contains the *trp* regulatory elements consists of a polycistronic messenger transcribed from the structural genes, and possibly the regulatory region, and a separate RNA species (called *trp* regRNA) which is transcribed from the regulatory region. This conclusion is based on hybridization experiments with *trp* RNA synthesized *in vitro* and the separate DNA strands of *trp* transducing strains of λ with and without the *trp* regulatory elements. The length of *trp* regRNA determined by filtration on Sephadex G-200 is 110–180 nucleotides. From the amount and the length of *trp* regRNA we have calculated that 8–20 copies of *trp* regRNA are synthesized per copy of polycistronic *trp* mRNA. We conclude that during transcription of the *trp* operon RNA polymerase frequently is rejected at a specific site ahead of the first structural gene, *trpE*. The termination factor Rho is not involved in this process. A different protein fraction, which specifically stimulates the synthesis of *trp* enzymes in an *in vitro* protein-synthesizing system (Pouwels and Van Rotterdam, 1975), was found to antagonize the abortive synthesis of *trp* mRNA.

A model is proposed for the control of transcription of the *trp* genes, which operates through a mechanism of punctuation of RNA synthesis at a specific site on the DNA template and anti-termination of RNA synthesis by means of a positive control factor.

Introduction

Recently a system has been developed for the synthesis *in vitro* of mRNA transcribed from the tryptophan (*trp*) operon of *Escherichia coli* (Pannekoek and Pouwels, 1973; Rose *et al.*, 1973). The synthesis of *trp* mRNA is carried out with purified RNA polymerase from *E. coli* and with DNA from a *trp* transducing strain of ϕ80 as a template. Unlike the transcription *in vitro* of some bio-degradative operons (de Crombrugghe *et al.*, 1971) this synthesis does not require accessory factors.

We have now investigated whether the regulatory elements of the *trp* operon, which include the promotor, operator and a DNA region between operator and the first structural gene, *trpE* (Jackson and Yanofsky, 1973) are transcribed

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Abbreviations: SSC = 0.15 M NaCl, 0.015 M tri-sodium citrate; n × SSC = n fold concentrated SSC buffer; TCA = trichloroacetic acid; poly (U, G) = copolymer of uridylic- and guanylic acid; *tonB* = a gene adjacent to the *trpA* gene involved in the resistance to phage T1 and ϕ80; Δ (trp RNA) = the difference in hybridization values of [^{3}H]*trp* mRNA synthesized *in vitro* with l-λEA6 DNA and l-λEA-BG2 DNA, relative to that with l-λEA6 DNA; Δ*trp* RNA = the RNA species corresponding to the material which hybridizes with l-λEA6 DNA but not with l-λEA-BG2 DNA.

in vitro. As a result of these studies we have found that at least part of the regulatory region of the *trp* operon is transcribed more frequently than the structural genes.

The significance of this abortive RNA synthesis for the control of transcription of the *trp* genes was investigated by determining the effect on the punctuation of transcription of a protein fraction, isolated from *E. coli*, which specifically stimulates the synthesis *in vitro* of *trp* enzymes (Pouwels and Van Rotterdam, 1975). Our findings are consistent with a model for the control of transcription of the *trp* genes, which operates through a mechanism of punctuation of RNA synthesis at a specific site on the DNA template and antitermination of RNA synthesis by means of a positive control factor.

Materials and Methods

Bacteriophages

The structure of the *trp* transducing strains of $\phi 80$ and λ used in these studies is depictured in Fig. 1. All the strains are plaque-forming phages. The propagation of the phages and their purification by differential centrifugation and equilibrium centrifugation in CsCl gradients have been described previously (Pouwels and Van Rotterdam, 1972). Isolation of bacteriophage DNA was accomplished by extraction with redistilled phenol, followed by extensive dialysis of the DNA against sterile 25 mM Tris-HCl (pH 7.9), 0.1 mM EDTA.

RNA Polymerase and Rho Factor

E. coli RNA polymerase (holo-enzyme) and Rho factor were purified from *E. coli MRE600* (RNAaseI$^-$) as described previously (Pannekoek *et al.*, 1974). Both protein preparations were shown to be free of detectable RNAase acting on single-stranded or double-stranded RNA and of DNAase.

Synthesis of ^{3}H-labelled RNA in vitro

Incubations were carried out for 1 hr at 37°C unless otherwise specified. The reaction mixture (0.10–1.0 ml) contained: 25 mM Tris-HCl (pH 7.9), 8 mM $MgCl_2$, 0.15 M KCl, 0.1 mM dithiothreitol, 45–50 μg/ml $\phi 80$ *trp* DNA, 0.2 mM of each of ATP, GTP and CTP and 0.1 mM [^{3}H]UTP (specific radioactivity 1000–2400 counts/min/pmole) and 4 μg/ml Rho (saturating amount). These components were pre-incubated for 10 min at 37°C and the reaction was started by addition of 20 μg/ml RNA polymerase (holo-enzyme). The synthesis of ^{3}H-labelled RNA was arrested by the addition of an equal volume of redistilled phenol saturated with 2×SSC*, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. 20×SSC* was added to a final concentration of 2×SSC. Aliquots of extracted [^{3}H]RNA were directly used for DNA-RNA hybridization assays, after separation of the aqueous phase from the phenol phase.

Separation of Complementary Strands of Phage DNA

Denaturation of λEA6, λEA-BG2, λBA24 and λ DNA with alkali was as described by Shapiro *et al.* (1969). Separation of the complementary strands was performed according to the procedure of Hradecna *et al.* (1967). Removal of poly (U, G)* after separation of complementary strands and self-annealing of separated strands was performed as described previously (Pannekoek and Pouwels, 1973).

DNA-RNA Hybridization

DNA-RNA hybridization was carried out in solution, with ^{3}H-labelled $\phi 80$ *trp* RNA and at least a ten-fold excess of the codogenic l-DNA strand of either λEA6, λEA-BG2, λBA24 or λ. The procedure for processing, collecting and counting of ^{3}H-labelled DNA-RNA hybrids was that of Goff and Minkley (1970). Hybridizations were carried out in duplicate or tri-

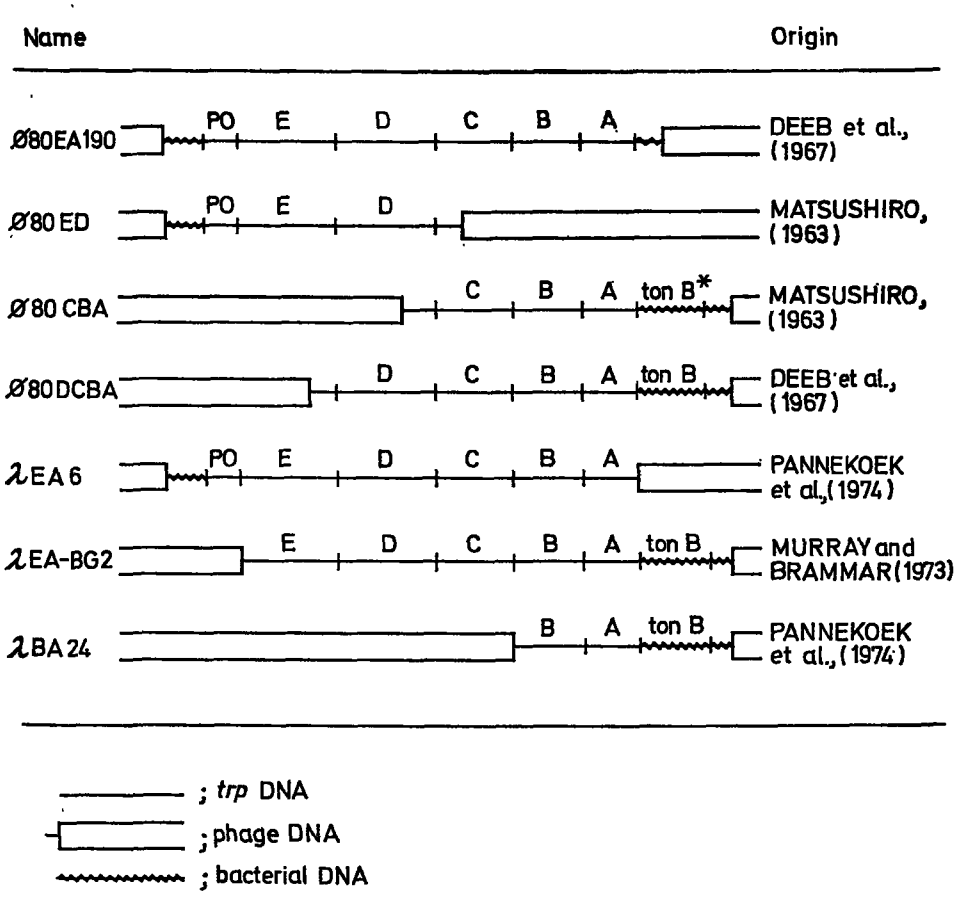

Fig. 1. Structure of *trp* transducing strains of $\phi 80$ and λ

plicate. The hybridization of [^{3}H]$\phi 80$ *trp* RNA with l-λ DNA was performed to obtain a background value. When RNA synthesis was carried out in the presence of Rho factor, the background amounted to about 0.5% of the input [^{3}H]RNA. For RNA synthesized in the absence of Rho, background hybridization with l-λ DNA was about 1% of the input [^{3}H]RNA. All results presented have been corrected for the background.

Chemicals

[^{3}H]UTP (50 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, England). Poly (U,G) (2:1) was a product of General Biochemicals (Chagrin Falls, U.S.A.). Pancreatic RNAase and T1 RNAase were from Worthington (Freehold, U.S.A.).

Results

Kinetics of Synthesis of trp mRNA

The kinetics of synthesis of *trp* mRNA on DNA from three *trp* transducing strains of $\phi 80$ (for the structure of the phages see Fig. 1) is given in Fig. 2. The intact *trp* operon consists of a regulatory area with promotor and operator and

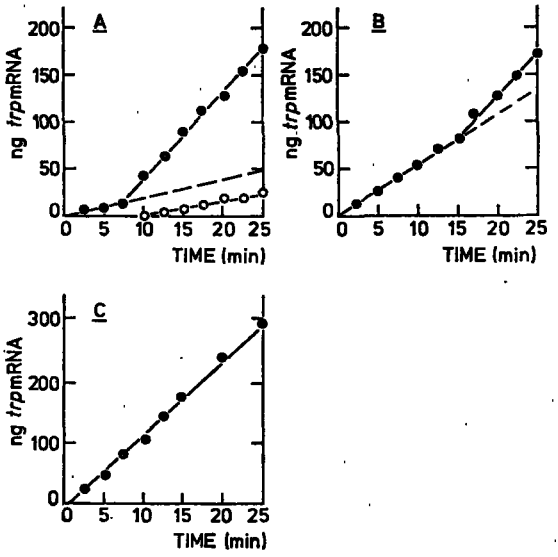

Fig. 2A—C. Kinetics of *trp* mRNA synthesis with different ϕ80 *trp* DNA templates. RNA synthesis was carried out at 25°C in a volume of 2.0 ml. The following components were pre-incubated for 10 min at 25°C: 25 mM Tris-HCl (pH 7.9), 8 mM $MgCl_2$, 0.15 M KCl, 0.1 mM dithiothreitol, 0.2 mM ATP, GTP and CTP, 0.1 mM [^{3}H]UTP (specific radioactivity 1500 counts/min/pmole), ϕ80 *trp* DNA (45 μg/ml) and Rho (0.45 μg/ml). The reaction was started by the addition of RNA polymerase; 20 μg/ml. Aliquots (160 μl) were taken at the indicated times and the reaction was stopped by the addition of 160 μl redistilled phenol (saturated with 2×SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA). 3—50 ng of [^{3}H]RNA was hybridized in duplicate for 16 hr at 67°C with either 0.5 μg l-λEA6 DNA or with 0.5 μg l-λ DNA (background determination) in 0.125 ml 2×SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20% saturated with phenol. Hybridization efficiency was 85–90%. Panel A: (•—•) [^{3}H]ϕ80EA190 RNA hybridized with l-λEA6 DNA, (o—o) [^{3}H]ϕ80EA190 RNA hybridized with l-λBA24 DNA. Panel B: [^{3}H]ϕ80ED RNA hybridized with l-λEA6 DNA. Panel C: [^{3}H]ϕ80CBA RNA hybridized with l-λEA6 DNA. The results have been corrected for the background hybridization. On the ordinate the amount of *trp* mRNA (ng) synthesized per ml reaction mixture is plotted which is calculated from the amount of incorporated [^{3}H]UMP. The assumption is made that *trp* mRNA contains 25% of incorporated UMP

five structural genes *E*, *D*, *C*, *B* and *A* which are transcribed in this order *in vivo*. The synthesis of RNA corresponding to the entire *trp* operon was studied by performing DNA-RNA hybridization with the l-strand of λEA6 DNA and that of the promotor-distal genes *trpB* and *trpA* only by hybridization with l-λBA24 DNA.

On ϕ80EA190 DNA, which contains the entire *trp* operon, synthesis of RNA hybridizing with l-λEA6 DNA is biphasic (Fig. 2A). Synthesis starts without a lag period and continues linearly for approximately 7 min; then the rate of synthesis suddenly increases and remains constant for at least 10 min. Synthesis of *trpBA* mRNA on this DNA shows a lag-period of about 9 mm suggesting that during the transcription at 25°C 9 min are required for RNA polymerase to reach the *trpB* gene. This result per se is an indication that the *trp* genes are transcribed

in the correct order. A similar profile of *trp* mRNA synthesis is obtained with DNA from ϕ80ED, which contains the *trp* promotor and operator together with the two promotor-proximal genes (Fig. 2B).

In contrast with these results a constant rate of *trp* mRNA synthesis is observed with ϕ80CBA DNA, which contains the promotor-distal genes of the *trp* operon only (Fig. 2C).

Our results with ϕ80EA190 DNA confirm those obtained by McGeoch *et al.* (1973) and by Zalkin *et al.* (1974), who used a less purified transcription system. The linear time course of the synthesis of *trp* mRNA which we obtained with DNA lacking the *trp* promotor, compared with the biphasic kinetics of RNA synthesis obtained with DNA templates, containing the *trp* promotor, suggests that on the latter DNA templates RNA synthesis starts at two promotors; the genuine *trp* promotor and a phage promotor, presumably the leftward promotor p_L^{80}, which is located upstream of the *trp* promotor. From the biphasic kinetics of *trp* mRNA synthesis on ϕ80EA190 DNA McGeoch *et al.* (1973) and Zalkin *et al.* (1974) have reached the same conclusion.

ϕ80EA190 RNA Hybridizing with l-λEA6 DNA but not with l-λEA-BG2 DNA

When RNA synthesized on ϕ80EA190 DNA in the presence of Rho factor is hybridized with l-λEA6 DNA containing the entire *trp* operon, including the regulatory elements, the amount of [^{3}H]RNA hybridizing is approximately 25% greater than the amount that hybridizes with l-λEA-BG2 DNA which contains the *trp* operon but lacks the regulatory elements (Table 1).

This difference may result from:

a) transcription of gene(s) beyond *trpA* present on ϕ80EA190 DNA and λEA6 DNA but not on λEA-BG2 DNA,

b) transcription of bacterial DNA preceding the *trp* operon, common to ϕ80EA190 and λEA6 DNA,

c) transcription of the regulatory region of the *trp* operon.

We have shown previously that [^{3}H]ϕ80EA190 RNA, synthesized in the presence of Rho, could be fully competed in the *trp* specific hybridization with l-λEA6 DNA by RNA isolated from a strain of *E. coli* which constitutively

Table 1. Hybridization of [^{3}H]ϕ80EA190 RNA with l-λEA6 DNA and l-λEA-BG2 DNA

DNA	Hybridization (counts/min)	*trp* mRNA (%)	Δ(*trp* RNA) (%)
l-λEA6	4350; 4397; 4346	12.1	26.0
l-λEA-BG2	3213; 3307; 3165	8.9	

Hybridizations were carried out for 15 hr at 67° C in 0.120 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% saturated with phenol. 38 ng [^{3}H]RNA (3.6×10^4 counts/min) were mixed with a large excess (2.7 μg) of either l-λEA6 DNA or l-λEA-BG2 DNA. Hybridization efficiency was nearly 100%. Δ(*trp* RNA) represents the difference of the hybridization of [^{3}H]ϕ80EA190 RNA with l-λEA6 DNA and l-λEA-BG2 DNA, relative to that with l-λEA6 DNA.

synthesizes *trp* mRNA, but not by RNA isolated from an isogenic strain grown under conditions of repression of the *trp* operon (Pannekoek *et al.*, 1974). This result already suggested that virtually all RNA which hybridizes with l-λEA6 DNA represents *trp* mRNA and, consequently, that the above possibilities a) and b) do not apply. In the following paragraphs experiments will be described which show that the [^{3}H]RNA hybridizing with l-λEA6 DNA, but not with l-λEA-BG2 DNA, indeed is *trp* specific.

Transcription on φ80EA190 DNA does not Proceed beyond the trp Genes

The *tonB* gene, located adjacent to *trpA* is partly or even completely deleted from φ80EA190 and λEA6 DNA, but is present on λEA-BG2 DNA (Gratia, 1971; Murray and Brammar, 1973; Franklin, 1974). The difference of the hybridization values can thus not be due to transcription of *tonB*. Moreover, if part of the *tonB* gene were still present on φ80EA190 but not on λEA6 DNA and if this part were transcribed *in vitro* from the l-strand, starting at its own promotor or as a result of read-through transcription, this would cause a difference in the opposite direction. This means that in case *tonB* is transcribed the actual difference of the hybridization values between l-λEA6 DNA and l-λEA-BG2 DNA would be even more significant. The following observations, however, indicate that *tonB* is not transcribed under the conditions used for our *in vitro* synthesis.

I) *tonB* is present on λBA24 DNA. From the results presented in Fig. 2A it is clear that φ80EA190 RNA synthesized during the first 9 min of transcription does not hybridize with l-λBA24 DNA, which contains the *tonB* gene. Therefore, during this period there is no transcription of *tonB* from the l-strand starting at its own promotor.

II) From the length determination of *trp* mRNA synthesized in the presence of Rho, we have previously concluded that transcription is terminated either at the end of the *trp* operon or at a site within the operon, and does not proceed into *tonB* (Pannekoek *et al.*, 1974). This conclusion is reinforced by the results of an experiment which are presented in Table 2. When [^{3}H]RNA synthesized

Table 2. Hybridization of [^{3}H]φ80EA190 RNA with l-λEA6 DNA, with l-λEA-BG2 DNA and with a mixture of both

l-λEA6 DNA (μg)	l-λEA-BG2 DNA (μg)	Hybridization (counts/min)
0.27	—	2444
0.68	—	2652
—	0.28	1954
—	0.70	1949
0.27	0.70	2457
0.68	0.70	2523

Hybridizations were carried out for 22 hr at 67° C in 0.125 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20% saturated with phenol. The input of [^{3}H]RNA was 38 ng which corresponds to 2.3×10^4 counts/min of 5% TCA precipitable material. The hybridization efficiency was 90–95%.

on ϕ80EA190 DNA in the presence of Rho factor was hybridized with l-λEA6 DNA (without *tonB*) or with a mixture of l-λEA6 DNA and l-λEA-BG2 (with *tonB*), the hybridization values were not significantly different. From this result it is clear that ϕ80EA190 RNA does not contain RNA species other than *trp* mRNA which specifically hybridize with l-λEA-BG2 DNA. Therefore, we can conclude that *tonB* is not transcribed by read-through transcription either.

ϕ80EA190 RNA Hybridizing with l-λEA6 DNA but not with l-λEA-BG2 DNA is trp Specific

The results presented in a preceding section indicate that transcription of the *trp* genes of ϕ80EA190 DNA starts at two promotors, the *trp* promotor and also at a promotor which is located upstream of the *trp* promotor. If a bacterial DNA region ahead of the *trp* genes is present on ϕ80EA190 and on λEA6 DNA, the difference in hybridization values between l-λEA6 DNA and l-λEA-BG2 DNA (Table 1) could be explained by transcription of this region. The following arguments, however, make this notion very unlikely.

In a previous paper (Pannekoek *et al.*, 1974) we have shown that RNA taken from a wild-type strain of *E. coli*, grown under conditions of repression of the *trp* operon, does not compete with ^{3}H-labelled RNA synthesized *in vitro* on ϕ80EA190 DNA, with regard to the *trp* specific hybridization with l-λEA6 DNA, while RNA from *E. coli trpR* competes fully under the same conditions. This result indicates that a bacterial gene preceding the *trp* operon, which might be transcribed *in vivo* from the l-strand, certainly is not transcribed from the l-strand *in vitro*, neither started at its own promotor, if present, nor by read-through starting at the phage promotor.

Another argument can be advanced to exclude read-through transcription on the l-strand of bacterial DNA ahead of the *trp* genes, viz. the fact that the difference between l-λEA6 DNA and l-λEA-BG2 DNA in hybridization with [^{3}H]ϕ80EA190 RNA synthesized *in vitro* is maintained when transcription started at the phage promotor is arrested before reaching any bacterial genes (Table 3). To arrest the read-through transcription RNA synthesis was carried out in a buffer of low ionic strength in the presence of saturating amounts of Rho factor. Evidently, the transcription starting at a phage promotor is arrested before it reaches the *trp* operon, as can be seen by comparing the results obtained under

Table 3. Hybridization of [^{3}H]ϕ80EA190 RNA, synthesized in 0.05 M KCl, with l-λEA6 DNA and l-λEA-BG2 DNA

DNA	Hybridization (counts/min)	Δ(*trp* RNA) (%)
l-λEA6	977	29
l-λEA-BG2	695	

Hybridizations were carried out for 16 hr at 67° C in 0.100 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% saturated with phenol. 0.4 μg of either l-λEA6 DNA or l-λEA-BG2 DNA was mixed with 24 ng [^{3}H]RNA (1.8×10^4 counts/min of 5% TCA precipitable material). The hybridization efficiency was about 80%.

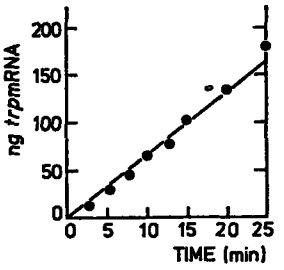

Fig. 3. Kinetics of *trp* mRNA synthesis with ϕ80EA190 DNA in a buffer of low ionic strength. RNA synthesis was carried out as described in the Legend to Fig. 2, except for the concentration of KCl which was 0.05 M

these conditions (Fig. 3) with those of Fig. 2A. The synthesis of *trp* mRNA on ϕ80EA190 DNA still starts without a lagperiod, but now the rate remains constant for at least 20 minutes. The disappearance of the biphasic kinetics indicates that *trp* mRNA starting at the leftward phage promotor, p_L^{80}, is arrested at a site ahead of the *trp* genes. This site probably is the leftward termination site, t_L, since *trp* mRNA is not reduced by Rho in buffers of low ionic strength on a DNA template which lacks this site (Pannekoek and Pouwels, manuscript in preparation).

Our finding that the difference in hybridization values of ϕ80EA190 RNA with l-λEA6 DNA and l-λEA-BG2 DNA is maintained under conditions that exclude *trp* mRNA synthesis starting at the leftward promotor of ϕ80 indicates that the RNA which hybridizes with l-λEA6 DNA, but not with l-λEA-BG2 DNA, is *trp* specific. We shall refer to this value as Δ(*trp* RNA)*.

Δtrp RNA is Transcribed from the trp Regulatory Region*

The only difference in the structure of the *trp* operon on λEA6 and λEA-BG2 is the absence of the *trp* regulatory elements from the latter phage. In the preceding sections we have presented evidence that the *Δtrp* RNA is not transcribed from bacterial DNA outside the *trp* operon. Consequently, it appears logical to assume that the *Δtrp* RNA originates from the *trp* regulatory region. We have tested this hypothesis by determining the Δ(*trp* RNA) values for RNA preparations synthesized on two more ϕ80 *trp* DNA's. In one DNA (ϕ80ED) the promotor-distal genes *trpCBA* are deleted. For this DNA one would expect a higher proportional Δ(*trp* RNA) value than for ϕ80EA190 DNA since the relative contribution of RNA transcribed from the structural genes is diminished. In the second DNA (ϕ80DCBA) all regulatory elements and the promotor-proximal gene *trpE* have been deleted. For RNA synthesized on this DNA one would not expect to find a difference in *trp* specific hybridization with l-λEA6 DNA and l-λEA-BG2 DNA.

As can been seen from Table 4 these expectations are borne out by the experimental data: RNA synthesized on ϕ80ED DNA shows a much higher Δ(*trp* RNA) value than ϕ80EA190 RNA, but with ϕ80DCBA RNA a slightly lower percentage

Table 4. The formation of *Δtrp* RNA on different ϕ80 *trp* DNA templates

Template DNA	Hybridization with		*Δ(trp* RNA) (%)
	l-λEA6 DNA (counts/min)	l-λEA-BG2 DNA (counts/min)	
ϕ80EA190	4364	3228	26.0
ϕ80ED	2765	1045	63.2
ϕ80DCBA	13534	14989	−10.7

Hybridizations were carried out for 16 hr at 67° C in 0.100 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% saturated with phenol. 38 ng [^{3}H]ϕ80EA190 RNA (3.6×10^4 counts (pH 7.5), 1 mM EDTA, 10% saturated with phenol. 38 ng [^{3}H] ϕ80EA190 RNA (3.6×10^4 counts/min), 65 ng [^{3}H]ϕ80ED RNA (6.2×10^4 counts/min) and 27 ng [^{3}H]ϕ80DCBA RNA (3.7×10^4 counts/min) were mixed with respectively 0.25 μg of either l-λEA6 DNA or l-λEA-BG2 DNA. The hybridization efficiency was about 90%.

of hybridization is obtained with l-λEA6 DNA than with l-λEA-BG2 DNA. We therefore conclude that *Δtrp* RNA is transcribed from the regulatory region of the *trp* operon DNA that is not represented in λEA-BG2 DNA.

The Size of Δtrp RNA

The regulatory elements of the *trp* operon are thought to comprise not more than a few percent of the operon. The *Δ(trp* RNA) value, however, is much larger than could be accounted for on this assumption, if all parts of the *trp* operon were transcribed with equal efficiency. This could either mean that the size of the regulatory elements is much larger than was previously thought, or that the frequency of transcription of the regulatory elements is much higher than that of the structural genes. In the latter case the majority of the transcripts of the regulatory region must be present as separate RNA molecules, probably of discrete length, which are much smaller than the polycistronic *trp* mRNA molecules.

To test this prediction we have characterized the RNA synthesized on ϕ80EA-190 DNA by filtration on Sephadex G-200. The results (Fig. 4) show that RNA which specifically hybridizes with l-λEA6 DNA (*trp* mRNA) is present in two discrete peaks. The majority of *trp* specific RNA elutes together with the bulk of the RNA (representing mainly ϕ80 RNA) but a minor fraction of *trp* specific RNA is eluted as a separate peak, just after the void volume. By hybridizing the material from separate fractions to l-λEA6 DNA and l-λEA-BG2 DNA the position of *Δtrp* RNA could be determined.

From the results presented in Fig. 4 it is clear that the *Δ(trp* RNA) values in the second peak are much higher than in the first peak. For the RNA in the first peak these values are lower than those found for the unfractionated [^{3}H]-ϕ80EA190 RNA preparation while for the second peak these values are higher. From the position of the material in the second peak relative to that of *E. coli* tRNA and [^{3}H]UTP we have estimated that the size of this material is 110–180 nucleotides. From these results we conclude that *Δtrp* RNA is synthesized as a discrete RNA species of a length of 110–180 nucleotides.

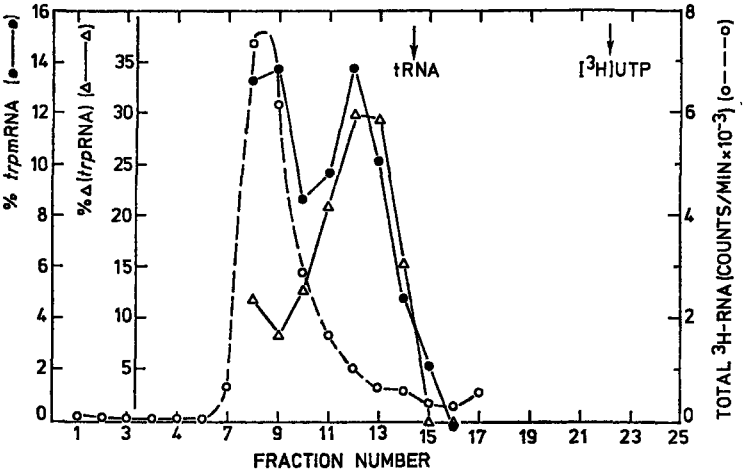

Fig. 4. Sephadex G-200 chromatography of [³H]ϕ80EA190 RNA. The synthesis of [³H]ϕ80 EA190 RNA was as described in Materials and Methods. The reaction was stopped by the addition of an equal volume of redistilled phenol saturated with 2×SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. 6 µl 1 M Tris-HCl (pH 7.9) and 6 µl 0.5 M EDTA (pH 7.5) were added to 60 µl of the ³H-labelled RNA. To prevent aggregation of small RNA species the RNA was heated for 2 min at 100°C and the mixture was quickly cooled in ice. Marker *E. coli* tRNA (10 µl of a solution containing 4 mg/ml) was added and the mixture was layered on a Sephadex G-200 column (length 27 cm) which was equilibrated and eluted with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA; fractions of 0.35 ml were collected. The optical density at 260 nm of all fractions was measured to determine the position of marker *E. coli* tRNA. Aliquots (40 µl) were applicated on DEAE-cellulose paperstrips, washed with 5% TCA* and 96% ethanol and counted to measure the presence of [³H]RNA in the fractions. Recovery of ³H-labelled RNA was 96% which corresponds with 1.9×10^5 counts/min of 5% TCA precipitable material. Of the [³H]RNA containing fractions 90 µl samples (1.5×10^3–1.8×10^4 counts/min of 5% TCA precipitable material) were adjusted with 20×SSC to a final concentration of 2×SSC and hybridized for 22 hr at 67°C with 0.5 µg l-λEA6 DNA or with 0.5 µg l-λEA-BG2 DNA in 0.125 ml 2×SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20% saturated with phenol. The hybridization efficiency was 80–90%. The percentage of Δ (*trp* RNA) in the [³H]ϕ80EA190 RNA preparation before chromatography was 24.0%. A background of 30 counts/min was substracted from all values. Total ³H RNA (o---o); percentage of *trp* mRNA (●—●); percentage of Δ (*trp* RNA) (Δ---Δ). The arrows indicate the positions of *E. coli* tRNA and [³H]UTP

The Amount of trp mRNA Synthesized on Different ϕ80 trp DNA Templates

Our results so far indicate that for most of the *trp* transcripts synthesized on a DNA template containing the *trp* regulatory elements synthesis is arrested at the end of the regulatory region. In order to determine whether this abortive transcription is limited to transcription initiated at the *trp* promotor, or takes place as well when *trp* mRNA is started at p_L, we have compared the synthesis of *trp* mRNA on DNA templates which contain both the *trp* promotor and the phage promotor with the transcription on DNA templates which contain the phage promotor only. Our results (Table 5) show that *trp* mRNA synthesis on DNA templates which lack the *trp* regulatory elements is 4–10 times higher than that

Table 5. The amount of *trp* mRNA synthesized on different ϕ80 *trp* DNA templates

Template DNA	*trp* mRNA (%)	% of *trp* operon present on DNA	Comparative ratio
ϕ80EA190	10.1	100	1.0
ϕ80ED	3.2	50	0.6
ϕ80DCBA	38.2	84	4.5
ϕ80CBA	31.5	50	6.2

Hybridizations were carried out for 16 hr at 67° C in 0.090 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% saturated with phenol. 27–32 ng of [^{3}H]RNA (4.9×10^4–5.8×10^4 counts/min of 5% TCA precipitable material) was mixed with 0.3 μg l-λEA6 DNA. The hybridization efficiency was about 90%. In column 4 the ratio is given of the percentage of each *trp* mRNA and of ϕ80EA190 *trp* mRNA, after correction for the fraction of the *trp* operon present in the particular *trp* DNA.

on DNA templates which contain the *trp* regulatory elements. These results strongly suggest that the presence of the *trp* regulatory elements truncate the synthesis of *trp* mRNA initiated at the phage promotor, probably because of the presence of a transcriptional barrier at the end of the *trp* regulatory elements.

Rho Factor is not Involved in the Synthesis of Δtrp RNA

In order to determine whether Rho factor is involved in the formation of *Δtrp* RNA we have synthesized RNA on *ϕ*80EA190 DNA in the presence or absence of Rho and hybridized these RNA preparations with l-λEA6 DNA and l-λEA-BG2 DNA (Table 6). The relative *Δ*(*trp* RNA) value for [^{3}H]*ϕ*80EA190 RNA synthesized in the absence of Rho is approximately 50% lower than that for RNA synthesized in the presence of Rho. In the presence of this factor, the synthesis of *trpBA* mRNA is strongly reduced, due to intra-operon termination of transcription (Pannekoek *et al.*, 1974). This results in a decrease of the total amount of *trp* mRNA and thus in a increase of the *Δ*(*trp* RNA) value. We therefore conclude that Rho factor is not involved in the synthesis of *Δtrp* RNA.

Table 6. The influence of the presence of Rho factor on the formation of *Δ*(*trp* RNA) during synthesis of [^{3}H]ϕ80EA190 RNA

Rho	Hybridization with		*Δ*(*trp* RNA) (%)
	l-λEA6 DNA (counts/min)	l-λEA-BG2 DNA (counts/min)	
−	8200	7073	13.7
+	5535	4168	24.7

Hybridizations were carried out for 16 hr at 67° C in 0.100 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20% saturated with phenol. 0.5 μg of either l-λEA6 DNA or l-λEA-BG2 DNA was mixed with either 68 ng [^{3}H]ϕ80EA190 RNA, synthesized in the absence of Rho (6.8×10^4 counts/min of 5% TCA precipitable material), or 54 ng [^{3}H]ϕ80EA190 RNA, synthesized in the presence of Rho (5.4×10^4 counts/min of 5% TCA precipitable material). The hybridization efficiency was about 90%.

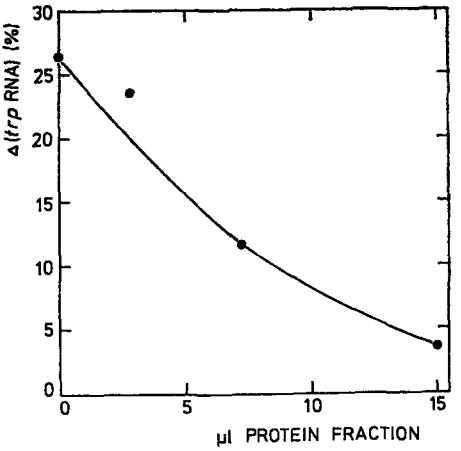

Fig. 5. The effect of the protein fraction from *E. coli* on the *Δ (trp* RNA) value. The conditions for the synthesis of [^{3}H]*ϕ*80EA190 RNA and hybridization of it with l-*λ*EA6 DNA and l-*λ*EA-BG2 DNA were as described in the Legend to Table 7

The Effect of a Protein Fraction from E. coli on the Synthesis of Δtrp RNA

Pouwels and Van Rotterdam 1975 have recently isolated a partially purified protein fraction from *E. coli* extracts which specifically stimulates the formation of *trp* enzymes in an *in vitro* protein-synthesizing system. They also showed that the stimulating effect is due to an increase of the rate of transcription of the *trp* operon.

Table 7. The effect of the protein fraction of *E. coli* on the synthesis of *trp* mRNA

Protein fraction (µl)	[^{3}H]ϕ80EA190 RNA synthesized (counts/min)	Hybridization with l-λEA6 DNA (counts/min)	*trp* mRNA (%)
0	6.3×10^5	3264	10.3
2.8	8.8×10^5	4131	8.7
7.1	9.5×10^5	4483	9.0
15.0	15.2×10^5	6180	7.8

RNA synthesis was carried out in a volume of 0.050 ml as described in Materials and Methods. The indicated volumes of the protein fraction were present during the pre-incubation step before the start of RNA synthesis by the addition of RNA polymerase. The protein fraction was partially purified from extracts of *E. coli* 514 *trpAE*^{del} *trpR* by chromatography on DEAE-cellulose and filtration through Amicon XM-100. A more detailed description is given in an accompanying paper (Pouwels and Van Rotterdam, 1975). After the synthesis the reaction mixture was diluted with 0.150 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. Aliquots (10 µl) of [^{3}H]RNA (3.1×10^4—7.6×10^4 counts/min of 5% TCA precipitable material, i.e. 41–103 ng [^{3}H]ϕ80EA190 RNA) were hybridized in triplicate for 16 hr at 67° C with 0.5 µg l-λEA6 DNA in 0.100 ml 2 × SSC, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% saturated with phenol. The hybridization efficiency was about 90%.

We have now investigated the effect of the partially purified protein fraction on the rate of *trp* mRNA synthesis on ϕ80EA190 DNA and on the formation of *Δtrp* RNA. Our results (Table 7; Fig. 5) show that the addition of increasing amounts of the partially purified protein fraction results in an increase of both total and *trp* mRNA (measured by hybridization with l-λEA6 DNA) to approximately the same extent. At the highest concentration used, the protein fraction stimulates RNA synthesis 2.4 fold and *trp* mRNA synthesis 1.9 fold.

Most strikingly, however, is the effect of the protein fraction on the formation of *Δtrp* RNA. With the increase of the concentration a decrease of the values for *Δ*(*trp* RNA) is observed (Fig. 5). This result strongly suggests that the protein fraction, which was characterized in the *in vitro* protein-synthesizing system as a positive control factor since it stimulates the synthesis of the *trp* enzymes, functions as an anti-terminator of RNA synthesis by allowing the progress of RNA polymerase beyond the transcriptional barrier in the regulatory region.

Discussion

Transcription of the *trp* operon of *E. coli* is frequently arrested at a termination site early in the operon. This site must be passed in order to transcribe the structural genes. These conclusions are based on the difference which we have found between the hybridization values of ϕ80EA190 RNA, synthesized *in vitro*, with DNA containing the *trp* operon including the regulatory elements and with DNA containing the *trp* operon without the regulatory elements (Table 1) and on the characterization of the length of the RNA transcribed from the *trp* regulatory region. Most of the transcripts from the regulatory region of the *trp* operon, for which we propose the abbreviation *trp* regRNA, are present as separate RNA molecules of discrete length, estimated at 110–180 nucleotides. Yanofsky and collaborators have shown that in *trp* mRNA the initiation codon for the translation of the first structural gene, *trpE*, is preceded by at least 165, but less than 400 nucleotides, in the *trp* messenger RNA (Bronson *et al.*, 1973; Cohen *et al.*, 1973; Yanofsky, personal communication). Hiraga and Yanofsky (1972) have presented evidence that the DNA region transcribed *in vivo*, preceding the first structural gene, is less than 200 nucleotides in length. Assuming that the section of the *trp* regulatory region from which *trp* regRNA is transcribed includes the region between the operator and the first structural gene, the good agreement between the determined length of *trp* regRNA synthesized *in vitro* and the length of the message *in vivo* from this DNA region suggests that the site where RNA synthesis is stopped must be close to the translation initiation codon of *trpE*.

From the difference between the results obtained for hybridization of RNA transcribed from ϕ80EA190 DNA with DNA containing the regulatory region and DNA lacking this region, and from the size of *trp* regRNA we estimate that the frequency of *in vitro* transcription of the regulatory region is 8–20 times higher than that of the structural genes. The relative abundance of *trp* regRNA indicates that RNA polymerase is blocked during transcription at a particular site preceding the first structural gene, *trpE*. Whether transcription of the structural genes is the consequence of reinitiation of transcription beyond this site or simply represents a continuation of transcription cannot be concluded from our

experiments. However, data from *in vivo* experiments indicating that the message transcribed from the DNA region preceding the first structural gene is covalently linked to the transcript of the first structural gene (Bronson *et al.*, 1973), favour the latter hypothesis.

Jackson and Yanofsky (1973) have isolated mutants of *E. coli* in which the expression of the *trp* operon is enhanced as a consequence of the deletion of a region between the operator and the first structural gene, *trpE*. These authors conclude that this region may have a regulatory function in the expression of the *trp* genes, either by regulating the rate of initiation of transcription or by modulating the continuation of transcription through a mechanism of expulsion of RNA polymerase. From our finding that transcription of the *trp* operon in *vitro* is frequently terminated at the end of the regulatory region we conclude that the latter hypothesis is correct and that the expulsion occurs at a specific site located between the operator and *trpE*.

A control-mechanism which functions by modulating the frequency of transcription of the structural genes could account for the differences in rate of constitutive *trp* mRNA synthesis observed when *E. coli* bacteria with a defective repressor are grown in different media (Rose and Yanofsky, 1972; Pannekoek and Pouwels, unpublished experiments). The frequency of transcription could be modulated by the presence of ligands which interact with RNA polymerase. Alternatively, positive control factors—functionally comparable to the *N* protein of phage lambda—(Protass and Korn, 1966; Kourilsky *et al.*, 1968; Roberts, 1969; Luzzati, 1970) might interact with DNA or with the DNA-RNA polymerase complex and prevent abortive RNA synthesis. Our finding that a protein fraction, which specifically stimulates the synthesis *in vitro* of *trp* enzymes prevents abortive transcription of the *trp* operon lends strong support to the second alternative.

Control of expression of genes by modulation of the frequency of attenuation of transcription probably is not unique for the *trp* operon, and might represent a more general type of control of transcription. Dahlberg and Blattner (1973) concluded from experiments on *in vitro* transcription of DNA of phage lambda that barriers exist at specific sites in the DNA template which impede the progress of RNA polymerase. Kasai (1974) has presented evidence for the presence, in *Salmonella typhimurium*, of a transcriptional barrier which affects the expression of the histidine operon. He proposed that progress of RNA synthesis beyond the barrier was regulated by a positive control factor which acts as an anti-terminator. Whether bio-synthetic operons such as the *his* and *trp* operon share the same cofactor, as is the case with some bio-degradative operons (de Crombrugghe *et al.*, 1971; Nissley *et al.*, 1971; Lee *et al.*, 1974) or that each operon has its own factor remains to be seen.

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SAMENVATTING EN DISCUSSIE

De resultaten van de experimenten, die in dit proefschrift zijn beschreven, zullen hieronder worden samengevat en, voor zover dat niet in de publicaties heeft plaatsgevonden, worden besproken.

INVLOED VAN DE IONSTERKTE OP DE SPECIFICITEIT VAN TRANSCRIPTIE

1.1. De ionsterkte bepaalt in hoge mate de specificiteit van transcriptie: de bindingsaffiniteit van RNA-polymerase voor DNA is groter naarmate de ionsterkte lager is (1), maar bij lage ionsterkte neemt de specificiteit van transcriptie af (2-4). Deze verlaagde specificiteit manifesteert zich door een toename van de symmetrie van transcriptie en door de vorming van mRNA-ketens met heterogene nucleotide-sequenties aan het 5' uiteinde. In overeenstemming hiermee zijn onze waarnemingen dat alleen specifieke synthese van *trp* mRNA wordt gevonden, wanneer de transcriptie wordt uitgevoerd in een buffer van hoge ionsterkte (Publicatie I: Tabel 1 en Fig. 5).

1.2. De ionsterkte beïnvloedt tevens de continuïteit van het transcriptieproces *in vitro*: bij een relatief hoge zoutconcentratie vindt er, na de voltooiing van de synthese van een mRNA-keten, dissociatie plaats van het transcriptie-complex (5), gevolgd door reïnitiatie van transcriptie. Aangetoond kon worden, dat er tijdens de synthese van Φ80 *trp* RNA ook in aanwezigheid van Rho reïnitiatie plaatsvindt (Publicatie III: Tabel 1 en Fig. 2), wanneer de synthese werd uitgevoerd in een buffer van hoge ionsterkte.

1.3. De ionsterkte heeft een grote invloed op de specificiteit van de werking van Rho-factor. Uit de resultaten van de experimenten, die in Publicatie III (Fig. 3 t/m 6 en Tabel 3) zijn vermeld, kan worden geconcludeerd, dat Rho-factor in 0.15 M KCl in staat is zowel de intra-operon terminatie-plaats (t_2) in het *trpC* gen als de terminatie-plaats aan het einde van het operon (t_1) te herkennen. Evenwel, bij deze ionsterkte is Rho niet in staat de faag-terminatieplaats t_L, gelegen tussen de faag-promotor p_L en de *trp* promotor p_T, te herkennen en aldaar de transcriptie te doen stoppen. Experimenten, uitgevoerd met als matrijs λ *trp* DNA's waarvan de *trp* promotor (p_T) gedeleteerd is, zodat de synthese van *trp* mRNA uitsluitend start op de faag-promotor (p_L), toonden aan dat de transcriptie van het *trp* operon door Rho gestopt wordt, mits de synthese wordt

gedaan bij lage ionsterkte (0.05 M KCl). Door gebruik te maken van λ *trp* DNA waarin de terminatie-plaats t_L ontbreekt, kon aangetoond worden dat Rho-factor de synthese van *trp* mRNA op deze plaats onderbreekt. Experimenten, waarbij Φ80 *trp* DNA wordt gebruikt als matrijs voor de *trp* mRNA synthese leidden tot dezelfde conclusie (H. Pannekoek, ongepubliceerde experimenten en Publicatie V: Fig. 2C).

PROMOTOREN VOOR DE TRANSCRIPTIE VAN HET trp OPERON

2.1. Uit de experimenten die beschreven zijn in de vorige paragraaf, alsmede uit het bifasische patroon van de kinetiek van *trp* mRNA synthese (Publicatie V: Fig. 2A-B), kan worden afgeleid, dat de transcriptie van het *trp* operon op Φ80 *trpEA-190* DNA start op twee promotoren, i.e. de faag-promotor p_L en de *trp* promotor p_T. Deze bevindingen werden m.b.v. minder gezuiverde *in vitro* transcriptie-systemen bevestigd (6,7). De bijdrage van de transcriptie gestart op p_L aan de vorming van *trp* mRNA moleculen is afhankelijk van het type Φ80 *trp* DNA, dat als matrijs wordt gebruikt (vergelijk Publicatie V: Fig. 2A en 2B en Tabel 5).

2.2. De resultaten van verschillende experimenten geven aanleiding voor de stelling, dat de op p_L gestarte transcriptie van het *trp* operon niet middels "read-through" synthese tot stand komt, d.w.z. het transcript van de Φ80 genen, gelegen tussen p_L en p_T, is niet covalent gebonden aan het *trp* mRNA molecuul.

a) De lengte-bepaling van *trp* mRNA, gesynthetiseerd in aanwezigheid van een verzadigende hoeveelheid Rho, m.b.v. polyacrylamide-gelelectrofo-rese wees uit, dat het aantal nucleotiden waaruit dit transcript bestaat ca. 4,400 is (Publicatie III: Fig. 3). Deze mRNA moleculen bevatten de genetische informatie gecodeerd door de genen *trpE, trpD* en een deel van *trpC* (Publicatie III: Tabel 3 en Fig. 6), terwijl de lengte correspondeert met de som van het aantal baseparen van deze genen (8). De lengte van de Φ80 genen tussen p_L en p_T kan op grond van kinetische experimenten geschat worden op tenminste 3,000 baseparen (H. Pannekoek, ongepubliceerde experimenten). Derhalve zou een transcript van deze genen, wanneer dit covalent gebonden was aan het *trpEDC* DNA transcript, een lengte hebben die belangrijk groter is dan 4,400 nucleotiden. Daarom concluderen we, dat *trp* mRNA geen of vrijwel geen Φ80 nucleotide-sequenties bevat.

b) In Publicatie V werd aangetoond, dat de frequentie van transcriptie

van de *trp* regulatie-elementen (*trp* regRNA) 8-20 x hoger is dan van de structurele genen, doordat het merendeel der RNA-polymerase moleculen op een specifieke plaats verhinderd wordt de *trp* mRNA synthese te continueren. De lengte van de *trp* regRNA moleculen bedraagt 110-180 nucleotiden (Publicatie V: Fig. 4). Uit deze gegevens blijkt ondubbelzinnig, dat er geen Φ80 RNA covalent gebonden kan zijn aan *trp* regRNA-ketens.

MODEL VOOR *trp*-mRNA SYNTHESE GESTART OP p_L

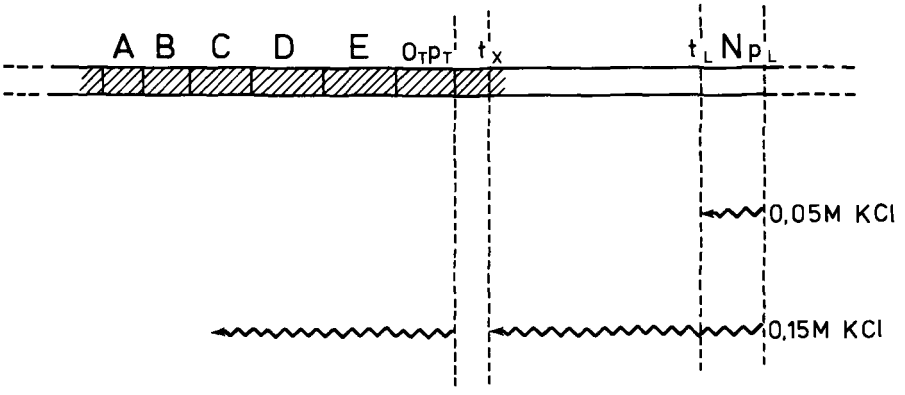

Fig. 1 Verklaring: ═══ = Φ80 DNA; ▨ = bacteriëel DNA; ∿∿∿ = mRNA; p_T = promotor van het *trp* operon; o_T = operator van het *trp operon; E, D, C, B* en *A* = structurele genen van het *trp* operon; t_X = transcriptie-terminatieplaats; p_L = promotor van het *N* operon; t_L = transcriptie-terminatieplaats van het *N* operon.

c) Uit het "twee-staps" DNA-RNA·hybridisatie-experiment (Publicatie I: Fig. 4), dat werd uitgevoerd bij lage temperatuur om fragmentatie van het RNA te voorkomen, kan dezelfde conclusie worden getrokken. Hierbij werd {^{3}H}Φ80 *trp* DNA gepre-hybridiseerd met gedenatureerd Φ80 DNA dat op nitrocellulose filters was geïmmobiliseerd, waarna het niet-gehybridiseerde materiaal werd gehybridiseerd met *l*-Φ80 *trp* DNA. Uit het resultaat, dat er zich RNA bevindt in het gepre-hybridiseerde Φ80 *trp* RNA preparaat, dat in de tweede hybridisatie-stap

specifiek hybridiseert met *l*-Φ80 *trp* DNA (en niet met *l*-Φ80 DNA) kan eveneens worden afgeleid, dat *trp* mRNA niet covalent gebonden is aan Φ80 RNA.

Op grond van de gepresenteerde argumenten kan voorgesteld worden, dat de transcriptie gestart op p_L stopt op een plaats, genoemd t_X, nabij de *trp* promotor (p_T). De resultaten geven geen uitsluitsel over de vraagstelling of de plaats t_X en p_T fysisch en functioneel gescheiden loci zijn. In Fig. 1 zijn deze gegevens samengevat.

HET PATROON VAN TRANSCRIPTIE VAN DE STRUCTURELE GENEN VAN HET trp OPERON

3.1. Een ander criterium voor de specificiteit van transcriptie van het *trp* operon dan de asymmetrie van *trp* mRNA synthese (Publicatie I: Fig. 5) is de volgorde, waarin de genen worden overgeschreven. De volgorde van het verschijnen van *trpED* mRNA (promotor-proximaal) en van *trpBA* mRNA (promotor-distaal) vertoont een karakteristiek patroon, n.l.: *trpED* mRNA wordt gesynthetiseerd direct vanaf de start van de reactie, terwijl de synthese van *trpBA* mRNA enige tijd na de start aanvangt (Publicatie V: Fig. 2A). Hieruit kan de conclusie worden getrokken, dat de start van transcriptie van het *trp* operon geschiedt op een plaats gelegen vlak voor het *trpE* gen (i.c. de *trp* promotor) en dat de structurele genen in de volgorde *trpE*, *D*, *C*, *B*, *A* tot expressie komen. Dit patroon van gepolariseerde expressie komt overeen met resultaten van *in vivo* experimenten. Uit dit experiment, alsmede uit competitie-hybridisatie experimenten tussen *in vivo* gemaakte {^{3}H}*trpEA* mRNA en ongelabeld, *in vitro* gesynthetiseerd Φ80 *trp* RNA (Publicatie I: Fig. 5), blijkt dat er *in vitro* een populatie mRNA moleculen gevormd wordt, waarin de genetische informatie van ieder *trp* gen vertegenwoordigd is.

3.2. In afwezigheid van Rho of bij lage concentraties van deze factor worden er equivalente hoeveelheden mRNA, afkomstig van elk van de *trp* genen, gesynthetiseerd (Publicatie III: Fig. 6). Wanneer Rho in hoge concentratie (b.v. 5 µg/ml) aan het transcriptie-mengsel toegevoegd wordt stopt de RNA synthese binnen in het *trp* operon, waardoor er minder *trpBA* mRNA gemaakt wordt dan *trpED* mRNA. Op grond van differentiële hybridisatie van *in vitro* gesynthetiseerd *trp* mRNA met *l*-λ *trpEA-6* DNA en *l*-λ *trpBA-24* DNA, alsook uit lengtemetingen van *trp* mRNA hebben wij geconcludeerd, dat bij hoge concentraties aan Rho-factor de transcriptie stopt na de genen *trpE*, *trpD*

en ca. 2/3 deel van het *trpC* gen overgeschreven te hebben, terwijl bij lage concentraties aan Rho de transcriptie stopt aan het einde van het operon bij t_1 (Publicatie III: Tabel 3, Fig. 5 en 6).

3.3. In afwezigheid van Rho-factor zijn de *trp* mRNA moleculen veel langer dan overeenkomt met de lengte van het operon, terwijl toevoeging van kleine hoeveelheden Rho aan het reactie-mengsel leidt tot de synthese van *trp* mRNA van een lengte overeenkomend met die van het *trp* operon. *Trp* mRNA moleculen, die langer zijn dan het *trp* operon kunnen Φ80 transcripten bevatten van: a) genen gelegen na het *trp* operon en b) genen gelegen voor het *trp* operon. Mogelijkheid b) kan uitgesloten worden op grond van argumenten die in paragraaf 2.2. zijn besproken. Hierin werd aangetoond, dat Φ80 RNA, afkomstig van genen gelegen voor het *trp* operon niet covalent gebonden is aan *trp* mRNA. Uit deze experimenten concluderen wij, dat mogelijkheid a) de juiste is en dus dat Rho-factor betrokken is bij terminatie van transcriptie aan het einde van het operon. Deze conclusie wordt gesteund door de uitkomsten van een experiment waarin het effect gemeten werd van Rho op de synthese van RNA afkomstig van genen gelegen na het *trp* operon. In dit Φ80 gebied komen nucleotide-sequenties voor, die homologie vertonen met λ DNA-sequenties in het overeenkomstige gebied (9). Dientengevolge kan continuering van transcriptie na het *trp* operon aangetoond worden door de aanwezigheid van "laat" Φ80 RNA te bepalen m.b.v. een DNA-RNA hybridisatie-test tussen {^{3}H}Φ80 *trp* RNA en *l*-λ DNA. In een competitie-hybridisatie experiment werd gebruik gemaakt van *l*- λ DNA, {^{3}H}Φ80 *trp* RNA, gesynthetiseerd in aan- of afwezigheid van Rho en als competitor-RNA ongelabeld Φ80 RNA, gemaakt *in vitro* in afwezigheid van Rho. In het competitor-RNA komt RNA voor afkomstig van de "late" genen van Φ80 DNA (Publicatie II: Tabel 2). Uit het experiment bleek, dat Φ80 RNA uitsluitend competeert met {^{3}H}Φ80 *trp* RNA wanneer laatstgenoemd RNA in afwezigheid van Rho werd gesynthetiseerd. Hieruit kan worden afgeleid, dat door de aanwezigheid van Rho de transcriptie van de "late" genen van Φ80 wordt verhinderd, in overeenstemming met de aanname dat Rho een terminatie-plaats t_1 aan het einde van het *trp* operon herkent.

De waarneming, dat Rho een specifieke terminatie-plaats herkent aan het einde van het *trp* operon, doet vermoeden dat Rho-factor een actieve rol speelt bij de terminatie van transcriptie van bacteriële operons *in vivo*. Fig. 2 geeft een schematische voorstelling van de rol van Rho bij de transcriptie van de structurele *trp* genen.

MODEL TRANSCRIPTIE *trp* GENEN ONDER INVLOED VAN RHO

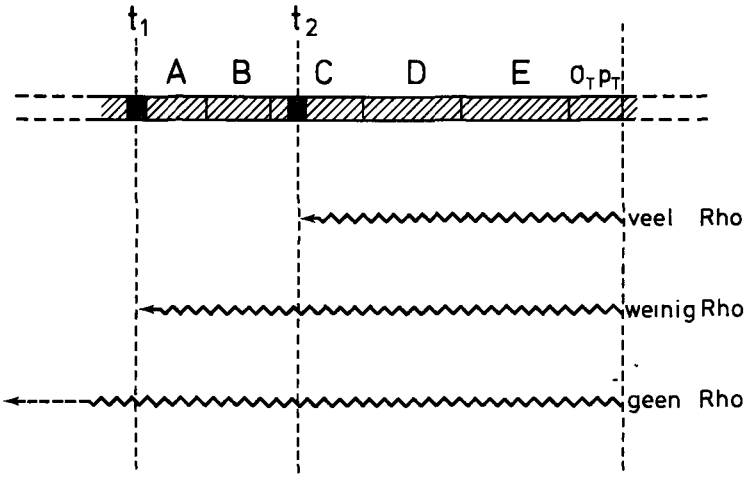

Fig. 2 Voor een verklaring van de gebruikte symbolen zie Fig. 1.

REGULATIE VAN TRANSCRIPTIE DOOR TERMINATIE/ ANTI-TERMINATIE

4.1. In Publicatie V zijn argumenten aangevoerd voor een nieuw mechanisme voor de regulatie van transcriptie van het *trp* operon. Kort samengevat kan dit mechanisme als volgt gekarakteriseerd worden:

a) 85-95% van de RNA-polymerase moleculen, die hetzij op p_T hetzij op p_L zijn gestart, bereiken de structurele genen van het *trp* operon niet. Door de aanwezigheid van een transcriptie-barrière, gelegen in de *trp* regulatie-elementen vóór het *trpE* gen, wordt de RNA-synthese gestopt. Dit terminatie-proces is onafhankelijk van de aanwezigheid van Rho.

b) 5-15% van de RNA-polymerase moleculen "ontsnapt" aan dit mechanisme en kan zorg dragen voor de synthese van *trp* mRNA. Doordat het merendeel der RNA-polymerase moleculen stopt voordat de structurele *trp* genen bereikt zijn, resulteert dit in een frequentere (8-20x) transcriptie van de *trp* regulatie-elementen t.o.v. de transcriptie van de structurele genen.

c) Een eiwitfractie uit *E. coli*, genoemd At-factor (anti-terminatie factor), die in een gekoppeld transcriptie-translatie systeem de synthese

in vitro van *trp* enzymen stimuleert (10), is in staat de transcriptie-barrière op te heffen. Dit heeft tot gevolg, dat er in aanwezigheid van At-factor equivalente hoeveelheden *trp* regRNA en *trp* mRNA gesynthetiseerd worden (Publicatie V: Fig. 5). Deze anti-terminatie factor At stelt derhalve de RNA-polymerase moleculen in de gelegenheid de structurele *trp* genen tot expressie te brengen. Voor dit mechanisme t.b.v. de regulatie van de transcriptie *in vitro* van het *trp* operon kan het volgende model voorgesteld worden:

MODEL TERMINATIE/ANTI-TERMINATIE VOOR DE REGULATIE VAN DE TRANSCRIPTIE VAN HET *trp* OPERON

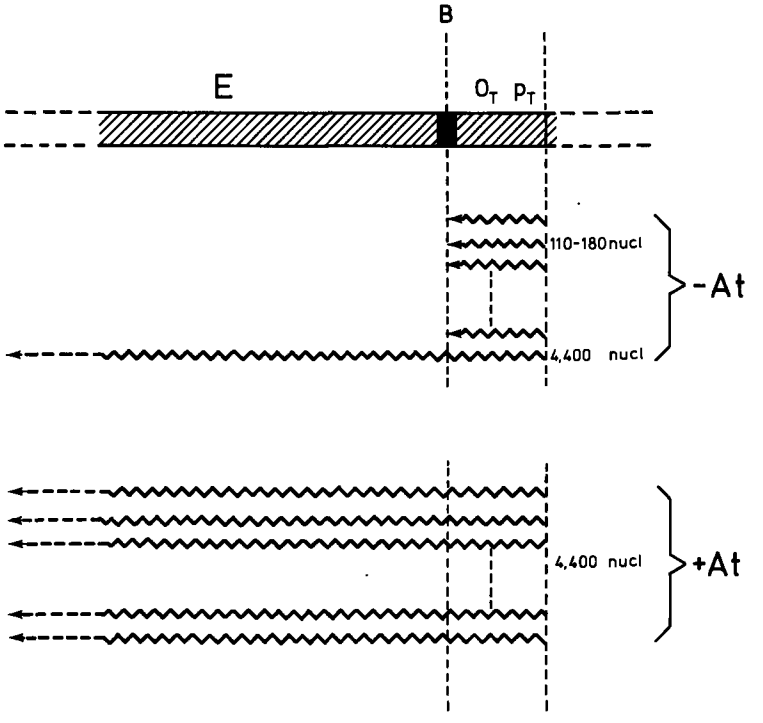

Fig. 3 Verklaring: *B* = transcriptie-barrière; voor een verklaring van de andere symbolen zie Fig. 1.

DE FUNCTIE VAN HET MECHANISME VAN TERMINATIE/ ANTI-TERMINATIE VAN TRANSCRIPTIE VOOR DE REGULATIE VAN DE EXPRESSIE VAN HET trp OPERON

5.1. Er zijn er aantal aanwijzingen, dat het voorgestelde nieuwe mechanisme voor de regulatie van transcriptie *in vitro* van het *trp* operon eveneens functioneel is in de levende cel. Hieronder volgt een opsomming van deze aanwijzingen.

a) Jackson en Yanofsky (11) hebben mutanten geïsoleerd van *E. coli*, die deleties bevatten binnen het *trp* operon welke een deel van het *trpD* gen en het gehele *trpE* gen beslaan, doch de *trp* operator intact laten. Deze mutanten bleken, onder condities van derepressie, een hogere biosynthese van tryptofaan-synthetase (gen-product van *trpB* en *trpA*) dan wild-type bacteriën te bewerkstelligen, hetgeen toegeschreven kon worden aan een frequentere transcriptie van het *trp* operon. Dit resultaat kan verklaard worden door aan te nemen, dat deze deleties de transcriptie-barrière, die gelegen is direct voor het *trpE* gen, verwijderen, zodat RNA-polymerase na te zijn gestart op de *trp* promotor ongehinderd de synthese van een polycistronische messenger kan voltooien.

b) Yanofsky en medewerkers (12-14) hebben gerapporteerd, dat in *trp* mRNA, geïsoleerd uit *E. coli*, het initiatie-codon voor de translatie van het eerste structurele gen (*trpE*) wordt voorafgegaan door een sequentie van tenminste 165 nucleotiden ("leader-sequence"). Tevens bleek (14), in deze studie gericht op het ophelderen van de nucleotide-volgorde van *trp* mRNA, dat er *in vivo* meer RNA gemaakt wordt dat nucleotide-sequenties bevat afkomstig van de "leader-sequence" dan *trp* mRNA volgend op het eerste initiatie-codon voor de translatie. Deze resultaten corresponderen met de in dit proefschrift vermelde bevindingen t.a.v. de regulatie van transcriptie *in vitro* van het *trp* operon, zowel voor wat betreft de lengte van *trp* regRNA-ketens als ook t.a.v. de meer frequente transcriptie van de regulatie-elementen t.o.v. de structurele genen.

5.2. Over de mogelijke functie van het voorgestelde mechanisme voor de regulatie van transcriptie is niets bekend. Hieronder volgen een tweetal argumenten ten gunste van een rol van het mechanisme van terminatie/ anti-terminatie.

a) Vullen het terminatie-mechanisme en het repressie-mechanisme elkaar aan? Een vergelijking van de eigenschappen van gezuiverde repressor voor het *E. coli* lactose (*lac*) operon (15) met die van de repressor

voor het *trp* operon (16) levert de volgende gegevens op. De dissociatie-constante (K_d) van het complex *lac* repressor - *lac* operator is een factor 3.5 x 10^2 lager dan die van de *trp* repressor - *trp* operator, waarbij de halveringstijden, onder gelijke proefomstandigheden, van het *lac* repressor - *lac* operator- en het *trp* repressor - *trp* operator-complex resp. 10 min en 2 min zijn. Klaarblijkelijk is de complexvorming tussen de *lac* repressor en diens operator aanmerkelijk effectiever dan die voor de repressie van het *trp* operon.
Deze gegevens suggereren, dat het minder effectieve repressie-mechanisme van het *trp* operon een bepaalde mate van "ontsnappings-synthese" van *trp* mRNA zal toelaten. Als bescherming tegen ongewenste synthese van *trp* mRNA en *trp* enzymen zou een transcriptie-barrière, gelegen vóór de structurele genen, de resterende *trp* mRNA synthese kunnen verhinderen.

b) Vullen het anti-terminatie mechanisme en het derepressie-mechanisme elkaar aan? Onder condities van derepressie heft de At-factor de transcriptie-barrière op en verhoogt daardoor de synthese van *trp* mRNA (Publicatie V: Fig. 5 en Tabel 7). Onder condities van derepressie *in vivo* is het aantal copiën *trp* mRNA per genoom afhankelijk van de kweekomstandigheden voor de bacterie ("metabolic regulation") (17). Deze waarneming suggereert, dat er onafhankelijk van regulatie van expressie van de *trp* genen door repressie/ derepressie, een tweede mechanisme is dat de expressie van de *trp* genen controleert. Het is mogelijk, dat het terminatie/ anti-terminatie mechanisme gerelateerd is aan het door Rose en Yanofsky (17) gevonden mechanisme van "metabolic regulation".

5.3. Ook bij de bestudering van de transcriptie *in vitro* van het biosynthetische histidine (*his*) operon van *Salmonella typhimurium* is onlangs gevonden, dat er zich een transcriptie-barrière bevindt op een gedeelte van het *his* operon, gelegen voor het eerste structurele gen (*hisG*) (18). Deze auteur stelde eveneens voor, dat er een positieve factor zou bestaan, die de barrière opheft en daardoor de expressie van het *his* operon zou verhogen. Derhalve kan de hypothese voorgesteld worden, dat het mechanisme van terminatie/ anti-terminatie een wijze van regulatie van gen-expressie is, die niet slechts beperkt is tot het *trp* operon, maar tevens functioneert voor andere biosynthetische operons.
Toekomstig onderzoek naar de regulatie van transcriptie van het *E. coli*

arginine (*arg*) operon, waarvoor eveneens een *in vitro* transcriptie-systeem beschikbaar is (zie Publicatie IV), zal deze hypothese kunnen toetsen.

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SUMMARY

The specificity and the regulation of transcription *in vitro* of the tryptophan (*trp*) operon of *Escherichia coli* was studied with a preparation consisting of:

- purified DNA from *trp* transducing strains of Φ80 (Φ80 *trp* DNA)
- purified RNA-polymerase (holo-enzyme) from *E. coli*
- purified transcription termination factor Rho from *E. coli*
- a buffer of relatively high ionic strength (8 mM $MgCl_2$, 0.15 M KCl).

The results of the experiments can be summarized as follows:

1) Specific transciption of the *trp* operon can be demonstrated when RNA synthesis is carried out in a buffer of high ionic strength with RNA-polymerase saturated with σ factor in the presence of the termination factor Rho. In the absence of σ- or Rho factor and/or in buffers of low ionic strength (0.05 M KCl) synthesis of *trp* mRNA was less specific.
2) Accessory factors appear not to be essential for specific transcription of the *trp* operon. Another biosynthetic operon, the bipolar arginine (*argECBH*) cluster of genes, also was found to be transcribed specifically without additional factors.
3) The Rho factor preferentially reduces transcription of both the "late" genes of Φ80 (*trp*) DNA and of the *r*-strand of the "early" genes, whereas transcription of the "early" region on the *l*-strand is almost unaffected.
4) The Rho factor causes an increase of the dissociation-rate of the transcription-complex under our standard transcription conditions.
5) Transcription *in vitro* of the *trp* operon on Φ80 *trpEA-190* DNA is initiated at two different sites, i.e. the genuine *trp* promotor (p_T) and the phage promotor for leftward Φ80 transcription (p_L).
6) Transcription *in vitro* of the *trp* genes occurs in a sequential, polarized fashion; the promotor-proximal genes *trpE* and *trpD* are transcribed before the promotor-distal genes *trpB* and *trpA*.
7) In the absence of Rho or at low concentrations of this factor all the *trp* genes are transcribed with nearly equal efficiency; at high concentrations of Rho, however, more than 90% of the *trp* mRNA originates from the promotor-proximal genes *trpE*, *trpD* and part of the *trpC* gene.
8) The presence of Rho greatly affects the length of the *trp* DNA transcript. At low concentrations of Rho the length of *trp* mRNA is 5,000-7,000 nucleotides which corresponds with the length of the *trp* operon. Thus Rho recognizes a transcription termination site (t_1) at or near the end of the

trpA gene.

At high concentrations of Rho also a termination site (t_2) within the *trpC* gene is recognized which causes the appearance of *trp* mRNA molecules which are shorter (4,400 nucleotides) than the operon. When RNA synthesis is carried out without Rho most of the *trp* mRNA was much longer than the length of a full polycistronic *trp* mRNA.

9) The *trp* DNA transcript synthesized with Φ80 *trp* DNA which contains the *trp* regulatory elements consists of a polycistronic messenger transcribed from the structural genes, and possibly the regulatory region, and a separate RNA species (called *trp* regRNA) which is transcribed from the regulatory region.

10) The length of *trp* regRNA is 110-180 nucleotides; the frequency of transcription of the *trp* regulatory region is 8-20 fold higher than that of the structural genes.

11) After the onset of transcription of the *trp* operon RNA-polymerase frequently is rejected at a specific site within the regulatory region ahead of the first structural gene *trpE*. The termination factor Rho does not participate in this process.

12) A protein fraction from *E. coli* (called At) which specifically stimulates the synthesis of *trp* enzymes in an *in vitro* protein-synthesizing system (Pouwels, P.H., Van Rotterdam, J. Molec. gen. Genet. *136*, 185-197 (1975)) was found to antagonize the abortive synthesis of *trp* mRNA; in the presence of a saturating concentration of At equimolar amounts of *trp* regRNA and *trp* mRNA are made.

CURRICULUM VITAE

Op verzoek van de Faculteit der Wiskunde en Natuurwetenschappen volgen hier enkele persoonlijke gegevens.

juli 1962 - eindexamen H.B.S.-B aan het Casimir Lyceum te Amstelveen

okt. 1962 - eerste inschrijving in de Faculteit der Wiskunde en Natuurwetenschappen aan de Universiteit van Amsterdam

okt. 1966 - kandidaatsexamen; studierichting schei- en natuurkunde met biologie (Letter g)

dec. 1969 - doctoraalexamen; hoofdrichting biochemie (Prof. E.C. Slater en Prof. P. Borst), bijvak radiochemie (Prof. A.H.W. Aten)

mrt. 1970 - detachering bij de afdeling Biochemie van het Medisch Biologisch Laboratorium TNO te Rijswijk in het kader van de vervulling van militaire dienstplicht. In deze periode werd deelgenomen aan een project, dat ten doel had de bestudering van een proces dat bijdraagt tot het herstel van beschadigd DNA

okt. 1971 - toekenning van een beurs voor de duur van $1\frac{1}{2}$ jaar voor de in dit proefschrift beschreven onderzoek door de Commissie voor Moleculaire Biologie en Radiobiologie van de Europese Gemeenschap te Brussel. Dit onderzoek werd door mij begonnen op de Service de Biochimie van het Centre d'Etudes Nucléaires de Saclay te Gif ^{S}/ Yvette (Frankrijk)

mrt. 1973 in tijdelijke diensttreding aan de Rijksuniversiteit te Leiden als wetenschappelijk medewerker van de afdeling Moleculaire Genetica van het J.A Cohen Instituut voor Radiopathologie en Stralenbescherming. Het onderzoek werd vanaf maart 1973 voortgezet op het Medisch Biologisch Laboratorium TNO te Rijswijk.

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