# In Vitro Transcription of the Viral-Specific Sequences Present in the Chromatin of Cells Transformed by Simian Virus 40

### (RNA polymerase/RNA·DNA hybridization)

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ABSTRACT    Separated strands of simian virus 40 (SV40) DNA fragments were used in hybridization experiments to study the RNA transcribed by Escherichia coli RNA polymerase from the chromatin of cells transformed by SV40. The template activity of chromatin of the transformed cell line 11A8 (mouse-embryo cells) examined is about 17% that of purified DNA, suggesting that most of the chromatin DNA is repressed by chromosomal proteins. The SV40-specific RNA present in the RNA transcribed in vitro from 11A8 chromatin hybridizes specifically with the minus strand of SV40 DNA. Little or no reaction occurs with the plus strand of viral DNA. The SV40-specific RNA transcribed in vitro from chromatin of transformed cells shares sequences with the RNA produced during the early phase of SV40 lytic infection, and is similar to that present in the 11A8 cell line in vivo. Although the influence of chromosomal proteins on this pattern of transcription was not definitely determined, preliminary evidence indicates that an asymmetric pattern of transcription may also occur when 11A8 DNA is transcribed by E. coli RNA polymerase.

One to three copies of simian virus 40 (SV40) DNA per cell appear to be covalently linked to chromosomal DNA in virus-transformed cells (1, 2). The entire viral genome is present, since infectious virions can be recovered after cultivation with permissive cells (3–6). Viral gene activity however, is somehow repressed since many viral-specific functions, detectable in green monkey cells infected with SV40, fail to occur in established transformed cell lines. Synthesis of tumor (T) antigen, tumor-specific transplantation antigen, and U antigen (7), as well as virus-specific RNA (8–11) occurs in all virus-free transformed cells. The replication of SV40 DNA, production of viral capsid proteins, and the appearance of progeny virions are somehow aborted. At present, the level at which expression of viral genetic information is regulated in these cells is unclear. However, it has been recently shown that the stable species of viral-specific RNA present in lytically infected or SV40-transformed cells are quite distinct. Virtually no stable transcripts complementary to the plus strand of SV40 DNA are present in most transformed cells (12, 13).

Although gene regulation in eukaryotic cells is still poorly understood, numerous studies have indicated that isolated chromatin is a more restrictive template for in vitro transcription than purified DNA (14). DNA complexed with histones, or their model cationic polypeptides, is quantitatively blocked from acting as a template for RNA synthesis (15, 16). It is, therefore, implied that chromosomal proteins may regulate the transcription of DNA. Since the viral genomes in transformed cells are integral parts of cellular chromosomes, the possible

regulation of the integrated viral genomes by chromosomal proteins can be evaluated by studying the transcriptional properties of chromatin. In this paper, we report the detection of viral-specific sequences transcribed from chromatin of transformed cells by Escherichia coli RNA polymerase. The viral-specific RNA synthesized in vitro was found to be complementary to the same strand (minus) of SV40 DNA as the viral-specific RNA present in vivo.*

## MATERIALS AND METHODS

Cell Cultures. A stable line, 11A8 (kindly furnished by Dr. Helene Smith), of mouse-embryo cells (Balb/3T3) transformed by small-plaque SV40 was grown in Dulbecco–Vogt medium supplemented with 10% fetal-calf serum. Three to four days after seeding, confluent monolayers of growing cells were scraped off the bottles and harvested by low-speed centrifugation. In some cases cells were frozen and stored at −60° in medium containing 5% glycerol. No obvious differences were noted between fresh and frozen cells with respect to the properties of chromatin examined.

Preparation of Chromatin. The procedure of Marushige and Bonner (18) for the isolation of rat-liver chromatin was modified for the transformed cells grown in tissue culture. The cell pellet (about 0.5–1.5 ml packed volume) was washed with 20 ml of saline–EDTA [75 mM NaCl–24 mM EDTA (pH 8.0)], and centrifuged at 3000 × g for 15 min. The pellet was resuspended in 20 ml of fresh saline–EDTA containing 0.1 ml of 2-octanol, and was homogenized with a small blender (Dumore homogenizer, Racine, Wisc.) at 5000 rpm for three 1-min intervals in an ice-water bath (step 1). The nuclear fraction was pelleted at 3000 × g for 15 min, washed with saline–EDTA, and lysed in 10 ml of 10 mM Tris·HCl (pH 8.0) with a hand Teflon homogenizer. Chromatin was pelleted at 12,000 × g for 20 min and washed three times with 10 mM Tris·HCl (step 2). The crude chromatin was then suspended in 10 ml of 10 mM Tris·HCl (pH 8.0) with gentle hand homogenization, and 5 ml of suspension was layered on 25 ml of 1.7 M sucrose–10 mM Tris·HCl (pH 8) with stirring to mix the contents of the upper one-third of the centrifuge tube. After centrifugation at 22,000 rpm (40,000 × g) for 3 hr in a Spinco SW 25.1 rotor, the purified chromatin pellet was washed with 10 mM Tris·HCl (step 3) or dialyzed to remove the sucrose for chemical determinations. It was further sheared with a Virtis homogenizer at 17,000 rpm for 90 sec. This purified chromatin

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Abbreviation: SV40, simian virus 40.

* The minus strand of SV40 DNA is the strand complementary to the early lytic RNA and the RNA synthesized in vitro with E. coli RNA polymerase and the supercoiled form of SV40 DNA. The plus strand is the other strand of SV40 DNA.

TABLE 1. *Chemical composition of purified chromatin of transformed cells*

| Component | Weight ratio* |
|---|---|
| DNA | $1.00 \pm 0$ |
| Total proteins | $2.49 \pm 0.09$ |
|   Histone | 1.01 |
|   Nonhistone | 1.48 |
| RNA | $0.05 \pm 0.01$ |

\* Average values of three preparations ± standard deviations.

was used for most of the experiments. All of the above procedures were carried out at 4°.

The DNA was isolated by dissolving chromatin in 4 M CsCl followed by centrifugation at 114,000 × *g* overnight. The DNA at the bottom of the tubes was further deproteinized by phenol extraction.

*Transcription of the Chromatin with DNA-Dependent RNA Polymerase.* DNA-dependent RNA polymerase was isolated from mid-logarithmic phase *E. coli* B (General Biochemicals, Chagrin Falls, Ohio) to fraction 4, as described by Chamberlin and Berg (19, 15). It was essentially free of DNA ($A_{280}/A_{260} = 1.85$). The endogenous template activity of the enzyme in the absence of added template was about 1 % of its full activity at the enzyme concentrations used in the present experiments. The RNA synthetic mixture contained 0.8 mM each of ATP, GTP, CTP, and UTP, 40 mM Tris·HCl (pH 7.9), 4 mM MgCl₂, 1 mM MnCl₂, 12 mM 2-mercaptoethanol, either 150 mM NaCl or 2 mM spermidine phosphate (Sigma Chem. Co., St. Louis, Mo.), 1.4–1.7 mg of RNA polymerase (calculated by $A_{280}^{1\%} = 6.5$), and 1.1 mg of DNA in the form of purified chromatin in a total volume of 10 ml. The reaction mixture was incubated at 30° for 60 min. At the end of the incubation, sodium dodecyl sulfate was added to a final concentration of 0.5%, and the NaCl concentration was adjusted to 0.15 M. The mixture was immediately extracted twice with equal volumes of water-saturated phenol. Nucleic acids were precipitated from the aqueous phase after ether extraction with 2 volumes of ethanol in the presence of 0.1 M sodium acetate (pH 5) at −20°, overnight. The precipitate, redissolved in 5 ml of 10 mM Tris·HCl (pH 7.5) plus 1 mM MgCl₂, was digested with 100 μg of DNase I (RNase free, Worthington Biochem. Corp., Freehold, N.J.) at 25° for 15 min. The mixture was deproteinized by phenol, precipitated as above, and redissolved and passed through a Sephadex G-25 column in 0.1 M NaCl. The RNA in the excluded fraction was precipitated by ethanol. DNA contamination was about 5%, as determined by the diphenylamine method (20). The final yield of the RNA was about 4 mg.

*SV40 Virus and Viral DNA.* Virus and viral DNA were purified as described (21, 22). Small-plaque purified SV40 was grown in Vero cells and labeled with carrier-free [³²P]orthophosphate (100 μCi/ml). Viral DNA was purified by isopycnic banding in CsCl–ethidium bromide followed by chromatography on a Dowex 50–Sephadex G-100 column. The specific activity of SV40 DNA was about 0.5–1.5 × 10⁶ cpm/μg.

*Separation of SV40 DNA Strands and DNA·RNA Hybridization.* SV40 DNA was sheared in a Ribi cell fractionator at 50,000 lb./inch² to a mean molecular size of 3 × 10⁵ daltons (1). The plus and minus strands of the fragmented

SV40 DNA were separated by incubation with an excess of SV40 complementary RNA, synthesized *in vitro* by *E. coli* RNA polymerase with SV40 DNA I as a template (12). The SV40 DNA strand complementary to the *in vitro* RNA is designated as the minus strand, while the other is the plus strand.

Increasing concentrations of RNA (0.5–6.5 mg/ml) transcribed from chromatin were incubated with 1–2 ng (about 300 cpm) of either the plus or minus strand of SV40 DNA fragments in 0.1–0.25 ml of 1.0 M NaCl, 30 mM phosphate buffer (pH 6.8), and 0.1% sodium dodecyl sulfate for 12–20 hr at 68°. The percentage of SV40 [³²P]DNA fragments in duplex molecules was determined by hydroxyapatite chromatography (12). Single-stranded DNA molecules were eluted in 0.14 M, while DNA·RNA hybrids were eluted in 0.4 M sodium phosphate buffer (pH 6.8), both at 60°.

*Chemical Determinations and Other Analytical Methods.* The DNA content of chromatin was determined by the diphenylamine method (20) after hydrolysis in 0.5 N HClO₄ (95°, 10 min), with calf-thymus DNA as a standard ($A_{260}^{1 \text{ mg}} = 20$). The RNA content was determined by the orcinol method (23) on the supernatant of a 0.3 N KOH chromatin hydrolysate (37°, 18 hr), with ATP as standard ($E_{259} = 15.4 \times 10^3$ at pH 7). Total protein content was determined by Lowry's method (24) on the chromatin residue after DNA hydrolysis (10% trichloroacetic acid, 95° for 5 min). The histone fraction was extracted from chromatin with 0.2 N and then 0.4 N H₂SO₄, and its concentration was determined by a micro-biuret method (25), while the residual nonhistone protein was determined by the Lowry procedure. Bovine-serum albumin was used as a standard.

## RESULTS

*Chromatin of SV40-Transformed Cells.* A procedure for the purification of chromatin must preserve the native template properties imposed by the associated chromosomal proteins. The purification scheme outlined in *Methods* avoids the use of detergent in order to preserve the interactions between DNA and chromosomal proteins. Instead of intact nuclei being isolated, the cell suspension was homogenized in saline–EDTA, and the crude nuclear fraction was isolated by centrifugation. As determined by DNA content of the purified chromatin (step 3), about 75% of the cell DNA was recovered by the procedure outlined in *Methods*. The relatively high yield of chromatin minimizes artifactual fractionation due to the purification procedure and ensures that the isolated material is representative of cellular genetic information.

The DNA:protein:RNA ratios of the purified chromatin are 1.00:2.49:0.05 (Table 1). Although this composition is reasonably reproducible, the protein content is somewhat higher than that of chromatin isolated from other mammalian tissues, e.g., calf thymus and rat liver, and determined by comparable methods (27, 18). This higher protein content is entirely accounted for by the presence of larger amounts of nonhistone proteins.

Melting profiles and their derivative curves, determined for sheared chromatin, clarified by centrifugation, showed recognizable major thermal transitions at 70° and 78° due to histone-stabilized segments, as well as a total hyperchromicity of about 30% in 0.25 mM EDTA, pH 8.0 (26). The melting profiles of chromatin provided an important and convenient characterization of isolated chromatin, and were routinely

TABLE 2. *In vitro transcription of SV40-specific sequence*

| Experiment* | Template | Condition of RNA synthesis | Condition of hybridization | | %SV40 DNA fragments hybridized† | |
|---|---|---|---|---|---|---|
| | | | RNA concentration (mg/ml) | Hr of incubation | (−) Strand | (+) Strand |
| I | Chromatin (sheared) | NaCl (0.15 M) | 5.6 | 16 | 21 | 2 |
| | Chromatin (sheared) | Spermidine (2 mM) | 5.2 | 16 | 17 | 2 |
| | Chromatin (unsheared) | Spermidine (2 mM) | 5.7 | 16 | 9 | 2 |
| II | Chromatin (unsheared) | Spermidine (2 mM) | 4.2 | 16 | 17 | 2 |
| III | 11A8 DNA | NaCl (0.15 M) | 4.0 | 18 | 10 | 2 |
| IV | 11A8 DNA | Same | 5.1 | 36 | 9 | 0 |
| V | 11A8 DNA | Same | 6.0 | 36 | 16 | 1 |
| VI | Mouse DNA | NaCl (0.15 M) | 1.5 | 26 | 2 | 1 |
| | | | | 48 | 1 | — |

\* Numbers indicate different sets of experiments.

† 2% background for hybridization of minus- or plus-strand fragments in the absence of RNA has been subtracted from these values.

used to assess the quality of chromatin preparations. Preparations of exceptionally low hyperchromicity or altered major thermal transitions were not used as template for RNA synthesis.

A comparison of the template activity of chromatin and isolated DNA is shown in Fig. 1. Template efficiency was assayed by the rate of RNA synthesis when increasing concentrations of either DNA or chromatin were added to a constant amount of *E. coli* RNA polymerase, in the presence of an excess of ribonucleotide triphosphates. The template activity of chromatin appears to be about 17% that of pure DNA as determined by $V_{max}$ or $V_{max}/K$ (15). The possibility that an RNase activity associated with chromatin was responsible for the apparently low template activity was ruled out. Incubation of 23 μg of [³H]RNA (5800 cpm) with purified chromatin (15 or 30 μg of chromatin DNA) under the experimental conditions shown in Fig. 1 resulted in no detectable solubilization of the labeled RNA. We therefore conclude that the template activity of 11A8 chromatin is about 17% that of the purified DNA and that most of the chromatin DNA is repressed.

*Transcription of Viral-Specific Sequences from Chromatin of SV40-Transformed Cells.* The transcription of SV40-specific sequences from the chromatin of 11A8 cells was analyzed by hybridizing the *in vitro* RNA product with the separated strands of ³²P-labeled SV40 DNA. The results of reactions between the separated strands of SV40 DNA fragments and increasing concentrations of RNA transcribed from chromatin are shown in Fig. 2. The fraction of DNA in hybrid molecules was determined by hydroxyapatite chromatography. In the absence of RNA, there was essentially no retention of labeled DNA by hydroxyapatite. The extent of hybridization between the minus-strand fragments of SV40 DNA and the increasing RNA concentrations used in this experiment is almost linear and has not reached a plateau (Fig. 2). Nevertheless, at the highest RNA concentrations used, about 20% of the minus-strand fragments appear in duplex molecules while essentially none of the plus-strand DNA fragments are found in hybrids. The amount of SV40-specific RNA transcribed *in vitro* from purified chromatin is far less than that found in monkey-kidney cells after lytic infection with SV40 (21), and is somewhat less than that present in transformed 11A8 cells (12). The RNA synthesized and isolated under these experimental conditions is very heterogeneous in size, and the majority of the molecules appear to be quite small (1–3S; Shih, T. Y. & Lebowitz, J., unpublished observation). Whether the size of these RNA molecules is related to the low levels of hybridization in these experiments remains to be determined.

Table 2 summarizes the results of hybridization experiments with RNA transcribed from chromatin under various condi-
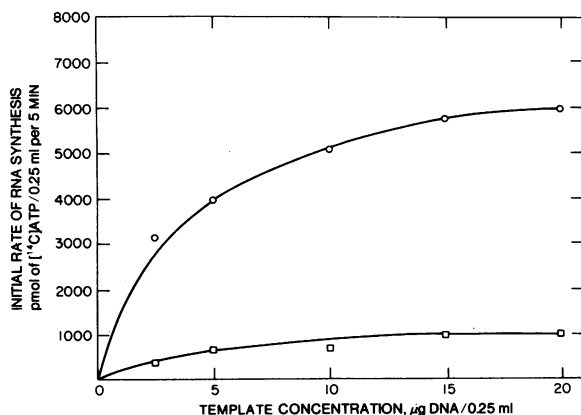


FIG. 1. Template activity of chromatin from SV40-transformed 11A8 cells. The indicated concentrations of sheared purified chromatin (□) and isolated DNA (○) were incubated with 33 μg of *E. coli* DNA-dependent RNA polymerase at 37°, in the presence of 40 mM Tris·HCl (pH 8.0), 4 mM MgCl₂, 1 mM MnCl₂, 12 mM 2-mercaptoethanol, 0.4 mM each of GTP, CTP, UTP, and [¹⁴C]ATP (1 μCi/μmol) in a total volume of 0.25 ml. The reaction was terminated after 5 min of incubation by addition of 5 ml of 10% trichloroacetic acid. The incorporated [¹⁴C]ATP was collected on nitrocellulose membrane filters and counted in a liquid scintillation system. Each point represents the average of duplicate determinations. The incorporation of [¹⁴C]ATP by the enzyme in the absence of template (1% of the maximal incorporation) was subtracted from each point.

TABLE 3. *Comparison of in vitro RNA from 11A8 chromatin with early lytic SV40 RNA*

| RNA | Concentration of RNA (mg/ml) | Percent minus-strand fragments in duplex molecules after hybrid-ization* |
|---|---|---|
| 11A8 Chromatin | 5.6 | 21 |
| Early lytic | 1.2 | 37 |
| 11A8 Chromatin plus early lytic | 5.0 / 1.2 | 40 |

\* Hybridization reactions were performed between fragments of the minus strand of SV40 DNA (6.5 ng/ml, about 300 cpm) and the indicated RNA preparations. 2% background, for the reaction of minus-strand fragments in the absence of RNA, has been subtracted from these values.

tions. Although plateau values for hybridization were not obtained, the percentage of hybridization at similar RNA concentration can be compared. It is evident that the transcription of SV40-specific sequences in each case is limited primarily to the minus strand. The situation is similar to that observed in transformed 11A8 cells (12). RNA made from purified chromatin that was resuspended with a hand Teflon homogenizer or sheared with a Virtis homogenizer (17,000 rpm for 90 sec) was compared in these experiments. Virtis homogenization was used to disperse the chromatin gel and to minimize aggregation of the template. RNA transcribed from sheared chromatin hybridized to a slightly greater extent with the minus DNA strand than a similar amount of RNA transcribed from unsheared chromatin (Table 2, exp. I), but no significant difference was noted in other experiments. The effect of both high and low ionic strength on the transcription of 11A8 chromatin was also examined, since chromatin in high salt concentrations (0.15 M) has a greater tendency to aggregate, whereas at low salt concentrations (2 mM spermidine added to stimulate the RNA synthesis), nonspecific binding of RNA polymerase to DNA has been described (28). Variation of the ionic strength, however, had little effect on the transcription of integrated SV40 DNA (Table 2). In an attempt to determine whether the limitation of transcription of 11A8 chromatin to the minus DNA strand is determined by chromosomal proteins, we investigated transcription of purified 11A8 DNA *in vitro*. For these experiments, proteins were removed from 11A8 chromatin as described in *Methods*. It is clear from results presented in Table 2 that the product transcribed from 11A8 DNA contains even less SV40-specific RNA than does the RNA transcribed from chromatin. Nevertheless, the hybridization pattern observed is similar to that seen when chromatin was used as the template, i.e., transcription primarily from the minus DNA strand. This result suggests that chromosomal proteins, themselves, are not responsible for the asymmetric transcription of the integrated SV40 sequences in transformed cells. In control experiments, the RNA transcribed from purified mouse-embryo DNA did not hybridize to a significant extent with fragments of either strand of SV40 DNA.

It has been shown that the SV40-specific RNA in transformed cells includes those sequences synthesized "early"

(i.e., before viral DNA synthesis) in a lytic infection (12). In an attempt to determine whether the sequences transcribed *in vitro* contain these "early" SV40 RNA sequences, the experiment described in Table 3 was performed. When high concentrations of the RNA preparations used in the experiments described in Fig. 2 were added to saturating amounts of "early" lytic SV40 RNA, little or no increase in the reaction with the minus strand was observed. Thus, the RNA synthesized from a chromatin template appears to contain sequences present in early lytic SV40 RNA, the predominant SV40-specific RNA sequences found in transformed 11A8 cells (Khoury *et al.*, unpublished results).

## DISCUSSION

It has previously been shown that a specific polypeptide is synthesized *in vitro* using a coupled system containing purified chromatin as a template (17). Our results confirm other reports that indicate that chromatin is a restrictive template for RNA synthesis *in vitro* (14, 29–34). The purified chromatin used in these experiments had less than 20% of the template activity of purified transformed cellular DNA. In these experiments, we were able to detect a specific SV40 RNA product which is presumably transcribed from DNA sequences that are an integral part of the cell genome. The viral-specific RNA prepared *in vitro* with the chromatin template is complementary to the minus strand of SV40 DNA. This RNA product is thus transcribed from the same strand of the viral DNA as the RNA present in this transformed cell line *in vivo*. The portion of the viral genome that contains the genetic information for "early" viral function is expressed in this transformed line and is also transcribed *in vitro* from the purified chromatin template.

The experiments described in Table 2 indicate that when purified cellular DNA is used as the template *in vitro*, only the minus strand of the integrated SV40 DNA is transcribed. This observation suggests that the preference for the minus strand as the template for synthesis *in vitro* may be intrinsic
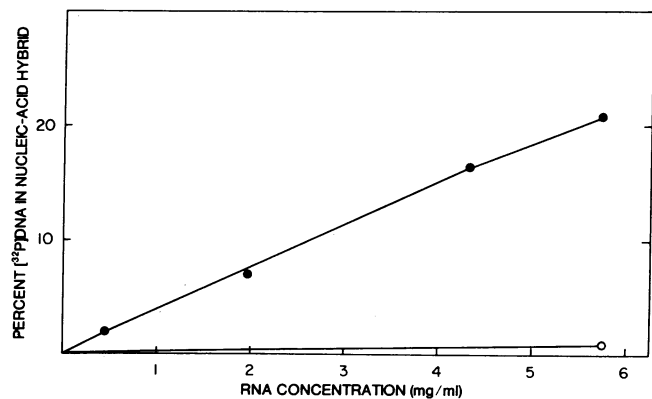


FIG. 2. Hybridization of RNA transcribed from chromatin to the separated strands of ^{32}P-labeled SV40 DNA fragments. Increasing concentration of RNA, synthesized *in vitro* from 11A8 chromatin, were incubated with small amounts (10 ng/ml, about 250 cpm) of the plus (O)- or minus (●)-strand fragments of SV40 DNA in a volume of 0.1 ml. After 20 hr of incubation at 68°, the samples were analyzed by hydroxyapatite chromatography to determine the percent ^{32}P-labeled DNA in duplex molecules. A background value of 2% for the reaction of either DNA strand in the absence of RNA has been subtracted from these values.

to the DNA; the chromosomal proteins may have little, if any, role to play in the strand-selection process. Alternatively, since the minus strand of purified SV40 DNA I has been previously shown to be the template for *E. coli* RNA polymerase *in vitro*, the pattern of transcription observed may be an intrinsic property of the enzyme used. However, the role of chromosomal proteins in regulation of the extent of transcription of the minus strand cannot be ruled out by the present data. Further improvement of the RNA-synthesizing system *in vitro* (e.g., by using homologous RNA polymerase) and the development of methods for concentrating viral-specific RNA sequences in the vast excess of cellular RNA (35) should resolve many of these points.

## NOTE ADDED IN PROOF

After this manuscript was submitted for publication, S. M. Astrin, using chromatin prepared from a different transformed cell line, reported a similar pattern of transcription, (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2304–2308.

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