RNA polymerase II ternary transcription complexes generated *in vitro*

Steven Ackerman, David Bunick, Ruben Zandomeni and Roberto Weinmann

The Wistar Institute of Anatomy and Biology, Philadelphia, PA 19104, USA

Received 21 April 1983; Revised and Accepted 3 August 1983

ABSTRACT

Ternary transcription complexes have been formed with a HeLa cell extract, a specific DNA template, and nucleoside triphosphates. The assay depends on the formation of sarkosyl-resistant initiation complexes which contain RNA polymerase II, template DNA, and radioactive nucleoside triphosphates. Separation from the other elements in the *in vitro* reaction is achieved by electrophoresis in agarose - 0.25% sarkosyl gels. The mobility of the ternary complexes in this system cannot be distinguished from naked DNA. Formation of this complex is dependent on all parameters necessary for faithful *in vitro* transcription. Complexes are formed with both the plasmid vector and the specific adenovirus DNA insert containing a eucaryotic promoter. The formation of the complex on the eucaryotic DNA is sequence-dependent. An undecaribonucleotide predicted from the template DNA sequence remains associated with the DNA in the ternary complex and can be isolated if the chain terminator 3'-O-methyl GTP is used, or after T1 ribonuclease treatment of the RNA, or if exogenous GTP is omitted from the *in vitro* reaction. This oligonucleotide is not detected in association with the plasmid vector. Phosphocellulose fractionation of the extract indicates that at least one of the column fractions required for faithful runoff transcription is required for complex formation. A large molar excess of abortive initiation events was detected relative to the level of productive transcription events, indicating a 40-fold higher efficiency of transcription initiation vs. elongation.

INTRODUCTION

Eucaryotic transcription involves three distinct processes: initiation, elongation, and termination of the RNA molecule. Initiation can be further delineated as RNA polymerase locating the proper control region sequence of DNA; binding of RNA polymerase to this region to form a specific transcription complex; some as yet undefined energy-dependent step(s); unwinding of DNA; incorporation of the first nucleotide; incorporation of the next few nucleotides; and a commitment to productive RNA synthesis. The steps involved in the initiation process of RNA synthesis are a primary target for the control mechanism(s) of gene regulation.

The recent development of *in vitro* transcription systems prepared

from cellular extracts (1, 2), which are dependent upon exogenous DNA and nucleoside triphosphates, enable us to study some of the regulatory elements involved in eucaryotic transcription. The assay for these systems depends on the correct initiation of transcription, and on RNA elongation. Discrete truncated RNA runoffs are obtained due to a restriction enzyme cut of the DNA template. Studies have concentrated on the biochemical fractionation of the protein factors (3-5) which, in addition to RNA polymerase II, are required for synthesis of the runoff RNA.

To investigate initiation mechanisms we must be able to separate them from RNA elongation processes. We have established a requirement for hydrolysis of the β - γ bond of ATP for faithful formation of ternary initiation complexes (6). This same step seems to be the one affected by DRB in in vitro transcription systems (7, 8). These studies were feasible because the ternary complexes resulting from RNA polymerase II initiation are stable in vitro. A ternary transcription complex is defined by the association of RNA polymerase, the DNA template, and one or more ribonucleoside triphosphates (rNTP). If initiation and some elongation events occur such that a short oligoribonucleotide is synthesized, then this transcription complex is also termed a ternary complex with a nascent RNA chain (9). The resulting transcription complex can then be studied to determine how various transcription inhibitors either block the formation of the transcription complex or affect RNA elongation.

Sarkosyl, a mild ionic detergent, has been extensively studied as an inhibitor of RNA polymerase-mediated initiation of transcription (10, 11). Viral transcription complexes are stable and can elongate preinitiated RNA chains in the presence of sarkosyl (10, 11). We have developed a system exploiting the sarkosyl resistance of RNA polymerase II transcription events, concentrating on the functional requirements for the assembly of transcription initiation complexes. Results on the isolation of eucaryotic RNA polymerase II transcription ternary complexes resistant to dissociation by sarkosyl and some of the requirements for their assembly in vitro are presented. We can demonstrate that the binding of $\alpha^{32}\text{P}$ -rNTPs in the ternary transcription complex is both specific for the DNA sequence of a specific gene's initiation region and stable in the presence of the ionic detergent sarkosyl. The predicted 5'-end oligonucleotide can be isolated from complexes containing the eucaryotic RNA polymerase II promoter. A molar excess of initiation events over the amounts of full length runoff RNA products suggests that abortive initiations are the prevalent kind

detected.

MATERIALS AND METHODS

Radioisotopes were purchased from Amersham. Agarose was from BRL (Maryland) and sarkosyl from ICN (California). Nucleoside triphosphates and analogues were from PL Biochemicals (Wisconsin). All chemicals were reagent grade. Radioactive precursors were from Amersham (Chicago, Illinois).

Whole cell extracts were prepared as described by Manley (2), except that initial DTT concentrations were 5 mM when the cells were homogenized and the final dialysis buffer contained 20 mM Hepes, pH 7.9, 20% glycerol, 5 mM MgCl₂, 75 mM KCl, 0.1 mM EDTA, and 2 mM DTT. DNA templates were purified by plasmid purification techniques as described (12).

Transcription reactions and transcription complex formation were carried out in 8-10 mM Hepes (pH 7.9), 60 mM KCl, 7.5 mM MgCl₂, 8-10% glycerol, 2 mM DTT; unlabeled rNTPs were as indicated. Labeled rNTPs ($\alpha^{32}\text{P}$, 5 μCi per reaction) were added to a final concentration of 20 μM unless indicated otherwise. Creatine phosphate was added to all reactions, to 4 mM. The DNA concentration selected (20 or 25 μg DNA/ml) gave the optimum levels of transcription in the runoff assay. Reaction mixtures for runoff RNA synthesis were incubated at 30°C for 60 min and processed as described (1).

For electrophoretic analysis of transcription complexes in agarose gels, 20- μl reactions were incubated for 20 min at 30°C. EDTA and sarkosyl were added (10 mM, 0.25%, respectively) and the reaction mixtures loaded on 2% agarose gels in 40 mM Tris, 2 mM EDTA, 10 mM Na acetate (TAE) buffer supplemented with 1 μg ethidium bromide/ml and 0.25% sarkosyl. Electrophoresis was at 25 mA (60-80 V) for 16-20 h at room temperature and the chamber buffer was TAE. At agarose concentrations lower than 2%, the gels were found to be extremely soft due to the presence of sarkosyl and the sample wells sometimes collapsed. Gel electrophoresis was performed without overlaying the gel with buffer to avoid the high background resulting from the unincorporated ^{32}P -rNTPs diffusing into the surrounding buffer and recirculating through the agarose. Gels were dried on Whatman 3 MM paper and exposed at -70°C to Kodak XRP film using DuPont Lightning-Plus screens.

For oligonucleotide analysis, transcription complexes were isolated by electrophoresis through 2% agarose-0.25% sarkosyl as above. The plasmid pBR313 DNA and insert Ad2 DNA were visualized by UV light and the bands

excised. After incubation in 16 volumes of 0.05% SDS-10 μ g proteinase K/ml-1 μ g tRNA/ml for 2 h at 20°C, the gel slices were dissolved by heating to 100°C and immediately shaken with one-fourth volume of water saturated phenol. The solutions were placed in ice water for 10 min and then centrifuged at 10,000 rpm for 20 min. The aqueous phase was re-extracted with phenol-CHCl₃ and then CHCl₃ alone, passed through a 1-ml column of DEAE-cellulose and the RNA eluted with 30% triethylamine carbonate (pH 8-9). Triethylamine carbonate was lyophilized away and the RNA resuspended in water (50 μ l) and precipitated with ethanol. An aliquot was applied to an 8 M urea-18% polyacrylamide gel (run in 90 mM Tris base, 90 mM boric acid, 2.8 mM EDTA; pH 8.3) and electrophoresed until the bromophenol blue migrated half the gel length. The undecanucleotide band in the acrylamide gel was excised and placed in 8 ml water containing RNases T2 and A, and incubated for 16 h at 37°C. The eluted nucleotides were bound to DEAE-cellulose and removed with 30% triethylamine carbonate (pH 8-9). After lyophilization the nucleotides were incubated with both RNases again. Nucleotides were analyzed by PEI plate chromatography in 1.2 M LiCl after RNase digestion and subjected to autoradiography (14).

RESULTS

We used the whole-cell extract described by Manley et al. (2), which transcribes specific genes at readily detected levels, to investigate the functional requirements for assembly of transcription complexes. The template was a cloned gene of Ad2, which contains the major late promoter (map unit 16.45) inserted into plasmid pBR313 (1). An RNA runoff transcript 536n in length can be detected when the template is digested with the restriction enzyme SmaI. Incubation for 20 min is more than sufficient for one round of transcription (Zandomeni, unpublished results). Normally a visible precipitate can be detected during the transcription reaction. This precipitate contains newly synthesized RNA and greater than 90% of the DNA template. Purification of the initiation complex requires that it remain in solution, and that most of the proteins, which probably bind nonspecifically to the complex, be released.

To solubilize this precipitate formed after incubation, several detergents were tested. Non-ionic detergents such as Triton X-100 and Nonidet P-40 did not aid in the solubilization of the large amount of template precipitate but 0.4% of either detergent can be added to the transcription reaction prior to initiation without any ill effects on the

Fig. 1. Effect of detergent and/or transcription inhibitor on *in vitro* transcription. Transcription reactions (25 μ l) were as described in Materials and Methods, except that GTP was omitted. Lane 1, after a 20-min pre-incubation α - 32 P-GTP was added (5 μ Ci, 20 μ M) and incubation continued for 60 min. Lane 2, as in lane 1, except that α -amanitin was also added (1 μ g/ml). Lane 3, as in lane 1, except that EDTA (10 mM) was also added. Lane 4, as in lane 1, except that EDTA (10 mM) and sarkosyl (to 0.25%) were also added. Lane 5, as in lane 1, except that sarkosyl (to 0.25%) was added with the GTP. Lane 6 contains size markers of end-labeled ϕ x 174-HaeIII fragments (1353, 1078, 872, and 603 nucleotides).

amount of transcripts synthesized. Sarkosyl is an ionic detergent that removes most proteins from DNA but leaves RNA polymerase II preinitiated transcription complexes bound to the DNA and allows elongation to proceed (10, 11). When added to our incubation buffers, the sarkosyl formed a precipitate with the divalent cation. We therefore routinely added EDTA with the sarkosyl to chelate the divalent cation, as EDTA alone does not solubilize the transcription complexes. In an assay using 32 P-end labeled DNA we have quantitated the amount of nucleoprotein that remains soluble or is precipitated during incubation at 30°C. If sarkosyl is omitted after the incubation, the major portion of the DNA template (90%) precipitates with protein. When sarkosyl is added at the end of the *in vitro* incubation, most of the adenovirus *Sma*I template and pBR313 vector DNAs remain in the supernatant (>90%); the pellet contains only a minor portion of the DNA in the reaction (<10%).

To demonstrate that sarkosyl does not disrupt preinitiated RNA polymerase II complexes, reaction mixtures were preincubated for 20 min in the

presence of ATP, CTP, and UTP (the first 10 nucleotides of the specific transcript are 5' ACUCUCUCC, ref. 15), and for an additional 60 min in the presence of $\alpha^{32}\text{P}$ -GTP (Fig. 1, lane 1) to allow elongation to occur. In addition to labeled GTP, α -amanitin (lane 2), EDTA (lane 3), EDTA and sarkosyl (lane 4), or sarkosyl (lane 5) were included. α -Amanitin did not allow RNA polymerase II-dependent transcription, whether it was added before (with all four rNTPs) (results not shown) or after the preincubation period (lane 2). Sarkosyl added before the preincubation abolished RNA synthesis (10, 11; results not shown). In contrast, if the initiation complex is preformed, elongation proceeds efficiently in the presence of sarkosyl (lane 5) but seemingly stops in the presence of EDTA (lanes 3 and 4). EDTA also prevents initiation when added before preincubation by chelating the Mg^{2+} . When GTP is omitted, RNA synthesis cannot be detected (results not shown). The autoradiogram of lane 1 is greatly overexposed and represents multiple rounds of transcription as compared with the single round of elongation observed in the presence of sarkosyl (lane 5), where further initiations were blocked by sarkosyl.

Having established that sarkosyl allows elongation but not initiation in this transcription system, we next approached the problem of separating the transcription complexes from other cellular proteins. The procedure for the separation of ternary complexes involves sarkosyl-agarose gels in Tris-acetate-EDTA buffer. The existence of the ternary complexes was monitored by the comigration of $\alpha^{32}\text{P}$ -rNTPs with the respective DNA templates. In this way only oligonucleotides bound to the DNA by proteins of the ternary transcription complex will be sarkosyl-resistant and therefore we expect the radiation observed to be specific for a transcription initiation event. Both protein and DNA are unlabeled. Sarkosyl concentrations between 0 and 0.5% were used to determine the optimum for the separation of ternary complexes in agarose gels (Fig. 2). In Fig. 2 we compared the left lanes containing the transcription complexes with the right lanes containing purified labeled DNA, for each sarkosyl concentration indicated. The amount of precipitated material in the loading well increases with decreasing sarkosyl concentrations; at less than 0.125% sarkosyl, none of the radioactive nucleotides associated with the template (in incubations using ATP, $\alpha^{32}\text{P}$ -CTP, and UTP) migrates. From 0.125% to 0.5% sarkosyl, there appears to be little difference in the amount of radioactive nucleotides associated with the DNA bands. Nucleotides become associated both with the eukaryotic viral DNA and with the plasmid pBR313 vector,

Fig. 2. The effect of different sarkosyl concentrations on the migration of transcription complexes. Transcription complexes (20 μ l) were labeled after incubation with ATP, UTP, and α - 32 P-CTP. A series of parallel 2% agarose gels was prepared, each one supplemented with the indicated concentrations of sarkosyl, and samples (20 μ l) containing the same concentration of sarkosyl and 10 mM EDTA were loaded into their respective wells. The sarkosyl concentrations are indicated above the lanes. To the right of each sample, end-labeled SmaI cut template DNA was run with the same sarkosyl concentration as the sample to account for the differences in migration in the presence of detergent and to detect differences in the migration of ternary complexes versus naked DNA. Arrows on the right denote the migration of pBR313 and SmaI-F-labeled DNA fragments.

which are clearly separated. Sarkosyl is not included in the buffer chamber, as this seems to cause a smearing effect. Clear separation between transcription complexes and free forms of DNA is not achieved in these low resolution gels. Differences in sarkosyl concentration, which make the gels increasingly soft as the detergent concentration increases, also cause a difference in the migration of the naked DNA markers. The actual fate of the DNA can also be followed by staining the gel with ethidium bromide (1 μ g/ml). Addition of sarkosyl to greater than 0.5% does not change the migration rate of the DNAs, nor is there a decrease in the amount of radioactive nucleotides associated with the complexes (results not shown).

The formation of the complex is dependent on the incubation conditions. If the concentration of extract or DNA is either above or below

Fig. 3. A. The temperature dependency of ternary transcription complex formation. Transcription complexes were formed (in 20 μ l) using α - 32 P-CTP as precursor at a final concentration of 20 μ M. Lane 1 represents a reaction mixture incubated at 0°C for 20 min. Lane 2 represents a reaction incubated at 20°C for 20 min. Lane 3 represents a reaction incubated at 30°C for 20 min. Lane 4 represents a reaction incubated at 37°C for 20 min. Lane 5 represents a reaction incubated at 56°C for 20 min. After the 20-min incubation period, all samples were treated with EDTA and sarkosyl, and analyzed by electrophoresis on 2% agarose - 0.25% sarkosyl gels. The autoradiogram of the dried gel is shown. The upper arrow indicates the migration of pBR313 DNA and the lower arrow is *Sma*I F DNA. B. The *Sma*I-F bands of the dried agarose gel were excised, counted (Cerenkov), and plotted as a percent of the optimal value (30°C, 1,258 cpm).

the optimum for runoff transcription of this gene, the amount of radioactivity associated with the complex decreases in parallel. When other parameters such as the ion and/or metal concentrations vary above or below the runoff transcription optimum, the amount of radioactivity associated with the complexes decreases accordingly.

The temperature dependency for the formation of transcription complexes is shown in Fig. 3. Lane 1 depicts a reaction incubated at 0°C for 20 min, the time during which at least one round of transcription is completed at 30°C. No binding of nucleotides to the transcription complex is observed when incubations are carried out at 0°C and it is possible that transcription complexes do not even form. Lanes 2, 3, 4, and 5 depict gel analysis of ternary complexes formed at 20°C, 30°C, 37°C, and 56°C, respectively. Again, radioactive nucleotides associated with both vector (pBR) DNA and Ad2 DNA (*Sma*I-F) (from top to bottom of the figure). The quantitative analysis of radioactive nucleotides associated with the

Fig. 4. Functional analysis of the transcription complexes. The 5'-end sequence of the adenovirus major late promoter transcripts (15) is indicated at the top. Incubations were performed in 20 μ l reaction volumes and incubated at 30°C for 20 min. Sarkosyl was added to 0.25% and EDTA to 10 mM and the reactions were electrophoresed in a 2% agarose - 0.25% sarkosyl gel. In lane 1 is a reaction in the absence of DNA template. Lane 2 represents a reaction with ATP and labeled α^{32} P-UTP; lane 3, with ATP and α^{32} P-CTP; lane 4, with UTP and α^{32} P-CTP; lane 5, ATP, UTP and α^{32} P-CTP; lane 6 is identical to lane 5 except for the addition of 1 μ g/ml of α -amanitin; lane 7 contains all four rNTPs with α^{32} P-CTP. In the reaction in lane 8, EDTA and sarkosyl were added prior to the addition of the cell extract. The open arrow indicates the position of the ethidium bromide-stained adenovirus SmaI-F restriction enzyme fragment. Electrophoresis was from right to left.

specific template is summarized in panel B. The optimum at 30°C coincides with the temperature optimum for transcription (1, 2). Thus, the temperature parameter that affects specific in vitro transcription also affects complex formation.

Having established that the optimal incubation conditions for analysis of transcription complexes coincide with those required for the synthesis of runoff RNAs, we investigated the specificity of transcription complex formation. The sequence for transcription initiation on the SmaI-F fragment promoter is indicated at the top of Fig. 4. In Fig. 4, lane 1, the DNA-dependence of the system is demonstrated since omission of DNA from the reaction results in the absence of radioactive nucleotides in the transcription complex area of the gel. Omitting all rNTPs does not alter the migration of the DNA (not shown). If the ATP is unlabeled and CTP is

the radionucleotide, the amount of labeling associated with the DNA is extensive (Fig. 4, lane 3). When CTP is omitted, but ATP and ^{32}P -UTP are included (lane 2), the vector DNA region is labeled but the SmaI-F DNA region is not, demonstrating a correlation with the specific template sequence. No labeled complexes are seen with CTP and UTP (where either or both are radioactive) (Fig. 4, lane 4), indicating that the labeling is completely ATP and/or sequence-dependent and that the initiations observed are at the specified major late promoter region. Transcription complex formation using three instead of two nucleotides (cf. lanes 3 and 5) demonstrates some increase in the amount of incorporated radionucleotide in the A + C + U reaction ($\alpha^{32}\text{P}$ -CTP in both reactions). The increased radioactivity is due to possible formation on an undecanucleotide when adding A + C + U, whereas A + C results only in a dimer being formed except for the endogenous UTP in the extract and the contaminating UTP in the commercial ATP and CTP, which would allow further elongation. Addition of α -amanitin (1 $\mu\text{g/ml}$) and ATP, UTP, and ^{32}P -CTP before the extract is added does not inhibit complex formation (Fig. 4, lane 6), which is not unexpected since α -amanitin inhibits elongation of RNA polymerase II transcripts (16) after the formation of the first dinucleotide bond (17). Note that the levels of radiation associated with SmaI-F DNA are decreased somewhat since, only the dimer should be synthesized by RNA polymerase II instead of the possible undecanucleotide. Lane 7 shows the results of ternary complex formation under the conditions leading to formation of runoff transcripts, i.e., in the presence of all four rNTPs. The amount of complex formed is not increased, because longer RNAs (runoff transcripts) are probably not retained in the complex. EDTA and sarkosyl added prior to the cell extract prevent complex formation (Fig. 4, lane 8). We have not been able to isolate similar ternary complexes formed by RNA polymerase I on human rDNA or with RNA polymerase III on adenovirus VA DNA when we have challenged with the appropriate nucleotide combinations, possibly because the complexes are not resistant to sarkosyl.

To eliminate the possibility that nonspecific binding to the ends of the DNA occurred, we used supercoiled DNA in the assay, cutting the DNA after complex formation with any of several restriction enzymes. Restriction of this DNA is inefficient because proteins binding to the DNA during incubation block restriction recognition sequences (see also refs. 18 and 19). Adding the restriction enzyme after the sarkosyl renders the enzyme inactive. Since RNA polymerase II is known to initiate at

Fig. 5. Comparison of β - γ -imido derivatives of CTP and UTP in transcription complex formation. Assays were as in Materials and Methods, where the assay in lane 1 contains α - ^{32}P -CTP (5 μCi , 20 μM , $^*\text{C}$), UTP (300 μM), and ATP (500 μM); lane 2 assay contains the same as lane 1 but UMP-PNP (300 μM , Ui) was added instead of UTP; lane 3 assay contains α - ^{32}P -UTP (5 μCi , 20 μM , $^*\text{U}$), CMP-PNP (300 μM , Ci), and ATP (500 μM); and lane 4 assay contains the same as lane 3 except that CMP-PNP was replaced by CTP (300 μM).

single-stranded regions and at the ends of double-stranded DNA templates, linear ^{32}P -DNA templates (5'-termini labeled) and ATP, CTP, and UTP were incubated with the extract for 20 min at 30°C, and treated with calf intestinal phosphatase. The ^{32}P was readily removed, indicating that RNA polymerase was not binding to the ends of the DNA, and thereby protecting the label from the action of the phosphatase. The possibility that initiation and elongation along the termini of the DNA molecule occurred is not completely ruled out but seems unlikely since the sequence recognized by the SmaI blunt cutting restriction enzyme is 5'CCC $^+$ GGG3'. Thus,

GGG CCC

transcripts initiated at the ends should contain G residues. However, GTP is either omitted from the reaction mix, is unlabeled, or is substituted by the chain terminator 3'-O-methyl-GTP.

We observe a decreased amount of specifically labeled (SmaF) transcription complexes using ^{32}P -UTP with ATP and CTP. Substituting the imidotriphosphate CMP-PNP for CTP results in increased labeling of the ternary complexes with ^{32}P -UTP (Fig. 5, cf. lanes 3 and 4), to the same levels as with labeled CTP when cold UTP is used (Fig. 5, lane 1). The use of UMP-PNP, however, does not affect the level of CTP labeling of the ternary complexes (Fig. 5, lane 2). This could be due to contaminants in the commercial sources of unlabeled CTP since reducing the CTP concentrations partially reduced this difference. However CTP from different manufacturers did not alleviate this problem. The effect of the CMP-PNP might also be related to the slower rate of elongation detected with the imidotriphosphates (6) and stabilization of the ternary complex after formation of the

Fig. 6. Analysis of the oligoribonucleotide associated with ternary transcription complexes. Reactions (250 to 500 μ l) were incubated for 20 min at 30°C and then EDTA (10 mM) and sarkosyl (to 0.25%) were added. After electrophoresis on sarkosyl (0.25%)-agarose gels, as described in Materials and Methods, the oligonucleotides were extracted from the corresponding excised bands as visualized by ethidium bromide fluorescence under a UV light. The purified oligonucleotides were analyzed on a 18% polyacrylamide-urea gel (Fig. 7, panel A). The lanes correspond to oligonucleotides from the pBR313 vector (lanes a, c, g, i, and k), the *Sma*I adenovirus DNA (lanes b, e, h, j, and l), the 18S ribosomal RNA (lane d) and the bromophenol blue dye (lane f). To avoid transcription, either GTP was omitted (lanes a and b), the RNA was treated with ribonuclease T1 (which cleaves after G, lanes c through f), or the RNA chain terminator 3'-O-methyl GTP (lanes g through l) was used. Lanes a through h contain α^{32} P-CTP-labeled oligoribonucleotides and i through l α^{32} P-UTP-labeled oligoribonucleotides. Lanes i and j contained CTP, whereas lanes k and l contained 400 μ M β - γ -imido-cytidine-triphosphate. The markers are an end-labeled oligodeoxyribonucleotide 11 bases long (lane m) and a ladder separated by two nucleotide steps containing RNA synthesized in vitro with

E. coli RNA polymerase and poly dAT in the presence of 3'-me-ATP (35, lane n). The synthetic undecadeoxyribonucleotide, synthesized by the phosphoramidite method and end-labeled with polynucleotide kinase, was a generous gift from Dr. M. Concino (38). Nearest neighbor analysis of the undecanucleotide isolated from Ad2 transcription complexes is shown in panel B. The acrylamide gel slice from the undecanucleotide region from a reaction similar to lane h, Fig. 7A, was crushed with a glass rod and incubated in 8 ml H₂O at 37°C, also containing RNases T2 and A. The solubilized radioactive nucleotides were concentrated by binding to DEAE-cellulose and eluted with triethylamine carbonate (30%, pH 8-9). After lyophilization the radioactive material was resuspended in 5-10 μ l H₂O, further digested with RNases T2 and A and applied to a PEI plate that was then developed with 1.2 M LiCl. Markers for rNMPs are from E. coli RNA synthesized in vitro using calf thymus DNA template and T2-digested.

first dinucleotide bond. We have no explanation for why CTP inhibits ternary complex formation while not inhibiting runoff transcription nor why the imido analog CMP-PNP can replace CTP and thus eliminate the observed inhibition. This phenomenon is under further investigation using different promoters.

Addition of SDS to the reaction mixture after a 20-min incubation (final concentration 0.1%) completely removes associated α ³²P-labeled nucleotides from the DNA bands in the agarose gel, demonstrating that the labeled nucleotides are not covalently associated with the DNA template. Additional evidence against covalent association comes from an SDS-polyacrylamide gel that revealed no protein bands containing ³²P label derived from the α -position of the precursor ribonucleotides (not shown).

The SmaI-F region of an agarose gel was next analyzed for the presence of the predicted oligonucleotide. RNAs from the bands containing the pBR313 vector and the SmaF template regions were extracted after melting the gel and concentrated on a DEAE-cellulose column. These fractions were analyzed on 18% polyacrylamide gels as shown in Fig. 6A. To limit the length of the RNA and maintain a high level of radioactivity in the complex, we either omitted GTP (lanes a and b), digested the RNA extracted from the complexes with T1 ribonuclease (lanes c through f), or incubated with the RNA chain terminator 3'-O-methyl-GTP (lanes g through l). Under these particular conditions, an undecanucleotide is found associated with the SmaF template DNA but not with the pBR313 vector (compare lanes b, e, h, j, and l with all the others). This undecanucleotide would be predicted based on the sequence of the major late promoter transcript. One more nucleotide would be present at the 5'-end if capping were occurring at this stage. We were able to obtain an undecanucleotide independent of whether

the label was $\alpha^{32}\text{P}$ -UTP or $\alpha^{32}\text{P}$ -CTP and whether transcripts were made in the presence of 3'me-GTP (lanes h, j, and l) or in the presence of GTP and T1 RNase (lane e). The double band seen in lane b might reflect the presence of low levels of GTP in the extract, resulting in penta-decanucleotide (upper band, if the G at position 11 is incorporated) and decanucleotide (lower band, if it is not incorporated) synthesis. The amount of the undecanucleotide is increased when CMP-PNP and labeled UTP were used (compare lanes j and l in Fig. 6A). This undecanucleotide association is specific because agarose gel slices containing 18S rRNA (lane d) or the bromophenol blue marker (lane f) did not reveal the presence of similar size oligonucleotides. Oligonucleotides smaller than 8n and eluted from the complex would be lost during the preparation procedure at the ethanol precipitation step.

We performed nearest neighbor analysis of the undecanucleotide transcript (from a reaction such as the one in Fig. 6, lane h) to confirm that this oligonucleotide is derived from the adenovirus major late promoter (Fig. 6B). The undecanucleotide was excised from the 18% polyacrylamide gel, eluted, concentrated again on DEAE-cellulose, and digested with RNases T2 and A (Fig. 6B). Most of the ^{32}P is transferred from the parent CMP residue to the 5'-neighbor, UMP. A small amount of the label (about one-third of the UMP level) transferred to a 5'-neighbor, CMP, as would be expected. Near the origin we detect a spot that comigrates with pppAp marker, the expected 5'-end nucleotide tetrphosphate if accurate initiation and no capping of the undecanucleotide occurs. Some larger RNase-resistant oligonucleotides due to incomplete digestion remain at the origin. Other spots that possibly contain capped and/or methylated nucleotides, with intermediate mobilities, have not been identified. Thus, the undecanucleotide associated with the ternary complexes containing the adenovirus major late promoter contains nucleotide sequences compatible with faithful initiation in vitro.

Since the cell lysate transcription system contains most cellular proteins, we fractionated a nuclear extract (20) by chromatography on phosphocellulose (3). As shown in Fig. 7, the whole cell extract (lane 1) forms transcription complexes more readily than a nuclear extract (lane 3), prepared as described (20). This particular nuclear extract has about one-third the protein content of the whole cell extract, and the amount of Ad2 runoff is about one-half that of the whole cell extract. The ability of this particular nuclear extract to form tenary complexes is also reduced

Fig. 7. Comparison of whole cell transcription lysate with a fractionated nuclear extract. Transcription complexes were formed under standard assay conditions (20 μ l) except that GTP was omitted and CTP was the labeled nucleotide. Lanes 1, 3, 5-9 are 16-h exposures to demonstrate the normal levels of radioactivity associated with the DNA bands, whereas lanes 2, 4, and 10-14 are the corresponding 2-week exposures. Lanes 1 and 2 are with the whole cell extract (WCE), lanes 3 and 4 are with the nuclear extract (NE). Lanes 5-14 are the fractions obtained after phosphocellulose chromatography (PC) of the nuclear extract. Fraction A is the flow-through protein; fraction B is the 0.3 M KCl eluate; fraction C is the 0.5 M KCl eluate; and fraction D is the 1 M KCl eluate. RNA polymerase II (lane 16) was purified from the whole cell extract by chromatography through heparin-agarose, DEAE-Sephadex and phosphocellulose, as described previously (6) and exposed for 2 weeks.

about ten-fold. No such differences were observed in two other nuclear extracts. This nuclear extract was applied to a phosphocellulose column. The unbound protein (A), and protein fractions step-eluted with 0.3 M, 0.5 M, and 1 M KCl (B, C, and D, respectively) (3) were tested for their ability to transcribe and form ternary complexes. Fractions A + C + D allowed specific runoff transcription to occur at low levels without the addition of exogenous RNA polymerase II compared to the nuclear extract

(ref. 3; not shown). Lanes 5 through 9 represent complexes formed by the fractions eluted from the phosphocellulose column. Although a short exposure (16 h) demonstrates no complex formation, as monitored by radioactivity associated with the DNA bands (lanes 5 through 9), and only a small amount with the SmaI-F band and the nuclear extract (lane 3), a longer exposure (14 days) reveals extensive labeling of the DNA bands by the nuclear extract (lane 4). Lane 2, WCE, is greatly overexposed. Lanes 10 through 14 are longer exposures of lanes 5 through 9.

Whereas fractions A and D alone are inactive in labeling the SmaI-F band (lanes 11 and 14), fractions B and C label both bands well (lanes 12 and 13). Thus, whereas we observe that fractions A, C, and D are required for RNA synthesis, and this can sometimes be enhanced by addition of purified RNA polymerase II as observed by others (3), only fractions B or C are sufficient for transcription complex formation, at a low level. Since fraction B contains most of the RNA polymerase II (3, 37), complex formation here could be due to the RNA polymerase and small amounts of fraction C initiation factors contained in fraction B. The combination of fractions C and D yields a greater level of ternary complex formation than fraction C alone (lane 10). Purified RNA polymerase II does not form sarkosyl-resistant transcription complexes (lane 16) while the cruder enzyme contained in fraction B does (lane 13). Thus, in addition to RNA polymerase II, other factors are required for ternary complex formation, but not as many as are required for in vitro runoff transcription. The fractions described here are heterogeneous mixtures of many proteins and their identification awaits further chromatographic purification.

The amount of labeled triphosphate that incorporated into ternary complexes seemed to be much greater than the amount predicted from an analysis of runoff RNAs. To investigate this further, reactions with different extracts were analyzed quantitatively for production of the 536 nucleotide runoff RNA and for the amount of nucleotide-labeled specific ternary complex formed. To limit the size of the molecules in ternary complexes and to assure uniformity in the measurements, all reactions were done in the presence of A, C, U and 3'-O-meGTP. Thus, ternary complexes should all contain the undecanucleotide. The bands corresponding to the Ad2 SmaI-F transcription complexes or the 536-nucleotide RNA transcript were excised from dried gels and counted in scintillation fluid. The fmoles of initiations for each were calculated from the specific activity of the isotope and the number of specific nucleotide residues per oligo-

Table I

Extract	SmaI-F initiation events in transcription complex (fmoles)			SmaI-F RNA runoff molecules from in vitro transcription (fmoles)			Ratio of initiation events transcript (A*UGm/A*UG)
	A*UGm	ACU*Gm	C/U	A*UG	AC*UG	C/U	
A	116	17	6.8	1.4	0.4	3.5	83
B	55	14	3.9	1.6	1.4	1.1	34
C	44	9	4.9	1.0	0.6	1.7	44
D	103	14	7.4	1.8	1.1	1.6	57
E	60	10	6.0	1.5	1.4	1.1	40
F	83	11	7.5	0.9	1.2	0.8	92
G	109	20	5.5	3.0	0.9	3.3	36
H	60	17	3.5	1.0	0.6	1.7	60
I	31	9.5	3.3	2.4	1.3	1.8	13
J	1.8	ND		0.1	0.1	1.0	18
K	5.1	ND		0.23	0.22	1.0	22
L	17	ND		0.33	0.32	1.0	52
mean $\pm$ SD			5.4 $\pm$ 1.6			1.6 $\pm$ 0.9	46 $\pm$ 24

Transcription complex and runoff reactions in a volume of 20 μ l contained either 3'-O-methyl-GTP (Gm) or GTP, respectively. The radioactive precursors were either 32 P-CTP (*C) or 32 P-UTP (*U) at 2.5×10^4 cpm/pmole. Experiments A through L represent different extracts assayed at the same time (rows A through I) or at different times (J through L). Transcription complex reactions were stopped after 20 min by adding EDTA and sarkosyl, and electrophoresed through agarose-sarkosyl gels. Transcription reactions were allowed to proceed for 60 min and then processed to isolate RNA. Transcripts were analyzed in 2% agarose-HgOHCH₃ gels.

nucleotide (assuming this is the 5'-undecaribonucleotide and contains 5 C's and 4 U's) or transcript (the 536n RNA runoff contains 141 C's and 119 U's, ref. 15). Thus, shorter oligonucleotides in the ternary complexes would result in an underestimation of the number of initiation events. The results of this analysis are summarized in Table I. The number of molecules of complex detected with C is five-fold larger than with U (see also

Fig. 6). Although we do not understand the basis for the preferential labeling using CTP, excess initiations were also detected with UTP (Table I). This pattern is also reflected in the undecanucleotide when labeled with UTP. No significant difference in the labeling with C or U was detected in the formation of the runoff RNA (Table I). A significant difference between the number of molecules which participate in complex formation and the number of runoff RNAs was detected. The average difference is 46-fold if we compare the CTP values, or 15-fold if we compare the UTP values. In either case, a large molar excess of transcription complex formation over runoff transcript can be detected.

In order to assure that this molar excess of initiation events calculated from the total radioactivity in the SmaI-F ternary complexes is not due to other RNA polymerase activity or other initiation sites, quantitation of the undecanucleotide specific for this RNA polymerase II promoter also revealed a 40-fold molar excess compared to 536n RNA runoff transcripts. Though the undecanucleotide constitutes a major portion of the RNA associated with the DNA band, it does not account for all of the radioactivity associated with the ternary complex. Depending upon the whole cell extract used, anywhere from 50 to 80% of the SmaI-F DNA-associated radiation corresponds to the undecanucleotide.

In a congeneric manner we have analyzed transcription complexes formed on other genes, where the initiation sequences are different from the Ad2 major late promoter. The Ad2 early 4 (E4) promoter region transcripts initiated with any one of 5 U residues or an A residue [UUUUUACACUG...; (15)], and murine leukemia virus long terminal repeat (LTR) gene transcript initiated with a G residue (GCGCCAGU...), adenovirus E1a (ACUCUUG...), E1b (ACAUCUG...), and pIX (AUCUG...) gene transcripts have all been analyzed for transcription complex formation. In all instances, 1) transcription complex formation is sequence-dependent (results not presented) as shown for Ad2 SmaI-F, and 2) ATP is required for initiation to occur although the initiating nucleotide is not always an A residue.

DISCUSSION

The results presented here demonstrate that eucaryotic transcription ternary complexes can be prepared in substantial quantities by gel filtration or analyzed qualitatively in agarose gels. Our analysis was based on the in vitro formation of stable initiation complexes; b) their monitoring by the use of ³²P-labeled nucleoside triphosphates and, c) the use of the

ionic detergent sarkosyl. Without sarkosyl, the DNA remains covered with protein and more than 90% of the DNA will precipitate from solution rather than enter the gel and separate by electrophoresis in agarose gels. The stability of the complexes in the presence of sarkosyl is expected since others have observed that this anionic detergent can solubilize enzyme-DNA complexes and allow RNA elongation to proceed in preformed viral DNA complexes (10) and purified nuclei systems (11). Our results are in agreement with the general observation that addition of sarkosyl prior to extract addition prevents initiation of RNA synthesis, whereas the addition of sarkosyl after pre-incubation allows elongation to occur, but not re-initiation. Recently Sindha et al. (21) investigated nucleoprotein structures formed by in vitro transcription systems and observed that these structures are insoluble. We have overcome this problem by using sarkosyl to remove most of these DNA binding proteins and to solubilize transcription complexes. The ability to separate transcription complexes from the other proteins in the extract enables us to study the functional and structural elements of the complex, as well as the requirements for assembly and stability. The indistinguishable differences in migration between ternary complexes and naked DNA suggest that very few proteins are needed to retain labeled oligonucleotides in association with the complex, at least after initiation.

The use of agarose gel electrophoresis for monitoring transcription complexes has been documented previously (22, 23) for bacterial RNA polymerase and for pure eucaryotic RNA polymerase II (17, 24-27). However, pure RNA polymerase II does not selectively transcribe eucaryotic genes. In the yeast system formation of ternary complexes with partially purified RNA polymerase II was detected on both the eucaryotic insert and the vector (26, 27). We have detected the formation of sarkosyl-resistant ternary complexes on the pBR313 and on the adenovirus-specific insert. Weak RNA polymerase II promoters have been identified on pBR322, portions of which are derived from pBR313 (28, 29). Initiation of in vitro transcription from TATAA boxes located at positions 4321 and 2612 in pBR322 was demonstrated (29). Other TATAA boxes located at positions 97, 443, 3233, 3271 and 3947 also function as weak RNA polymerase II promoter sites (29). These putative promoters account for only a part of the sequence in the vector pBR313, which is four times larger than the adenovirus sequence. Thus, the ternary complexes formed on pBR313 would be due to the presence of these eucaryotic-like sequences, in amounts proportional to the number

of putative promoters in this vector.

The oligonucleotides synthesized during restricted nucleotide challenges (ACU vs. AC vs. AU) as well as the undecanucleotide synthesis experiments indicate that the adenovirus major late promoter control region to which the RNA polymerase II and factors bind is indeed the main region of initiation, and that a specific initiation event accounts for most of the radioactivity associated with the viral DNA template. Further evidence of specificity in the initiation events is provided by our ability to isolate the predicted undecanucleotide RNA from the specific SmaF DNA in the agarose gel, but not from the vector pBR313 band. The nearest neighbor analysis of the undecanucleotide is compatible with the 5' undecanucleotide of the major late promoter (1, 2). The ternary complexes formed with the whole cell extract as a source of transcriptional activity seems to be specific for RNA polymerase II because: a) all parameters which affect runoff transcription, like salt concentration, DNA concentration, extract concentration and incubation temperature have coincidental optimas for the formation of ternary complexes; b) α -amanitin, a highly specific RNA polymerase II inhibitor affects the formation of ternary complexes both when measured by the agarose gel assay (Fig. 4) and when the specific undecanucleotide is analyzed (not shown); c) a subset of the fractions required for in vitro transcription is required for ternary complex formation and d) when elongation by RNA polymerase II is unrestricted, i.e., in the presence of all four nucleotide triphosphates (none of them 3'-O-methylated) no undecanucleotide can be detected.

We have also tried isolating ternary complexes by using gel filtration in the presence of sarkosyl. While we can demonstrate co-chromatography of ^{32}P -DNA (3 μg), ^3H -UTP (less than 0.1% of the input), and ^{35}S -methionine-labeled protein (0.1% of the input, 3 μg) the expected undecanucleotide from the Ad2 major late promoter could not be detected in association with the complex purified by Sepharose 2B chromatography. However, an undecanucleotide was present in large amounts in the leading edge of the included column fractions, suggesting dissociation of the oligonucleotide during chromatography, probably due to dilution. Hence, gel filtration in the presence of sarkosyl of RNA polymerase II ternary complexes results in dissociation of the synthesized oligonucleotide and at best leaves the binary complex intact.

Ternary complexes appear to be fragile as they are disrupted upon dilution and manipulation. Mechanical mixing seems to prevent complex

formation and we therefore gently mix the reaction components by hand. It has been suggested that the gel matrix may help to stabilize bacterial DNA:protein complexes (36). A 2- to 10-fold dilution of the reaction mixture eliminates transcription (Ackerman, unpublished observations). Electrophoresis on the gel rather than by Sepharose 2B chromatography helps to maintain the ternary complexes in a concentrated form.

In an attempt to separate proteins required for transcription complex formation from proteins required for runoff transcription we prepared a nuclear extract (20). This extract has fewer proteins than the whole cell extract. Fractionation of this nuclear extract by phosphocellulose chromatography resulted in four salt elution steps (3). Salt elution steps C + D (0.5 M and 1 M KCl) of the phosphocellulose column, and non-bound protein (A) are required for runoff transcription. In contrast fraction B (95% of the RNA polymerase II [37]) and fraction C (2% of the RNA polymerase II [37]) appear to be sufficient for ternary complex formation, in the absence of exogenous RNA polymerase II. Recently, Davison et al. (37) presented results which support our data on ternary complex formation. Using the S100 transcription system (1) fractionated on phosphocellulose (3) they observed that formation of preinitiation complexes (binary complexes in the absence of nucleotides) requires phosphocellulose fractions C and D, while to obtain runoff transcripts they had to add to this RNA polymerase II and fraction A. This agrees with our results which indicate that fraction B or C alone can form ternary complexes and fraction D helps to enhance ternary complex formation in the absence of exogenous RNA polymerase II. Thus, ternary complexes contain DNA; specific nucleotides related to faithful initiation on the eucaryotic template; and some of the machinery required for specific RNA polymerase II initiation directly involved in complex formation.

In bacterial systems, the numerous aspects of RNA polymerase-DNA interactions have been described (30-32). The *E. coli* enzyme apparently binds to the DNA template promoter region, without requiring the presence of rNTPs, to form a closed complex and then an open complex. The initiation of RNA synthesis occurs only from the open complex and there are normally many non-productive interactions for every productive initiation event (30, 32). Though *E. coli* RNA polymerase forms only the closed complex at 0°C, it is not known whether the eucaryotic RNA polymerase binds specifically to DNA at 0°C. Our results indicate that there are no ternary (open) complexes formed at 0°C, but the RNA polymerase may still be bound

to the DNA in a closed binary complex similar to the bacterial model. At 30°C the eucaryotic RNA polymerase binds and initiates RNA synthesis, as does the bacterial system (Fig. 3). Thus, enzymic reactions and/or melting of the DNA double helix are required for formation of the ternary complex. We have previously described a requirement for hydrolysis of the β - γ bond of ATP for ternary complex formation (6). Similarly, there is an ATP requirement for formation of ternary complexes, even when this nucleotide is not a part of the initiation sequence of the gene (results not shown).

In our investigation of eucaryotic transcription complexes, we observed that the levels of radionucleotide associated with the SmaI-F DNA in Fig. 4 were substantially greater than expected compared to the amount of RNA synthesized from the major late promoter in a runoff assay of 60 min. A large molar excess (almost 50-fold) of initiation events on the specific SmaF template does not appear to lead to the formation of the 536n runoff product. The ratio for non-productive:productive initiation events might represent an underestimate since some of the RNA may be released (Fig. 4, lane 7). Since synthesis of an undecanucleotide was assumed for the calculations in Table I, smaller oligonucleotides could be present. This has been minimized by the presence of non-limiting amounts of nucleotides and incubation in the presence of 3'-O-meGTP, so that mostly the undecanucleotide should accumulate. This molar excess does not represent incorrect initiations, since a molar excess of undecanucleotide (about 40-fold) was also detected in 18% polyacrylamide gels. The number of initiation events measured is approximately half the number of eukaryotic templates, so if only one event/template occurs half of the templates are occupied by initiating RNA polymerase II. Less than 2.5% of all of the in vitro initiations produce RNAs that can later be measured as runoff RNAs. One could speculate that these are abortive initiations which occur only in vitro; that the in vitro system is deficient in some factor(s) required for efficient elongation but not for initiation; or that a similar phenomenon occurs in vivo. The reasons for these abortive initiation phenomena still remain unclear but similar observations on the bacterial RNA polymerase (32-34) and for the mammalian in vitro system (35) have been reported. We have also shown that short promoter proximal RNAs in excess of the full sized runoff RNAs are synthesized under normal conditions (8). The relationship between these short RNAs and the initiation events measured here has not yet been clearly established. Alteration of the balance between the non-productive initiations and those that lead to the synthesis of full

size runoff RNAs could represent a significant regulatory mechanism.

Using the whole cell lysate transcription system, this novel transcription complex assay provides evidence that gene regulation can now be investigated at the initial sequence of events - selective binding of RNA polymerase to a gene control region and formation of the first nucleotide(s) bond(s) of the transcript.

ACKNOWLEDGEMENTS

S. A. was supported by 2T-32-CA-09171 and D. B. by 5T-32-CA-07922. This work was supported by grants AI-13231, CA-21124 and CA-10815 to R. W.

REFERENCES

1. Weil, P. A., Luse, D. S., Segall, J. and Roeder, R. G. (1979) *Cell* 18, 469-484.
2. Manley, J. L., Fire, A., Cano, A., Sharp, P. A. and Geyer, M. L. (1979) *Proc. Natl. Acad. Sci. USA* 77, 3855-3859.
3. Matsui, T., Segall, J., Weil, P. A. and Roeder, R. G. (1980) *J. Biol. Chem.* 255, 11992-11996.
4. Tsai, S. Y., Tsai, M.-J., Kops, L. E., Minghetti, P. P. and O'Malley, B. W. (1981) *J. Biol. Chem.* 256, 13055-13059.
5. Dynan, W. S. and Tjian, R. (1983) *Cell* 32, 669-680.
6. Bunick, D., Zandomeni, R., Ackerman, S. and Weinmann, R. (1982) *Cell* 29, 877-886.
7. Zandomeni, R., Mittleman, B., Bunick, D., Ackerman, S. and Weinmann, R. (1982) *Proc. Natl. Acad. Sci. USA* 79, 3167-3170.
8. Zandomeni, R., Bunick, D., Ackerman, S., Mittleman, B. and Weinmann, R. (1983) *J. Mol. Biol.* (in press).
9. Gamper, H. B. and Hearst, J. E. (1982) *Cell* 29, 81-90.
10. Shmookler, R. J., Buss, J. and Green, M. H. (1974) *Virology* 57, 122-127.
11. Gariglio, P., Buss, J. and Green, M. H. (1974) *FEBS Letters* 44, 330-333.
12. Birnboim, H. C. and Doly, J. (1979) *Nucl. Acids Res.* 7, 1513-1522.
13. Maxam, A. M. and Gilbert, W. (1980) In: *Methods in Enzymology*, Grossman, L. and Moldave, K. Eds., Vol. 65, pp. 499-560, Academic Press, New York.
14. Randerath, K. and Randerath, E. (1967) In: *Methods in Enzymology*, Grossman, L. and Moldave, K. Eds., Vol. XII, part A, pp. 323-347, Academic Press, New York.
15. Baker, C. and Ziff, E. B. (1981) *J. Mol. Biol.* 149, 189-221.
16. Cochet-Meilhac, M. and Chambon, P. (1974) *Biochim. Biophys. Acta* 353, 160-184.
17. Vaisius, A. C. and Wieland, T. (1982) *Biochemistry* 21, 3097-3101.
18. Beard, P. and Nyfeler, K. (1982) *EMBO* 1, 9-14.
19. Beard, P. (1978) *Cell* 15, 955-967.
20. Luse, D. S., Haynes, J. R., Van Leeuwen, D., Schon, E. A., Cleary, M. L., Shapiro, S. G., Lingrel, J. B. and Roeder, R. G. (1981) *Nucl. Acids Res.* 9, 4339-4354.

21. Sindha, S. N., Hellwig, R. J., Allison, D. P. and Niyogi, S. K. (1982) *Nucl. Acids Res.* 10, 5532-5552.
22. Chelm, B. K. and Geiduschek, E. P. (1979) *Nucl. Acids Res.* 7, 1851-1867.
23. Garner, M. M. and Revzin, A. (1981) *Nucl. Acids Res.* 9, 3047-3060.
24. Crepin, M., Traidou, P., deLong, J.-C. and Gros, F. (1981) *Eur. J. Biochem.* 118, 371-377.
25. Dynan, W. S. and Burgess, R. R. (1981) *J. Biol. Chem.* 256, 5866-5873.
26. Ballario, P., Buongiorno-Nardelli, M., Carnevali, F., DiMauro, E. and Pedone, F. (1981) *Nucl. Acids Res.* 9, 3959-3978.
27. Carnevali, F., Caserta, M. and DiMauro, E. (1982) *Nucl. Acids Res.* 10, 3195-3209.
28. Bolivar, F., Rodriguez, R. L., Betlach, M. C. and Boyer, H. W. (1977) *Gene* 2, 75-93.
29. Sassone-Corsi, P., Corden, J., Kédinger, C. and Chambon, P. (1981) *Nucl. Acids Res.* 9, 3941-3958.
30. Kadesch, T. R., Rosenberg, S. and Chamberlin, M. J. (1982) *J. Mol. Biol.* 155, 1-29.
31. Rosenberg, S., Kadesch, T. R. and Chamberlin, M. J. (1982) *J. Mol. Biol.* 155, 31-51.
32. Hansen, U. M. and McClure, W. R. (1980) *J. Biol. Chem.* 255, 9556-9563; 255, 9564-9570.
33. Melancon, P., Burgess, R. R. and Record, M. T., Jr. (1982) *Biochemistry* 21, 4318-4331.
34. Gralla, J. D., Carpousis, A. J. and Stefano, J. E. (1980) *Biochemistry* 19, 5864-5869.
35. Lescure, B., Bennetzen, J. and Sentenac, A. (1981) *J. Biol. Chem.* 256, 11018-11024.
36. Fried, M. and Crothers, D. M. (1981) *Nucl. Acids Res.* 9, 6505-6525.
37. Davison, B. L., Egly, J.-M., Mulvihill, E. R. and Chambon, P. (1983) *Nature* 301, 680-686.
38. Concino, M., Goldman, R. A., Caruthers, M. and Weinmann, R. (1983) *J. Biol. Chem.* (in press).