Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase II Specific Initiation and Elongation Factors from Calf Thymus†

S. R. Spindler*

ABSTRACT: Two protein factors (termed SF-1 and SF-2) were previously purified from calf thymus which specifically stimulate the activity of RNA polymerase II on native DNA [Benson, R. H., Spindler, S. R., Hodo, H. G., & Blatti, S. P. (1978) *Biochemistry 17*, 1387]. The apparent association of several of the small subunits of purified RNA polymerase II with the purified factors suggests that these factors interact with the polymerase in vivo. We describe experiments here which test the effects of purified SF-1 and SF-2 on transcription by purified enzyme II in vitro. Elongation rate studies were performed which made use of the time- and temperature-dependent RNA polymerase II–DNA complex to synchronously initiate polymerase molecules in the absence and presence of SF-1 and SF-2. Form I SV40 DNA was used as the template because it is free of double- and single-stranded scissions, as well as single-stranded regions. The lengths of the RNAs synthesized after various time intervals during the linear phase of RNA synthesis were examined by zonal sedimentation in Me_2SO–sucrose gradients. The advantages in using this elongation rate assay over using the assays which employ γ-labeled nucleotide triphosphates or rifampicin derivatives are discussed. SF-1 had no detectable effect on the elongation rate of enzyme II, while enhancing the initial rate of RNA synthesis sevenfold. Further, nitrocellulose filter binding studies suggest that under these conditions SF-1 is not required for efficient DNA binding by RNA polymerase II. These findings suggest that SF-1 is an initiation factor. In contrast, SF-2 enhanced the elongation rate of enzyme II to the same degree as it stimulated enzyme activity, suggesting that SF-2 is an elongation factor. Taken together, our previous data cited above and those presented here suggest that two groups of subunits of RNA polymerase II dissociate from the rest of the enzyme during the early stages of polymerase purification. One group of polypeptides, SF-1, enhances the ability of the enzyme to initiate RNA synthesis; a second group of polypeptides, SF-2, enhances the elongation rate of the enzyme on native templates. The possibility must be considered that these factors, especially SF-1, are involved in the transcriptional specificity of polymerase II in a manner analogous to the effect of the σ subunit on the bacterial core RNA polymerase.

The cells of both higher and lower eucaryotes contain three structurally (Sklar et al., 1975; Valenzuela et al., 1976; Spindler et al., 1978a,b; D'Alessio et al., 1979) and functionally distinct classes of DNA-dependent RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) [for a review see Roeder (1976)]. RNA polymerase I transcribes rRNA precursor (Blatti et al., 1970; Reeder & Roeder, 1972), polymerase II synthesizes pre-mRNA (Blatti et al., 1970; Zylber & Penman, 1971), and polymerase III transcribes 5S ribosomal RNA as well as tRNA precursors (Weinmann & Roeder, 1974; Weil & Blatti, 1976).

Despite the demonstrated in vivo specificity of these enzymes, purified eucaryotic RNA polymerases have little if any specificity when transcribing deproteinized DNA templates [Roeder et al. (1970) and Roeder (1974), and see Chambon (1974)], suggesting the participation of ancillary factors in the initiation and/or termination specificity of these enzymes. Although in vitro studies employing *Escherichia coli* RNA polymerase seemed to implicate the acidic chromosomal proteins in the specificity of transcription (Axel et al., 1973; Barrett et al., 1974; Tsai et al., 1976), the validity of these experiments has been questioned in more recent studies (Zasloff & Felsenfeld, 1977a,b; Giesecke et al., 1977). In this regard, Wu (1978) has recently shown that the elements necessary for the specific transcription of certain regions of purified adenovirus DNA by RNA polymerase III are not chromatin components but rather soluble cellular components. This work suggests that transcription factors exist in eucaryotes which may be analogous to elements such as the σ factor (Burgess et al., 1969) and the other protein specificity factors which are found in procaryotes [see Losick & Pero (1976)].

Extracts of calf thymus contain a factor which specifically stimulates the transcription of double-stranded DNA templates by RNA polymerase II (Stein & Hausen, 1970). Recently, this stimulatory activity was purified and shown to be composed of structurally distinct protein elements, termed SF-1 and SF-2 (Benson et al., 1978). Stimulatory factor SF-1 is apparently a dimer composed of a 27 000 and a 36 000 or 38 000 molecular weight species. The 36 000 and 38 000 molecular weight species appear to be subunits of purified polymerase II. The second activity, SF-2, is due to two proteins, one of 19 000 and the other of 34 000 molecular weight. In addition to the components described above, each of the purified factors also contains small amounts of several of the other low molecular weight subunits of RNA polymerase II. The apparent association of these small subunits of RNA polymerase II with highly purified SF-1 and SF-2 suggests that these stimulatory factors physically associate with enzyme II in vivo. For this reason we undertook a study of the functional role of these factors in the RNA polymerase II reaction.

We have determined the elongation rate of the polymerase in the presence and absence of the stimulatory proteins. This rate was calculated from measurements of the lengths and quantities of the RNAs produced at various times during the first few minutes of linear synthesis. The data also provide

† From the Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, Texas 77030, and the Endocrine Research Division, University of California, San Francisco, San Francisco, California 94143. *Received January 16, 1979; revised manuscript received May 31, 1979.* This work was supported by National Institutes of Health Grant No. 5RON GM 19494.

* Present address: Endocrine Research Division, University of California, San Francisco, San Francisco, CA 94143.

0006-2960/79/0418-4042$01.00/0

an estimate of the number of polymerase molecules which initiate synthesis in the presence and absence of the factors. This method of investigating the role of eucaryotic transcription factors in the polymerization reaction avoids the complications which arise in the use of both γ-^{32}P-labeled nucleotide triphosphates (high levels of lable incorporation into protein and low levels of incorporation into RNA) and rifampicin derivative AF/05 (direct inactivation of the stimulatory factors).

Materials and Methods

Materials. All chemicals were reagent grade. [5′-^{3}H]UTP (>20 Ci/mmol) and [*methyl*-^{14}C]thymidine (>33 Ci/mmol) were purchased from New England Nuclear. Calf thymus DNA (grade I) and dithiothreitol were from Sigma Chemical Co. α-Amanitin (A grade) and antibiotics were obtained from Calbiochem. Dimethyl-d_6 sulfoxide (>99% deuterated) was purchased from J. T. Baker Chemical Co. Tissue culture media and fetal bovine serum were from Gibco.

Cells and Virus. The established cell line BSC-1 was grown in Dulbecco's modified Eagle's minimum essential medium[1] with 10% fetal bovine serum in the presence of ampicillin (25 μg/mL), penicillin (120 μg/mL), and streptomycin (35 μg/mL). Virus stocks were prepared from a plaque-purified stock of SV40 virus (strain 307L) by infection at a multiplicity of 0.01 pfu per cell. Original virus stocks were kindly provided by Dr. D. R. Dubbs.

Solutions. All solutions were prepared by using reverse-osmosis purified water. Dithiothreitol, when present, was added immediately before use. Solutions containing ammonium sulfate were adjusted to the desired concentration by the addition of 4 M ammonium sulfate, titrated to pH 7.9 at 20 °C with NH_4OH.

SV40 Form I DNA Preparation. BSC-1 cells were infected at a multiplicity of 40 pfu per cell and incubated with DMEM and 2% fetal bovine serum containing 4 μM thymidine and 25 nCi/mL ^{14}C-labeled thymidine. After 70 h SV40 DNA[2] was released by the method of Hirt (1967), extracted 3 times with phenol and 2 times with chloroform–isoamyl alcohol (24:1), and centrifuged to equilibrium in CsCl (density 1.60 g/mL)–ethidium bromide (300 μg/mL). Ethidium bromide was removed from the sample by four extractions with isoamyl alcohol. After dialysis and ethanol precipitation, the DNA was sedimented in a 5–20% sucrose gradient containing 0.5 M NaCl, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA. The form I peak was exhaustively dialyzed against 25 mM $(NH_4)_2SO_4$, 10 mM Tris-HCl (pH 7.9), and 1 mM EDTA and stored at 4 °C. The specific activity of the DNA was (3–4) × 10^6 cpm/mg.

Protein and DNA Determinations. Protein concentrations were determined by the method of Lowry et al. (1951) after dialysis against 10 mM Tris-HCl (pH 7.9) or precipitation with cold 10% trichloroacetic acid. DNA concentration was estimated by adsorption at 260 nm or by the method of Burton (1956).

RNA Polymerase II Purification. RNA polymerase II was purified from calf thymus either by the method of Weil & Blatti (1975) through the second DEAE-Sephadex column and then subjected to sucrose density gradient sedimentation (Blatti et al., 1970) or by the method of Hodo & Blatti (1977). When the method of Hodo & Blatti was employed, the agarose A-1.5M step was not used, and enzyme purified through the phosphocellulose step was further purified by the density gradient centrifugation method described (Hodo & Blatti, 1977). RNA polymerase II was >96% pure as judged by $NaDodSO_4$ gel electrophoresis and had a specific activity of between 150 and 200 units/mg of protein. It was also free of detectable ribonuclease, ribonuclease H, and DNA endonuclease activity when assayed under stimulatory factor assay conditions.

RNA Polymerase Assay. One unit of RNA polymerase activity represents the incorporation of 1 nmol of UMP in 10 min at 37 °C in a 60-μL assay containing 3 μmol of Tris-HCl (pH 7.9), 100 nmol of $MnCl_2$, 60 nmol of DTT, 20 μg of calf thymus DNA, 36 nmol each of GTP, CTP, and ATP, 6 nmol of UTP, and 0.5 μCi of ^{3}H-labeled UTP. Reactions were terminated, washed, and counted as described (Spindler et al., 1978a).

Purification of Stimulatory Factors. SF-1, provided by Dr. R. H. Benson, was purified by a modification of the previously described procedure[3] (Benson et al., 1978). SF-1 purified by this technique is about 10% pure as judged by $NaDodSO_4$ gel electrophoresis. SF-2 was purified as previously described (Benson et al., 1978) to 95% homogeneity as judged by $NaDodSO_4$ gel electrophoresis. SF-1 and SF-2 purified by these techniques are free of detectable DNA endonuclease activities as assayed by the SV40 allomorph conversion assay when incubated under assay conditions with or without RNA polymerase II. The factors are free of RNase activity as assayed by the reduction in size of 28S ribosomal RNA and are also free of detectable ribonuclease H activity (Benson et al., 1978).

Assay of the Elongation Rate of RNA Polymerase II in the Presence and Absence of SF-2. Reactions contained components at the concentrations described in the legend to Figure 5B, except that the final volume was 180 μL and 0.15 unit of RNA polymerase II was used. Samples of 50 μL were withdrawn at 40, 80, and 120 s. RNA synthesis was terminated and UMP incorporation determined as described in the legend to Figure 2. The molecular weights of the RNA products were then determined as described in Figures 3 and 4.

Results

Characterization of SF-1 Activity Utilizing SV40 Form I DNA as the Template. Form I SV40 DNA was used as the template in these studies because it is free of double- and single-stranded scissions as well as single-stranded regions, which might interrupt the propagation of RNA chains during the assay. Both the RNA polymerase II stimulatory proteins from calf thymus (Stein & Hausen, 1970; Benson et al., 1978) and the SV40 DNA template (Mandel & Chambon, 1974) dramatically alter the ionic strength requirements of enzyme II. For this reason we examined the effect of ammonium sulfate concentration on the transcription of SV40 DNA in the presence of SF-1. Figure 1 shows the ammonium sulfate concentration vs. activity profile for the transcription of SV40 DNA in the presence and absence of SF-1. Maximal enzyme activity on SV40 DNA was obtained at 20 mM ammonium sulfate, in contrast to the 100 mM optimum found with native calf thymus DNA as the template. This unusual ammonium

[1] Abbreviations used: DMEM, Dulbecco's modified Eagle's minimum essential medium; pfu, plaque-forming units; Me_2SO, dimethyl sulfoxide; SV40 virus, simian virus 40; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

[2] The term SV40 DNA refers hereafter to only the supercoiled form I SV40 DNA (>96% supercoiled).

[3] Dr. R. H. Benson, personal communication.

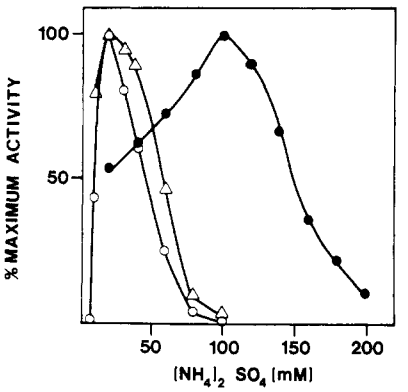

FIGURE 1: Effect of ammonium sulfate concentration on RNA polymerase II activity in the presence or absence of SF-1 with SV40 form I DNA as the template. In a final volume of 70 μL, each reaction contained 0.3 μg of calf thymus RNA polymerase, 5 μmol of Tris-HCl (pH 7.9), 125 nmol of $MnCl_2$, 25 nmol of $MgCl_2$, 9 nmol of EDTA (pH 7.9), 30 nmol of DTT, and 10% glycerol (v/v). In addition, the reactions contained 5 μg/mL SV40 DNA and 2 μg of SF-1 (△) or no SF-1 (○) or 280 μg/mL calf thymus DNA (●) as indicated in the figure. The $(NH_4)_2SO_4$ concentration in each reaction was as indicated. The assays were incubated at 37 °C for 10 min, and RNA synthesis was begun by the addition of a 3-μL aliquot containing 0.6 nmol of UTP (500 mCi/mol) and 30 nmol each of GTP, CTP, and ATP. After 10 min of synthesis at 37 °C, 60-μL aliquots were removed from each reaction and spotted onto DEAE-cellulose filter paper and washed and counted as described (Spindler et al., 1978a).

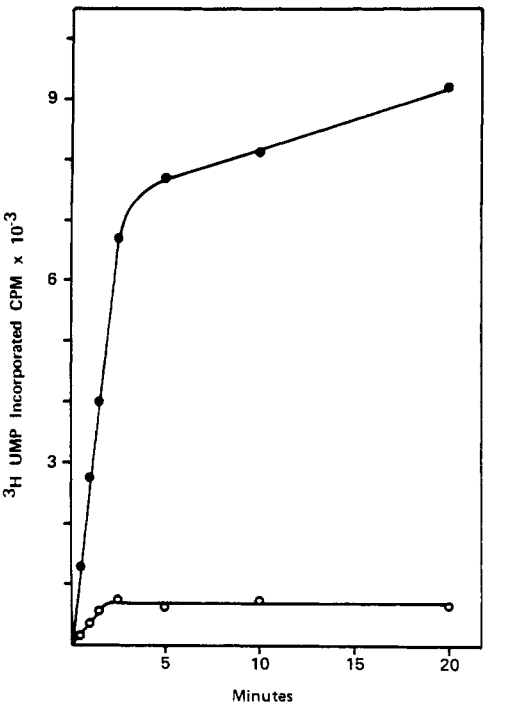

FIGURE 2: Kinetics of RNA polymerase II RNA synthesis on SV40 DNA in the presence and absence of SF-1. In a final volume of 215 μL, one reaction contained 6 μg of stimulatory factor, while the other reaction contained no SF-1. In addition, each reaction contained 3.5 μg of SV40 DNA (form I), 0.5 μg of calf thymus RNA polymerase II, 5.3 μmol of $(NH_4)_2SO_4$ (pH 7.9), 14.5 μmol of Tris-HCl (pH 7.9), 420 nmol of $MnCl_2$, 70 nmol of dithiothreitol, 55 nmol of EDTA (pH 7.9), and 10% glycerol (v/v). The assay was incubated for 10 min at 37 °C. RNA synthesis was begun by the addition of 5 μL containing 12 nmol of UTP (2000 mCi/mmol) and 70 nmol each of GTP, CTP, and ATP. At 30-, 60-, and 90-s intervals, 60-μL aliquots of the reaction were added to 5 μL of a solution containing 4% $NaDodSO_4$ (w/v), 64 μg of α-amanitin, and 500 nmol of EDTA (pH 7.9) and immediately placed into boiling H_2O for 1 min to terminate the reaction. The aliquots were cooled to room temperature, and 5-μL samples of each were spotted on DEAE-cellulose filter paper. The remaining volume of each sample was analyzed by zonal sedimentation on Me_2SO–sucrose gradients (see Figure 3). The later time points were obtained by spotting 5 μL of the reaction mixture onto DEAE-cellulose filter papers at the indicated times and then immediately immersing them in 1 L of 5% Na_2HPO_4 (w/v). All filters were washed and counted as described under Materials and Methods. The amount of incorporation at the early time points was corrected for dilution. (○) represents synthesis in the absence of factor; (●) represents synthesis in the presence of factor.

sulfate requirement may be related to ionic strength dependent changes in the structure of the supercoiled SV40 DNA (Mandel & Chambon, 1974). As can be seen, SF-1 did not significantly alter this optimum on SV40 DNA. For this reason we performed the studies with SF-1 described below near this ammonium sulfate optimum. Interestingly, SF-1 reduced the ammonium sulfate optimum of enzyme II on native calf thymus DNA from 100 to 10–30 mM.[3]

Although SF-1 did not alter the ionic strength optimum for transcription of SV40 DNA, it did stimulate RNA polymerase activity on this template. Figure 2 illustrates the kinetics of RNA synthesis in the presence of SF-1. As can be seen, there was marked enhancement in activity during the first few minutes of linear RNA synthesis. Thereafter, the rate of synthesis declined rapidly in both the presence and absence of SF-1. Thus, SF-1 stimulated both the rate and the extent of the reaction. The large difference in synthetic rates during the early linear phase of synthesis was utilized to investigate the step in the reaction enhanced by SF-1.

Measurement of RNA Polymerase II Elongation Rate in the Absence and Presence of SF-1. When RNA polymerase II and double-stranded DNA are incubated together under appropriate conditions, they form a stable, time- and temperature-dependent enzyme–DNA preinitiation complex (Meilhac et al., 1972). The formation of this binary complex is thought to involve the opening of the double helix and insertion of a part of the polymerase between the strands. This complex appears to be equivalent to the preinitiation complex found with bacterial RNA polymerase (Chamberlin, 1974). A 10-min preincubation is sufficient time for the formation of a maximum number of preinitiation complexes by enzyme II on SV40 DNA at a DNA concentration 60-fold below the concentration used in the present study (Meilhac et al., 1972). We have confirmed that 10 min is also sufficient preincubation time under our experimental conditions. In order to synchronize the initiation event in the experiment shown in Figure 2, we incubated RNA polymerase II and SV40 DNA together for 10 min at 37 °C in the presence or absence of SF-1 to allow the formation of binary complexes. Under these conditions, when the reaction was synchronously initiated by the addition of nucleotide triphosphates, a seven- to eightfold stimulation in total incorporation took place in the presence of SF-1 during the first few minutes of linear activity.

To compare the elongation rates of RNA polymerase II in the presence and absence of SF-1, we analyzed the sizes of the RNA products obtained at 30, 60, and 90 s in this experiment by centrifugation in 99% Me_2SO–sucrose gradients. Me_2SO has been shown to destroy all RNA and DNA secondary structure at this concentration (Strauss et al., 1968), and zonal sedimentation in 99% Me_2SO was utilized for this reason. The marker RNAs used in these gradients were 4S tRNA and 18S rRNA, which migrate at 1.21 s_{25,Me_2SO} and 3.25 s_{25,Me_2SO}, respectively (Strauss et al., 1968). The results obtained are shown in Figure 3. As can be seen, the product RNAs transcribed in the presence or absence of SF-1 had similar sedimentation coefficients at each time point, indicating similar rates of chain elongation.

Figure 4 presents the results obtained when the molecular weights of the product RNAs obtained at each point were

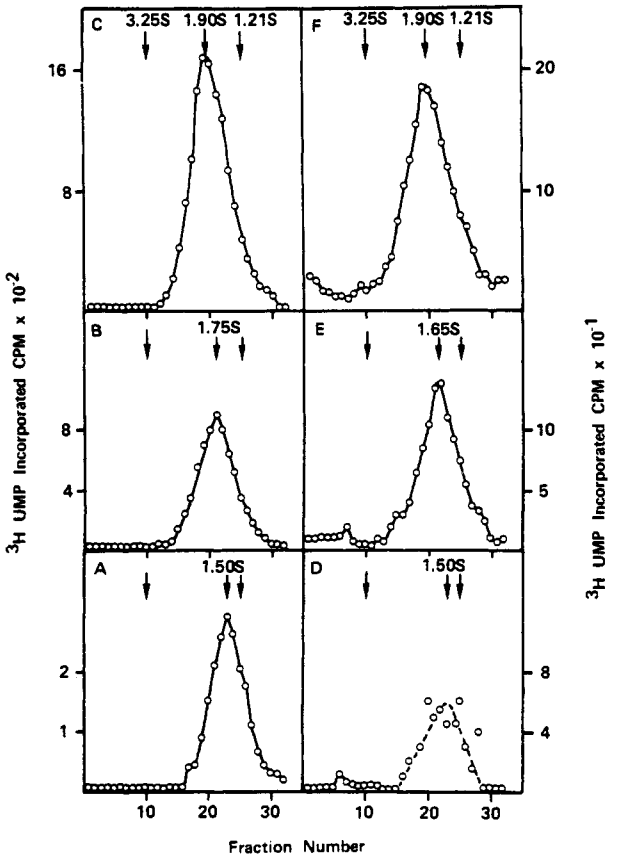

FIGURE 3: Sedimentation analysis of product RNAs made in the presence and absence of SF-1 on 99% Me_2SO–sucrose gradients. The remaining aliquots from the 30-, 60-, and 90-s assays described in Figure 2 were combined with ^{14}C-labeled rat liver 18S rRNA and 4S tRNA and layered directly onto 99% Me_2SO and 1 mM EDTA (pH 7.4) stabilized by linear gradients of 3–10% sucrose and 0–99% Me_2SO-d_6 (Strauss et al., 1968). The gradients were centrifuged for 20 h at 20 °C at 49 500 rpm in a Spinco SW50.1 rotor. Fractions were collected and processed as described under Materials and Methods. The sedimentation coefficients were determined in relation to the marker values (David & Chase, 1972). The reactions in parts A–C were carried out in the presence of SF-1, and the reactions in parts D–F were performed in the absence of the factor. Parts A and D were 30-s time points; parts B and E were 60-s time points; parts C and F were 90-s time points.

plotted as a function of the reaction time. As can be seen, the sizes of the RNAs synthesized in the presence and the absence of the factor increased linearly during the first 90 s of synthesis. Essentially identical elongation rates were found in both the presence and absence of SF-1. Under these conditions, where UTP was limiting (60 μM UTP), the elongation rate calculated from the data presented in Figure 4 is 4.5 nucleotides per s. At higher UTP concentrations (120 μM UTP), the elongation rate increased to six nucleotides per s. The stimulatory factor again increased RNA synthesis without affecting the elongation rate. These results suggest that the factor acts by increasing the number of RNA polymerase II molecules which are able to initiate RNA synthesis.

The apparent enhancement of initiation in the presence of SF-1 might have resulted from a stabilization of the RNA polymerase against inactivation during the 37 °C preincubation rather than from an actual enhancement of the preinitiation or initiation events. However, this does not appear to be the case. Enzyme II was found to be very stable under the preincubation conditions used here. We compared the kinetics of UMP incorporation when the reaction was begun by the addition of RNA polymerase to the complete reaction cocktail (i.e., without any preincubation) with the kinetics obtained

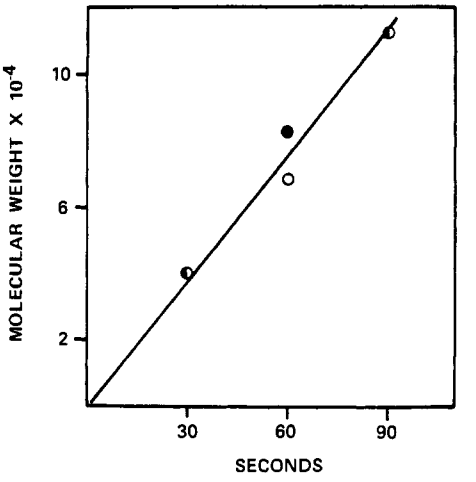

FIGURE 4: Rate of RNA chain elongation in the presence and absence of SF-1. The molecular weight of the in vitro RNA at each time point was calculated (Strauss et al., 1968) from the sedimentation coefficients obtained in Figure 3. (○) represents synthesis in the absence of factor; (●) represents synthesis in the presence of factor.

when the formation of preinitiation complexes was allowed to proceed for 10 min at 37 °C before the addition of nucleotide triphosphates (data not shown). Both the rate and extent of RNA synthesis were similar in each case. These results suggest that no loss in polymerase activity occurred during the 10-min preincubation used. In addition, the presence of 1 mg/mL BSA during the preincubation had little effect on the initial rate of the reaction. This result indicates that the presence of the additional protein of SF-1 did not nonspecifically stabilize the polymerase during the preincubation, again suggesting that SF-1 promotes either the preinitiation or the initiation event.

Apparent Stoichiometry of the Initiation Reaction in the Presence and Absence of SF-1. An estimate of the percentage of the RNA polymerase II molecules which initiated RNA synthesis in the absence and presence of SF-1 can be calculated from the data presented in Figures 2 and 4. Assuming a native molecular weight of 6.0 × 10^5 for calf thymus RNA polymerase II (Kedinger et al., 1974) and a molecular weight of 1.1 × 10^5 for the RNA chains synthesized at 90 s (Figure 4), the approximate percentages of the RNA polymerase molecules which initiated synthesis in the presence and absence of SF-1 were 25 and 3.5%, respectively. Although these numbers are approximate due to the assumptions made, the absolute difference between synthesis in the presence and absence of SF-1 is sevenfold (Figure 2).

Characterization of SF-2 Activity Utilizing SV40 DNA as Template. We investigated the mechanism of action of stimulatory factor SF-2 using the elongation rate assay described above. To do this we first determined the kinetics of the enzyme II reaction on SV40 DNA in the presence of SF-2. Figure 5A shows the kinetics of RNA synthesis on SV40 DNA in the presence and absence of SF-2 under reaction conditions similar to those used for the SF-1 assay shown in Figure 2. The relative kinetics observed in this study are clearly different from those obtained with SF-1. Synthesis in the presence of SF-2 was stimulated only slightly initially, while a greater enhancement was observed at later times due to the marked decline which occurred in the rate of unstimulated RNA synthesis. These kinetics are essentially identical with those found with SF-2 by using native calf thymus DNA as the template. Because the rate of RNA synthesis was only stimulated slightly at early times, when the unstimulated rate of synthesis was linear, several parameters of the reaction were

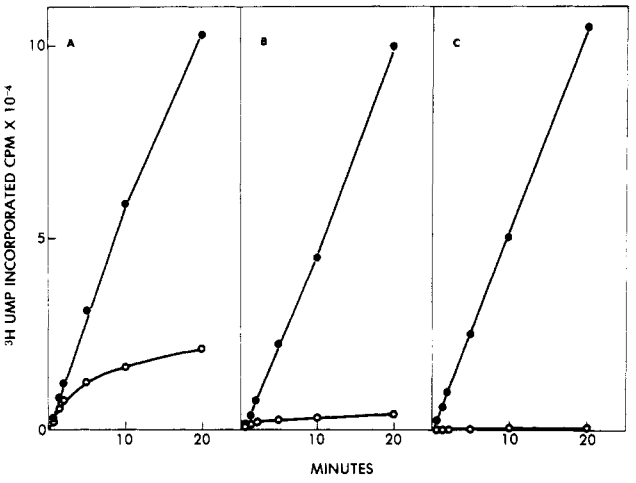

FIGURE 5: (A) The kinetics of RNA synthesis on SV40 DNA at 20 mM ammonium sulfate in the presence or absence of saturating levels of SF-2. Standard reactions (legend to Figure 2) contained 50 μg/mL SV40 DNA, 0.3 mM GTP, CTP, and ATP, 0.05 mM UTP, 20 mM ammonium sulfate, and 0.08 unit of enzyme II in a final volume of 36 μL. Aliquots were spotted onto DEAE-cellulose filter disks at the indicated times. (●) represents synthesis in the presence of saturating levels of SF-2; (○) represents synthesis in the absence of SF-2. (B) The kinetics of RNA synthesis on SV40 DNA at 12 mM ammonium sulfate in the presence or absence of saturating levels of SF-2. Reaction conditions were the same as those described in the legend to Figure 5A except that the reaction mixture contained 12 mM ammonium sulfate. (●) represents synthesis in the presence of SF-2; (○) represents synthsis in the absence of SF-2. (C) The kinetics of RNA synthesis on SV40 DNA at 6 mM ammonium sulfate in the presence or absence of saturating levels of SF-2. Reaction conditions were the same as those described in the legend to Figure 5A except that the reaction mixture contained 6 mM ammonium sulfate. (●) represents synthesis in the presence of SF-2; (○) represents synthesis in the absence of SF-2.

investigated to attempt to maximize this difference.

We found that the relative difference in the initial rates of stimulated vs. nonstimulated RNA synthesis was maximal at limiting UTP concentrations, such as those used with SF-1 (data not shown). Increasing the UTP concentration to 0.2 mM progressively decreased the difference in the relative initial rates of RNA synthesis. The relative difference in initial rates was also maximal at low ammonium sulfate concentrations. Parts A, B, and C of Figure 5 represent RNA synthesis at 20, 12, and 6 mM ammonium sulfate, respectively, each at limiting UTP concentration. As can be seen, essentially no RNA synthesis occurred in the absence of SF-2 at 6 mM ammonium sulfate under these conditions. As the ammonium sulfate concentration increased, the amount of RNA made in the absence of SF-2 also increased. However, it remained linear for only a few minutes in all the cases tested. In contrast, there was essentially a constant level of linear synthesis in the presence of SF-2 at all the ammonium sulfate concentrations tested. From this data we determined that 12 mM ammonium sulfate and a limiting UTP concentration (50 μM) would be optimal for studying the effect of SF-2 on the elongation rate of RNA polymerase II.

Measurement of the RNA Polymerase II Elongation Rate in the Absence and Presence of SF-2. In order to determine which step in the RNA polymerization reaction is enhanced by SF-2, we measured the elongation rate of the enzyme in the presence and the absence of the factor. The kinetics of RNA synthesis obtained are similar to those shown in Figure 5B. RNA synthesis was linear for 20 min in the presence of SF-2. In contrast, in the absence of SF-2 it was linear for only a few minutes and then declined rapidly. Twice as much RNA was made in the presence of SF-2 at each early time point as

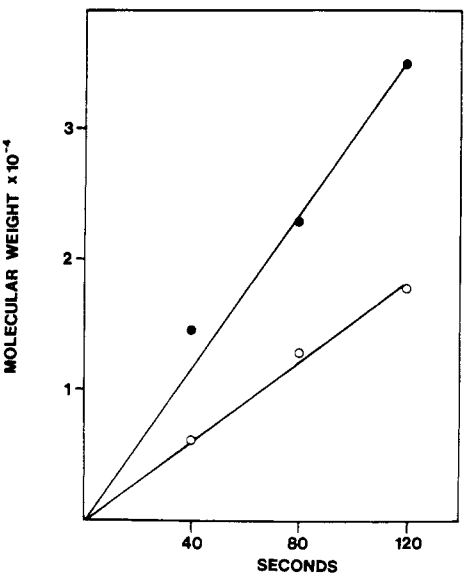

FIGURE 6: Rates of RNA chain elongation in the presence and absence of SF-2. The molecular weights of the RNAs synthesized in the presence and the absence of SF-2 after 40, 80, and 120 s were determined by sedimentation in Me_2SO–sucrose gradients, as described in Figure 3. (●) represents synthesis in the presence of saturating levels of SF-2; (○) represents synthesis in the absence of SF-2.

was synthesized in its absence.

The sizes of the early RNAs produced in this experiment were determined by sedimentation in 99% Me_2SO–sucrose gradients as described under Materials and Methods (data not shown). Values of 1.68, 2.04, and 2.30 s_{25,Me_2SO} were obtained in the absence of SF-2 at 40, 80, and 120 s, respectively. Values of 2.08, 2.46, and 2.77 s_{25,Me_2SO} were obtained in the presence of SF-2 at 40, 80, and 120 s, respectively. When the molecular weights derived from these values were plotted as a function of time, the results presented in Figure 6 were obtained. As can be seen, the initial rate of RNA elongation in the presence of SF-2 was double that observed in its absence. Since the amount of RNA synthesized during the linear phase of RNA synthesis was also doubled in the presence of SF-2, this factor apparently increased the elongation rate of RNA polymerase II. In three separate determinations the increase in elongation rate was always within 20% of the increase found in the RNA synthetic rate. The apparent elongation rates calculated from the data in Figure 6 are about 10 and 15 nucleotides per s in the presence and absence of SF-2, respectively.

Discussion

Calf thymus RNA polymerase II stimulatory factors SF-1 and SF-2 were tested for their effects on the elongation rate of enzyme II. This work involved the use of an elongation rate assay not used previously in studies of transcription factors. This elongation rate assay was developed because of ambiguities in the results obtained by previously published assay procedures.

The number of initiation events occurring in an RNA polymerase reaction has usually been determined by measuring the ratio of nucleotide triphosphates to nucleotide monophosphates incorporated into RNA by using γ-^{32}P-labeled GTP or ATP. Since the γ-phosphate is incorporated only at the 5′ end of RNA chains, this technique allows the calculation of average chain length and from this length the number of initiation events which occur. But this technique has not yielded satisfactory results in studies of mammalian transcription factors. Even highly purified eucaryotic RNA

polymerases often contain contaminating protein kinase activity [e.g., Bell et al. (1977)] which can obscure increased γ-phosphate incorporation into RNA. Because of this activity, careful controls must be performed when doing γ-^{32}P-labeled incorporation studies, and in general, adequate controls have not been reported. The high backgrounds of incorporation obtained are difficult to eliminate, even with extensive proteinase treatment.[3] In addition, rather large amounts of polymerase activity are required to obtain sufficient ^{32}P incorporation into 5′ ends, and the low levels of incorporation obtained make the results difficult to interpret.

Using a different approach, Sugden & Keller (1973) performed kinetic studies utilizing the rifampicin derivative AF/05 to investigate the effects of the HeLa and KB cell stimulatory factors on the polymerization reaction. Rifampicin AF/05 selectively inhibits RNA chain initiation on native DNA by class I and II RNA polymerases (Meilhac et al., 1972). Both the primary binding of enzyme II to DNA and the time- and temperature-dependent steps of initiation are inhibited. The elongation reaction is apparently unaffected by the drug over the concentration range used. Rifampicin AF/05 completely abolished the effects of both of the HeLa and KB cell stimulatory factors when the drug and factors were added simultaneously to the polymerase reaction. Since these factors were unable to stimulate RNA synthesis when RNA chain initiation was blocked, the factors were assumed to stimulate the initiation reaction. However, the conclusions drawn from the AF/05 experiments cannot be regarded as definitive. Unlike rifampicin, derivates such as AF/05 are not specific for the DNA-dependent RNA polymerases, since the RNA-dependent DNA polymerases of tumor viruses (Gurgo et al., 1971) and other enzymes such as hexokinase (Riva et al., 1972) are also inhibited over the same concentration range. In addition, the chemical structure of these drugs suggests that they may act as detergents, and this property may be the source of their inhibitory activity. In this regard, it is interesting to note that these derivatives are required at much higher concentrations to inhibit eucaryotic polymerases than the concentration of rifampicin required to inhibit *E. coli* RNA polymerase, suggesting a nonspecific effect on the enzyme. Thus, the data can also be explained by assuming that AF/05 directly inactivated the stimulatory factors, thus preventing their action independent of the effect of the drug on chain initiation.

In the work reported here we avoided the problems with γ-labeled nucleotides and rifampicin derivatives discussed above and investigated the effect of SF-1 and SF-2 on the elongation rate in a more direct way. γ-Labeled nucleotides were not used, and the radioactive label used was not subject to transfer to protein. In addition, no initiation inhibitors were necessary, and no significant reinitiation occurred during the time interval of interest.

No change in the elongation rate of polymerase II was found in the presence of SF-1. Only about 3.5% of the enzymes initiated RNA synthesis in the absence of SF-1, while about 25% initiated synthesis in the presence of the factor under the conditions used. These results suggest that SF-1 enhances either the preinitiation or the initiation event. It is difficult to distinguish between these possibilities since SF-1 binds to DNA under the preincubation conditions and interferes with DNA filter binding studies. However, at a polymerase to SV40 DNA ratio of 1:1, after incubation under the preinitiation conditions used here, about 30% of the DNA was bound to nitrocellulose filters. This apparent binding of polymerase to SV40 DNA was both time and temperature dependent, suggesting it represents the formation of preinitiation complexes.[4] In view of these results, and the sevenfold enhancement of RNA synthesis observed at early times, it seems unlikely that SF-1 enhances the preinitiation reaction. Thus, SF-1 probably enhances the initiation of RNA chains.

We do not know how to interpret the DNA binding of SF-1, since at the ionic strength used in these studies SF-1 also binds to several cation exchangers (Benson et al., 1978). Thus, the binding may not be a specific affinity interaction. However, SF-1 could act directly on the template to destabilize or completely open regions of the double helix at which the polymerase may more easily initiate RNA synthesis. Alternatively, it may act directly on the enzyme to promote the initiation reaction. It is not presently possible to distinguish between these possibilities.

We also tested the effect of SF-2 on the elongation rate of polymerase II. We found that SF-2 promotes a doubling in both the initial rate of RNA elongation and the initial rate of RNA synthesis. The close correspondence between these rates strongly suggests that SF-2 is an elongation factor. The effect of SF-2 on the elongation rate is interesting in view of the presence of the helix destabilizing protein UP-1 (Herrick & Alberts, 1976a,b) as a component of SF-2 (Benson et al., 1978). UP-1 has been shown to stimulate the activity of eucaryotic DNA polymerase α (Herrick et al., 1976); thus, it may have a role in both DNA and RNA synthesis.

The similarities between bacterial core RNA polymerase and purified eucaryotic RNA polymerase II are striking: neither polymerase initiates RNA synthesis efficiently on double-stranded DNA (Chambon, 1975; Burgess, 1971); both enzymes lack transcriptional specificity in vitro (Chambon, 1975; Burgess, 1971); both initiate RNA synthesis preferentially at nicks or gaps in the DNA (Chambon, 1975; Chamberlin, 1974); both polymerases consist of two large subunits accompanied by a number of smaller subunits (Roeder, 1976; Burgess, 1971). In addition, both the eucaryotic and procaryotic enzymes may lose subunits during purification (Huet et al., 1975; Spindler et al., 1978b; Burgess, 1971). The σ subunit of the bacterial enzyme is easily dissociated from the enzyme during polymerase purification, and it is this subunit which is required for the efficient and specific initiation of transcription in vitro and in vivo (Burgess, 1971). In view of the similarities between the two systems, the possibility must be considered that RNA polymerase II components similar to the σ subunit exist in eucaryotes. Indeed, the endogenous class II RNA polymerase in nuclei isolated from several cell types is apparently unable to reinitiate (Bitter & Roeder, 1978; Stallcup et al., 1979; S. R. Spindler, J. A. Martial, H. M. Goodman, and J. D. Baxter, unpublished experiments) or elongate (Cox, 1976) efficiently. These results are interesting since SF-1, SF-2, and RNA polymerase II stimulatory factors from several other cell types are readily lost from nuclei after cell disruption (unpublished experiments). Now that cloned DNAs containing well characterized eucaryotic transcription units are available, it is possible to test whether these factors are involved in the efficient and specific transcription of eucaryotic genes.

Acknowledgments

I am grateful to Dr. Stanley Blatti for providing facilities and support for this research, as well as many interesting discussions. I also thank Dr. John D. Baxter for many helpful

[4] C. Jones, personal communication; unpublished experiments.

discussions during the preparation of the manuscript.

References

Axel, R., Cedar, H., & Felsenfeld, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 4713.
Barrett, T., Maryanka, D., Hamlyn, P. H., & Gould, H. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 5057.
Bell, G. I., Valenzuela, P., & Rutter, W. J. (1977) *J. Biol. Chem. 252*, 3082.
Benson, R. H., Spindler, S. R., Hodo, H. G., & Blatti, S. P. (1978) *Biochemistry 17*, 1387.
Bitter, G. A., & Roeder, R. G. (1978) *Biochemistry 17*, 2198.
Blatti, S. P., Ingles, C. J., Lindell, T. J., Morris, P. W., Weaver, R. F., Weinberg, F., & Rutter, W. J. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 649.
Burgess, R. R. (1971) *Annu. Rev. Biochem. 40*, 711.
Burgess, R. R., Travers, A. A., Dunn, J. J., & Bautz, E. K. F. (1969) *Nature (London) 221*, 43.
Burton, K. (1956) *Biochem. J. 62*, 315.
Chamberlin, M. J. (1974) *Annu. Rev. Biochem. 43*, 721.
Chambon, P. (1975) *Annu. Rev. Biochem. 44*, 613.
Cox, R. F. (1976) *Cell 7*, 455.
D'Alessio, J. M., Spindler, S. R., & Paule, M. R. (1979) *J. Biol. Chem. 254*, 4085.
David, I. B., & Chase, J. W. (1972) *J. Mol. Biol. 63*, 217.
Giesecke, K., Sippel, A. E., Nguyen-Huis, M. C., Croner, B., Hynes, N. E., Wurtz, T., & Schutz, G. (1977) *Nucleic Acids Res. 4*, 3943.
Gurgo, C., Ray, R. K., Thiry, L., & Green, M. (1971) *Nature (London) 229*, 111.
Herrick, G., & Alberts, B. (1976a) *J. Biol. Chem. 251*, 2124.
Herrick, G., & Alberts, B. (1976b) *J. Biol. Chem. 251*, 2133.
Herrick, G., Delius, H., & Alberts, B. (1976) *J. Biol. Chem. 251*, 2142.
Hirt, B. (1967) *J. Mol. Biol. 26*, 365.
Hodo, H. G., & Blatti, S. P. (1977) *Biochemistry 16*, 2334.
Huet, J., Buhler, J., Sentenae, A., & Fromageot, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3024.
Kedinger, C., Gissinger, F., & Chambon, P. (1974) *Eur. J. Biochem. 44*, 421.
Losick, R., & Pero, J. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) p 227, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Mandel, J.-L., & Chambon, P. (1974) *Eur. J. Biochem. 41*, 367.
Meilhac, M., Tysper, Z., & Chambon, P. (1972) *Eur. J. Biochem. 28*, 291.
Reeder, R. H., & Roeder, R. G. (1972) *J. Mol. Biol. 67*, 433.
Riva, S., Fietta, A., & Silvestri, L. G. (1972) *Biochem. Biophys. Res. Commun. 49*, 1263.
Roeder, R. G. (1974) *J. Biol. Chem. 249*, 249.
Roeder, R. G. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 285–329, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Roeder, R. G., Reeder, R. H., & Brown, D. D. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 727.
Sklar, V. E. F., Schwartz, L. B., & Roeder, R. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 348.
Spindler, S. R., D'Alessio, J. M., Duester, G. L., & Paule, M. R. (1978a) *J. Biol. Chem. 253*, 6242.
Spindler, S. R., Duester, G. L., D'Alessio, J. M., & Paule, M. R. (1978b) *J. Biol. Chem. 253*, 4669.
Stallcup, M. R., Ring, J. C., Ucker, D. S., & Yamamoto, K. R. (1979) in *Hormones and Cell Culture*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (in press).
Stein, H., & Hausen, P. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 709.
Strauss, J. H., Kelly, R. B., & Sinsheimer, R. L. (1968) *Biopolymers 6*, 793.
Sugden, B., & Keller, W. (1973) *J. Biol. Chem. 248*, 3777.
Tsai, S. Y., Harris, S. E., Tsai, M.-J., & O'Malley, B. W. (1976) *J. Biol. Chem. 251*, 4713.
Valenzuela, P., Hager, G. L., Weinburg, F., & Rutter, W. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1024.
Weil, P. A., & Blatti, S. P. (1975) *Biochemistry 14*, 1636.
Weil, P. A., & Blatti, S. P. (1976) *Biochemistry 15*, 1500.
Weinmann, R., & Roeder, R. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1970.
Wu, G.-J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2175.
Zasloff, M., & Felsenfeld, G. (1977a) *Biochem. Biophys. Res. Commun. 75*, 598.
Zasloff, M., & Felsenfeld, G. (1977b) *Biochemistry 16*, 5135.
Zylber, E. A., & Penman, S. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2861.