Mapping of the SV40 Specific Sequences Transcribed in Vitro from Chromatin of SV40 Transformed Cells†

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ABSTRACT: Chromatin was isolated from SV40 transformed mouse cells (SV3T3) and transcribed with *Escherichia coli* RNA polymerase. The SV40 specific transcripts were analyzed by annealing the RNA to the minus strands of purified fragments of SV40 DNA produced by cleavage of the DNA with a restriction enzyme isolated from *Hemophilus aegyptius*. Quantitation of the frequency of transcription from the regions represented by the fragments showed that the early region (fragments A and D) was transcribed five to ten times more frequently than the remaining regions. These results are in good agreement with the transcription pattern observed in the transformed cell. In contrast, transcription of purified SV3T3 DNA by *E. coli* polymerase produced roughly equal frequencies of transcription from all regions of the integrated SV40 DNA. Comparison of the results with the known distribution of initiation sites for *E. coli* RNA polymerase on linear SV40 DNA indicates that the major initiation site is relatively unavailable in SV3T3 chromatin whereas other sites are available. This restriction is not observed when purified SV3T3 DNA is used as a template and must therefore result from the association of protein or other macromolecules with the DNA of the chromatin.

Evidence is accumulating in support of the concept that the structure of chromatin plays an important role in regulation of transcription in eucaryotic cells. Early evidence indicated that, with respect to the repetitive sequences of the genome, the DNA sequences of isolated chromatin that were available for transcription by added heterologous polymerase were the same sequences that were transcribed in vivo (Huang and Huang, 1969; Paul and Gilmour, 1968; Smith et al., 1969; Tan and Miyagi, 1970). This finding has now been extended to unique sequences of the DNA, i.e., the SV40 genes in SV3T3 cells (Astrin, 1973; Shih et al., 1973) and the globin genes in hemopoietic tissue (Axel et al., 1973; Gilmour and Paul, 1973; Steggles et al., 1974). Chromosomal proteins have been directly implicated in the transcriptional specificity observed in vitro (Paul et al., 1973), and one mechanism for transcriptional regulation in vivo may be that these proteins prevent transcription of the region of the DNA to which they are bound. Such a mechanism probably accounts for the observation that mouse satellite DNA is not transcribed in vivo nor in isolated chromatin but is transcribed when deproteinized DNA is used as a template (Reeder, 1973).

One system that may be used to advantage to study transcriptional regulation is that of the integrated SV40 genes in the chromatin of SV3T3 cells, a line of mouse fibroblasts transformed by SV40 virus. The SV40 genome is integrated into cellular DNA (Sambrook et al., 1968) and although the entire viral genome is present, less than 70% of the genome is transcribed in transformed cells (Tonegawa et al., 1970; Sambrook et al., 1972). The major transcript, which apparently codes for proteins involved in the expression of antigens specific for transformed cells, is transcribed from about 50% of the "−" strand of the integrated genome (Khoury et al., 1975). Previous findings, utilizing radioactive transcripts of isolated SV40 DNA as probes, have indicated that this same region is transcribed in vitro when SV3T3 chromatin is used as a template for *E. coli* RNA polymerase (Astrin, 1973). In the present study, the SV40 specific sequences transcribed from chromatin have been mapped with respect to DNA fragments produced from the cleavage of SV40 DNA with a restriction endonuclease isolated from *Hemophilus aegyptius*, endonuclease R·HaeIII (Edgell et al., 1972). The results indicate that the major in vitro transcripts map in the region that is transcribed in vivo. In addition, a model is proposed with respect to the availability in chromatin of the known initiation sites for *E. coli* RNA polymerase within the SV40 genome.

Materials and Methods

Cells and Virus. The origins and method of cultivation of cell lines 3T3 (Todaro and Green, 1966) and SV3T3 (Sambrook et al., 1968) have been described. SV40 small plaque virus was obtained from Dr. John Newbold, University of North Carolina at Chapel Hill.

Enzymes. E. coli DNA polymerase purified to homogeneity was a gift of Dr. Lawrence Loeb. *E. coli* RNA polymerase was purified according to Burgess (1969) on agarose columns. Endonuclease R·HaeIII was a gift of Dr. John Newbold.

In Vitro Labeling of SV40 DNA. DNA was prepared from small plaque SV40 virus as described by Huang et al. (1973). The DNA was labeled in vitro by the method of J. Summers (manuscript in preparation). Single-stranded circular SV40 DNA was produced by limited endonucleolytic digestion of component I DNA with pancreatic DNase and separation of circles from linears on an alkaline sucrose gradient. Purified circular DNA was used as a template for *E. coli* DNA polymerase I with the addition of random oligonucleotide primers (6 per circle) produced by the method of Dumas et al. (1971). The reaction mixture contained 0.1 *M* Tris (pH 7.5), 7 m*M* $MgCl_2$, 100 μM each of dATP,

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dGTP, and dCTP, 20 μM [^{3}H]dTTP (50 Ci/mmol), 1 μg of DNA polymerase, and 1 μg of single-stranded circular SV40 DNA in a final volume of 0.1 ml. The reaction was incubated for 4 hr at 15°. The product consisted of 2 μg of double-stranded DNA containing one nonradioactive strand (the template strand) and one newly synthesized radioactive strand. The product sedimented as SV40 component II DNA (double stranded open circular form) in neutral sucrose gradients and had a specific activity of 1.0×10^7 cpm ^{3}H/μg.

Preparation of [^{3}H]DNA Probes. ^{3}H-labeled SV40 DNA prepared as described above was digested with endonuclease R·HaeIII in a reaction volume of 0.02 ml containing 10 mM $MgCl_2$, 100 mM Tris (pH 7.5), 5 mM dithiothreitol, and 5 μg of enzyme. DNA was digested for 12 hr at 37°. The resulting fragments were separated by electrophoresis through 17 × 0.6 cm cylindrical gels of 3.5% polyacrylamide as described by Edgell et al. (1972). Digests of 2 μg of SV40 were visualized by staining the DNA with 5 μg/ml of ethidium bromide in distilled water for 15 min, then viewing under a long wavelength uv light. Individual DNA fragments were well resolved allowing the portions of the gel containing DNA bands to be cut out with a razor blade and crushed by passing them through an orifice prepared by cutting off a 23 gauge needle. The DNA was eluted from the crushed gel slices with 0.05 M EDTA (pH 7.0) and stored at 0°. The separation of "+" and "−" strands by annealing to complementary RNA and purification of "−" strands of each fragment was performed as described by Khoury et al. (1972) except that the "+" strand of each fragment was degraded by S-1 treatment of the annealing reaction in which RNA "+" strands were annealed to the denatured DNA. The purified DNA "−" strands of each fragment were less than 1% S-1 resistant after self-annealing. (S-1 digestions were performed as described below for hybridization reactions.)

Preparation and Transcription of Chromatin. Chromatin was prepared from isolated nuclei by the method of Seligy and Miyagi (1969) and sedimented through 1.7 M sucrose. Chromatin (at a DNA concentration of 1 mg/ml) was sheared for 30 sec at a setting of 6 in a Sorvall Omni-mix fitted with a micro cup. The size of the DNA in the sheared chromatin was distributed in a rather broad molecular weight range with a mean of 10^7 as estimated by sedimentation in neutral sucrose gradients. Less than 5% of the DNA had a molecular weight of less than that of intact SV40 DNA (3×10^6). RNA was synthesized from a chromatin template by the use of equal weight ratios of DNA and *E. coli* RNA polymerase in a standard 10-ml reaction mixture containing 150 mM KCl, 40 mM Tris (pH 7.9), 0.1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, and 4 mM ATP, GTP, CTP, and UTP (P-L Biochemicals). The reaction was incubated at 37° for 4 hr and then EDTA was added to a final concentration of 0.1 M and sodium dodecyl sulfate added to a concentration of 1%. The mixture was extracted twice with phenol–chloroform (1:1) and once with chloroform. The RNA was precipitated with cold 5% trichloroacetic acid to remove triphosphates, redissolved in 1 M Tris (pH 7.9)–0.1 M NaCl, and precipitated with ethanol overnight at −20°. The precipitate was dissolved in 0.1 M sodium acetate (pH 5.3) and 0.01 M $MgCl_2$ and treated with RNase-free DNase (Worthington Biochemical) at a concentration of 20 μg/ml for 0.5 hr. EDTA was added to a concentration of 0.1 M and sodium dodecyl sulfate added to a concentration of 1% and the reaction was phenol–chloroform extracted and ethanol precipitated as described above. The RNA was dissolved in distilled water and the concentration determined by absorbance at 260 nm.

Preparation and Transcription of SV3T3 DNA. SV3T3 DNA was prepared from sheared chromatin by addition of sodium perchlorate to a final concentration of 1 M and extraction four times with phenol–chloroform and once with chloroform. The DNA was precipitated with ethanol and dissolved in 10 mM Tris (pH 7.9)–1 mM EDTA. DNA concentration was determined by absorbance at 260 nm. RNA transcribed from SV3T3 DNA was synthesized and purified as described above for SV3T3 chromatin except that the DNase treatment was done in the reaction mixture at the end of the reaction.

Hybridization. Annealing reactions were performed in sealed capillary tubes in 1 M NaCl–0.001 M EDTA–0.01 M Tris (pH 7.4) at 65° for 72 hr. Annealings of RNA transcribed from chromatin were performed in a total volume of 25 μl, annealings of RNA transcribed from DNA were performed in a total volume of 10 μl. The time course of annealing indicated that the time period chosen was sufficient to allow the annealing reaction to go to at least 95% of completion in all cases. More than 70% of the [^{3}H]DNA probe remained hybridizable after 72 hr at 65°. The quantity of RNA added to each annealing reaction was chosen so that the DNA probe would be in excess over the quantity of SV40 specific RNA added. This determination was made by annealing various quantities of RNA to two different concentrations of each probe and choosing conditions such that addition of more RNA gave additional annealing, but the addition of DNA probe did not.

S-1 Digestion. After hybridization, samples were diluted tenfold into buffer containing 0.05 M sodium acetate (pH 4.0), 0.005 M $ZnSO_4$, and 0.2 M NaCl. Half of the sample was spotted onto glass fiber filters and precipitated with 0.5 N HCl to determine total counts present. To the remainder was added 250 cpm of denatured ^{14}C-labeled mouse kidney DNA and 900 units of S-1 nuclease (Ando, 1966). After digestion at 37° for 1 hr the samples were spotted onto glass fiber filters and acid precipitated. The extent of digestion of the denatured mouse kidney DNA (added as a control) was always greater than 95% whereas, under the conditions used, less than 1% of double stranded DNA was digested.

Results

Transcription of Chromatin. Chromatin was prepared from SV3T3 cells and transcribed with *E. coli* RNA polymerase as described under Materials and Methods. The kinetics of transcription are shown in Figure 1. At the end of a 4-hr reaction, 8 mg of RNA was produced from 4 mg of chromatin DNA template. Thus each region of the chromatin DNA has been transcribed twice, on the average. However, since there are fewer binding sites for polymerase on chromatin than on naked DNA (Cedar and Felsenfeld, 1973), and all sequences of the DNA in chromatin are not available for transcription by *E. coli* polymerase (Astrin, 1973), each available region of the chromatin has probably been transcribed many times.

Synthesis of Probes for the Detection of SV40 Specific RNA. The probes used for the detection of SV40 specific transcripts from various regions of the genome were the "−" strands of purified fragments of SV40 DNA produced by the cleavage of in vitro labeled DNA with endonuclease R·HaeIII (Edgell et al., 1972). High specific activity ^{3}H-labeled DNA probes (10^7 cpm/μg) were obtained by using

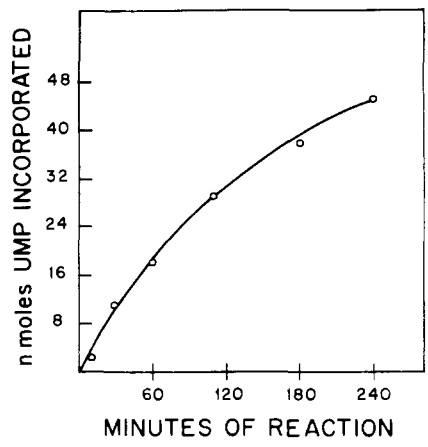

FIGURE 1: Time course of transcription of SV3T3 chromatin with *E. coli* RNA polymerase; 5 μCi of [^{3}H]UTP (17 Ci/mmol) was added to 100 μl of the standard reaction mixture described under Materials and Methods. At the times indicated, duplicate aliquots were removed for the determination of total cpm and acid precipitable cpm.

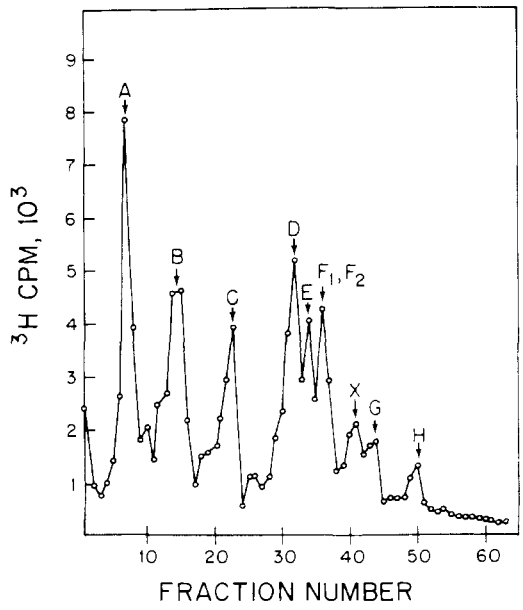

FIGURE 2: Migration of HaeIII fragments of SV40 DNA in a 3.5% polyacrylamide gel. Electrophoresis was carried out as described by Edgell et al. (1972). The gel was cut into 2.5-mm segments and the DNA eluted as described under Materials and Methods. An aliquot of each fraction was counted in Aquasol scintillation fluid. Migration is from left to right.

DNA synthesized in vitro by *E. coli* DNA polymerase I using a template of single-stranded circular SV40 DNA primed by oligodeoxynucleotides as described under Materials and Methods. The fragment pattern produced by cleavage of the in vitro labeled SV40 DNA is shown in Figure 2. The fragment pattern of the in vitro labeled DNA is indistinguishable from that of DNA labeled in vivo (Huang et al., 1973); ten large fragments were produced, as well as six small fragments which do not appear on the gel in Figure 2. The DNA "−" strands of the seven largest fragments were prepared as described under Materials and Methods for use as probes for the detection of virus specific RNA. These fragments in sum constitute greater than 85% of the SV40 genome.

Quantitation of SV40 Specific Transcripts. In order to measure the frequency of transcription from each region of the integrated SV40 genome, a small amount of RNA transcribed in vitro was annealed to an excess of the labeled "−" DNA strand of each of the following HaeIII fragments: A, B, C, D, and an equimolar mixture of E, F_1, and F_2. The absolute amount of each probe that annealed to the RNA was determined by digestion with the single-strand specific nuclease, S-1. Under the conditions of probe excess employed in these experiments, all the RNA transcribed from a given region of the DNA should hybridize to the probe representing that region and may be quantitated by determining the amount of S-1 resistant probe.

SV3T3 Chromatin Transcripts are from the Same Regions as are Transcribed in Vivo. The results of the annealings of RNA transcribed from SV3T3 chromatin with the various probes are shown in Table I. The results, tabulated in the right-hand column, indicate that regions A and D are transcribed preferentially; regions B, E, F_1, and F_2 are transcribed eight times less frequently than the A region. As the data show, the absolute amount of RNA hybridized to each probe does not change even when the amount of probe is doubled. Thus, the hybridization reactions have gone to completion and all the complementary RNA sequences have been detected.

Preferential transcription in vitro of the A and D regions is good agreement with the transcription pattern observed

Table I: Annealing of RNA Transcribed from SV3T3 Chromatin to Endo R·HaeIII Fragments of SV40 DNA.[a]

Probe				
Fragment	Cpm	μmoles of Fragment $\times 10^{-12}$[b]	% S-1 Resistance[c]	μmoles of Transcript $\times 10^{-12}$/120 μg of RNA
A	500	104	10	210
A	1000	208	5	210
B	500	208	13	27
B	1000	416	6	25
C	500	303	20	61
C	1000	606	11	67
D	500	476	25	120
D	1000	952	15	142
E, F_1, F_2	500	175	16	28
E, F_1, F_2	1000	350	9	31
Control A	500	104	6	7

[a] The table shows the results of annealing the minus strand of labeled genome fragments of SV40 with RNA transcribed from chromatin. Annealings were performed under conditions of probe excess as described under Materials and Methods. In each case, 120 μg of input RNA was used except in the case of fragment A in which 6 μg of RNA was used. The annealing of fragment A to 120 μg of RNA gave 80% S-1 resistance at both probe concentrations indicating that the RNA was in excess over the probe. Only at a much lower input of RNA (6 μg) were conditions of probe excess achieved allowing a calculation of the absolute amount of RNA complementary to fragment A. The right-hand column gives the absolute amount of each fragment that annealed to 120 μg of input RNA. In the case of fragment A, this number is taken as 20 times the μmoles of transcript in 6 μg of RNA. The data designated control is an annealing to 120 μg of RNA isolated from a reaction to which no polymerase had been added. Yeast carrier RNA was added to this reaction in an amount equal to the amount of RNA synthesized in a reaction which included polymerase. [b] These numbers were calculated from the specific activity of the in vitro labeled DNA serving as probe and from the nucleotide length of each fragment as determined by Lebowitz et al. (1974). The radiolabeled nucleotide may not be equally distributed in the cleavage products causing slight errors in these figures. From the data of Danna and Nathans (1972), it can be calculated that values for the A + T content for the HaeIII fragments range from 58 to 64%. The error is therefore probably small and does not significantly affect the conclusions. [c] Initial S-1 resistance of each probe (less than 1%) has been subtracted.

Table II: Annealing of RNA Transcribed from SV3T3 DNA to Endo R·HaeIII Fragments of SV40 DNA.[a]

Probe				
Fragment	Cpm	μmoles of Fragment × 10^{-12}[b]	% S-1 Resistance[c]	μmoles of Transcript × 10^{-12}/120 μg of RNA
A	500	104	20	21
A	1000	208	11	23
B	500	208	9	19
B	1000	416	5	21
C	500	303	12	36
C	1000	606	7	42
D	500	476	10	48
D	1000	952	6	57
E, F_1, F_2	500	175	7	12
E, F_1, F_2	1000	350	4	14

[a] The table shows the results of annealing the minus strand of labeled genome fragments of SV40 with RNA transcribed from SV3T3 DNA. In each case, 120 μg of input RNA was used. The right-hand column gives the absolute amount of each fragment that annealed to the RNA. [b] These numbers were calculated as described in Table I, footnote *b*. [c] Initial S-1 resistance of each probe (less than 1%) has been subtracted.

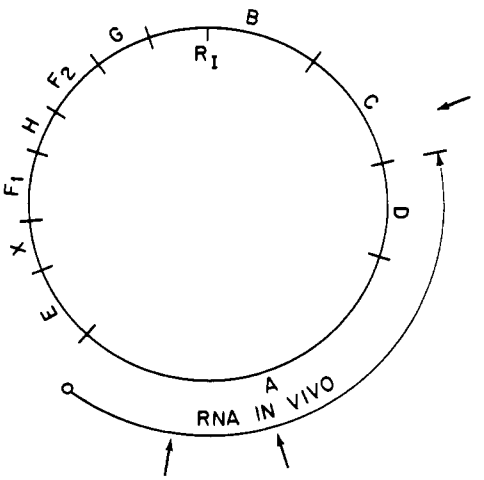

FIGURE 3: Map of the HaeIII fragments of SV40 DNA as determined by Lebowitz et al. (1974). The positions of fragments X, F_1, and F_2 have been determined by Newbold (1973).

in this line of transformed cells. The primary in vivo transcript appears to come from the same region as is transcribed early in lytic infection, that is regions A and D (Khoury et al., 1975). This region comprises about 40% of the genome, a number that is in good agreement with previous data on the percentage of the "−" strand of SV40 that is transcribed in these cells (Astrin, 1973).

Also shown in Table I are the results of a control experiment in which RNA isolated from a reaction with SV3T3 chromatin to which no polymerase had been added was annealed to fragment A. A low level of annealing of the control RNA to fragment A was observed. This annealing represents the level of in vivo synthesized RNA contaminating the isolated chromatin as well as any RNA synthesized by endogenous RNA polymerase in vitro. The value for the quantity of A region specific transcripts in the control experiment is about 3% of the value for the reaction with added polymerase indicating that the added polymerase was responsible for 97% of the observed synthesis.

One possible explanation for the pattern observed with transcripts from chromatin is that selective degradation of the transcripts from the B, and E, F_1, F_2 regions is taking place resulting in a lower apparent frequency of transcription from those regions. Such processing of SV40 specific sequences has not been reported in transformed cells but processing does take place during the lytic cycle of SV40 (Aloni, 1972). Therefore an experiment was carried out to try to detect such processing in the in vitro system. ^{3}H-labeled transcripts of the entire "−" strand of SV40 (prepared as described by Lindstrom and Dulbecco, 1972) were added to a complete reaction mixture containing chromatin and RNA polymerase. During a 4-hr incubation, no acid solubilization of the [^{3}H]RNA was observed. This result indicates that the pattern of transcription observed in vitro from chromatin is not the result of selective degradation of transcripts.

Transcription of SV3T3 DNA Produces a Different Pattern from that of Chromatin. SV3T3 DNA purified from isolated chromatin as described under Materials and Methods was transcribed with *E. coli* RNA polymerase and the SV40 specific transcripts were quantitated as described above. The results are shown in Table II. As can be seen from the figures in the right-hand column, there is no longer preferential transcription of region A. In fact, all regions are transcribed with about equal frequency. Thus, the major transcripts from SV3T3 chromatin closely resemble the in vivo transcripts, but the transcripts of DNA come from all regions.

Discussion

The transcription patterns can be best interpreted with the aid of the fragment map shown in Figure 3. This figure shows the relative positions and sizes of the various fragments used as probes. The arrows indicate the positions of initiation sites for *E. coli* RNA polymerase on linear SV40 DNA (Westphal et al., 1973; Lebowitz et al., 1974). Transcription proceeds counterclockwise from these sites. The major initiation site is in fragment C; about 55% of initiations take place there when linear SV40 DNA is used as template (Westphal et al., 1973). The region transcribed in the transformed cell is indicated and is comprised of regions A and D (Khoury et al., 1975). This region is the same region that is transcribed most efficiently in chromatin (see Table I). The major transcripts from chromatin appear to be initiated at the sites in fragment A and terminate in fragments D and C. Only 13% of the transcripts appear to be initiated at the site in fragment C as indicated by a comparison of the transcription frequency of fragment A with that of fragment B. Thus, one likely interpretation is that the major initiation site for *E. coli* polymerase is relatively unavailable in chromatin. The structure of chromatin appears to be such that those regions that are transcribed in the cell (A + D) are available for transcription in chromatin, but the regions that are repressed in vivo are complexed with protein in such a way as to make them unavailable to added polymerase. The major initiation site for *E. coli* polymerase may be in such a repressed region. When the proteins are removed from the DNA, the repressed regions then become equally available to the polymerase. This conclusion is supported by the data on transcription of SV3T3 DNA. In that case initiation appears to be taking place with equal efficiency at both the B region sites and the A region sites. Only the E, F_1, F_2 region is transcribed relatively inefficiently, presumably because of its lack of proximity to the initiation site.

The experiments reported here indicate that in vitro transcription of the SV40 sequences in SV3T3 chromatin shows a high degree of fidelity. In the present studies, 87% of the transcription taking place off the SV40 genome in chromatin took place in the region that is expressed in vivo. The remainder of the synthesis took place from regions that are apparently repressed in vivo. This small amount of generalized transcription may reflect the relative native state of the chromatin or the efficiency with which *E. coli* polymerase recognizes the cellular transcriptional controls. Alternatively, it may reflect the actual efficiency with which cellular transcriptional controls operate. Thus the SV40 specific in vivo transcript characterized in SV3T3 cells may represent the combined result of transcriptional and processing controls. No processing was detected in the in vitro system, an observation which might explain the presence in the in vitro RNA of some transcripts from repressed regions.

The findings reported here add to the evidence that the association of protein and other macromolecules with the DNA in chromatin plays an important role in transcriptional regulation. Not only are the same regions of SV40 genome transcribed from chromatin as are transcribed in vivo but, as Swetly and Watanabe (1974) have reported, chromatin isolated from SV40 transformed cells at different stages in the cell cycle shows the same temporal specificity of SV40 transcription as is observed in vivo. The finding that transcriptional specificity is lost and generalized transcription is observed when purified SV3T3 DNA is used as a template indicates that proteins or other macromolecules bound to the DNA may be involved in restricting the transcription.

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