Transcription of Reconstituted Simian Virus 40 Nucleoprotein Complexes

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The regulatory effects of host cellular histones on the transcription of simian virus 40 (SV40) DNA were investigated by using reconstituted and native SV40 nucleoprotein complexes (NPCs). Reconstituted NPCs were prepared from SV40 DNA and the combination fraction of five histones, H1, H2A, H2B, H3, and H4, isolated from the nuclei of permissive (CV-1) or nonpermissive (BALB/c 3T3, rat liver, and calf thymus) cells. Native NPCs were prepared by alkali disruption of purified SV40 virions. Nuclease digestion of these NPCs gave regular patterns of bands similar to those of SV40 NPCs from SV40-infected CV-1 cells, suggesting the presence of a nucleosomal structure. Transcription of NPCs was analyzed in vitro by using *Escherichia coli* DNA-dependent RNA polymerase. Both histone H1 and the fraction consisting of all five histones inhibited transcription of SV40 DNA by about 90 to 95%. The fraction consisting of four histones lacking H1 reduced the transcription by 30 to 35%, to a level similar to that of transcription with native NPCs. Transcription was inhibited regardless of whether the origin of histones was permissive or nonpermissive cells. Gel electrophoretic patterns of RNA products transcribed from SV40 DNA and reconstituted and native NPCs showed several identical peaks between 13S and 28S. The patterns were identical whether NPCs reconstituted with H1 alone, all five histones, or four histones lacking H1 were used.

Nucleoprotein complexes (NPCs) consisting of viral DNA and host cellular histones H2A, H2B, H3, and H4 have been isolated by alkali disruption of simian virus 40 (SV40) (5), polyoma virus (9), bovine papilloma virus (8), and human papilloma virus (8). Similar complexes have also been isolated by Triton treatment of nuclei lytically infected with SV40 or polyoma virus (7). The viral NPCs have a structure similar to that of host cell chromatin (2, 5, 8, 27). They consist of circular molecules of 20 to 21 nucleosomes (approximately 10 nm in diameter) interconnected by naked DNA strands (2, 5, 7, 27). Nucleosomes in viral NPCs are composed of segments of DNA approximately 200 base pairs long wound about cores composed of histones H2A, H2B, H3, and H4. The arrangement of nucleosomes is nonrandom at least with regard to sequences near the origin of DNA replication (33, 40, 41). The replication origin is much more accessible to probing by single-cut restriction endonucleases than other sites in the SV40 genome (35, 40, 42).

The SV40 NPCs isolated from nuclei of lytically infected cells with 0.15 M NaCl contain not only histones H2A, H2B, H3, and H4 but histone H1 as well (5, 38). On the other hand, the SV40 NPCs isolated from purified SV40 virions by high pH treatment apparently do not contain

histone H1 (5). Histone H1 is thought to be involved in virus chromosome condensation in cell nuclei (2, 27, 39). Details of the organization of the NPCs, the individual capsid polypeptides, and the internal proteins are not clear at present. Moreover, little is known about the function of the four cellular histones associated with SV40 DNA in SV40 gene expression.

SV40 DNA is transcribed efficiently by DNA-dependent RNA polymerase isolated from calf thymus (23, 24) and *Escherichia coli* (19, 20, 43, 44). Moreover, the DNA is transcribed asymmetrically and extensively in vitro under standard (44) and restricted (19) polymerization conditions by *E. coli* RNA polymerase. Although the production of mRNA in eucaryotic cells is quite complex (6), it appears that *E. coli* RNA polymerase is also able to recognize "active" chromatin and to produce functional mRNA (28). RNA synthesized by *E. coli* RNA polymerase produces SV40 T-antigen and stimulates DNA synthesis when transferred by microinjection into cultures of epithelioid primary mouse kidney cells (11). A cell-free, linked transcription-translation system has been developed by using *E. coli* RNA polymerase and wheat germ extracts (34). These features suggest that *E. coli* RNA polymerase is useful for investigating the regulatory mechanisms involved in SV40 gene

expression. Consequently, studies on the transcription of SV40 DNA and in vitro-assembled SV40 NPCs that use *E. coli* RNA polymerase should provide information useful for investigating the function of the four cellular histones associated with SV40 DNA in SV40 gene expression.

SV40 NPCs containing a characteristic nucleosomal configuration can be reconstituted in vitro from SV40 DNA and histones H2A, H2B, H3, and H4 (11, 15, 27). Several studies have shown that the NPCs can be used as templates for transcription (13, 43). Their small size should make them useful model systems for the study of the effects of histones, nonhistone nuclear proteins, and SV40 T-antigen products on SV40 gene expression. We have used in vitro-assembled SV40 NPCs for studying the effects of nonhistone chromosomal proteins of host cells on transcription of SV40 DNA (14). We have shown that a 0.35 M NaCl-soluble fraction of host cell chromatin contains proteins which specifically control SV40 gene expression (14). The present communication extends studies on the effects of host cellular histone H1 alone, all five histones, or four histones lacking H1 on in vitro transcription of SV40 DNA.

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MATERIALS AND METHODS

Virus propagation. CV-1 cells (a cell line established from African green monkey kidney cells) were grown in Eagle minimal essential medium supplemented with 10% calf serum inactivated at 60°C. Confluent cellular monolayers were infected with SV40 (strain 777 obtained from a small plaque type) at a multiplicity of 0.01 to 0.05 PFU per cell and harvested 7 to 9 days after infection.

Virus purification. SV40-infected cells were gently scraped off the monolayer with a silicon rubber and immediately centrifuged at $10,000 \times g$ for 30 min at 4°C. The supernatant was kept at 4°C. The packed cells were suspended in 10 volumes of 0.01 M Tris-hydrochloride (pH 8.0) containing 1% sodium deoxycholate by means of homogenization in a loosely fitting Potter homogenizer. The resulting cell suspension was sonicated eight times for 15 s in an ice-water bath with an ultrasonic disintegrator and then incubated for 30 min at 37°C. The protease inhibitor, sodium bisulfite (NaHSO_3), was added to all of the solutions used throughout the present study at a final concentration of 0.01 M. A 3-volume amount of cold 0.01 M Tris-hydrochloride (pH 8.0) was then added to the suspension, and centrifugation was performed at $10,000 \times g$ for 20 min at 4°C. The resulting supernatant was combined with the original supernatant from cell harvesting. The combined supernatants, containing SV40 virions, were then centrifuged at $80,000 \times g$ for 90 min

at 4°C. The precipitate, containing crude virions, was taken up in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.25% Triton X-100.

The suspension of crude virions was layered onto a bilayer system consisting of 1.5 ml of 15% (wt/vol) sucrose (top layer) and 3.0 ml of 1.33 g of CsCl (bottom layer) per ml in cold phosphate-NaCl buffer (0.01 M sodium phosphate [pH 7.4]-0.15 M NaCl), and centrifuged at $100,000 \times g$ for 5 h at 4°C in an RPS-65TA rotor with a Hitachi 65P centrifuge. The purified SV40 virions banded in the CsCl layer. The virions were collected with a syringe and dialyzed against 100 volumes of 0.01 M Tris-hydrochloride (pH 7.4)-0.15 M NaCl twice before SV40 DNA and NPC extractions.

Preparation of SV40 DNA. SV40 DNA was prepared from purified virions as described by Marmur (25). SV40 DNA I (supertwisted circular-form SV40 DNA) was purified by electrophoresis in 0.7% (wt/vol) agarose gels in cylindrical glass tubes of 2.7-cm internal diameter (25-cm length) as previously described (37). Electrophoresis was performed for 24 to 30 h at 4°C and 1.5 V/cm of gel in a vertical tank with gels fully immersed in the buffer. Separated SV40 DNA I was recovered from gels by reelectrophoresis.

Preparation of cellular histones. Cellular histones were extracted from isolated nuclei by the method of Johns (17). The nuclei were isolated from cultured cells (CV-1 and 3T3 cells) and tissues (rat liver and calf thymus) by the methods described by Blobel and Potter (3). Histone H1 alone, a mixture of H2A, H2B, H3, and H4, and a mixture of H1, H2A, H2B, H3, and H4 were used for the preparation of in vitro-reconstituted SV40 NPCs.

Preparation of reconstituted SV40 NPCs. SV40 DNA and histones were each dissolved in a solvent consisting of 2.0 M NaCl, 5.0 M urea, 10 mM NaHSO_3 , and 10 mM Tris-hydrochloride (pH 7.9) at a concentration of 200 $\mu\text{g}/\text{ml}$. The DNA and histones were mixed at a ratio of histone to DNA (wt/wt) of 2.0. The mixture of DNA and histones was dialyzed for 8 h at 4°C against 200 volumes of the same solvent. Reconstitution was then performed by successive dialyses every hour at 4°C against 200 volumes of the step-gradient solvent with decreasing NaCl-urea concentrations: 1.5 M NaCl-2.5 M urea; 1.0 M NaCl-1.0 M urea; 0.8 M NaCl-0.5 M urea; 0.6 M NaCl-0.1 M urea; 0.4 M NaCl alone; 0.3 M NaCl; and 0.15 M NaCl, all in 10 mM NaHSO_3 and 10 mM Tris-hydrochloride (pH 7.9). Reconstituted NPCs were dialyzed for 8 h at 4°C against 500 volumes of 10 mM Tris-hydrochloride (pH 7.9), 1.0 mM EDTA, 0.5 mM dithiothreitol (DTT), and 10 mM NaHSO_3 . Urea was used to reduce histone aggregation.

The SV40 NPCs thus formed were separated from unbound histones by passage through a Bio-Gel A5m column (1.8 by 24.0 cm). The column was washed and equilibrated with 10 mM Tris-hydrochloride (pH 7.9), 1.0 mM EDTA, 0.5 mM DTT, and 10 mM NaHSO_3 before use. The flow rate of gel filtration was 3.5 ml/h, and 2-ml fractions were collected. Fractions 7 and 8 containing the SV40 NPCs were pooled, dialyzed against a solution of 10 mM Tris-hydrochloride (pH 7.9), 1 mM DTT, and 10 mM NaHSO_3 , and concentrated by polyethylene glycol (molecular weight, 15,000). These procedures were performed at 0 to 4°C.

Preparation of native SV40 NPCs. SV40 virions were disrupted by incubation at pH 9.8 for 5 min at 37°C by the method of Christiansen et al. (5). Briefly, equal volumes of dialyzed virions were mixed with 0.2 M glycine (pH 9.8)–1.0 mM DTT and incubated for 5 min at 37°C. The resulting suspension was then passed through a Bio-Gel A5m column (1.8 by 24.0 cm) equilibrated with 10 mM Tris-hydrochloride (pH 7.9), 1.0 mM EDTA, 0.5 mM DTT, and 10 mM NaHSO₃. This chromatographic step permits separation of NPCs from unbound histones and contaminating substances. The eluted fractions containing SV40 NPCs were pooled, dialyzed, and concentrated as described above. These procedures were performed at 0 to 4°C.

Determination of DNA and histone contents in SV40 NPCs. The concentration of isolated SV40 DNA was determined by UV absorption at 260 nm. The SV40 DNA content of the NPCs was determined by the diphenylamine method (4), with calf thymus DNA as the standard. The protein contents of isolated histone fractions and of NPCs were determined by the method of Lowry et al. (22) with bovine serum albumin as the standard.

Gel electrophoresis of histone fractions associated with SV40 DNA. The extent of the binding of histone fractions and individual components of histone fractions to SV40 DNA under the present NPC reconstitution conditions was determined by gel electrophoresis. The histone fractions before and after reconstitution were analyzed on 15% polyacrylamide gels containing urea as described by Panyim and Chalkley (30). Gels were 6 cm in diameter and 10 cm in length and were run for 3 h at 1.5 mA per tube. Staining of gels was carried out for 3 h with 0.1% amide black in 20% methanol and acetic acid.

Nuclease digestion of SV40 NPCs. Reconstituted and native SV40 NPCs were tested for the presence of a nucleosomal arrangement by using staphylococcal nuclease. The reconstituted and native SV40 NPCs were suspended in 0.34 M sucrose-buffer A (consisting of 60 mM KCl, 5 mM MgCl₂, 15 mM 2-mercaptoethanol, 15 mM NaCl, and 15 mM Tris-hydrochloride [pH 7.4]) as described (29). The suspension (10 µg of DNA per ml) was made 1 mM in CaCl₂ and digested with 60 U of staphylococcal nuclease per ml (Worthington Biochemicals Corp., Freehold, N.J.; 6,000 U/mg) for 30 s at 37°C. Nondigestible DNA fragments were extracted twice with an equal volume of a mixture of chloroform and isoamyl alcohol (24:1; vol/vol). The aqueous phase containing DNA fragments was dialyzed overnight against 20 mM Tris-hydrochloride (pH 7.4) and 1 mM EDTA at 2 to 4°C.

Electrophoretic analysis of nondigestible DNA fragments. The DNA fragments (10 µg/ml) were analyzed on a 0.5% agarose-2.5% polyacrylamide composite gel in a glass tube (0.6 cm in diameter; 13.0 cm in length) prepared by the method of Peacock and Dingman (31). Electrophoresis was performed for 1 h at 8 V/cm of gel and 4°C in a vertical tank with the gel fully immersed in Tris-EDTA-borate buffer (31). The gel was extruded into ethidium bromide staining solution (1 µg/ml). After 30 min, the stained bands were visible as fluorescence over long-wavelength UV light (about 365 nm).

In vitro transcription of SV40 DNA and SV40

NPCs. *E. coli* DNA-dependent RNA polymerase holoenzyme (100 U/0.14 mg of protein) was purchased from Boehringer Mannheim Corp., New York, N.Y., and [α -³²P]UTP (50 to 125 Ci/mmol) was purchased from Radiochemical Centre, Amersham, England. SV40 cRNA was synthesized essentially by the method of Ketner and Kelly (18). The reaction mixture contained 10 mM MgCl₂; 150 mM KCl; 33 mM Tris-hydrochloride (pH 7.9); 1 mM DTT; 0.26 mM ATP, GTP, and CTP; 0.02 mM [α -³²P]UTP; 10% (vol/vol) glycerol; 124 µg of bovine serum albumin per ml; 0.10 mM EDTA; 8 µg of SV40 DNA I or SV40 NPCs per ml; and 12 µg of *E. coli* RNA polymerase per ml. After a 10-min incubation at 37°C, the reaction was terminated with 10 volumes of cold 10% trichloroacetic acid–0.1 M sodium pyrophosphate. The acid-precipitated material was collected on glass fiber filters (Whatman GF/C), washed, dried, and then counted in a liquid scintillation counter.

The preceding reaction mixtures were incubated for 30 min at 37°C to prepare SV40 cRNA for gel. Purified DNase (Worthington Biochemicals) was added to a final concentration of 30 µg/ml, and the incubation at 37°C was continued for 10 min. Reactions were stopped by sodium dodecyl sulfate-phenol extraction and the synthesized cRNA was freed from radioactive substrates on a Sephadex G-100 column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; 0.4 by 15.0 cm) as described by Lebowitz et al. (21). Column fractions 5 to 14 containing cRNA were pooled, and the cRNA was precipitated at –20°C in the presence of 200 µg of phenol-treated carrier *E. coli* tRNA per ml, 2% potassium acetate, and 66% ethanol.

Agarose-acrylamide gel analysis of the SV40 cRNA. The SV40 [³²P]cRNA transcribed from SV40 DNA and NPCs was analyzed by electrophoresis on a slab gel (15.0 by 17.0 by 0.2 cm) containing 0.5% agarose, 2.0% acrylamide, and 0.5% sodium dodecyl sulfate in the Tris-EDTA-borate system of Peacock and Dingman (31). Electrophoresis was performed at 50 mA at room temperature until the marker dye ran off the distal end of the gel. The gel was exposed with Sakura medical X-ray film for 48 h at 4°C to detect radioactive cRNA bands.

RESULTS

Content of DNA and histone in reconstituted and native SV40 NPCs. A gel filtration method was adopted to separate SV40 NPCs from unbound histones. Bio-Gel A5m was chosen for the gel material so that the NPCs were eluted at void volume, and unbound histones or contaminating substances were eluted near total volume (Fig. 1). The purified NPCs were eluted within fractions 6 to 10, and the unbound histones were eluted into fractions 15 to 20 (Fig. 1). The NPCs in fractions 7 and 8 were pooled and used as purified SV40 NPCs for analysis.

The histones in the reconstitutes were examined by acid-urea gel electrophoresis (Fig. 2). Histones bound to SV40 DNA were extracted with 0.4 N H₂SO₄ from SV40 NPCs and analyzed on 15% polyacrylamide gels containing urea (Fig.

FIG. 1. Purification of SV40 NPCs by gel filtration. The complexes were prepared from SV40 DNA and CV-1 histones as described in the text and subjected to gel filtration. The DNA content in each fraction was determined spectrophotometrically by absorbance at 260 nm. Fractions 7 and 8 containing SV40 NPCs were pooled, and the five histones associated with SV40 DNA were analyzed by gel electrophoresis as described in the text (see Fig. 2). Other complexes were also purified by the same procedures.

FIG. 2. Gel electrophoretic patterns of five histone fractions (H1, H2A, H2B, H3, and H4) associated with SV40 DNA using *in vitro* reconstitution procedures as described in the text. Samples were subjected to electrophoresis on 15% acid-urea polyacrylamide gels for 3 h at 1.5 mA/tube and room temperature. (a) CV-1 histone fractions before reconstitution of SV40 NPCs. (b) CV-1 histone fractions extracted with 0.4 N H_2SO_4 from SV40 NPCs after reconstitution.

2). There was no selective loss of any histone during the reconstitution; moreover, the histones bound to SV40 DNA were not degraded or altered after reconstitution. The weight ratio of histone to DNA for NPCs was estimated by the method of Lowry et al. (22) and using absorbance at 260 nm (4) (Table 1). The ratio for reconstituted NPCs consisting of SV40 DNA and histones H2A, H2B, H3, and H4 was approximately 0.68 to 0.72. This is close to the ratio for native SV40 NPCs isolated from purified virions. Histone H1 bound to SV40 DNA at ratios of 0.27 to 0.31. The weight ratio for NPCs consisting of SV40 DNA and all five histones, H1, H2A, H2B, H3, and H4, was approximately 0.90 to 0.92.

Staphylococcal nuclease digestion of reconstituted and native NPCs. The presence of a nucleosomal structure has been confirmed by electron microscopy (2, 7, 10) and by the use of staphylococcal nuclease (2), which digests preferentially the DNA between nucleosomes. The limited nuclease digestion yields a series of high multimers of the DNA of the biochemical units (2). These multimers are obtained by mild digestion of SV40 NPCs. The series of high multimers can be demonstrated by gel electrophoresis as described (2). We used staphylococcal nuclease as the probe for confirming the presence of the nucleosomal structure. Nuclease digestion patterns of reconstituted and native SV40 NPCs are shown in Fig. 3. Distinct DNA bands corresponding to multimers higher than the monomer were observed similarly in both reconstituted and native SV40 NPCs. The presence of a nucleosomal arrangement on the SV40 DNA molecules was biochemically confirmed.

***In vitro* transcription of reconstituted and native SV40 NPCs.** SV40 DNA and NPCs were transcribed *in vitro* by *E. coli* RNA polymerase under assay conditions similar to those described by Ketner and Kelly (18). The reaction was carried out in 150 mM KCl, a salt concentration which is believed to increase the specificity of the attachment of the enzyme to the DNA (32). A 1:9 weight ratio of enzyme to template DNA was chosen to minimize nonspecific binding to template SV40 DNA.

The quantity of RNA synthesized on naked SV40 DNA was found to increase continually during 60 min of synthesis (Fig. 4). The results presented in Table 1 show that all 10 templates supported the incorporation of [α - ^{32}P]UMP into RNA catalyzed by *E. coli* RNA polymerase. We examined the role of histone H1 in SV40 DNA gene expression. The transcription of purified SV40 DNA was inhibited by about 90 to 92% in the presence of CV-1 or 3T3 histone H1. The transcription of SV40 NPCs containing histones

TABLE 1. *Template activities of SV40 DNA and SV40 NPCs for in vitro transcription by E. coli DNA-dependent RNA polymerase*

Template ^a	Histone-DNA ratio (wt/wt)	[α - ³² P]UMP incorporated (cpm)	% of incorporation
SV40 DNA	0	85,937	100
Reconstituted			
SV40 DNA + CV-1 histone H1	0.31	8,601	10
SV40 DNA + CV-1 histones H2A-H4	0.69	56,767	66
SV40 DNA + CV-1 histones H1-H4	0.90	4,297	5
SV40 DNA + 3T3 histone H1	0.27	6,881	8
SV40 DNA + 3T3 histones H2A-H4	0.71	59,296	69
SV40 DNA + 3T3 histones H1-H4	0.92	2,580	3
SV40 DNA + R.L. histones H2A-H4	0.72	57,577	67
SV40 DNA + C.T. histones H2A-H4	0.68	60,154	70
Native			
SV40 DNA + CV-1 histones H2A-H4	0.68	55,859	65

^a Abbreviations: H1-H4, consisting of H1, H2A, H2B, H3, and H4; H2A-H4, consisting of H2A, H2B, H3, and H4; R.L., rat liver; and C.T., calf thymus.

FIG. 3. Nuclease digestion patterns of native and reconstituted SV40 NPCs. The SV40 NPCs were digested with staphylococcal nuclease and subjected to electrophoresis. The DNA bands in gels were stained with ethidium bromide and photographed with a yellow filter. (a) Native SV40 NPC consisting of SV40 DNA and the four CV-1 histones, H2A, H2B, H3, and H4. (b) Reconstituted SV40 NPC consisting of SV40 DNA and the four CV-1 histones, H2A, H2B, H3, and H4.

H2A, H2B, H3, and H4 was also inhibited by approximately 95 to 97% in the presence of the same histone H1. The SV40 NPC containing histone H1 was a relatively poor template which supported incorporation of only approximately 15% as much [α -³²P]UMP as reconstituted and native SV40 NPCs containing histones H2A, H2B, H3, and H4. Purified SV40 DNA was at least 10 times as efficient a template as the DNA complexed with histone H1. Moreover, the SV40 DNA was approximately 1.5 times as effective as the DNA complexed with histones H2A, H2B, H3, and H4. The four histones lacking H1 reduced transcription of SV40 DNA by 30 to 35%, a level similar to that for transcription of native

FIG. 4. Rate of incorporation of radioactive UMP into RNA. The reaction was started by addition of *E. coli* DNA-dependent RNA polymerase and stopped at various times to determine the amount of [α -³²P]UMP incorporated into RNA. Incorporation was determined as described in the text.

SV40 NPCs. Transcription of SV40 DNA was inhibited regardless of whether the origin of the histone fraction was permissive or nonpermissive cells.

Gel electrophoretic analysis of SV40 cRNA molecules transcribed from SV40 DNA and SV40 NPCs. Radiolabeled SV40 cRNA was separated from radioactive substrates on a Sephadex G-100 column. Sephadex G-100 was chosen for the column material to insure that labeled cRNA was eluted at void volume and that radioactive substrates were eluted near total volume (Fig. 5). The labeled cRNA was eluted within fractions 5 to 14 as shown in Fig. 5. The labeled cRNA in fractions 5 to 14 was pooled and used as purified SV40 cRNA for gel electrophoretic analysis. The possibility of RNA degradation by contaminating RNase activity can be completely eliminated by these procedures.

The association of histone H1 with SV40 DNA results in strong inhibition of transcription of the DNA as presented in Table 1. We compared the size and quantity of RNA made on SV40 DNA-histone H1 complexes with those made on

purified SV40 DNA to determine whether RNA chain elongation was inhibited by the association of H1. The size of cRNA molecules from SV40 DNA and NPCs showed a heterogeneous distribution with several peaks around 28S, 23S, 19S, 16S, and 13-5S. Gel electrophoretic patterns of RNA made on SV40 DNA-3T3 histone H1 complexes differed in the quantity of 19-23S RNA from the patterns of control DNA (Fig. 6g and h). Gel patterns in Fig. 6g and h showed that an approximately 19-23S RNA made on SV40 DNA-3T3 histone H1 complexes was less than the 19-23S RNA made on control DNA. This difference suggests that RNA chain elongation may be inhibited by the association of histone H1 with SV40 DNA. However, the small difference in the 19-23S RNA quantity does not quantitatively account for the 10-fold difference in total RNA synthesis (Table 1). We cannot therefore conclude from our results that the decreased template activity of the NPCs relative to that of control SV40 DNA is due to inhibition of elongation alone of RNA chains. Further experiments should be done to conclude whether the decreased activity of NPCs is due to inhibition of initiation or elongation of RNA chains or both.

Although the template activity of SV40 NPCs was less than that of control DNA, the size and species of RNA molecules transcribed from the NPCs were similar to those from the control DNA. Gel patterns were identical whether NPCs were reconstituted with H1 alone, with all five histones, or with the four histones lacking H1. The patterns were also identical regardless of whether the histones associated with SV40 DNA were derived from permissive or nonpermissive cells.

DISCUSSION

The contention that *E. coli* RNA polymerase transcribes SV40 DNA with specificity has rested on the presence of discrete RNA species (19) and transcription from a preferred initiation site (45) under conditions that greatly restrict total RNA synthesis. *E. coli* RNA polymerase initiates transcription at a restricted number of sites on SV40 DNA (12, 19) and initiates transcription at meaningful sites on the DNA (1, 11, 12, 26, 36). We also have previously reported the same observation for initiation of transcription at a restricted number of sites on SV40 DNA (14). It is known that SV40 DNA is transcribed asymmetrically and extensively in vitro under standard (44) and restricted (19) polymerization conditions by *E. coli* RNA polymerase. Our results obtained from the electrophoretic analysis of the transcription products were consistent with this conclusion (Table 1 and Fig. 6a). Figure

FIG. 5. Elution pattern of Sephadex chromatography of radioactive SV40 cRNA transcribed in vitro from SV40 DNA. The SV40 cRNA was prepared as described in the text and was chromatographed on a Sephadex G-100 column. The column was washed and equilibrated with 150 mM KCl and 10 mM Tris-hydrochloride (pH 7.6) before use. The flow rate of chromatography was 3.6 ml/h, and 0.3-ml fractions were collected. Portions of 50 μ l from each fraction were counted in scintillation fluid as described in the text. Fractions 5 to 14 containing SV40 cRNA were pooled and recovered by ethanol precipitation at -20°C as described in the text. Other cRNA samples were also recovered by the same procedures for gel electrophoretic analysis.

6 shows that SV40 NPCs as well as SV40 DNA were transcribed extensively *in vitro* by *E. coli* RNA polymerase.

Wasylyk et al. (43) have reported evidence that there is no preferential release of transcribing *E. coli* RNA polymerase due to the presence of nucleosomes. The arrangement of nucleosomes is random without the origin of SV40 DNA replication (33, 40, 41). Hall (13) has shown that SV40 NPCs are not physically altered after transcription with saturating amounts of *E. coli* RNA polymerase. This strongly suggests that the *in vitro* transcription process does not cause nucleosome disruption. Consequently, these findings lead to the proposal that SV40 NPCs might be ideal models for studying the effects of histones, nonhistone chromosomal proteins, and SV40 T-antigen products on SV40 gene expression. We have suggested that a 0.35 M NaCl-soluble fraction of host chromosomal proteins specifically controls SV40 gene expression (14). We observed that the cellular histones bound to SV40 DNA inhibited transcription of the DNA regardless of whether the origin of the bound histones was permissive or nonpermissive cells (Table 1).

The presence of urea during reconstitution procedures prevented the aggregation of histones or reconstituted SV40 NPCs or both (Fig. 1). Reconstituted and native SV40 NPCs were

purified by removing unbound histones and contaminating substances in a Bio-Gel column (Fig. 1); then the weight ratio of histone to DNA in the NPCs was calculated (Table 1). There was no selective loss of any histone during the reconstitution; moreover, the histones bound to SV40 DNA were not degraded or altered after reconstitution (Fig. 2). The chromatin-like repetitive structure of NPCs containing histones H2A, H2B, H3, and H4 was confirmed by the gel patterns of discrete DNA segment products by nuclease digestion. The gel electrophoretic patterns of discrete DNA segments were similar to the patterns described by Bellard et al. (2) for SV40 NPCs isolated from the nuclei of lytically infected cells (Fig. 3). These NPCs as well as purified SV40 DNA were used as templates for *in vitro* transcription using *E. coli* RNA polymerase (Table 1).

Transcription analysis indicated that histone H1 strongly inhibits transcription and, moreover, that the inhibitory effect of histone H1 on transcription is markedly different from that of the other four histones (Table 1). We have shown by electron microscopy that the complex fibers of SV40 DNA and histone H1 consist of twisted macrofibers and partially condensed configurations 15 to 35 nm in width (15). Germond et al. (10) and Muller et al. (27) have also demonstrated that histone H1 bound to SV40

FIG. 6. Autoradiography of analytical patterns of ^{32}P -labeled RNA products synthesized *in vitro* on SV40 DNA and SV40 NPCs by *E. coli* RNA polymerase. The RNA products purified by column chromatography were subjected to gel electrophoresis and detected by autoradiography as described in the text. The order of SV40 DNA and complexes as template used for RNA synthesis was as follows: (a and g) Control SV40 DNA alone; (b) complex of SV40 DNA and histone H1 derived from CV-1 cells; (c) SV40 DNA and histones H2A-H4 (consisting of H2A, H2B, H3, and H4) from CV-1 cells; (d) DNA and histones H1-H4 (consisting of H1, H2A, H2B, H3, and H4) from CV-1 cells; (e) DNA and histones H2A-H4 from calf thymus cells; (f) DNA and four histones H2A-H4 from rat liver cells; (h) DNA and H1 from 3T3 cells; (i) DNA and histones H2A-H4 from 3T3 cells; (j) DNA and histones H1-H4 from 3T3 cells; and (k) native SV40 NPCs consisting of SV40 DNA and histones H2A-H4 from CV-1 cells.

NPC alters the superhelix density of the NPC. H1 may, therefore, inhibit transcription by facilitating the aggregation or condensation of complex fibers.

Our results indicated that reconstituted SV40 NPCs containing histones H2A, H2B, H3, and H4 are transcriptionally active, although at a reduced rate compared with that of the purified SV40 DNA (Table 1). This activity was not related to whether the histones were derived from permissive or nonpermissive cells. Our findings on the transcription of reconstituted SV40 NPCs containing histones H2A, H2B, H3, and H4 are compatible with those of previous reports on the transcription of reconstituted SV40 NPCs (14) and the native SV40 NPCs isolated from permissive cell nuclei (16). Similarly, in a study of crude and purified native SV40 NPCs by Hall (13), crude SV40 NPCs were almost totally inactive as templates for RNA synthesis, whereas purified SV40 NPCs were transcriptionally active. He has suggested that, in addition to restriction of transcription by histones present in the SV40 NPCs, the crude preparations also contained cellular inhibitors of RNA synthesis or RNase. In our study using the *in vitro* reconstitution system, the RNA synthesis of SV40 NPCs lacking histone H1 increases approximately fivefold compared with that of NPCs containing H1 (Table 1). Therefore, it is possible that histone H1 contained in the crude extracts of SV40 NPCs was one of the other inhibitory substances. It may be difficult to confirm the effect of contamination in SV40 NPCs isolated from permissive cell nuclei. Further analyses are required to conclude whether inhibitory substances are present and, if so, their possible influence on transcription of SV40 NPCs.

The size of RNA molecules from SV40 DNA and NPCs showed a heterogeneous distribution with several peaks from 28S to 5S (Fig. 6). This result is in agreement with the results reported previously (11, 26, 43). Electrophoretic analysis of RNA products synthesized on SV40 DNA, reconstituted, and native NPCs showed several identical peaks between 13S and 28S (Fig. 6). We inferred from our results that the species of RNA molecules transcribed was not related to the species of histone fractions bound to SV40 DNA. The results of our study also suggest that the RNA species transcribed from SV40 DNA was not related to whether histones bound to the DNA were derived from either permissive or nonpermissive cells (Fig. 6). We found that the histones bound to SV40 DNA inhibited transcription of SV40 DNA regardless of whether the origin of the histones was permissive or nonpermissive cells (Table 1). It thus appeared

that the H2A, H2B, H3, and H4 complex composed of nucleosomes did not specifically regulate SV40 gene expression, at least in terms of the present *in vitro* transcription system using *E. coli* RNA polymerase. Further experiments using mammalian RNA polymerase are required to determine the role of histone H1 in SV40 gene expression. We are investigating the role of H1 by using a nuclear RNA polymerase-SV40 DNA complex (SV40 transcriptional complex) from infected monkey cells.

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LITERATURE CITED

1. Astrin, S. M. 1973. *In vitro* transcription of simian virus 40 sequences in SV3T3 chromatin. *Proc. Natl. Acad. Sci. U.S.A.* 70:2304-2308.
2. Bellard, M., P. Oudet, J. E. Germond, and P. Chambon. 1976. Subunit structure of simian-virus-40 minichromosome. *Eur. J. Biochem.* 70:543-553.
3. Blobel, G., and V. R. Potter. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. *Science* 154:1662-1665.
4. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62: 315-323.
5. Christiansen, G., T. Landers, J. Griffith, and P. Berg. 1977. Characterization of components released by alkali disruption of simian virus 40. *J. Virol.* 21:1079-1084.
6. Cohn, W., and E. Volkin (ed.). 1976. mRNA: the relation of structure to function. *Prog. Nucleic Acid Res. Mol. Biol.* 19:1-511.
7. Cremisi, C., P. F. Pignatt, O. Croissant, and M. Yaniv. 1976. Chromatin-like structures in polyoma virus and simian virus 40 lytic cycle. *J. Virol.* 17:204-221.
8. Favre, M., F. Breitburd, O. Croissant, and G. Orth. 1977. Chromatin-like structures obtained after alkaline disruption of bovine and human papilloma-viruses. *J. Virol.* 21:1205-1209.
9. Friedman, T., and D. David. 1972. Structural roles of polyoma virus proteins. *J. Virol.* 10:776-782.
10. Germond, J. E., B. Hirt, P. Oudet, M. Gross-Bellard, and P. Chambon. 1975. Folding of the DNA double helix in chromatin-like structures from simian virus 40. *Proc. Natl. Acad. Sci. U.S.A.* 72:1843-1847.
11. Graessmann, M., and A. Graessmann. 1976. "Early" simian-virus-40-specific RNA contains information for tumor antigen formation and chromatin replication. *Proc. Natl. Acad. Sci. U.S.A.* 73:366-370.
12. Hale, P., and J. Lebowitz. 1978. Binding and transcription of simian virus 40 DNA by DNA-dependent RNA polymerase from *Escherichia coli*. *J. Virol.* 25:298-304.
13. Hall, M. R. 1977. Transcription and reisolation of the simian virus 40 nucleoprotein complex. *Biochem. Biophys. Res. Commun.* 76:698-704.
14. Hidaka, H., M. Ocho, N. Tanabe, and T. Oda. 1977. Regulation of transcription of simian virus 40 DNA by chromosomal proteins of host cells, p. 62-64. *Proceedings of the 1977 Molecular Biology Meeting, Japan. Kyoritsu Shuppan Co., Ltd., Tokyo.*
15. Hidaka, H., S. Omura, N. Tanabe, and T. Oda. 1977. Fine structure of SV40 DNA-chromatin protein com-

- plexes. *J. Electron Microsc.* **26**:226.
16. Huang, E. S., M. Nonoyama, and J. S. Pagano. 1972. Structure and function of the polypeptides in simian virus 40. II. Transcription of subviral deoxynucleoprotein complexes *in vitro*. *J. Virol.* **9**:930-937.
 17. Johns, E. W. 1964. Studies on histones. 7. Preparative methods for histone fractions from calf thymus. *Biochem. J.* **92**:55-59.
 18. Ketner, G., and T. J. Kelly. 1976. Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. *Proc. Natl. Acad. Sci. U.S.A.* **73**:1102-1106.
 19. Lebowitz, P., and R. Bloodgood. 1975. Transcription of SV40 DNA by *E. coli* RNA polymerase: synthesis of a DNA-RNA intermediate and discrete RNA transcripts. *J. Mol. Biol.* **94**:183-201.
 20. Lebowitz, P., R. Stern, P. K. Ghosh, and S. M. Weissman. 1977. Specificity of initiation of transcription of simian virus 40 DNA I by *Escherichia coli* RNA polymerase: identification and localization of five sites for initiation with [γ - 32 P]ATP. *J. Virol.* **22**:430-445.
 21. Lebowitz, P., S. M. Weissman, and C. M. Radding. 1971. Nucleotide sequence of a ribonucleic acid transcribed *in vitro* from phage lambda deoxyribonucleic acid. *J. Biol. Chem.* **246**:5120-5139.
 22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
 23. Mandel, J. L., and P. Chambon. 1974. Animal DNA-dependent RNA polymerases. Studies on the reaction parameters of transcription *in vitro* of simian virus 40 DNA by mammalian RNA polymerases AI and B. *Eur. J. Biochem.* **41**:367-378.
 24. Mandel, J. L., and P. Chambon. 1974. Animal DNA-dependent RNA polymerases. Analysis of the RNAs synthesized on simian virus 40 superhelical DNA by mammalian RNA polymerase AI and B. *Eur. J. Biochem.* **41**:379-395.
 25. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**:208-218.
 26. Mueller, C., A. Graessmann, and M. Graessmann. 1978. Mapping of early SV40-specific functions by microinjection of different early viral DNA fragments. *Cell* **15**:579-585.
 27. Muller, U., H. Zevtgraf, I. Eicken, and W. Keller. 1978. Higher order structure of simian virus 40 chromatin. Histone H1 is responsible for the condensation of intranuclear viral DNA-protein complexes. *Science* **201**:406-415.
 28. Neirlich, D., W. Rutter, and C. F. Fox (ed.). 1976. Molecular mechanisms in the control of gene expression. ICN-UCLA Symposia on Molecular and Cellular Biology, vol. 5. Academic Press Inc., New York.
 29. Noll, M., J. O. Thomas, and R. O. Kornberg. 1975. Preparation of native chromatin and damage caused by shearing. *Science* **187**:1203-1206.
 30. Panyim, S., and R. Chalkley. 1969. High resolution acrylamide gel electrophoresis of histones. *Arch. Biochem. Biophys.* **130**:337-346.
 31. Peacock, A. C., and C. W. Dingman. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. *Biochemistry* **7**:668-674.
 32. Pettijohn, D., and T. Kamiya. 1967. Interaction of RNA polymerase with polyoma DNA. *J. Mol. Biol.* **29**:275-295.
 33. Ponder, B. A. J., and L. V. Crawford. 1977. The arrangement of nucleosomes in nucleoprotein complexes from polyoma virus and SV40. *Cell* **11**:35-49.
 34. Prives, C., E. Gilboa, M. Revel, and E. Winocour. 1977. Cell-free translation of simian virus 40 early messenger RNA coding for viral T-antigen. *Proc. Natl. Acad. Sci. U.S.A.* **74**:457-461.
 35. Scott, W. A., and D. J. Wigmore. 1978. Sites in simian virus 40 chromatin which are preferentially cleaved by endonucleases. *Cell* **15**:1511-1518.
 36. Shih, T. Y., G. Khoury, and M. A. Martin. 1973. *In vitro* transcription of the viral-specific sequences present in the chromatin of cells transformed by simian virus 40. *Proc. Natl. Acad. Sci. U.S.A.* **70**:3506-3510.
 37. Tanabe, N., H. Hidaka, S. Watanabe, and T. Oda. 1978. A simple large-scale method for separating closed circular form DNA by gel electrophoresis. *Acta Med. Okayama* **32**:379-385.
 38. Varshavsky, A. J., V. V. Bakayev, P. M. Chumackov, and G. P. Georgiev. 1976. Minichromosome of simian virus 40: presence of histone H1. *Nucleic Acids Res.* **3**:2101-2113.
 39. Varshavsky, A. J., S. A. Nedospasov, V. V. Schmatchenko, V. V. Bakayev, P. M. Chumackov, and G. P. Georgiev. 1977. Compact form of SV40 viral minichromosome is resistant to nuclease: possible implications for chromatin structure. *Nucleic Acids Res.* **4**:3303-3325.
 40. Varshavsky, A. J., O. H. Sundin, and M. J. Bohn. 1978. SV40 viral minichromosome: preferential exposure of the origin of replication as probed by restriction endonucleases. *Nucleic Acids Res.* **5**:3469-3478.
 41. Varshavsky, A. J., O. H. Sundin, and M. Bohn. 1979. A stretch of "late" SV40 viral DNA about 400 bp long which includes the origin of replication is specifically exposed in SV40 minichromosomes. *Cell* **16**:453-466.
 42. Waldeck, W., B. Fohring, K. Chowdhury, P. Gruss, and G. Sauer. 1978. Origin of DNA replication in papovavirus chromatin is recognized by endogenous endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* **75**:5964-5968.
 43. Wasyluk, B., G. Thevenin, P. Oudet, and P. Chambon. 1979. Transcription of *in vitro* assembled chromatin by *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **128**:411-440.
 44. Westphal, H. 1970. SV40 DNA strand selection by *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **50**:407-420.
 45. Zain, B. S., R. Dhar, S. M. Weissman, P. Lebowitz, and A. M. Lewis, Jr. 1973. Preferred site for initiation of RNA transcription by *Escherichia coli* RNA polymerase within the simian virus 40 DNA segment of the nondefective adenovirus-simian virus 40 hybrid viruses Ad2*ND₁ and Ad2*ND₃. *J. Virol.* **11**:682-693.