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Binding and Transcription of Simian Virus 40 DNA by DNA-Dependent RNA Polymerase from *Escherichia coli*

PATRICIA HALE AND JACOB LEBOWITZ*

Department of Microbiology and Comprehensive Cancer Center, University of Alabama in Birmingham, Birmingham, Alabama 35294

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Supercoiled simian virus 40 was transcribed more efficiently than was nonsupercoiled DNA. The effect was increased from two- to fivefold by the addition of rifampin with triphosphates. The number and locations of polymerase binding sites with respect to *Hin* II-III restriction fragments were determined. The total number of binding sites was nine, as determined by UV difference spectroscopy. The locations of these binding sites were on the A, B, D, E, F, and G fragments, as determined by gel electrophoresis. The number of sites was the same for both supercoiled and relaxed or *Hin* II-III-digested DNA, and the point of saturation of supercoiled DNA by polymerase remained the same with increasing concentrations of rifampin from 0 to 8 μg/ml.

Several features of transcription of simian virus 40 (SV40) DNA I by *Escherichia coli* RNA polymerase suggest similarities between in vitro and in vivo transcripts. RNA made by *E. coli* polymerase produces T antigen and stimulates DNA synthesis when transferred by microinjection into epithelioid primary mouse kidney cultures (6). A cell-free linked transcription-translation system has been developed, using *E. coli* RNA polymerase and wheat germ extracts. Authentic viral polypeptides are synthesized with viral polypeptide I in relative abundance. A number of polypeptides are precipitated with antiserum to SV40 T antigen (17). Although the production of mRNA in eucaryotic cells is quite complex (3), it appears that *E. coli* RNA polymerase is also able to recognize "active" chromatin and to produce functional mRNA (15). Consequently, the SV40 system should allow a comparison between transcription by bacterial and mammalian RNA polymerases.

In vitro transcription of supercoiled SV40 DNA has been studied by a variety of methods. However, the locations and number of polymerase binding sites have not yet been completely determined. Five ATP initiation sites for *E. coli* RNA polymerase have been mapped by Lebowitz et al. (12), but the locations of three or four GTP initiation sites are still unknown. In addition, it has not yet been shown that any of these initiation sites also corresponds to a polymerase binding site.

We have used difference spectroscopy, rifampin inhibition, and gel electrophoresis to determine the number, relative spectral changes, and locations of *E. coli* RNA polymerase binding sites on supercoiled and nonsupercoiled SV40 DNA to answer some of these questions. The results suggest that the difference in template ability between supercoiled and nonsupercoiled DNA may be due to a difference in strength of polymerase binding. Further evidence to support this hypothesis is described in the accompanying paper, where a reagent has been used to determine the involvement of single-strand-like, or hairpin, regions in this process.

MATERIALS AND METHODS

Polymerase binding to *Hin* II-III fragments of SV40 DNA. Polymerase binding to *Hin* II-III fragments was a modification of the procedure developed by Jones and Reznikoff (11). SV40 DNA I (100 μg) was digested by 25 U of *Hin* II-III restriction endonuclease in a reaction buffer containing 5 mM NaCl, 6 mM Tris (pH 7.4), 6 mM $MgCl_2$, and 200 mg of bovine serum albumin per ml.

After *Hin* digestion, 30 μg of the digested DNA was incubated with 20 μl (100 μg) of *E. coli* polymerase in a buffer containing 6 mM Tris (pH 7.9), 6 mM $MgCl_2$, 6 μM dithiothreitol, 60 μM EDTA, 3 mg of bovine serum albumin per ml, 0.2 M KCl, and 8% glycerol.

This mixture was incubated for 30 min at 37°C, 150 μl (870 mg) of heparin was then added to inactivate free polymerase, and the incubation was continued for 10 min more at 37°C. The incubation mixture was then filtered gently on a nitrocellulose B-6 Schleicher & Schuell filter. The filter was washed four times with 1 ml of 10 mM Tris (pH 7.9)-10 mM $MgCl_2$-0.1 mM dithiothreitol-0.1 mM EDTA-0.05 M KCl. After the washes, the filter was incubated in 1.5 ml of 2% sodium dodecyl sulfate (SDS) for 3 h at 37°C with gentle shaking. This step results in dissociation of the DNA fragments from the polymerase and their release from the filter. The SDS was then removed by applying

the sample to a 50-ml Sephadex G-25 column. The DNA fragments were then concentrated to 20 μl by vacuum concentration. Five microliters of 5% SDS–50% glycerol was then added to the sample, and it was incubated for 30 min at 65°C and applied to a 4% polyacrylamide gel. One microgram of *Hin* II-III-digested DNA was given the same SDS-heat treatment and run on a gel at the same time to be used as a marker for identification of the *Hin* II-III fragments. The samples were applied to 10-cm gels, which were run for 16 h at 40 V. Gels were removed and stained for visualization in 0.5 μg of ethidium bromide per ml.

Preparation of SV40 DNA. CV-1 cells were grown in minimum essential medium (Eagle) with Hanks salts, 2 mM glutamine, and 10% fetal calf serum. Confluent monolayers were infected with SV40 (small-plaque strain, obtained from D. Nathans) at an input multiplicity of 5 to 20 PFU per cell in minimum essential medium, 2 mM glutamine, and 2% fetal calf serum. After a 36-h incubation at 37°C, supercoiled SV40 DNA I was recovered according to the Hirt procedure (9) followed by ethanol precipitation, subsequent sedimentation to equilibrium in CsCl-ethidium bromide gradients, and storage in 6 mM Tris-hydrochloride (pH 7.3)–3 mM EDTA at −20°C.

Polymerase binding by difference spectra. The procedure for polymerase binding by difference spectra was a slight modification of that described by Sarocchi and Darlix (18, 19). The experiment was conducted with Beckman tandem double-sector cells, i.e., cells split by a quartz plate perpendicular to the light path. Two such tandem cells were placed in a recording spectrophotometer (Beckman Acta V), one in the reference, the other in the sample compartment.

Initially, both cells were filled in the same manner. Buffer was placed in one sector, and DNA was placed in the other. To perform the binding titration, RNA polymerase was added to the DNA sector of the sample compartment and to the buffer sector of the reference compartment. In this configuration, reference and sample beams pass through exactly the same materials, the only difference being that in the sample compartment RNA polymerase binds to the DNA. Consequently, we obtain the difference spectra involved in the interaction of the enzyme with the DNA.

E. coli RNA polymerase (20.5 μg per point) was added to 42 μg of DNA per ml in a total volume of 1 ml of binding buffer, consisting of 0.04 M Tris (pH 7.9), 0.01 M $MgCl_2$, and 0.05 M NaCl. DNA and polymerase were incubated together for 10 min after each polymerase addition before the optical density was measured and recorded at 260 nm. Temperature was controlled at 37°C throughout each binding experiment by using thermostated cuvette holders controlled by a Lauda water bath.

RNA polymerase. RNA polymerase was purified from *E. coli* cells according to the procedure of Burgess and Jenrisak (2). Sigma content was determined by rifampin inhibition, according to the method of Mangel and Chamberlin (14). All enzyme preparations had a sigma content of >85%.

Enzymes. *Hin* II-III endonuclease was purchased from New England Biolabs, Beverly, Mass., or prepared by the method of Smith (21). *Hpa* II was also purchased from New England Biolabs.

Transcription assay. The assay mixture consisted of: 40 mM Tris-hydrochloride (pH 8.0); 8 mM $MgCl_2$; 0.1 mM dithiothreitol; 0.8 mM K_2HPO_4; 100 mM KCl; 0.5 mg of bovine serum albumin per ml; 2.5 mM each of ATP, UTP, GTP, and CTP. [^{3}H]ATP was added to the triphosphates such that a 1:12.5 dilution of the triphosphates gave 1 nmol of ATP to between 8 and 12,000 cpm. Length of assay and DNA, RNA polymerase, and rifampin concentrations were as described in the figure legends. In all experiments, DNA and polymerase were incubated together for 10 min at 37°C before the triphosphates were added.

RESULTS

In vitro transcription of SV40 DNA by *E. coli* RNA polymerase in the presence or absence of rifampin. The antibiotic rifampin may be used to select for strong promoter sites (sites that have a very strong binding affinity for polymerase) by its irreversible binding and inactivation of the polymerase through the β subunit of the enzyme. When rifampin is added to DNA and polymerase before addition of triphosphates, neither supercoiled (FI) nor nicked-circle (FII) DNA will be transcribed. If, however, the antibiotic is added in conjunction with the triphosphates, after the DNA and polymerase have been incubated together to allow the formation of "pre-initiation complexes," FI and FII transcription may be differentiated (8, 14). Figure 1 shows these effects on the transcription of SV40 DNA by RNA polymerase from *E. coli*. In the absence of rifampin, FI DNA was approximately a 2.5 times better template than FII DNA. The effect was increased to fivefold when 4 μg of rifampin per ml was added with the triphosphates. Little difference was observed between FI with rifampin added with the triphosphates and FI with no rifampin added. These results are in agreement with those of Mandel and Chambon (13) for SV40 and those obtained in the φX174, PM2, and λ systems, where, respectively, 20-, 10-, and 5-fold differences were observed for transcription of FI DNA over FII DNA in the presence of rifampin (1, 5, 8, 16).

Binding of *E. coli* RNA polymerase to Hin II-III-digested SV40 DNA fragments. The sites for *E. coli* RNA polymerase binding may be determined by binding the polymerase to *Hin* II-III SV40 DNA fragments followed by filtration on nitrocellulose filters. The polymerase is trapped by nitrocellulose, and thus any fragments that have polymerase bound to them will also be trapped. The fragments are then dissociated from the polymerase by SDS and run on 4% polyacrylamide gels. *E. coli* RNA polymerase holoenzyme is used to ensure no binding to fragment ends. Free polymerase is inactivated by heparin after polymerase-DNA

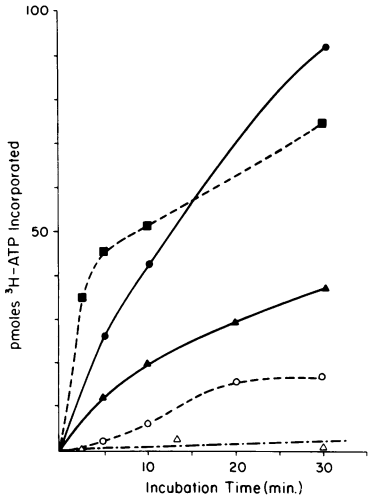

FIG. 1. *In vitro transcription of SV40 DNA by E. coli RNA polymerase in the presence or absence of 4 μg of rifampin per ml. Transcription assay was as described in the text. Symbols:* (△) *FI or FII DNA with rifampin added before the triphosphates;* (○) *FII DNA with rifampin added with the triphosphates;* (▲) *FII DNA with no rifampin added;* (■) *FI DNA with rifampin added with the triphosphates;* (●) *FI DNA with no rifampin added. All assays contained 2 μg of DNA and 25 μl of a 1:75 dilution of E. coli RNA polymerase (~0.8 mg/ml).*

binding has taken place. The results of these binding experiments are shown in Fig. 2. Polymerase bound to fragments A, B, D, E, F, and G. Binding to fragment F was found to be less consistent than to the other fragments and, in some experiments, could only be observed as a light band.

RNA polymerase was also bound to SV40 DNA I, and *Hin* II-III digestion was attempted. This proved unfeasible for two reasons. The heparin that was added to inactivate unbound polymerase interfered with *Hin* II-III digestion. In addition, polymerase appeared to block *Hin* II-III digestion as well. Consequently, restriction endonuclease treatment had to be performed before RNA polymerase binding to determine the fragments containing promoter sites.

Determination of number of binding sites by difference spectroscopy. Binding fragments and analysis by nitrocellulose filtration and electrophoresis allow location of binding sites to *Hin* fragments but do not allow determination of the number of binding sites in each fragment. Difference spectroscopy may be used to determine the number of binding sites as well as the relative spectral changes produced by the various forms of SV40 DNA. As RNA polymer-

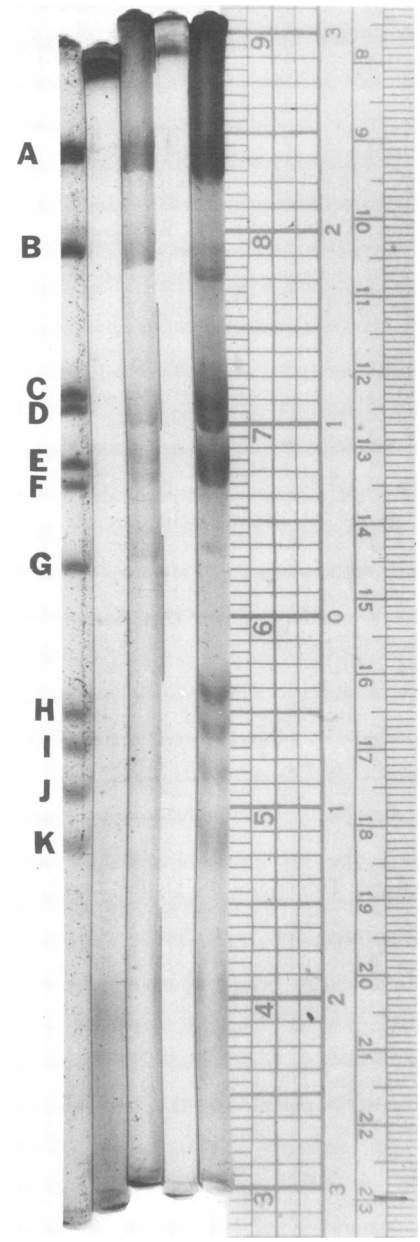

FIG. 2. *Identification of E. coli RNA polymerase-bound Hin II-III endonuclease-digested SV40 DNA fragments. E. coli RNA polymerase was incubated with 30 μg of Hin II-III-digested SV40 DNA, filtered on nitrocellulose filters, and subjected to electrophoresis on 4% polyacrylamide gels as described in the text. The leftmost and rightmost gels represent controls of 1 μg of Hin-digested SV40 DNA. The center gel represents the polymerase-bound SV40 DNA fragments. The other two gels represent nondigested SV40 FI DNA. The six bands observed correspond to Hin fragments A, B, D, E, F, and G.*

ase binds to DNA, a hypochromic shift at 260 nm may be observed (18, 19). This shift is observed until all binding sites on the DNA are saturated. At saturation, a break in the hypochromic shift is observed, and the number of polymerase molecules per DNA molecule bound at saturation may be determined from this breaking point.

This technique has been used to determine the number of polymerase binding sites on T7 DNA (18, 19) and is in agreement with the number determined by other methods (4). Figure 3 shows the application of this technique to SV40 DNA. The hypochromic shift for supercoiled DNA was approximately two to three times that for relaxed, or linear, DNA. This difference in hypochromicity is in agreement with the increased amount of transcription of supercoiled DNA, as compared with relaxed DNA, shown in Fig. 1, where the transcriptional ability of supercoiled DNA was also two- to threefold that of nonsupercoiled DNA. In these experiments, linear DNA was prepared by digestion with *Hpa* II endonuclease, which cuts in *Hin* II-III fragment C, a fragment that does not contain an *E. coli* RNA polymerase binding site (see Fig. 2). This enzyme was used to ensure that no binding sites might be lost by endonuclease cleavage.

Both hypochromicity plots in Fig. 3 break at approximately 9 molecules of RNA polymerase per DNA molecule. This result reproducibly fluctuated between 8.8 and 9.1 molecules, with maximum errors in optical density readings of no more than 2%. Break points in the hypochromicity plots were determined by least-squares fittings of the lines. Line fitting was reproducible within 3%.

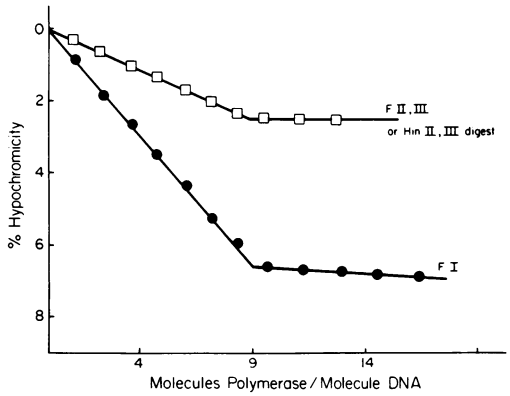

FIG. 3. *Hypochromic shifts observed due to E. coli RNA polymerase binding to SV40 DNA. SV40 DNA (42 μg) was incubated with increasing concentrations of enzyme as described in the text. Symbols: (●) SV40 I DNA; (□) FII, FIII, or Hin II-III-digested SV40 DNA.*

Number of polymerase binding sites on *Hin* II-III-digested SV40 DNA. The location of polymerase binding sites was done on *Hin* II-III-digested SV40 DNA fragments (Fig. 2). To be sure that no binding sites are lost due to *Hin* II-III cleavage of FI DNA, the number of binding sites on *Hin* II-III DNA fragments was also determined by hypochromicity changes, using the same method as that for FI and FII or FIII DNA. Figure 3 also shows nine binding sites for both the *Hin* II-III fragments and the supercoiled DNA at the same DNA concentrations. No binding sites are, apparently, lost by the *Hin* II-III cleavage. The hypochromicity for binding of polymerase to the fragments shows the same amount of shift as for linear or nicked SV40 DNA. These results suggest that the polymerase binding sites observed by binding polymerase to *Hin* II-III fragments and filtration may also represent the locations of these sites in supercoiled DNA.

Polymerase saturation of SV40 DNA in the presence of increasing amounts of rifampin. The antibiotic rifampin (when added with the triphosphates) may be used to select for binding tightness of polymerase to DNA. As rifampin concentration is increased, polymerase molecules that are bound at weaker binding sites may be selected out, resulting in a lower saturation point for the DNA. This method was used to measure the relative binding tightnesses of polymerase sites on supercoiled SV40 DNA. Figure 4 shows the results of this experiment. At increasing rifampin concentrations from 0 to 8 μg/ml, the saturation point for supercoiled SV40 DNA remained the same. These results suggest that all binding sites on the supercoiled DNA molecule have similar binding tightnesses at 37°C and that the number of binding sites in the presence or absence of rifampin remains the same. However, it will be shown in the accompanying paper (7) that when SV40 DNA I is chemically modified at polymerase binding sites, increasing rifampin concentrations result in lower saturation points, indicating that binding sites may show different affinities when the DNA is specifically modified.

DISCUSSION

We have determined nine polymerase binding sites, as measured by hypochromicity shifts, and have located these binding sites in *Hin* II-III endonuclease fragments A, B, D, E, F, and G. These results are compared to those obtained by fingerprint analysis of *E. coli* RNA initiation

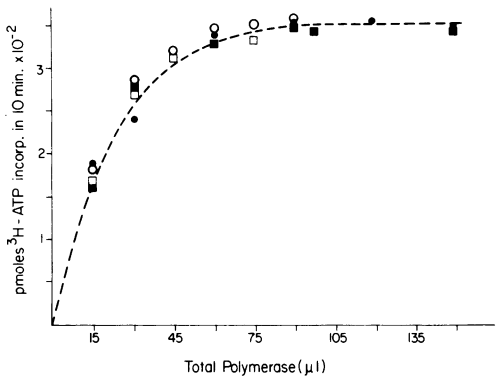

FIG. 4. *E. coli RNA polymerase saturation of SV40 FI DNA in the presence of different amounts of rifampin. Transcription assay was as described in the text. Symbols:* (○) *No rifampin;* (□) *2 μg of rifampin per ml;* (■) *4 μg of rifampin per ml;* (●) *8 μg of rifampin per ml. Rifampin was always added with triphosphates. All assays contained 1 μg of SV40 FI DNA and given amounts of a 1:75 dilution of E. coli RNA polymerase (~6 mg/ml).*

sites by Lebowitz et al. (12) in Fig. 5. The arrows represent the locations of five ATP initiation sites for *E. coli* RNA polymerase that they have mapped in *Hin* II-III fragments A and G. They have also observed three to four GTP initiation sites, which they did not attempt to locate. From a comparison of their results and ours, these GTP sites may be located in *Hin* II-III fragments D, E, F, and G, which we observe to also contain polymerase binding sites. Experiments are now planned to confirm these binding sites as GTP initiation sites.

In our hypochromicity experiments, we have found that, although the number of molecules of polymerase bound per DNA molecule remains the same, the amounts of hypochromic shift observed with polymerase addition differ significantly between supercoiled and nonsupercoiled DNA. Although the nature of the hypochromism has not been fully explored, a comparison of the binding of spermine, albumin, rho factor, core polymerase, and holopolymerase reveals that the more specific the binding of the molecule to DNA, the greater the magnitude of the hypochromic effect (18, 19). Consequently, the decrease in hypochromic shift for nonsupercoiled DNA suggests a weaker binding of polymerase to these forms of DNA. The hypochromic shift observed may be explained by a stacking of unpaired bases by polymerase binding. Unpaired regions in supercoiled DNA may be stacked by polymerase binding, allowing a larger hypochromic shift. With nonsupercoiled DNA, the smaller hypochromic shift may be due to the necessity for an unwinding of the DNA at these same binding sites before base stacking by polymerase may be allowed.

Recently, Shen and Hearst (20) have mapped hairpin sites that occur in single stranded SV40 DNA by trimethylpsoralen cross-linking. These sites are represented by the solid lines in the middle circle of Fig. 5. A total of six sites were observed, all of which occur in or very near fragments containing *E. coli* RNA polymerase binding sites.

Hsu and Jelinek (10) have also recently mapped hairpin regions in single-stranded DNA by electron microscopy; these are represented by the solid lines in the innermost circle of Fig. 5. Their results are similar to those of Shen and Hearst (20) except that they also report a hairpin region at 0.47 to 0.52 map units in the *Hin* A fragment. These investigators have also reported "rabbit ear" structures in the region of the B-G

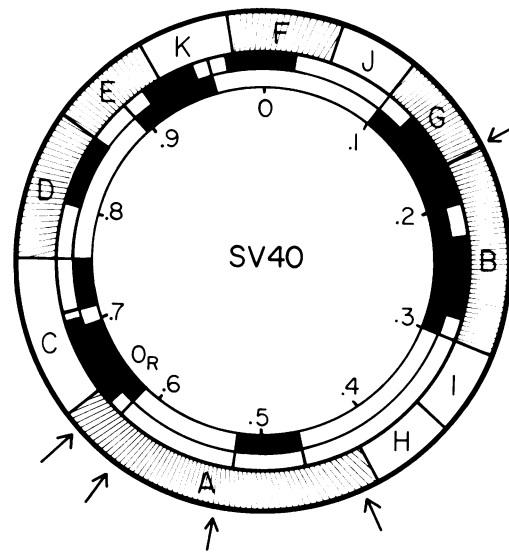

FIG. 5. *SV40 map of hairpin regions in single-stranded DNA and Hin II-III fragments binding E. coli RNA polymerase. Hatched regions in the outermost circle represent Hin fragments binding E. coli RNA polymerase. Arrows represent [γ-^{32}P]ATP initiation sites mapped by Lebowitz et al. (12). Solid regions on the innermost circle represent sites where hairpin regions have been mapped in single-stranded DNA by Hsu and Jelinek (10). Solid regions in the middle circle represent sites where hairpin regions have been mapped in single-stranded DNA by trimethylpsoralen cross-linking by Shen and Hearst (20). Early in vivo RNA transcripts have been mapped from 0.65 to 0.17 map units and are transcribed counterclockwise on the map from the DNA minus strand, whereas late in vivo mRNA's have been mapped from 0.65 to 0.17 map units and are transcribed clockwise on the map from the plus strand of the DNA. (See Shen and Hearst [20] for references on in vivo mRNA's.)*

junction. This structure would be in complete agreement with the polymerase binding sites we observe in this region. If this structure stradles the B-G *Hin* cleavage site, the binding sites would not be destroyed by *Hin* cleavage, as we have observed to be the case. This rabbit ear structure may provide two polymerase binding sites in close conjunction, one in the B fragment and one in the G fragment. Figure 4 shows the effects of increased rifampin concentration on saturation of SV40 DNA by *E. coli* RNA polymerase. No effect was observed when concentrations of up to 8 μg/ml were used. These results suggest that all of the polymerase binding sites have similar affinities for polymerase under these conditions, also supporting the proposal that, under standard conditions, the increased transcription observed from the B-G region (22) may be due to the existence of two adjacent binding sites rather than to one binding site with greater affinity for polymerase than binding sites located in other regions of the DNA.

A recent report of the use of a linked transcription-translation system for the analysis of SV40 shows that viral polypeptide I is produced in substantial amounts (17). This can only occur if in vitro transcription is partially symmetrical. Evidence that 10 to 20% of the mRNA is symmetrically transcribed (17) supports the possibility that these transcripts are for viral polypeptide I and that this synthesis from the late region is preferential. Our ability to detect an *E. coli* RNA polymerase binding site in the E fragment in addition to the mapping in this fragment of a single-strand hairpin close to the 5′ end of the late 16S in vivo mRNA suggests that authentic viral polypeptide I could be made in vitro by initiating a late class of mRNA in the E fragment (Fig. 5). It is clear that a correspondence exists between binding sites recognized by *E. coli* RNA polymerase and the 5′ ends of late 19S (fragment D) and 16S (fragment E) mRNA's. This is also true of early in vivo 19S mRNA, although other binding sites exist in the early region as well. It is possible that the hairpins observed in single-stranded SV40 DNA (10, 20) may serve the precursors of cytoplasmic mRNA by acting as recognition sites for processing. If *E. coli* RNA polymerase recognizes such sites exposed in SV40 DNA FI and initiates transcription, a functional mRNA may be produced. These speculations are open to experimental analysis.

Further support for the involvement of single-strand regions in the binding of polymerase to DNA is given in the accompanying paper (7). A single-strand-specific reagent was used to determine the effect of modification of single-strand regions in supercoiled DNA on RNA transcription. We will show that this affects the binding of polymerase to the DNA template (7). Potential hairpin structures may provide polymerase recognition sites for specific strong promoter complexes. These types of structures may also be involved in binding of mammalian polymerase, although initiation sequences for RNA synthesis may differ between the two systems.

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