Specific Binding and Protection of Form II SV40 Deoxyribonucleic Acid by Ribonucleic Acid Polymerase II from Wheat Germ†

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ABSTRACT: The introduction of an artificial single-strand break at 0.725 map unit on the simian virus 40 (SV40) genome directs wheat germ RNA polymerase II to bind specifically to this region of DNA. The binding site includes the single-strand break in a specific location within the enzyme–DNA complex. The binary complexes formed at single-strand breaks provide protection of the DNA from the action of deoxyribonucleases. Approximately 38 base pairs of DNA are protected against deoxyribonuclease I digestion, and 34 base pairs are protected against micrococcal nuclease digestion. The evidence for this consists of electrophoretic analysis of nuclease-protected DNA under native and denaturing conditions. Surprisingly, protection of form II SV40 DNA by *Escherichia coli* RNA polymerase is very similar to protection by wheat germ RNA polymerase, as evidenced by the appearance of very similar protected DNA species using both native and denaturing electrophoretic analysis. That is, binary complexes are shown to protect similar sized DNA segments of ~35–41 base pairs from nuclease digestion and are inferred to contain the single-strand break in a similar, specific location within the binding site. Thus, despite their extreme evolutionary divergence and dissimilar subunit structure, the procaryotic and eucaryotic enzymes can participate in superficially very similar complexes with DNA. We discuss the relationship between the observed specific binding to form II SV40 DNA and the form of SV40 DNA in in vivo preinitiation complexes. We infer from this discussion that the involvement of specific single-strand breaks in transcription initiation cannot be excluded at the present time.

The class II RNA polymerase in eucaryotes is responsible for the transcription of mRNA [for a review, see Roeder (1976)]. The mechanism of DNA site selection and subsequent initiation of transcription by RNA polymerase II is almost completely unknown. Highly stable complex formation between enzyme and double-stranded DNA has not been demonstrated unequivocally [see below for a discussion of Seidman et al. (1979)]; stability requires either transiently denatured regions (Hossenlopp et al., 1974; Lilley & Houghton, 1979) or addition of initiating nucleotides (Lescure et al., 1978; Saragosti et al., 1979). However, since the constituents and properties of in vivo preinitiation complexes are unknown, the physiological significance of these observations is uncertain.

This behavior contrasts with that of *Escherichia coli* RNA polymerase which binds double-stranded DNA to form binary complexes at promoter sites with lifetimes commonly measured in hours [for examples, see Chamberlin (1976) or Seeberg et al. (1977)]. These procaryotic complexes protect ~40 base pairs of DNA from digestion with DNase I[1] [Heyden et al., 1972; Okamoto et al., 1972; for references, see Siebenlist (1979a)], involve DNA–protein contacts over 55–75 base pairs (Johnsrud, 1978; Schmitz & Galas, 1979), and include ~11 base pairs of specifically located melted DNA (Siebenlist, 1979b). *E. coli* core enzyme, which lacks sequence specificity, also forms stable binary complexes with double-stranded DNA (Mueller, 1971; Hinkle & Chamberlin, 1972a,b).

Tight binding of RNA polymerase II to DNA with single-strand breaks (nicks) or denatured regions rather than to relaxed double-stranded DNA can be taken as evidence for a nonphysiological interaction only by comparison with the contrasting properties of *E. coli* RNA polymerase holoenzyme cited above. However, under certain circumstances the behavior of the *E. coli* holoenzyme mimics this behavior of RNA polymerase II. For example, in at least one case *E. coli* RNA polymerase fails to form a stable binary complex at a physiologically significant promoter (Küpper et al., 1976). *E. coli* holoenzyme has also been shown to form highly stable complexes with nicked DNA (Hinkle et al., 1972).

The enzymatic and chemical probe experiments cited above, which have led to a detailed description of the interaction between *E. coli* RNA polymerase and DNA, have not been done on RNA polymerase II–DNA complexes. Even the extent of the tight DNA binding site as measured by DNase I protection experiments remains unknown. Therefore, it is important to probe eucaryotic binary complexes both to establish the fundamental interactions which govern formation of individual complexes and to compare these to the (presumably analogous) procaryotic system.

We have chosen, for this purpose, to investigate the properties of heterologous complexes formed between the highly purified eucaryotic RNA polymerase II from wheat germ (Jendrisak & Burgess, 1975) and DNA from the mammalian papovavirus SV40. The SV40 genome directs host RNA polymerase II transcription in vivo as evidenced by the sensitivity of late RNA synthesis to α-amanitin (Jackson & Sugden, 1972). However, highly stable binary complex formation in vitro has not been demonstrated by using relaxed double-stranded SV40 DNA. Partially purified mammalian RNA polymerase II forms somewhat stable complexes with

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[1] Abbreviations used: DNase I, deoxyribonuclease I from bovine pancreas; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; *Alu*, restriction endonuclease *Alu* from *Arthrobacter luteus*; *Eco*RI, restriction endonuclease *Eco*RI from *Escherichia coli* RY 13; *Hae*III, restriction endonuclease *Hae*III from *Haemophilus aegyptius*; *Hpa*I, restriction endonuclease *Hpa*I from *Haemophilus parainfluenzae*; *Hpa*II, restriction endonuclease *Hpa*II from *H. parainfluenzae*; *Msp*I, restriction endonuclease *Msp*I from *Moraxella* sp.; *Hin*dIII, restriction endonuclease *Hin*dIII from *Haemophilus influenzae* Rd; SV40, Simian virus 40; form I, closed circular supercoiled DNA; form II, nicked circular DNA; form III, linear DNA.

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supercoiled (form I) DNA but not with linear (form III) DNA (Hossenlop et al., 1974). The highly purified enzyme from wheat germ forms complexes of moderate stability when nicked (form II) SV40 DNA is involved (D. W. Chandler and J. Gralla, unpublished experiments).

The form of SV40 DNA in the physiologically relevant transcription *preinitiation* complex remains unknown, since it has not been possible to isolate such complexes from infected cells. Form I DNA is a possible candidate since it does participate in at least a fraction of elongation complexes (Birkenmeier et al., 1977; Green & Brooks, 1978). Form II DNA is an alternate possibility since it is produced in nuclear extracts by an enzymatic activity which nicks SV40 DNA specifically (Scott & Wigmore, 1978; Waldeck et al., 1978) in the vicinity of the putative sites of transcription initiation (Lai et al., 1978; Mueller et al., 1978; Laub et al., 1979).

We report here that artificially induced single-strand breaks direct the formation of specific binary complexes between RNA polymerase II from wheat germ and SV40 DNA. These complexes are sufficiently stable to provide protection from digestion by both DNase I and micrococcal nuclease. The region of protected DNA contains a precisely located single-strand break. Furthermore, we show that *E. coli* RNA polymerase binds single-strand breaks in a very similar manner.

Materials and Methods

Cells, Virus, and DNA Isolation. The TC7 subline of monkey kidney cells was grown on Dulbecco's modified Eagle's medium supplemented with 7% calf serum. SV40 virus, strain 776, was originally obtained from G. Fareed. Virus stocks were produced by infection at a multiplicity of 0.01 plaque forming unit per cell, and the lysate was collected after cytopathic effects were observed in ~90% of the cells.

SV40 DNA was prepared by infection at 1–10 plaque forming units per cell followed by extraction of supercoiled low molecular weight DNA. Briefly, 3 days after infection cells were lysed with 0.01 M Tris-HCl, pH 7.5, 0.007 M EDTA, 0.10 M sodium chloride, and 1% (w/v) sodium dodecyl sulfate. SV40 DNA was separated from high molecular weight DNA by centrifugation at 30000*g* for 30 min (Hirt, 1967). RNA and protein were removed by treatment with ribonuclease A (Sigma, heated at 90 °C for 10 min) and Pronase (Sigma, self-incubated to destroy DNase activity), extraction with chloroform–isoamyl alcohol (24:1), and precipitation in 70% ethanol at −20 °C. The redissolved SV40 DNA was banded twice in cesium chloride–ethidium bromide (300 μg/mL) gradients (Radloff et al., 1967) to obtain pure closed circular DNA (form I). Ethidium bromide was removed by passage over Dowex 50, and the DNA was finally dialyzed into 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA and stored in small aliquots at −20 °C. Agarose gel electrophoresis (Dugaiczyk et al., 1975) was used to monitor the structural integrity of the DNA.

Enzymes. E. coli RNA polymerase was prepared by the method of Burgess & Jendrisak (1975) and was stored at −70 °C in 50% glycerol solution. Wheat germ RNA polymerase was prepared by the method of Jendrisak & Burgess (1975) except that storage conditions were modified as follows. Purified RNA polymerase II was precipitated by slowly adding ammonium sulfate to 50% saturation and then stirring for 1 h. The protein precipitate was collected by centrifugation (20000*g* for 30 min). The pellet was redissolved at 0.5 mg/mL protein in 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, and 50% (v/v) glycerol. Small aliquots were stored at −70 °C until used. Wheat germ for this preparation was generously provided by General Mills. Both enzymes were judged of high purity by the high specific activity (>250 units/mg for the wheat germ enzyme and >600 units/mg for the *E. coli* enzyme) and the absence of significant contaminants appearing on sodium dodecyl sulfate–acrylamide gels.

DNase I was obtained from Worthington (Grade DPFF), dissolved at 5 mg/mL in 10^{-4} M HCl, and stored in aliquots at −20 °C. Micrococcal nuclease (Worthington) was diluted to 0.5 mg/mL in 1.3 mg/mL bovine serum albumin solution and stored in aliquots at −70 °C until used. Restriction enzymes were obtained from New England Biolabs. Bovine serum albumin (Sigma) as a 20 mg/mL solution was prepared in water and was heated for 20 min at 80 °C to eliminate nuclease activity.

Labeling of Form II Nicked DNA. Form II SV40 DNA was prepared and ^{32}P-labeled by nick translation using a procedure similar to that of Maniatis et al. (1975a). Briefly, DNase I stocks (5 mg/mL in 10^{-4} M HCl) were diluted in 10^{-4} M HCl to 10 ng/mL immediately before use. Typically, 10 pg was added to 0.05 mL of 50 mM Tris-HCl, pH 7.8, 5 mM magnesium chloride, and 5 mM 2-mercaptoethanol containing 2 μM [α-^{32}P]dATP, 100 μM each dCTP, dGTP, and dTTP, 50 μg/mL bovine serum albumin, and 20 μg of SV40 DNA. This amount of DNase I produced on the average 1 nick/SV40 DNA as measured by agarose gel electrophoresis. Five units of DNA polymerase I was added, and the reaction proceeded for 45 min at 15 °C. About one-half of the ^{32}P was incorporated into material which was excluded from Sephadex G50-80, which corresponds to replacement of less than 2% of the adenosine phosphate with ^{32}P. Thus, the specific radioactivity is very high in the vicinity of the nick and very low elsewhere. DNA was separated from the nucleotides by centrifugation through a small column of Sephadex G50-80 (Penefsky, 1977) equilibrated with 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA and stored at −20 °C when necessary.

Unlabeled SV40 DNA was nicked specifically with restriction enzyme *Msp*I (New England Biolabs) (or *Hpa*II, an isoschizomer of *Msp*I) by incubating 25 μg of SV40 DNA in 250 μL of a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 6 mM KCl, 1 mM dithiothreitol, 100 μg/mL bovine serum albumin, and 60 μg/mL ethidium bromide with 50 units of *Msp*I (*Hpa*II). After 30 min at 25 °C, 20 additional units of *Msp*I (*Hpa*II) was added and the incubation was continued for 30 min longer. The reaction mixture was extracted with butanol and 1% Sarkosyl (which was previously saturated with water) to remove ethidium bromide and with phenol–chloroform–isoamyl alcohol (50:50:2) to remove the proteins. DNA was precipitated in 70% ethanol and redissolved in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA.

Nicked DNA was 5′-labeled with ^{32}P in a manner similar to that described by Weiss et al. (1968). Five micrograms of DNA treated as above was incubated with 0.07 unit of bacterial alkaline phosphatase (Worthington) for 30 min at 65 °C in 100 mM Tris-HCl buffer, pH 8.0. This procedure removes phosphate of both nicks and ends. The dephosphorylated DNA was extracted 3 times with phenol–chloroform–isoamyl alcohol (50:50:2) and precipitated with 0.3 M sodium acetate and 3 volumes of 95% ethanol. The 5′-hydroxyl groups were labeled by using [γ-^{32}P]ATP and polynucleotide kinase (Boehringer) as described by Maxam & Gilbert (1977), and the labeled DNA was separated from [γ-^{32}P]ATP by centrifugation through a Sephadex G50-80 column. ^{32}P, which may have labeled any contaminating form III DNA, was removed by incubation with 0.2 unit of bacterial alkaline phosphatase as above except that the incubation temperature

was 37 °C. This treatment removes phosphate from double-stranded ends but not from internal nicks (Weiss et al., 1968).

DNA Binding and Protection. All binding and protection reactions were at 37 °C. A 1.5–2-μg amount of DNA was diluted into 0.070 mL of buffer containing 100 mM Tris-HCl, pH 7.9, 7 mM thioglycerol, 0.1 mM EDTA, 24 mM ammonium sulfate, 2.6 mM manganese chloride, and 0.4 mg/mL bovine serum albumin. The reaction was prewarmed to 37 °C, and RNA polymerase II was added (5–12.5 μg). The binding reaction was allowed to proceed for 2 min at 37 °C, and then a 10-fold weight excess of denatured calf thymus DNA (5 mg/mL, Sigma) was added. Digestion was then initiated by addition of magnesium chloride to 10 mM and addition of 40 μg of DNase I. Samples were removed at the times indicated in the figure legends, diluted to 0.5 mL in 100 mM Tris-HCl, pH 7.9, 7 mM thioglycerol, 0.1 mM EDTA, and 24 mM ammonium sulfate, and filtered slowly through a 13-mm diameter nitrocellulose filter (Schleicher and Schuell BA-85). The filter was rinsed twice with 1 drop of the diluent buffer and was then transferred immediately to a tube containing 0.25 mL of 0.5 M ammonium acetate, 0.1% (w/v) sodium dodecyl sulfate, 10 mM magnesium acetate, and 0.1 mM EDTA. DNA was dissociated from bound protein and eluted from the filter by soaking for 30 min at 37 °C. After the filter was removed, DNA was precipitated in the presence of 20 μg of carrier transfer RNA (Sigma) with 3 volumes of cold 95% ethanol. The pellet was taken up into the appropriate solution for gel electrophoresis.

The protocol for protection from micrococcal nuclease is similar except that calcium chloride (to 10 mM) and micrococcal nuclease (10 μg) were added to the wheat germ RNA polymerase II–DNA complex instead of magnesium chloride and DNase I.

The procedures are the same for complexes formed by using *E. coli* RNA polymerase except that the complexes are formed in 0.05 mL of 10 mM Tris-HCl, pH 8.0, 10 mM magnesium chloride, 50 mM sodium chloride, 10 mM 2-mercaptoethanol, and 1 mM EDTA with 40 μg of bovine serum albumin. Samples to be filtered were diluted in the same buffer without bovine serum albumin.

Experiments involving binding without subsequent digestion were begun by cleaving 6 μg of unlabeled form II DNA with endonuclease *Hin*dIII in 10 mM Tris-HCl, pH 7.4, 7 mM magnesium chloride, 60 mM sodium chloride, and 0.1 mg/mL bovine serum albumin. The DNA fragments were extracted with phenol–chloroform–isoamyl alcohol (50:50:2) and diethyl ether, and the DNA was concentrated by ethanol precipitation. Two micrograms of this DNA was used in a binding reaction with the same buffer described for RNA polymerase II above. The binding, filtration, and elution are as described above except that no magnesium chloride or DNase I is added. Fragments which bound RNA polymerase II were identified by electrophoresis as described below.

Gels and Autoradiography. Acrylamide gels were prepared and run in a manner similar to that described by Maniatis et al. (1975b) and contained an acrylamide/bis(acrylamide) ratio of 29:1. Fifteen percent native acrylamide gels were 18 × 18 × 0.15 cm and contained 100 mM Tris–borate, pH 8.3, and 5 mM magnesium chloride as buffer. Samples were loaded in 1:10 diluted buffer containing 0.05% (w/v) each xylene cyanol FF and bromophenol blue and 10% (v/v) glycerol. Native gels of 3.5% contained 2.5 mM EDTA in place of magnesium chloride. Twelve percent acrylamide denaturing gels were 18 × 18 × 0.15 cm and contained 100 mM Tris–borate, pH 8.3, 2.5 mM EDTA, and 7 M urea (Schwarz/Mann, ultrapure). Electrophoresis buffer did not contain urea. Denatured samples were prepared by redissolving protected DNA in formamide and heating to 100 °C for 3 min. Electrophoresis was carried out at 7–9 W. One percent agarose gels which separate quantitatively the various forms of SV40 DNA were cast and run horizontally as described by Dugaiczyk et al. (1975). They were placed on several sheets of filter paper and allowed to dry before autoradiography.

Autoradiography was carried out with Kodak No Screen or Du Pont Cronex 4 X-ray film. Wet or frozen acrylamide gels wrapped in saran (Dow) or dry agarose gels were placed against film and exposed at −70 °C with or without an intensifier screen (Du Pont Quanta II). Gels were calibrated by coelectrophoresis of fragments of known sequence (Reddy et al., 1978) from restriction digests of SV40 and also from the *E. coli* lac operon (gift of M. Chao). These marker fragments were labeled either by nick translation or by [γ-^{32}P]ATP and polynucleotide kinase.

Results

RNA Polymerase II Binds Specifically to a Single-Strand Break Created by a Restriction Enzyme and Protects the DNA against Nuclease Digestion. The experiment shown in Figure 1 demonstrates that the introduction of a single nick at 0.725 map unit in the SV40 genome directs RNA polymerase II to bind tightly and preferentially to the region containing the nick. Form I SV40 DNA was first treated with endonuclease *Hpa*II in the presence of ethidium bromide. This treatment (Parker et al., 1977) leads with high frequency to form II DNA with a single-strand break at map position 0.725 (Sack & Nathans, 1973); the preponderance of form II DNA was confirmed by quantitation of labeled DNA forms separated by agarose gel electrophoresis (not shown). The DNA was then cleaved into several fragments with another restriction enzyme, and RNA polymerase II was added to the mixture of fragments under conditions which allow formation of binary complexes. The solution was then passed through a nitrocellulose filter which retains DNA–protein complexes but not DNA alone. Those fragments which have bound RNA polymerase II will be retained on the filter selectively and may be eluted subsequently and identified by polyacrylamide gel electrophoresis. Figure 1 shows that when a specific nick is introduced into the SV40 genome at 0.725 map unit, the DNA fragment containing this site binds RNA polymerase II and is therefore preferentially retained by the filter. Under these conditions no fragments are retained when unnicked DNA is used. Thus, the introduction of a single nick in this position is sufficient to direct stable RNA polymerase II binding. Similar results are obtained in a buffer containing Mg^{2+} in place of Mn^{2+}.

Next, we wished to determine whether the binary complex formed at the nick would protect DNA from digestion by nucleases. The 5′ side of the nick was labeled with ^{32}P by using the procedure of Weiss et al. (1968), which labels nicks in preference to termini. Agarose gel electrophoresis followed by autoradiography confirmed that greater than 90% of the radioactivity was in form II DNA. Binary complexes with RNA polymerase II were formed and digested with either DNase I or micrococcal nuclease. The protected DNA was then subjected to polyacrylamide gel analysis.

Figure 2 shows that RNA polymerase II protects nicked DNA from digestion by both micrococal nuclease and DNase I, as evidenced by the appearance of specific radioactive bands on the gel autoradiograph. At the earliest digestion time of 0.5 min the predominant protected species are in discrete bands greater than 40 base pairs long for both DNase I and mi-

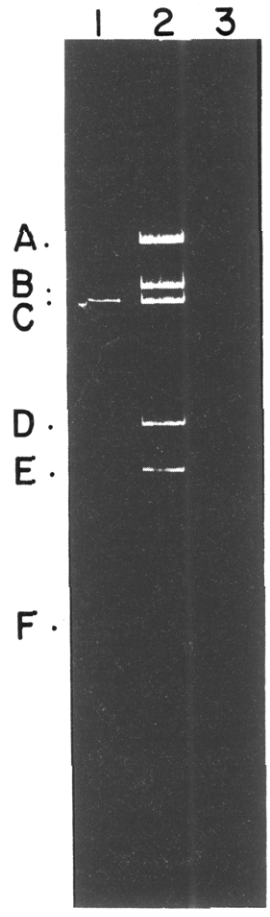

FIGURE 1: Specific binding of wheat germ RNA polymerase II to a restriction fragment containing a single-strand break. A 3.5% acrylamide gel stained with ethidium bromide is shown. A unique single-strand break was introduced into the SV40 genome at map position 0.725 as described in the text. The DNA was then digested with endonuclease *Hin*dIII into six fragments labeled A–F (lane 2); fragment C includes map position 0.725 and therefore contains the single-strand break. The mixture of fragments (2 μg) was incubated with RNA polymerase II (2.5 μg) as described, and those fragments which were retained on nitrocellulose filters as a consequence of bound enzyme are displayed in lane 1. Lane 3 shows an identical experiment using DNA which was not nicked.

crococcal nuclease. As the digestion proceeds the protected DNA is reduced to the limit size of ~38 base pairs for DNase I and ~35 base pairs for micrococcal nuclease. Smaller protected species accumulate on the autoradiograph as the digestion proceeds, suggesting that the protected DNA may be degraded slowly by nonrandom internal cleavages.

In order to investigate the internal structure of the binary complex, we subjected the nuclease-protected DNA to denaturing gel electrophoresis. Again, a specific limit-digest band of protected DNA (~10 bases long) is obtained after digestion with either nuclease (Figure 3). This band must represent a predominant *single-stranded* species which has as its 5′ terminus the ^{32}P introduced at the site of nicking and as its 3′ terminus a nuclease-sensitive cleavage site. Thus, RNA polymerase in the binary complex must be bound specifically at the nick in a manner which places a nuclease-sensitive site ~10 base pairs in the 3′ direction from the nick on the broken strand.

The remaining internal structure of binary complexes must be probed by using a different assay. A preferred method would be to isolate unlabeled protected DNA and ^{32}P-label all the denatured single strands at their 5′ termini with polynucleotide kinase. This assay proved not to be feasible due

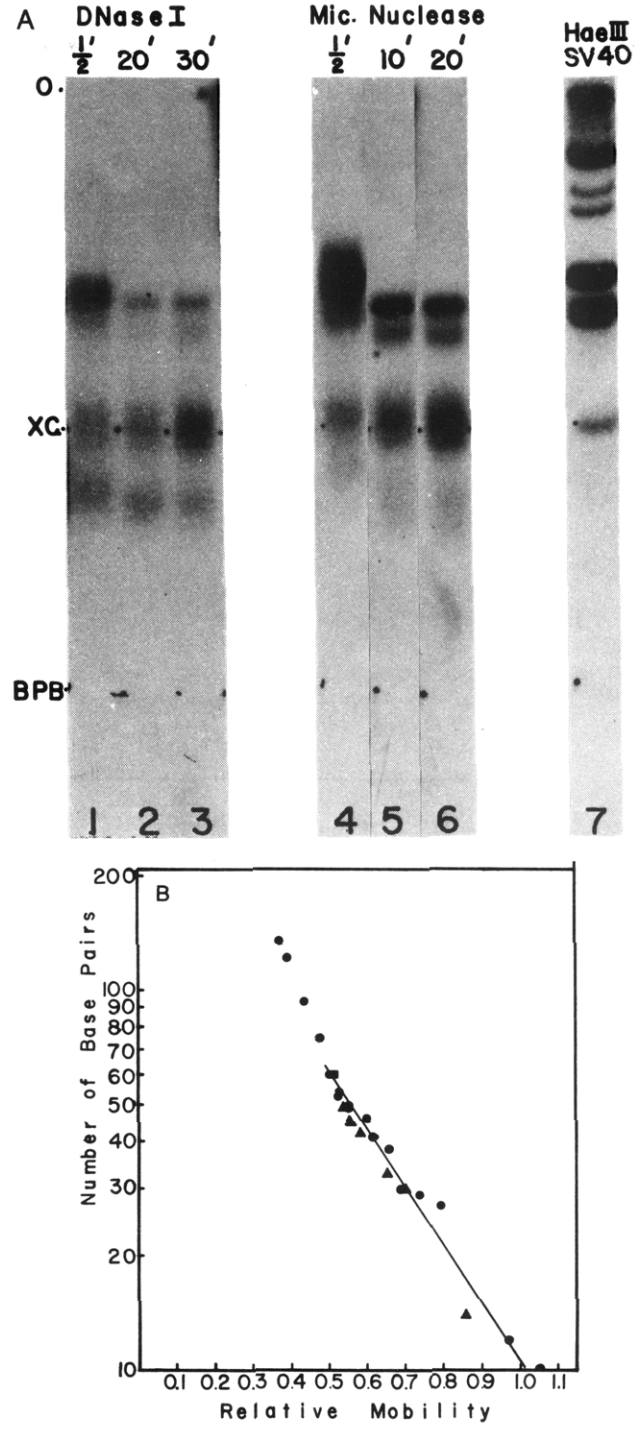

FIGURE 2: (A) Protection from nuclease digestion of a ^{32}P-labeled nick site on SV40 DNA by bound wheat germ RNA polymerase II. An autoradiogram of a Tris–borate–magnesium–15% native acrylamide gel is shown. Lanes 1, 2, and 3 display the results of digestion of 2 μg of ^{32}P-labeled nicked SV40 DNA–wheat germ RNA polymerase (5 μg) complexes by DNase I for 0.5, 20, and 30 min, respectively. Lanes 4, 5, and 6 show DNA protected by wheat germ RNA polymerase from micrococcal nuclease digestion for 0.5, 10, and 20 min. No bands were observed even after long film exposures when wheat germ RNA polymerase was omitted from the reaction mixture. The DNA was labeled specifically at the nick site by the method of Weiss et al. (1968). Lane 7 shows an overexposed *Hae*III digest of SV40 DNA which has been kinase labeled. The positions of xylene cyanol FF (XC) and bromophenol blue (BPB) are marked by dots. 0 marks the top of the gels. (B) Calibration of native 15% acrylamide gels. Digests of SV40 DNA by *Hae*III (▲) and *Alu*I (●) were run and the sizes estimated from the known nucleotide sequence used to calibrate all gels. Relative mobility refers to the distance migrated relative to xylene cyanol FF.

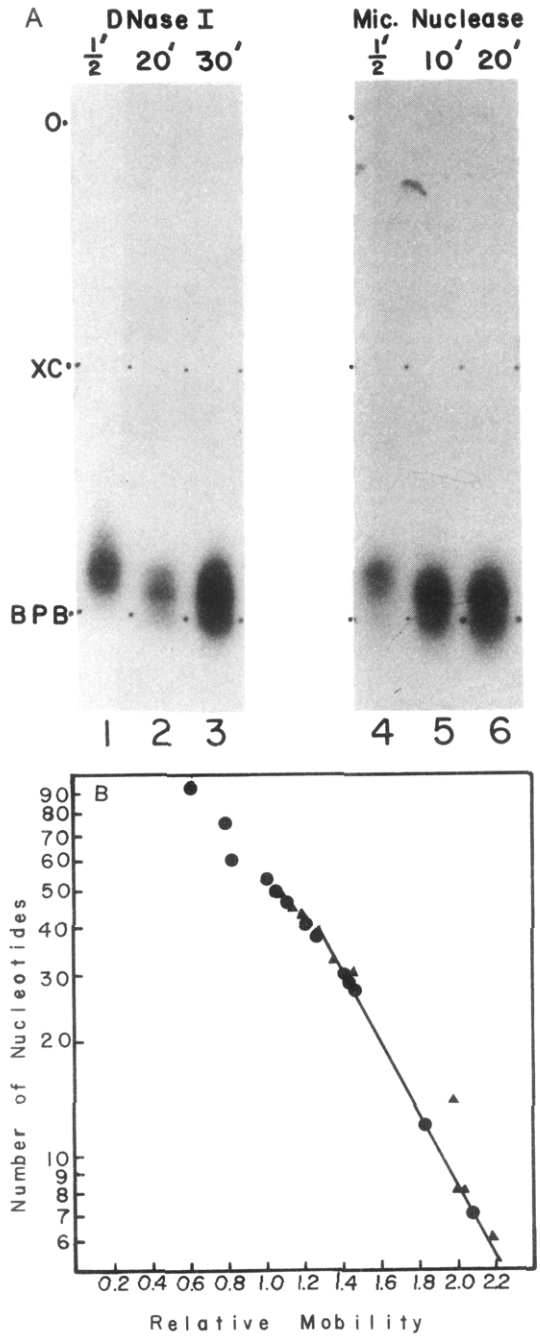

FIGURE 3: (A) Denaturing gel analysis of SV40 DNA kinase labeled at the nick and protected from nuclease digestion by wheat germ RNA polymerase II. Five micrograms of SV40 DNA which had been nicked at map position 0.725 and labeled with polynucleotide kinase was mixed with 5 μg of wheat germ RNA polymerase II in the buffer described. The complexes were digested with DNase I for 0.5, 20, or 30 min (lanes 1–3) or micrococcal nuclease for 0.5, 10, or 20 min (lanes 4–6) and collected by binding to nitrocellulose filters, and the DNA was eluted. Samples were analyzed by electrophoresis on denaturing 12% acrylamide–Tris–borate–EDTA–7 M urea gels. Control experiments with storage buffer substituted for wheat germ RNA polymerase II showed no protection even on long film exposures. XC and BPB mark the upper xylene cyanol FF and lower bromophenol blue tracking dyes. (B) Calibration of denaturing 12% acrylamide–urea gels. Digests of labeled SV40 DNA with endonucleases *Hae*III (▲) and *Alu*I (●) were used to calibrate all gels. Relative mobility refers to the distance migrated relative to xylene cyanol FF. Sizes were calculated from the known nucleotide sequence.

to the presence of excess 5′ ends from the nucleotide and oligonucleotide products of nuclease digestion. Therefore, the alternative method of labeling DNA by nick translation was used.

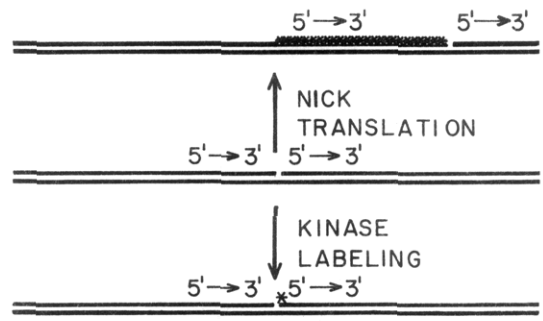

FIGURE 4: A schematic diagram showing the two means of labeling SV40 DNA in the vicinity of a single-strand break used in the experiments described. Nick translation replaced an average of ~20 adenosine phosphates with [α-^{32}P]deoxyadenosine per SV40 molecule. The 5′ → 3′ direction refers to the top strand in all cases. The region labeled by nick translation is indicated by (X). The position of kinase labeling is indicated by (*).

RNA Polymerase II Protection of Nick-Translated DNA. Labeling DNA by nick translation leads to high specific radioactivity 5′ to the final nick (see Figure 4). The location of this translated nick is no longer specific with respect to DNA sequence, regardless of whether the initial DNA is nicked specifically or randomly. DNA for this experiment was nicked randomly and labeled until only 1 to 2% of the adenosine phosphates were replaced with ^{32}P. This leads to DNA which is labeled to very high specific activity in the vicinity of the nick and negligible specific activity far from the nick.

When binary complexes formed between RNA polymerase II and nick-translated SV40 DNA are challenged with nucleases, a prominent radioactive DNA band appears after electrophoresis on a native polyacrylamide gel. During the early phases of digestion with DNase I (Figure 5) the band is rather broad and represents DNA in the size range 38–46 base pairs. At longer times the band becomes narrower and reaches a limit size of 37 base pairs. The length of the micrococcal nuclease protected DNA (Figure 5) approaches 33 base pairs, after the longest times of digestion, slightly shorter than that obtained by using 5′-^{32}P-labeled DNA (Figure 2). Longer exposures reveal a minor band of ~60 base pairs during the very earliest digestion time points and a minor band of ~20 base pairs accumulating during the latest times (not shown). The observed kinetics are consistent with successive digestion from 60 to 33 to 20 base pairs, but we have not investigated whether there exists a true precursor–product relationship.

The use of nick-translated DNA demonstrates again that binary complexes at nicks protect DNA from nuclease digestion and also allows the protection of the nicked strand to be probed further. That is, if these binary complexes are digested with nuclease and then subjected to denaturing polyacrylamide gel electrophoresis, all radioactive protected DNA bands revealed must originate 5′ to the nick on the broken strand (see Figure 4).

Figure 6 shows such an experiment and displays the radioactive single-stranded bands protected from nuclease digestion by bound RNA polymerase II. DNase I digestion (Figure 6) shows two resolved bands with lengths of ~10 and ~14 nucleotides. The ratio of radioactivity in these bands approaches approximately equimolar during the time course of digestion, suggesting that they are derived from the same class of RNA polymerase II binding sites. Micrococcal nuclease digestion reveals a single radioactive band of intermediate size, ~12 nucleotides long (Figure 6). These two experiments suggest that the internal structure of the binding site may be slightly more complex 5′ to the nick (Figure 6)

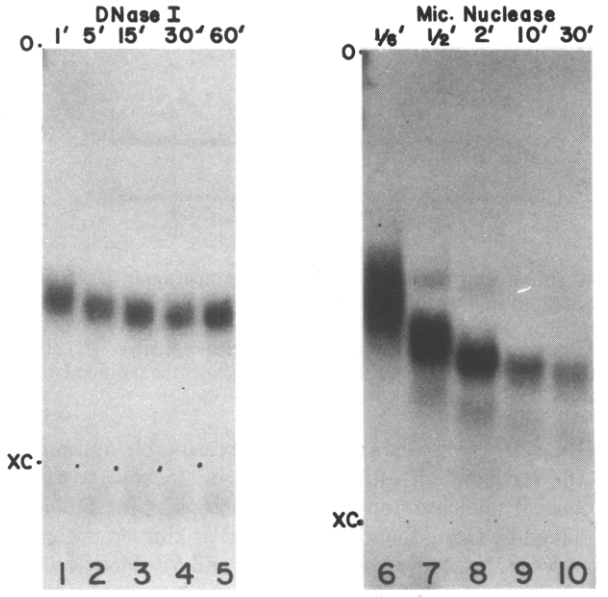

FIGURE 5: RNA polymerase II protection of nick-translated DNA. An autoradiograph of a 15% native acrylamide–Tris–borate–magnesium gel of nick-translated SV40 DNA protected from DNase I digestion by wheat germ RNA polymerase II is shown. Complexes of RNA polymerase II (12 μg) and SV40 DNA (1 μg) were digested and prepared for electrophoresis as described. Samples were removed after 1, 5, 15, 30, and 60 min of digestion for lanes 1–5, respectively. 0 marks the gel origin. A dot marks the position of xylene cyanol FF. Lanes in which storage buffer was substituted for RNA polymerase showed no protected species even after gel exposures 8 times that shown. Lanes 6–10 show an autoradiograph of an identical experiment except that the complexes were challenged with micrococcal nuclease. The times of digestion were 1/6, 0.5, 2, 10, and 30 min for lanes 6–10, respectively.

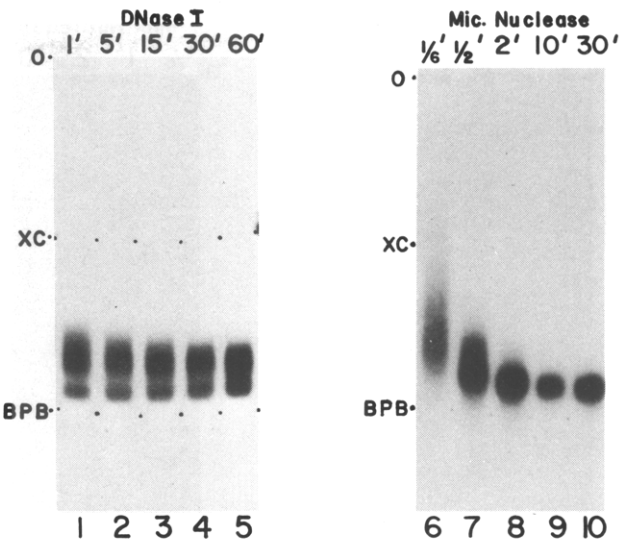

FIGURE 6: Denaturing gel electrophoresis of nick-translated protected DNA. Lanes 1–5 show DNA fragments protected from DNase I digestion by wheat germ RNA polymerase II as in Figure 5 which were analyzed on a denaturing 12% acrylamide–Tris–borate–EDTA–7 M urea gel. Lanes 1–5 contain DNA from samples digested for 1, 5, 15, 30, and 60 min, respectively. The ratio of radioactivity in the upper and lower bands decreased from 2.1 to 1.4 (equimolar) during the 60-min time course. Lanes 6–10 show a similar autoradiograph of DNA protected from micrococcal nuclease digestion by wheat germ RNA polymerase II as in Figure 5. Lane numbers 6–10 correspond to 1/6, 0.5, 2, 10, and 30 min of digestion, respectively. Dots mark upper xylene cyanol FF and lower bromophenol blue tracking dyes.

than 3′ to the nick (Figure 3); later we derive a self-consistent model which accommodates all of the data by inferring an internal single-strand nuclease cleavage site (see Discussion).

E. coli RNA Polymerase Protects Nicked DNA in a Very Similar Manner. Since there is very little information available comparing the tight binding of procaryotic and eucaryotic RNA polymerases, we used the above structural probes to make such a comparison. To our surprise, we found the pattern of both native and denatured protected DNA to be basically unchanged when *E. coli* RNA polymerase was substituted for RNA polymerase II (Figure 7). Specifically, the procaryotic enzyme also binds nicked DNA so as to provide protection of a slightly longer segment from DNase I digestion (38–44 base pairs) than from micrococcal nuclease digestion (33–36 base pairs). This is similar to the type of protection observed with the eucaryotic enzyme (Figure 5). The denaturing gel also reveals the characteristic pair of bands (14 and 10 bases long) for DNase I protected DNA and the single band of intermediate size (12 bases long) for micrococcal nuclease protected DNA. Thus, the procaryotic and eucaryotic enzymes, despite their extreme divergence in terms of subunit structure (Jendrisak & Burgess, 1977), are directed by single-strand breaks to protect DNA in a specific and nearly identical manner.

Discussion

Nicks Direct the Specific Placement of RNA Polymerase II. These results show that a single-strand DNA break can direct RNA polymerase II to bind SV40 DNA specifically (Figure 1). The binding site must include this nick since ^{32}P introduced at the site of nicking is protected against nuclease digestion by bound enzyme (Figure 2). Moreover, the nick occupies a specific position within the binding site. The evidence for this is that when binary complexes are challenged with nucleases and the protected DNA analyzed on denaturing gels, specifically sized segments of single-stranded DNA appear (Figures 3 and 6). The size of the protected single-stranded DNA must represent the distance between the nick and a nuclease cleavage site. If the placement of the nick were random, all sizes between 0 and 35 base pairs would be represented, contrary to the results obtained. This can be seen in Figure 3 where the distance between the nick and a DNase I cleavage site is found to be 10 base pairs.

A Model for Placement of RNA Polymerase II at Single-Strand Breaks. A model for the placement of RNA polymerase II at single-strand breaks, which derives from and is consistent with all our data, is presented in Figure 8. The most important features of this model are the concepts of specific placement of bound RNA polymerase II with respect to a single-strand break and the identification of approximate RNA polymerase II–DNA binding domains as defined by regions resistant to nuclease action. We consider the precise location of enzyme cleavage sites to be less certain as well as less important.

Briefly, this model (Figure 8) was derived as follows. The distances between the nick and the two external cleavages on the nicked (top) strand were estimated independently. The length 3′ to the nick is set at 10 base pairs as evidenced by the species appearing on the denaturing gel of protected DNA labeled with 5′-^{32}P at the site of the nick (Figure 3). The state of the remainder of the top strand is inferred from the species appearing on denaturing gels of nick-translated protected DNA (Figure 6). Our model places an internal single-stranded DNase I cleavage so as to produce a terminal 14-base species and an internal 10-base species. This arrangement is preferred because the shortening of the native protected DNA during the early stages of digestion, which we presume reflects trimming at ends, is reflected in the 14-base but not the 10-base single-strand species (see Figure 6). The point of internal

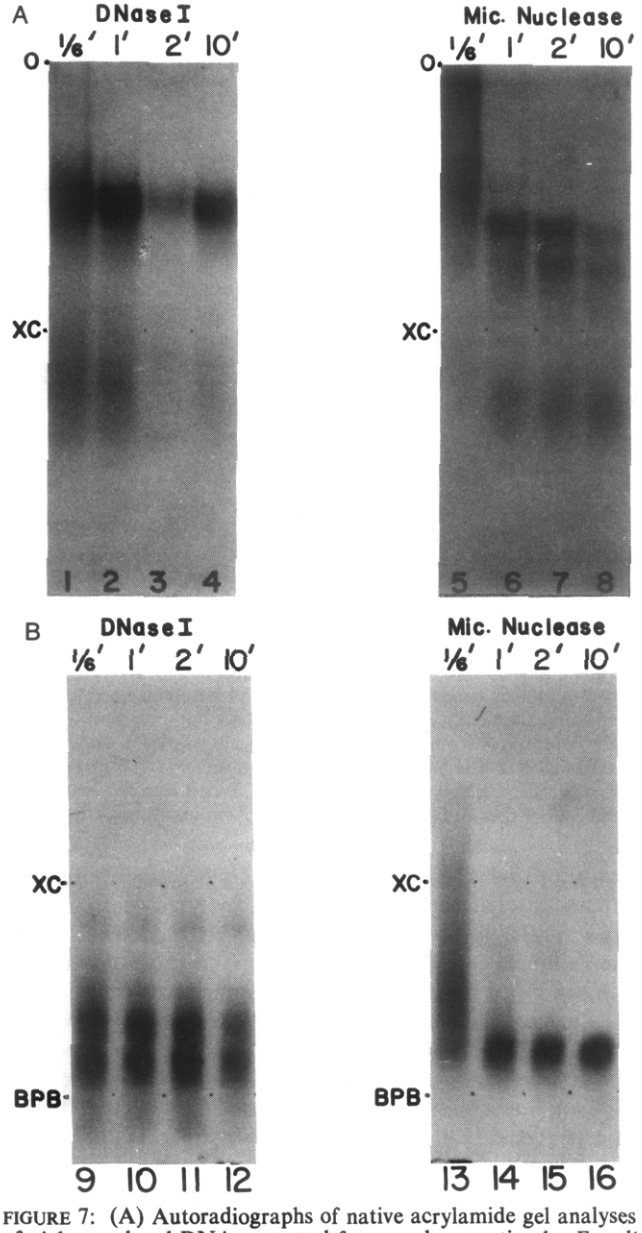

FIGURE 7: (A) Autoradiographs of native acrylamide gel analyses of nick-translated DNA protected from nuclease action by *E. coli* RNA polymerase are shown. Samples were prepared as in Figure 5 except that *E. coli* RNA polymerase and the appropriate buffer system replaced wheat germ RNA polymerase II. Lanes 1–4 display results obtained after digestion of complexes with DNase I for 1/6, 1, 2, and 10 min. Lanes 5–8 show bands representing DNA fragments protected from micrococcal nuclease digestion after 1/6, 1, 2, and 10 min. Dots mark the xylene cyanol FF tracking dye. DNA was analyzed on Tris–borate–magnesium containing 15% acrylamide gels. (B) Denaturing gel analysis of the same experiments for which native gels are shown in (A). Lanes 9–12 show nick translation labeled DNA protected from DNase I digestion by *E. coli* RNA polymerase. Times of digestion were 1/6, 1, 2, and 10 min. Lanes 13–16 show nick-translated DNA protected from micrococcal nuclease digestion by *E. coli* RNA polymerase for 1/6, 1, 2, and 10 min. Autoradiographs of 12% acrylamide–Tris–borate–EDTA–7 M urea gels are shown. Dots mark the upper xylene cyanol FF and lower bromophenol blue tracking dyes.

cleavage of micrococcal nuclease is placed so as to accommodate the appearance of two unresolved 12-base species (Figure 6). This site is consistent with the appearance of the ~20 base pair protected species which accumulate during long digestion times (Figures 2, 5, and 7).

If the bottom strand cleavages are arranged similarly, the average double-strand length of native protected DNA is predicted to be 34 base pairs for both micrococcal nuclease and DNase I. These estimates are in reasonable agreement with the independent estimates of 33 or 35 base pairs for micrococcal nuclease and 37 or 38 base pairs for DNase I determined by sizing the nick-translated or kinased protected native species, respectively (Figures 2, 4, and 5). Models which do not include internal cleavage sites cannot be reconciled with these latter results. In constructing the model, we have assumed for the sake of simplicity that the cleavages give "flush" DNA termini. This assumption is necessary since we have no independent information regarding the length of the bottom strand. Since the measured length of DNase I protected DNA appears to be 3 to 4 base pairs longer than presented in our model, it is possible that the bottom strand is slightly longer for protection from this enzyme. Therefore, the model derived simply represents an approximation where the complicating effects of specific DNA sequence and staggered nuclease cleavages are not taken into account. Although more complicated models may be constructed, any model must include the nick in a specified position within the binding site.

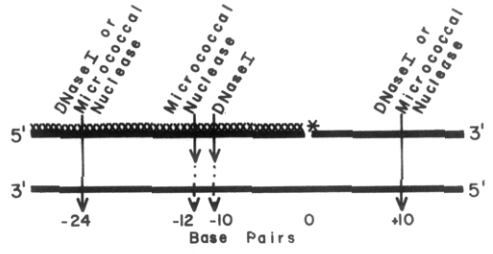

FIGURE 8: An approximate model for the protection of DNA from nuclease digestion by RNA polymerase bound at a site containing a single-strand break. Solid arrows refer to high-frequency cleavage sites. Dotted arrows refer to low-frequency cleavage sites. (X) shows the region labeled by nick translation and (*) shows the position labeled by polynucleotide kinase.

Similar Binding Domains of a Eucaryotic and Procaryotic RNA Polymerase in Binary Complexes. We have shown that the domains of tightly bound DNA in binary complexes, as defined by the nuclease-protected regions shown in Figure 8, are very similar for the procaryotic and eucaryotic RNA polymerases isolated from *E. coli* and wheat germ, respectively. The evidence for this is the identical patterns of both double-strand and single-strand DNA protected from digestion by DNase I and micrococcal nuclease. This suggests that a basic structural feature of binary complexes is preserved for the two enzymes, despite their evolutionary divergence and dissimilar subunit structure. RNA polymerase II isolated from wheat germ consists of two very large subunits and a pattern of smaller subunits (Jendrisak & Burgess, 1977) which is typical of eucaryotic RNA polymerase II [see Chambon (1975)]. *E. coli* RNA polymerase also includes among its subunits two very large species, β and β', which have been shown to be that part of the core enzyme which may be chemically cross-linked to DNA (Hillel & Wu, 1978; Simpson, 1979). Perhaps the common occurrence of these very large subunits is responsible for the similar binding domains of the procaryotic and eucaryotic binary complexes.

There are also some similarities between binary complexes formed at *E. coli* promoters and those found at nicks in this study. For example, the length of nicked native DNA protected from DNase I digestion is close to that protected by promoter-bound *E. coli* RNA polymerase [see, for example, Siebenlist (1979b)]. It seems likely that the introduction of a nick substitutes for some property of promoter sequence which can direct RNA polymerase to bind DNA in a manner which protects 37–40 base pairs from DNase I digestion. Weaker single-stranded internal cleavage sites could also be

identified in the promoter–*E. coli* RNA polymerase complex when probed carefully by partial DNase I digestion (Schmitz & Galas, 1979). Thus, binary complexes formed at nicks and promoters are similar superficially, suggesting that the process of DNA site selection and binding at nicks and promoters have in common a basic subset of molecular interactions. Since the eucaryotic and procaryotic enzymes protect at nicks in a similar and highly nonrandom way, it is reasonable to expect that the two enzymes may share a subset of interactions with DNA which are necessary but perhaps not sufficient for promoter binding.

Role of the Single-Strand Break. Our analysis has shown that the nick occupies a specific position within binary complexes. Since formation of the highly stable binary complexes studied here depends on the presence of nicks (see Figure 1), the specific positioning of the nick must facilitate a mechanistic step required for tight binding which this preparation of RNA polymerase II cannot normally accomplish on double-stranded SV40 DNA.

What aspect of binary complex formation might be facilitated by a single-strand break? One possibility is that the nick might facilitate the local melting of the double helix. This is suggested since *E. coli* RNA polymerase unwinds and locally denatures DNA in binary complexes (Wang et al., 1977; Siebenlist, 1979b) and nicks have been shown to promote melting of DNA (Paleček, 1976). Moreover, it has been proposed that local melting is the rate-limiting step in binary complex formation (Chamberlin, 1976; Seeburg et al., 1977). A specifically located nick could in principle serve as a nucleation site for local melting, facilitating the most difficult step involved in formation of binary complexes.

Formation of a binary complex does not necessarily imply transcriptional competence. For example, single-strand breaks are known to stimulate binding more dramatically than transcription for both *E. coli* RNA polymerase (Hinkle et al., 1972) and RNA polymerase II (Dreyer & Hausen, 1976; D. W. Chandler and J. Gralla, unpublished experiments). Whether stable binary nicked complexes can efficiently initiate a transcript probably depends on elements of DNA sequence (Stefano & Gralla, 1979; Dynan & Burgess, 1979).

Physiological Binding by RNA Polymerase II. We have argued that RNA polymerase II and *E. coli* RNA polymerase share certain determinants of binary complex formation since they protect nicked DNA in the same manner and since this protection is superficially similar to promoter protection by *E. coli* RNA polymerase. The logical extension of this argument would be to infer that since of the two only RNA polymerase II cannot form stable complexes with *intact* double-stranded DNA in vitro, this enzyme does not represent a holoenzyme. Functionally, this would mean simply that it is incapable of binding tightly DNA sequences which direct proper initiation of transcription. This could be due to subunits damaged or lost during purification or simply because the wheat germ enzyme is heterologous with respect to the template.

Recently, it has been reported that wheat germ RNA polymerase II forms complexes with heterologous adenovirus DNA exhibiting a half-life of 15 min (Seidman et al., 1979). However, two lines of evidence concerning wheat germ RNA polymerase II raise the possibility that these complexes may have formed at nicks, since the authors report that their DNA template contains on the average 1 nick/molecule. First, our results (Figure 1 and unpublished experiments) show that if a single nick is introduced into the smaller SV40 genome, binding at this site predominates under equilibrium conditions which are similar to those used for adenovirus DNA. Second, a transcription assay with phage λ DNA suggests that heparin-resistant complexes form preferentially at nicks and that these binary complexes decay with a half-time of 10–20 min (Dynan & Burgess, 1979). Taken together, these data suggest that nicks are the preferred sites for both binding and transcription and that 15 min may be an appropriate half-life for such interactions. Since the adenovirus DNA contained a single nick and directed formation of complexes with a 15-min half-life, we infer that these complexes may include nicks and the formation of stable complexes with double-stranded DNA remains to be demonstrated unequivocally.

There is evidence, nevertheless, that highly purified RNA polymerase II exhibits some sequence preference in association with DNA. Two electron microscopic studies have revealed such a preference by using polyoma DNA to observe nonrandom sites of transcription initiation (Lescure et al., 1978) or adenovirus DNA to observe nonrandom sites of association (Seidman et al., 1978). The latter study used an excess of RNA polymerase, 7 times the ratio (to DNA) used to obtain a complex half-life of 15 min as measured by the filter binding assay (discussed above). Therefore, the multiple binding sites observed after fixing for electron microscopy are not necessarily the same as those exhibiting the 15-min half-life. In both studies the binary complexes were of uncharacterized stability and kinetically trapped. Such interactions could be of significance or could simply represent preferences of incomplete enzymes for interaction with regions which might be melted easily or otherwise resemble promoters.

The observation that purified RNA polymerase II binds nicks specifically but binds intact double-stranded DNA poorly raises the question of whether the eucaryotic enzyme has evolved in some cases to recognize specifically placed single-strand breaks in DNA. There is at present no direct evidence excluding this possibility. In the case of RNA polymerase II transcription of SV40, two studies suggest that form I DNA is associated with certain elongation complexes (Birkenmeier et al., 1977; Green & Brooks, 1978). However, the methods used to isolate and detect these complexes would lead to disruption of any elongation complexes containing form II DNA and thereby prohibit their detection (Champoux & McConaughy, 1975). Moreover, nicks could direct the formation of preinitiation complexes in vivo which could be sealed prior to elongation by the rapid and abundant DNA sealing enzyme activity present in nuclei which acts on SV40 DNA (DePamphlis et al., 1975). In fact, specifically located nicks appear in SV40 chromatin during incubation of infected cell nuclear extracts (Scott & Wigmore, 1978; Waldeck et al., 1978). The location of this endogenous nuclease action on the SV40 genome has been shown to be between 0.67 and 0.73 map unit (Scott & Wigmore, 1978), which is also the region most likely to contain promoters (Lai et al., 1978; Mueller et al., 1978; Laub et al., 1979) as well as altered chromatin structure (Varshavsky et al., 1979) and the origin of replication (Fareed, 1972). We have shown that a specifically located nick at 0.725 map unit can indeed direct specific RNA polymerase II binding in vitro. Although these connections are tantalizing, there is no evidence at present to relate single-strand breaks to eucaryotic transcription in vivo. Such a relationship is unprecedented in procaryotic transcription and therefore must be considered unlikely in the absence of strong positive evidence.

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References

Birkenmeier, E. H., Radonovich, M. F., Shani, M., & Salzman, N. P. (1977) *Cell 11*, 495.
Burgess, R., & Jendrisak, J. (1975) *Biochemistry 14*, 4634.
Chamberlin, M. J. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 159–191, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Chambon, P. (1975) *Annu. Rev. Biochem. 44*, 613.
Champoux, J. J., & McConaughy, B. L. (1975) *Biochemistry 14*, 307.
DePamphlis, M. L., Beard, P., & Berg, P. (1975) *J. Biol. Chem. 250*, 4340.
Dreyer, C., & Hausen, P. (1976) *Eur. J. Biochem. 70*, 63.
Dugaiczyk, A., Boyer, H. W., & Goodman, H. M. (1975) *J. Mol. Biol. 96*, 171.
Dynan, W. S., & Burgess, R. R. (1979) *Biochemistry 18*, 4581.
Fareed, G. C., Garon, C. F., & Salzman, N. P. (1972) *J. Virol. 10*, 484.
Green, M. H., & Brooks, T. L. (1978) *J. Virol. 26*, 325.
Heyden, B., Nusslein, C., & Schaller, H. (1972) *Nature (London), New Biol. 240*, 9.
Hillel, Z., & Wu, C. W. (1978) *Biochemistry 17*, 2954.
Hinkle, D., & Chamberlin, M. J. (1972a) *J. Mol. Biol. 70*, 157.
Hinkle, D., & Chamberlin, M. J. (1972b) *J. Mol. Biol. 70*, 187.
Hinkle, D., Ring, J., & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 197.
Hirt, B. (1967) *J. Mol. Biol. 26*, 365.
Hossenlopp, P., Oudet, P., & Chambon, P. (1974) *Eur. J. Biochem. 41*, 397.
Jackson, A., & Sugden, B. (1972) *J. Virol. 10*, 1086.
Jendrisak, J., & Burgess, R. (1975) *Biochemistry 14*, 4639.
Jendrisak, J., & Burgess, R. (1977) *Biochemistry 16*, 1959.
Johnsrud, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5314.
Küpper, H., Contreras, R., & Khorana, H. G. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) p 473, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Lai, C. J., Dhar, R., & Khoury, G. (1978) *Cell 14*, 971.
Laub, O., Bratosin, S., Horowitz, M., & Aloni, Y. (1979) *Virology 92*, 310.
Lescure, R., Chester, A., & Yaniv, M. (1978) *J. Mol. Biol. 124*, 73.
Lilley, D. M. J., & Houghton, M. (1979) *Nucleic Acids Res. 6*, 507.
Maniatis, T., Jeffrey, A., & Kleid, D. (1975a) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1184.
Maniatis, T., Jeffrey, A., & van de Sande, H. (1975b) *Biochemistry 14*, 3787.
Maxam, A., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560.
Mueller, C., Graessmann, A., & Graessmann, M. (1978) *Cell 15*, 579.
Mueller, K. (1971) *Mol. Gen. Genet. 111*, 273.
Okamoto, T., Sugiura, M., & Takanami, M. (1972) *Nature (London), New Biol. 237*, 108.
Paleček, E. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 18*, 151.
Parker, R. C., Watson, R. M., & Vinograd, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 851.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891.
Radloff, T., Bauer, W., & Vinograd, J. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1514.
Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M. L., & Weissman, S. M. (1978) *Nature (London) 200*, 494.
Roeder, R. G. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 285–329, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sack, G. H., & Nathans, D. (1973) *Virology 51*, 517.
Saragosti, S., Lescure, R., & Yaniv, M. (1979) *Biochem. Biophys. Res. Commun. 99*, 1077.
Schmitz, A., & Galas, D. J. (1979) *Nucleic Acids Res. 6*, 111.
Scott, W. A., & Wigmore, D. (1978) *Cell 15*, 1511.
Seeburg, P., Nüsslein, C., & Schaller, H. (1977) *Eur. J. Biochem. 74*, 107.
Seidman, S., Surzycki, S. J., DeLorbe, W., & Gussin, G. N. (1979) *Biochemistry 18*, 3363.
Siebenlist, U. (1979a) *Nucleic Acids Res. 6*, 1895.
Siebenlist, U. (1979b) *Nature (London) 279*, 651.
Simpson, R. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3233.
Stefano, J. E., & Gralla, J. (1979) *Biochemistry 18*, 1063.
Varshavsky, A., Sundin, O., & Bohn, M. (1979) *Cell 16*, 453.
Waldeck, W., Fohring, B., Chowdhury, K., Gruss, P., & Sauer, G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5964.
Wang, J. C., Jacobson, J. H., & Saucier, J. (1977) *Nucleic Acids Res. 4*, 1225.
Weiss, B., Live, T., & Richardson, C. (1968) *J. Biol. Chem. 243*, 4530.