

STUDIES ON THE MECHANISM OF  
TERMINATION OF TRANSCRIPTION.

by Kevin M. O'Hare

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Department of Molecular Biology  
University of Edinburgh  
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### Summary

The termination of RNA synthesis by purified E.coli RNA polymerase, at the principal termination site for host-mediated early transcription of coliphage T7 DNA, has been studied in detail. Recognition of the "stop" signal and cessation of RNA chain growth are highly efficient. A nitrocellulose binding assay has shown that the subsequent release of RNA product is rapid, but unexpectedly the separation of DNA and enzyme is slow, such that it becomes rate limiting for further transcription.

Conditions which inhibit DNA release have been defined so that enzyme remains bound at or near the termination site. Digestion of the DNA by restriction endonucleases followed by filtration on nitrocellulose has then allowed the identification and enrichment of restriction fragments which contain the termination site. This affords a novel route for mapping restriction sites near this and perhaps other termination sites.

Using an antiserum prepared against purified sigma protein, the possible involvement of sigma in termination has been investigated. It was hypothesised that sigma might bind to core enzyme at or near the termination site, and might have an essential role in some or all steps in termination. The results indicate that sigma has no such role in the termination of transcription in vitro.

Preliminary studies with the transcriptional termination factor rho suggest that rho may increase the rate of RNA release, but does not affect the rate of DNA release.



Attempts were made to extend the known nucleotide sequence around this termination site. RNA was synthesised in vitro, hybridised to the complementary single strand of DNA and used as a primer for DNA polymerase I of E.coli. However, DNA synthesis at nicks and ends of DNA so obscured the results that only tentative progress was made in determining the sequence of the DNA formed by extension of the RNA primer, and in locating restriction sites within this DNA.



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## CHAPTER 1.

### INTRODUCTION

#### 1. Transcription and gene expression

The study of microbial genetics has shown that the chromosomes of bacteria and bacteriophages consist of units of transcription called operons (Jacob and Monod, 1961) comprising a single gene or groups of co-ordinately expressed, related genes. Genetic information, encoded as a sequence of bases in double-stranded DNA is expressed by producing an RNA complementary to one strand (transcription) which is then used as a template or messenger for synthesis of the gene product (translation). The frequency of synthesis of messenger RNA molecules is strictly controlled by signals encoded in the DNA which determine when and where transcription starts and stops (Chamberlin, 1974).

There is usually a cluster of regulatory sites at the points where transcription starts. They include the promoter, which is the site where RNA polymerase, the enzyme responsible for transcription, binds and initiates the synthesis of an RNA molecule. There may also be sites at which other proteins can bind to stimulate the transcription of an operon, or to interfere with the binding of RNA polymerase and so inhibit transcription. The activity of these regulatory proteins varies in response to changes in the environment. The control of transcriptional initiation, though of great interest, is outside the scope of this study. However, the mechanism of initiation of RNA synthesis will be discussed below as it may bear some relationship to the mechanism of



termination of RNA synthesis.

At the distal end of the operon, there must be a site at which RNA synthesis terminates with release of the RNA product and enzyme from the DNA. It is now clear that in certain operons, a termination site can precede even the first gene (Bertrand et al, 1975; Roberts, 1975). In such cases, regulation of termination provides a further means to control the expression of the operon. Sites between successive genes of an operon at which the termination of transcription and consequently gene expression can be regulated are also known (Roberts, 1969).

2. The study of transcription in vitro

In the bacterium, Escherichia coli, a single enzyme is responsible for virtually all RNA synthesis (di Mauro et al, 1969). This DNA dependent RNA polymerase (EC. 2.7.7.6) can be readily purified to near homogeneity by a number of methods (e.g. Berg, Barrett and Chamberlin, 1971; Burgess, 1969; Burgess and Jendrisak, 1975). The properties of the purified enzyme have been intensively studied and are reviewed in "RNA polymerase" (ed. Losick and Chamberlin, 1976). The complete molecule or holoenzyme has a molecular weight of about 470,000 and consists of four polypeptides in the stoichionetry  $\alpha_2\beta\beta'\sigma$  (Burgess, 1969; Berg and Chamberlin, 1970). A further small polypeptide,  $\omega$ , is often found in preparations of RNA polymerase but is not needed for enzyme activity. The core enzyme  $\alpha_2\beta\beta'$  is catalytically competent and can unspecifically transcribe single-stranded DNA and denatured or damaged double-



stranded DNA but is relatively inactive on intact native DNAs (Burgess et al, 1969; Hinkle, Ring and Chamberlin, 1972). The sigma subunit ( $\sigma$ ) confers on core the ability to interact effectively with double-stranded templates and to initiate at promoter sites (Bautz et al, 1969; Chamberlin and Ring, 1972).

The  $\beta$  subunit is the target for the drugs rifampicin and streptolydigin (Heil and Zillig, 1970) which suggests its direct participation in the initiation and elongation steps of transcription (Lill et al, 1970; Cassani et al, 1971). The properties of  $\beta'$  are consistent with its having a major role in the binding of DNA (Zillig et al, 1970). Though  $\alpha$  is an essential subunit of RNA polymerase, its role in transcription is not clearly defined (see Krakow et al, 1976).

DNA can be readily extracted from bacteriophages, including transducing phages, in native form for use as a template for transcription in vitro. However, even bacteriophage templates have a number of promoters and terminators for E.coli RNA polymerase so that their transcription can be complex. This complexity can now be avoided by using restriction endonucleases to dissect the DNA such that fragments carrying a single operon, a single promoter or a single termination site can be purified and studied.

Using these components, there is often a strong correlation between transcription in vitro and in vivo (Chamberlin, 1974). This shows that the purified enzyme can select some initiation and termination sites with



fidelity, though at other sites additional protein factors may be required in vitro and in vivo. Numerous proteins have been described which affect transcription in vitro. Most of these affect one or a few operons only, and so cannot be considered as general components of the transcription machinery: e.g. the lac repressor (Chen et al, 1971), the ara activating protein (Greenblatt and Schleif, 1971) and the catabolite activator protein (de Crombrughe et al, 1971). However, a number are reported to affect transcription in vitro in a more general way. In most cases, it is not known whether these have any physiological significance. The single case where a factor is known to be active in vivo is the transcription termination protein, rho ( $\rho$ ). This was first isolated by Roberts (1969) and is a polypeptide of molecular weight 50,000 capable of existing as multimers which are thought to be the active form (Minkley, 1973). It leads to termination of transcription at specific sites in vitro. Much of the evidence that rho is active in vivo has come from the study of the regulation of transcription in phage  $\lambda$  and <sup>of</sup> polarity in bacterial operons, recently strengthened by the discovery of rho mutants.

### 3. Rho, polarity and the termination of transcription

Some mutations in a structural gene of an operon have two effects. They not only affect the product of the mutated gene, but also reduce or abolish the expression of the distal genes located "downstream". This phenomenon, called polarity, was first described in the lac operon



(Jacob and Monod, 1962) and has since been reported for other systems, notably the trp operon (Yanofsky et al, 1971).

There are two types of polar mutations - nonsense and insertion <sup>/deletion</sup> mutations. When an in-phase nonsense codon is produced within a gene by mutation, translation of the mRNA for this gene will be terminated at this site in the absence of suppressor.

Thus, except where re-initiation of translation occurs (Platt et al, 1972), RNA sequences distal to the mutation will no longer be traversed or sequestered by ribosomes (Stretton and Brenner, 1965; Brenner and Beckwith, 1965; Zipser, 1967). Insertion mutations arise by interjection of a foreign piece of DNA (usually a transposable "insertion sequence") within the structural gene (Jordan, Saedler and Starlinger, 1968; Shapiro, 1969; Malamy, 1970). The polar effect of insertion <sup>/deletion</sup> mutations can be explained as above if they either contain nonsense codons, or generate them by changing the phase of reading of distal DNA sequences.

That the polar effect occurs primarily at the level of transcription rather than translation has been shown in the trp operon. Transcription proceeds normally for some distance beyond a polar mutation, but the RNA produced is "hyperlabile" and is mostly confined to the gene actually carrying the mutation. Distal genes in the operon are transcribed at an abnormally low rate (Morse and Yanofsky, 1969; Hiraga and Yanofsky, 1972). There is a rough correlation between the reduction of expression of distal genes and the location of the polar mutation; a gradient of polarity such that the further the mutation is from



the start of the next gene, the stronger is the polarity (Newton et al, 1965; Yanofsky et al, 1971). This implies that there is a factor which can detect naked RNA sequences and interacts with the transcribing complex of enzyme, DNA and nascent RNA in such a way as to cause termination of transcription at specific sites.

Mutations of E.coli that suppress polarity, without restoring the function of genes carrying polar mutations have been described; suA (Beckwith, 1963; Morse and Primakoff, 1970) and psu (Korn and Yanofsky, 1976a). These mutants have recently been shown to produce altered rho factors (Richardson et al, 1975; Ratner, 1976; Korn and Yanofsky, 1976b) which is strong evidence that rho has a role in vivo. This suggests that rho can interact with the transcribing complex in vivo, acting through untranslated RNA sequences so as to cause termination of transcription. However, ribosomal and transfer RNAs are not translated in vivo so it is clear that untranslated RNA is not a sufficient, although probably a necessary condition for rho action. A specific sequence encoded in the DNA must also be recognised (either directly or in the RNA product) by RNA polymerase and/or rho factor.

Rho-dependent termination sites have been detected by in vitro studies within a number of operons (de Crombrughe et al, 1973; Shimizu and Hayashi, 1974). The identity of such in vitro sites with the sites at which transcriptional termination occurs as a result of polar mutations in vivo has not yet been demonstrated. A rho-dependent termination



site has also been detected within the insertion sequence IS2 (de Crombrughe, et al., 1973).

Further evidence for the physiological role of rho comes from the study of  $\lambda$  transducing phages carrying bacterial operons in positions such that they can be transcribed from the  $\lambda$  promoter  $P_L$  (Adhya et al., 1974; Franklin, 1974; Segawa and Imamoto, 1974). During the lytic cycle of  $\lambda$ , when transcription is initiated at  $P_L$  in the presence of active  $\lambda$  N gene product, rho-dependent termination sites in  $\lambda$  are readthrough (see Chapter 1, section 4d (i) and Fig. 1). It is clear that N antagonises rho action although its mechanism is not fully understood. N seems to act at or near the promoter (Friedman et al., 1973) perhaps by inducing a conformational change in RNA polymerase at the time of initiation, or by binding to RNA polymerase and accompanying the transcribing complex, such that the polymerase cannot interact with rho when it reaches rho-dependent termination sites; however, less direct explanations cannot yet be excluded (Franklin and Yanofsky, 1976; Adhya et al., 1976). It has recently been demonstrated that N protein may be co-precipitated with RNA polymerase from extracts of  $\lambda$  infected cells by antisera prepared against purified RNA polymerase (Epp and Pearson, 1976). Whatever the mechanism of N action, in the transducing phages described above, transcription initiated at  $P_L$  in the presence of N protein proceeds through bacterial operons containing polar mutations which would normally cause termination of transcription. Thus N can suppress polarity, as expected if the termination of transcription



responsible for polarity is catalysed by rho.

The mechanism by which rho causes termination is still not fully understood. There is evidence that it can bind to RNA, DNA and RNA polymerase, though it is thought to interact at least initially with RNA. Lowery-Goldhammer and Richardson (1974) have shown that rho cleaves ATP to ADP in the presence of RNA even if DNA and RNA polymerase are absent. Moreover, there is a requirement for such cleavage in rho mediated termination which is quite distinct from the cleavage of ATP to AMP which occurs in RNA synthesis. Thus NTP analogues which can be cleaved at the  $\alpha$ - $\beta$  position, but not at the  $\beta$ - $\gamma$  position permit transcription but not rho-dependent termination (Howard and de Crombrugge, 1976; Galluppi et al, 1976).

Rho and RNA polymerase migrate together through non-denaturing polyacrylamide gels (Darlix et al, 1971). However, it is not clear whether this is a specific interaction or is due to the aggregation of rho into multimers (Minkley, 1973).

It has been reported that rho can bind DNA and thus lead to its retention on nitrocellulose filters (Beckman et al, 1971). This could represent a specific interaction or a low affinity of rho for all polynucleotides. Oda and Takanami (1972) presented electron microscopic evidence for the binding of rho to a few sites on double-stranded bacteriophage fd DNA, suggesting a specific interaction, but the identification of the bound protein as rho was somewhat uncertain.



However, Goldberg and Hurwitz (1972) have shown that the efficiency with which rho catalyses the termination of transcription is not affected by a large excess of DNA, whereas the ratio of RNA polymerase to rho is critical. If rho does interact with DNA to cause termination, it cannot be a stoichiometric interaction. It is difficult to reconcile this result with any model for rho action which does not involve interaction with the RNA polymerase. Goldberg and Hurwitz (1972) also showed that rho can catalyse termination without being present at the time of initiation of an RNA molecule.

Adhya et al (1976) have put forward a model for the termination of transcription which accommodates much of the evidence about rho action. The basis of this model is that rho can interact with untranslated RNA between genes, or at the end of an operon or following a polar mutation. It next moves along the RNA in a 5' to 3' direction, perhaps at the expense of ATP hydrolysis, until it reaches the transcribing complex. It then accompanies the complex until it reaches a nucleotide sequence at which it can catalyse termination of transcription, perhaps primarily by causing release of the RNA. This model offers a simple explanation for the existence of polarity gradients. However, it has not yet been proved that rho can move along a polynucleotide. It is equally possible that there is some sequence or structure in the RNA adjacent to RNA polymerase at a rho-dependent termination site to which the factor binds and catalyses RNA release in a reaction which requires ATP hydrolysis. On either of these models,



the extent to which a given site is rho-dependent could be governed both by the affinity of rho for the sequences present and by the degree to which RNA polymerase tends to "pause" in this region. There is evidence that RNA polymerase does "pause" at sites on T7 DNA where rho-dependent termination can occur (Darlix and Horaist, 1975).

In general, the RNA is released at rho-dependent termination sites (Roberts, 1969; Goldberg and Hurwitz, 1972). However, a well-known effect of rho in vitro is to reduce the net incorporation of substrates into RNA. Partly this is because rho causes smaller RNAs to be produced, but it suggests that the enzyme is not released, or is released in an inactive form (Roberts, 1976). Rho effects are usually studied at low ionic strength because at many sites, the factor is most active under such conditions. However, low ionic strength does not favour enzyme release (Bremer and Konrad, 1964; Maitra and Hurwitz, 1967). Thus it has been suggested that in vivo the ionic strength may be high enough to allow enzyme release, but still low enough to allow rho to act with some efficiency which probably varies from site to site and is rarely 100%.

#### 4. The transcription cycle.

Transcription in vitro by RNA polymerase involves four main steps, each of which is a complex set of events

- (a) binding of RNA and selection of a promoter
- (b) initiation
- (c) elongation
- (d) termination and release of RNA product and DNA.



Each of these steps will be discussed with special emphasis on the transcription of T7 DNA by E.coli RNA polymerase.

a) Binding of DNA and selection of a promoter

RNA polymerase can interact with DNA in the absence of substrates to form binary complexes. With bacteriophage DNA as template, and holoenzyme, these complexes are formed primarily at promoters (for review see Chamberlin, 1976). The strength of the interaction varies with the template, presumably depending on the nucleotide sequence at the promoter. With at least some templates, the complexes formed are extremely stable. RNA chains can be initiated by such complexes without dissociation, showing that they are true intermediates in the transcription cycle (Hinkle and Chamberlin, 1972a; Chamberlin and Ring, 1972).

A widely used working hypothesis was put forward by Zillig and co-workers for the binding and selection of promoters by E.coli RNA polymerase (Walter et al, 1967). They postulated that repeated association-dissociation events occur until RNA polymerase associates with a promoter region. A specific binary complex is then formed in which the DNA maintains its helical configuration (the closed promoter complex). This complex is in equilibrium with the open promoter complex wherein the DNA strands have separated to allow RNA polymerase direct access to the template bases. This latter is able to initiate an RNA chain.

Though holoenzyme has a weak affinity for all regions of DNA, there are about 8 sites on T7 DNA where extremely stable complexes can be formed (Hinkle and Chamberlin,



1972a, b). Core has an intermediate affinity for all regions of DNA so that in the absence of sigma, enzyme would take several hours to find a tight binding site. Sigma reduces the affinity of RNA polymerase for most regions of DNA but promotes binding at a few sites. As sigma alone does not bind detectably to DNA, it presumably acts by allosterically altering the conformation of core enzyme from a form suitable for transcribing DNA to one better able to bind at promoters.

The kinetics of formation of stable binary complexes show that the rate-limiting step is first order (Hinkle and Chamberlin, 1972b). This suggests that the dissociation of holoenzyme from non-promoter regions is rate-limiting since the binding of holoenzyme to DNA must be a second order reaction. Given the association-dissociation kinetics for holoenzyme binding to non-promoter regions, and the number of such regions, the formation of a stable complex is remarkably rapid. Thus, a substantial number of trial bindings may occur by ligand displacement reactions, wherein holoenzyme is transferred from one site to another without dissociation (Chamberlin, 1976). This model requires at least two sites on the holoenzyme for interaction with DNA, and there are several sites on RNA polymerase with a significant affinity for polynucleotides (Krakow, Rhodes and Jovin, 1976). Alternatively RNA polymerase might bind initially as a dimer (So *et al*, 1967). Ligandtransfer reactions have been postulated for the binding of core to DNA at single stranded breaks (Hinkle,



Ring and Chamberlin, 1972) and in the lac repressor-operator interaction (von Hippel et al, 1974).

When holoenzyme arrives at a promoter, it is thought to interact with parts of the nucleotides exposed in the grooves of the DNA duplex to form a closed promoter complex. Complexes are formed by the lac and  $\lambda$  repressors at their operators without opening up the DNA duplex (Wang, Barkley and Bourgeois, 1974; Maniatis and Ptashne, 1973). Moreover, alteration of the bases in promoter regions such as to change their appearance from outside the helix without affecting their base pairing properties can inactivate promoters (Stahl and Chamberlin, 1976). This is evidence for the preliminary formation of a closed promoter complex. It has proved difficult to demonstrate that such complexes are formed at some promoters, as they may be rapidly converted to open complexes (e.g. at the major T7 promoters).

Stable complexes are not formed on T7 DNA at low temperatures or in high salt, suggesting that the DNA duplex must be opened in these complexes. Mangel and Chamberlin (1974a, b, c) have exploited such conditions to search for closed complexes. They found evidence that at 0°C holoenzyme does become associated with T7 DNA at the same sites where stable complexes are formed at 25°C. The low temperature complexes seem to have the properties expected for closed complexes. They dissociate quite slowly, with a half-life (constant between 0° and 15°C) of about 40 minutes. Dissociation is stimulated by polyanions, probably by displacement reactions. Their



properties are comparable to those of core-DNA complexes which are also thought to be closed complexes.

There is no marked effect of temperature on the binding of core to DNA, and it is much more sensitive to attack by polyanions than is holoenzyme in open complexes (Hinkle and Chamberlin, 1972a). The effect of sigma on such core-DNA complexes is to dissociate them rather than convert them to open complexes (Hinkle and Chamberlin, 1972a). It is probable that core only initiates at ends or single strand breaks in double stranded phage DNA (Vogt, 1969; Hinkle, Ring and Chamberlin, 1972). Transcription by core is stimulated by single strand breaks, whereas holoenzyme is inhibited. Presumably such breaks act both as binding sites and initiation sites for core, but only as binding sites for holoenzyme (Dausse et al, 1972; Hinkle, Ring and Chamberlin, 1972).

It is thought that only open promoter complexes are competent for RNA chain initiation and that their formation from closed promoters involves the unwinding of the DNA duplex. It is known that the binding of RNA polymerase to  $\lambda$  DNA at 37°C leads to a detectable unwinding of the helix, equivalent to the opening of some 10 base pairs per holoenzyme (Saucier and Wang, 1972; Wang, 1974). No such unwinding was detected at 0°C, when only the closed complexes are believed to form.

The properties of open complexes have been reviewed by Chamberlin (1974, 1976). They are extremely stable under standard conditions but their stability is greatly reduced at low temperatures or high ionic strengths



(Hinkle and Chamberlin, 1971, 1972a, b; Seeburg and Schaller, 1975). They can rapidly initiate an RNA chain (Mangel and Chamberlin, 1974a). They have, by comparison to free holoenzyme, relatively low affinity for the initiation inhibitors rifampicin and polyanions (Hinkle, Mangel and Chamberlin, 1972; Hinkle and Chamberlin, 1972a, b); this property is the basis of an assay for the fraction of enzyme in these complexes (Mangel and Chamberlin, 1972a). Finally, they protect DNA within the complex from attack by restriction endonucleases (Allet *et al.*, 1974; Ludwig and Summers, 1976) and pancreatic DNAase (Heyden, Nüsslein and Schaller, 1972; Okamoto, Sugiura and Takanami, 1972).

When rifampicin and substrates are simultaneously added to preformed holoenzyme-DNA complexes, a fraction presumed to represent open promoter complexes rapidly initiates RNA synthesis and so escapes inactivation. Chamberlin and Ring (1972) showed that about 8 RNA polymerase molecules bound per T7 DNA molecule could rapidly initiate whereas other enzyme molecules (known to be less tightly bound, presumably at non-promoter sites) could not. By varying the rifampicin concentration they could distinguish three enzyme molecules which initiated more rapidly than the other five. The former are now believed to correspond to the three major promoter sites on T7 DNA and the rest to minor promoters (see Chapter 1, section 5). The dependence of rifampicin-resistant synthesis on the temperature at which enzyme and DNA were allowed to interact before drug addition is marked, and reminiscent of a DNA melting



profile (Hinkle, Mangel and Chamberlin, 1972). The mid-point temperature of this dependence curve is thought to measure the efficiency with which RNA polymerase can separate the DNA strands at a given promoter and is affected by ionic conditions and glycerol in a manner consistent with this hypothesis (Mangel and Chamberlin, 1974b, c). There is evidence that the ease of "melting" may control the selection of a particular promoter from a group of promoters (Stahl and Chamberlin, 1977).

A number of promoter regions have now been sequenced (Gilbert, 1976; Ptashne et al, 1976; Walz, Pirotta and Ineichen, 1976; Sanger et al, 1977) and the following conclusions have been drawn (reviewed in Gilbert, 1976; see Fig. 4).

1. In open promoter complexes, a DNA segment some 40 base pairs long is protected against nucleases. The initiation point lies near the centre of this segment.
2. A sequence similar to TATAAT is found in the non-coding strand centred about 10 bases before the initiation point. Mutations which produce base changes in this sequence have been described which either increase or decrease the efficiency of the promoter.
3. A sequence similar to TGTTGACAATT is found in the non-coding strand centred about 35 bases before the initiation point. Mutations which produce base changes in this region and decrease the efficiency of the promoter have been described.

It should be noted that RNA polymerase can neither bind to nor select the correct initiation point on the



40 base pair fragments purified from promoter complexes. This has been interpreted as showing that sequences outside the protected fragment are needed for the initial binding reaction. Thus it is possible that the formation of a closed complex involves the "recognition site" some 35 base pairs before the initiation site. The conversion to an open complex could involve the simultaneous opening of the DNA helix (presumably around the initiation site) and a conformational change in the enzyme so that it now binds to the 40 base pair sequence it protects. The second common sequence some 10 base pairs before the initiation point could be required for this change. It has been suggested (Pribnow, 1975) that this A-T rich sequence may be held in an unpaired configuration in the open complex. If this were so, then some 15 base pairs would have to be opened up, which seems to be greater than the current estimates (Dausse, Sentenac and Fromageot, 1976; Wang, 1974). Perhaps this sequence is not itself denatured, but is required for RNA polymerase to open the DNA around the initiation site.

b) Initiation

Initiation occurs in vitro at open promoter complexes by binding of a purine nucleoside triphosphate. The triphosphate group is conserved at the 5' end of the RNA because transcription proceeds in a 5' to 3' direction (Bremer et al, 1965; Maitra, Nakata and Hurwitz, 1967). As the apparent  $K_m$  for initiation is higher than that for elongation (Anthony et al, 1969), it is possible to define conditions such that only elongation can occur. Initiation



may then be primed by adding either dinucleotides or a higher concentration of one nucleoside triphosphate (Downey and So, 1970; Minkley and Pribnow, 1973; Dausse et al, 1972). The dinucleotides required vary between different promoters on T7 DNA. A comparison of the dinucleotides active at the major promoter A2 with the sequence of this promoter shows that there is a limited flexibility regarding the position of initiation (Minkley and Pribnow, 1973; Pribnow, 1975)

CU

AC            dinucleotides which prime

CA            from this site

AU

pppAUGAA    normal RNA sequence

ACCACATGAA    DNA sequence around

TGGTGTACTT    initiation site

Indeed transcription from the lac promoter in vitro starts with either pppAAUU (85%) or pppGAAUU (15%) (Maizels, 1973; Majors, 1975).

In general, the 5' sequences found in vitro are the same as those found in vivo.

### c) Elongation

By comparison to template binding and initiation, very little is known of the mechanism of elongation. Ribonucleoside 5' triphosphates are added sequentially to the 3' end of the growing chain, as directed by base pairing with the coding strand of the DNA. Pyrophosphate



is released. Shortly after the initiation of transcription the sigma factor is released, due perhaps to displacement by the RNA chain itself (Krakow and von der Helm, 1970). Released sigma can be recycled for further initiations (Travers and Burgess, 1969; Darlix et al, 1969). Release of sigma may convert RNA polymerase to a form more advantageous for elongation, having a moderate affinity for all DNA regions.

There must be considerable conformational changes involved in the transition from holoenzyme in an open promoter complex to core in an elongating complex. Indeed whereas some 40 base pairs of DNA are protected in an open complex, only about 26 are protected in an elongating complex (Rohrer and Zillig, 1977). Some 22 bases of RNA are also protected in elongating complexes on T7 DNA. All models for transcription assume that the nascent RNA is hybridised with the DNA and this hybrid is subsequently denatured with simultaneous reformation of the DNA duplex and release of free RNA. The length of hybrid remains unknown. Richardson (1975) has postulated the existence of a site within the elongating complex where this displacement reaction occurs so that free RNA is released from the complex rather than an RNA-DNA hybrid. Evidence has been presented (Rohrer and Zillig, 1977) that the non-coding strand of the DNA is less well protected than the coding strand against attack by a single-strand specific nuclease.

There must be some mechanism for checking the complementarity of the incoming nucleoside triphosphate with the



selecting base in the DNA and rejecting those substrates which do not fit. It is known from model reactions that RNA polymerase can make mistakes, though the figures quoted vary considerably with the template and conditions. The incorporation of GMP instead of AMP directed by poly d(A-T) has been reported as 1:1000 (Bick, 1975) or 1:40,000 (Springgate and Loeb, 1975). All non-complementary nucleoside triphosphates and even polyphosphate inhibit the incorporation of AMP directed by poly d(A-T) equally strongly (Rhodes and Chamberlin, 1974). This suggests that the site to which incoming nucleoside triphosphates initially bind does not have very stringent requirements, perhaps demanding merely a triphosphate group. Base pairing evidently plays no part in the initial binding. It is hypothesised that after binding, a conformational change occurs to bring the nucleotide into alignment for base pairing and that the catalytic centre is only brought into the correct position if base pairing occurs.

Studies on transcription in vitro have revealed sequence specific effects on the rate of elongation, although these can only be detected at low substrate concentrations. "Pausing" of RNA polymerase has been observed in the transcription of the lac operon, and the sequences at which "pauses" occur have been determined (Maizels, 1973). There was no common sequence at the 3' end of "paused" transcripts, but there was a G-C rich region in the transcript 8 to 10 bases before the 3' end. The effects of base changes in this region have been determined (Gilbert, Maizels and Maxam, 1974; Gilbert,



1976) and suggest that G:C to A:T changes in this region weaken or abolish "pausing", while A:T to G:C changes strengthen it. Thus, sequences already copied into RNA and located one turn of the DNA helix behind the growing point, affect the rate at which RNA polymerase can elongate. A model has been suggested based on the relative strengths of G-C rich RNA-DNA hybrids and DNA duplexes, holding that the enzyme cannot elongate if the RNA-DNA hybrid has not been disrupted (Gilbert, 1976). This could be significant for the termination of transcription and will be discussed below.

A similar phenomenon has been described for the transcription of T7 DNA (Darlix and Fromageot, 1974; Darlix, 1974). The position of elongating complexes within the early region of T7 was determined by electron microscopy (Darlix and Horaist, 1975). The "pauses" seem to occur at sites where rho can mediate the termination of T7 transcription (Darlix, 1973). Although comparable information is not available in other systems, it is possible that rho acts primarily as an RNA release factor at sites where transcription has "paused". Evidence for this will be discussed below.

In vitro "pausing" is clearly sequence-specific in a way not significantly related to the nature of the incoming nucleoside triphosphate. In vivo, where substrate concentrations are not limiting, the step-time for addition of a further nucleotide to U, C and G residues has been determined for E.coli (Manor, Goodman and Stent, 1969) from the frequency at which such residues are found at the



3' end of RNA chains. Though G is "covered" fastest and C slowest, there is only a threefold range. If this is to be a factor in the construction of termination sites, it seems necessary to postulate extended runs of pyrimidines, producing a deceleration sufficient to help RNA polymerase and/or rho factor to respond to the nucleotide sequence which actually codes for termination.

d) Termination and release of RNA product and DNA

The study of transcriptional termination has in the past been most concerned with determining the DNA sequences at which transcription stops. The release of RNA product and DNA has been less well examined. It was originally thought that there were two classes of termination - those where termination could occur in the absence of accessory proteins, and those at which such factors were required. It now seems that this distinction is artificial, and that sites may differ only in the extent to which termination occurs in the absence of protein factors.

A number of specific termination events have been studied in detail. Although they show some similarities, there seems to be no single model which fits all the results. These systems are discussed below.

(i) Lambdoid phages

In the absence of other protein factors, the RNA transcribed from  $\lambda$  DNA by E.coli RNA polymerase in vitro originally appeared to be rather heterogeneous in size and, at 0.1 M KCl, little RNA was released. Analyses providing better resolution have since revealed many transcripts of



discrete sizes, including 4S and 6S (Lebowitz, Weissman and Radding, 1971; Blattner and Dahlberg, 1972) and large transcripts of molecular weights  $6 \times 10^5$ ,  $1 \times 10^6$  and  $2.3 \times 10^6$  (Roberts, 1975). The origin and orientation of these transcripts on the  $\lambda$  map have been determined by hybridisation studies (Fig. 1). A protein factor, rho, was discovered which profoundly affected the pattern of transcription in vitro (Roberts, 1969) such that it more closely resembled N-independent transcription in vivo (Kourilsky et al, 1970). In the presence of rho, the 4S and 6S species were still synthesised but new transcripts of size 7S and 12S were made rather than the larger molecules described above (Roberts, 1969, 1970, 1975). Evidence was presented that these new species arose by rho-induced termination of transcription at specific sites, which were read through in the absence of rho. There is now considerable evidence that the positive regulatory protein N (which is needed for normal expression of all  $\lambda$  late genes) acts by antagonising rho-induced termination to allow efficient read through in vivo (Heinemann and Spiegelman, 1970; Adhya et al, 1974). Inoko and Imai (1975) have shown that E.coli mutants which allow expression of  $\lambda$  late genes in the absence of N produce an altered rho protein.

Two classes of termination sites were thus postulated, rho dependent and rho independent. It was suggested that RNA can be released from rho independent sites, but that in vitro few enzyme molecules reach distant sites due perhaps to random inactivation. Thus, the small amount



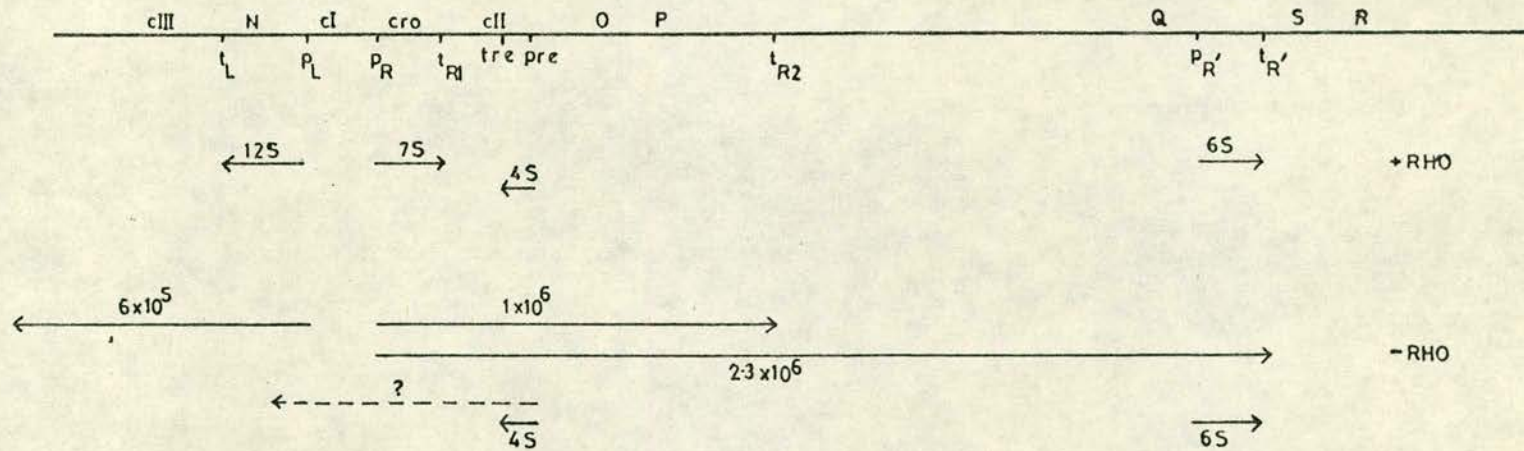


Fig.1 TRANSCRIPTION ON LAMBDA IN VITRO



of RNA released in vitro in the absence of rho, consisted mainly of the short 4S and 6S RNAs. In the presence of rho, much more RNA was released, presumably mainly from rho dependent sites (Roberts, 1970).

Using DNA from a  $\lambda$  mutant which had a new promoter beyond the rho dependent site  $t_{R1}$ , Roberts (1970) found that rho increased the frequency of in vitro termination at  $t_{R2}$ , but did not affect termination at  $t_R$  (see Fig. 1). The intermediate requirement for rho at  $t_{R2}$  showed that the original description of two classes of termination site was too simple. There is now considerable evidence that all  $\lambda$  termination sites are more or less affected by rho. Rosenberg et al (1975) have shown that rho increases the yield of  $\lambda$  4S RNA in vitro. They point out that this is not necessarily due to more efficient stopping: it might instead reflect faster recycling of enzyme due to rho catalysed release of RNA and/or enzyme from the DNA. It is also clear that some termination sites are inefficient even in the presence of rho. For example, there must be readthrough of  $t_{R1}$  in vivo (although rho is presumably active) since  $\lambda$  mutants with deletions of  $t_{R2}$  do not depend on N for late gene expression (Roberts, 1975).

The nucleotide sequences for the 4S and 6S RNAs produced in vitro have been determined (Lebowitz, Radding and Weissman, 1971; Dahlberg and Blattner, 1973). Remarkably, both have the 3' terminal sequences RUUUUUUA<sub>OH</sub> preceded by a G-C rich region which can be formed into a hairpin loop. An identical sequence has been found for the 3' end of the  $\lambda$  4S RNA produced in vivo (Smith and



Hedgpeth, 1975). Moreover, there was addition of a variable, small number of A residues (not coded in the template) at the 3' end, both in vivo and in vitro (Smith and Hedgpeth, 1975; Rosenberg, Weissman and de Crombrughe, 1975). The effect of rho in vitro on these sequences has been examined, and whilst the 3' addition of A residues was not affected, transcripts were found with an extra 3' U residue which was template coded, giving the sequences  $\text{RUUUUUUAU}_{\text{OH}}$ . The effect on the 6S RNA was slight, with perhaps 20% of the molecules carrying the extra U, but 70% of the 4S RNA molecules showed this alteration (Rosenberg et al, 1975). In fact, the increment in 4S RNA molecules produced in the presence of rho correlated quite well with the number carrying an extra U.

The sequence of the  $\lambda$  7S RNA is being studied, and that portion which codes for the cro protein has been published (Roberts et al, 1977). Within this sequence there is a G-C rich region followed by  $\text{AUUUUUUA}_{\text{OH}}$ . There is no evidence that termination ever occurs at this site in vitro. It might therefore be significant that the G-C rich sequence cannot form a hairpin loop whereas the analogous sequences near the  $\lambda$  4S and 6S termini can. The sequence for  $t_{\text{R1}}$  has not been published yet, but it is reported to be quite different from that at the 4S and 6S termination sites. By comparison with another sequence at which rho dependent termination occurs in vitro, the sequence CAATCAA may be important in  $t_{\text{R1}}$  (Kupper et al, 1978; Rosenberg et al, 1978; see Fig. 4). Furthermore, it has been reported in Bertrand et al, (1977) that mutations which alter the



efficiency of termination at  $t_{RL}$  have base changes in a region 20 to 30 bases before the 3' end of the RNA.

Small RNA molecules analogous to the  $\lambda$  4S and 6S RNAs have been described for the phages  $\phi 80$  and P22 (Roberts et al, 1976). All seem to be "leader" molecules whose termination in vivo is controlled so that under certain conditions genes distal to the termination site are expressed. The  $\lambda$  4S RNA is thought to arise by initiation at the promoter  $p_{pre}$ , and readthrough in the presence of the cII and cIII gene products (Spiegelman et al, 1972) allows the expression of the gene for  $\lambda$  repressor (Reichardt and Kaiser, 1971). The  $\lambda$  6S RNA appears to be initiated at the promoter  $p_R'$  and under the influence of a positive regulator (the product of gene Q), readthrough occurs to allow expression of late genes (Roberts, 1975). The  $\phi 80$  RNA equivalent to the  $\lambda$  4S RNA has the 3'-terminal sequence CUUUU<sub>OH</sub>, preceded by a G-C rich region which can form a hairpin loop (Pieczenik, Barrell and Geftter, 1972). P22 mutants have been described which lysogenise their hosts with extremely high efficiency (Hong, Smith and Ames, 1971). When DNA from such mutants is transcribed in vitro, the RNA thought to be the "leader" for repressor synthesis is either not synthesised (perhaps because the termination site has been deleted) or it has an altered sequence (Roberts et al, 1976).

In conclusion, a number of termination sites have been described in lambdoid phages. Termination at these sites depends to a varying degree on rho in vitro, but sequence analysis has shown that even at apparently rho independent



sites, rho can affect the nucleotide at which termination occurs. It should be noted that under appropriate conditions, all the sites can be readthrough, and this may impose constraints on the sequences at these sites in addition to those necessary for termination of transcription. The factors affecting the balance between readthrough and termination differ at the sites -

$t_{R1}$ , $t_{R2}$ and $t_L$	rho and $\lambda$ N protein
$t_R'$	$\lambda$ Q protein (and rho?)
tre	$\lambda$ cII and cIII proteins and rho

Sequence analysis has shown that there are common features in the 3' termini of the RNAs produced at some but not all these sites (see Fig. 4), and that there is little in common in the DNA sequences beyond the last nucleotide (Rosenberg, de Crombrughe and Musso, 1976). Indeed, sequences some way back into the RNA may be instrumental in causing the termination of transcription. Until base changes which alter the efficiency of termination have been determined in a sufficient number of cases it will be difficult to identify the features crucial in defining a termination site.

(ii) The trp operon of E.coli

The control of trp transcription has been studied in great detail by Yanofsky and co-workers. Two regulatory sites have now been described (Fig. 2). One, the operator, is a site at which the trp repressor protein can bind, in the presence of tryptophan, to block transcription from the trp promoter (Rose et al, 1973). However, repression is



Figure 2. The attenuator site in the *trp* operon of *E. coli*

The horizontal arrows beneath the DNA represent symmetrical sequences at the attenuator site. Their function (if any) in termination of transcription at this site is unclear, though at least part of the symmetrical sequence beyond the termination site can be deleted without affecting termination in vivo and in vitro, or the control of termination in vivo. The vertical arrows above and below the RNA show the positions where termination occurs within this site. Their lengths are proportional to the frequency of termination at that base pair in the DNA.



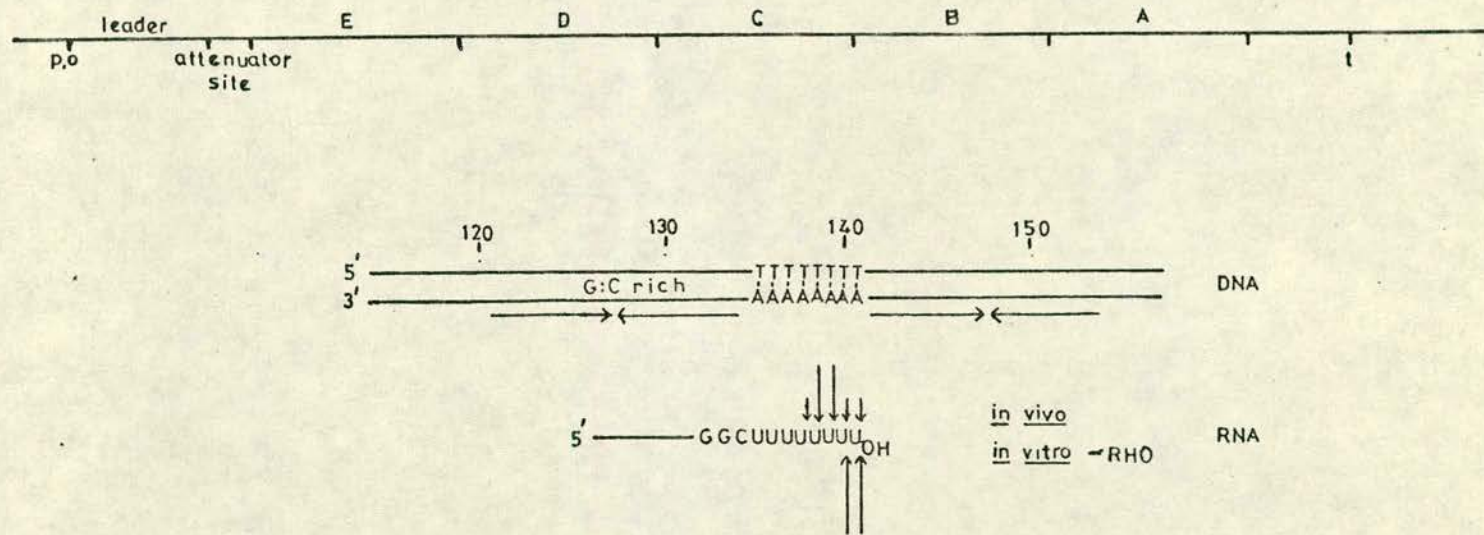


Fig. 2 The attenuator site in the *trp* operon of *E. coli*



not absolute, and the residual (or basal) transcription is increased in some internal deletion mutants of the trp operon (Jackson and Yanofsky, 1973). These deletions do not affect the initiation of transcription (nor its repression), but define a site, the attenuator, which lies within a leader sequence preceding the first gene, trpE (Bronson et al, 1973; Bertrand et al, 1975). The attenuator appears to be a site at which in the presence of excess tryptophan in vivo, some 85% of the RNA polymerase molecules terminate (Bertrand et al, 1975, 1976; Squires et al, 1976; Pannekoek, Brammar and Pouwels, 1975). Starvation for tryptophan (but not other amino-acids) interferes with the termination of transcription at this site, allowing more transcription to proceed into the structural genes (Bertrand and Yanofsky, 1976). The major effect of starvation for tryptophan is the derepression of the operon, but even in trpR<sup>-</sup> strains (where repression cannot occur so that expression of the operon is constitutive), starvation has a pronounced effect on trp gene expression, provided that the attenuator site is present (Bertrand and Yanofsky, 1976).

Termination of transcription at the attenuator occurs efficiently in vitro, in the absence of accessory proteins (Lee et al, 1976). The 3' terminal sequences - C(U)<sub>6</sub>U<sub>OH</sub> and - C(U)<sub>7</sub>U<sub>OH</sub> were detected in about equal amounts, and the sequence preceding the terminus includes a G-C rich region capable of forming a hairpin loop (Fig. 2), as also found for the small  $\lambda$  RNAs. It was reported that the ternary complex of enzyme, RNA and DNA did not dissociate



in vitro (Lee et al, 1976). Possible effects of rho have not been examined.

There is considerable evidence that in vivo, other protein factors affect the efficiency of this termination site. Korn and Yanofsky (1976a) isolated a number of mutants which suppressed polarity in both the trp and lac operons simultaneously. These mutants produce an altered rho factor (Korn and Yanofsky, 1976b). In some of these strains, readthrough of the trp attenuator site was increased several-fold. This is strong evidence that rho mediates the transcriptional termination at this site in vivo even though it is not required in vitro.

Pouwels and van Rotterdam (1975) have reported the partial purification of a protein factor needed for increased readthrough in vitro.

Morse and Morse (1976) and Yanofsky and Soll (1977) have presented evidence that the effect of tryptophan starvation on the frequency of readthrough in vivo is mediated by the concentration of tryptophanyl tRNA<sup>trp</sup>. Treatments which reduced the extent to which tRNA<sup>trp</sup> was charged with tryptophan reduced the efficiency of termination at the attenuator. There was evidence that trp tRNA synthetase was not directly involved in this control; only the level of trp tRNA<sup>trp</sup>. Morse and Morse (1976) also presented evidence for a possible link between translation and the control of trp attenuation. In relA<sup>-</sup> mutants (which have an altered ribosomal protein, and do not produce ppGpp on amino-acid starvation), starvation for tryptophan does not cause as much readthrough as in



relA<sup>+</sup> strains. The molecular mechanisms of the trp tRNA<sup>trp</sup> and relA effects remain unclear, but they might be related to the discovery of Platt et al (1976) that there is a ribosome binding site (and potentially translatable tryptophan codons) in the leader sequence (Lee et al, 1976).

The 3' terminal sequences of the leader RNA found in vivo differ from those found in vitro, in that most terminate with - C(U)<sub>4</sub>U<sub>OH</sub> and - C(U)<sub>5</sub>U<sub>OH</sub> with only a little (Bertrand et al, 1977).  
- C(U)<sub>3</sub>U<sub>OH</sub>, - C(U)<sub>6</sub>U<sub>OH</sub> and - C(U)<sub>7</sub>U<sub>OH</sub> This may reflect the presence in vivo of the factors discussed above.

The effects of various deletions with known end points have been reported in the same paper. The results show that sequences beyond position 150 (see Fig. 2) are not needed for termination in vitro or in vivo, nor for the in vivo effects of rho and tryptophan starvation, whereas deletions extending back to position 120 eliminate all these processes. Most interestingly a mutant which deletes the last four of the eight A:T base pairs in which termination normally occurs shows partial attenuator function. Leader RNA molecules with the 3' sequences - C(U)<sub>3</sub>U<sub>OH</sub> and - C(U)<sub>4</sub>G<sub>OH</sub> are found in vivo but the efficiency of termination is only 50% of normal. No termination could be detected at this region of the mutant DNA in vitro, even in the presence of rho. This latter finding was consistent with the observation that rho mutations do not increase readthrough in vivo with this deletion, whereas tryptophan starvation does (Bertrand et al, 1977). Apparently sequences important for rho dependent termination have been deleted whereas sequences needed for the control of termination by



tryptophan starvation have been conserved. Conceivably the residual termination detected in vivo corresponds solely to the tryptophan starvation mechanism, whilst termination by a distinct rho mechanism is abolished. The former mechanism may generate the shorter of the 3' terminal sequences detected in vivo while rho dependent termination may be responsible for the longer sequences. According to this model, in vitro termination would correspond closely to the mechanism which responds to rho in vivo.

In the case of the small  $\lambda$  RNAs, terminated at sites where both rho dependent and rho independent termination can occur, the rho mechanism leads to the production of transcripts longer by one nucleotide. It would be of great interest to determine whether the 3' sequences of the trp leader RNAs are altered in rho mutants: in particular whether the levels of - C(U)<sub>6</sub>U<sub>OH</sub> and especially - C(U)<sub>7</sub>U<sub>OH</sub> are reduced.

Mitchell, Reznikoff and Beckwith (1976) have defined a transcriptional terminator at the distal end of the trp operon, some distance beyond the end of the final gene, trpA. Guarente, Mitchell and Beckwith (1977) have made deletions which fuse the lac operon of E.coli to trp such that expression of the lac operon depends on readthrough of this trp terminator. Mutations to a Lac<sup>+</sup> phenotype were selected. Some of these mapped in the trp region and might be deletions or base changes of the trp terminator which allow increased readthrough. Another mutation to Lac<sup>+</sup> mapped at the rho gene, suggesting that rho acts at this site at the end of the trp operon in vivo.



Sites analogous to the trp attenuator are thought to be present in other amino-acid biosynthetic operons including his in Salmonella (Kasai, 1974; Lewis and Ames, 1972) and ilv in E.coli (Wasmuth and Umbarger, 1973).

(iii) T7 and T3 phages

In the absence of other proteins, most of the RNA transcribed from T7 DNA by E.coli RNA polymerase is of discrete size, about  $2.4 \times 10^6$  (Maitra et al, 1970; Millette et al, 1970; Peters and Hayward, 1974a, b). This polycistronic RNA arises by initiation at three closely grouped sites near 1.3% on the T7 map and termination at about 19.3% (Studier, 1972, 1973; Hyman, 1971; McDonell, Simon and Studier, 1977). These figures represent distances from the genetically defined left end of the T7 DNA molecule (Fig. 3). The T7 RNAs made in vivo by host RNA polymerase are monocistronic and can be reproduced in vitro by cleavage of the polycistronic RNA with RNAaseIII (Dunn and Studier, 1973a). The large RNA can also be found in vivo in RNAaseIII deficient hosts (Dunn and Studier, 1973b).

If the 19.3% termination site, t1, is deleted, a second site, t2 is revealed. This maps at about 30% (Studier, 1972). Peters and Hayward (1974a) have shown that t2 is also functional in the absence of other protein factors in vitro, although less efficiently than t1. Termination of transcription at t1 appears to be about 90% efficient in vitro.

Rho independent termination has been reported at sites to the left of t1. At high E: DNA ratios and in the



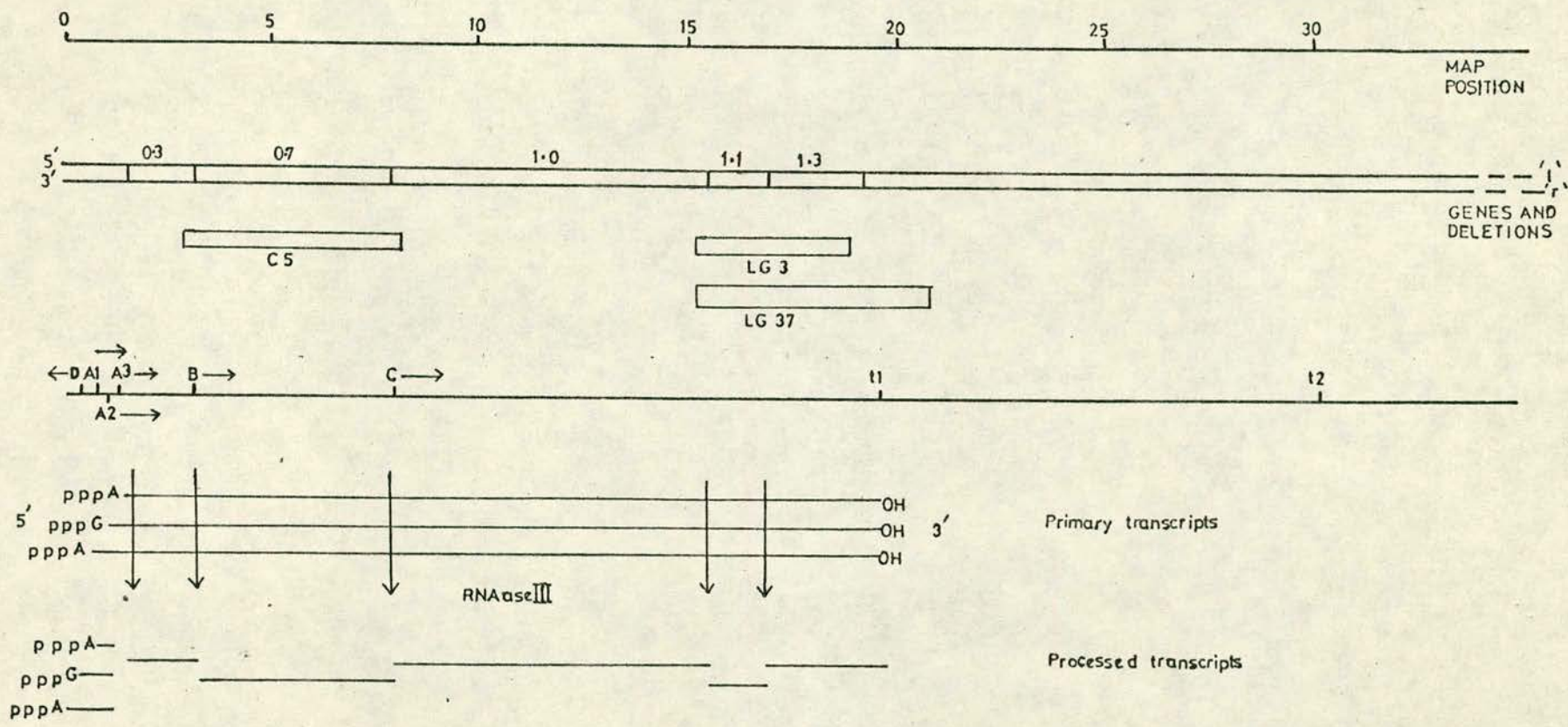


Fig.3 Transcription of T7 early region by *E. coli* RNA polymerase



presence of rifampicin, Minkley and Pribnow (1973) found evidence that termination occurred between some of the early genes. Such termination is probably artefactual. Stahl and Chamberlin (1977) have shown that when enzyme is bound at a minor promoter site between two of the early genes, it is relatively slow to initiate an RNA chain. Thus, when rifampicin and substrates were added together, the enzyme is inactivated before it can initiate. However, such inactivation does not lead to dissociation of the E-DNA complex, which then acts as a physical barrier to elongating enzymes and mimics a termination site.

The effect of rho on the transcription of T7 DNA has been examined by a number of groups, but the results are conflicting. Darlix (1973) has presented evidence that rho may cause termination at sites between the genes, possibly close to sites at which RNAaseIII cleavage occurs. Indeed, cleavage of T7 RNA produces 3' sequences CC(C)UUUAU<sub>OH</sub> which are reminiscent of those found on the  $\lambda$  4S and 6S RNAs (Rosenberg, Kramer and Steitz, 1974). Darlix and Horaist (1975) have shown that RNA polymerase "pauses" within these same regions if the nucleoside triphosphate concentrations are low, and that rho can act to release the RNA from the "paused" complexes. It has been suggested that the sequences responsible for "pausing", cleavage and rho induced termination may be evolutionarily related, but there is little evidence for this in other systems.

Dunn and Studier (1973a) reported that rho did not allow production of T7 transcripts of defined size



in vitro, but it did prevent the formation of full-sized RNA. Moreover, DNA with deletions of the region to the left of gene 1 showed less rho stimulated termination. These results are not irreconcilable with those of Darlix and Horaist (1975). Rho dependent termination to the left of gene 1 could also explain the observed variations in the stoichiometry of T7 early RNAs in vivo (Hercules et al, 1976).

Though T7 and T3 are closely related (Davis and Hyman, 1971), the pattern of transcription on T3 is significantly different. The T3 terminator analogous to t1 functions in vivo, but not in vitro (Davis and Hyman, 1970; Dunn, McAllister and Bautz, 1972a; Chakrabarty et al, 1974). Evidence has been presented that in the presence of rho, in vitro transcription is restricted to the early region (Dunn, McAllister and Bautz, 1972b).

Mutant strains of E.coli producing altered rho protein are now available (see Chapter 1, section 3). Kiefer, Neff and Chamberlin (1977) have examined the effect of such mutations on the extent of readthrough of the t1 sites of both T7 and T3. Readthrough was monitored by the synthesis of late proteins following infection of non-permissive hosts with phages which have a gene 1 amber mutation. Gene 1 codes for an RNA polymerase which is required for normal expression of late genes, so that expression of late genes by a gene 1 mutant should only be by readthrough of t1. Kiefer et al concluded that the rho mutations tested did not significantly increase readthrough.



However, it is known that the effectiveness of a particular rho mutant varies markedly depending on the termination site examined. It would be desirable to repeat these experiments with other rho mutants, and to examine the effects directly by studying the RNA produced at various times after infection. The effect of one rho mutation (suA) on T7 early RNAs was actually examined before the nature of the lesion was fully understood (Summers, 1971). Gel analyses of the RNAs showed some significant effects; in particular, new large RNA species (larger than any of the monocistronic RNAs) were produced in the mutant.

The release of polycistronic T7 RNA from t1 in vitro has been reported by several groups to occur only at high ionic strength (Millette et al, 1970; Maitra et al, 1970). Though the release of enzyme was not directly examined, reinitiation occurred, implying that at least some enzyme was released in an active form. More recently, Schäfer and Zillig (1973) reported that the release of RNA occurs at both high and low ionic strength, but that at low ionic strength enzyme is not released. Furthermore they reported that sigma was associated with the enzyme at t1 following release of RNA at low ionic strength. Finally, using  $\gamma$ -<sup>32</sup>P-NTPs to assay initiations, in conditions of low ionic strength and low E:DNA ratios, Chamberlin and Ring (1972) observed an early burst of initiations followed by a lag equivalent to the time taken for transcription of the T7 early region. There were then more initiations, which is evidence that at least some of the enzyme molecules are released and can re-initiate. The discrepancies between



these studies may be a function of the detailed conditions used, and the nature of the DNA and enzyme preparations. Much of the work to be presented below is an investigation of RNA and enzyme release from T7 DNA.

The complete sequence of t1 has not been determined. The 3' terminal nucleoside of the polycistronic RNA made in vitro was originally reported to be mainly U (Maitra et al, 1970; Millette et al, 1970). The latter group also reported the generation by T<sub>1</sub> RNAase of a unique 3' terminal decanucleotide, implying the sequence - G(X)<sub>9</sub>U<sub>OH</sub> (where X = A, C or U). This result appears to be compatible with the type of U-rich sequences reported for the λ 4S and 6S RNAs. However, attempts made by the same group to extend the known sequence appear to have run into unexplained difficulties (R. Millette, personal communication). On the other hand, studies carried out in Edinburgh have indicated that the 3' terminal RNA sequence is - CCC<sub>OH</sub> and that the next base coded by the DNA at t1 is G (Peters and Hayward, 1974a, b). A 3' terminal nucleoside of C was found for RNA terminated at t2 with A as the next base coded by the DNA (Peters and Hayward, 1974a). These results were highly specific. It remains difficult to reconcile such contrasting findings. The availability of more direct and rapid methods for sequencing DNA (Sanger and Coulson, 1975; Maxam and Gilbert, 1977) should soon allow the complete sequence to be determined.



(iv) The *supF* gene coding for tRNA<sup>tyr</sup> in *E. coli*

The study of transcription on T7 DNA is relatively simple, as there is in essence only one operon for *E. coli* RNA polymerase present. This is not generally the case, for example in the  $\lambda$  and  $\phi 80$  transducing phages (and deletion derivatives) which have been extensively used to study transcription. Now that templates can be dissected with restriction endonucleases, fragments of DNA carrying a single operon, promoter or termination site can be purified and studied.

One study of this kind has been made with fragments of DNA containing the promoter for the *supF* gene of *E. coli* (Küpper et al, 1976). More recently, a rho dependent termination site has been located some distance beyond the end of the region coding for mature tRNA (Küpper et al, 1978). The site is partially effective even in the absence of rho, while essentially all transcription terminates there in the presence of rho. The RNA produced in vitro shows variable termination within a sequence CAAUCAAAUUAU, the principal termini being - CAAAUA<sub>OH</sub> and - CAAAUAU<sub>OH</sub> (Fig. 4).

The sequence beyond that coding for the mature tRNA has been determined. It shows a remarkable triple reiteration of a 178 base pair sequence, with very few differences between the repeats. The site at which transcriptional termination occurs is in the second repeat, and the observed results suggest that whereas very little termination occurs at the sequence CAATTAAATAT, the sequence CAATCAAATAT gives efficient rho dependent termination.



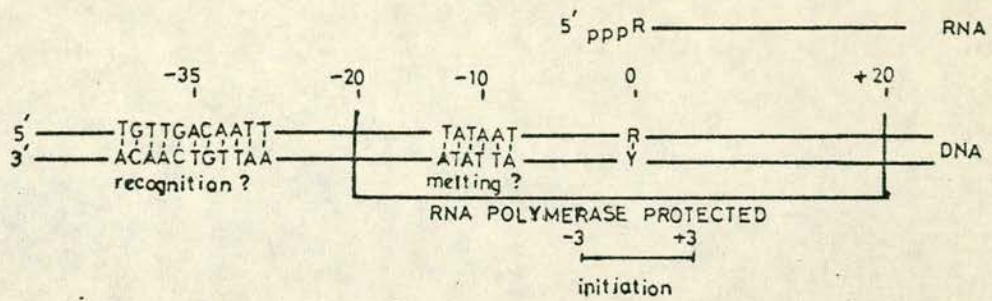
Figure 4. General structure of a promoter

Sequences, found at many promoter sites, which are thought to have a role in promoter function are shown relative to the point where initiation of transcription occurs. Also shown are the limits within which initiation can take place and the extent of the sequences which RNA polymerase bound at the promoter site protects from DNAase.

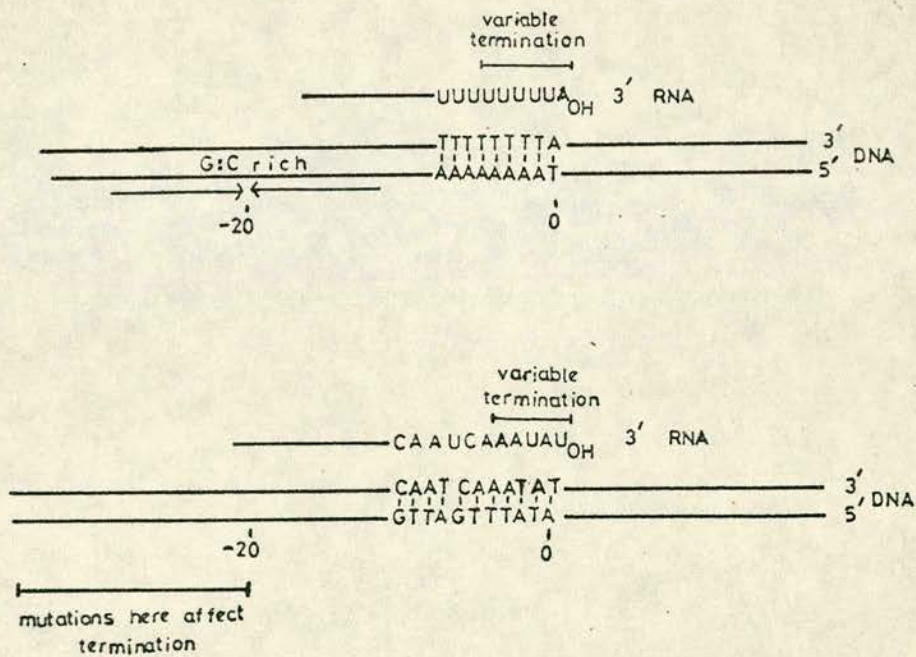
Structures found at some termination sites

Sequences and symmetry elements (shown by horizontal arrows) which seem to be important for termination of transcription are shown. The limits within which termination occurs are also shown. The upper structures are found at several sites where termination can occur in vitro in the absence of rho, though rho may act at such sites in vivo. The lower structure has been found at two termination sites including  $t_{R1}$  of  $\lambda$  where termination seems completely dependent on rho in vitro and in vivo. The region where base changes affect termination at  $t_{R1}$  in vivo is shown.





General structure of a promoter



Structures found at some termination sites

Fig. 4



(The sequence CAATCAA has also been found at a rho dependent  $\lambda$  terminator,  $t_{RL}$  (Rosenberg et al, 1978).) In contrast to the  $\lambda$  4S and 6S, and the trp attenuator RNA termini, no hairpin loop can be envisaged in the RNA just preceding the 3' terminus. The nearest potential hairpin is some 50 bases back from the 3' end, in contrast to the 10-15 bases at the other sites.

By increasing the ionic strength, termination at the above site can be suppressed, and it then occurs at the analogous point in the third repeat. This has the same CAATCAAATAT sequence as the second repeat, but a hairpin loop could be formed by the RNA immediately preceding this sequence.

The release of RNA and enzyme from these sites have not been reported, and there is no evidence about the activity of these terminators in vivo.

#### (v) Other systems

Much early work on transcription used T4 DNA as a template. Unfortunately, the termination of T4 transcription is still a confusing subject. At low ionic strength, for example, in 0.05 M KCl, RNA synthesis reaches a plateau, consistent with a single round of transcription, and the RNA product and enzyme are not released (Bremer and Konrad, 1964; Maitra and Hurwitz, 1967). At higher ionic strength, for example 0.2 M KCl, RNA synthesis is maintained for long periods. Both RNA and enzyme are released, allowing reinitiation to occur (So et al, 1967; Maitra and Barash, 1969). The T4 RNA produced at low ionic strength is



shorter than that which accumulates as free RNA at high ionic strength (Richardson, 1970a, b; Schäfer and Zillig, 1972). Thus there are sites on T4 DNA where E.coli RNA polymerase stops transcription at low but not at high ionic strength. The physiological significance of these sites, if any, is unknown.

It is clear that ionic strength can markedly affect the termination of transcription in vitro, and it is interesting to note that rho activity is affected by ionic strength, but that the extent of inhibition of rho at high ionic strength varies with the template. Rho is active with T4 DNA only at low ionic strength (Richardson, 1970b), but is active with  $\lambda$  and T7 DNA up to 0.1 M KCl (Roberts, 1969; Goff and Minkley, 1970) and with fd RF1 and T3 DNA at 0.2 M KCl (Takanami et al, 1971; Dunn, McAllister and Bautz, 1972b). The ATPase activity of rho with synthetic polynucleotides is reduced in parallel with its ability to catalyse termination on T7 DNA as the salt concentration is increased (Galluppi et al, 1976). This suggests that at least some of the effects of salt on the termination activity of rho may be due to effects on RNA-rho interaction.

Sequences similar to those on the  $\lambda$  4S and 6S RNAs may be observed at the 3' termini of an E.coli 6S RNA transcribed in vivo (- (U)<sub>6</sub>A<sub>OH</sub> : Ikemura and Dahlberg, 1973) and a Bacillus subtilis 5S rRNA precursor produced in vivo (- (U)<sub>6</sub>G(U)<sub>6</sub>G<sub>OH</sub> : Sogin et al, 1976).

It is also of interest that yeast 5S rRNA transcription may be terminated in a sequence of 29 successive T-A base pairs (Maxam et al, 1977).



Because of their small size, the double stranded form I DNA from single stranded DNA coliphages seemed suitable templates for the study of transcription. However, the pattern of transcription on such templates is rather complex. A number of promoters and termination sites have been described (Axelrod, 1976a, b; Hayashi et al, 1976). McMahon and Tinoco (1978) have searched the known DNA sequence of  $\phi$ X174 (Sanger et al, 1977) for sequences which resemble those found at other termination sites. Some 5 sequences were found, which map with termination sites active in vitro (Axelrod, 1976a). Together with the known positions of the promoters, these sites can account for the synthesis of most of the  $\phi$ X174 RNAs found in vivo (Hayashi et al, 1976). Moreover, from the frequency of the RNAs in vivo, the efficiencies of each of these termination sites has been calculated and correlated with their nucleotide sequences. Their calculations fit the model of Gilbert (1976), where the stability of the template duplex DNA (or of the mRNA-DNA hybrid, or both) determine termination of transcription, so that a termination site consists of a G-C rich region in the DNA followed by an A-T rich region.

##### 5. Bacteriophage T7

Bacteriophage T7 is a small virulent coliphage with a linear double stranded DNA genome whose size is estimated to be 40,000 base pairs (McDonell, Simon and Studier, 1977). For recent reviews see Studier (1972) and Hausmann (1976). The sequence of bases is unique with a small terminal



repetition estimated as about 110 base pairs in length on the basis of restriction site mapping (Ludwig and Summers, 1976).

Some 20 essential and 8 non-essential genes have been mapped and numbered from left to right along the genome (Studier, 1972; Fig. 3). Non-essential genes have been given decimal numbers indicating their positions relative to the essential genes. For example the non-essential gene for T7 lysozyme is called 3.5 as it maps between the essential genes 3 and 4.

The proteins induced by T7 can be classified into 3 groups according to the time course of their synthesis during infection. Class I are synthesised up to 8 minutes after infection at 30°C and are the products of genes 0.3 to 1.3, which occupy the leftmost 20% of the T7 map. They include a DNA ligase, an RNA polymerase and several proteins which inhibit host functions, including transcription and the DNA restriction and modification systems. Class II are synthesised from 6 to 15 minutes post infection and are the products of genes 1.7 to 6, which code for proteins which degrade the host DNA and synthesise phage DNA. Class III are synthesised from 6 minutes till lysis and are the products of genes 7 to 20. They code for the proteins found in the phage particle, and proteins responsible for DNA maturation (Studier and Maizels, 1969; Studier, 1972).

The appearance of all class II and III proteins is almost, if not wholly dependent upon a functional gene 1 product. This has been identified as an autonomous RNA



polymerase with a high specificity for T7 DNA and is necessary for the normal transcription of class II and III genes (Chamberlin et al, 1970). The class I genes are transcribed by E.coli RNA polymerase. All detectable transcription of T7 in vivo corresponds to one DNA strand, since all RNA is transcribed rightwards with respect to the standard genetic map (Summers and Szybalski, 1968; Fig. 3). Some 12 or 13 discrete transcripts appear during T7<sup>+</sup> infection (Summers, 1969). However, only 5 of these are made with a T7 gene 1 mutant, corresponding to T7 early RNAs for the class I genes (Siegel and Summers, 1970). By analysing the RNA and protein made in vivo by various deletion mutants, it has been shown that each early RNA codes for one protein, though perhaps 0.3 gene RNA codes for two (Summers et al, 1973; Studier, 1973).

Early RNA can compete with about 20% of the total phage specific RNA for hybridisation to "r" strand DNA (Siegel and Summers, 1970). Direct visualisation of hybrids between early RNA and "r" strand DNA showed that the RNA hybridised to a region from 1 to 20% along the genome (Hyman, 1971). The size of this region is that expected from the sum of the molecular weights of the early RNAs.

Examination of the transcription of T7 DNA by E.coli RNA polymerase by electron microscopy has shown that there is one major starting point at 1.3%, and a termination site at about 20% (Davis and Hyman, 1971; Delius et al, 1973). A similar analysis has identified positions where RNA polymerase can bind in the absence of substrates at 0.5, 1.0, 1.3, 1.6, 3.1, 8.0 and 92.8% (Bordier and Dubochet, 1974;



Koller et al, 1974; Delius et al, 1973). These sites are probably the tight binding sites of Hinkle and Chamberlin (1972). It should be noted that under normal conditions and at low E:DNA ratios, only the sites at 1.0, 1.3 and 1.6% seem to be active as promoters (Stahl and Chamberlin, 1977).

The termination observed at 20% by Delius et al (1973) occurred in the absence of rho, whereas that observed by Davis and Hyman was sometimes dependent on rho (see discussion in Summers, 1972). Whether Davis and Hyman's results were due to peculiarities of the phage, enzyme preparations or conditions used, all subsequent reports have indicated that termination <sup>occurs</sup> efficiently (85-95%) at this site in vitro in the absence of rho (e.g. Maitra et al, 1970; Millette et al, 1970). The RNA product has the molecular weight  $2.4 \times 10^6$ , consistent with transcription from 1 to 20% on the T7 genome. Moreover, this RNA codes for the cell-free synthesis of a T7 class I protein, T7 RNA polymerase (Maitra, 1970) though it should be admitted that the synthesis of a class II protein, T7 lysozyme, was also detected (Millette et al, 1970). However the lysozyme assay used was very sensitive, and this result almost certainly reflects a low level readthrough of the terminator, leading to some transcription of class II genes by E.coli RNA polymerase. Such readthrough has been observed, both in vitro (Peters and Hayward, 1974a) and in vivo (McAllister and Barrett, 1977).

Studier has described ligase deletion mutants, some of which have also deleted the 20% terminator t1 (Studier,



1972, 1973; Simon and Studier, 1973). With t1 deleted, there is a higher level of expression of class II and III genes by E.coli RNA polymerase. From the endpoints of deletions which do or do not affect t1, it was originally mapped by heteroduplex analysis to  $20.2 \pm 0.2\%$  (Simon and Studier, 1973). Recently this mapping has been revised on the basis of analysis with restriction endonucleases to  $19.3 \pm 0.2\%$  (McDonnell, Simon and Studier, 1977). From the size of RNA produced in vivo when t1 is deleted, a second terminator t2, has been mapped at about 30% (Studier, 1973). This site is also active in vitro in the absence of rho (Minkley and Pribnow, 1973; Peters and Hayward, 1974a). These deletion studies provide strong evidence that t1 functions in vivo and in vitro, and with similar efficiency.

Dunn and Studier (1973a) discovered a factor (which on purification was shown to be RNAase III) which cleaved the large polycistronic RNA synthesised in vitro into 5 major monocistronic RNAs corresponding to those found in vivo. When the 5' ends of the RNA were labelled in vitro (using  $-^{32}\text{P}$  ATP and GTP), three small RNAs were found after cleavage. They are generated by initiation of transcription at 1.0, 1.3 and 1.6% followed by RNAase III cleavage to the left of gene 0.3 (Fig. 3). These initiation sites are the promoters A1, A2 and A3. Pribnow (1975) has presented sequences for A2 and A3.

The 5' and 3' sequences of the molecules produced by cleavage in vitro are essentially identical to those of the in vivo transcripts (Kramer, Rosenberg and Steitz, 1974;



Rosenberg, Kramer and Steitz, 1974). There was variable addition of a few A residues to the 3' end of the RNAs found in vivo, as on the  $\lambda$  4S and 6S RNAs (see Chapter 1, section 4 d)(i)). This work provides strong evidence that post-transcriptional cleavage is the major mechanism for the generation of T7 early RNAs.

The function of this cleavage is less clear. It had been claimed that the unprocessed early RNA is not functional (Hercules et al, 1974), even though T7 does grow in hosts which have little RNAase III activity, producing a large polycistronic early RNA (Dunn and Studier, 1973b). However, a thorough study by Dunn and Studier (1975) indicated that both in vivo and in vitro, the absence of cleavage affects only the expression of gene 0.3 detectably. Steitz and Bryan (1977) have shown that there are two ribosome binding sites on gene 0.3 RNA and that cleavage is needed for full activity of the leftmost site. The existence of two sites may explain why two functions have been ascribed to gene 0.3 - one which inhibits host translation (Herrlich et al, 1974) and one which blocks the bacterial restriction and modification system (Studier, 1975).

Kinetic studies on the UV inactivation of T7 early genes also support a model in which these genes are transcribed from effectively one promoter, with the possible exception of gene 0.3 (Brautigan and Sauerbier, 1973).

The simple model predicts that equal molar amounts of the early RNAs should be synthesised in vivo, but this seems not to be the case (Summers et al, 1973; Young and Smith,



1973; Minkley, 1974). Though the RNAs for genes 0.7, 1.0, 1.1 and 1.3 are approximately equimolar, that for gene 0.3 may be present in ten-fold molar excess. Hercules *et al* (1976) have investigated the half-lives of the RNAs, and excluded variable messenger stability as a major explanation of this phenomenon. Their results suggest *in vivo* termination sites at the distal ends of genes 0.3 and 0.7. Termination at these sites has been observed *in vitro* in the presence of rho (Darlix, 1973; Darlix and Horaist, 1975). However, no discrete small RNA species were found in a RNAase III deficient host (Dunn and Studier, 1973b). This might imply that rho cannot produce specific termination *in vivo* at the above sites, or that the termination is greatly affected by differences in growth conditions between the various studies. On the basis of their *in vitro* results, Dunn and Studier (1973a) concluded that rho causes unspecific termination to the left of gene 1. They suggested that cleavage with RNAase III could account for the observed stoichiometry.

The simple model also predicts that none of the T7 early messenger RNAs found *in vivo* should have 5' triphosphate, as their 5' termini are generated by cleavage rather than initiation of transcription. A low level of 5' triphosphate has been detected on gene 1 RNA *in vivo* (Young and Smith, 1973) and Kramer *et al* (1974) detected a little of the 5' triphosphate groups on the messenger RNAs *in vivo* though 90% were on the initiator fragments. Minkley and Pribnow (1973) using specific dinucleotide primers were able to initiate transcription *in vitro* from



the minor promoters B and C. These promoters correspond to the binding sites at 3.1 and 8.0% (Fig. 3), and if they are used in vivo they could generate gene 0.7 and 1 mRNAs bearing 5' triphosphate groups.

It is clear that the major pathway for synthesis of T7 early RNAs is by RNAase III cleavage of RNAs initiated at the major promoters and terminated at t1. However, some of the RNAs may be terminated at rho dependent sites within the early region, and a small proportion may be initiated at minor promoters.



### 1. Bacteria and Bacteriophage

The strains of bacteria and bacteriophage used are described in Table 1.

### 2. Media

T-broth (Studier, 1969) contained 10 g Bacto Tryptone and 5 g NaCl in 1 litre distilled water.

L-broth (Lennox, 1955) contained 10 g Difco Tryptone, 5 g yeast extract, and 10 g NaCl in 1 litre distilled water.

High GCM medium (Ray and Schekman, 1969) contained 0.5% glucose, 1 mg/ml vitamin free casamino acids and 0.2 mM  $\text{Na}_2\text{HPO}_4$  in a 10-fold dilution of "concentrated minimal salts". "Concentrated minimal salts" contained 120 g Tris base, 3.5 g  $\text{Na}_2\text{SO}_4$ , 20 g  $\text{NH}_4\text{Cl}$ , 20 g KCl and 5 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  in 900 ml distilled water. pH was adjusted to 7.0 with HCl and the volume brought to 1 litre with distilled water.

Phage buffer was 3 g  $\text{KH}_2\text{PO}_4$ , 7 g  $\text{Na}_2\text{HPO}_4$ , 5 g NaCl, 1mM  $\text{MgSO}_4$ , 0.1 mM  $\text{CaCl}_2$  and 0.001% gelatin in 1 litre distilled water.

Cell buffer was 3 g  $\text{KH}_2\text{PO}_4$ , 7 g  $\text{Na}_2\text{HPO}_4$ , 4 g NaCl and 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in 1 litre distilled water.

For plating T7 phage, bottom agar was 10 g Bacto agar in 1 litre T-broth, and top agar 7 g Bacto agar in 1. litre T-broth.

For plating MS2 phage, bottom agar was 15 g Bacto agar in 1 litre L-broth. L-C top agar was 10 g Difco Tryptone, 5 g yeast extract, 5 g NaCl, 7 g Bacto agar, 5 ml 20% glucose and 10 ml 0.5 M  $\text{CaCl}_2$  in 1 litre distilled water.



Table I.

STRAIN	DESCRIPTION	SOURCE	REF.
<u>E.coli</u> B	su <sup>-</sup> , prototroph.	F.W.Studier	1,2.
<u>E.coli</u> 011	su <sup>+</sup> , Khsp, thy <sup>-</sup>	F.W.Studier	1,2.
<u>E.coli</u> BBW/1	B, su <sup>+</sup> , prototroph.	R.Hausmann	3.
<u>E.coli</u> K 38	K12, Hfr, su <sup>-</sup> , prototroph.	D.Ray	4.
<u>E.coli</u> 1113	K12, RNAase I <sup>-</sup> , RNAase II <sup>ts</sup> , PNPase deficient.	W.Gilbert	5.
<u>E.coli</u> MRE 600	RNAase I <sup>-</sup>	R.Elsworth	6.
T 7	wild type	F.W.Studier	7.
T 7 C5 LG3 am342a	C5 deletion 32-80%. LG3 deletion 14.6-18.2%. gene <sub>1</sub> amber 342a	F.W.Studier	7.
T 7 LG37	LG37 deletion 14.6-19.4%.	F.W.Studier	7.
MS 2	wild type	N.Willetts	8.

1. F.W.Studier *Virology* 39, 562, (1969)
2. F.W.Studier *Science* 176, 367, (1972)
3. R.Hausmann and B.Gomez *J.Virol.* 1, 779, (1967)
4. D.S.Ray *Virology* 30, 397, (1966)
5. W.Gilbert *J.Biol.Chem.* 247, 5404, (1972)
6. K.A.Cammack and H.E.Wade *Biochem J.* 96, 671, (1965)
7. M.N.Simon and F.W.Studier *J. Mol. Biol.* 79, 249, (1973)
8. J.H.Strauss jr. and R.L.Sinsheimer *J. Mol. Biol.* 7, 43, (1963)



### 3. Enzymes

1. RNA polymerase from E.coli (EC 2.7.7.6) was prepared as described below.
2. Highly purified DNA polymerase I of E.coli (EC 2.7.7.7.) was a generous gift from Drs W.Kelley and H.Whitfield Jnr. (Kelley and Whitfield, 1971). It was stored frozen at  $-50^{\circ}\text{C}$ .
3. Fragment A of E.coli DNA polymerase I, lacking 5' to 3' exonuclease activity (Klenow, 1971) was purchased from the Boehringer Corporation (London) Ltd, Lewes, Sussex, U.K. It was stored in 50% glycerol at  $-20^{\circ}\text{C}$ .
4. Deoxyribonuclease I from bovine pancreas (EC 3.1.4.5) was grade D from Worthington Biochemical Corporation, Freehold, New Jersey 07728, USA.
5. Ribonuclease A from bovine pancreas (EC 2.7.7.16) was grade RASE from Worthington. A 0.1 mg/ml stock solution in 0.05 M  $\text{NH}_4\text{HCO}_3$  was heated at  $100^{\circ}\text{C}$  for 5 min to destroy contaminating DNAase. It was stored frozen at  $-20^{\circ}\text{C}$ .
6. Phosphodiesterase I from snake venom (EC 3.1.4.1) was grade VPH from Worthington. A 1 mg/ml stock solution in 10 mM Tris-HCl (pH 8.5) was stored frozen at  $-20^{\circ}\text{C}$ .
7. Alkaline phosphatase from E.coli (EC 3.1.3.1) was from Whatman Biochemicals Ltd, Maidstone, Kent, U.K. A 0.5 mg/ml stock solution in 0.05 M  $\text{NH}_4\text{HCO}_3$  was heated at  $100^{\circ}\text{C}$  for 15 min, and stored frozen at  $-20^{\circ}\text{C}$ .
8. Polynucleotide kinase purified from T4 infected E.coli (EC 2.7.1.78) was either kindly given by A. Morrison and Professor K.Murray, or purchased from P-L Biochemicals, Inc., Milwaukee, Wisconsin 53205, U.S.A. It was stored in 50% glycerol at  $-20^{\circ}\text{C}$ .



9. Restriction endonuclease HpaI was purified from Haemophilus parainfluenzae and generously donated by J.C. Boothroyd. It was stored in 10% glycerol at 4°C.

10. Restriction endonuclease HpaII from Haemophilus parainfluenzae was also provided by J.C. Boothroyd. It was stored in 50% glycerol at -20°C.

11. Restriction endonuclease HindII from Haemophilus influenzae, strain d was kindly given by R. Thompson. It was stored in 50% glycerol at -20°C.

12. Termination factor rho ( $\rho$ ) from E.coli was a generous gift from L. Finger (Department of Chemistry, University of Indiana, Bloomington, Indiana 47401, USA). It was stored in 50% glycerol at -20°C.

13. Antibodies raised in rabbit against the sigma subunit of E.coli RNA polymerase were kindly given by Dr A. Ishihama. It was stored as a suspension in 50% saturated  $(\text{NH}_4)_2\text{SO}_4$  at 4°C.

14. Lysozyme (EC 3.2.1.17) from chicken egg white was grade LYS F from Worthington.

15. Albumin from bovine serum was step V from Armour Pharmaceuticals Company Ltd, Eastbourne, U.K. A 20 mg/ml solution in 50 mM Tris-HCl (pH 7.8) was frozen and thawed several times to destroy contaminating nucleases. It was stored frozen at -20°C.

#### 4. Nucleotides

Ribonucleoside- and 2'-deoxyribonucleoside-5'-triphosphates were purchased from P-L Biochemicals Inc. Solutions were neutralised with  $\text{NaHCO}_3$ , their concentrations checked by U.V. absorption spectrophotometry and stored at -20°C.



Ribonucleoside 2'-(3')-monophosphates were also from P-L. The 2'-deoxyribonucleoside-5'-monophosphates were from Sigma (London) Chemical Company, Kingston-upon-Thames, Surrey, U.K. 2'-deoxyguanosine was also a Sigma product.

Salmon sperm DNA from Calbiochem Ltd., Bishops Stortford, Hertfordshire, U.K., was dissolved at 2.5 mg/ml in 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA and stored at 4°C. The alternating copolymer, poly d(A-T), (sodium salt) was from Research Products, Miles Laboratories Ltd, Slough, U.K. It was dissolved at 1.25 mg/ml in 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA and stored frozen at -20°C. Poly r(U,G) containing randomly polymerised guanylic and uridylic acids in a ratio of 1:1 to 1:1.5 was from Miles and was stored at -50°C.

#### 5. Radiochemicals

The following isotopically labelled compounds were all purchased from the Radiochemical Centre, Amersham, Buckinghamshire, U.K.

1. ( $\gamma$ -<sup>32</sup>P) adenosine-5'-triphosphate, triethylammonium salt, PBl68, 2000 Ci/mmole.
2. ( $\alpha$ -<sup>32</sup>P) 2'-deoxyguanosine-5'-triphosphate, triethylammonium salt, PBl66, 140 Ci/mmole.
3. (U-<sup>14</sup>C) adenosine-5'-triphosphate, ammonium salt, CFB91, 0.6 Ci/mmole.
4. (5-<sup>3</sup>H) uridine-5'-triphosphate, ammonium salt, TRK289, 22 Ci/mmole.
5. (5-<sup>3</sup>H) cytidine-5'-triphosphate, ammonium salt, TRK339, 22 Ci/mmole.





6. (Methyl-<sup>3</sup>H) thymidine, aqueous solution, TRK120, 19 Ci/mmole.

7. (Methyl-<sup>3</sup>H) thymidine-5'-triphosphate, ammonium salt, TRK354, 15 Ci/mmole.

#### 6. Scintillation Chemicals

Butyl-PBD (5-(4-biphenyl)-2-(4-t-butyl phenyl)-1-oxa-3, 4 diazole) was from Fisons Scientific Apparatus, Loughborough, U.K.

PPO (2,5 diphenyl oxazole) was from International Enzymes Ltd, Windsor, U.K.

Soluene 350 tissue solubiliser and dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyl oxazolyl)-benzene) were from Packard Instruments, Ltd, Caversham, Berkshire, U.K.

NCS tissue solubiliser from Amersham/Searle was obtained through Hopkins and Williams Ltd, Chadwell Heath, Essex, U.K.

#### 7. Chromatographic and Electrophoretic Materials

DEAE-cellulose (DE52), phosphocellulose (P11), 3 MM filter paper, and the ion-exchange papers AE81 and DE81 were from Whatman. Sephadex Gr-75 was from Pharmacia (Great Britain) Ltd, 75 Uxbridge Road, London, U.K. Biogel A1.5m was from BIO-RAD Laboratories Ltd, Bromley, Kent, U.K. DNA-agarose was prepared from calf thymus DNA (Sigma, type V) and agarose (Sigma, type II) as in Schaller *et al* (1972). A mixture of xylene cyanol FF, orange Gr, and acid Fuschin dyes from G.T. Gurr Ltd, London, SW6, U.K., were used as markers for paper electrophoresis. N, N' methylene bisacrylamide, 2x recrystallised acrylamide and



N,N,N',N' tetramethyl ethylenediamine (TMED) were from Serva, obtained through Uniscience Ltd, Fulham, London, U.K. Ammonium persulphate (Analar grade) and coomassie brilliant blue were from BDH Chemicals Ltd, Dorset, U.K. Bromophenol blue was from Sigma. Ethidium bromide was grade B from Calbiochem.

#### 8. Other materials

Diethyl pyrocarbonate, dithiothreitol and 2-mercapto-ethanol were from Sigma. Sodium dodecyl sulphate (SDS), "specially pure" and Aristar grade urea were from BDH.

GF/C filter discs were from Whatman. Nitrocellulose filters (0.45  $\mu\text{m}$  pore size) were from Sartorius Membran-Filter GmbH, 34 Göttingen /FRG, Postfach 142, W. Germany.

Streptolydigin was a generous gift from Dr G.B. Whitfield, Jnr, of the Upjohn Company, Kalamazoo, Michigan 49001, USA. Rifampicin was kindly donated by Lepetit Pharmaceuticals Ltd, Maidenhead, Berkshire, U.K. Stock solutions of rifampicin and streptolydigin at 100 mg/ml in dimethyl-formamide were stored at  $-20^{\circ}\text{C}$ . Dilutions into 50 mM Tris-HCl, (pH 7.8), 50 mM NaCl, 1 mM EDTA were kept at  $4^{\circ}\text{C}$  and discarded after 4 weeks.

Polyethylene glycol was Carbowax 6000 from Union Carbide Ltd, Hythe, Southampton, U.K. Spermidine from Mann Research Laboratories Products was obtained through V.A. Howe, 88 Peterborough Road, London, SW6, U.K.

"Repelcote", a 0.2% solution of dimethyl dichlorosilane in carbon tetrachloride was from Hopkins and Williams.

Glass beads were Jencons Ballotini No. 11. Visking dialysis



tubing was from the Scientific Instrument Centre Ltd, Leake Street, London WC1, U.K. Sarkosyl NL35 (a 35% (w/v) solution of sodium lauryl sarcosinate) was from Geigy (U.K.) Ltd, Manchester, U.K.

All other products used were of Analar grade if possible.

#### 9. Special treatment of chemicals

Analar grade phenol was redistilled over metallic zinc in a system flushed with nitrogen. It was collected under 50 mM Tris-HCl (pH 7.8) which had been recently degassed, was saturated with nitrogen and stored under buffer in the dark at  $-20^{\circ}\text{C}$ .

CsCl was baked at  $180^{\circ}\text{C}$  for 18 hrs and stored in a dessicator.



### CHAPTER 3. METHODS

#### 1. Handling nucleic acids

To minimise degradation by trace nucleases, solutions to be used in nucleic acid work were if possible autoclaved, and apparatus was autoclaved or baked at 180°C for at least 4 hours. Solutions used for RNA were treated with 0.02% diethyl pyrocarbonate at 50°C for 3 hours and then autoclaved. Provided that they would not interfere with subsequent procedures, SDS or sarkosyl were added to 0.2%. Volatile or unstable solutions which could not be autoclaved were filtered through Millipore HA nitrocellulose filters (0.45 µm pore) into sterile bottles.

Polyallomer tubes were boiled in 10 mM EDTA for 10 min, rinsed with distilled water and autoclaved. Glassware was cleaned with persulphuric acid, rinsed with distilled water and dried. It was then siliconised by immersion in "Repelcote", dried at 120°C and rinsed with distilled water. Dialysis tubing was boiled in 10% NaHCO<sub>3</sub> and rinsed with distilled water. Bags were tied, filled with 10 mM EDTA and dialysed for 18 hours at 4°C in distilled water. The bags were finally stored in 0.1 mM EDTA at 4°C.

Disposable plastic gloves were worn throughout.

#### 2. Centrifugation

All high speed centrifugation was carried out in Beckman model L, L2-50 or L2-65B ultracentrifuges. Low and medium speed centrifugations were in the Sorvall RC2-B centrifuge. The rotors used, time, temperature and average "g" value are stated in the text.



### 3. Alcohol precipitation

Nucleic acid solutions in corex centrifuge tubes were made 0.3 M in potassium acetate (pH 5.4) and two volumes of absolute ethanol added. The mixture was kept at  $-20^{\circ}\text{C}$  for at least 2 hours, preferably overnight, and the precipitate collected by centrifugation (SS34 rotor, 20,000 x g, 20 min,  $4^{\circ}\text{C}$ ). By this procedure, solutions as dilute as 0.2  $\mu\text{g}$  nucleic acid/ml could be precipitated with acceptable efficiency.

### 4. Acid precipitation and counting of radioisotopes.

The incorporation of isotopically labelled substrates into nucleic acids was routinely assayed by acid precipitation. Aliquots up to 0.05 ml were absorbed onto 2.1 cm diameter Whatman GF/C filter discs and immediately immersed in ice-cold 5% (w/v) trichloroacetic acid containing 10 mM sodium pyrophosphate, in a specially designed plastic block. The filters were washed by gentle rotation for at least 20 min, and then the acid was replaced by 80% (v/v) ethanol for a further 10 min. The block and discs were dried at  $120^{\circ}\text{C}$  for 15 min. The discs were transferred to vials containing 5 ml of 0.5% (w/v) butyl-PBD in toluene and radioactivity measured in a Packard Tri-Carb scintillation counter. This method was also used to locate nucleic acids, e.g. after gel filtration or after centrifugation through a glycerol gradient.

To ensure accurate conversion of scintillation counts to nucleotide concentrations, the efficiency of counting was investigated. Aliquots of labelled nucleotide dried onto filter discs were counted as above and compared with similar aliquots in a water compatible scintillant



(Petri, 1972).  $^{14}\text{C}$  was counted with equal efficiency in both systems, but  $^3\text{H}$  on filter discs was only detected 66% as efficiently as in the Petri scintillant. However,  $^{14}\text{C}$  and  $^3\text{H}$  labelled nucleic acids were detected with equal efficiency in either system. The incorporation of  $^3\text{H}$  and  $^{14}\text{C}$  NTP in identical in vitro transcription reactions were compared and found to be equivalent provided that the specific activity of  $^3\text{H}$  NTP (as determined above) was appropriately corrected.

Presumably nucleic acid is precipitated on the surface of the filter, whereas smaller molecules can penetrate the filter, reducing the efficiency of counting of the weak  $\beta$  particles emitted by  $^3\text{H}$ .

##### 5. Growth of bacteriophage T7

Infectious T7 phage suspended in phage buffer were assayed by the usual double layer plaque method (Adams, 1959) with T-broth top and bottom agar. A stationary culture of the appropriate host in T-broth provided the indicator bacteria. A single culture could be used for up to one week.

Stocks of phage were produced by inoculating a single plaque (produced as above) into 5 ml of a fresh overnight T-broth culture of bacteria mixed with 15 ml of fresh broth in a 200 ml flask. Shaking at  $37^\circ\text{C}$  led to lysis in 3-4 hours. A drop of chloroform was then added and 0.5 g NaCl dissolved with shaking. Cell debris was removed by centrifugation (10,000 x g, 10 min, room temperature) and the supernatants stored at  $4^\circ\text{C}$ . Stocks usually had  $5 \times 10^9 - 10^{11}$  plaque forming units (pfu)/ml.



Large scale lysates (up to 5l) were grown as 500 ml portions in 2l flasks in a New Brunswick Gyrotory shaker. The pre-warmed T-broth was inoculated with one twentieth volume of overnight bacterial culture and vigorously shaken at 37°C till the cell density (measured with a Petroff-Hauser slide) reached  $10^9$  cells/ml. Bacteriophage were then added to  $10^8$  pfu/ml, and shaking continued till lysis (usually 2-3 hours).

To prepare  $^3\text{H}$ -thymidine labelled phage, the bacteria were grown in high GCM medium to  $5 \times 10^8$  cells/ml. The cultures were then made 8 mM in  $\text{MgSO}_4$  and 4 mM in  $^3\text{H}$ -thymidine deoxyguanosine (to inhibit the breakdown of thymidine) and / (18 mCi/mmmole) was added to 0.1 mM. Phage was then added to  $10^8$  pfu/ml and incubation continued till lysis, usually in 2 hours. DNA extracted from phage grown in this way had a specific activity of 4000-5000 cpm/ $\mu\text{g}$ .

To concentrate and purify the phage, the lysates were made 1% (v/v) with chloroform and 0.5 M in NaCl. After 10 min shaking at 37°C, the cell debris was either removed by centrifugation, (GSA rotor, 10,000 x g, 10 min, 4°C) or left to settle for 18 hours at 4°C and the supernatant siphoned off. The supernatant was made 6% (w/v) in polyethylene glycol (PEG) and after several hours stirring at 4°C, the phage-PEG precipitate was collected by centrifugation as above, or left to settle for 48 hours at 4°C and the supernatant siphoned off (Yamamoto et al, 1970). The phage-PEG precipitate was twice extracted by resuspension in phage buffer and centrifugation (SS34 rotor, 5,000 x g, 10 min, 4°C). The combined supernatants were



recentrifuged (SS34, rotor, 10,000 x g, 5 min, 4°C) and the final turbid phage suspension, (usually 75 ml from 5 litres) was purified and concentrated on CsCl gradients.

CsCl was dissolved in phage buffer. (The light precipitate formed was removed by a brief centrifugation.) The quantity of CsCl required for a given density ( $\rho$ ) of solution was calculated using the equation -

$$\% \text{ CsCl (w/w) } = 137.48 - (138.11/\rho)$$

Densities were checked by Zeiss refractometry.

The phage suspension was layered onto pre-formed step gradients in 60 ml nitrocellulose tubes for centrifugation in the SW25.2 rotor (85,000 x g, 18 hours, 10°C). The gradients comprised 5 ml of density 1.70 gm/cm<sup>3</sup>, 5 ml of 1.60, 3 ml of 1.50, 6 ml of 1.45 and 6 ml of 1.40. The visible phage bands were withdrawn through the side of the tube by syringe, diluted with CsCl solution ( $\rho = 1.50$ ) to 50 ml and centrifuged to equilibrium in the SW25.1 rotor (50,000 x g, 48 hours, 10°C). The phage band was collected through the bottom of the tube into a sterile bottle, and stored at 4°C. If such phage were required for DNA isolation after being stored for longer than one month, they were again centrifuged to equilibrium. The yield from 5 litres was typically  $5 \times 10^{13}$  pfu.

#### 6. Growth of bacteriophage MS2

Infectious phage were titrated as above but using L-broth bottom agar and L-C top agar supplemented with CaCl<sub>2</sub> to 5 mM and glucose to 0.1% (w/v). The plating bacteria, E.coli K38, were used in the log phase of growth; an



overnight culture was diluted 20-fold into fresh L-broth and grown to  $3-5 \times 10^8$  cells/ml.

Large and small scale stocks of phage were prepared in essentially the same way. Overnight cultures of bacteria were diluted 20-fold into L-broth, grown with shaking at  $37^\circ\text{C}$  to  $5 \times 10^8$  cells/ml and then inoculated with MS2 (to  $10^8$  pfu/ml). After 5 hours shaking at  $37^\circ\text{C}$ , lysozyme ( $5 \mu\text{g/ml}$ ) and EDTA ( $1 \text{ mM}$ ) were added, and the cultures cooled on ice for 30 min. Chloroform ( $1\%$  (v/v)) and NaCl ( $0.5 \text{ M}$ ) were then added and cell debris removed by centrifugation (GSA rotor,  $10,000 \times g$ , 10 min,  $4^\circ\text{C}$ ). The supernatants, usually  $10^{11} - 10^{12}$  pfu/ml, were stored at  $4^\circ\text{C}$  or purified further as follows.

PEG was added to  $6\%$  (w/v) and the phage concentrated as for T7. The resuspended phage were centrifuged on pre-formed CsCl gradients in the SW25.2 rotor ( $85,000 \times g$ , 18 hours,  $10^\circ\text{C}$ ), comprising 7 ml of CsCl solution of density  $1.70 \text{ gm/cm}^3$ , 5 ml of 1.50, 4 ml of 1.40, 4 ml of 1.35, 4 ml of 1.30 and 4 ml of 1.20. The phage band was collected, diluted with <sup>solution</sup> CsCl ( $\rho = 1.40$ ) and centrifuged to equilibrium in the SW25.1 rotor ( $50,000 \times g$ , 48 hours,  $10^\circ\text{C}$ ). The final phage preparation, typically  $10^{15}$  pfu from 5 litres, was collected and stored at  $4^\circ\text{C}$ .

#### 7. Nucleic acid extraction

Purified phage were diluted to  $A_{260 \text{ nm}}^{1 \text{ cm}} = 20$ , and dialysed against  $0.1 \text{ M}$  Tris-HCl (pH 8),  $0.1 \text{ M}$  NaCl,  $1 \text{ mM}$  EDTA to remove CsCl. They were then extracted twice with an equal volume of redistilled phenol (pre-equilibrated



with 50 mM Tris-HCl (pH 8), 50 mM NaCl, 1 mM EDTA) by continuous gentle mixing (15 min, room temperature). The phases were separated by centrifugation (1,000 x g, 5 min, room temperature). The combined phenol layers were re-extracted with buffer. The combined aqueous phases were then extracted once more with phenol and the phenol discarded.

MS2 RNA (see Fig. 5) was precipitated with ethanol, resuspended in buffer and reprecipitated twice before storing at  $-20^{\circ}\text{C}$  as a precipitate in ethanol containing 0.1% (w/v) SDS, or as aliquots in 50 mM Tris-HCl (pH 8), 50 mM NaCl, 1 mM EDTA at  $-70^{\circ}\text{C}$ .

T7 DNA was dialysed against 50 mM Tris-HCl (pH 7.8), 0.5 M NaCl, 1 mM EDTA for 24 hours and then against several changes of the same buffer containing 50 mM NaCl for 24 hours. The first dialysis prevented cloudiness of the final DNA preparations.

The yields were usually around 0.6-0.8 mg nucleic acid per 20  $A_{260}$  units.

#### 8. T7 DNA strand separation

The strands of T7 DNA can be separated by exploiting the preferential association of one strand with guanine-rich polymers (Summers and Szybalski, 1968; Szybalski et al, 1971). Purified phage stocks were dialysed against 1 mM EDTA (pH 8) to remove CsCl, and diluted in 1 mM EDTA to an absorbance at 260 nm of 10.0. Aliquots of 0.5 ml were placed in sterile polyallomer tubes ( $\frac{5}{8}$  in x 3 in) and to each were added 0.04 ml of 5% (v/v) sarkosyl NL35



Figure 5. Panel A: 25  $\mu\text{g}$  MS2 RNA electrophoresed on 2.4% polyacrylamide gel at 7.5 V/cm for 90 min. Soaked in distilled water for 30 min and then scanned at 260 nm.

Panel B: Separation of the strands of T7 DNA. 5  $A_{260}$  units of phage ( $10^6$  cpm  $^3\text{H}$ ) were denatured in alkali, 200  $\mu\text{g}$  poly r(U,G) were added and the mixture was neutralised. It was brought to  $\rho = 1.73$  with saturated CsCl solution, and centrifuged (50 Ti rotor, 95,000 x g, 60 hrs,  $10^\circ\text{C}$ ). 0.2 ml fractions were collected, diluted to 0.4 ml with 1 mM EDTA and their  $A_{260}$  determined. Aliquots were also acid-precipitated.



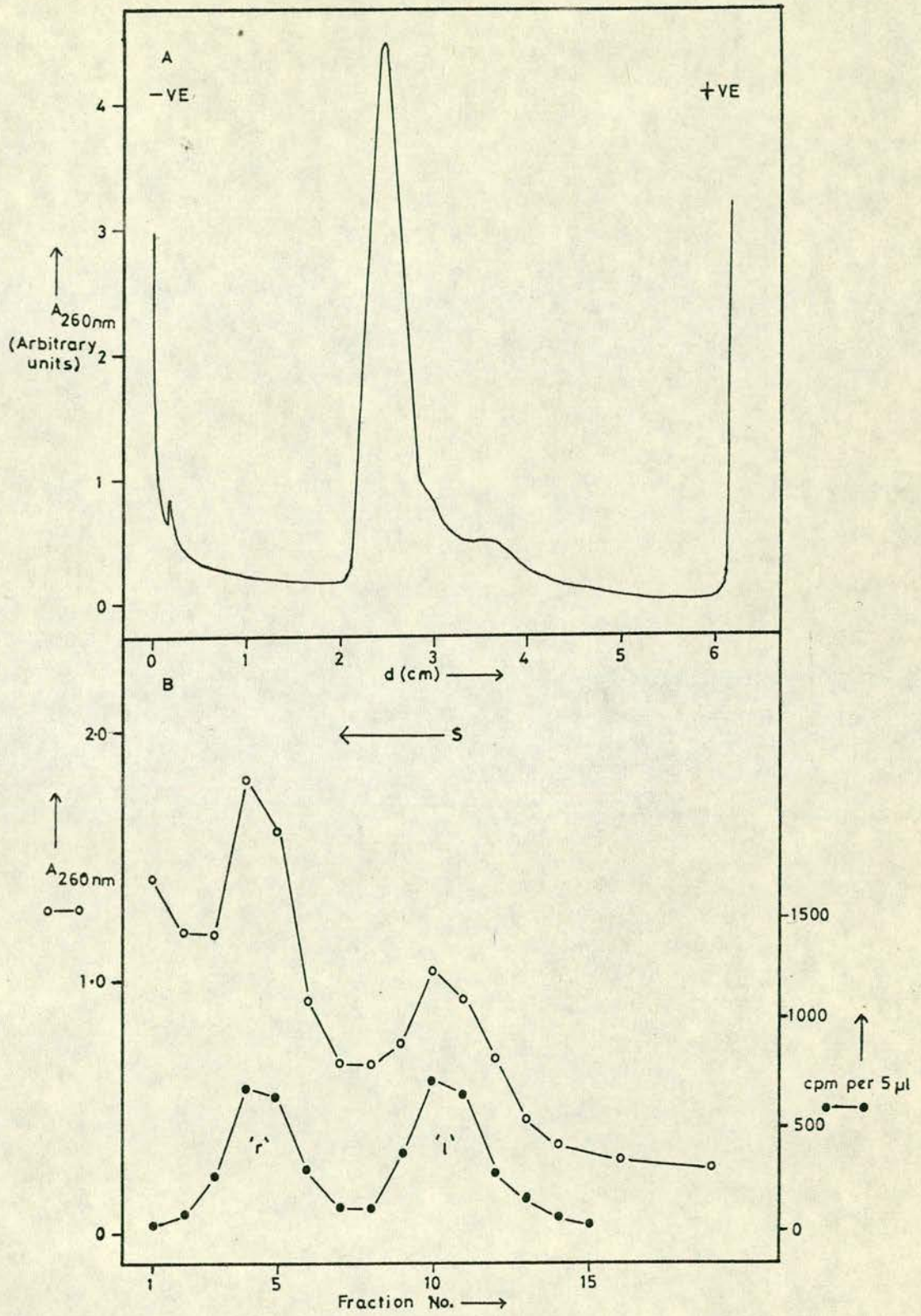


Fig. 5



and 0.06 ml of 1 M NaOH to produce a pH of about 12.5. After 9 min at room temperature, 200  $\mu$ g of poly r(U,G) were added. One minute later, the mixture was neutralised by the addition of 0.06 ml of 2 M  $\text{NaH}_2\text{PO}_4$ . The volume was adjusted to 1.2 ml with 1 mM EDTA and 4.8 ml of a saturated solution of CsCl added to make the overall density 1.72 gm/cm<sup>3</sup>. The tubes were filled with liquid paraffin and centrifuged to equilibrium in the 50 Ti rotor (95,000 x g, 60 hours, 10°C).

The gradients were fractionated by collecting 0.2 ml aliquots from the bottom into sterile siliconised glass tubes. An equal volume of 1 mM EDTA was added so that their absorbance at 260 nm could be determined using micro-cuvettes. If the DNA was radioactive, then aliquots were acid precipitated and counted. A typical separation is shown in Fig. 5.

The peak fractions were pooled, made 0.1 M in NaOH and the ribopolymer hydrolysed at 37°C for 5 hours. After neutralisation with HCl, the DNA was dialysed against 10 mM Tris-HCl (pH 8), 10 mM NaCl, 1 mM EDTA and stored in siliconised glass vials at -50°C. Aliquots were thawed only once, immediately before use.

The recovery was typically about 30%.

#### 9. Concentration and molecular weights of nucleic acids

Unit absorbance at 260 nm in low ionic strength buffers was taken to be equivalent to 40  $\mu$ g/ml for MS2 RNA (Eoyang and August, 1968), 40  $\mu$ g/ml for T7 single stranded DNA (as for MS2 RNA) and 47  $\mu$ g/ml for T7 double stranded DNA



(Richardson, 1966). The molecular weights were assumed to be

- 1.05 x 10<sup>6</sup> for MS2 RNA (Strauss and Sinsheimer, 1963)
- 25.0 x 10<sup>6</sup> for T7<sup>+</sup> DNA (Freifelder, 1970)
- 22.9 x 10<sup>6</sup> for T7 C5 LG3 DNA (McDonnell et al, 1977)
- 23.8 x 10<sup>6</sup> for T7 LG37 DNA (McDonnell et al, 1977)

#### 10. Autoradiography

Dried gels or electrophoretograms were marked at the edges with radioactive ink to allow accurate alignment with the developed film. They were then placed in contact with the film (Kodak X-O mat XH-1) in a lead-backed folder and kept in the dark at room temperature.

Wet gels were placed on a glass plate and covered with snap-wrap (Empress Products Ltd, Middlesex, U.K.). Filter discs marked with radioactive ink were affixed to aid re-alignment. The photographic film was placed between the snap-wrap and a further glass plate. The assembly was taped together and kept in the dark at room temperature.

If tungstate intensifying screens (Ilford fast tungstate) were to be used, the film was given a brief light exposure according to a pre-determined calibration, and then placed between the sample and the tungstate surface of the screen. The assembly was wrapped in foil and kept at -70°C.

#### 11. Gel electrophoresis

A great variety of both rod and slab gels have been used in these studies. Slab gels were generally preferred because many samples could be directly compared. However, low percentage polyacrylamide gels were often made as rods,



because their physical properties made it difficult to excise a track from a slab for analysis.

Rectangular glass plates were 16 cm x 40 cm x 0.3 cm or 16 cm x 20 cm x 0.3 cm and Perspex spacers were 0.15 or 0.3 cm thick. Plates were first wetted with distilled water to test for freedom from grease. Grease free plates (one with a rectangular notch, 2.5 cm x 11 cm) and spacers (lightly greased with white paraffin) were assembled and held together with "butterfly" clamps. Acrylamide gel mixtures were then carefully poured between the plates. A Perspex well-former could then be inserted. However, if another gel was to be poured on top, as in discontinuous systems, the lower gel mixture was overlaid with distilled water and allowed to set. The surface of the lower gel was then rinsed with upper gel mixture and the upper gel poured as above. The whole assembly was often placed in a container filled with water to the level of the gel to minimise any tendency to leak during setting.

A. 2.4% polyacrylamide gels for analysis of RNA  
(Peacock and Dingman, 1968)

A stock solution of acrylamide and bisacrylamide (20:1) was diluted to 2.4% in E-buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, adjusted to pH 7.2 with glacial acetic acid), and thoroughly degassed at a water pump. TMED was added to 0.04%, and ammonium persulphate to 0.1%. The mixture was poured into Perspex tubes (0.3 cm<sup>2</sup> x 7.5 cm) sealed with parafilm to make rod gels, or into a glass plate assembly to produce a slab, and allowed to set. Because of the physical weakness of 2.4% gels they had to



be supported with pieces of net or dialysis tubing for rod gels, or with a small 8% polyacrylamide gel (made up as above) for slabs. The rods were inverted to expose a flat surface on which to load the samples. The gels were electrophoresed in E-buffer containing 0.2% (w/v) SDS at 5 V/cm for 1 hr before loading. Samples were then electrophoresed for at least 2 hrs under the same conditions.

Samples were usually 1-20  $\mu$ g RNA containing 10% glycerol, 0.5% (w/v) SDS and 0.1% bromophenol blue. They were heated at 47°C for 10 min before loading.

After electrophoresis, the gels were soaked in distilled water for 30 min and then scanned at 260 nm in a Unicam SP800 spectrophotometer fitted with a Gilford scanning attachment. Alternatively, the gels were soaked in 1  $\mu$ g ethidium bromide /ml for 30 min, washed with water for 30 min and photographed under short-wave length ultra-violet radiation. Fluorescence due to bound ethidium could be visualised in this way, even with denatured DNA or RNA. Presumably binding can occur by electrostatic interaction between the ethidium and polynucleotide phosphate groups, even when the more usual intercalation cannot occur. For quantitation of radioactively labelled nucleic acids, the gels were frozen with dry ice and divided into 1 mm segments using a Mickle gel slicer. The slices were taken, usually in pairs, to 5 ml of Petri's scintillant (0.35 gm PPO, 10.1 mg dimethyl POPOP, 0.9 ml 1% (w/v) SDS, 10 ml NCS or soluene 350 tissue solubiliser and 90 ml toluene). The samples were incubated at 37°C till the scintillant had completely penetrated the gel slices (usually 18 hrs), then cooled and counted.



B. 1.8% polyacrylamide gels containing 0.5% agarose (Peacock and Dingman, 1968).

A stock solution of acrylamide and bisacrylamide (20:1) was diluted to 3.6% in E-buffer and degassed. A 1% solution of agarose in E-buffer was made by bringing it to boiling with vigorous stirring on a hot plate. Equal volumes of agarose and acrylamide solutions were mixed, made 0.04% in TMED and 0.1% in ammonium persulphate, and poured as usual. Such composite gels are physically stronger than a 2.4% polyacrylamide gel, and slabs may be poured without a support; polythene o-rings were inserted into the bases of perspex tubes used, to prevent the gels from slipping out.

The gels were pre-run, and the samples prepared and analysed as for 2.4% polyacrylamide gels.

C. Agarose gels for analysis of DNA (Sharp, Sugden and Sambrook, 1973)

The agarose solution (usually 1.5%) was made in gel buffer (40 mM Tris, 33 mM sodium acetate, 2 mM EDTA adjusted to pH 8.3 with glacial acetic acid) by bringing it to boiling point with vigorous stirring as above. The solution was kept at 50°C till used. A small amount of this solution was diluted 10-fold, spread over one of the glass plates and baked at 120°C for 10 min, to provide a roughened surface. The glass plates and spacers were assembled without a bottom spacer, clamped to a slab gel apparatus (Raven Scientific Ltd, Haverhill, Suffolk, U.K.) and tilted back to an angle of about 60° to the horizontal. Gel mixture was poured into the bottom tank and allowed to set, forming a wedge to support the gel



proper. Gel buffer was added to the bottom tank and the gel poured as usual. Electrophoresis was usually for 16-18 hrs in gel buffer at 5 V/cm.

Typical samples were 1  $\mu$ g DNA in 10-20  $\mu$ l of gel buffer containing 20% glycerol and 0.1% bromophenol blue. The DNA was visualised using ethidium bromide as above.

D. SDS-8.75% polyacrylamide gels for analysis of protein (Laemmli, 1970)

A stock solution of acrylamide and bisacrylamide (37.5:1) was diluted to 8.75% in lower gel buffer (0.375 M Tris-HCl (pH 8.8), 0.1% SDS) and degassed. TMED was added to 0.025% and ammonium persulphate to 0.045% and the gel poured. An upper 3% stacking gel was made with the same acrylamide stock diluted in upper gel buffer (0.125 M Tris-HCl (pH 6.8), 0.1% SDS). TMED was added to 0.1% and ammonium persulphate to 0.03%. Electrophoresis was at 8 mA for 16-18 hrs in 25 mM Tris, 0.19 M glycine, 0.1% SDS, 0.1% 2-mercaptoethanol, adjusted to pH 8.6 with concentrated ammonium hydroxide.

Samples were 1-20  $\mu$ g protein in 62.5 mM Tris-HCl (pH 6.8), 3% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue. They were denatured at 75°C for 15 min before loading.

After electrophoresis the gels were fixed in 9% acetic acid, 45% methanol for 15 min at 37°C, then stained in fixative containing 0.1% coomassie brilliant blue for 15 min. Destaining was in 7% acetic acid, 5% methanol at 37°C, usually overnight. For quantitation, the gels were scanned at 585 nm using a Vitatron TLD100 densitometer.



E. Polyacrylamide gels in 7M urea for denatured nucleic acids (Maniatis, Jeffrey and van de Sande, 1975)

The gels were made by dissolving Aristar urea to 7M in a mixture of acrylamide-bisacrylamide (29:1) and 10 x buffer (0.9 M Tris, 25 mM EDTA adjusted to pH 8.3 with boric acid), and brought to volume with distilled water. In this way, a 30% stock could be used to make gels 13% or less in acrylamide. The mixture was degassed, made 0.033% in TMED and 0.05% in ammonium persulphate, and poured. Electrophoresis was at 5V/cm for 16-18 hrs in 1 x buffer.

Samples were denatured by heating at 100°C for 5 min in the presence of at least 50% freshly deionised formamide, containing 0.1% bromophenol blue, 0.1% xylene cyanol FF and 15 mM EDTA. They were then cooled in ice-water and loaded. Gels were stained with ethidium bromide, and markers were a mixture of 4S and 5S RNAs from Xenopus laevis ( gift from Dr P. Ford).

F. Gradient gels

Gels with a linear gradient of polyacrylamide concentration were made up from equal volumes of two acrylamide-bisacrylamide mixtures, prepared as above. A standard gradient maker was used, consisting of two connected chambers, one magnetically stirred and fitted with an outlet. Care was taken to use amounts of TMED and ammonium persulphate such that the gels could be poured before setting took place.



## 12. Enzyme reactions

### A. RNA polymerase

Routine assays (Burgess, 1969) were carried out at 37°C with salmon sperm DNA (500 µg/ml) in 40 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol and 0.4 mM of each rNTP, one being labelled at 1 Ci/mole. Assays of crude preparations also included 2 mM potassium phosphate (pH 7.0) to inhibit polynucleotide phosphorylase, and 10 µg/ml yeast RNA as an alternative substrate for nucleases. Incubation was usually for 10 min. One unit of enzyme catalyses the incorporation of one nmole of labelled substrate per hour.

Transcription studies with highly purified polymerase were in 50 mM Tris-HCl (pH 7.8), 150 mM KCl, 1 mM EDTA, 12.5 mM MgCl<sub>2</sub>, 1 mM DTT (high salt) or 40 mM Tris-HCl (pH 8), 25 mM KCl, 1 mM EDTA, 10 mM MgCl<sub>2</sub>, 1 mM DTT (low salt). Nucleoside triphosphates were 0.4 mM, with one labelled at 1-50 Ci/mole. Templates were T7 DNA (25-125 µg/ml), poly d(A-T) (25 µg/ml) or salmon sperm DNA (500 µg/ml). RNA polymerase concentrations up to 100 µg/ml were used.

### B. *E. coli* DNA polymerase I and Klenow fragment A

Routine assays (Richardson et al., 1964) were at 37°C with poly d(A-T) at 25 µg/ml in 66 mM Tris-HCl (pH 7.8), 6.6 mM MgCl<sub>2</sub>, 1 mM DTT and 0.1 mg/ml BSA (bovine serum albumin). Deoxyribonucleoside triphosphates were at 30 µM, one labelled at 25 Ci/mole. Enzyme concentrations of up to 0.5 u/ml were used and incubations were typically 30 min. One unit of enzyme catalyses the incorporation of one nmole of labelled substrate per hour.



Reactions primed by T7 RNA-DNA hybrids used 5-10 pmole hybrid/ml, buffers as above and enzyme at 30 u/ml. Unlabelled dNTPs were at 30  $\mu$ M but  $\alpha$ -<sup>32</sup>P labelled dNTPs were at 1-10  $\mu$ M, about 100 Ci/mmmole.

#### C. Pancreatic DNAaseI

Digestion was carried out in 10 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub> at 37°C for 3 hrs. DNA was at 20-100  $\mu$ g/ml and enzyme at 50  $\mu$ g/ml.

#### D. Snake venom phosphodiesterase

Reactions were in 10 mM Tris-HCl (pH 8.5) at 37°C for 1 hr. DNA was at 5  $\mu$ g/ml and enzyme at 100  $\mu$ g/ml.

#### E. Ribonuclease A

Digestion was carried out in 10 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 0.3 M NaCl at 37°C for 30 min. RNA was at 10-50  $\mu$ g/ml and enzyme at 1  $\mu$ g/ml.

#### F. Alkaline phosphatase

Reactions were in 20 mM Tris-HCl (pH 8) at 37°C for 2 hrs. Nucleic acid was at 50-200  $\mu$ g/ml and enzyme at 20  $\mu$ g/ml.

#### G. Polynucleotide kinase

Reactions were carried out in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 15 mM 2-mercaptoethanol at 37°C for 1 hr. DNA 5'-OH termini were present at 2-50 pmole/ml and enzyme at 5-10 u/ml. High specific activity  $\gamma$ -<sup>32</sup>PATP was used at 1-5  $\mu$ M.

#### H. Restriction endonucleases

HpaII and HindII digestions were carried out in 10 mM Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol at 37°C for several hours. HpaI was used in the same buffer,



but with 80 mM NaCl added. The DNA concentration was 20-50  $\mu\text{g/ml}$  and enough enzyme was added to give complete digestion in 3-5 hrs.

### 13. Paper electrophoresis (Murray, 1970)

To determine  $^{32}\text{P}$  transferred from dNTPs into ribonucleotides when DNA synthesis was primed by RNA, the reaction mixtures were first gel filtered on Sephadex G-75 to remove unincorporated nucleotides. An aliquot was then hydrolysed in 0.2 M KOH for 16-20 hrs at  $37^{\circ}\text{C}$ . The mixture was neutralised with perchloric acid, chilled to precipitate  $\text{KClO}_4$  and briefly centrifuged. The supernatant was dried down on polythene and resuspended in a small volume of distilled water for loading. Enzymic digestion of DNA was usually performed in small volumes, and the whole reaction loaded.

The samples were dried as spots onto AE cellulose ion exchange paper alongside a mixture of dyes and 0.2 nmole aliquots of marker nucleotides. Electrophoresis was in 10% acetic acid, 1% pyridine (pH 3.5) at 2.5 KV until the dyes had migrated sufficiently (usually several hours). The marker nucleotides were located on the dried paper under short-wave ultra-violet illumination, and radioactive material by autoradiography. Radioactive nucleotides were identified by comparison with markers or by their mobility relative to that of xylene cyanol FF ( $R_f$ ).

For two dimensional analysis, "tracks" from the AE paper were sewn onto DEAE cellulose paper and electrophoresed in 8.75% acetic acid, 2.5% formic acid (pH 2)



at 2 KV for several hours. Electrophoresis was again monitored with dyes.

14. Nitrocellulose filter binding assay (Hinkle and Chamberlin, 1972a)

The filters (Sartorius, SM11306) were boiled in distilled water for 10 min, thoroughly rinsed with distilled water and stored at 4°C in 10 mM Tris-HCl (pH 7.8), 10 mM 2-mercaptoethanol, 1 mM EDTA. Buffers used for filtering and washing were either 0.5 or 0.05 M NaCl in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA. The filters were prewashed with 1 ml of buffer at reduced pressure (2-4 cm Hg) before the sample was filtered, usually in 2 ml buffer. The filters were then twice washed with 1-2 ml buffer and taken to clean scintillation vials. The filters were dried at 120°C for 20 min, cooled and 5 ml of 0.5% (w/v) butyl-PBD in toluene added. Radioactivity was determined as before.



## CHAPTER 4. RNA POLYMERASE - PURIFICATION AND PROPERTIES

### 1. Purification of RNA polymerase from E.coli

So many different methods have been published for the purification of E.coli RNA polymerase that it has become a question of choosing the approach most suited to a particular study, such as amino-acid sequencing, in vitro transcription or chemical modification (see Burgess, 1976). For studies on in vitro transcription it is essential to use polymerase which is free of ribonucleases, and which contains stoichiometric amounts of sigma factor. To prepare enzyme with these characteristics, the procedure of Burgess (1969) was adopted up to chromatography on DEAE-cellulose, followed by affinity chromatography on DNA-agarose (Nüsslein and Heyden, 1972). Final purification was by gel filtration on Biogel A1.5 m (Burgess and Jendrisak, 1975) or by chromatography on phosphocellulose (Burgess, 1969).

Enzyme activity was determined as described under Methods, and protein concentration was determined according to the equation

$$A_{280} \times 1.54 - A_{260} \times 0.76 = \text{mg protein/ml}$$

where  $A_{280}$  and  $A_{260}$  are the absorbances at 280 and 260 nm respectively (Warburg and Christian, 1941).

Enzyme was purified from two widely different strains of E.coli, K12.1113 and MRE600, each chosen because of their deficiencies in nucleases (see Table 1 in Materials). Large scale cultures were grown into late log phase and the cells harvested by continuous centrifugation. The cell paste could be stored at  $-70^{\circ}\text{C}$  for at least one year without



ill effect. Enzyme was extracted from 200 g batches according to the methods outlined above. The cells were disrupted in a Waring blender using a dry ice/ethanol bath to cool the blender vessel. All subsequent steps were at 2-4°C.

After fractionation by ammonium sulphate precipitation, the extract was loaded onto a DEAE cellulose column (7.5 cm<sup>2</sup> x 13.5 cm) in Burgess buffer A at 100 ml/hr. RNA polymerase was eluted in Burgess buffer C containing 0.23 M KCl. (If necessary, enzyme was precipitated by making it 50% saturated with ammonium sulphate, pelleted by centrifugation (10,000 g, 20 min) and redissolved in freezing buffer (10 mM Tris-HCl (pH 8), 0.1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 1 mM DTT) for storage at -70°C.)

The eluate was usually diluted to 1 mg protein/ml with 10 mM Tris-HCl (pH 8), 0.25 M KCl, 1 mM EDTA, 0.1 mM DTT, 5% glycerol, and pumped at 20 ml/hr onto a DNA-agarose column (5 cm<sup>2</sup> x 7 cm) pre-equilibrated with the same buffer. The calf thymus DNA-agarose had been made up according to Schaller et al (1972), and usually retained 0.6-1 mg DNA/ml. It was poured in a Whatman Multi-System column (MS-PC 2520) and was used with upwards elution to avoid serious compression of the agarose. Elution of RNA polymerase was in two steps; first, with 2 column volumes of buffer containing 0.55 M KCl followed by 2 column volumes of buffer containing 1.25 M KCl. The 0.55 M KCl eluate contained 'core' enzyme contaminated with ribonucleases while the 1.25 M KCl eluate contained 'holoenzyme' essentially free of ribonucleases. (This procedure was



preferred to gradient elution as it avoided the concentration of extremely dilute enzyme eluates.) The two eluates, usually 0.3-0.5 mg protein/ml were concentrated about fourfold by dialysis against storage buffer (10 mM Tris-HCl (pH 7.9), 10 mM MgCl<sub>2</sub>, 0.1 M KCl, 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol), and stored at -20°C. Their enzyme activity was stable for at least 9 months.

For further purification, up to 10 mg RNA polymerase (DNA agarose holoenzyme) was dialysed against buffer P (50 mM Tris-HCl (pH 7.8), 50 mM KCl, 0.1mM EDTA, 0.1 mM DTT, 5% glycerol) and pumped at 10 ml/hr onto a phosphocellulose column (1 cm<sup>2</sup> x 7 cm) pre-equilibrated with buffer P. After standing for 1 hr the column was washed with buffer P to remove trace contaminants, and the 'trail' of sigma removed by prolonged washing was passed through a 1 ml DEAE cellulose in buffer P. After passage of at least 5 column volumes, core polymerase was eluted from the phosphocellulose with a linear gradient of 0.2-0.6 M KCl in buffer P. Sigma was eluted from the DEAE cellulose with buffer P containing 0.4 M KCl. The proteins were concentrated and stored as above.

Alternatively, up to 10 mg RNA polymerase (DNA-agarose holoenzyme) was purified by gel filtration on a Biogel A1.5 m column (3 cm<sup>2</sup> x 60 cm) in 10 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 0.1 mM EDTA, 0.1 mM DTT, 5% glycerol. The enzyme, eluting with the peak of absorbance at 280 nm, was concentrated by dialysis against storage buffer and stored at -20°C.



These two methods give similar recoveries (only 30-60%) but gel-filtration was preferable as it avoided dissociation of holoenzyme into core and sigma, whilst giving a good purification from ribonucleases.

## 2. Analysis of purified enzyme on SDS/polyacrylamide gels

To determine protein purity and subunit stoichiometry, samples of the enzymes were examined by electrophoresis on SDS/polyacrylamide gels as described in Methods (Fig. 6). Unknown molecular weights were determined by comparing their mobilities with those of marker polypeptides. Proteins of known amino-acid sequence were preferred as their molecular weights are precisely known. The molecular weights of the polymerase subunits were:

$\beta'$	165,000	(Burgess, 1969)
$\beta$	155,000	(Burgess, 1969)
$\sigma$	85,000	(this work)
$\alpha$	38,000	(this work)

Assuming that the subunits were stained proportionately by coomassie blue, their stoichiometry determined by densitometry was  $\beta + \beta' : \sigma : \alpha = \overset{1.0+1.0}{1.0} : 0.99 : 2.01$  in DNA-agarose holoenzyme and  $\overset{1.0+1.0}{1.0} : 0.35 : 2.0$  for DNA-agarose core. Nüsslein et al (1971) report that the sigma found in DNA-agarose core is inactive, but evidence will be presented below that at least some is active. Preparations from E.coli K12 often have a polypeptide of molecular weight similar to that of sigma, especially in DNA-agarose core. After dissociation into core and sigma by phosphocellulose chromatography, sigma preparations were usually contamin-



Figure 6. Analysis of RNA polymerase preparations by SDS/  
polyacrylamide gel electrophoresis

The lower gel was 7-17% polyacrylamide, with a 3% stacking gel, prepared with the buffers of Laemmli (1970).

Electrophoresis was at 5 V/cm for 18 hrs. Staining was with Coomassie Blue.

Track	Enzyme	Amount ( $\mu$ g)
a	MRE600 - Phosphocellulose holoenzyme	2
b	MRE600 - Phosphocellulose core	2
c	MRE600 - Phosphocellulose sigma	1.5
d	K12 - DNA-agarose 'core'	3
e	K12 - DNA-agarose holoenzyme	3
f	MRE600 - DNA-agarose 'core'	5
g	MRE600 - DNA-agarose holoenzyme	5
h	K12 - Biogel filtration of DNA-agarose 'core'	5
i	K12 - Biogel filtration of DNA-agarose holoenzyme	5



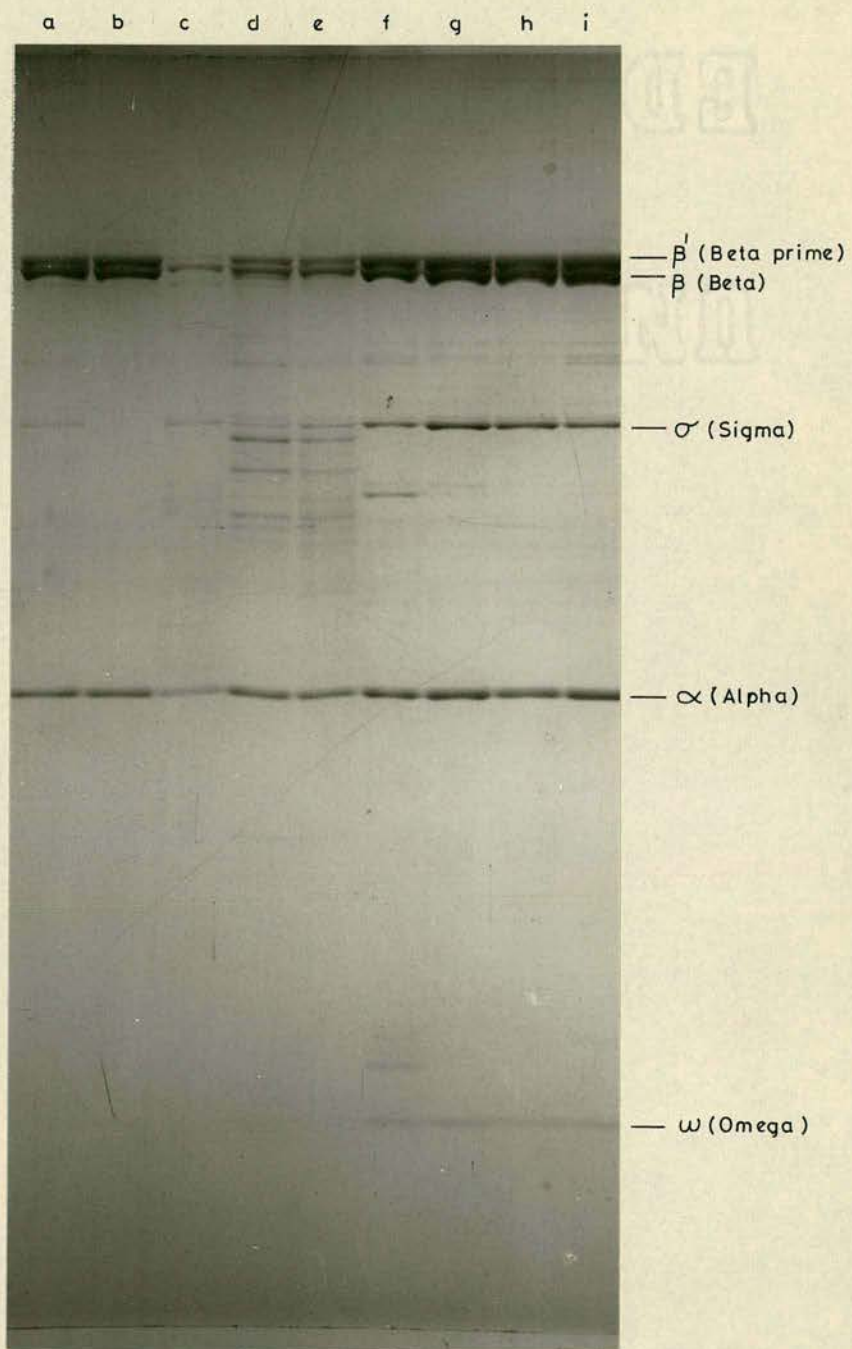


Fig. 6



ated with  $\alpha$  and a little  $\beta$ , while core showed  $\beta + \beta' : \alpha = 1:1$ . Thus reconstituted holoenzyme usually contained an excess of  $\alpha$ . More than 95% of all protein in the final enzyme preparations was RNA polymerase.

Other polypeptides were usually detectable, even in the purest of preparations. They include proteins of molecular weights 115,000, 110,000, 82,000, 76,000, 72,000, 69,000. The polypeptide of molecular weight 69,000 is reported to be an ATPase (Paetkau and Coy, 1972) and is a common contaminant of RNA polymerase preparations. Most of these contaminants were removed in the final purification step. The polypeptide known as  $\omega$ , (Burgess, 1969) was not removed by gel filtration and presumably is bound firmly to RNA polymerase. Enzymatically active enzyme can be reconstituted from the separated, purified subunits  $\alpha$ ,  $\beta$ ,  $\beta'$  and  $\sigma$  alone (Heil and Zillig, 1970), so the true function of  $\omega$  remains unknown.

### 3. Estimation of absolute enzyme activity

RNA polymerase preparations vary in activity despite apparently similar polypeptide compositions. Chamberlin and Ring (1972) have estimated that a completely active preparation should have a specific activity of 37,000 units/mg on poly d(A-T). The preparations used in the present study showed activities around 6,000 units/mg with poly d(A-T), indicating about 16% active enzyme.

A more direct estimate of activity comes from the number of RNA chains initiated on T7 DNA. Initiations can be assayed using  $\gamma$ -<sup>32</sup>PATP or GTP, or by the total incorporation of nucleotides when re-initiation is inhibited.



In the original work, (Mangel and Chamberlin, 1974a) assays were prepared with T7 DNA in excess, and NTPs and rifampicin then added together. This approach was necessary to exclude any additional initiations due to recycling of sigma because the preparations used had limiting amounts of sigma. In the present work sigma was not limiting (see below), so rifampicin was usually added one minute after NTPs. The results showed that my polymerase preparations were 10-20% active.

This agreement with the poly d(A-T) assays suggests that the active core and active holoenzyme titres are the same, as expected if the core is 10-20% fully active, 80-90% inactive, and sigma is saturating.

#### 4. Estimation of sigma activity

If the final step of purification had been chromatography on phosphocellulose, core and sigma preparations were combined to reconstitute holoenzyme. In pilot experiments, increasing amounts of sigma were added to a fixed amount of core, and the activity of the mixture on T7 DNA determined. The assays (usually 0.1 ml) contained 1  $\mu\text{g}$  T7<sup>+</sup> DNA, 2  $\mu\text{g}$  core and up to 1  $\mu\text{g}$  of sigma. The mixtures were incubated in the absence of magnesium ions (in 2.5 mM EDTA, 37°C, 5 min) and transcription started by addition of MgCl<sub>2</sub> to 12.5 mM. The incorporation of radioactive nucleotide was determined during the next 10 min. A typical result is shown in Fig. 7. A 3-4 fold stimulation was usually produced by saturating amounts of sigma. Holoenzyme could then be reconstituted by mixing core with enough sigma to achieve maximal activity.



Figure 7. Sigma assay

100  $\mu$ l transcription reactions were prepared at high ionic strength in the absence of DNA, containing 2.5  $\mu$ g MRE600 Phosphocellulose-purified core and varying amounts of sigma. After 5 min at 37°C, T7<sup>+</sup> DNA (10  $\mu$ g/ml) was added, and 10 min later two 40  $\mu$ l aliquots were acid-precipitated. <sup>3</sup>H UTP was at 2 mCi/mmol.

Sigma preparation 1 (o) was prepared from DNA-agarose holoenzyme and preparation 2(●) from DNA-agarose 'core'.



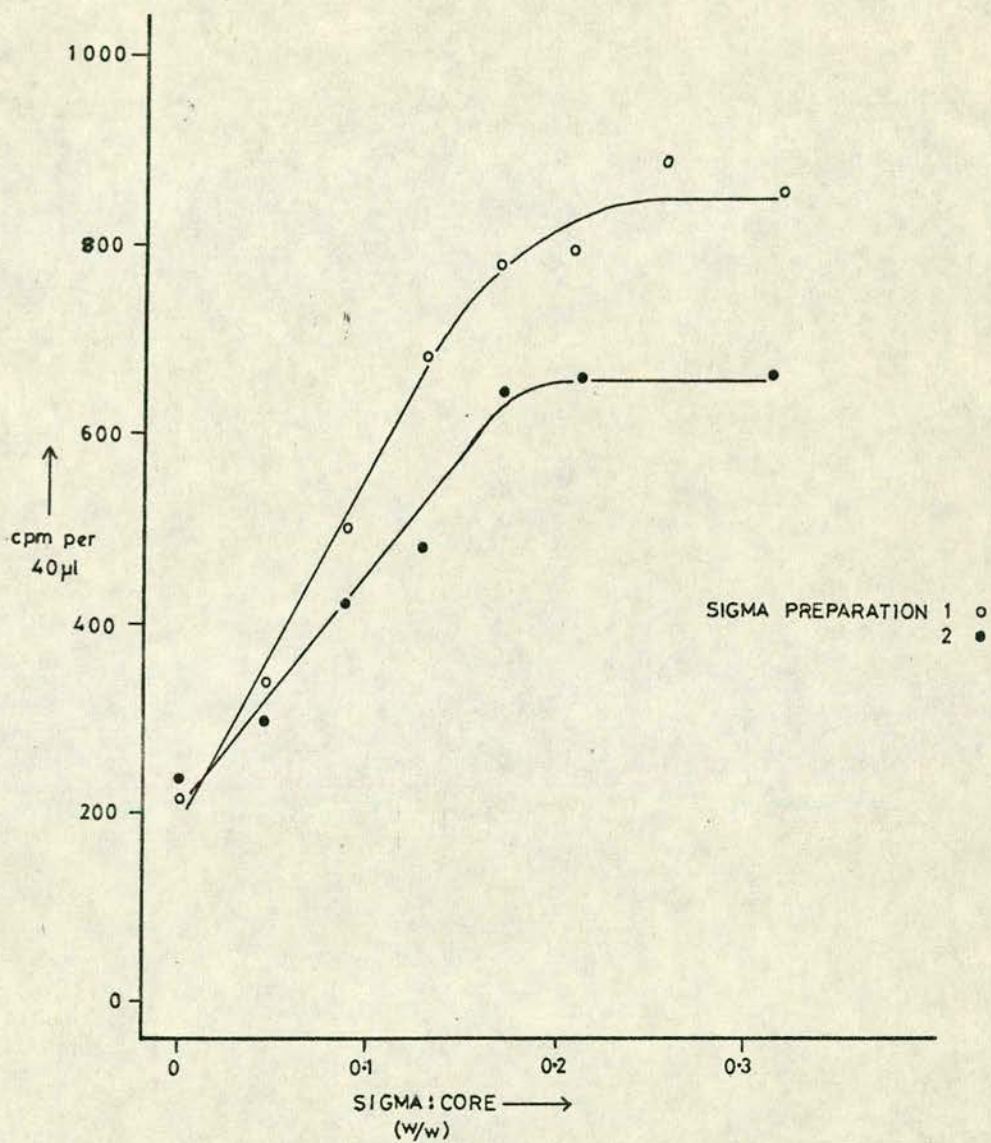


Fig. 7



Berg, Barrett and Chamberlin (1971) defined a unit of sigma as that amount which increased the incorporation of substrate into RNA in an assay similar to the above by one nmole per hour. They calculated that fully active sigma has an activity of 32,000 units/mg. The specific activities of the sigma preparations used in this study were around 11,000 units/mg, but were typically only 30% sigma protein.

Complementary information was obtained by immunoprecipitation experiments whose main purpose will be described in chapter 7. A fixed amount of holoenzyme was incubated with various amounts of anti-sigma antiserum in reaction mixtures lacking only DNA (5 min, 37°C). T7<sup>+</sup> DNA (10 µg/ml) or salmon sperm DNA (500 µg/ml) was then added and the incorporation of nucleotide determined as usual (Fig. 8). The activity on salmon sperm DNA, in contrast to that with T7<sup>+</sup> DNA was only affected at very high levels of antiserum. This titration with T7 DNA is not quite the complement to the sigma titration (see Fig. 7), as all activity can be abolished, not just the 75-80% which is due to sigma dependent transcription. This may be because antibody prepared against sigma will react with both sigma and holoenzyme. The antiserum used did precipitate holoenzyme (T. Linn and K. O'Hare, unpublished observations). The equilibria may be represented as -

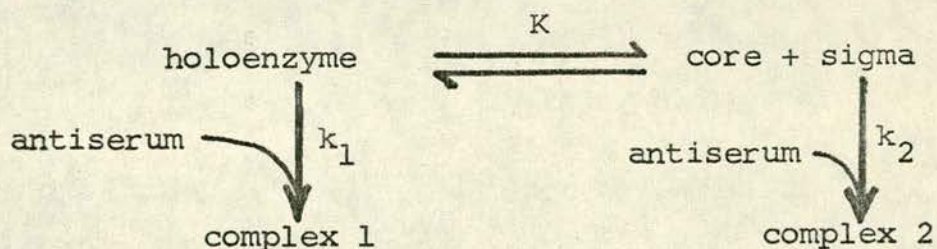




Figure 8. Titration of antisigma antiserum

50  $\mu$ l transcription reactions were prepared at high ionic strength in the absence of DNA, containing 1.5  $\mu$ g MRE600 DNA-agarose holoenzyme and varying amounts of antiserum. After 5 min at 37<sup>o</sup>C, DNA was added; T7 C5 LG3 DNA (10  $\mu$ g/ml) or salmon sperm DNA (500  $\mu$ g/ml). 20  $\mu$ l aliquots were acid-precipitated after 5 and 10 min synthesis. <sup>3</sup>H CTP was at 10 mCi/mmmole.

Panel A: T7 C5 LG3 DNA

B: salmon sperm DNA

C: % activity on T7 C5 LG3 DNA vs. amount of antiserum.

- o no antiserum
- 30 nl antiserum/ $\mu$ g enzyme
- 100
- 300
- △ 750
- ▲ 1500



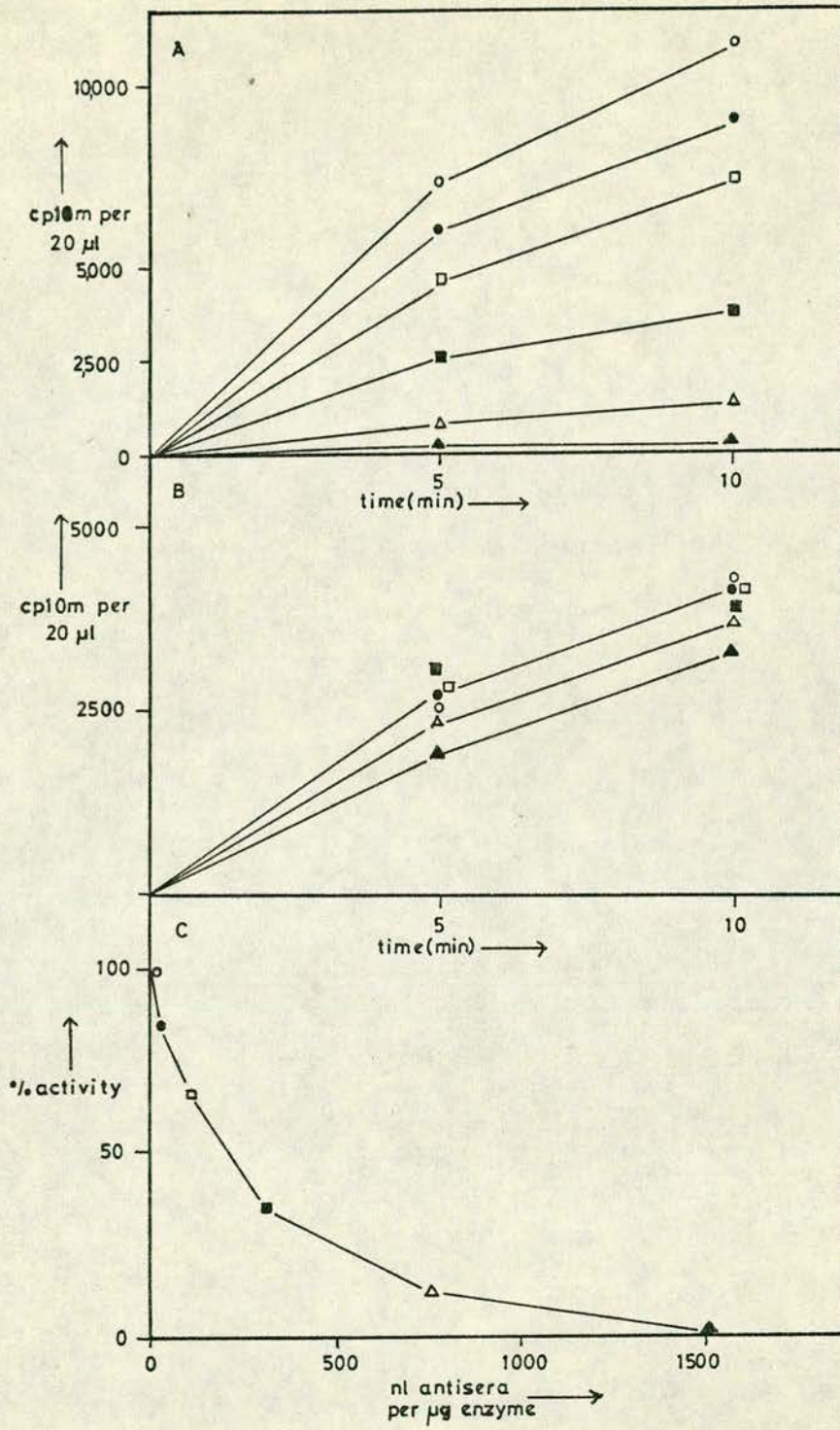


Fig. 8



However, 90% of the activity on T7<sup>+</sup> DNA could be abolished before the activity on salmon sperm DNA was significantly affected. This may indicate that  $k_2 > k_1$ .

The first experiments described in this section showed that sigma could stimulate the activity of phosphocellulose core on T7 DNA up to 4-fold whereas the immunoprecipitation data suggest that removal of sigma from holoenzyme could reduce the activity by about 10-fold. This discrepancy could be due to sigma contamination of the core preparations used, or more probably due to over-estimation of sigma inactivation because of precipitation of holoenzyme in the latter experiments. It also seems likely that the antiserum has some activity against core subunits as the activity on salmon sperm DNA is affected at very high levels of antiserum.

##### 5. Estimation of enzyme activity in the binding of DNA

RNA polymerase can interact with DNA to form complexes which, unlike free DNA, are retained on nitrocellulose filters (Jones and Berg, 1966). This filter binding assay has been used extensively by Chamberlin and co-workers to investigate the binding of E.coli RNA polymerase to T7<sup>+</sup> DNA (Hinkle and Chamberlin, 1971, 1972a,b; Chamberlin and Ring, 1972; Hinkle, Mangel and Chamberlin, 1972; Hinkle, Ring and Chamberlin, 1972). The binary complexes are retained on filtration in 50 mM NaCl but not in 0.5 M NaCl, presumably because the complexes dissociate at high ionic strength. Addition of NTPs to binary complexes allows them to be converted to transcribing complexes which are



retained in both 50 mM and 0.5 M NaCl. To further characterise the enzyme preparations used in the present study, the formation of both sorts of complex has been investigated.

Mixtures (usually 50  $\mu$ l) containing 1.25  $\mu$ g [ $^3$ H]-T7<sup>+</sup> DNA and up to 1  $\mu$ g of holoenzyme were incubated for 10 min in the absence of NTPs at 37°C. A 10  $\mu$ l sample was then diluted in 2 ml of buffer containing 50 mM NaCl for subsequent filtration. Transcription was started in the residual mixtures by adding NTPs, and after a further 2 min incubation a similar sample was diluted in 0.5 M NaCl buffer and filtered. The titration curves are shown in Fig. 9. At all enzyme concentrations (including saturating levels) the binary complexes are less efficiently retained than the transcribing complexes, but the curves are very similar in shape. This strongly suggests that all the polymerase molecules which form binary complexes can rapidly initiate an RNA chain, consistent with their being holoenzyme-promoter complexes (Chamberlin and Ring, 1972). It also suggests that the release of sigma when transcription proceeds does not lead to an increase in the amount of DNA bound. This indicates that sigma is not limiting in the preparations of holoenzyme used in this study.

The shape of the titration curves shows that a DNA molecule need bind only one enzyme molecule in order to be retained (Hinkle and Chamberlin, 1972a). Thus at low enzyme:DNA ratios, the retention gives a measure of the activity of the polymerase. The results show that my



Figure 9. Binding of  $^3\text{H-T7}^+$  DNA by RNA polymerase.

50  $\mu\text{l}$  transcription reactions at high ionic strength contained  $^3\text{H-T7}^+$  DNA (1.25  $\mu\text{g}$ , 5600 cpm  $^3\text{H}$ ) and varying amounts of MRE600 holoenzyme (purified through Biogel filtration). After 10 min at  $37^\circ\text{C}$ , 20  $\mu\text{l}$  were taken to 2 ml of filtration buffer containing 50 mM NaCl at room temperature, and filtered. NTPs were then added to the reactions and 2 min later, 20  $\mu\text{l}$  were taken to 2 ml of ice-cold filtration buffer containing 0.5 M NaCl, and filtered.

- ★ 50 mM NaCl - binary complexes ●
- ★★ 0.5 M NaCl - elongating complexes ○



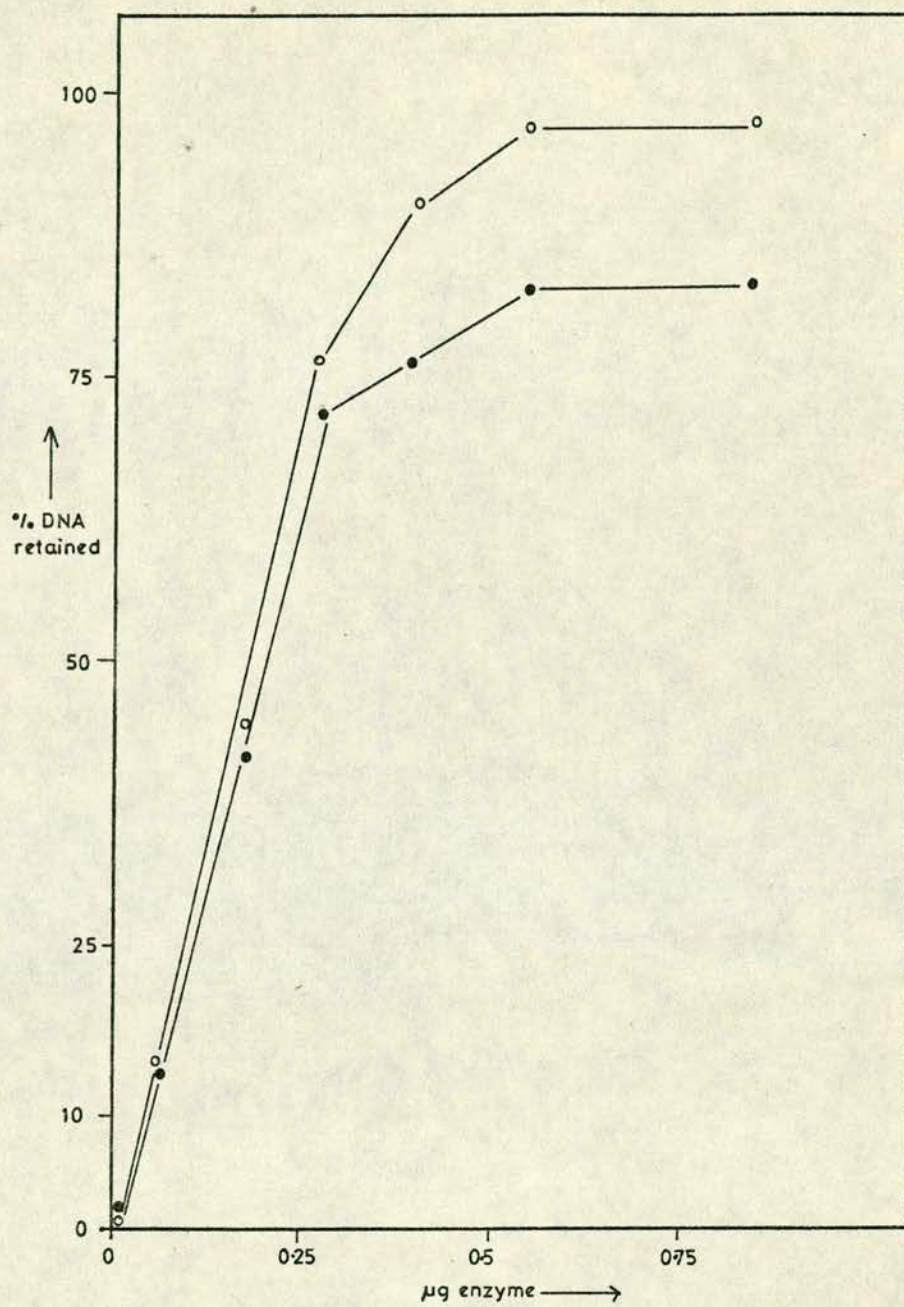


Fig. 9



preparations were typically 10% active, in reasonable agreement with estimates based on transcriptional activity (see section 3).

#### 6. Ribonuclease assays

Polymerase preparations were routinely assayed for endoribonucleases by two methods, both involving the examination of RNA on 2.4% polyacrylamide gels. The effect of exonucleases would be to change the profile of the RNA peaks, so that only severe contamination could be detected (and not quantitated).

In the first method, enzyme was incubated with MS2 RNA and samples of the RNA analysed after various times. The amount of full sized RNA was then quantitated by scanning the rod gels at 260 nm. Alternatively, slab gels were stained with ethidium bromide, photographed and the photograph scanned. In general, holoenzyme was almost free of endoribonuclease after DNA-agarose chromatography (no detectable RNA breakdown by an equal weight of protein in 1hr). Cruder fractions were heavily contaminated. DNA-agarose core enzyme was usually slightly more contaminated than holoenzyme.

The second method was applied only to highly purified enzyme preparations (and is discussed in chapter 5). In this assay, the effect of prolonged incubation of enzyme with in vitro synthesised T7 RNA was determined. Again DNA-agarose holoenzyme seemed free of endonucleases. As DNA-agarose 'core' appeared not to synthesise full-sized T7 RNA, Schaller et al (1972) concluded that there was no



active sigma in such preparations. However, the present work showed that some DNA-agarose 'core' preparations could synthesise T7 RNA (Fig. 10). This indicates that such 'core' preparations contain some active sigma <sup>may</sup> but/be sufficiently contaminated with ribonucleases to prevent detection of full-sized in vitro transcripts. When samples of DNA-agarose 'core' and holoenzyme were examined on isoelectric focussing gels, both showed bands with the acidic pI characteristic of sigma. Furthermore, sigma purified from DNA agarose 'core' by phosphocellulose chromatography was active (Fig. 7).

Though the preparations were not assayed directly for exoribonuclease, there is probably very little, if any, of this activity present in the most highly purified preparations. No change in acid precipitable RNA could be determined over at least one hour after the completion of incorporation in the presence of rifampicin (e.g. see Fig. 11.).

#### 7. Comparison of MRE600 and K12 enzymes

Enzyme preparations from these two strains were broadly similar, but did display some differences. Their yields, purity, specific activities and subunit stoichiometry were very similar, though the contaminating polypeptides differed somewhat (see Fig. 6).

The antisigma antiserum had been prepared against K12 material, but showed the same reactivity with the enzymes from either strain.



Figure 10. Size of RNA synthesised by MRE600 RNA polymerase  
(DNA-agarose-eluates, before and after Biogel  
filtration).

50  $\mu$ l transcription reactions at high ionic strength contained 6  $\mu$ g T7 C5 LG3 DNA, and enzyme at E:DNA = 4.  $^3$ H-CTP was present at 10 mCi/mmole. After 4 min at 37°C, MgCl<sub>2</sub> (12 mM) was added. Rifampicin (10  $\mu$ g/ml) was added 12 min later and 2 x 20  $\mu$ l aliquots taken for analysis after a further 3 min incubation.

Analysis was by electrophoresis on a 2.4% polyacrylamide slab gel at 5 V/cm for 2 hrs. The gel was stained in 1  $\mu$ g ethidium bromide per ml, and photographed through a red filter using short wave ultra violet illumination as described in chapter 3.

- sample heated 47°C for 10 min
- + sample heated 100°C for 10 min
- a MS2 RNA            1  $\mu$ g
- b DNA-agarose 'core'
- c DNA-agarose holoenzyme
- d Biogel-filtered DNA-agarose 'core'
- e Biogel-filtered DNA-agarose holoenzyme



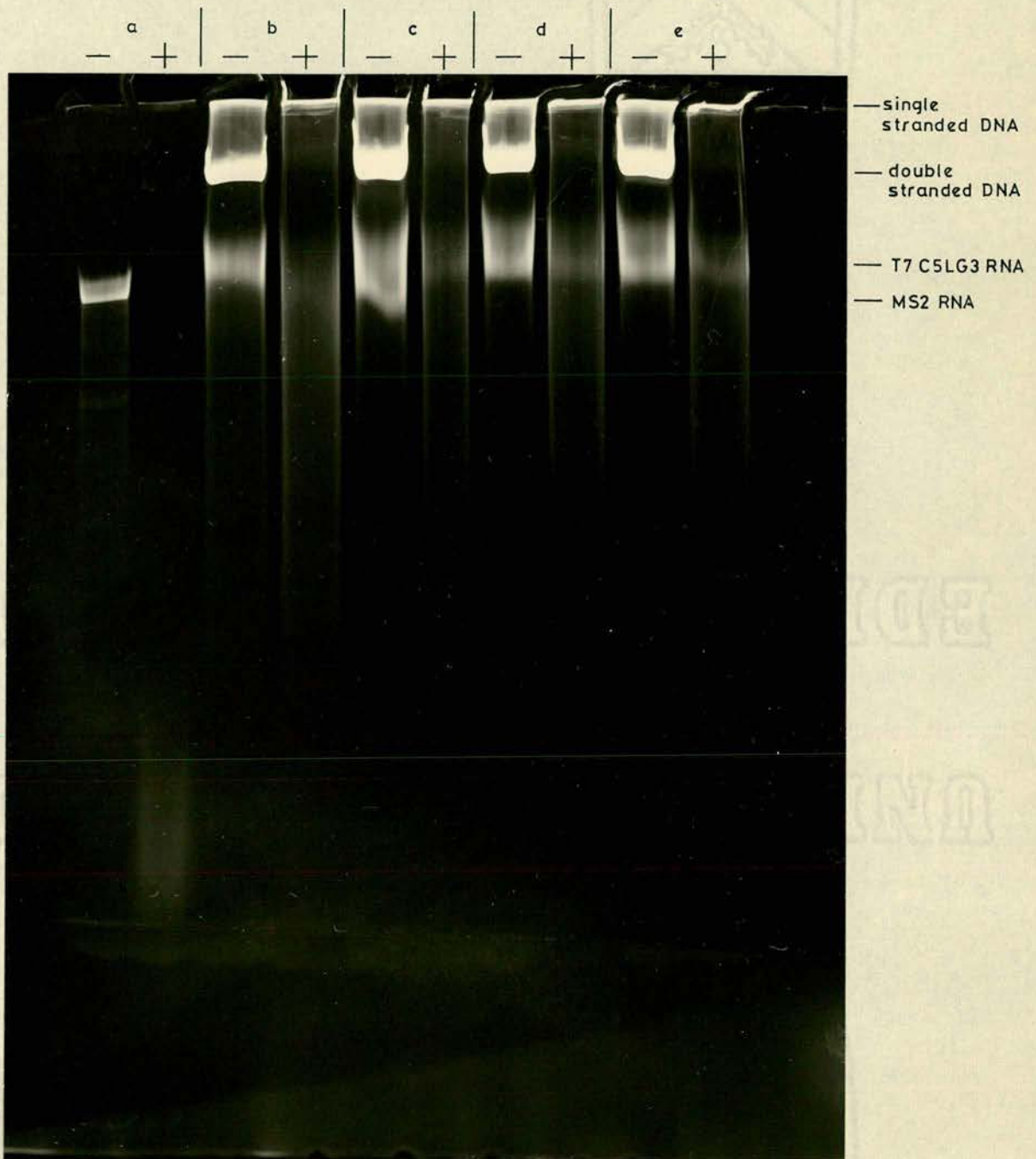


Fig.10



CHAPTER 5. In vitro TRANSCRIPTION OF T7 DNA BY E.coli RNA  
POLYMERASE

1. Kinetics of transcription

The absolute rate of transcription of T7 DNA by E.coli RNA polymerase is determined by many factors. It depends directly on the concentrations of enzyme, template and substrates. Moreover, the rates of DNA binding, initiation, elongation and termination are all strongly affected by pH, ionic conditions and temperature. Interpretations of the kinetics of transcription should take account of all these factors.

Most of the studies to be reported here concern transcription at low enzyme (E):DNA ratios\* (typically 2-2.5). As discussed in chapter 1, though there are some 7 promoters on T7 DNA for E.coli RNA polymerase, essentially all transcription at low E:DNA ratios initiates from the three major promoters to the left of the early region (see Fig. 3).

In a typical reaction, mixtures at 0.15 M or 0.025 M KCl were set up with low E:DNA ratios and incubated at 37°C in the absence either of magnesium ions ( $Mg^{2+}$ ) or ribonucleoside-5'-triphosphates (NTPs) for 4 or 5 mins. This preincubation allows the enzyme to locate and bind to a promoter so that on adding the missing component, there is a nearly synchronous initiation of transcription. After the first cycle of RNA synthesis the synchrony will be reduced, and eventually a steady state may be established. The kinetics of incorporation of isotopically labelled NTPs into acid precipitable material were followed as described in chapter 3.

\* Here and throughout this thesis, all E:DNA ratios are active enzyme to DNA ratios. They should be multiplied by 5 to obtain total enzyme to DNA ratios.



The results obtained using T7<sup>+</sup> and T7 C5 LG3 DNA\* are presented in Fig. 11. The profiles show that the initial rates of incorporation were the same for either template. This rate was maintained for 2 mins with T7 C5 LG3 DNA and for 4 mins with T7<sup>+</sup> DNA. In each case this is the time required for RNA polymerase to transcribe as far as t1 at the end of the early region. Evidence for this conclusion based on the rate of growth of nascent RNA will be presented later (section 3). It is supported by kinetic studies using the drug rifampicin.

Rifampicin is a potent inhibitor of the initiation of RNA synthesis by free RNA polymerase, although it is slower to act if the enzyme has already bound to a promoter (Chamberlin and Ring, 1972; Mangel and Chamberlin, 1974a). It can bind to RNA polymerase in elongating complexes, but does not inhibit them (Yarborough *et al.*, 1976). When rifampicin is added to a transcription reaction it will soon inhibit any further initiations, but it will not interfere with the completion of nascent RNA molecules. The effect of adding rifampicin some 30 seconds after NTPs is shown in Fig. 11. It is clear that there is very little incorporation after 2 mins with T7 C5 LG3 DNA or 4 mins with T7<sup>+</sup> DNA. The results are consistent with an elongation rate of 30 nucleotides per second in 0.15 M KCl and 25 nucleotides per second in 0.025 M KCl, assuming that the RNA was initiated at the major promoters and terminated at t1.

Incorporation of nucleotides in the presence of rifampicin after the end of the first cycle of transcription

\* The T7 C5 LG3 DNA was prepared from a strain also carrying a gene 1 amber mutation, am 342a. For simplicity the DNA will be described as T7 C5 LG3 DNA, the amber mutation being irrelevant to these studies.



Figure 11. Kinetics of transcription

0.6 ml transcription reactions contained DNA (2.5 pmole/ml), and enzyme at E:DNA = 1.5. After 4 min at 37°C, NTPs (<sup>3</sup>H-CTP at 50 mCi/mmole) were added and 30 seconds later, 0.3 ml taken to tubes at 37°C containing 3 µg rifampicin. 50 µl aliquots were taken at various times for acid-precipitation.

- Panel A: T7<sup>+</sup> DNA at 25 mM KCl (low ionic strength)  
B: T7<sup>+</sup> DNA at 0.15 M KCl (high ionic strength)  
C: T7 C5 LG3 DNA at 25 mM KCl  
D: T7 C5 LG3 DNA at 0.15 M KCl
- control
  - rifampicin (10 µg/ml)

The C5 and LG3 deletions remove 8.4 T7 units  
From a transcribed early region of 17.8 T7 units.



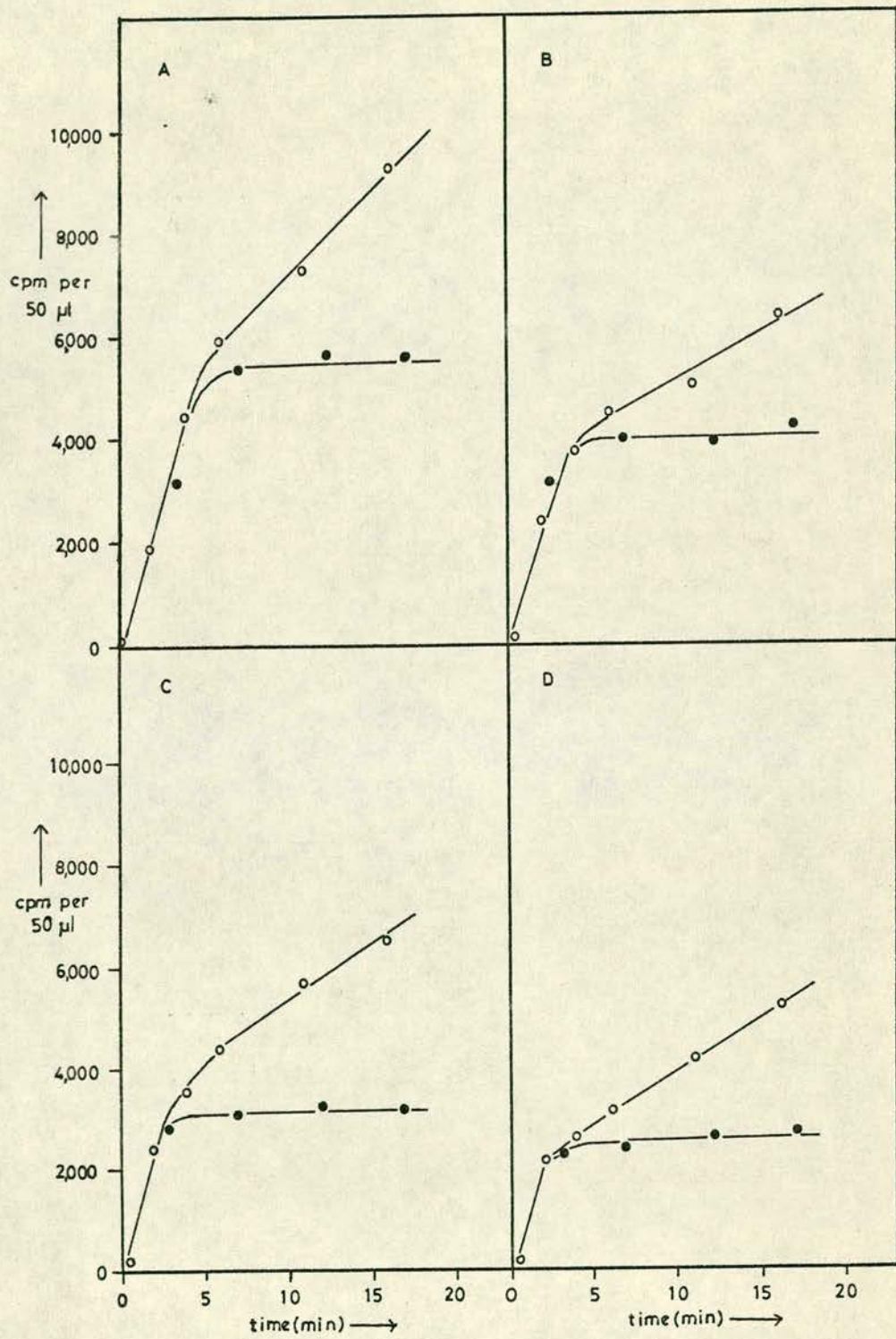


Fig.11



may be due to readthrough of the termination site. A comparison of the rates before and after this time allows estimation of the efficiency of the site to be 90-95% at 0.15 M and 0.025 M KCl.

In the absence of rifampicin, there was a reduction in the rate of incorporation following the first round of transcription. This could have several causes:-

- (1) inactivation of the template DNAs
- (2) the mean rate of elongation during the first round of transcription might be significantly greater than that of successive rounds, due to deterioration of (some) polymerase molecules
- (3) there might be total inactivation of some enzyme molecules after the completion of the first cycle
- (4) there might be a relatively slow step between the completion of the first round and the initiation of a second round of transcription. The synchrony of the first round would then be rapidly lost and a reduced steady state rate of transcription could be established without the rate of elongation or the number of active enzyme and DNA molecules being significantly changed.

A massive inactivation of the template DNAs has to be hypothesised, as each DNA molecule has several promoters, and each promoter can be used by many polymerase molecules in a very short period of time (Chamberlin and Ring, 1972). It is highly unlikely that the enzyme preparations used in this study were badly contaminated with DNAase, and such contamination would lead to a progressive inactivation of the templates, which does not fit the observed kinetics.



There is evidence that the mean rate of elongation did not change. When rifampicin was added later than the end of the first round, the rate of incorporation of NTPs fell gradually to zero. The time taken for complete inhibition was 2 mins with T7 C5 LG3 DNA and 4 mins with T7<sup>+</sup> DNA. This is as expected if initiations were occurring asynchronously and incorporation stops when those enzyme molecules which had initiated just prior to drug addition completed elongation of RNA, at the normal rate. Experiments in which a different isotopically labelled NTP was added simultaneously with the drug gave consistent results.

It is probable that some enzyme molecules become inactivated during the incubation. However, it is difficult to imagine a mechanism whereby enzyme molecules would be inactivated specifically after the first round. . Instead, one would expect a certain percentage loss after every round, leading to a gradual falling off in the rate of incorporation. This does not agree with the observation that a new steady state is rapidly established. It therefore seems likely that the rate of enzyme inactivation is too low to be significant in the kinetics under consideration.

Assuming that there is a slow step after the first round, it could be one (or more) of the following:-

1. synthesis of the 3' terminal RNA sequences
2. release of RNA
3. release of DNA
4. rebinding of sigma



5. selection of a promoter site
6. initiation of RNA synthesis at the promoter

The similarity of the kinetics at low and high ionic strength suggests, but does not prove, that the same slow step occurs under both conditions. Though the rate of elongation at high ionic strength is 20% greater than at low salt (see section 3), the measured rate of incorporation over the first few minutes is actually lower. This suggests that a higher proportion of enzyme molecules initiate successfully at low ionic strength. The yield of RNA from the first round suggests that 16% of the enzyme molecules are active at high and 21% at low ionic strength.

## 2. Molecular weights of T7 RNA molecules synthesised.

Samples from transcription reactions were routinely analysed by electrophoresis on 2.4% polyacrylamide gels (see chapter 3). Typical profiles are presented in Fig. 12. The molecular weights of the RNAs can be calculated from their mobility. A calibration curve was constructed using yeast ribosomal RNA (molecular weights  $1.3 \times 10^6$  and  $0.7 \times 10^6$ ), mouse ribosomal RNA ( $1.7 \times 10^6$  and  $0.7 \times 10^6$ ) and MS2 RNA ( $1.05 \times 10^6$ ). (The ribosomal RNAs were gifts from Dr U. Loening and the MS2 RNA was prepared as described in chapter 3. The positions of these RNAs were determined by scanning the gels at 260 nm.) As expected, mobility varied linearly with the logarithm of molecular weight (Bishop et al, 1967) up to at least  $1.7 \times 10^6$ . Assuming that RNA mobility is solely determined by size, the molecular weights of the in vitro transcripts appear



Figure 12. Size of RNA synthesised  
at high ionic strength  
50  $\mu$ l transcription reactions/contained DNA (5 pmole/ml) and  
MRE600 Phosphocellulose-purified holoenzyme at E:DNA = 4.  
 $^3$ H-UTP was at 3 mCi/mmol. After 4 min at 37°C, MgCl<sub>2</sub>  
(12 mM) was added, and 10 min later rifampicin (50  $\mu$ g/ml).  
After a further 4 min incubation at 37°C, the products  
were analysed by electrophoresis on 2.4% polyacrylamide  
gels for 3 hrs at 5 V/cm. Marker RNAs were added (1-2  $\mu$ g  
each) and their positions (shown by arrows) determined  
by scanning at 260 nm.

Panel A: T7<sup>+</sup> DNA

B: T7 C5 LG3 DNA



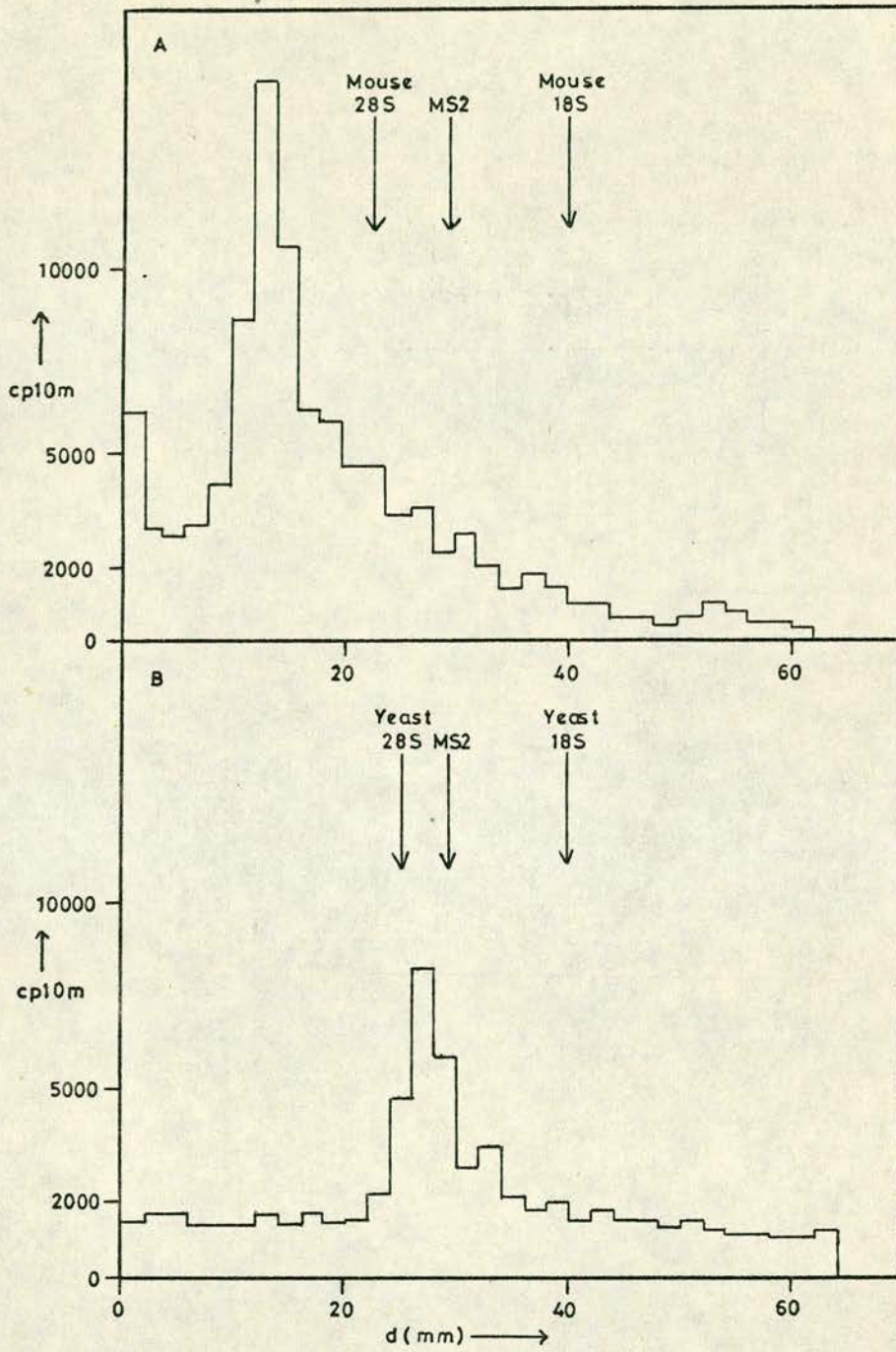


Fig. 12



to be  $1.26 \times 10^6$  for T7 C5 LG3 RNA and  $2.5 \times 10^6$  for T7<sup>+</sup> RNA. The accuracy of the latter estimate is uncertain, as it depends on an extrapolation, and the correlation of mobility and size on these gels does not hold for very large RNAs of this size (Peacock and Dingman, 1968).

These figures agree well with those expected for transcription of the early region of T7 DNA. Assuming that initiation of the average molecule occurred at 1.5%, the data indicate termination at 20.2% on T7<sup>+</sup> and at 19.2% on T7 C5 LG3 DNA. The latter estimate takes account of the most recent estimates for the size of the deletions (McDonnell et al, 1977). These authors now place the termination site t1 at 19.3 ( $\pm 0.2$ )%.

The recovery of labelled RNA on these gels was usually greater than 85%, including a variable amount at the top of the gel. This may reflect aggregation of the RNA, possibly with other reaction components, although the samples were heated in 0.1% SDS before loading (47<sup>o</sup>C, 10 min). It should be noted that RNA which had been purified on glycerol gradients following a phenol extraction did not have material at the top of the gel on electrophoretic analysis (chapter 9).

From a comparison of the baselines before and behind the RNA peaks, there is a clearly detectable, but low, readthrough of the termination site. The resulting RNA is mainly heterogeneous, with occasional evidence of a peak with molecular weight  $2.5 \times 10^6$  in the T7 C5 LG3 transcripts. This could be from termination at t2. As indicated before, the kinetics of transcription following rifampicin addition are also consistent with some 5-10% readthrough.



### 3. Rate of elongation

The size of nascent RNA molecules can be determined as for mature RNAs by analysing samples taken during the first round of transcription (Fig. 13). Such results indicated elongation rates of 30 nucleotides per second at 0.15 M KCl and 25 nucleotides per second at 0.025 M KCl. These estimates agree with those calculated from the time required for complete inhibition following rifampicin addition (section 1).

### 4. Detection of endonuclease activity in the transcription reaction

Transcription reactions were performed as usual, but at a higher E:DNA ratio to increase the sensitivity of the assay. Rifampicin was added after 10 minutes, and samples taken for analysis after further incubation (Fig. 14). The profiles show little change for up to 15 minutes at least after adding rifampicin. The recovery of labelled RNA in the peak could be used to quantitate endonuclease activity, but this assay is neither direct nor sensitive. In particular, internal breaks could be made without detectably affecting the apparent size of the RNA owing to its secondary structure. Such "hidden breaks" could be revealed by examination of the RNA on denaturing gels or on native gels after denaturation. Such experiments showed that the RNA synthesised by DNA-agarose holoenzyme could have a significant number of breaks even at the earliest times (e.g. see Fig. 35). Thus it is clear that significant endonuclease activity <sup>can be</sup> / present in such preparations,



Figure 13. Rate of elongation

150  $\mu$ l transcription reactions at low ionic strength, contained T7 C5 LG3 DNA (2.5 pmole/ml) and MRE600 Biogel-purified holoenzyme at E:DNA = 1.5. After 4 min at 37<sup>o</sup>C, NTPs (<sup>3</sup>H-CTP at 50 mCi/mmmole) were added and 30  $\mu$ l samples taken for analysis at various times. Electrophoresis was on 2.4% polyacrylamide gels for 3 hrs at 5 V/cm.

Panel A: 1 min after NTP addition

B: 2 min

C: 5 min

The position of marker MS2 RNA is indicated by the arrows.



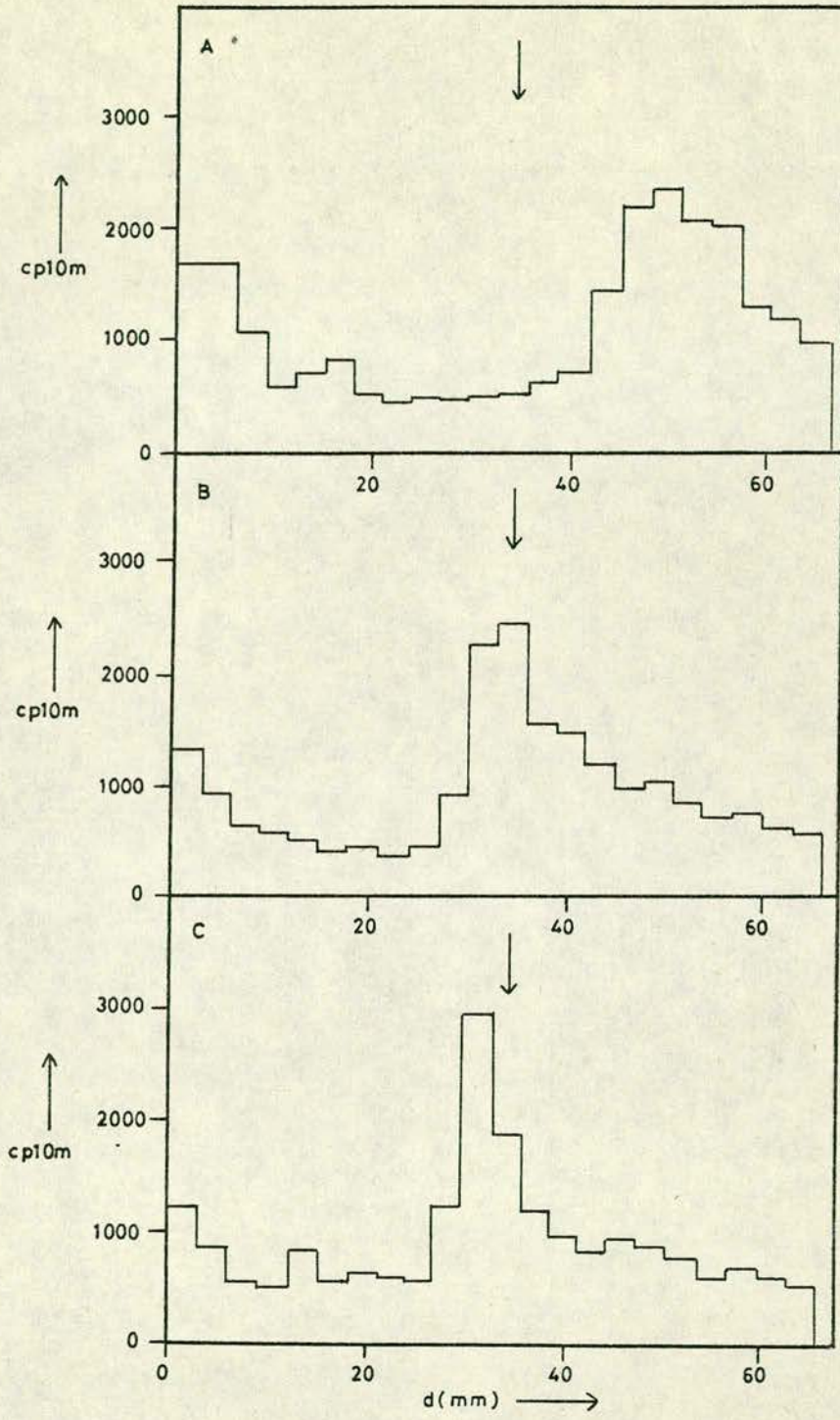


Fig. 13



Figure 14. Endonuclease assay using T7 C5 LG3 RNA

100  $\mu$ l transcription reactions at high ionic strength contained T7 C5 LG3 DNA (5 pmole/ml) and K12 DNA-agarose holoenzyme at E:DNA = 4.  $^3$ H-CTP was at 3 mCi/mmole. After 4 min at 37 $^{\circ}$ C, MgCl $_2$  (12 mM) was added and rifampicin (50  $\mu$ g/ml) was added 10 min later. 25  $\mu$ l aliquots were taken at various times, for analysis by electrophoresis on 2.4% polyacrylamide gels for 3 hrs at 5 V/cm.

Panel A: 4 min after MgCl $_2$

B: 14 min

C: 25 min

The position of marker MS2 RNA is shown by the arrows.



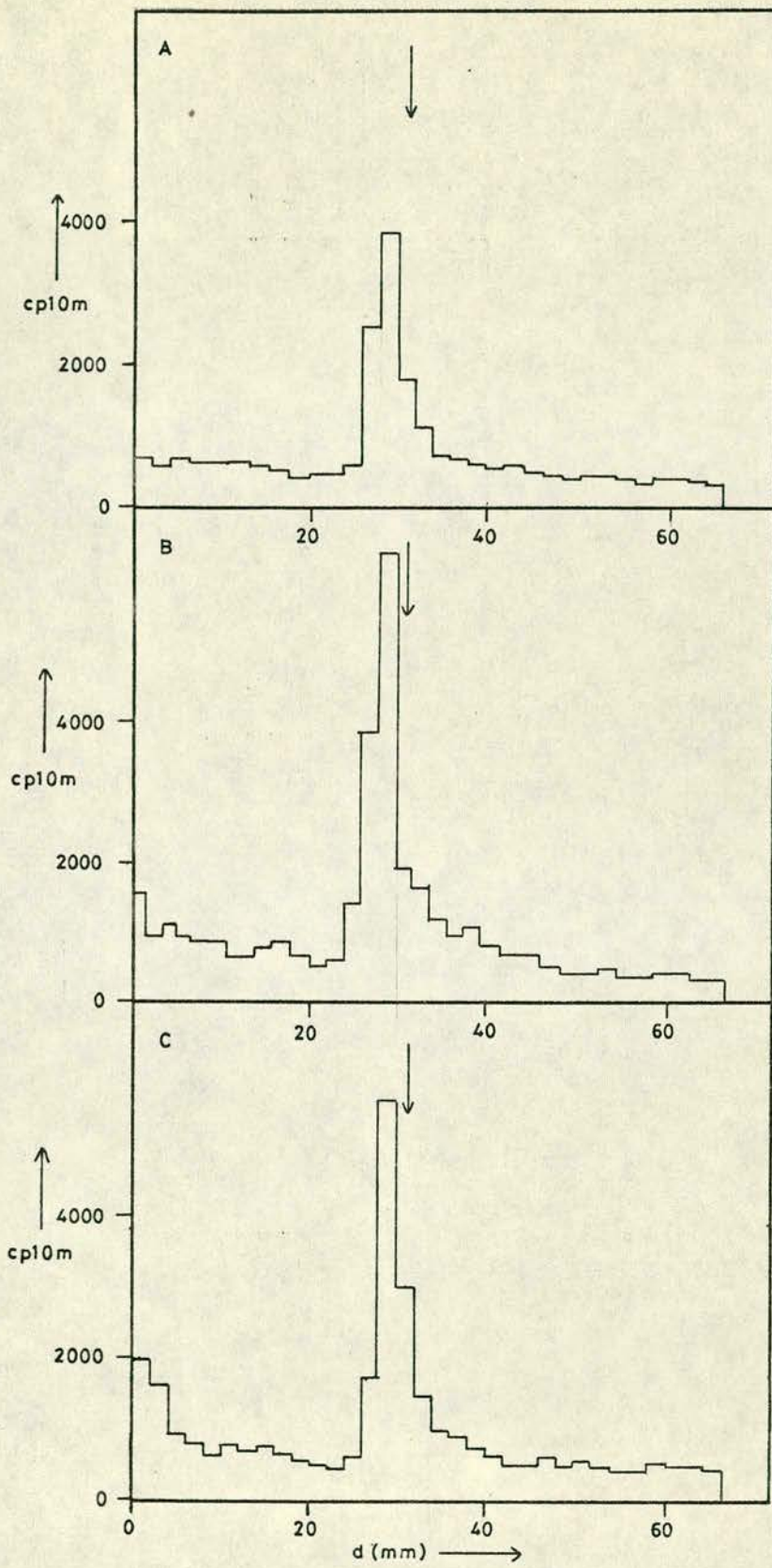


Fig 14



but that it is not sufficient to change the mobility of "native" RNA detectably in 25 mins. Accordingly, the molecular weights determined for the RNAs should be reliable.

It should be noted that further purification by gel filtration essentially removed this endonuclease activity, so that there were very few "hidden" breaks in the RNA synthesised by the most highly purified enzyme preparations (see Fig. 10).

#### 5. An assay for DNA release

The filter binding assay of Hinkle and Chamberlin (1972a) was adapted for use as an assay for DNA release. Binary complexes (including those formed in the presence of rifampicin) are stable in 50 mM NaCl, but not in 0.5 M NaCl. Transcribing (or elongating) complexes are stable in either case. Termination of RNA synthesis should therefore, sooner or later, lead to loss of salt-stable complexes and hence to reduced retention of DNA on nitrocellulose filters at 0.5 M NaCl.

Reactions were generally set up in 0.15 M KCl at low E:DNA ratio and incubated without NTPs (37°C, 4 or 5 min). On addition of NTPs transcribing complexes were formed. Rifampicin was added 30 seconds after NTPs, to prevent subsequent re-initiations. The binding of [<sup>3</sup>H]labelled DNA to nitrocellulose when filtered in the presence of 0.5 M NaCl was followed (Fig. 15).

The figure shows that prior to the addition of NTPs there is very little DNA retained at 0.5 M NaCl. Note however that binary complexes are efficiently formed and



Figure 15. Binding of T7 DNA during transcription

A 0.3 ml transcription reaction at high ionic strength contained  $^3\text{H-T7}^+$  DNA (35,000 cpm, 1 pmole/ml) and Biogel-purified holoenzyme at E:DNA = 2.5. After 4 min at  $37^\circ\text{C}$ , NTPs were added; 2 min later 100  $\mu\text{l}$  were taken to rifampicin (10  $\mu\text{g/ml}$ ) and 30 seconds later 100  $\mu\text{l}$  to heat-denatured salmon sperm DNA (500  $\mu\text{g/ml}$ ).

At various times, 10  $\mu\text{l}$  samples were diluted with 2 ml of ice-cold buffer containing 0.5 M NaCl, and filtered.

- control
- rifampicin
- salmon sperm DNA



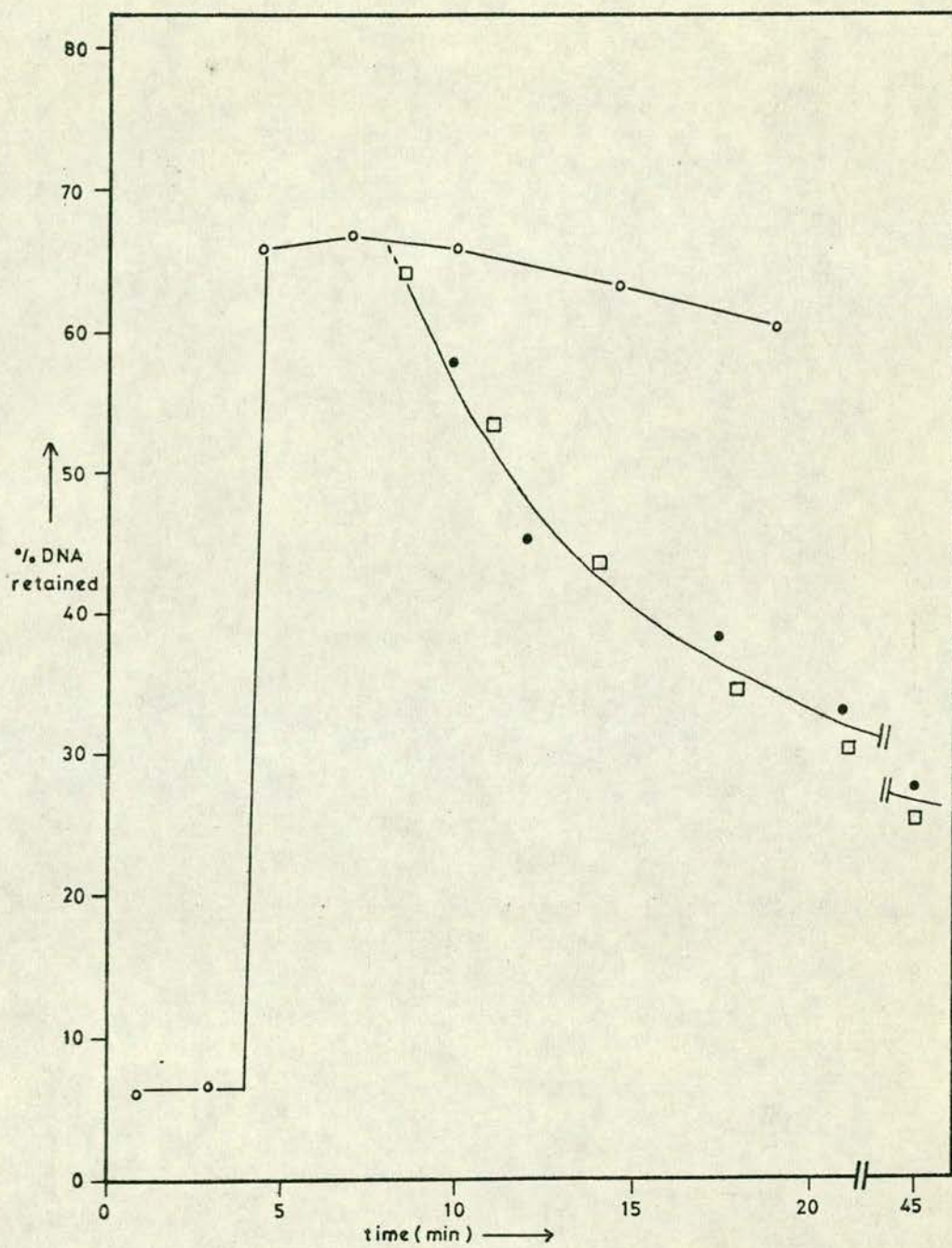


Fig. 15



can be detected by filtration in 50 mM NaCl (see Fig. 9). In the absence of polymerase, some 3% of DNA is retained in 50 mM NaCl and 7% in 0.5 M NaCl. This baseline was consistently observed and has been subtracted from all the data presented here.

After addition of NTPs, initiation and elongation occupies about 4 mins on T7<sup>+</sup> DNA. During this period much of the DNA is retained with a progressive fall thereafter. This fall could reflect dissociation of DNA and polymerase following termination; or release of RNA leaving a binary complex which might not itself dissociate until transferred to 0.5 M NaCl; or a gradual deterioration of the polymerase. (Results concerning the release of RNA are presented later.) The following evidence does not support the latter hypothesis. If rifampicin is not added, then much of the DNA continues to be retained after 4 min, and the decline is greatly slowed. This suggests that re-initiation can occur after 4 mins, implying release of active polymerase from the termination site during the transcription reaction. Moreover, if excess unlabelled DNA (native T7 DNA or heat denatured salmon sperm DNA) is added instead of rifampicin, the "release" profile for [<sup>3</sup>H] DNA is essentially unchanged, presumably because most of the released enzyme next binds to unlabelled DNA (Fig. 15). This result also shows that rifampicin is neither required for, nor an inhibitor of, the observed release.

The rate of decline of DNA retained has a half-life ( $t_{1/2}$ ) of 20 mins at active enzyme to DNA ratios (E:DNA) of 2-2.5, but is very sensitive to this ratio. Each T7 DNA



molecule has several sites where holoenzyme can bind tightly (Hinkle and Chamberlin, 1972a). At low E:DNA ratios, this number is effectively equal to the number of promoters. If all these sites had the same affinity for RNA polymerase, then the distribution of enzymes over the DNA population prior to NTP addition would follow the Poisson law. A certain percentage of DNA molecules would have no enzymes bound, while some would carry one, two, three, etc. polymerases. In fact, the sites have different affinities, which will somewhat bias the distribution. It should be noted however, that at a given E:DNA ratio the percentage of DNA retained during transcription agrees well with that predicted by the Poisson law. Since only one transcribing enzyme is required for retention of DNA on the filter, the rate of release measured is that at which the last enzyme-DNA complex becomes unstable in 0.5 M NaCl. To determine the rate of dissociation of a single enzyme, the reactions should ideally be at a sufficiently low E:DNA ratio that most of the DNA carries either one or no enzyme.

Experiments similar to that of Fig. 15 were performed at an E:DNA ratio of 0.8. In this case, much less DNA becomes retained on transcription, but greater than 60% of the DNA which is retained should have only one enzyme bound, compared to the 25% at an E:DNA ratio of 2.0. The profile was little changed, although the points were more scattered, but the half-life for release was reduced to 4-5 mins. Note that this is the rate at which the E-DNA complex becomes unstable in 0.5 M NaCl, and may not be directly



related to the rate of DNA release. It is possible that the DNA release reaction has intermediates which may be unstable in 0.5 M NaCl. It is easy to imagine that an E-DNA complex at the termination site might after RNA release be unstable in 0.5 M NaCl, by analogy to binary complexes.

Even if there are no intermediates which are unstable in 0.5 M NaCl, this may not be an accurate estimate of the rate of DNA release at the termination site  $t_1$ . Some of the DNA molecules will still have bound more than one enzyme, which would lead to underestimation of the rate of release. Secondly, readthrough of  $t_1$  followed by termination and release at some distal site, would lead to underestimation of the rate of release.

The results obtained for the rate at which the E-DNA complexes become unstable in 0.5 M NaCl at different E:DNA ratios could fit either of two models.

1. Release of DNA occurs by the dissociation of an E-DNA complex at  $t_1$  (which is stable in 0.5 M NaCl) to give free enzyme and DNA. This reaction has a half-life of 4-5 mins, but at high E:DNA ratios, the DNA becomes free of all enzymes at a much slower rate, as each DNA molecule carries several enzyme molecules.

2. Release of DNA occurs by the dissociation of an E-DNA complex at  $t_1$  which is not stable in 0.5 M NaCl. The prolonged retention of DNA (in complexes stable in 0.5 M NaCl) at an E:DNA ratio of 2-2.5 is because most of the DNA molecules which have bound any RNA polymerase molecules have bound several. Release (i.e. dissociation



of an E-DNA complex at t1) is slow, so that transcribing complexes are stopped from reaching t1 because of enzyme already bound there, and it is these blocked transcribing complexes which lead to DNA retention in 0.5 M NaCl, while the enzyme actually bound at t1 dissociates from the DNA at this ionic strength. At lower E:DNA ratios, much of the DNA retained has only one enzyme molecule bound, so that although the E-DNA complex at t1 dissociates slowly, it is not stable during the filtration, so DNA release appears to be much quicker. The rate determined may in fact measure the rate at which enzyme at t1 becomes unstable in 0.5 M NaCl, and this may be the rate of RNA release, i.e the rate at which E-DNA-RNA at t1 (stable in 0.5 M NaCl) is converted to E-DNA at t1 (unstable in 0.5 M NaCl). In this way, the profile of DNA retention at an E:DNA ratio of 2-2.5 would be made up of at least two components.

a) release of RNA by those DNA molecules carrying only one enzyme molecule -  $t_{1/2}$  4-5 min.

b) release of an enzyme molecule from t1 on those DNA molecules carrying two enzymes.

The true rate at which an E-DNA complex at t1 (terminator complex) dissociates would be nearer a  $t_{1/2}$  of 20 min according to this model.

Evidence for this latter model will be presented below.

## 6. Properties of the DNA release reaction

The effect of various treatments on the release of DNA



was examined, usually by allowing reactions to proceed for 6-7 mins, after the addition of NTPs, on T7<sup>+</sup> DNA at an E:DNA ratio of 2-2.5 in the presence of rifampicin, before changing the conditions. The results are summarised below.

a) Detergents

The addition of detergents (SDS or sarkosyl, 1%) completely abolished the retention of DNA, showing that this retention is dependent upon the continued presence of native protein.

b) Ribonucleases

Reactions were performed with T<sub>1</sub> and pancreatic ribonucleases present throughout, at concentrations sufficient to degrade any RNA synthesised to very short oligonucleotides, although this was not directly tested. The rate of DNA release was unaffected, indicating that DNA retention did not require the formation of some complex with long RNA molecules.

c) Temperature

Reactions were performed as usual and allowed to proceed into the "release" phase. Aliquots were then incubated at different temperatures and the rate of release followed as usual (Fig. 16). The half-lives determined were:-

37°C	18 min
24°C	50 min
10°C	very long

According to model 1, this would be because the rate of dissociation of the terminator complex is decreased at



Figure 16. Effect of temperature on DNA release

A 0.2 ml transcription reaction, at high ionic strength, contained  $^3\text{H-T7}^+$  DNA ( 1 pmole/ml) and Biogel-purified holoenzyme at E:DNA = 2.7. After 4 min at  $37^\circ\text{C}$ , NTPs were added, and 2 min later heat-denatured salmon sperm DNA (500  $\mu\text{g/ml}$ ). At 10 min, 70  $\mu\text{l}$  were transferred to  $24^\circ\text{C}$ , and 30 seconds later 70  $\mu\text{l}$  to  $10^\circ\text{C}$ . 10  $\mu\text{l}$  samples were filtered as before (Fig. 15).

- $37^\circ\text{C}$
- $24^\circ\text{C}$
- $10^\circ\text{C}$



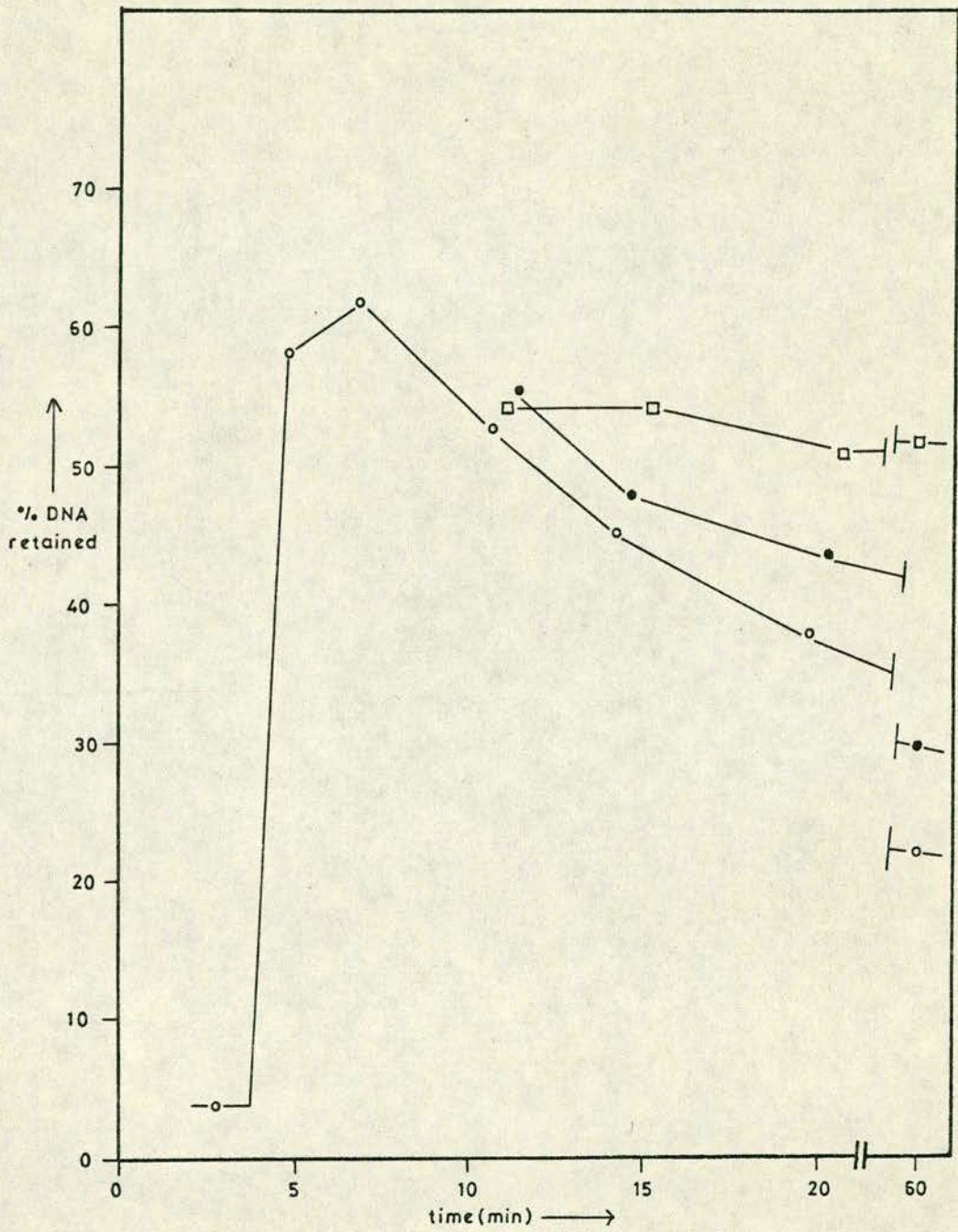


Fig. 16



reduced temperatures. Model 2 would suggest that the decreased rate of release is due to the decreased rate of elongation by enzymes up to t1 from a position just behind it, where they were blocked by an enzyme actually bound at t1. However, the rate of dissociation of salt stable complexes at 24°C is so slow that it seems likely that dissociation of the terminator complex is slower at reduced temperatures.

d) EDTA

When EDTA was added during the period of elongation it caused a total inhibition of release (Fig. 17). This is presumably due to chelation of  $Mg^{2+}$  ions, and consequent cessation of elongation so that enzyme molecules never reach the termination site.

The addition of EDTA during "release" also resulted in total inhibition of further release (Fig. 17). This is predictable by model 2, as only DNA molecules bearing more than one RNA polymerase are retained in 0.5 M NaCl during this "release" phase, and all but the first must continue transcription in order to reach the terminator. It is this transcription (and hence "release") that EDTA inhibits. If model 1 is valid, this result indicates that  $Mg^{2+}$  ions are essential for dissociation of terminator complexes.

e) Streptolydigin

The drug streptolydigin is known to inhibit the elongation of RNA polymerase by interacting with the  $\beta$  subunit. When it was added during the period of elongation, there was a total inhibition of subsequent release



Figure 17. Effects of EDTA and streptolydigin on DNA release

A 0.3 ml reaction at high ionic strength contained  $^3\text{H-T7}^+$  DNA (1 pmole/ml), and Biogel-purified holoenzyme at E:DNA = 2.7. After 4 min at  $37^\circ\text{C}$  NTPs were added, and 2 min later heat-denatured salmon sperm DNA (500  $\mu\text{g/ml}$ ). At  $6\frac{1}{2}$  min 50  $\mu\text{l}$  was taken to EDTA (25 mM) and 30 seconds later 50  $\mu\text{l}$  to streptolydigin (100  $\mu\text{g/ml}$ ). At 11 min 50  $\mu\text{l}$  was taken to EDTA (25 mM) and 30 seconds later 50  $\mu\text{l}$  to streptolydigin (100  $\mu\text{g/ml}$ ). 10  $\mu\text{l}$  samples were filtered as before (Fig. 15).

- control
- streptolydigin added during elongation
- streptolydigin added during release
- △ EDTA added during elongation
- ▲ EDTA added during release



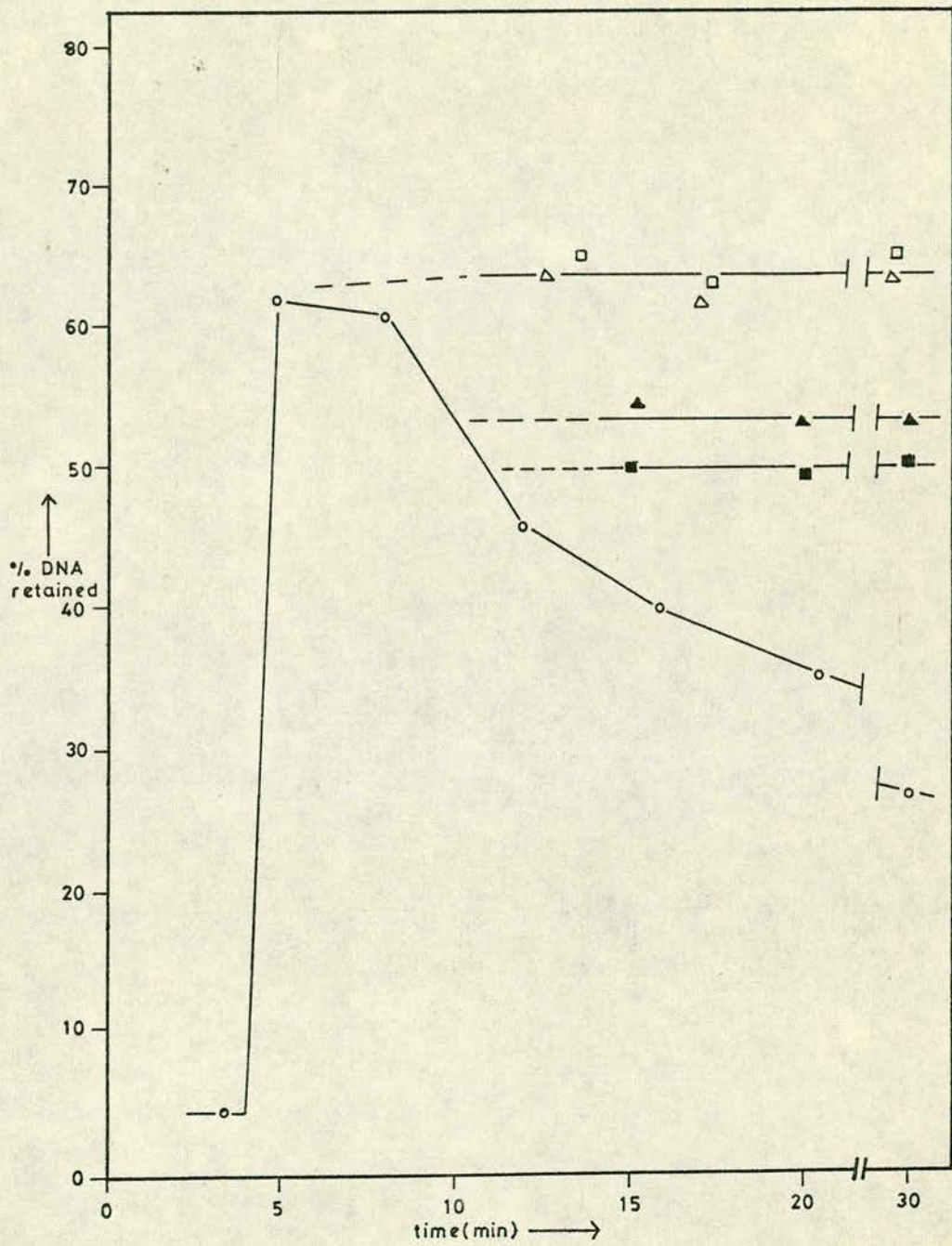


Fig. 17



be retained upon filtration at 0.5 M NaCl as they are complexed with enzyme and DNA.

Aliquots were taken from a transcription reaction, and total RNA assayed by acid precipitation while complexed RNA was detected by filtration. The results show that during the first 4 mins all the RNA is in complexes, and that RNA release occurs after this time (Fig. 18). Evidently endonuclease activity is negligible within the terms of this test. A steady state for bound RNA is established if re-initiations are allowed. From a comparison of the amount of RNA bound at 4 mins and at the steady state, it seems that the size of the average nascent RNA chain at the steady state is at least 70% of a full size transcript. Even allowing for readthrough, this strongly suggests that termination is rate-limiting. If any of the other steps of the transcription cycle (promoter binding, initiation or elongation) were rate-limiting, then all transcribing enzyme molecules should be distributed randomly throughout the early region, carrying on average a half-size RNA. This is direct evidence that some step of termination is rate-limiting, but it does not prove that it is RNA release. More than one enzyme can transcribe each DNA molecule. Hence, if dissociation of terminator complexes is slow, there could be a build-up of enzymes carrying nearly completed RNA just behind the termination site.

The rate of RNA release was studied as for DNA release, by inhibiting re-initiations with rifampicin and following the retention of RNA on nitrocellulose filters in 0.5 M



Figure 18. Total and filter-bound RNA

A 200  $\mu$ l transcription reaction at high ionic strength contained T7<sup>+</sup> DNA (1 pmole/ml), and Biogel-purified holo-enzyme at E:DNA = 2.0. After 4 min at 37°C, NTPs (<sup>3</sup>H-CTP at 50 mCi/mmole) were added. At 6<sup>3</sup>/<sub>4</sub> min, 60  $\mu$ l were treated with streptolydigin (100  $\mu$ g/ml).

10  $\mu$ l samples were taken both for acid-precipitation and for filtration in 2 ml of ice-cold buffer containing 0.5 M NaCl.

- control (acid-precipitable)
- control (filter-bound)
- streptolydigin (acid-precipitable)
- streptolydigin (filter-bound)



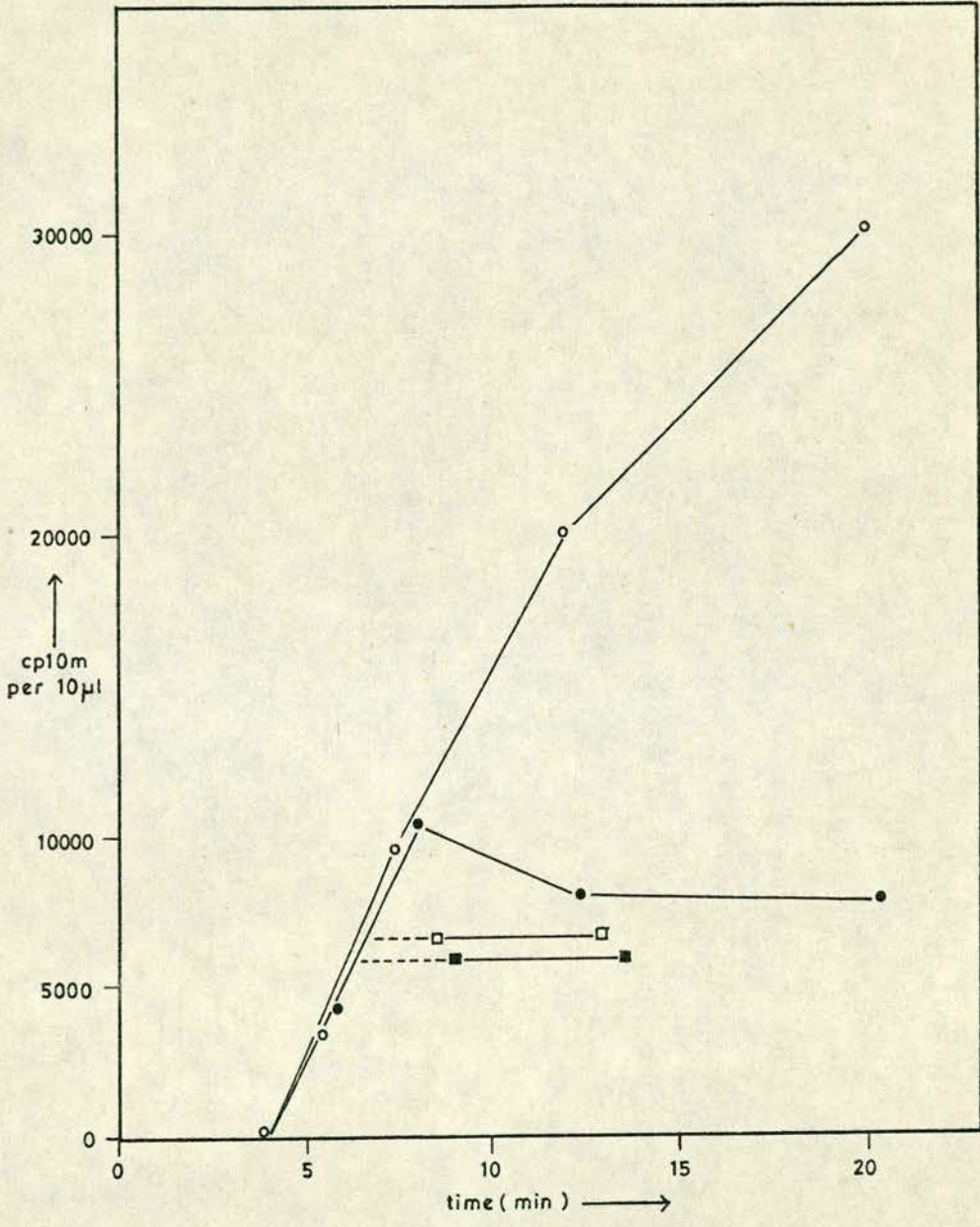


Fig. 18



NaCl. This is shown in Fig. 19. Model 2 predicts that the release of RNA will be made up of at least two components.

a) release of RNA on termination from all the DNA which had only bound one enzyme and from the leading enzymes on DNA molecules which had bound more than one enzyme.

b) release of RNA by subsequent enzymes on DNA molecules which had bound more than one enzyme only after dissociation of a termination complex.

In this way, some 65% of the RNA at an E:DNA ratio of 2-2.5 will be released at the true rate of RNA release, with the remainder only slowly released. This rate has a  $t_{1/2}$  of 4 mins.

It is unlikely that degradation of RNA has contributed significantly to the observed release, as when elongation is inhibited, there is very little release of nascent RNA (Fig. 18).

If there is significant readthrough of the terminator site, then in the presence of rifampicin, some of the DNA and RNA may be retained for longer than the rest. The profiles shown (Figs. 15 and 19) indicate that this might be the case, although quantitation of the degree of readthrough is not possible from these studies alone.

## 8. Conclusions

On the basis of the kinetics of transcription, a slow step was hypothesised at the end of the first round. Under conditions similar to those used in these studies the rate of promoter location and binding has a  $t_{1/2}$  of 20-30 seconds (Hinkle and Chamberlin, 1972a). The rate of initiation of



Figure 19. Retention of RNA during transcription

A 200  $\mu$ l transcription reaction, at high ionic strength, contained T7<sup>+</sup> DNA (1 pmole/ml) and Biogel-purified holo-enzyme at E:DNA = 2.4. After 4 min at 37<sup>o</sup>C, NTPs (<sup>3</sup>H-CTP at 50 mCi/mmole) were added. At 7 min 75  $\mu$ l were treated with rifampicin (10  $\mu$ g/ml).

10  $\mu$ l samples were diluted into 2 ml of ice-cold filtration buffer containing 0.5 M NaCl, and filtered.

- o control
- rifampicin



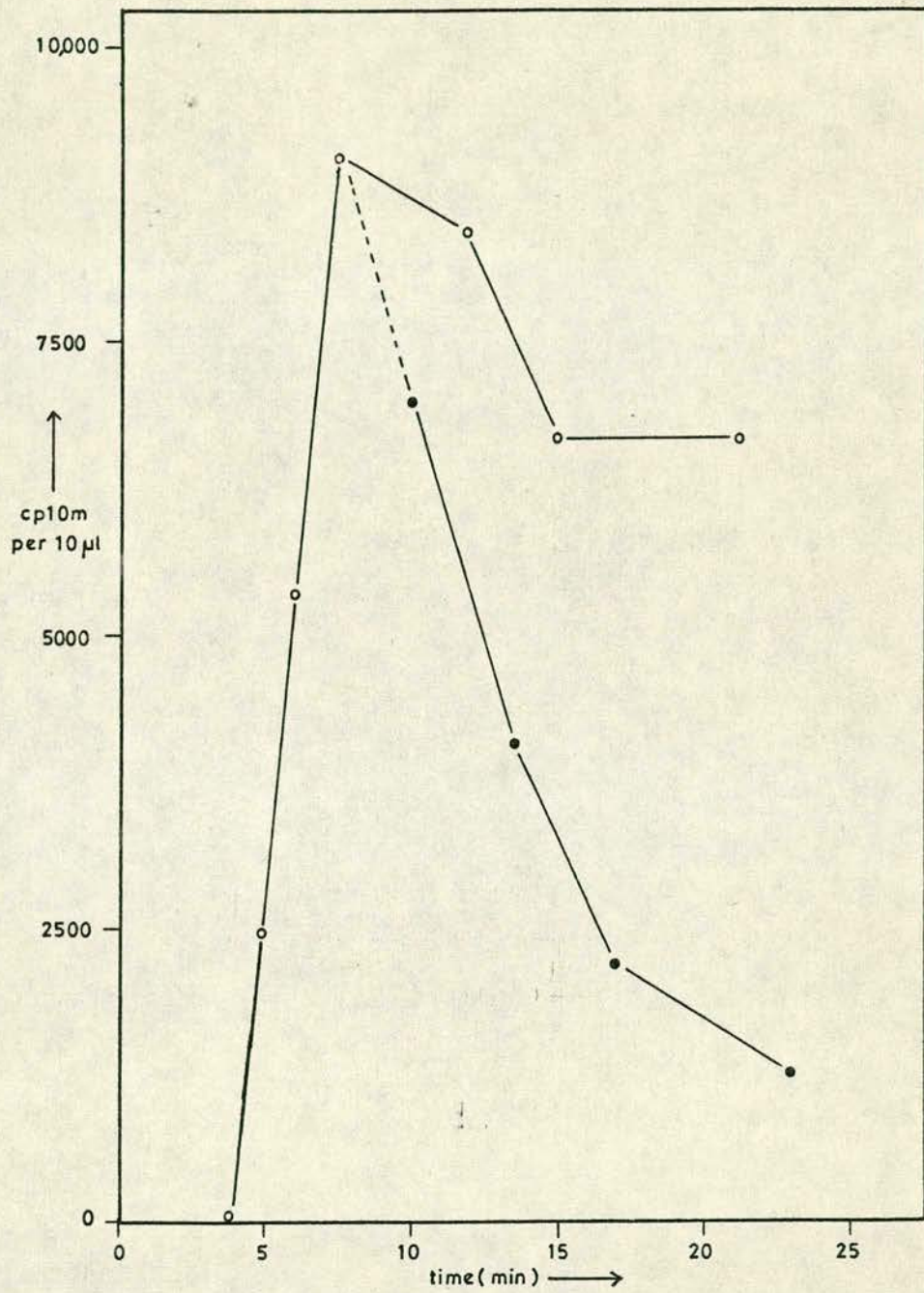


Fig. 19



such promoter complexes has a  $t_{1/2}$  of 0.2 second (Mangel and Chamberlin, 1974a).

There have been no detailed kinetic studies on the binding of sigma to core enzyme, so it is impossible to give a  $t_{1/2}$  for this process. However, it is clear that free sigma becomes rapidly associated with available core enzyme, and an equilibrium between core and holoenzyme is established which turns over at a significant rate (Travers, 1973).

Evidence has been presented here that the  $t_{1/2}$  of RNA release is 4 min and because of the nature of the assay used, the synthesis of the 3' terminal RNA sequences has to be at least as fast. Similarly, evidence has been presented that the  $t_{1/2}$  of DNA release is 20 min,\* and it seems likely that this is the rate-limiting step in the transcription of T7 DNA by E.coli RNA polymerase.

\* The relationship between this experimentally determined Figure and the rate at which an enzyme molecule (bound at 61 after completion of an RNA) dissociates from the DNA is not a direct one, and is discussed in chapter. 10.



CHAPTER 6. IDENTIFICATION OF RESTRICTION FRAGMENTS OF T7  
DNA CONTAINING PROMOTERS AND TERMINATION SITES

1. Introduction

DNA can be dissected into fragments using the site-specific type II restriction endonucleases. These enzymes bind to a sequence of nucleotides in the DNA and make breaks in both strands. Whether the breaks are opposite (to produce double stranded ends) or staggered (to produce single stranded ends) depends on the enzyme used (R.J. Roberts, 1976).

In these studies two enzymes have been used. They are:-

a) HindIII from Haemophilus influenzae strain d which recognises the sequence

↓  
GTYRAC

CARYTG  
↑

b) HpaII from Haemophilus parainfluenzae which recognises the sequence

↓  
CCGG

GGCC  
↑

Y is pyrimidine, R is purine, and the arrows indicate where the strands are broken.

Both of these enzymes cut T7<sup>+</sup> DNA into 50-60 fragments ranging in size from 20 to 2500 base pairs long. Some of the sites of attack on T7<sup>+</sup> DNA are known, although no complete map is available for either enzyme (Ludwig and Summers, 1975, 1976; Humphries et al, 1974; F.W. Studier, personal communication; J.C. Boothroyd, personal communication). A preliminary map of the early region is shown in Fig. 20.

Fragments of T7 DNA containing promoters and termin-



Figure 20. Preliminary map of restriction sites within the T7 early region

The positions of the sites are according to Studier (personal communication). The sizes of the fragments are shown in base pairs according to Ludwig and Summers (1975, 1976).

The strain of T7 is shown at the top (equivalent to the left end of the T7 map) and the restriction endonuclease at the bottom.

The positions of the promoters (A1, A2, A3, B, C and D) and terminator (t1) are shown on the left-hand line.



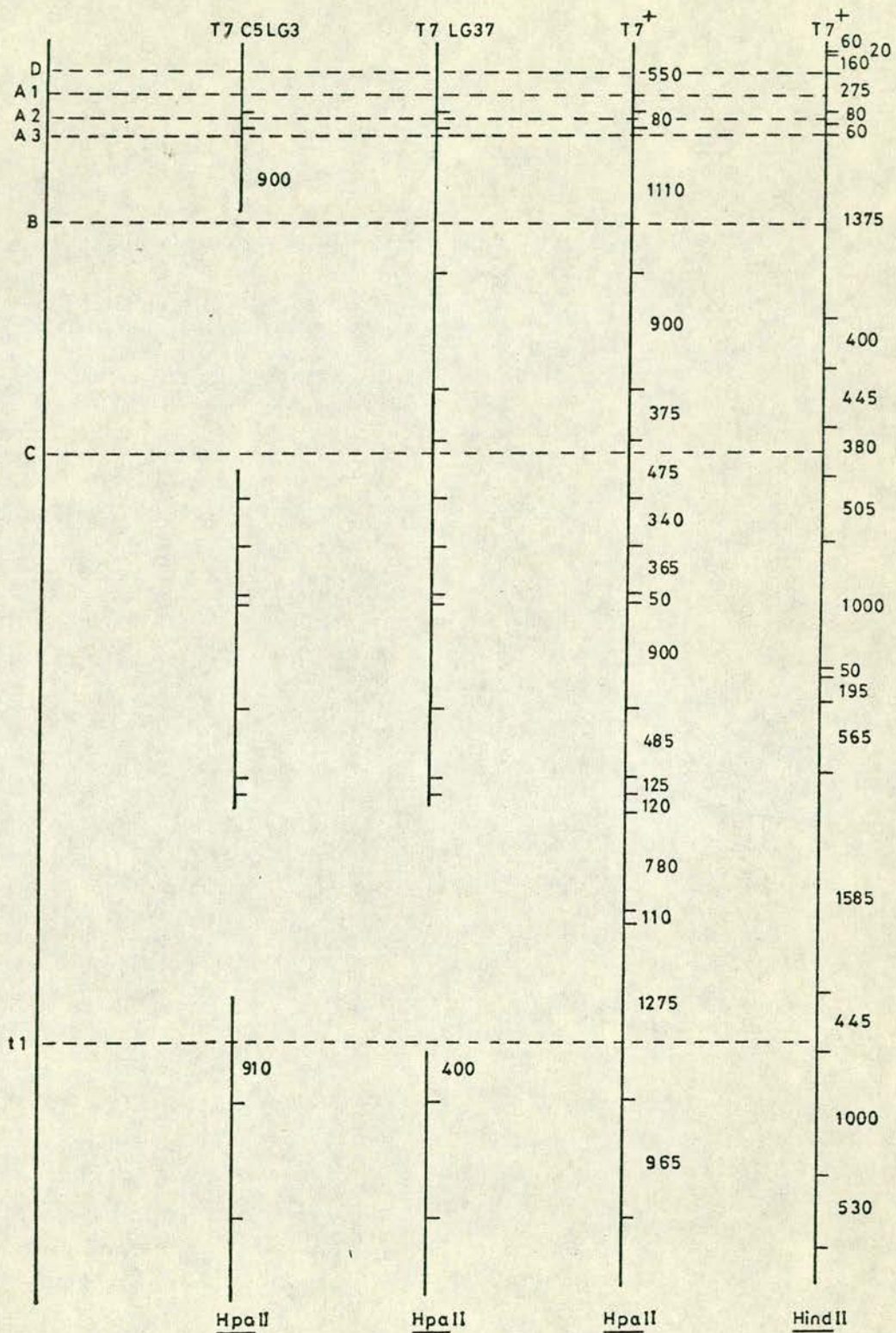


Fig. 20



ation sites have been purified using nitrocellulose filters to retain those fragments which have bound RNA polymerase. The retained fragments have been identified by recovering them off the filters followed by gel electrophoresis in parallel with those fragments which passed through the filter.

## 2. HindII fragments containing promoters

T7<sup>+</sup> DNA was digested to completion with HindII as described in chapter 3. The digests were then incubated (37°C) with RNA polymerase at low E:DNA ratios (1-5) in 0.15 M KCl and in the absence of NTPs. (As before, all E:DNA ratios are active enzyme to DNA ratios. They should be multiplied by about 5 to obtain total enzyme to DNA ratios.). After 10 mins incubation to allow enzyme to bind to promoters, the mixture was diluted to 0.2 ml with pre-warmed buffer containing 50 mM NaCl, and passed through a nitrocellulose filter at room temperature. The filter was washed with a further 0.2 ml, and the combined filtrates were precipitated with ethanol. The precipitate should be enriched for those fragments which do not contain tight binding sites for RNA polymerase.

The filter was then washed with at least 4 ml of buffer and transferred to a scintillation vial. Some 0.4 ml of buffer containing 1% sarkosyl NL35 (v/v) was overlaid on the filter and gently shaken (37°C, 30 min). The eluate (enriched for those fragments which do contain tight polymerase binding sites) was taken for ethanol precipitation.



The precipitates were collected by centrifugation, redissolved (in 25  $\mu$ l of 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA, 10% glycerol containing 0.1% bromophenol blue) and analysed by polyacrylamide gel electrophoresis. Ludwig and Summers (1975) have reported the sizes of all T7<sup>+</sup> DNA fragments produced by the restriction endonucleases HpaI, HpaII and HindII. More recently, they have determined the positions of some T7 promoters with respect to the HindII fragments by an analysis similar to that outlined above (Ludwig and Summers, 1976). For simplicity, the convention adopted by Ludwig and Summers to identify fragments by their size (in base pairs) and the enzyme producing them will be used below: e.g. HpaII 550, HindII 1375.

At an E:DNA ratio of 2-3, the T7<sup>+</sup> fragments HindII 275, 380 and 1375 were strongly retained (Fig. 21e). The fragments HindII 1880 and 2050 were weakly retained. Ludwig and Summers (1976) reported that HindII 80, 380 and 1375 were retained at an E:DNA ratio of 100. (The gels used here do not routinely allow detection of fragments smaller than 100 base pairs. Hence retention of HindII 80 would not have been detected.) From the map (Fig. 20) it is likely that HindII 80 contains the promoter site A2; HindII 275 the A1 promoter; HindII 380 the minor promoter C and HindII 1375 the minor promoter site B. In experiments where RNA polymerase was allowed to bind to T7<sup>+</sup> DNA and then digested with HindII, Ludwig and Summers (1976) detected new fragments of 435 and 1435 base pairs. These probably arise by binding of RNA polymerase to promoters



Figure 21. Fragments of T7 DNA which contain promoters and terminators

- + retained when filtered in 50 mM NaCl
- not retained when filtered in 50 mM NaCl

The samples were electrophoresed, on a 3-9% polyacrylamide gradient gel, at 7.5 V/cm for 18 hrs.

a. A HpaII digest of T7 C5 LG3 DNA (1  $\mu$ g) was incubated with Biogel-purified holoenzyme (E:DNA = 3.0) at high ionic strength for 10 min at 37°C, then fractionated by filtration through nitrocellulose as described in text.

b. A HpaII digest of T7 LG37 DNA (1  $\mu$ g) was incubated with RNA polymerase and fractionated as in (a).

c. A 25  $\mu$ l transcription reaction at high ionic strength contained T7 C5 LG3 DNA (2.5 pmole/ml), and Biogel-purified holoenzyme at E:DNA = 3.0. After 4 min at 37°C, NTPs were added followed 1 min later by rifampicin (10  $\mu$ g/ml). Streptolydigin (100  $\mu$ g/ml) was added at 8 min, and the mixture then digested with HpaII (3 hrs, 37°C) and fractionated by filtration.

d. As (c) except using T7 LG37 DNA. Streptolydigin was added at 11 min.

e. A HindII digest of T7<sup>+</sup> DNA (1  $\mu$ g) was incubated with RNA polymerase and fractionated as in (a). After electrophoresis, the gel was stained with ethidium bromide and photographed as described in chapter 3.



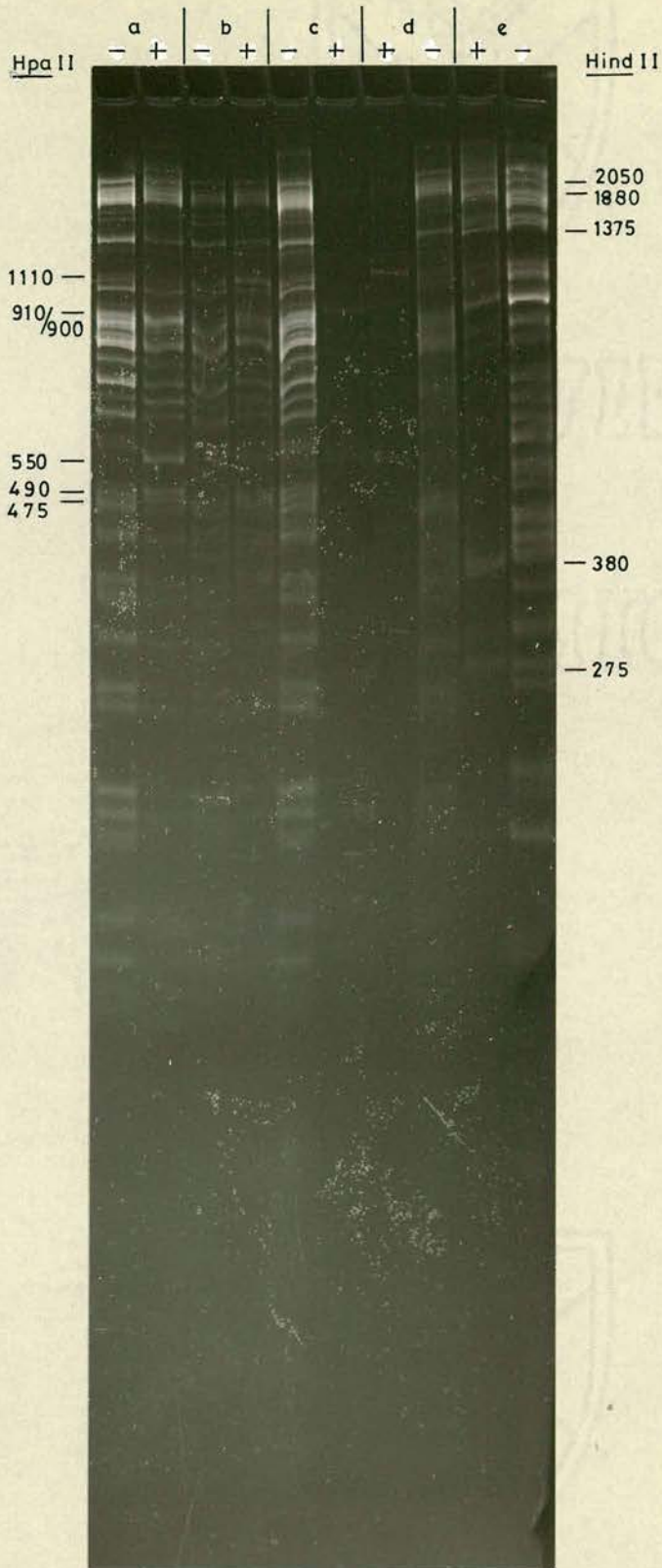


Fig. 21



D and A3 respectively, protecting HindII sites within or close to these promoters. Indeed, a HindII site has been found by sequence analysis near to A3 (Pribnow, 1975), in a region which may be needed for initial interaction of RNA polymerase with this promoter (see chapter 1). Ludwig and Summers (1976) analysed only those fragments which passed through the filters, and this may explain why they did not detect a promoter in HindII 275. There are two HindII fragments of 275 base pairs, so the retention of one of them might easily be missed unless the retained fragments were directly analysed, as in the present work.

HindII 1880 and 2050 appear to map outside the early region. At least one minor promoter site has been reported outside the early region (promoter E), and could be contained in one of these fragments.

The material recovered from the filter shows a background of all the fragments. This could possibly be due to binding of RNA polymerase and/or restriction endonuclease to the ends of the fragments, and has proved useful in the identification of strongly retained fragments.

### 3. HpaII fragments containing promoters

A number of experiments at E:DNA ratios in the range 2-5 have shown that the following fragments contain tight binding sites:-

<u>strain of T7</u>	<u>HpaII</u> fragments	<u>Fig</u>
T7 <sup>+</sup>	475, 490, 550, 1110	22, 23b
T7 C5 LG3	490, 550, 900 (?)	21a
T7 LG37	475, 490, 550, 1110	21c



Figure 22. Fragments of T7<sup>+</sup> DNA containing promoters

+ retained on filtration in 50 mM NaCl

- not retained on filtration in 50 mM NaCl

A 25  $\mu$ l transcription reaction at high ionic strength contained 1  $\mu$ g T7<sup>+</sup> DNA, and Biogel-purified holoenzyme at E:DNA = 2.5. After 10 min incubation at 37<sup>o</sup>C (in the absence of NTPs) 25  $\mu$ l of 10 mM Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol were added. HpaII was then added and the mixture was incubated for 4 hrs at 37<sup>o</sup>C and fractionated as before (Fig. 21).

Electrophoresis was at 7.5 V/cm for 18 hrs on a gel consisting of a 2.4% polyacrylamide slab above a 3-9% polyacrylamide gradient gel. The gel was stained and photographed as before.



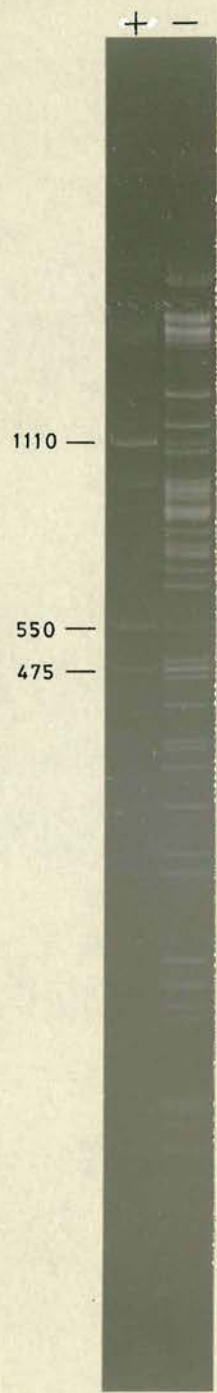


Fig. 22



Figure 23. Fragments of T7<sup>+</sup> DNA containing promoters and terminators.

+ retained on filtration in 50 mM NaCl

- not retained on filtration in 50 mM NaCl

a. A 25  $\mu$ l transcription reaction at high ionic strength contained 1  $\mu$ g T7<sup>+</sup> DNA, and Biogel-purified holoenzyme at E:DNA = 3.1. After 4 min at 37<sup>o</sup>C, NTPs were added, and 1 min later, rifampicin (10  $\mu$ g/ml). At 10 min streptolydigin (100  $\mu$ g/ml) was added, and the mixture then digested with HpaII.

b. A HpaII digest of T7<sup>+</sup> DNA (1  $\mu$ g) was incubated at high ionic strength with Biogel-purified holoenzyme RNA polymerase at E:DNA = 6.0 (37<sup>o</sup>C, 10 min), then fractionated as before (Fig. 21).

c. Unfractionated HpaII digest of T7<sup>+</sup> DNA (1  $\mu$ g). Electrophoresis was in 40 mM Tris, 2 mM EDTA, 20 mM sodium acetate (pH 7.2), on a gel consisting of a 2.4% polyacrylamide slab in the same buffer above a 3-9% polyacrylamide gradient in 100 mM Tris-borate (pH 8.3) 1 mM EDTA, at 7.5 V/cm for 18 hrs.

The gel was stained and photographed as before (Fig. 21).



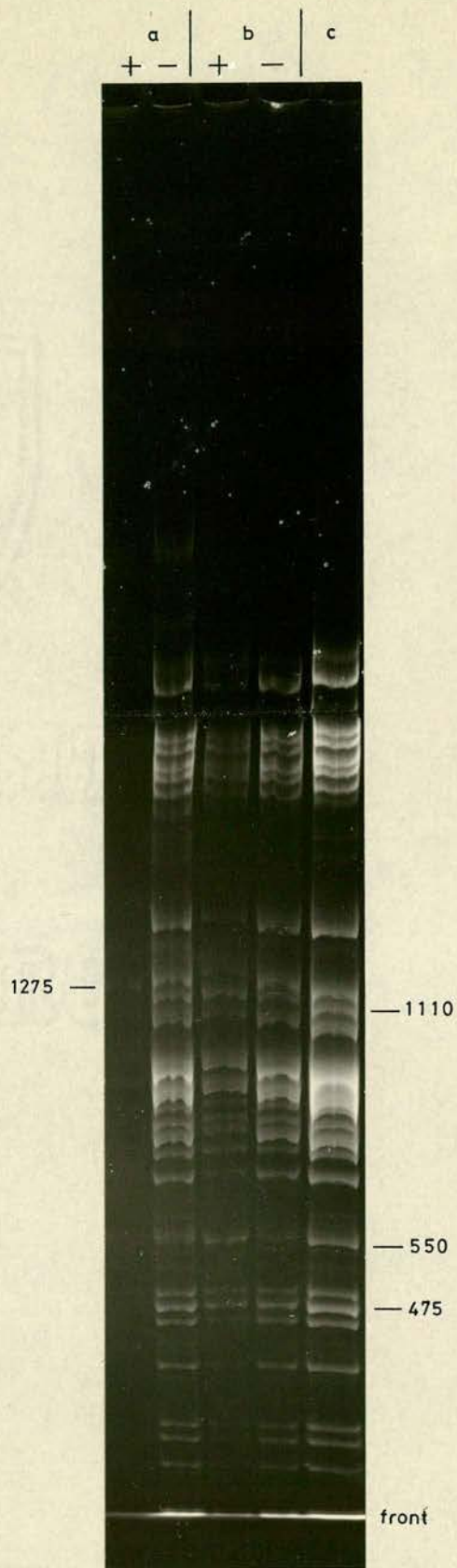


Fig. 23



As the effect of E:DNA ratio was not rigorously examined, the relative affinity that the sites within these fragments have for RNA polymerase is uncertain. From several experiments on T7<sup>+</sup>, the tentative order is:-

HpaII 550 ≡ 1110 > <sup>is</sup> 475 > 490 ≫ rest

The fragment HpaII550/the leftmost fragment (Ludwig and Summers, 1975) and probably contains the promoters A1 and D. From the map, it seems likely that HpaII 1110 contains the promoters A3 and B and HpaII 475 the promoter C. The promoter A2 is probably on a small HpaII fragment which would not be detected here. The fragment HpaII 490 does not map within the early region, and may contain the minor promoter E.

The LG37 deletion does not delete any of the known T7 promoters, and the pattern of retained fragments is the same as for T7<sup>+</sup>. Similarly the LG3 deletion does not affect any of the promoters, but the C5 deletion removes the minor promoters B and C (Stahl and Chamberlin, 1977). Unfortunately, the C5 deletion does not seem to create a fragment whose size is different from the rest of the HpaII fragments. The current map shows that HpaII 900 and 375 are deleted and HpaII 1110 and 475 fused to give a new fragment of about 900 base pairs which should contain the major promoter A3. There are many HpaII fragments of this size so it has not been possible to detect specific retention of a 900 base pair fragment above the background contributed by the many fragments of this size (see Fig. 21).

When T7<sup>+</sup> DNA was incubated with RNA polymerase to allow formation of promoter complexes, and then digested with HpaII



the same pattern of retention was observed. This suggests that none of the HpaII sites could be protected by RNA polymerase bound at the promoter sites.

#### 4. HpaII fragments containing termination sites

In chapter 5 it was concluded that following transcription, the release of RNA polymerase bound at or immediately behind the termination site at the end of the T7 early region could be inhibited by reducing the temperature to 10°C; or by adding EDTA to chelate Mg<sup>2+</sup>; or by adding streptolydigin. If so, it should be possible to demonstrate that enzyme is bound under those conditions to fragments of the DNA containing the termination site.

Reactions were set up at an E:DNA ratio of 2-2.5 as usual. After preincubation (4-5 min, 37°C), NTPs were added, followed 30 seconds later by rifampicin. Thus initiation and elongation should proceed for only one round of transcription. Streptolydigin was added later to inhibit release. It was usual to add the drug 2 mins after the end of the first round of transcription, viz. 6 min after NTPs for T7<sup>+</sup> DNA; 4 min after, for T7 C5 LG3 and 8 min after, for T7 LG37. The reactions were then diluted to 75 mM KCl, HpaII added and incubation continued for up to 5 hrs to achieve complete digestion of the DNA. The mixture was then fractionated by filtration in 50 mM NaCl through nitrocellulose filters to separate fragments with bound RNA polymerase from those free of enzyme

The fragments retained were:



<u>strain of T7</u>	<u>HpaII fragments</u>	<u>Fig</u>
T7 <sup>+</sup>	550, 1110, 1275	23a
T7 C5 LG3	550, 910	21c
T7 LG37	550, 1110	21d

The HpaII fragment of 1275 base pairs contains the right hand endpoints of both the LG3 and LG37 deletions (J.C. Boothroyd, personal communication) and so must contain t1. A new fragment HpaII 400 is generated by the LG37 deletion which does not contain the termination site. Similarly, the LG3 deletion generates a new fragment of 910 base pairs which does contain t1.

The protocol used would allow some release of RNA polymerase before the addition of streptolydigin, and the released enzyme molecules should be able to bind tightly to T7 promoters, despite the presence of rifampicin (Hinkle, Ring and Chamberlin, 1972). Any breakdown of the termination complex during the prolonged restriction reaction could also lead to the binding of promoters by the released enzyme. Thus the observed retention of HpaII 550 and 1110 is not unexpected upon filtration in 50 mM NaCl. The retention of these promoter-containing fragments was variable, and seemed to be related to the retention of fragments containing termination sites, i.e. the more HpaII 550 and 1110, the less HpaII 1275 using T7<sup>+</sup> (e.g. little HpaII 1110 in Fig. 23a). The retention of these fragments rather than HpaII 475 and 490 presumably reflects the relative affinities of the T7 promoter sites for E.coli RNA polymerase.



The large fragment retained using T7 C5 LG3 DNA has been identified above as HpaII 910, the new fragment generated by the LG3 deletion which contains t1. However, the C5 deletion seems to produce a new fragment of 900 base pairs which contains the major promoter A3. To unambiguously identify the fragment, these experiments would have to be repeated with the strains of T7 carrying only one of these deletions. If the retained fragment does carry t1, then this would show that the bound enzyme which is responsible for the retention lies within a region of the DNA from 450 base pairs to the left of t1 to 80 base pairs to the right. In any case, the above results confirm that for T7<sup>+</sup> DNA (at least), the fragment containing the termination site at the end of the early region can be specifically retained under the conditions used here.

In chapter 5, it was argued that streptolydigin stabilises complexes of RNA polymerase with T7 DNA at or immediately behind the termination site against dissociation in 0.5 M NaCl. However, such complexes apparently broke down to a considerable extent during the long incubation with HpaII, as very little DNA was retained after filtration in 0.5 M NaCl. Indeed, if complexes were incubated without HpaII, then again, very little DNA was retained on filtration in 0.5 M NaCl. Accordingly, the present experiments used filtration in 50 mM NaCl. This loss of 0.5 M NaCl-stable complexes might reflect release of polymerase from the terminator, followed by slow elongation of the previously "stalled" RNA polymerases up to the



terminator, with subsequent RNA release. In this way the salt-stable ternary complexes might be replaced by binary terminator complexes which, as argued before, may be unstable in 0.5 M NaCl. Slow elongation is possible in the presence of streptolydigin, as this drug is reasonably reversible in action (Cassani et al, 1971).

When streptolydigin was added 2 min before rather than after the end of the first round of transcription, and the complexes digested and analysed as above, no fragments were retained in 0.5 M NaCl, and only those fragments containing promoters and the termination site were retained in 50 mM NaCl (data not shown). The failure to detect any fragments from the central portion of the early region, where elongating complexes should have been blocked by streptolydigin, supports the suggestion that elongation continues at a significant rate during the HpaII digestion.

This could indicate that the binding of rifampicin gives some protection against the inhibition of elongation by streptolydigin. The experiments reported in Chapter 5 which showed that streptolydigin inhibited elongation (and perhaps release) used excess unlabelled DNA to prevent re-initiation on [<sup>3</sup>H]-T7<sup>+</sup> DNA, whereas the experiments described above using rifampicin to prevent re-initiation suggest that elongation (and perhaps release) occur at a significantly greater rate in the presence of both rifampicin and streptolydigin than with streptolydigin alone. Although both drugs have the same subunit,  $\beta$ , as target, it is not clear if their binding sites are independent or overlapping. The loci for resistance to



rifampicin and streptolydigin do both map in rpoB, the gene for  $\beta$ , but at different regions within the gene (Iwakura, Ishihama and Yura, 1973). Recent experiments to test the possibility of interference between these drugs indicate that rifampicin at up to 100  $\mu\text{g/ml}$  does not detectably affect inhibition of T7 DNA transcription by streptolydigin (50  $\mu\text{g/ml}$ ) added 30-60 secs later (R. Hayward, personal communication).

It might be argued that the retention of fragments HpaII 1110 and 550 in the above experiments is because polymerase molecules bound to them had never initiated. This seems unlikely, as each molecule active in binding DNA also appears to be able to initiate an RNA chain (see Chapter 4), and the yield of RNA per active polymerase molecule was routinely greater than 80% for the first round of transcription. If enzyme were bound in inactive complexes with promoters, they should prevent the passage of active, elongating complexes. This would reduce the molar yield of RNA, and lead to the production of small, specific products as detected at high E:DNA ratios by Minkley and Pribnow (1973) and Stahl and Chamberlin (1977). There is no evidence for such effects in the present work.

The retention of HpaII 1275 has been detected when streptolydigin was added as late as 20 min after the end of the first round of transcription. This confirms that release of RNA polymerase from the terminator t1 is inherently slow in vitro.

There seems to be no retention of a fragment containing a termination site from T7 LG37 DNA. It is possible that



dissociation of the terminator complex at t2 is much quicker than at t1. This could be tested directly using <sup>3</sup>H-labelled T7 LG37 DNA. Alternatively, even if dissociation is slow, the relevant fragment containing t2 may be too small to have been detected in these experiments.

Preliminary experiments to identify the HindII fragment containing the termination site were not continued, as the background from all fragments was too high to conclusively identify those fragments which were specifically retained.



CHAPTER 7. STUDIES ON THE ROLE OF SIGMA IN THE  
TRANSCRIPTION CYCLE

1. Introduction

In the experiments to be described here, sigma was inactivated using an antiserum prepared against the purified protein. The effect of this on various steps in the transcription of T7 DNA by E.coli RNA polymerase has been examined. In particular, the following hypothesis has been tested: that sigma might have a role in termination by binding to the core polymerase at the termination site (probably after RNA release) to facilitate its dissociation from the DNA. This hypothesis seemed plausible because sigma is believed to decrease the affinity of core enzyme for non-promoter DNA (Hinkle and Chamberlin, 1972a). Moreover, it might be physiologically desirable to ensure regeneration of holoenzyme prior to release.

Unless otherwise indicated the amount of antiserum used was sufficient to give at least a 95% reduction in the ability of polymerase to transcribe T7 DNA under the standard conditions, as judged in control experiments where enzyme was preincubated with antiserum (see chapter 4).

2. Initiation

Comparison of the activity of RNA polymerase core and holoenzyme with various templates led to the discovery that sigma is required for proper initiation and thus is essential for significant transcription of intact native templates (Bautz et al, 1969). Sigma is thought to convert the enzyme from a form which has a moderate affinity for



all DNA sequences (core) to holoenzyme, which has a high affinity for promoters and a low affinity for any other DNA sequence (Hinkle and Chamberlin, 1972a).

The effect of prior inactivation of sigma on the transcription of T7 and salmon sperm DNA has been shown in chapter 4 (Fig. 8). It is clear that transcription of T7 DNA but not of salmon sperm DNA is inhibited. The salmon DNA is believed to be considerably degraded, and should contain few, if any, promoters for bacterial holoenzyme.

Core is thought to be unable to open the duplex DNA structure as occurs in the initiation of transcription by holoenzyme at promoters (Hinkle and Chamberlin, 1972a,b). The transcription of native templates by core is believed to initiate at single stranded breaks or at the ends of the DNA (Hinkle and Chamberlin, 1972b; Vogt, 1969). However, that core does not have a greater affinity for promoter sites than other DNA sequences has not been directly demonstrated, as the lack of transcription from promoters could be related to its inability to open the duplex rather than an inability to recognise promoter sequences. It might be interesting to test whether core enzyme preferentially retains on nitrocellulose filters those fragments of T7 DNA which contain promoters. The time and scope of this study did not allow this idea to be pursued.

### 3. Elongation and the termination of transcription

The effect of removing sigma during the elongation step of the transcription cycle was investigated by adding



antiserum to a transcription reaction 30 seconds after the addition of NTPs.

a) Kinetics

The kinetic effects are shown in Fig. 24. There is essentially no difference between the control and experimental reactions for the first 4 min using T7<sup>+</sup> DNA or the first 2 min with T7 C5 LG3 DNA. As these are the times required for transcribing enzymes to reach the termination site, it suggests that the elongation phase of the transcription cycle is completely independent of sigma.

In all the reactions there was a change in the rate of incorporation of NTPs at the end of the first cycle, and if enough antiserum had been added there was no further incorporation. In this limit case, the kinetics closely resemble those observed when rifampicin was added instead of antiserum after 30 seconds transcription. This suggests that the enzyme recognises the termination site and stops the polymerisation of NTPs into RNA in the absence of sigma. At intermediate levels of antiserum, the rate after 4 min is less than in the control. Presumably some active sigma has survived to catalyse re-initiations.

If removal of sigma were to reduce the efficiency with which the elongating core enzyme recognised the termination site it should give rise to increased readthrough. This readthrough should be detectable as continued incorporation of NTPs after 4 min when rifampicin and antiserum were added simultaneously. The results given in Fig. 24 show that the removal of sigma does not increase readthrough. I conclude that recognition of the termination site is



Figure 24. Effect of antisigma antiserum on kinetics

50  $\mu$ l transcription reactions at high ionic strength contained T7 C5 LG3 DNA (5 pmole/ml) and Biogel-purified holoenzyme at E:DNA = 2.0.  $^3$ H-UTP was 10 mCi/mmole. After 4 min at 37 $^{\circ}$ C, MgCl $_2$  (12 mM) was added and 30 seconds later varying amounts of antiserum and/or rifampicin (10  $\mu$ g/ml) were added. 10  $\mu$ l aliquots were acid-precipitated as usual. Time is shown in min after addition of MgCl $_2$ .

nl antiserum per  $\mu$ g enzyme

o	0
●	400
△	800
▲	2000
□	0 plus rifampicin
■	1500 plus rifampicin



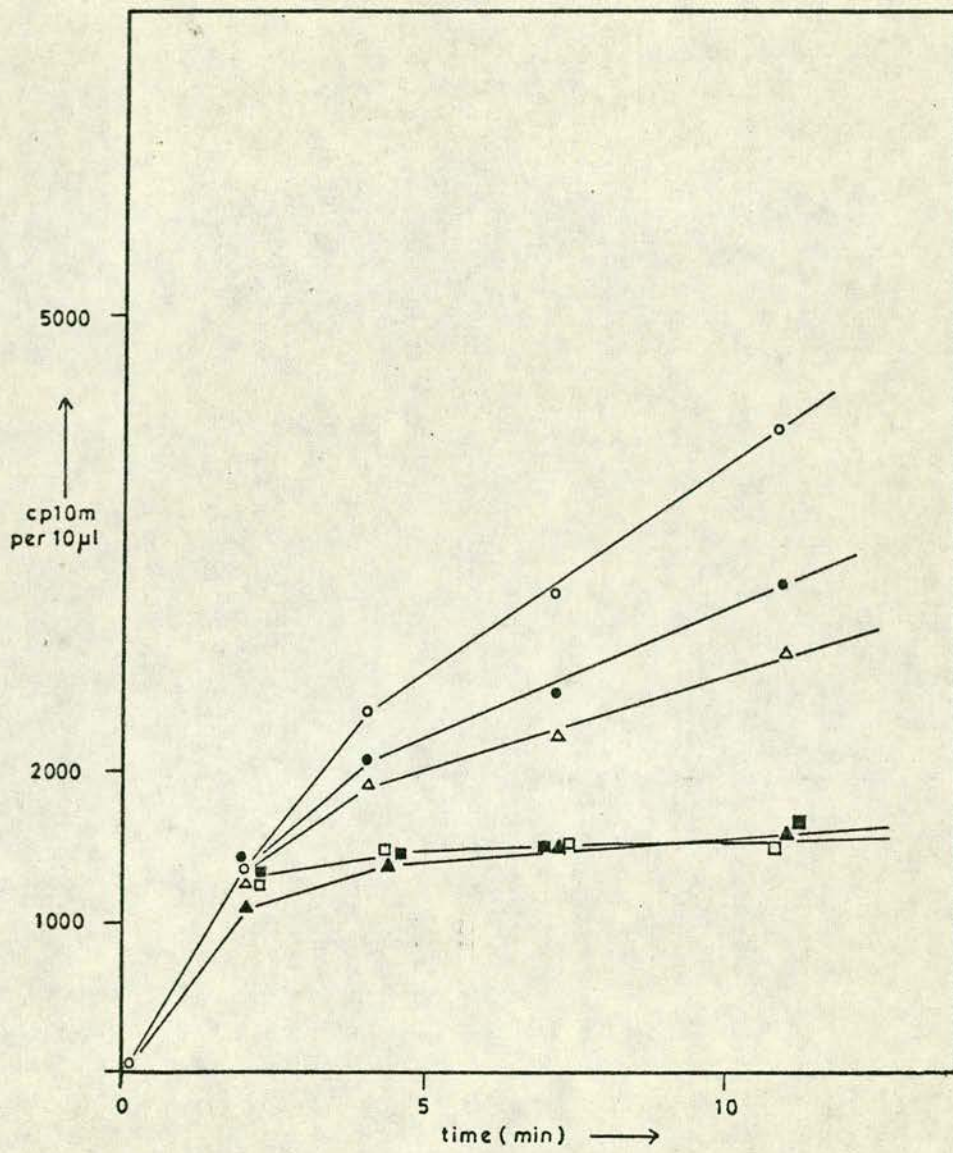


Fig. 24



independent of sigma. More rigorously, the conclusion should be that if sigma is needed for this step in termination, its turnover number must be at least 20-fold higher than in initiation, as this level of antiserum gave 95% inhibition of initiation after 4 min incubation, but no detectable increase in readthrough after a similar incubation.

b) Size of RNA synthesised

Aliquots from reactions similar to those of Fig. 24 were taken for analysis on 2.4% polyacrylamide gels. The profiles show that the size distribution of RNA molecules synthesised after addition of antiserum are the same as in the control reaction (Fig. 25). This is direct evidence that the termination site is recognised by core enzyme with normal efficiency despite the addition of antisigma. There is no evidence that large RNA molecules are produced.

The antiserum preparation does have detectable endonuclease activity. Thus when samples were taken for analysis later in the reaction, when readthrough might be easier to detect, the results were difficult to interpret with certainty (Fig. 25). However, RNA larger than T7 early RNA was never detected.

Similarly if less antiserum was added (in which case residual sigma activity was clearly considerable) the RNA made in the later phases of the reaction was either fully sized T7 early RNA or smaller. No larger RNA molecules were detected. These results are consistent with an unchanged efficiency of termination at t1 with some degradation of the RNA made.



Figure 25. Effect of antisigma antiserum on size of RNA synthesised

A 0.2 ml transcription reaction at high ionic strength contained T7 C5 LG3 DNA (5 pmole/ml) and Biogel-purified holoenzyme RNA polymerase at E:DNA = 1.8.  $^{14}\text{C}$ -ATP was at 3 mCi/mmole. After 4 min at  $37^{\circ}\text{C}$ ,  $\text{MgCl}_2$  (12 mM) was added, and 30 seconds later 0.1 ml was treated with antiserum (1500 nl per  $\mu\text{g}$  enzyme). At 7 min 30  $\mu\text{l}$  samples were taken for analysis by electrophoresis on 2.4% polyacrylamide gels, as usual. At  $8\frac{1}{2}$  min, 30  $\mu\text{l}$  samples were taken to  $^3\text{H}$ -CTP (10 mCi/mmole) and incubated for  $2\frac{1}{2}$  min. Rifampicin (10  $\mu\text{g}/\text{ml}$ ) was then added and incubation continued for a further  $2\frac{1}{2}$  min before analysis on gels. Electrophoresis was for  $2\frac{1}{2}$  hrs at 5 V/cm.

Panel A: control 7 min sample  
B: control  $^3\text{H}$  pulse from  $8\frac{1}{2}$ - $13\frac{1}{2}$  min  
C: antiserum 7 min sample  
D: antiserum  $^3\text{H}$  pulse from  $8\frac{1}{2}$ - $13\frac{1}{2}$  min

$^{14}\text{C}$  ———

$^3\text{H}$  - - - - -



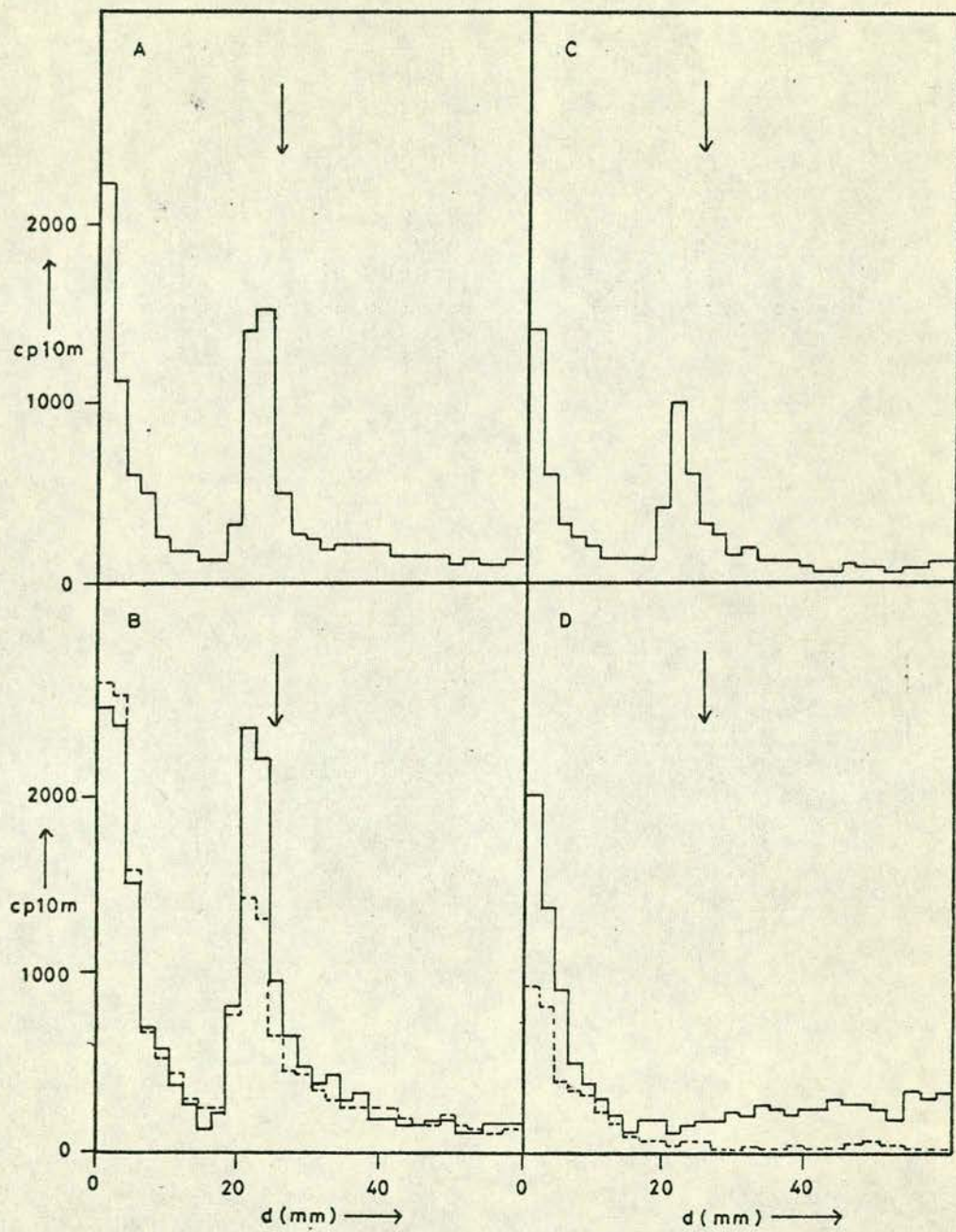


Fig. 25



#### 4. DNA release on termination of transcription

The release of DNA was assayed as described in chapter 5, using [ $^3\text{H}$ ]-T7<sup>+</sup> DNA and filtration through nitrocellulose. The effect of antiserum on DNA release is shown in Fig. 26. It is clear that release occurs at the same rate in the presence and absence of antiserum. As discussed in chapters 5 and 6, the dissociation of enzyme from the DNA at the termination site is rate-limiting in this assay, either from a terminator complex which is itself stable in 0.5 M NaCl or from one which is unstable in 0.5 M NaCl, but has blocked the elongation of RNA polymerase molecules up to the termination site. Evidence has been presented that the latter model is the correct one, but it is clear that the addition of antiserum does not change the rate-limiting step, dissociation of the termination complex. Thus it seems probable that sigma does not catalyse the dissociation of core enzyme from the DNA at t1. Alternatively, it must have a rapid turnover in this role, 20-fold higher than for initiation. In view of the overall slowness of DNA release, this alternative hypothesis is unappealing.

Hinkle and Chamberlin (1972a,b) examined the effect of adding sigma to core which had bound to T7 DNA in the absence of NTPs. The results showed that sigma could catalyse the dissociation of the DNA-core complex. It was hypothesised that in this system, sigma could interact with core on T7 DNA so that holoenzyme was released from the DNA, rather than that core was released before its conversion to holoenzyme. The evidence presented here suggests that the release of DNA by core at the end of the



Figure 26. Effect of antisigma antiserum on DNA release

A 0.2 ml transcription reaction at high ionic strength contained  $^3\text{H-T7}^+$  DNA (1 pmole/ml), and Biogel-purified holoenzyme RNA polymerase at E:DNA ratio = 2.0. After 4 min at  $37^\circ\text{C}$ , NTPs were added. At  $5\frac{1}{2}$  min rifampicin (10  $\mu\text{g/ml}$ ) was added, and at 6 min 80  $\mu\text{l}$  were treated with antisigma antiserum (3000 nl/ $\mu\text{g}$  enzyme).

10  $\mu\text{l}$  aliquots were filtered as before (Fig. 15).

- o control (rifampicin added at  $5\frac{1}{2}$  min)
- antisigma antiserum



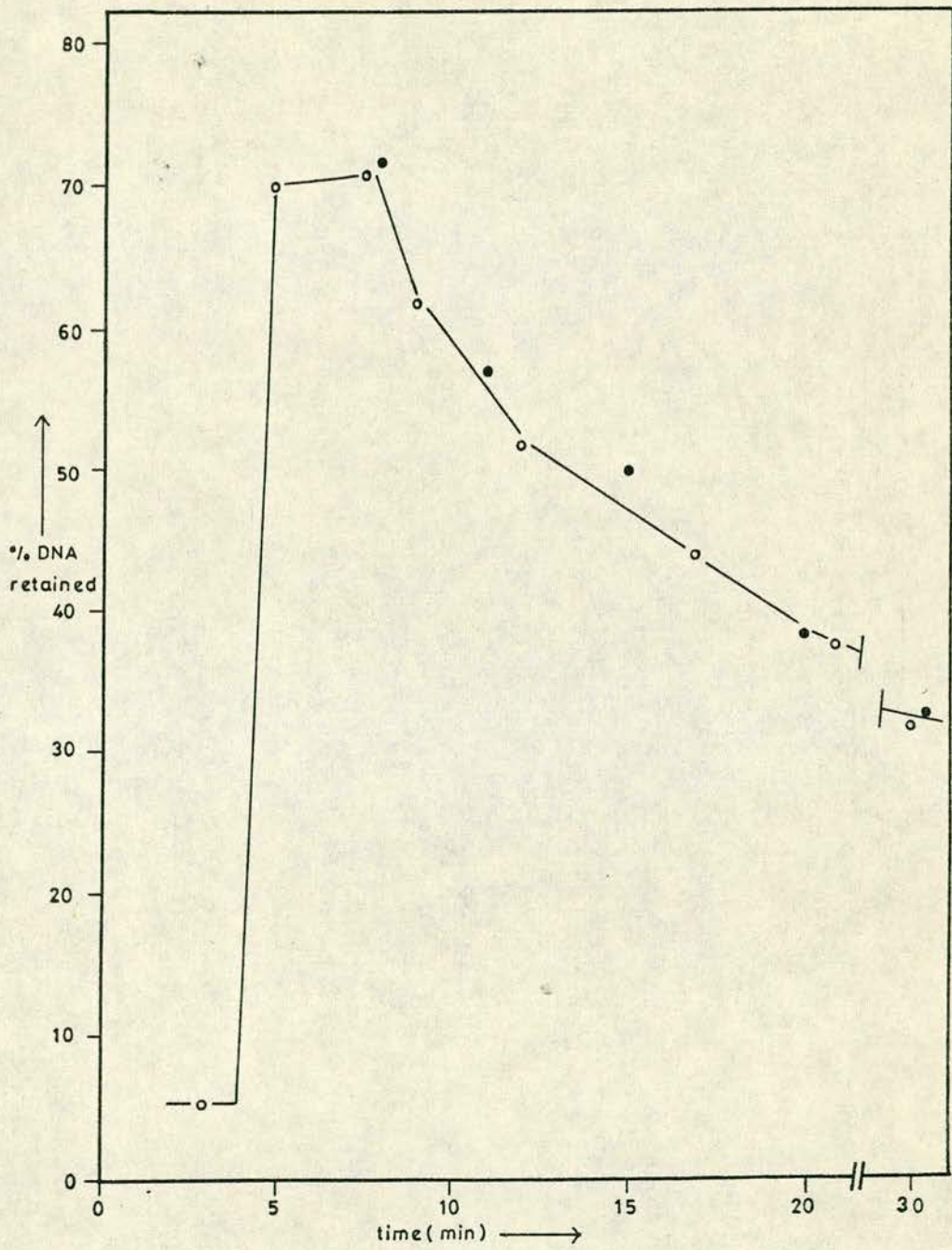


Fig. 26



T7 early region is independent of sigma. It may well be that core enzyme bound at t1 following transcription is in an "open" complex, ~~a conformation~~ rather different from that of the average "closed" unspecific/core-DNA complex. It should be noted that single stranded DNA displaces core from native T7 DNA (Hinkle and Chamberlin, 1972a) but has no effect on the dissociation of core enzyme from the termination site (see chapter 5).

It was not possible to show that release in the presence of antiserum involved the same HpaII restriction fragment as in the normal reaction (see chapter 6). Salt present in the antiserum preparation inhibited the restriction endonuclease. However release was presumably from the same site, as shown by the kinetics and size of RNA produced in the presence of antiserum.

##### 5. RNA release on termination of transcription

The release of RNA was difficult to assay in the presence of antiserum, because of the contaminating endonuclease activity. This could lead to apparent release of RNA even before the enzyme had reached the termination site. The contribution of degradation to the release of RNA can be estimated by using streptolydigin to inhibit elongation, so that the termination site is not reached (Fig. 27). No RNA is then released in the absence of antiserum, while in its presence all measured release can be attributed to degradation. The observed release in the presence of streptolydigin and antiserum is not sufficient to account for all the release seen in the presence of antiserum alone after the end of the first round of transcription. This is preliminary evidence that the release of RNA at t1 is independent of sigma.



Figure 27. Effect of antisigma antiserum on RNA release

A 0.4 ml transcription reaction at high ionic strength contained T7<sup>+</sup> DNA (1 pmole/ml) and Biogel-purified holoenzyme at E:DNA = 2.4. After 4 min at 37°C, NTPs were added (<sup>3</sup>H-CTP at 50 mCi/mmol). At 5½ min, 100 µl were taken to rifampicin (10 µg/ml) and 30 seconds later 100 µl were taken to antisigma antiserum (3300 nl/µg enzyme). At 6½ min, 50 µl were taken to streptolydigin (100 µg/ml) and 30 seconds later 50 µl were made to 100 µg/ml with streptolydigin and to 3300 nl/µg enzyme with antisigma antiserum. 10 µl aliquots were filtered as before (Fig. 19).

- rifampicin
- antisigma antiserum
- △ streptolydigin
- ▲ streptolydigin plus antisigma antiserum



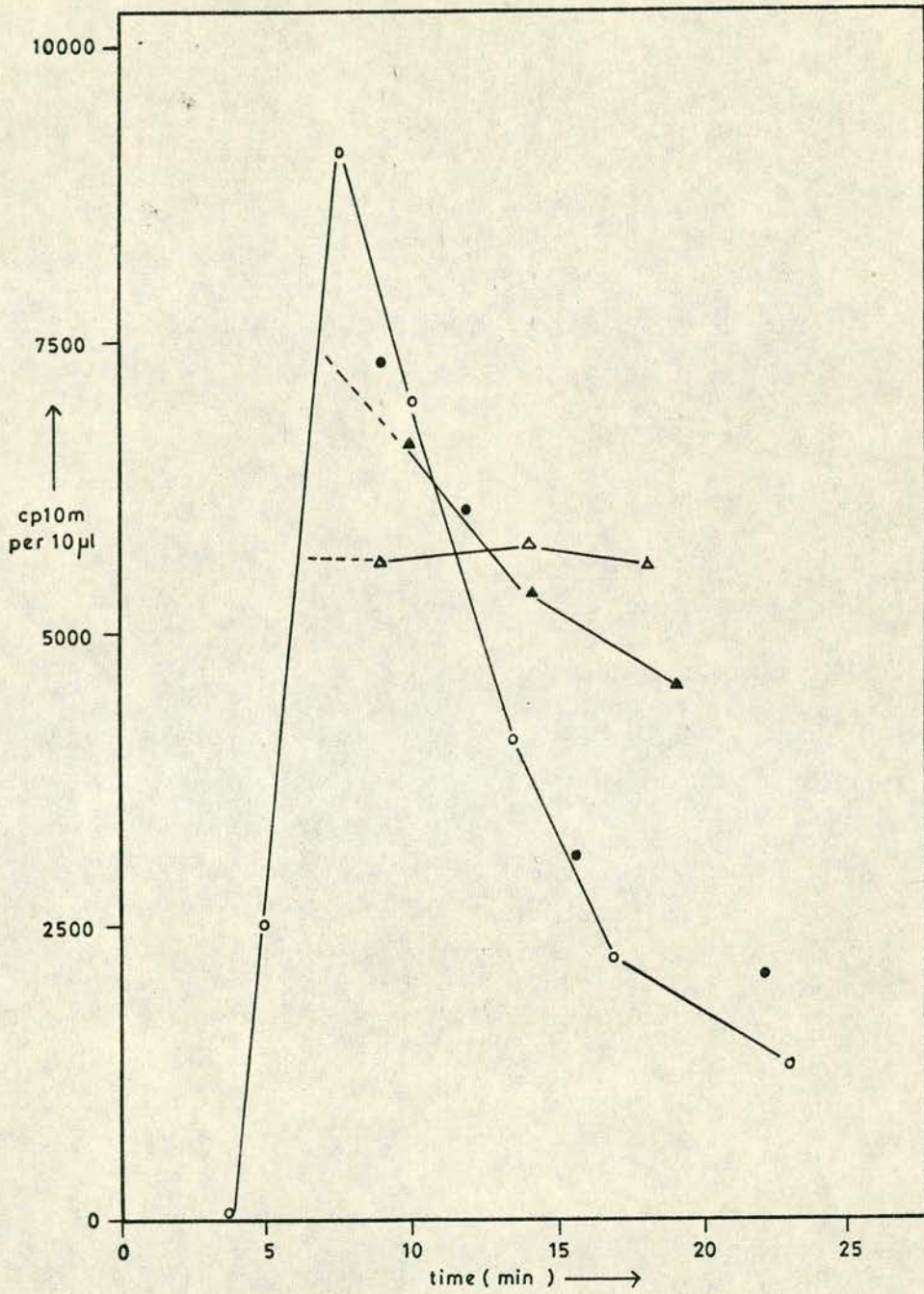


Fig. 27



CHAPTER 8. STUDIES ON THE ROLE OF rho IN THE TERMINATION  
OF TRANSCRIPTION AT t1

1. Introduction

Rho is normally assayed by measuring the inhibition of incorporation of NTPs using RNA polymerase and a template containing sites at which rho can act to terminate transcription (Galluppi et al, 1976). Preliminary assays showed that the rho preparation used in the present studies did, as expected, inhibit incorporation directed by T7<sup>+</sup> DNA at low ionic strength. On the basis of these assays, it was judged appropriate to use 1  $\mu$ g of rho protein per 3  $\mu$ g of polymerase protein. As the molecular weight of the active form of rho is not clearly known (Minkley, 1973), it has not been possible to estimate the molar ratio of active rho to active polymerase.

Rho cannot greatly accelerate the rate of polymerase release and reinitiation at low ionic strength in the case of those systems which contain known rho sites, as there would be no, or little, inhibition of incorporation in the standard assay if it did. Thus, it cannot be expected to eliminate the slowness of recycling at t1, even if it does act at this classically rho-independent site.

2. Kinetics

Rho was used in synchronously initiated transcription reactions with T7<sup>+</sup> and T7 C5 LG3 DNA at both 0.15 M and 25mM KCl. The results are shown in Fig. 28. Clearly, rho had an inhibitory effect on NTP incorporation in these reactions. The reaction most affected used T7<sup>+</sup> DNA at low ionic strength,



Figure 28. Effect of rho on the kinetics of transcription

0.6 ml transcription reactions were prepared at both low and high ionic strength, using either T7<sup>+</sup> DNA or T7 C5 LG3 DNA at 2.5 pmole/ml, and Biogel purified holoenzyme RNA polymerase at E:DNA = 1.5. After 4 min at 37<sup>o</sup>C NTPs were added (<sup>3</sup>H-CTP at 50 mCi/mmmole), and 30 seconds later, 250 μl were taken to 0.7 μg rho protein (E:rho = 4:1 by weight). 50 μl aliquots were acid-precipitated as usual.

o control

● plus rho

Panel A:	T7 <sup>+</sup> DNA	at low ionic strength
B:	T7 C5 LG3 DNA	at low ionic strength
C:	T7 <sup>+</sup> DNA	at high ionic strength
D:	T7 C5 LG3 DNA	at high ionic strength



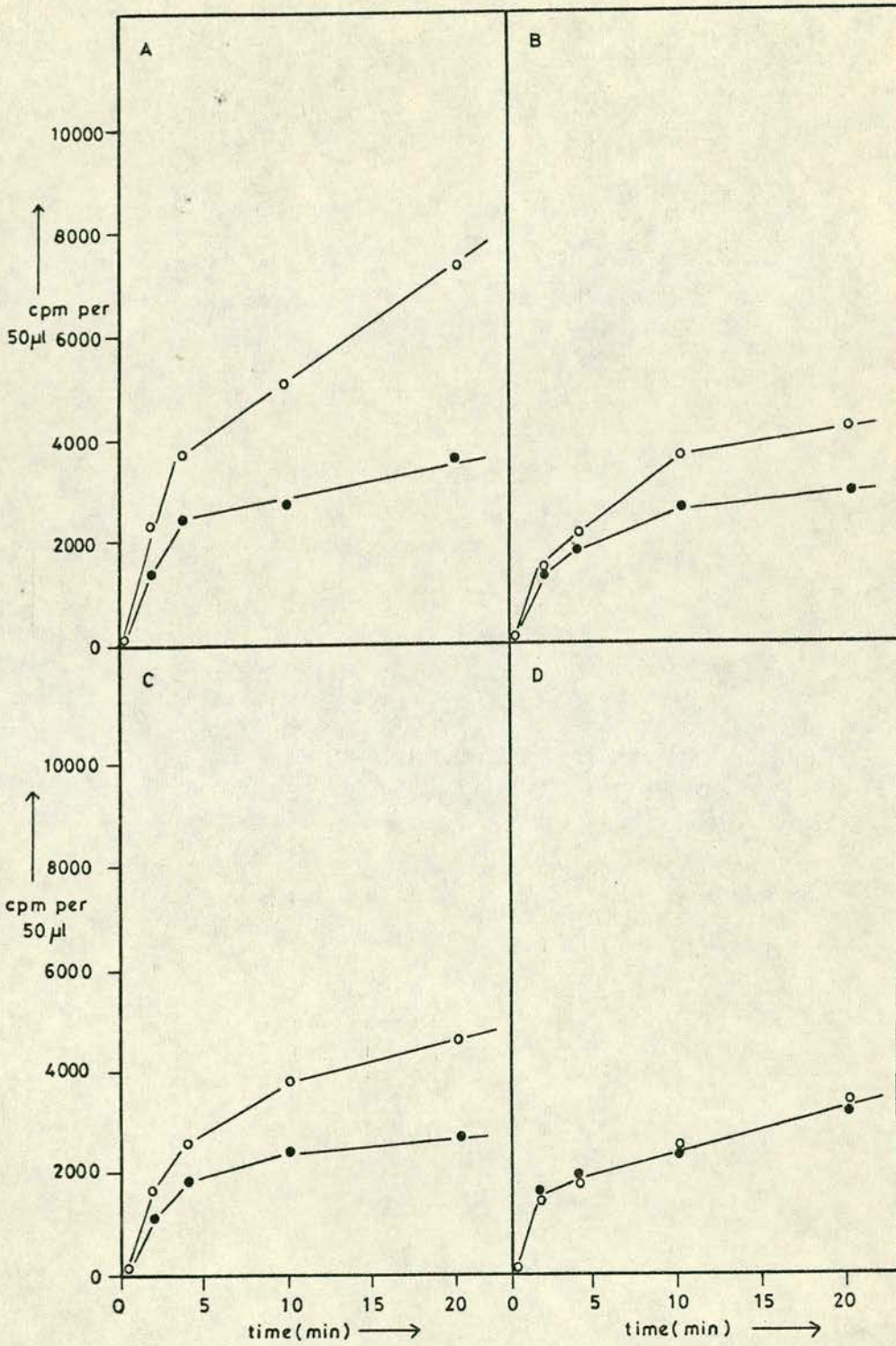


Fig. 28



and that least affected, T7 C5 LG3 DNA at high ionic strength. This was expected, as known rho sites are removed by the C5 and LG3 deletions (Dunn and Studier, 1973a; Darlix, 1974), and rho is known to be less active at higher ionic strength. Both rho's ATPase activity and its termination activity on T7<sup>+</sup> DNA are inhibited in parallel by salt (Galluppi *et al.*, 1976).

With T7<sup>+</sup> DNA at low ionic strength (Fig. 28, panel A), the inhibition is manifested well before the time needed to reach the termination site at the end of the early region. If rho has had no effect on the rate or extent of initiation, then this suggests that some termination of transcription is occurring within the early region, as expected. However, completion of termination and recycling of enzyme from these sites must be relatively slow for the inhibition to occur. The observed further reduction in the incorporation rate when RNA polymerase would reach t1 in the absence of rho suggests that some enzyme molecules still reach the end of the early region, and that the rate at which enzyme is recycled for subsequent rounds of transcription is not greatly increased. The steady state rate of incorporation established is lower than in the absence of rho, consistent with the synthesis of some shorter transcripts and slow recycling of enzyme. Thus the major effect of rho is to generate termination of transcription at sites within the early region: there is no indication that it increases the rate of recycling, for example by catalysing dissociation of the terminator complex. However, the latter effect might be difficult to detect in assays on T7<sup>+</sup> DNA.



Though the C5 and LG3 deletions do delete some of the rho sites present in T7<sup>+</sup> DNA, it is clear that some weak rho sites are not deleted. These residual rho sites seem to be effective only at low ionic strength (Fig. 28, panels B and D). Thus at 0.15 M KCl, when rho is active at sites which are deleted in T7 C5 LG3 DNA (Fig. 28, panel C), there is no indication that rho affects the change in rate of NTP incorporation which is argued here to be due to slow dissociation of RNA polymerase from t1 after termination of transcription.

### 3. Size of RNA synthesised

Reactions at low ionic strength were performed as usual, except that transcription was initiated by adding a mixture of NTPs and rifampicin to exclude re-initiations. The RNA made was analysed on 2.4% polyacrylamide gels (Fig. 29). No effect on the size of T7 C5 LG3 RNA could be detected. At first sight, this is contrary to the kinetic experiments described above. However, less full sized T7 C5 LG3 RNA is made in the presence of rho, which indicates that some of the RNA polymerase molecules must have terminated at sites other than t1, provided that rho did not affect initiation. The amount of radioactive material in the low molecular weight region of the gel is increased by rho. It is possible that rho mediated termination of transcription has led to the production of a very heterogeneous RNA population under these conditions.

Rho did have an obvious effect with T7<sup>+</sup> DNA even though full sized T7<sup>+</sup> RNA is poorly resolved from material at the top of the gel in this experiment. Little full sized



Figure 29. Effect of rho on size of RNA synthesised

50  $\mu$ l transcription reactions, with and without rho (0.15  $\mu$ g), at low ionic strength contained either T7<sup>+</sup> DNA or T7 C5 LG3 DNA at 2.5 pmole/ml and Biogel-purified holoenzyme at E:DNA = 1.5. The ratio of RNA polymerase to rho was 3:1 by weight. After 5 min at 37<sup>o</sup>C, a mixture of NTPs and rifampicin (10  $\mu$ g/ml finally) were added. <sup>3</sup>H-CTP was at 50 mCi/mmole. At 11 min the reactions were analysed, by electrophoresis on 2.4% polyacrylamide rod gels at 5 V/cm for 3 hrs.

The arrows show the position to which MS2 RNA migrated under these conditions.

- A T7<sup>+</sup> DNA
- B T7 C5 LG3 DNA
- C T7<sup>+</sup> DNA plus rho
- D T7 C5 LG3 DNA plus rho



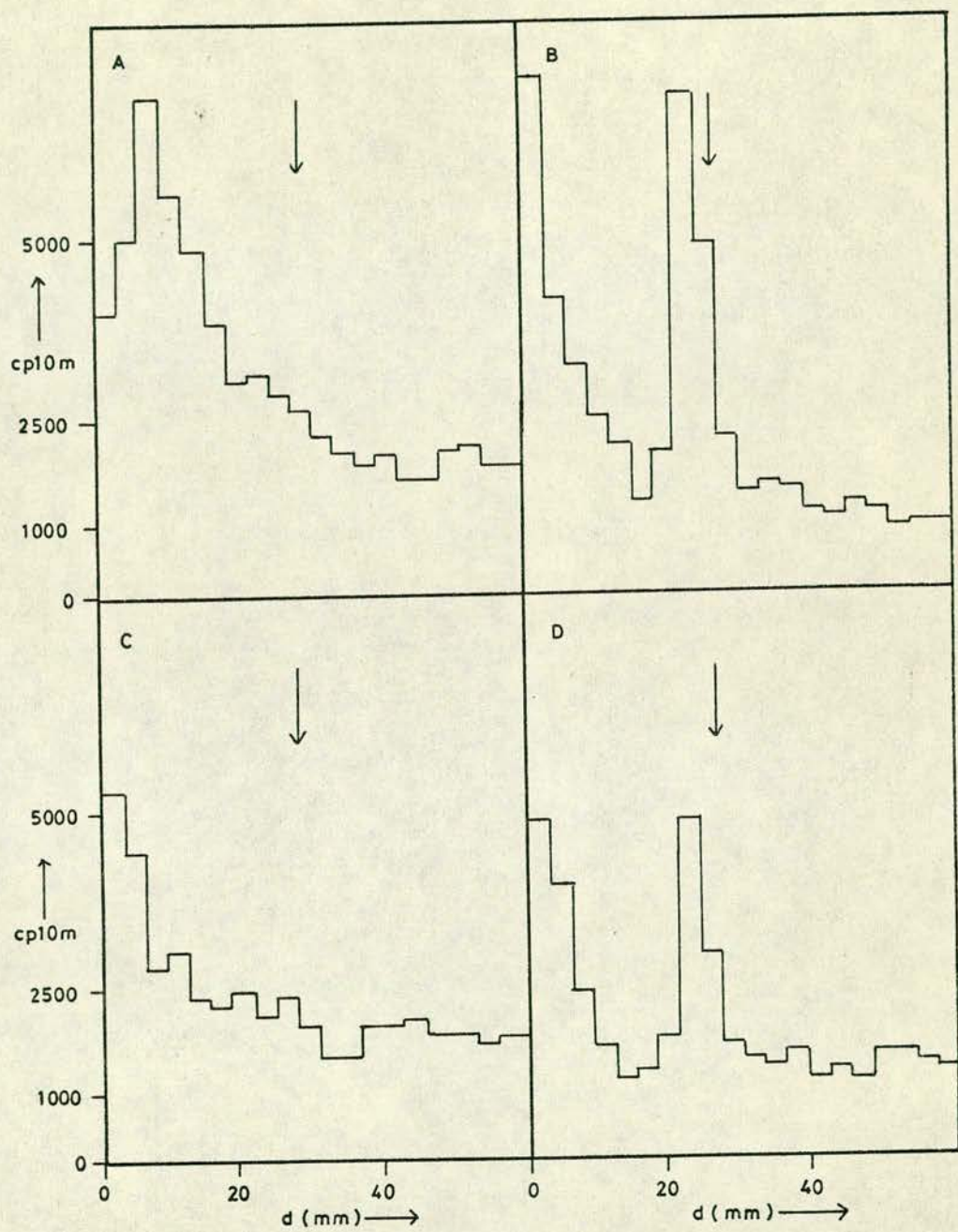


Fig. 29



RNA was produced in the presence of rho. The results using T7 C5 LG3 DNA make it unlikely that this absence of large RNA was due to endoribonuclease in the rho preparation, so that it must be due to the termination activity of rho. No specific short transcripts could be detected amongst the T7<sup>+</sup> products, a point also noted by Dunn and Studier (1973a). This is again consistent with there being a large number of rho sites producing a heterogeneous RNA population. However, this is not consistent with the detection by Darlix (1974) of rho mediated termination on T7<sup>+</sup> DNA to produce RNA molecules of discrete size classes, and it is clear that the number and/or efficiency of rho sites removed by the C5 and LG3 deletions is very different from that of those remaining.

#### 4. DNA release

From the kinetic experiments, it seems likely that rho does not increase the rate at which enzyme molecules become available for second and subsequent rounds of transcription after termination at t1. If rho does have an effect, then it is masked by the effect of termination within the early region. Experiments were performed to test directly if rho had any effect on the rate at which salt-stable E-DNA complexes break down after the termination of transcription on T7<sup>+</sup> DNA, using the filter binding assay described in chapter 5. The results are shown in Figure 30.

Transcription was initiated on [<sup>3</sup>H]-T7<sup>+</sup> DNA at low ionic strength by adding NTPs. Re-initiations were prevented by the addition of rifampicin and rho was added at various



Figure 30. Effect of rho on DNA release

A 0.25 ml transcription reaction at low ionic strength contained  $^3\text{H-T7}^+$  DNA (1 pmole/ml), and Biogel-purified holoenzyme at E:DNA = 1.5. After 4 min at  $37^\circ\text{C}$ , NTPs were added followed 2 min later by rifampicin (10  $\mu\text{g/ml}$ ). At 7 and  $10\frac{1}{2}$  min 75  $\mu\text{l}$  were taken to 0.15  $\mu\text{g}$  rho (E:rho = 2.5:1 by weight). 10  $\mu\text{l}$  aliquots were filtered as before (Fig. 15).

- o rifampicin (control)
- rho at 7 min
- Δ rho at  $10\frac{1}{2}$  min



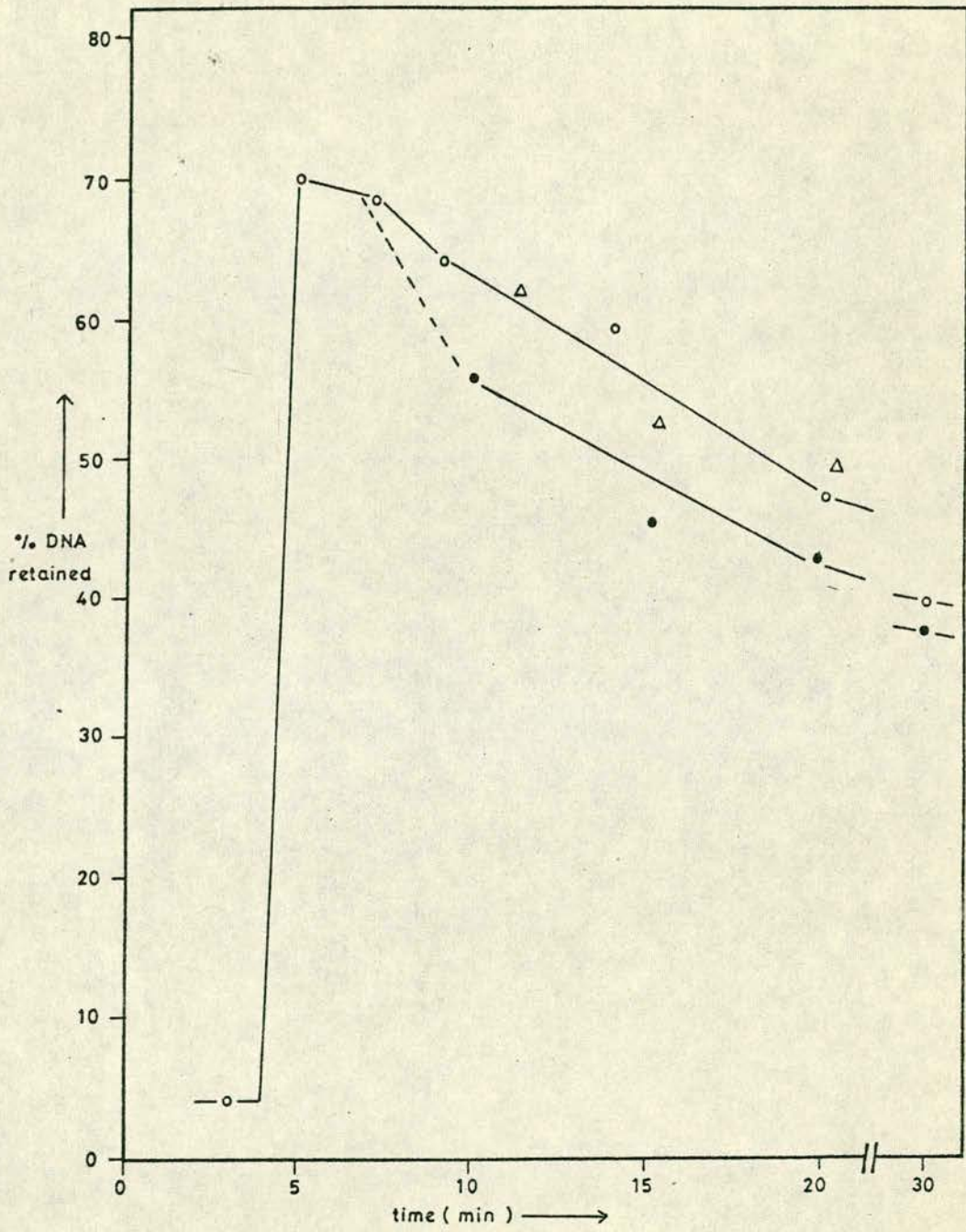


Fig. 30



times. When rho was present during transcription, then an initial rapid release of DNA was observed. This could be due to the release of DNA after rho had generated termination at sites within the early region. Alternatively, if the initial phase at which the E-DNA complexes become unstable in 0.5 M NaCl is limited by the rate of RNA release, then the results might suggest that rho catalyses the release of RNA at t<sub>1</sub>. However, the rate of release after this early period is very similar to that in both the control and a reaction where rho was added during the "release" phase. This suggests that rho does not directly affect the rate at which binary polymerase/terminator DNA complexes dissociate.

It was argued in chapters 5 and 6 that DNA is retained on nitrocellulose filters in 0.5 M NaCl only by ternary complexes, but that slow dissociation of terminator complexes at the lower ionic strength of the transcription reaction ensures that much of the DNA is retained in ternary complexes for a long time. Thus DNA molecules which had only bound one RNA polymerase would not be retained after the enzyme reached t<sub>1</sub> and released its RNA. Evidence has been presented that RNA release is not instantaneous. This is probably why there is not an immediate reduction in the retention of DNA after 4 min transcription, equivalent to those DNA molecules which had only bound one enzyme. Rho is known to catalyse the release of RNA at rho-dependent termination sites. Indeed, as discussed in chapter 1, this release of RNA may be the reason why transcription stops. It does not seem



improbable that RNA release at a site where transcription stops in the absence of rho could be catalysed by rho.

This conclusion remains highly tentative, as DNA and RNA release at low ionic strength have not been studied in detail in the present work. It would also be interesting to examine the effect of rho on DNA release at high ionic strength, even though other assays (inhibition of NTP incorporation, ATPase activity) indicate that rho becomes less effective as the salt concentration is increased. Time did not permit such experiments to be attempted.



CHAPTER 9. EXPERIMENTS TO DETERMINE NUCLEOTIDE SEQUENCES  
AT THE TERMINATION SITE

1. Introduction

From the study of transcription in vitro, it is clear that E.coli RNA polymerase can recognise both promoters and terminators. A knowledge of the nucleotide sequences of these sites should lead to a better understanding of the selectivity of transcription (Chamberlin, 1974; Gilbert, 1976). For various technical reasons, the sequencing of promoters had progressed in advance of the sequencing of terminators, but DNA sequencing has been revolutionised by the development of simple methods for the purification of specific DNA fragments and rapid techniques for sequence determination (Sanger and Coulson, 1975; Maxam and Gilbert, 1977). Thus the special techniques previously used to purify promoter DNA fragments are no longer essential, although still useful (e.g. chapter 6).

Earlier work in this and other laboratories was directed towards devising methods for the determination of sequences at the 3' end of RNA molecules (Dahlberg, 1968) and in the DNA template distal to the RNA stop site (Peters and Hayward, 1972). The latter approach used hybrids between the RNA and the complementary strand of DNA as a primed template for DNA polymerase I of E.coli. The DNA extensions so synthesised can then be sequenced. This technique was first applied to determine the nucleotides immediately before and after the RNA stop sites at t1 and t2 of T7 DNA, using hybrids made with RNA synthesised on



T7<sup>+</sup> and T7 LG37 DNA. If the first deoxyribonucleotide in the DNA extension is  $\alpha$ -<sup>32</sup>P labelled, subsequent alkaline hydrolysis will "transfer" <sup>32</sup>P to the 3' terminal mononucleotide of the RNA (see Fig. 31). At t1 only  $\alpha$ -<sup>32</sup>P dGTP showed such transfer and only to rCMP. At t2 the sequence CA was found rather than CG (Peters and Hayward, 1974a).

These experiments were extended using ribonucleases instead of alkaline hydrolysis and by using only 2 or 3 dNTPs during DNA extension, to show that the DNA sequence around t1 is  $\begin{matrix} \text{-CCCGA-} \\ \text{-GGGCT-} \end{matrix}$  (Hayward and Peters, 1974). However there are considerable technical problems involved in attempting to determine extensive sequences by this approach, though they have been overcome in another laboratory using much shorter RNAs, the  $\lambda$  4S and 6S RNAs (Rosenberg *et al*, 1976):

a) the release of DNA extensions by alkaline hydrolysis yields oligonucleotides with both 5' and 3' OH groups. The electrophoretic and chromatographic properties of such oligonucleotides are not well characterised, which makes sequence determination difficult (Brownlee, 1972).

b) the 3' OH of the DNA in the hybrid appears to act as a primer for DNA synthesis.

c) intactness of the RNA primer is a very important feature, but inherently difficult to achieve.

The investigations described below were designed to characterise these problems more clearly, and if possible to overcome them.



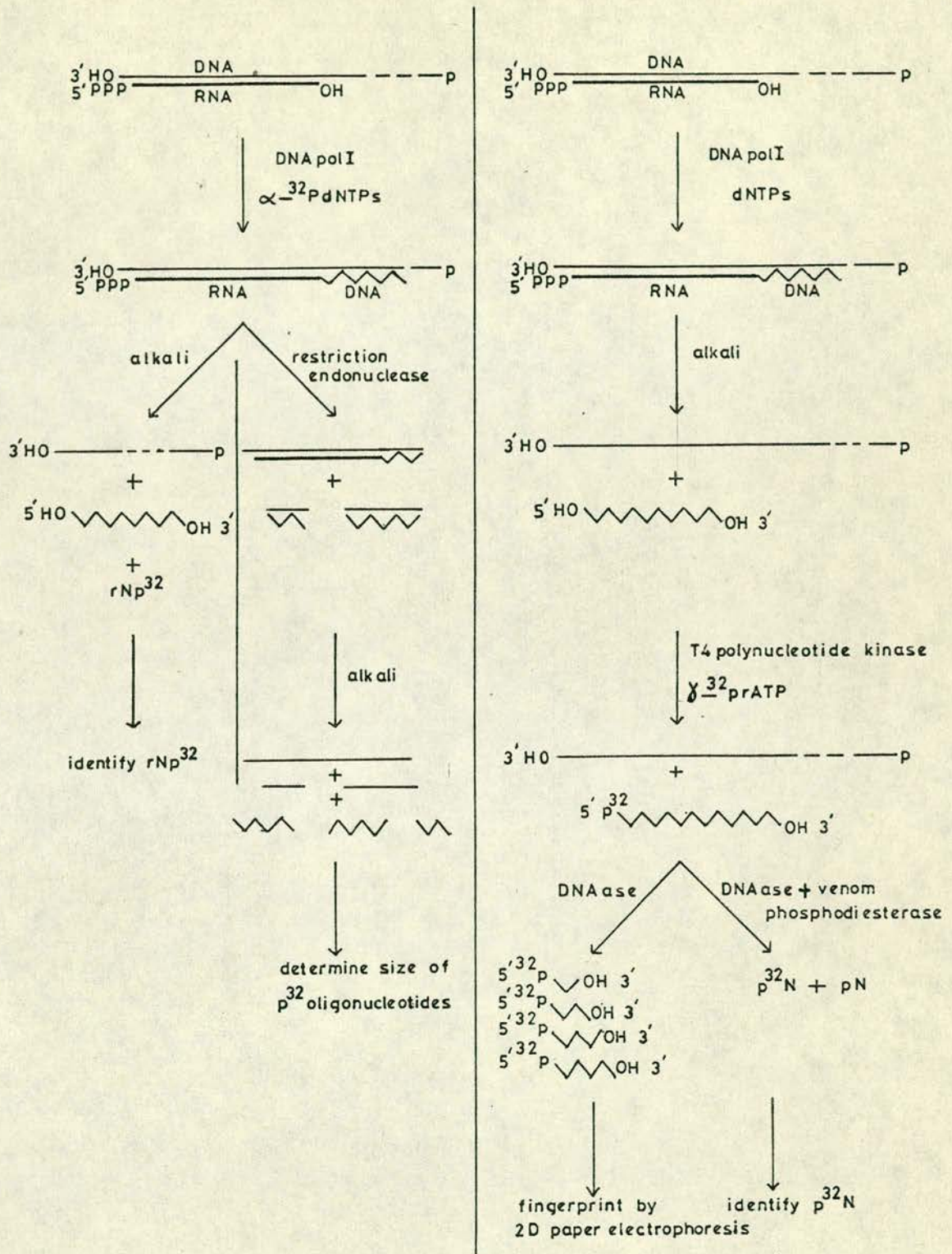


Fig. 31



## 2. Kinetics of hybridisation of RNA with "r" strand DNA

Glycerol gradient purified RNA (see below) was mixed in equimolar amounts with the heavy ("r") strand of T7 DNA and dialysed against 70% formamide, 1 M NaCl, 0.2 M Tris-HCl (pH 8), 20 mM EDTA at room temperature. Samples were taken at intervals and the formamide removed by dialysis against 0.1 M Tris-HCl (pH 8), 1 mM EDTA at 4°C. The extent of hybridisation was assayed by the protection of the RNA against attack by pancreatic RNAase (2 µg/ml for 30 min at 37°C). The results (Fig. 32) show that after as little as 2 hrs there was 100% protection.

It seemed plausible that though there is 100% protection of the RNA, the structure of the hybrids might be complex due to multimolecular interactions. Preliminary electron microscopic examination confirmed the presence of "networks", with few simple bimolecular hybrids (G.G. Peters, personal communication). Provided that the 3' OH RNA termini are efficiently paired with the DNA, the existence of networks need not affect the sequencing experiments.

## 3. Asymmetry of hybridisation

T7 early RNA is transcribed in vivo from the "r" strand (see chapter 1) so the asymmetry of hybridisation of in vitro synthesised T7 RNA may be used as a criterion of RNA purity. Hybridisation reactions were prepared as above using a six-fold molar excess of each of the single strands of T7 DNA mixed with glycerol gradient-purified RNA. A control of RNA with no added DNA was treated similarly. After dialysis against formamide for 18 hrs at room temp-



Figure 32. Kinetics of hybridisation

4  $\mu\text{g}$  T7<sup>+</sup> RNA in 25-30% glycerol, 0.1 M NaCl, 50 mM Tris-HCl (pH 8), 1 mM EDTA, .1% sarkosyl NL35 was mixed with 20  $\mu\text{g}$  "r" strand of T7<sup>+</sup> DNA in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, in a total volume of 0.6 ml. The RNA was labelled with <sup>14</sup>C-ATP to approx. 3500 cpm per  $\mu\text{g}$ . The mixture was dialysed at 25°C against 70% formamide, 1 M NaCl, 0.2 M Tris-HCl (pH 8), 20 mM EDTA. At intervals 50  $\mu\text{l}$  aliquots were withdrawn, diluted with 200  $\mu\text{l}$  of 0.1 M Tris-HCl (pH 7.8), 1 mM EDTA, and dialysed against this buffer at 4°C for 18 hrs.

To determine the percentage of RNA resistant to RNAase, 50  $\mu\text{l}$  aliquots were made 10 mM in MgCl<sub>2</sub> and pancreatic RNAase was added to 2  $\mu\text{g}/\text{ml}$ . They were incubated at 37°C for 30 min and then acid-precipitated. Undigested 50  $\mu\text{l}$  aliquots were also acid-precipitated.



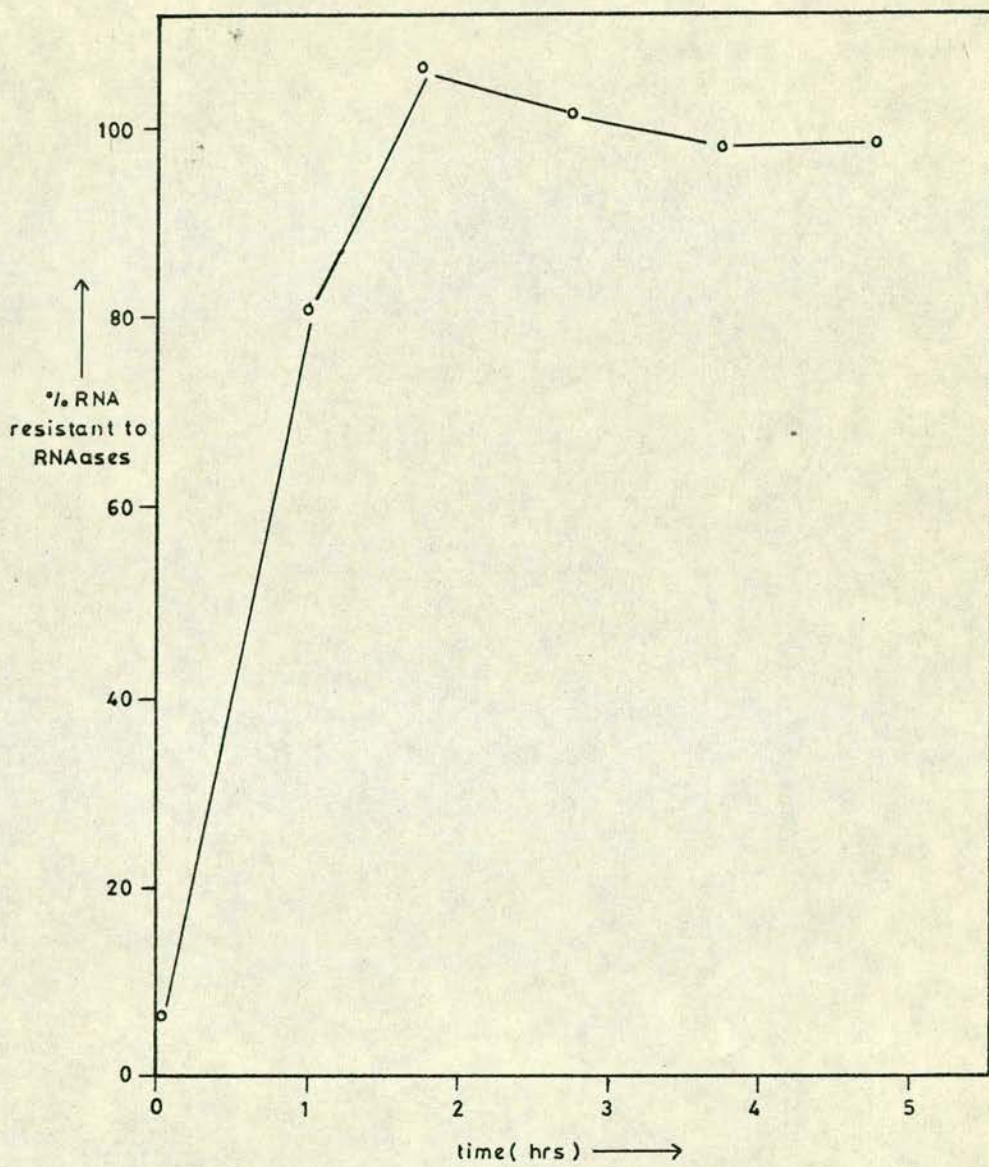


Fig. 32



erature, the formamide was removed and the extent of hybridisation determined as above. The results were:-

RNA alone	0.5%	resistant to RNAase
RNA plus "l" strand	3.7	" " "
RNA plus "r" strand	94.0	" " "

After correction for that amount of RNA which is resistant to RNAase, the asymmetry is expressed as  $(r-1)/(r+1)$  where  $r$  is the percentage resistant after hybridisation with "r" strand and  $l$  that after hybridisation with "l" strand. For glycerol gradient purified RNA the asymmetry was consistently close to 95%.

#### 4. Purity of hybrid components

Peters and Hayward (1974a) found approximately equal incorporation of each dNTP when used as sole substrate for extension of T7 DNA.RNA hybrids by DNA polymerase I, though only dGTP was added to the 3' end of the RNA. The alternative primers may be present from contaminating nucleic acids or be inherent features of the hybrids as prepared above.

The RNA used in these studies was synthesised in vitro, usually with T7 C5 LG3 DNA as template, in large scale (0.5 - 1 ml) reactions at 0.15 M KCl as described in chapter 3. The DNA was at 5 pmole/ml and the E:DNA ratio was 3-5. Rifampicin was added 10 mins after NTPs and the reactions incubated for a further 5 min at 37°C. EDTA (25 mM) and sarkosyl NL35 (1%) were added and the mixture was then phenol extracted before centrifugation on pre-formed 15-40% glycerol gradients in 50 mM Tris-HCl (pH 7.8), 0.1 M NaCl, 1% sarkosyl NL35 (SW25.1 rotor, 50,000 x g,



Figure 33. Analysis of glycerol gradient purification of in vitro synthesised RNA.

A 0.5 ml transcription reaction, at high ionic strength, contained T7 C5 LG3 DNA (2.5 pmole/ml) and Biogel-purified holoenzyme RNA polymerase at an E:DNA ratio of 4.  $^3\text{H}$ -CTP was at 5 mCi/mmole. After 4 min at  $37^\circ\text{C}$ ,  $\text{MgCl}_2$  (12 mM) was added and at 10 min, rifampicin (10  $\mu\text{g}/\text{ml}$ ). After 3 min further incubation, the mixture was made 25 mM EDTA and 1% sarkosyl NL35, and phenol-extracted. The aqueous phase was centrifuged on 30 ml preformed gradients of 15-40% glycerol in 10 mM Tris-HCl (pH 7.8), 0.1 M NaCl, 1 mM EDTA, 1% sarkosyl NL35 (SW25.1 rotor, 50,000 x g, 18 hrs,  $4^\circ\text{C}$ ). 1 ml fractions were collected, and the RNA located by acid-precipitation of 20  $\mu\text{l}$  aliquots of each fraction. 40  $\mu\text{l}$  aliquots were then examined by electrophoresis on a 2.4% polyacrylamide slab gel at 5 V/cm for 3 hrs. After electrophoresis the gel was stained and photographed as before (Fig. 10).

Track	sample
1	glycerol gradient fraction no. 11
2	12
3	13
4	14
5	15
6	16
7	17
8	18
9	2.5 $\mu\text{g}$ MS2 RNA



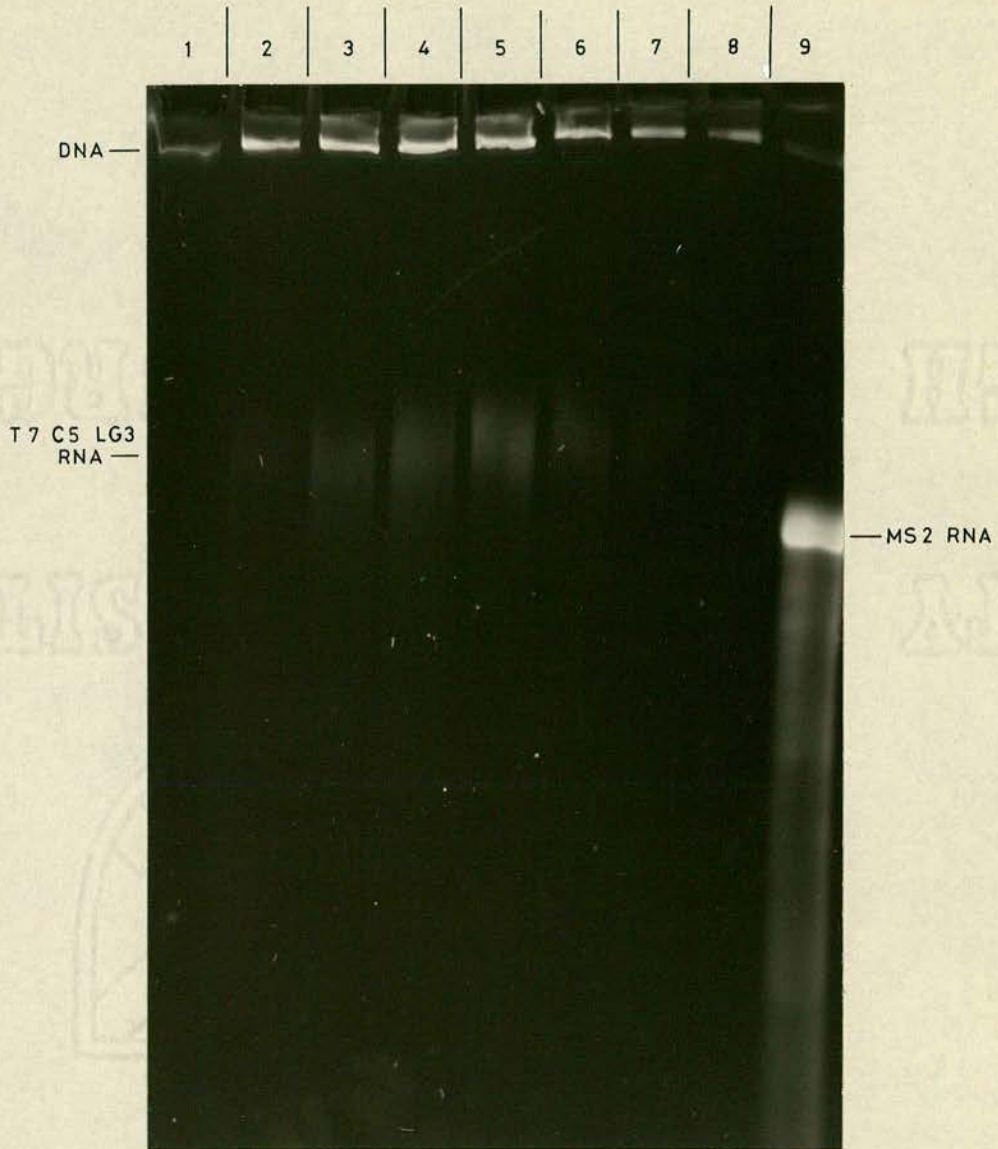


Fig. 33



ageous if the DNA extensions of RNA molecules could be purified or selectively labelled before sequence analysis. In principle, the simplest method for purifying the required RNA-DNA copolymers would be to fractionate them on the basis of size using polyacrylamide gel electrophoresis after denaturation. To test this, native and formamide denatured samples of glycerol gradient purified RNA and unextended hybrids were examined on non-denaturing gels (Fig. 34). The profiles show that the RNA in hybrid is considerably fragmented. Some of this damage can be ascribed to the procedures used in hybrid preparation, but the starting RNA itself is already badly fragmented. It is clear that the secondary structure of the RNA is concealing the damage, as reported for MS2 RNA subjected to mild RNAase treatment (De Wachter and Fiers, 1971). The gels were also scanned at 260 nm. This revealed that the double stranded DNA present in the hybrid was completely denatured by the formamide treatment and that little, if any, renaturation occurred during electrophoresis.

It seemed unlikely that the denaturation procedure itself could introduce breaks into the RNA. In confirmation of this view, it was found that heat denaturation alone produced similar gel profiles, and that these were not changed by incubation at 100°C for 30 min in the presence or absence of formamide. Freshly synthesised RNA, without purification by glycerol gradient centrifugation, also contained many hidden breaks, which were only revealed by denaturation.



Figure 34. Size of RNA in hybrid

Samples of T7 C5 LG3 RNA.DNA hybrid, and glycerol gradient-purified T7 C5 LG3 RNA, each containing 15,000 cpl0m, were examined by electrophoresis on 2.4% polyacrylamide gels at 5 V/cm for 3½ hrs. Similar samples were examined after denaturation by heating at 100°C in 50% formamide for 10 min, followed by rapid chilling on ice.

Panel A: RNA.DNA hybrid

B: RNA

C: denatured RNA.DNA hybrid

D: denatured RNA

The arrows show the position of marker MS2 RNA.



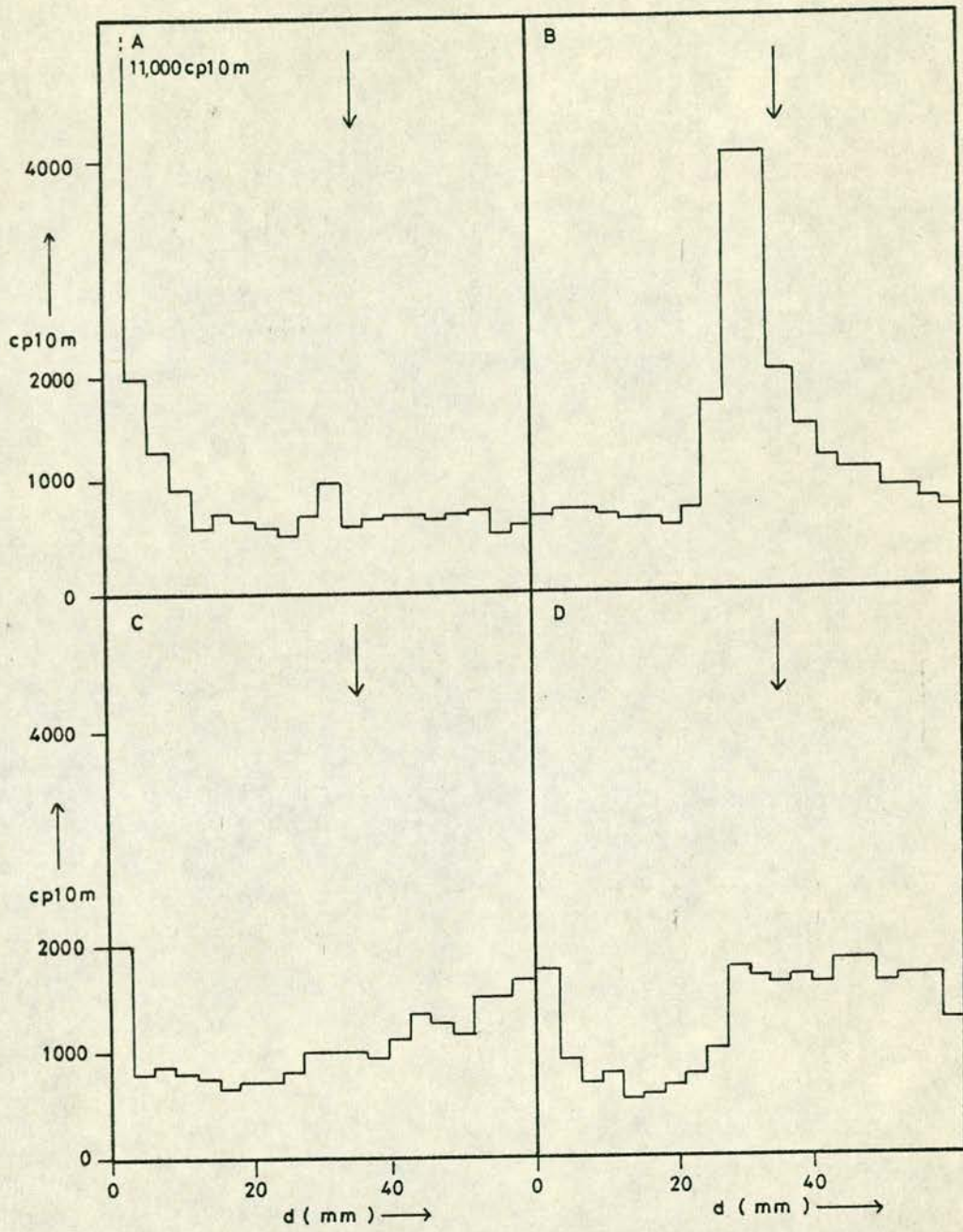


Fig. 34



The most likely cause of this damage is contaminating RNAase in the RNA polymerase preparations, despite the fact that they were routinely assayed for endonucleolytic activity and not used if detectably contaminated (chapter 4). However, the endonuclease assay originally used would not have detected breaks which did not disrupt the secondary structure. After the significance of hidden damage became clear, it was found that further purification of the enzyme by gel filtration gave preparations which could synthesise RNA with far fewer breaks (compare Figs. 10 and 34). Fig. 10 shows that the native samples contain the T7 C5 LG3 DNA template and RNA product which is slightly larger than MS2 RNA. After denaturation, the DNA no longer enters the gel and though some breaks in the RNA are revealed, a large proportion of the molecules are intact. This improvement in RNA polymerase and RNA quality was achieved in connection with experiments described in earlier chapters, and was not available when the sequence studies described below were being carried out.

As the RNA used in the preparation of hybrids was fragmented, purification of RNA-DNA extensions by gel electrophoresis appeared to be impracticable. It was feared that low yields and impurity would prohibit sequence analysis using this approach. Such preliminary work as was attempted confirmed this fear.

Another potential method for purification of RNA-DNA extensions relies on the different buoyant densities of RNA and DNA in caesium sulphate. Thus, short DNA extensions of long RNA molecules should band with RNA. This approach



was used by Hayward and Peters (1974) to confirm that A is the second base in the DNA extension of RNA terminated at t1. When extensions were made with unlabelled dGTP and one of the other three dNTPs labelled in the  $\alpha$  position with  $^{32}\text{P}$ , radioactivity appeared in the RNA peak only in the case of  $^{32}\text{P}$ dATP. However, this approach is limited by the need to avoid long DNA extensions and significant breakage of RNA, either of which would obscure the fractionation. It has not been exploited in these studies.

A simpler approach exploits the vulnerability of RNA to alkali: those oligonucleotides which are detectable only after alkaline hydrolysis should be DNA extensions of RNA. They should have free 5' OH ends allowing preferential labelling by T4 polynucleotide kinase (see below). This approach could not distinguish between DNA primed by fragmented and full-sized RNA, but there is evidence that fragmentation does not generate new RNA primers. Both alkaline hydrolysis and most common RNAases produce 3' phosphate termini which cannot be used as primers by DNA polymerase I (Kornberg, 1974). The specificity of  $^{32}\text{P}$  transfer observed by Peters and Hayward (1974a) confirmed that in this system fragments of RNA did not act as primers. Thus alkali release has been the method of choice in the present studies.

#### 6. $^{32}\text{P}$ transfer experiments

Preparations of T7 C5 LG3 RNA-DNA hybrid were used as primed templates for DNA polymerase I of *E. coli* (see Fig. 31), as described in chapter 3. Control reactions used the



"r" strand DNA as template. The substrates were  $\alpha$ - $^{32}\text{P}$  dGTP and the other three unlabelled dNTPs. Aliquots of the products were hydrolysed with alkali and the rNMPs released analysed for  $^{32}\text{P}$ . As expected from the results of Peters and Hayward (1974a) only rCMP was  $^{32}\text{P}$  labelled when hybrid made with RNA synthesised by MRE600 RNA polymerase was used, and there was no labelling of rNMPs when "r" strand was used as template. The yield of  $^{32}\text{P}$  rCMP indicated that about 10% of the RNA (judged as moles of intact molecules) had been extended with dGTP. This figure cannot be directly equated with the total number of extended RNA primers, as transfer from dATP, dCTP and dTTP was not analysed. However, the specificity of the transfer from dGTP suggests that as in the more complete experiments of Peters and Hayward (1974a) there would have been negligible transfer from the other 3 substrates.

When the RNA used in hybrid preparation had been synthesised by K12 RNA polymerase, then transfer was detected from dGTP to rCMP and rAMP. This might simply be due to a badly degraded RNA preparation. However, Rosenberg *et al* (1975) found that the  $\lambda$  4S and 6S RNAs synthesised *in vitro* by K12 RNA polymerase had a small, variable number of A residues added to the 3' ends. These oligo-A sequences were not template coded (Smith and Hedgpeth, 1975). It is possible that the transfer from dGTP to rAMP is a consequence of the addition of A residues to the 3' end of the T7 RNA by K12 RNA polymerase, provided that the RNA with A addition could form a primer with G as the next base directed by the template "r" strand DNA.



## 7. Rate of elongation

The kinetics of extension of RNA primers by DNA polymerase I cannot be measured by dNTP incorporation owing to simultaneous elongation of an unknown number of DNA primers. Samples were therefore taken from an extension reaction at various times after DNA polymerase addition, EDTA (25 mM) added to inhibit polymerisation and hydrolysed with alkali to remove the RNA primer. The  $^{32}\text{P}$  labelled DNA extensions were examined by electrophoresis on a 3-13% polyacrylamide gel in 7 M urea. After electrophoresis, the gel was soaked in 9% acetic acid, 45% methanol to fix the DNA and elute the urea. It was then dried and autoradiographed. A calibration curve relating mobility to size was constructed from the mobility of DNA fragments produced by digestion of T7 DNA with HpaI and HpaII which were electrophoresed in parallel and located by ethidium bromide staining. Scans of the autoradiograph are presented in Fig. 35.

$^{32}\text{P}$  is clearly associated with two classes of molecule:-

a) those which, like marker single stranded T7 DNA, do not enter the gradient gel and which are labelled throughout the reaction. These are interpreted as single stranded DNA molecules which have been extended at their 3' ends.

b) molecules which do penetrate the gel, and whose size increases with time. They are interpreted to be extensions made on RNA primers.

A little labelled material does enter the gel even if the samples were not hydrolysed. This is presumably because the RNA is fragmented so that some of the RNA-DNA molecules are small enough to penetrate the gel.



Figure 35. Rate of elongation by DNA polymerase I starting at RNA primers

6 pmole of T7 C5 LG3 RNA.DNA hybrid were used as primed template for DNA polymerase I of E.coli.  $\alpha$ -<sup>32</sup>PdGTP was present at 130 Ci/mmole. Aliquots containing 0.05 pmole hybrid were removed after 1, 2, 4 and 7 min, and EDTA added to 25 mM. They were phenol-extracted, and the aqueous layers passed through a 10 ml Sephadex G-100 column in 1 mM Tris-HCl (pH 7.8), 0.1 mM EDTA. The excluded material was concentrated to 100  $\mu$ l by dessication over P<sub>2</sub>O<sub>5</sub>/conc. H<sub>2</sub>SO<sub>4</sub>/NaOH. The samples were then alkali-hydrolysed, neutralised, and freed from KClO<sub>4</sub> as described in chapter 3. After denaturation in 90% formamide at 100°C for 10 min, they were electrophoresed (5 V/cm, 18 hrs) on a 3-13% polyacrylamide gradient gel in 7 M urea, 0.1 M Tris-borate (pH 8.3), 1 mM EDTA.

The gel was soaked in 9% acetic acid, <sup>45% methanol</sup>/to fix the DNA and remove the urea, before drying down at reduced pressure over a hot plate. It was then autoradiographed, and the autoradiograph scanned at 585 nm.

Markers were HpaII and HpaI digests of T7 C5 LG3 DNA which were located by staining with ethidium bromide.

The inset shows 'peak size' plotted against time.

———— 1 min  
— — — 2 min  
— . — . — 4 min  
— o — o — 7 min



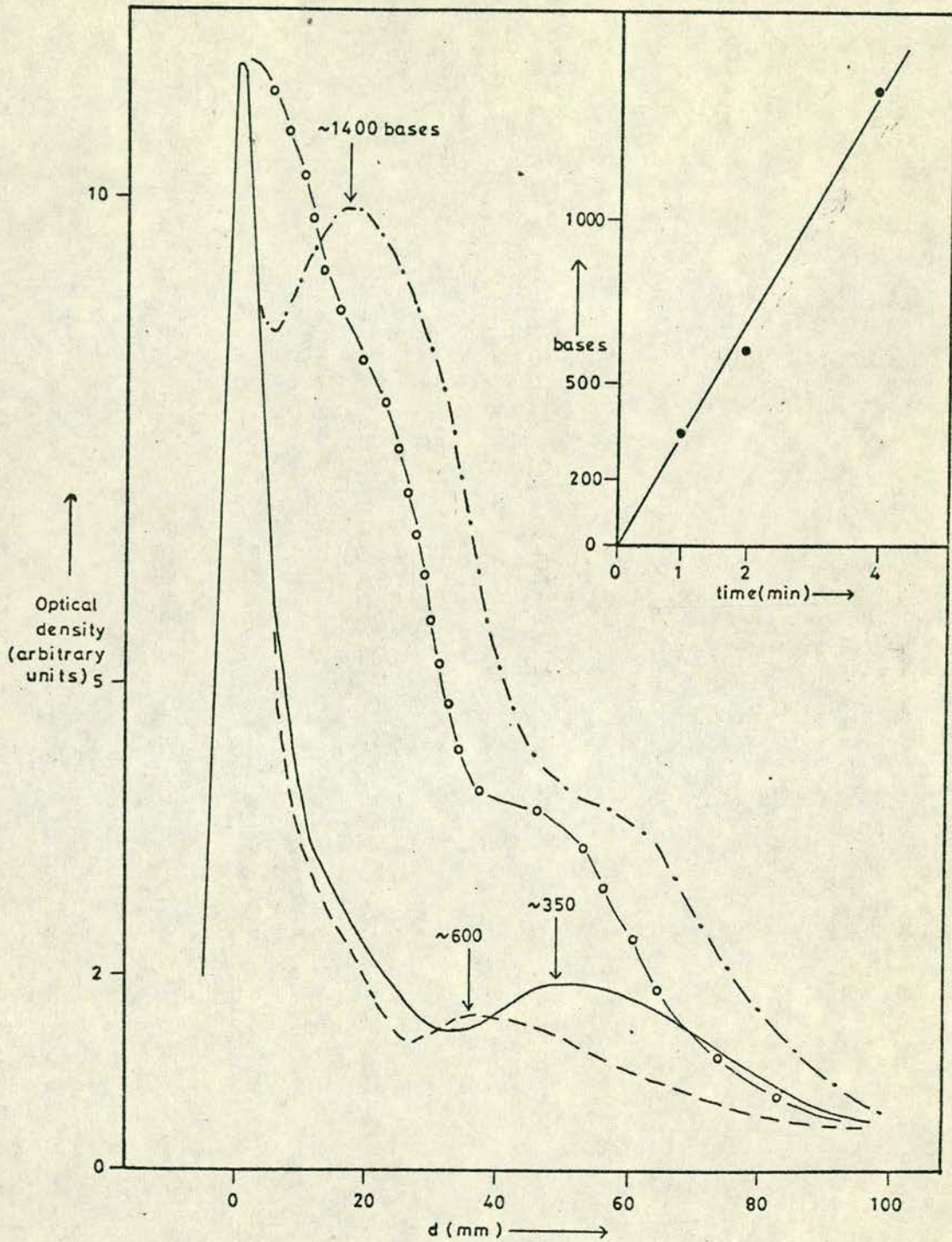


Fig. 35



The RNA-extensions have a large size range. Because a large molecule will contribute more to the blackening of the film than a small molecule, it is difficult to determine the true average size of the extensions. However as an estimate, the sizes which correspond to the peaks in the scans have been determined and plotted against reaction time (Fig. 35 inset). This suggests that the chains grow at approximately 400 nucleotides/min, which compares well with the figures quoted for the rate of elongation by DNA polymerase I on DNA templates (Kornberg, 1974).

Although at first the enzyme is adding nucleotides to an RNA primer, polymerisation and translocation will soon have effectively converted this to a DNA primer. Since there is no lag in the kinetics (Fig. 35) it may well be that the enzyme can use RNA and DNA primers with the same efficiency. However, a small difference in efficiency would not have been detected.

Knowing both the number of RNA primers extended and their approximate rate of elongation, the proportion of total incorporation ascribable to RNA primers can be estimated. The results suggest that in a typical reaction using DNA polymerase I, about 20% of the total incorporation is due to RNA priming. It seems clear that much of the incorporation is at the 3' end of the "r" strand DNA, probably arising from transient formation of primer structures by basepairing with a short complementary DNA sequence. This synthesis should be relatively unspecific. Some of the incorporation by DNA polymerase I can be attributed to synthesis at random single-stranded breaks



in the contaminating double stranded DNA ("nick-translation"). Klenow fragment A of DNA polymerase I has polymerase activity, but not the 5' to 3' exonuclease activity needed for the rapid "nick-translation" mode of polymerisation initiated at single stranded breaks. When Klenow fragment enzyme was used in these reactions there was less total incorporation of dNTPs, but unchanged extension of RNA primers, confirming that "nick-translation" by DNA polymerase I was responsible for at least some of the DNA priming observed.

#### 8. Studies with restriction endonucleases

As the T7 RNA extension reaction proceeds, there is synthesis of DNA to the right of the termination site. In this way, restriction sites to the right of t1 should be regenerated, offering a novel approach to the mapping of restriction sites in this region of T7 DNA. By combining digestion by a restriction endonuclease with subsequent alkaline hydrolysis, an oligonucleotide differing in size from any of the restriction fragments of whole DNA should be generated, unless the termination site is also a restriction site (see Fig. 31). The mapping of restriction sites in biologically significant regions of DNA is a necessary first step towards their sequencing by the rapid techniques now available.

Extensions were made as usual. The reaction was allowed to proceed for 20 min, so that the extensions should be about 8000 bases long. The restriction endonucleases HpaI, HpaII and HindII were used as described above and the



products electrophoresed on a 12% polyacrylamide gel in 7 M urea, after denaturation in formamide at 100°C. An autoradiograph of the wet gel is presented in Fig. 36. The unrestricted controls show that most of the denatured labelled material is too large to enter this gel, even after alkaline hydrolysis. (Nucleotides of less than 200 bases should be clearly resolved.)

HpaI cuts double stranded DNA at the centre of the sequence  $\begin{array}{c} \text{-GTTAAC-} \\ \text{-CAATTG-} \end{array}$ . It produces 19 fragments from T7<sup>+</sup> DNA, the smallest of which is about 280 b.p. The sites on T7 DNA where HpaI cuts have now been mapped (McDonnell *et al*, 1977). One of them is extremely close to the terminator, probably within 50 b.p., which is at the limit of the accuracy of the map. Preliminary experiments on hybrids extended with DNA polymerase I have given tentative evidence that this HpaI site is to the right of t1 rather than the left (unpublished experiments in collaboration with J.C. Boothroyd). These experiments (Fig. 36) showed that after digestion with HpaI a little labelled material enters the gel. Most noticeable is a band corresponding to an oligonucleotide of chain length around 8. Although detectable without alkali, full recovery of this band seems to require alkaline hydrolysis. RNAase contamination of the restriction enzyme might explain the apparent release of some oligonucleotide in the absence of alkali.

HindII has a specificity overlapping that of HpaI, cutting double stranded DNA at the centre of sequences  $\begin{array}{c} \text{-GTYRAC-} \\ \text{-CARYTG-} \end{array}$ . This produces some 60 fragments from T7<sup>+</sup> DNA whose size ranges from 20 to 2800 base pairs. The position



Figure 36. Studies of the products of RNA-extension using restriction endonucleases

T7 C5 LG3 RNA.DNA hybrid was extended (with  $\alpha$ -<sup>32</sup>PdGTP at 130 mCi/mmole) for 20 min at 37°C. Aliquots of 0.04 pmole were then treated as follows, and examined by electrophoresis on a 12% polyacrylamide gel in 7 M urea, 0.1 M Tris-borate (pH 8.3), 1 mM EDTA at 8 V/cm for 18 hrs. The gel was then autoradiographed.

Track	Treatment of aliquot
1	<u>HpaI</u> , followed by alkaline hydrolysis
2	<u>HpaI</u>
3	<u>HpaII</u> , followed by alkaline hydrolysis
4	<u>HpaII</u>
5	<u>HpaII</u> , followed by T <sub>1</sub> RNAase
6	<u>HindII</u>
7	<u>HindII</u> , followed by alkaline hydrolysis
8	control (not restricted)
9	control + alkaline hydrolysis

The samples were denatured in 50% formamide at 100°C for 10 min, then rapidly chilled on ice, before loading. The positions of molecular weight markers are indicated.



| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

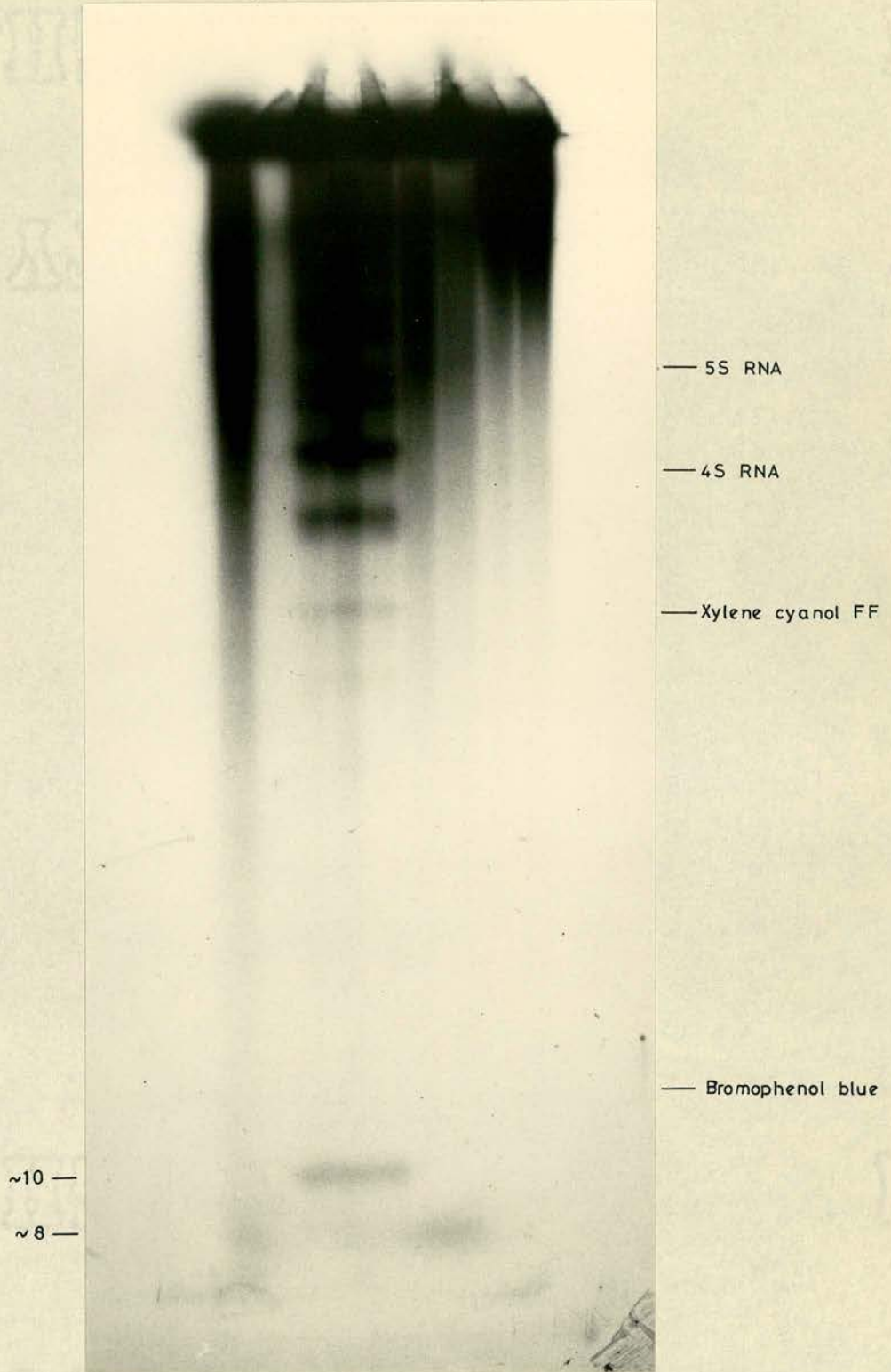


Fig. 36



of some of these fragments, (especially those from the early region) have been mapped (see Fig. 20; F.W. Studier, personal communication; Ludwig and Summers, 1975, 1976; Humphries et al, 1974). HindII generated a short oligonucleotide much as did HpaI, although its recovery now seemed less dependent on alkaline hydrolysis. This could reflect a higher level of RNAase in the HindII preparation used. No other specific labelled fragments were detected. Possibly there are no small HindII fragments in the region of the extension, i.e. between t1 and about 39% on the T7 map. It is known that the fragments immediately distal to the terminator site are 1000 and 530 base pairs, in that order.

HpaII cuts double stranded DNA at the arrows within the sequence  $\begin{array}{c} \downarrow \\ \text{-CCGG-} \\ \text{-GGCC-} \\ \uparrow \end{array}$  and produces some 56 fragments from T7<sup>+</sup> DNA ranging in size from 50 to 2850 base pairs. The sites where HpaII cuts within the early region have been mapped (see Fig. 20; F.W. Studier, personal communication). Redigestion of HpaI fragment C (which corresponds to 19.3-29.5% of the genome) with HpaII gave 5 fragments, the smallest of which was 350 b.p. (J.C. Boothroyd, unpublished experiments). When RNA extensions were digested with HpaII, many labelled DNA fragments less than 200 b.p. long were produced, and none seemed to require alkaline hydrolysis for their production. It now seems clear that the oligonucleotide from t1 to the first distal HpaII site would be around 350 b.p., too large to be resolved here. If all the observed bands arise from DNA extension of the RNA primers, then they must all map between 29.5 and about 39%

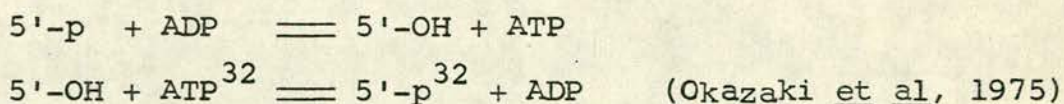


of the genome. It should be noted that one of these small oligonucleotides is only about 10 bases long and has not been previously reported.

It seems clear that this method can be used to map restriction sites following the 3' termini of RNA primers (e.g. transcripts) and DNA primers (e.g. restriction fragments). With RNA primers however, the restriction endonucleases used should preferably be free of RNAase, an activity which is rarely assayed in these enzyme preparations.

#### 9. Studies using T4 polynucleotide kinase

When hybrid preparations are used as primers for DNA polymerase I, much of the DNA synthesised is unrelated to RNA priming. Thus, a method for selective labelling of those extensions made at RNA primers would be of great value. Such labelling can in principle be achieved by the use of T4 polynucleotide kinase. This enzyme can specifically label 5'-OH termini of oligo- and polynucleotides with  $^{32}\text{P}$  using  $\gamma\text{-}^{32}\text{P}$  ATP as substrate. The conditions of labelling must be carefully controlled as the enzyme can also catalyse an exchange reaction to introduce  $^{32}\text{P}$  at 5'-phosphate termini:-



The 5' termini of T7 DNA are phosphorylated (Richardson, 1966) as are most of the ends produced by DNA fragmentation. Thus after alkaline hydrolysis of the products of an extension reaction, only the DNA extensions made at RNA



primers should have 5' OH groups. Once these have been labelled using polynucleotide kinase, methods are available for determining the sequence (Murray, 1970; Southern and Mitchell, 1971).

Hybrids were extended using  $^3\text{H}$  dTTP to follow the course of the reaction, and the RNA then hydrolysed by alkali. The monoribonucleotides were removed by gel filtration on Sephadex G-75, and the high molecular weight DNA was labelled using polynucleotide kinase as described in chapter 3. The reaction was at  $0^\circ\text{C}$  to minimise the introduction of  $^{32}\text{P}$  at 5'-p termini. A sample of hybrid which had not been extended was treated similarly as a control.

An aliquot of labelled material was digested to mononucleotides using pancreatic DNAase and snake venom phosphodiesterase (Fig. 31), and examined by electrophoresis on AE81 paper at pH 3.5. Autoradiography showed that the major 5' nucleotide present on DNA extensions made at RNA primers was G. There were trace amounts of A, C and T whilst the control showed trace amounts of all 4 nucleotides.

Pancreatic DNAase limit digests were examined by two dimensional electrophoresis on AE81 paper at pH 3.5 followed by DE81 paper at pH 2.0 as described in chapter 3. Labelled oligonucleotides were detected by autoradiography and those present only as a result of DNA synthesis on RNA primers were identified by comparing the control and experimental "fingerprints".



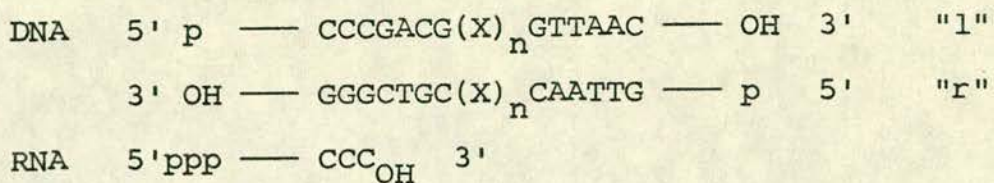
	R <sub>f</sub> AE	R <sub>f</sub> DE	Tentative identification
1	0.60	1.42	GA
2	0.35	1.19	GAC
3	0.24	0.54	?
4	0.07	0.44	GACG

These tentative identifications are based on published mobilities and "M" values (Murray, 1970). The sequence GACG agrees with the GA published by Hayward and Peters (1974) and further work which indicated that the next base was a pyrimidine (G.G. Peters, unpublished experiments). Nucleotide no. 3 is unlikely to be related to the others in view of its "M" values. It remains unidentified and unexplained.

Attempts were made to confirm and extend this sequence, but were inconclusive. Labelling with polynucleotide kinase was at 37°C, in an attempt to increase the efficiency of labelling of 5'-OH termini, but there was greatly increased labelling of <sup>oligo</sup>nucleotides in the control reaction. This made it impossible to identify with any certainty those oligonucleotides arising from DNA synthesis at RNA primers. The increased labelling was probably due to exchange of <sup>32</sup>P with 5'-p groups. Okazaki et al (1975) found that the exchange reaction is much reduced at 0°C compared with the kinase reaction. Unfortunately, this experiment was never repeated using 0°C for 5' labelling so the identification of the sequence GACG remains tentative. In particular it would be desirable to confirm that nucleotides no. 1, 2 and 4 are indeed related, by elution, partial digestion with venom phosphodiesterase and analysis of the products.



The information presently available from this laboratory, including the present studies and the published work by Peters and Hayward is consistent with the following sequences:



where n is probably 1( $\pm$ 2) from the results with HpaI and HindII in section 8.



## CHAPTER 10: DISCUSSION

1. The rate of DNA release following termination of transcription

It is well known that DNA is firmly bound to RNA polymerase during the elongation of an RNA chain. From the evidence presented above, it seems that enzyme may be slow to dissociate from the DNA after the completion of an RNA molecule. Does the filtration assay used in these studies give a true measure of the rate at which DNA is released following termination of transcription at t1?

It could be argued that the observed retention of DNA on filters is not due to enzyme bound at t1. It must be admitted that termination at this site is not 100% efficient either in vitro or in vivo. Estimates of the efficiency vary, between 75% in vivo (McAllister and Barrett, 1977) and 95% in vitro (Peters and Hayward, 1974a). The estimates for in vivo efficiency are derived from studies with gene 1 amber mutants, and the expression of late genes is equated with readthrough (e.g. Kiefer et al, 1977). However, the amber fragments of T7 RNA polymerase produced could have some activity, and there is often some degree of suppression of amber mutations even in non-suppressing strains, so it is possible that the readthrough of t1 has been over-estimated in such studies. From the rate of incorporation of NTPs after 4 mins transcription on T7<sup>+</sup> DNA in the presence of rifampicin, the frequency of readthrough of t1 in vitro in the present experiments appears to have been 5-10%.

Readthrough of t1 would lead to the retention of DNA on filters in elongating complexes at times after 4 min transcription on T7<sup>+</sup> DNA. However, there is a second termination



site t2 located about 30% on the T7 map, or about 2 min transcription time from t1. Again, this second site is less than 100% efficient (Peters and Hayward, 1974a), but if dissociation of enzyme after termination was rapid, then after 6 min transcription on T7<sup>+</sup> DNA at E:DNA = 2-2.5 in the presence of rifampicin, only 1-3% of the DNA should be retained by those few enzyme molecules which have readthrough / both t1 and t2. The reduction in the retention of DNA which commences after 4 min transcription seems to plateau at approximately 10% above background (Fig. 15). This is too high to be explained simply by readthrough of t1 and t2 so some of the DNA is probably retained in complexes with polymerase which has (in some way) been inactivated.

It seems unlikely that changes in the DNA structure as a consequence of transcription could per se lead to retention in 0.5 M NaCl. Little if any of the initial retention of DNA seems to be due to core activity in the RNA polymerase preparations used (see chapter 4). If many single stranded breaks were introduced during transcription, then core could initiate at such points. This core synthesis might lead to prolonged retention of DNA if the transcribing core complexes failed to reach a termination site and/or aborted transcription without dissociating from the DNA. For this to be significant, a large number of breaks would have to be made in the short time before rifampicin was added. The enzyme preparations used were not directly assayed for DNAase, although preparations of this purity are not usually contaminated with this activity (Burgess, 1976). It should be noted that the pattern on gels of restriction fragments



obtained by digestion in the presence of RNA polymerase for up to 5 hrs were as sharp as in control digests.

That the observed release is related to the termination of transcription at t1 is suggested by the kinetics of the reaction (e.g. Fig. 15). Release does seem to start some 4 min after the initiation of transcription on T7<sup>+</sup> DNA. The strongest evidence for such an interpretation comes from the observation that restriction fragments of T7 DNA which contain t1 can be specifically retained on nitro-cellulose filters after the termination of transcription (chapter 6).

Thus, it seems probable that the observed slow release of DNA is related to termination, but the detailed mechanism for retention in the assay used in these studies may be complex. The assay exploited filtration in 0.5 M NaCl, so that re-binding of released enzyme to promoters (which will take place, despite the presence of rifampicin) would not interfere with the detection of dissociation of enzyme and DNA after termination. However, it is possible that some of the intermediates in the termination of transcription are also unstable in 0.5 M NaCl. In particular, if RNA is released before the enzyme dissociates from the DNA, the resulting binary complex of enzyme with terminator DNA might be unstable under these conditions. There is some evidence for this view.

It was expected (and subsequently observed) that the binding of RNA polymerase to restriction fragments of T7 DNA containing promoter sites would be sensitive to 0.5 M NaCl. Unexpectedly the binding of enzyme to fragments



containing t1 was also sensitive to 0.5 M NaCl. If this reflects instability of binary RNA polymerase -t1 DNA complexes, then the retention of the DNA in the filter assay must be due to "stalled" ternary (RNA-synthesising) complexes which cannot reach t1 until the enzyme bound there is released (Fig. 37). This release is a slow process under the conditions used for transcription, but is not directly measured in the filter assay.

It proved impossible to detect enzyme bound to restriction fragments when filtered in 0.5 M NaCl, even when streptolydigin was added to block RNA elongation well before the termination of transcription. Ternary complexes of nascent RNA, DNA and enzyme are certainly stable in 0.5 M NaCl (Bremer and Konrad, 1964), so the ternary complexes whose elongation was <sup>expected to be</sup> blocked by streptolydigin in these studies must break down under the conditions used here. It seems that elongation and release must have continued despite the presence of streptolydigin during the incubation with restriction endonuclease, as the only fragments retained on filtration in 50 mM NaCl contained promoters or t1 (data not shown).

Thus, it seems that in the DNA release experiments, in which most DNA molecules were transcribed by two enzyme molecules, the first enzyme to reach t1 was only slowly released. This release involved the dissociation of a complex which was unstable in 0.5 M NaCl, but stable in the transcription reaction (0.15 M KCl) and in 50 mM NaCl. By analogy to binary complexes at promoter sites, it is hypothesised that this salt-sensitive terminator complex



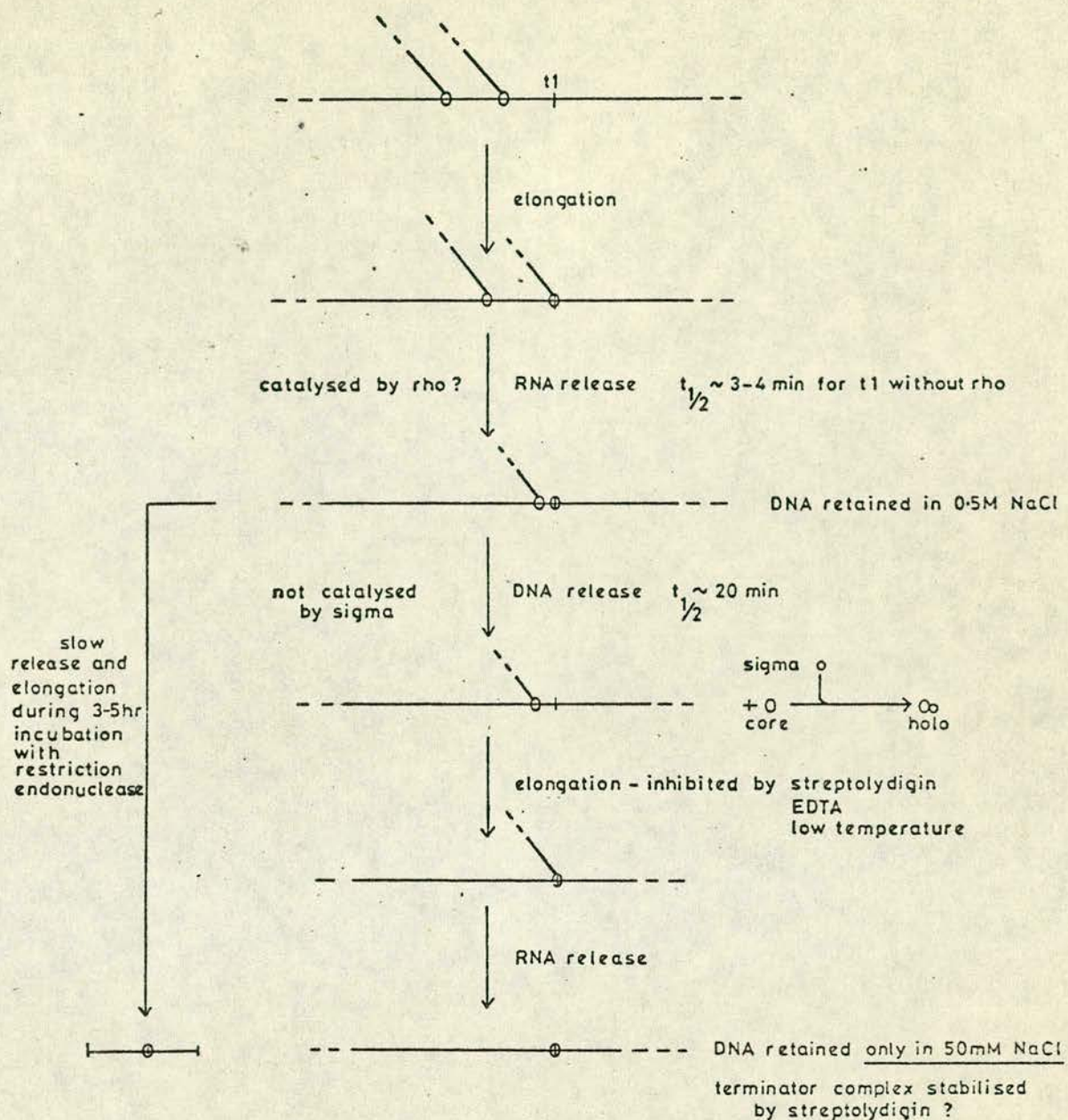


Fig. 37



is also binary i.e. RNA release has occurred (Fig. 37). Succeeding RNA polymerase molecules cannot reach t1 until the terminator complex has dissociated, and so the DNA is retained on nitrocellulose, on filtration in 0.5 M NaCl, by "stalled" ternary complexes. During the incubation with a restriction endonuclease in the presence of streptolydigin and rifampicin, elongation (and apparently dissociation of the terminator complex) was not completely inhibited. Thus, by the end of the incubation, RNA polymerase was bound only in binary complexes at promoters and at t1, allowing retention on nitrocellulose in 50 mM NaCl of fragments of DNA containing these sites.

This model agrees well with the results presented here. At low E:DNA ratios (e.g. E:DNA = 0.7) few of the DNA molecules should have more than one enzyme bound, so that the level of retention of DNA in 0.5 M NaCl at the end of the transcription cycle should fall rapidly. Indeed under such conditions the retention of DNA declines very much faster than at higher E:DNA ratios (e.g. E:DNA = 2.5).

According to the above interpretation, the rate of reduction in retention of DNA in 0.5 M NaCl is not a direct measure of the rate at which enzyme bound at t1 (in a complex which is unstable in 0.5 M NaCl) dissociates from the DNA. It would be a reasonably accurate measure if all DNA molecules had two or fewer polymerases bound, provided that translocation of the "stalled" ternary complexes up to t1 was a comparatively rapid process. It seems that the latter is likely to be true.

At E:DNA ratios of 2.0-2.5, some 85% of the DNA which



carries any polymerase should have two or fewer bound enzyme molecules. Under these conditions, the 0.5 M NaCl-stable complexes have an observed half-life of about 20 min. This figure is the most accurate estimate available from these studies for the rate of dissociation of RNA polymerase-terminator DNA binary complexes at 0.15 M KCl. This estimate is obviously highly dependent on the accuracy of the E:DNA ratios, and therefore on the accuracy of the estimates for the percentage of active RNA polymerase in the enzyme preparations. This latter has been determined in a number of independent ways (chapter 4), which all gave good agreement.

The slow dissociation of binary terminator complexes suggested by this analysis would lead to slow re-cycling of RNA polymerase and produce biphasic kinetics for transcription, as observed (Fig. 11).

## 2. The rate of RNA release following termination of transcription

If this model is correct, then what of the observed RNA release? The kinetics at E:DNA ratios = 2.0-2.5 show a rapid initial burst followed by a slower phase. The initial burst may reflect the release of an RNA molecule by each polymerase which has reached t1. Subsequent polymerase molecules cannot reach t1 until the binary terminator complex has dissociated; only then can they complete their transcript and release it. It is clear that no accurate estimate of the rate of RNA release at t1 after termination of transcription in 0.15 M KCl can be derived from the experiments reported above, because:



(i) the rapid loss of some RNA on filtration in 0.5 M NaCl may reflect the instability to high salt of a complex which could be considerably more stable at 0.15 M KCl.

(ii) the retention of some RNA in 0.5 M NaCl probably represents RNA in elongating complexes which had not yet reached t1 at 0.15 M KCl because of slow dissociation of the binary terminator complex.

It should be noted that there is not an instantaneous release of RNA after 4 min transcription on T7<sup>+</sup> DNA. This could be due to asynchronous termination of the first round followed by immediate release, though the kinetics of incorporation in the presence of rifampicin would argue against such an interpretation. Equally, there is no evidence for an instantaneous release of DNA after 4 min transcription, equivalent to that percentage of DNA which had only bound one enzyme molecule. These observations suggest that on reaching t1, the RNA-DNA-E complex (stable in 0.5 M NaCl) is converted to a complex which is not stable in 0.5 M NaCl in a reaction of half-life 3-4 min. It is hypothesised that this is the RNA release reaction, which produces free RNA and a DNA-E terminator complex which then dissociates with a half-life of 20 min (Fig. 37). It is possible that this reaction produces an RNA-DNA-E complex which is not stable in 0.5 M NaCl, but the above hypothesis does take account of the different salt stabilities of known binary and ternary complexes. RNA release could be catalysed by rho at this site, but more importantly, at other sites where <sup>RNA</sup>~~DNA~~ polymerase does not "pause" for so long (see below).



A more accurate estimate for the rate of RNA release might be obtained using very low E:DNA ratios and filtration in 0.15 M NaCl.

3. Is dissociation of the terminator complex inhibited by streptolydigin?

Conditions were defined in Chapter 5 which seemed to inhibit DNA release as assayed by filtration in 0.5 M NaCl. However, if the enzyme-DNA complexes responsible for retention are not complexes at the termination site, but are elongating complexes which cannot reach t1, then it could be that these conditions only inhibit elongation and not release.

Care was always taken to ensure that all detectable transcription had ceased before EDTA or streptolydigin were added, or the temperature changed. However, this may only have ensured that those DNA molecules which had been transcribed by a single enzyme molecule would by this time register as "free" DNA in the assay used, because of instability of binary enzyme-t1 DNA complexes in 0.5 M NaCl. Thus it is not possible to argue from the kinetics alone that release of t1 DNA by enzyme is inhibited by streptolydigin.

In the experiments with restriction endonucleases (Chapter 6), the absence of 0.5 M NaCl-stable complexes after a few hours incubation strongly suggests that elongation had continued under those conditions even in the presence of streptolydigin. Evidently all the polymerases are able to reach t1 by the end of the incubation so that those which remain bound to the DNA are in binary complexes. Without further study, including



the quantitation of the yield of terminator fragments, it is not possible to state with certainty that the observed results show that streptolydigin reduces the rate of dissociation of binary polymerase-terminator DNA complexes. However, it should be noted that streptolydigin does increase the stability of binary complexes of RNA polymerase with both poly d(A-T) and T4 DNA (von der Helm and Krakow, 1972). It might have a similar effect on the binary terminator complex.

The concentration of streptolydigin used (100  $\mu\text{g/ml}$ ) was sufficient to stop elongation as measured by NTP incorporation (e.g. Fig. 18). However, in the experiments with restriction endonucleases, it was added after rifampicin. Rifampicin binds to elongating complexes, but does not inhibit them (Yarborough *et al.*, 1976) and prior binding of rifampicin may to some extent have rendered the elongating complexes less susceptible to attack by streptolydigin. Indeed, rifampicin was reported to decrease the effect that streptolydigin had on the stability of binary complexes (von der Helm and Krakow, 1972). Unfortunately the effect of streptolydigin on the incorporation of NTPs in the presence of rifampicin was never tested in these studies.\* It is also possible that a higher concentration of streptolydigin (or streptolydigin in the absence of rifampicin) when added during the elongation phase of transcription would have allowed the retention of fragments from within the early region in 0.5 M NaCl.

Further studies may clarify the effect of streptolydigin

\* Recent experiments indicate that rifampicin (100  $\mu\text{g/ml}$ ) does not affect the inhibition of T7 DNA transcription by streptolydigin (50  $\mu\text{g/ml}$ ) added 1 min later.



on termination and show whether rifampicin and streptomycin can act simultaneously. However, the studies presented above do show how restriction fragments containing t1 can be identified. This should be of great practical use in the mapping of restriction sites around this and perhaps other termination sites.

#### 4. Is there a factor for DNA release *in vivo*?

Is there a factor present in the bacterium which accelerates the apparently slow dissociation of RNA polymerase from the t1 termination site? It is thought that the physiological ionic conditions resemble those used in vitro. As discussed in Chapter 7, it seemed reasonable to hypothesise that sigma might catalyse release. From a comparison of the properties of core and holoenzyme binary complexes on T7 DNA, Hinkle and Chamberlin (1972a) established that sigma reduces the affinity of core for all DNA except promoter sites. They also presented preliminary evidence that sigma can interact with core which has already bound to DNA; they detected a faster dissociation of core from T7 DNA in the presence of a four-fold sigma over core. Though there seems to be an excess of excess of/core subunits over sigma in vivo (Iwakura, Ito and Ishihama, 1974), much of the core is thought to be actually transcribing so that the true situation is unclear.

In the present studies there were approximately 1-2 moles of free sigma per mole of core bound at t1. However the inactivation of 95% of all sigma by antiserum had no effect on the rate of release of DNA as assayed by filter binding. This strongly suggests that there is no role for sigma in the dissociation of the DNA-enzyme complex at t1.



Schäfer and Zillig (1973a) have reported that following transcription of T7 DNA at low ionic strength (6 mM magnesium acetate, 50 mM  $\text{NH}_4\text{Cl}$ ) the RNA product is released but the enzyme is not. They also reported (but gave no evidence) that sigma became associated with the core enzyme bound at t1, but that release did not occur. This is not inconsistent with the results presented here.

The preliminary results reported in Chapter 8 indicate that rho has no significant effect on the rate of DNA release at t1. When rho was present before the elongating enzymes reached t1 there was an effect on the retention of DNA in the filter binding assay, but this could be because of termination and release at sites before t1: or because rho catalyses RNA release at t1, so accelerating the initial phase of the fall in retention of DNA (dependent on the rate of RNA release) without affecting the later phase (which reflects more accurately the dissociation of binary terminator complexes): or both. The effect of rho on RNA release has not been directly studied here.

It is of course possible that some factor other than rho or sigma catalyses DNA release in vivo. Several factors have been described which affect transcription in a relatively unspecific fashion, but their mechanisms and in vivo functions (if any) remain unclear (Davidson et al, 1970; Schäfer and Zillig, 1973b; Yang and Zubay, 1974).

It must be pointed out that once E.coli RNA polymerase has transcribed the early region of T7 DNA, it is no longer needed for phage development and indeed it is known



to be "switched off". Thus rapid release from t1 may not be required and so may not have been one of the constraints which have led to the evolution of t1. However, late T7 transcription by T7 RNA polymerase does involve readthrough of t1, so E.coli RNA polymerase cannot stay permanently bound to t1 (Skare, Niles and Summers, 1974).

Finally, the slow release of enzyme from t1 may be an in vitro artefact, although it has been observed with enzyme prepared from two very different strains of E.coli and several phage DNA preparations.

The generality of slow release at termination sites remains to be demonstrated. No restriction fragments containing t2 were detected in the present work using T7 LG37 DNA. It is possible that t2 is carried on a small HpaII fragment which would have escaped detection, or that release at t2 is intrinsically much more rapid than from t1. It would be of great interest to repeat these experiments using as template DNA a restriction fragment containing a single operon.

##### 5. The mechanism for termination of transcription and release of RNA and DNA at t1.

When an elongating complex reaches the termination site t1, RNA polymerase is able to recognise this and respond by stopping transcription. There is no evidence that either sigma or rho is needed for this primary recognition. The enzyme may recognise a sequence/structure in the DNA or the RNA. From comparison with other systems it seems possible that this might include a sequence in the DNA with two-fold symmetry, or a hairpin loop in the newly



synthesised RNA (which are different descriptions of the same signal). Such a signal is found at termination sites for many RNAs (see Chapter 1). At the end of the E.coli supF gene there are two nearly identical sequences, both of which are termination sites, but only the stronger terminator has such a signal. The self-complementary sequence is centred some 10-15 base pairs before the 3' end of the RNA, potentially well within reach of the RNA polymerase but not necessarily buried within the enzyme.

It had been thought that a G-C rich region followed by a sequence similar to  $-(U)_6A_{OH}$  in the RNA were necessary and sufficient to make a termination site. Though such sequences may be termination sites, it is now clear that there are alternatives. Indeed a G-C rich region followed by  $-(U)_6A_{OH}$  is found within the  $\lambda$  7S RNA where there seems to be no termination (Roberts et al, 1977). The observation that in this case no hairpin loop could be formed near the putative RNA terminus may be highly significant.

The sequences found at the 3' termini of transcripts from the  $\lambda$  cro gene and the E.coli supF gene appear to have some common features and are quite different from those found on the  $\lambda$  4S and 6S RNAs; the sequence  $-CCC_{OH}$  determined in Edinburgh for the 3' end of T7 early RNA is also different. It is clear that there may be considerable variation in the 3' terminal sequences of RNAs produced by the termination of transcription. Until the bases changed in terminator mutants have been identified, it will be difficult to determine which particular sequences are important for termination.



Sequence analysis has so far shown no detectable common features in the untranscribed regions of terminator sites (Rosenberg et al, 1976). Again, to establish that these regions have no significance for termination will require the analysis of terminator mutants.

As discussed above, the present studies have been unable to establish definitively that RNA release precedes DNA release. However it has been shown that enzyme may be bound at t1 after the termination of transcription in a complex which is stable in 50 mM NaCl but not 0.5 M NaCl. From the properties of binary enzyme-DNA complexes at promoters and of elongating complexes with nascent RNA, it seems highly probable that RNA polymerase can remain bound at t1 after releasing its completed RNA molecule. The release of RNA does not appear to be so rapid as to be unmeasurable by the filter binding assay, and is considerably faster than the rate of DNA release. RNA release is thought to require rho at some sites, perhaps because the elongating complexes only "pause" there, and in the absence of rho-catalysed RNA release tend to continue elongation of the RNA chain. The degree to which any site appears to depend on rho for termination would then be determined by the balance between the rate of RNA release and the duration of "pausing" at that site. In this way if the "pause" was significantly longer than the half-life for RNA release, then termination would appear to be rho-independent (e.g. t1), even though rho may not act to catalyse RNA release at such sites in vivo.

It seems plausible to assume that when RNA is released



the DNA duplex is reformed. This follows from the properties of core enzyme, which is thought only to form "closed" complexes when it binds DNA (Hinkle and Chamberlin, 1972a). In agreement with this, the complex of polymerase with t1 DNA has some of the properties of such "closed" core enzyme-DNA binary complexes.

The DNA is apparently released by the enzyme in a slow reaction, which is probably the rate-limiting step for continued transcription in this system. <sup>It has been argued above that the</sup> ~~The~~ half-life of the binary terminator complex at 37°C in 0.15 M KCl is about 20 min. The half-life of holoenzyme binary complexes is about one second at non-promoter sites and many hours at promoter sites on T7 DNA (Hinkle and Chamberlin, 1972a; Chamberlin, 1976). This suggests that sigma does not rebind to form normal holoenzyme prior to dissociation of the terminator complex in vitro. The half-life of core enzyme-DNA complexes is about 20 min (Hinkle and Chamberlin, 1972a), very similar to the figure determined here for the terminator complex. This suggests that the dissociation of enzyme from t1 is a passive process, not affected by any special features of the terminator DNA.

However, not all of the properties of the terminator binary complex are the same as those of core-DNA complexes. Core binary complexes rapidly dissociate when challenged with single stranded DNA; this property has been taken to be diagnostic of "closed" complexes. The dissociation of terminator complexes was unaffected by single stranded DNA (Chapter 5) which would suggest that the terminator complex is "open". Though core is thought to be unable to open



up the DNA duplex, it may be capable of maintaining it open after the termination of transcription and release of RNA. Alternatively, because of sequence specific effects, enzyme bound at t1 in a "closed" complex may be resistant to attack by single stranded DNA. At present it is impossible to distinguish between these two alternatives. It does not seem likely that enzyme at t1 is resistant as a result of having rebound sigma, because the rate of release of T7 DNA in the presence of single stranded DNA (as assayed by filter binding in 0.5 M NaCl) is not affected by antisigma antiserum.

## 6. Conclusions

From the kinetics of transcription of T7 DNA at low E:DNA ratios, it was hypothesised that there was a slow step at the end of the first round. The binding of both DNA and nascent RNA to RNA polymerase during a single round of transcription was investigated using a filter binding assay. The results obtained suggest that RNA release occurs before DNA release, and that the latter process is a comparatively slow one. Some of the properties of the enzyme-DNA complex at the termination site have been described. Conditions have been defined which allow detection of restriction fragments containing the termination site because they are complexed with enzyme after the end of transcription. This discovery should be of great practical use in the mapping of restriction sites around this termination site and, if slow DNA release is a general phenomenon, around other sites.



It has been shown that recognition of the termination site is independent of sigma, and that if sigma does become associated with the enzyme in the terminator complex, then it does not catalyse DNA release.

Preliminary experiments using the transcriptional termination factor rho suggest that rho may catalyse RNA release at this classically rho-independent termination site, but does not affect the rate of DNA release.



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