Conversion of simian virus 40 DNA to ordered nucleoprotein structures by extracts that direct accurate initiation by eukaryotic RNA polymerase II^{*†}

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ABSTRACT

Interaction of SV40 DNA with three different HeLa cell extracts capable of directing correct initiation of transcription leads to the formation of ordered nucleoprotein complexes that are structurally similar to SV40 minichromosomes and eukaryotic chromatin. These nucleoprotein complexes can be conveniently purified by band sedimentation or gel filtration. Their sedimentation and elution properties resemble those of SV40 minichromosomes. Electron microscopy of purified complexes shows beaded structures that are sensitive to proteases, resulting in recovery of naked, largely undegraded DNA. Contour lengths and compaction ratios of these nucleoprotein complexes are similar to those of authentic SV40 minichromosomes. Their digestion patterns with micrococcal nuclease and pancreatic DNase I resemble those of SV40 minichromosomes. Such nucleosome-like structures can also be obtained with linear SV40 DNA. Unlike nucleosomes, no histones could be detected in the purified nucleoprotein complexes. Non-histone chromosomal protein fractions (high mol. wt. and free of high mobility group proteins) prepared from the HeLa cell extracts can also generate similar ordered structures. We conclude that ordered nucleoprotein structures with certain common characteristics can be formed by interaction of DNA with non-histone chromosomal proteins as well as with histones. Only the former structures are generated in currently used cell-free transcription systems. It appears that only those purified nucleoprotein complexes containing the promoter can be actively transcribed in the presence of additional cell-free extract, suggesting that such structures and their protein components may be important in transcription.

INTRODUCTION

Specific, accurate initiation of transcription of class II eukaryotic genes has recently been achieved with the help of crude cell-free extracts with the addition of purified RNA polymerase II (1) or with the endogenous crude RNA polymerase II (2, 3). Using RNA polymerase II from various sources, Weil *et al.* (1) showed specific initiation at the major late adenoviral promoter if there were also present cytoplasmic extracts (S-100 fractions) derived from either virus-infected or uninfected human KB cells. A similar S-100 fraction from HeLa cells can also support accurate initiation at the SV40¹ early gene promoter (4). Four protein fractions required for the active,

selective initiation of transcription by RNA polymerase II were obtained by fractionation of the KB S-100 fraction (5). By fractionating concentrated homogenates (2) of chick oviduct and HeLa cells, Tsai et al. (6) reported the presence of common transcription factors from these two diverse sources.

Manley et al. (2) developed an extract from HeLa cells [similar to that described by Sugden and Keller (7)] that supports accurate initiation of transcription on various exogenously added templates, including SV40 DNA (8-10) and adenoviral DNA (11). Cepko et al. (12) showed that RNAs transcribed on restriction fragments of SV40 DNA with this extract were capable of directing the synthesis of several viral proteins when incubated with a reticulocyte lysate translation system. Weingärtner and Keller (13) showed that their whole cell extract (7) is capable of accurately initiating transcription on DNA templates carrying complete adenoviral transcription units, as well as the correct processing of the nascent transcripts.

Talkington et al. (3) prepared an RNA polymerase II-enriched S-100 extract by freezing a HeLa cell homogenate prior to the 100,000 X g centrifugation step. This extract could accurately initiate transcription on various exogenous templates (3).

Since different homologous and heterologous crude systems can direct accurate transcription on various DNA templates, it is likely that a mechanism common to all three extracts may be involved in the recognition process, possibly at the level of the template. Although all the investigations involved naked DNA as the template, the possible alteration of the template structure has not been reported. It is known that transcription (and replication) of SV40 DNA in vivo occurs in the form of the mini-chromosome (reviewed in Ref. 14), a complex of SV40 DNA with the core histones and nonhistone proteins. Jakobovits et al. (15) showed that the nucleoprotein nature of the template isolated from SV40-infected cells may be responsible for the accurate initiation of late transcription in vitro, albeit by Escherichia coli RNA polymerase, in contrast to the early strand transcripts from the naked SV40 DNA by the same enzyme. We find that all three transcription extracts convert SV40 form I DNA to ordered nucleoprotein complexes (NPCs) that exhibit biophysical properties similar to those of SV40 minichromosomes. Promoter-containing NPCs can be efficiently transcribed with additional extract. However, the extracts contain negligible amounts of histones, and neither do the complexes contain histones. It appears that the ordered SV40 NPCs are formed by nonhistone chromosomal proteins and the significance of these structures is discussed.

MATERIALS AND METHODS

Preparation of SV40 DNA

African green monkey kidney cells (BSC-1; gift of Dr. Maxine Singer, National Institutes of Health), free of mycoplasma, were grown and infected with SV40 (strain 776; gift of Dr. Robert Martin, National Institutes of Health) as described before (16). The infected cells were labeled with 50 $\mu\text{Ci/ml}$ [^3H]thymidine (80.1 Ci/mM, New England Nuclear) or 100 $\mu\text{Ci/ml}$ [^{32}P]orthophosphate (Amersham) 24 hr after infection. When early cytopathic effects appeared, the cells were harvested, lysed, and the labeled DNA was extracted by the procedure of Hirt (17), and purified by equilibrium centrifugation in CsCl containing ethidium bromide (18), as described by Khoury and Lai (19). The DNA was dialyzed against 10 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA. The SV40 DNA thus obtained was >98% form I, based on electron microscopy and gel electrophoresis.

DNA fragments of SV40 form I DNA cleaved by digestion with Pst I (Bethesda Research Labs.) were purified by phenol extraction, chloroform extraction, and ethanol precipitation. DNA was resuspended in 10 mM Tris-HCl (pH 7.9) containing 1 mM EDTA at a concentration of 200 $\mu\text{g/ml}$ and dialyzed against the same solvent.

Preparation of HeLa Cell Extracts

HeLa cells (strain HCAAT, suspension type; gift of Dr. James Regan of this Division), free of mycoplasma, were grown in suspension culture in 4-liter flasks at 37°C in Dulbecco minimal essential medium supplemented with 2.36 g/liter NaHCO_3 , 10.4 g/liter NaCl, 1.2 ml/liter gentamycin sulfate (Sigma), and 10% fetal calf serum (Grand Island Biological Co.) to a density of about $6-8 \times 10^5$ cells/ml. All further operations were done at 0-4°C. The cells were collected by centrifugation at 800 rpm for 10 min (Sorvall, GSA rotor), washed with phosphate-buffered saline and used directly.

The S-100 extract of the cells (extract A) was prepared according to Weil et al. (20). The soluble whole-cell extract (extract B) was prepared as described by Manley et al. (2) except that the final dialysis buffer contained 20 mM HEPES (pH 7.9), 12.5 mM MgCl_2 , 100 mM KCl, 0.1 M EDTA, 2 mM DTT, and 17% glycerol (9). Extract C was prepared according to Talkington et al. (3).

Nucleoprotein Formation

The reaction mixtures (50 μl) with each extract were similar to those described in the respective reports (1-3, 9) except that purified RNA polymerase II [used by Weil et al. (1) with extract A] and ribonucleoside triphosphates were omitted. Most reaction mixtures contained 1 μg of

^3H -labeled SV40 form I DNA and 15-30 μl of extract (150-250 μg protein). The reaction mixtures were incubated at 30°C for 60 min, as were controls without any extract. Then 10-20 μl of 0.1 N NaOH were added to raise the pH to 9.0, 200 μl of 10 mM Tris-HCl (pH 9.0) containing 0.2 mM EDTA and 0.5 mM DTT (Buffer A) were added, and the tubes were chilled in ice for 15 min.

Nucleoprotein formation with ^3H -labeled SV40 DNA cleaved with Pst I (cuts circular SV40 DNA at two sites to generate 4027 and 1216 bp fragments) was performed in a similar manner except that the reaction mixture (80 μl) contained 8 μg of ^3H -labeled SV40 form I DNA and 60 μl of extract (~1200 μg protein) and processed as above.

Isolation of NPC

(a) Velocity Sedimentation

The SV40 NPC was purified by sedimentation at 4°C of the reaction mixture in 12-ml sucrose gradients (5-30%, in buffer A) in a Beckman SW41 rotor at 39,000 rpm for 2 hr for SV40 form I DNA and 3 hr for Pst I-cleaved SV40 DNA. Fractions of 0.3 ml were collected from the bottom of the tubes, and aliquots were counted for radioactivity. Control samples (without protein) and appropriate molecular weight markers were centrifuged in separate tubes simultaneously.

(b) Gel Filtration

To obtain a large yield of NPC, nucleoprotein formation was carried out under identical conditions (see above) except that the reaction mixture was scaled up 10 fold. The mixture was then loaded on a Bio-Gel A-15m (Bio-Rad Laboratories) column (1.6 $\times$ 84 cm) equilibrated with buffer A. The column was then eluted with the same buffer and the eluant was monitored at 254 and 280 nm. Fractions (0.6 ml) were collected at a flow rate of 12 ml/hr. Aliquots were counted for determination of radioactivity. Standards of [^3H]SV40 form I DNA and [^3H]SV40 minichromosomes purified from infected cells (16) were run on similar columns.

Nuclease Digestion

(a) Digestion of NPC with Micrococcal Nuclease

[^{32}P]SV40 form I DNA (0.6 μg) was incubated with 14 μl of extract (DNA:protein ratio ~1:150) for 1 hr at 30°C. The crude NPCs were nuclease digested at 30°C for various times in 25 μl reactions containing 50 mM Tris-HCl (pH 7.9), 0.024 unit of micrococcal nuclease (Worthington), and 3.5 mM CaCl_2 (21). Reactions were terminated with 1.8 μl of 10% SDS containing 0.25 mM Na_2EDTA , followed with 3.2 μl of 10 mg/ml pronase, and incubation at 30°C for 1 hr. The samples were extracted twice with equal

volumes of phenol:chloroform:isoamyl alcohol (50:49:1), and adjusted to ~1.75% glycerol and ~0.001% bromophenol blue prior to electrophoresis in 1.5 mm × 135 mm long 0.5% agarose/3% polyacrylamide composite gels (22) [electrophoresis buffer was 40 mM Tris-HCl (pH 7.9), 20 mM Na acetate, and 2 mM Na₂ EDTA] for 6 hr at a constant 50 V. Autoradiography of dried gels was on Kodak XAR-5 X-ray film with a DuPont Cronex Lightening-Plus intensifying screen for 13.5 hr at -76°C. ϕ X174 RF DNA-HaeIII fragments (Bethesda Research Labs.) in the gels served as markers and were detected by ethidium bromide (1 µg/ml) staining.

(b) Digestion of NPC with Pancreatic DNase I

[³²P]SV40 form I DNA (0.5 µg) was incubated with 17 µl of the extract (DNA:protein ratio ~1:200) for 1 hr at 30°C. The crude NPCs were nuclease digested at 30°C for various times in 25 µl reactions containing 50 mM Tris-HCl (pH 7.4), 1.9 µg of pancreatic DNase I (Worthington), 6 mM MgCl₂ and 5 mM CaCl₂. Reactions were terminated and extracted as described above. Samples were then mixed with an equal volume of denaturation buffer (~0.05% xylene cyanol FF, ~0.05% bromophenol blue, 12 mM Na₂ EDTA, 10 M urea and 20% sucrose), incubated for 2 min at 100°C, and quick-cooled in an ice bath. Electrophoresis in 0.75 mm × 360 mm long 12% polyacrylamide - 7 M urea denaturing gels (electrophoresis buffer as above) was for 14 hr at a constant 350 V. Bromophenol blue and xylene cyanol FF were used as 13 and 58 base size markers, respectively (23). The wet gels were autoradiographed as above, but with 96 hr exposure.

Preparation of Non-histone Chromosomal (NHC) Proteins

NHC protein was prepared from HeLa cell extracts by the method of Elgin and Bonner (24), dialyzed against 10 mM Tris-HCl (pH 9.0) and concentrated by lyophilization to about 3.2 mg/ml.

Isolation of NHC Protein-DNA Complex

[³H]SV40 form I DNA (5 µg) was incubated with 128 µg of NHC protein using conditions described under "Nucleoprotein Formation", but with 100 mM NaCl added to the reaction mixture. After incubation and addition of alkali and buffer A as before, the mixture was loaded on a Bio-Gel A-15m column (1 × 46 cm) and eluted with buffer A containing 100 mM NaCl. Fractions (0.3 ml) were collected at a flow rate of 9 ml/hr. Other conditions were similar to those described earlier under "Gel Filtration."

Electron Microscopy

Peak fractions from sucrose gradients were pooled and dialyzed overnight against buffer A. Peak fractions from the Bio-Gel A-15m column were diluted

four fold with buffer A. Samples were treated with 15 mM glutaraldehyde buffered with 10 mM Tris-HCl (pH 7.5) for 5 min at room temperature. Electron microscopy was done following the method of Dubochet *et al.* (25), as modified by de Murcia *et al.* (26). Pure DNA samples were also visualized by the aqueous protein film technique (27). DNA contour length measurements and nucleosome counting were done directly by projecting the micrograph negative on a screen and tracing the DNA with a map measuring device (28).

In some experiments, purified NPCs were deproteinized by treatment with pronase (1 mg/ml) in the presence of 0.6% SDS, followed by phenol extraction, ethanol precipitation, and dialysis against 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA. Electron microscopy of the resulting DNA was according to Dubochet *et al.* (25) and Ferguson and Davis (27).

Electrophoresis of Proteins

Purified NPCs were dialyzed overnight against 1 mM Tris-HCl (pH 9.0), then lyophilized to dryness before dissolving in a minimum volume of sample loading buffer. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (29), in the microslab gel system (30).

Transcription Assays

Assays were carried out in a final volume of 50 μ l, using 10 μ l of extract and ionic conditions similar to those described by Handa *et al.* (9), except that 8–10 ng of ^{3} H-labeled circular SV40 DNA or linear SV40 DNA fragment, either naked or in the form of NPC (obtained from peak fractions in the sucrose gradients), were used as template, and poly(dI-dC) was added to the reaction (12) at a concentration of 22.5 μ g/ml. The reaction mixture was incubated at 30°C, 5- μ l aliquots were withdrawn at timed intervals and precipitated with cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. After 10 min at 0°C, the solution was filtered through a Whatman glass paper (GF/C) disc. The disc was then washed extensively with the same solvent, followed by cold ethanol, then dried under an infrared heat lamp, placed in a scintillation vial, and counted with BBOT-toluene solution (4 g of BBOT in a liter of toluene) in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Complexes are formed (as described below) by SV40 DNA after incubation with the three extracts used in these studies. Initial experiments indicated incomplete recovery of the complex in the peak position in sucrose gradients. The problem was traced to the insolubility of the complex, possibly due to aggregation or trapping. Increasing precipitation occurred with increasing

amounts of extract protein and/or decreasing pH. Therefore, after incubation at pH 7.9, dilute alkali was added to raise the pH to 9.0, and sucrose gradient centrifugation was performed at pH 9.0. Once purified through sucrose gradients, the complex was stable in the pH range of 7.5 to 9.5. Even under incomplete recovery conditions at the lower pH, the complexes displayed physical properties similar to those described below.

Isolation and Characterization of NPCs

(a) Sedimentation - The formation of NPCs was first evident from their sedimentation profiles showing a significant shift in the peak positions compared to naked SV40 form I DNA (Fig. 1). Practically all of the input DNA can be recovered as faster sedimenting material when incubated with the extracts. SV40 DNA, incubated under identical buffer conditions but without any extract, sediments like naked DNA. Using appropriate markers [Kilham rat virus (110 S) and T7 DNA (31.5 S) obtained respectively from Drs. Sankar Mitra and Warren Masker of this Division, and SV40 form I DNA (21 S)], one can calculate (from peak fractions) S values of 55, 64, and 68 for NPC-A, NPC-B and NPC-C, respectively. These S values are similar to those obtained for the SV40 minichromosome in its relaxed state (reviewed in 14; 16, 31). A small fraction of the SV40 DNA appears at the top of the gradient, possibly due to degradation by endogenous nucleases in the extracts. A rather broad peak is obtained with NPC-B (as compared to those with NPC-A and NPC-C) along with some fast-sedimenting material towards the bottom of the tube, possibly due to aggregation. Some aggregation is also apparent with the NPC-C (Fig. 1). The greater amount of nuclear proteins in B and C may account for the differences in the sedimentation behavior of these NPCs. NPCs of Pst I-generated SV40 DNA fragments could be isolated as two distinct peaks in sucrose gradients (results not shown).

(b) Gel filtration - Out of the several gel systems tried, Bio-Gel A-15m gave the desired separation. As seen in Figure 2, NPC-A and NPC-B are excluded from the column in the same region, slightly behind NPC-C. All are excluded a little ahead of SV40 minichromosomes isolated from infected cells (double arrow). As expected, naked SV40 form I DNA was retarded in the column. When the column eluants are monitored at 254 nm and 280 nm, there is a common peak at both wavelengths which coincides with the radioactivity peak shown in Figure 2. Beyond the void volume, a peak at 254 nm with low radioactivity is possibly due to degraded SV40 DNA and nucleic acids present in the extracts.

(c) Electron microscopy - The complexes, formed with all three extracts and purified on sucrose gradients, display (Figure 3) the familiar "beads-on-a-

Fig. 1. Sedimentation profiles in 5–30% sucrose gradients of complexes formed by SV40 DNA with (A) extract A, (B) extract B, and (C) extract C. The markers SV40 form I DNA (21 S), T7 DNA (32 S), and Kilham rat virus (110 S) are indicated by single, double, and triple arrows, respectively.

string" structure of SV40 minichromosomes (14, 33, 34). The beads are connected by coated spacers of various lengths. Complexes purified by Bio-Gel A-15m filtration displayed similar structures (not shown).

The electron micrographs (not shown) of deproteinized NPCs indicate the disappearance of beads with the recovery of naked DNA, suggesting the proteinaceous nature of the beads. Most of the DNA could be recovered as undegraded circles, suggesting protection of the DNA due to nucleoprotein formation. Along with predominantly closed circles, there were some open

Fig. 2. Elution profiles from Bio-Gel A-15m columns (1.6×84 cm) of complexes formed by SV40 DNA with (A) extract A, (B) extract B, and (C) extract C. Arrows indicate the peak positions of markers — single arrow: [^3H]SV40 DNA form I; double arrow: [^3H]SV40 minichromosome isolated from infected cells.

circles, suggesting some single strand nicking during the incubation and subsequent manipulations.

The average numbers of beads per DNA molecule for NPC-A, NPC-B and NPC-C were 12.8, 23.6 and 21.4, respectively, as compared to 21.1 for the SV40 minichromosome. The average diameters of the beads in NPC-A, NPC-B and NPC-C were 10.3, 9.9 and 10.0 nm, respectively, somewhat smaller than the average nucleosome diameter of 12.5 nm for the SV40 minichromosome. From measure-

Fig. 3. Electron micrographs of SV40 DNA incubated with (Control) buffer, (A) extract A, (B) extract B, and (C) extract C. The DNA and complexes were purified on sucrose gradients (Fig. 1) and samples prepared by the technique of Dubochet *et al.* (25). Magnification = 100,000.

ments of the DNA contour lengths, the compaction ratios were 2.41, 2.75 and 2.63, respectively, as compared to 2.70. Thus, in physical measurements, the NPCs are comparable to SV40 minichromosomes (34). The smaller number of beads in NPC-A is possibly due to the smaller amount of nuclear proteins in extract A.

(d) Enzymatic analyses - Digestion with micrococcal nuclease and pancreatic DNase I have been useful in elucidating nucleosomal structures (14, 36). When histone-SV40 DNA complexes assembled *in vitro* are digested with micrococcal nuclease, DNA fragments of about 145 bp are obtained (35). Similarly, digestion of crude NPC-A, NPC-B, and NPC-C yields DNA fragments of 145 ± 14 bp, 150 ± 18 bp, and 135 ± 11 bp, respectively. Thus, these values are similar in length to DNA in a nucleosome core particle (36, 37), but smaller than the 200 bp fragments obtained by digestion of chromatin in nuclei (36, 38). However, the micrococcal nuclease digestion patterns (shown for NPC-B in Fig. 4) of the NPCs, although showing a resistant core of defined length, showed no clear-cut multiple periodicity that is observed during chromatin digestion.

Pancreatic DNase I is known to cleave DNA within a nucleosome core in multiples of about 10 bp (39, 40). Figure 5 shows the electrophoretic patterns

Fig. 4. Autoradiogram of micrococcal nuclease digestion of NPC-B. Positions of an *Hae*III digest of ϕ X174 DNA are indicated to the left. The limit digest size is indicated to the right with an asterisk.

of DNA fragments obtained after digestion of crude NPC-B with pancreatic DNase I. Similar patterns were obtained with NPC-A and NPC-C. The distances between the DNA bands were 10.2 ± 1.5 bases, 9.7 ± 1.6 bases, and 10.6 ± 1.2 bases for NPC-A, NPC-B, and NPC-C, respectively, indicating an average interband distance of about 10 nucleotides — a characteristic property of eukaryotic chromatin.

Nature of Proteins in the Purified Complexes

It appears that these NPCs share some characteristic properties with nucleosomes. However, a marked difference was found in that although minute amounts of histones H3 and H2B can sometimes be found in the crude extracts, no histones could be detected in the purified complexes, using the acid-urea gels of Panyim and Chalkley (41) as well as high pH SDS gels (29), even with the use of the microslab gel system (30). The major bands (Fig. 6) are within the mol. wt. range of 200,000–50,000, based on mol. wt. markers (not

Fig. 5. Autoradiogram of pancreatic DNase I digestion of NPC-B. The positions of the markers xylene cyanol (XC) and bromophenol blue (BPB) are indicated. The dots indicate the positions of the DNA fragments.

shown). Even when overloaded with purified NPCs (30–40 μ g protein), the characteristic band pattern of histones could not be seen (gels not shown).

Since only small amounts of insoluble material remained at the top of the gel, it was unlikely that the NPCs were not completely dissociated. Nonetheless, to ensure complete dissociation, acid-urea gel electrophoresis was carried out in the presence of 0.01% cetyltrimethyl-ammonium bromide (42); even then, no histone bands were detected. The purified NPCs were electrophoresed on an SDS-polyacrylamide gel and the resultant gel was subjected to the highly sensitive silver staining method (43). Still there

Fig. 6. SDS-polyacrylamide gel electrophoresis of proteins in purified NPCs. Lanes A and B — NPC-A, 5 and 10 μ g protein; lanes C and D — NPC-B, 6 and 12 μ g protein; lanes E and F — NPC-C, 5 and 10 μ g protein; lane G — inner histones (chicken erythrocyte), 4.5 μ g; lane H — total histones (chicken erythrocyte), 2.5 μ g.

were no characteristic histone bands. These results all support the contention that histones are not present in these purified NPCs.

We turned our attention towards the second major group of proteins, i.e., the NHC proteins associated with eukaryotic chromatin. Fractions rich in NHC proteins were prepared by acid extraction (24) of the extracts. The preparations were checked for the absence of histones by gel electrophoresis, then incubated with SV40 DNA and the complexes purified by gel filtration on a Bio-Gel A-15m column. The NHC protein-SV40 DNA complexes exhibited the same gel filtration behavior as observed earlier in case of the NPCs. The presence of 100 mM NaCl throughout the operation was, however, helpful in maintaining the stability of the complex. The electron micrograph of purified NHC protein-SV40 DNA complexes (shown for NHC-C in Fig. 7) shows beaded structures similar to those obtained with the NPCs (Fig. 3). In general, the tendency of forming clumps on E.M. grids, always a problem with these complexes, was more pronounced in the case of NHC protein-DNA complexes, the reason for which is not clear at present.

The proteins in the purified NHC protein-DNA complex were separated on SDS-polyacrylamide gels utilizing the microslab gel system. The gel electrophoretic patterns are shown in Figure 8 (lanes B, C, D). It is clear that not all the NHC proteins in the crude preparation (lane A) are associated with the purified complexes. There are no bands in lanes B, C,

Fig. 7. Electron micrographs of SV40 DNA incubated with (A) buffer and (B) NHC protein and purified by chromatography on a Bio-Gel A-15m column. Both the DNA and the NPC were fixed with 15 mM glutaraldehyde and processed for electron microscopy as described under Materials and Methods. Magnification = 117,000.

and D that correspond to calf thymus HMG 1, 14, and 17 (gifts from Mr. Arthur E. Paton of this Division). It is interesting that the band patterns obtained from the NHC protein-DNA complexes and NPC-A, NPC-B and NPC-C have striking similarities (cf. Figs. 6 and 8). This is under further investigation. Therefore, it appears likely that the ordered structures observed are due to nonhistone proteins of the low mobility group.

Transcription Studies with DNA and NPC Templates

The HeLa cell extracts were transcriptionally active, and the transcription was dependent upon the addition of exogenous DNA. Extract A was optimally active with the addition of exogenous RNA polymerase II. Restriction fragments of cloned adenoviral DNAs, namely, Bal I-E and pFLBH (gifts of Dr. Malcolm Gefter and Mr. Andrew Fire of the Massachusetts Institute of

Fig. 8. SDS-polyacrylamide gel electrophoresis of proteins in purified NHC protein - SV40 DNA complex; lack of high mobility group proteins in purified NHC protein-SV40 DNA complex. Lanes A and E - crude NHC proteins, 16 μ g protein; lanes B, C and D - purified NHC protein-SV40 DNA complex, 15, 7.5 and 3 μ g protein, respectively; lane F - HMG1, 4 μ g; lane G - HMG14, 6 μ g; lane H - HMG17, 6 μ g. Lanes A-D are on one gel and lanes E-H are on another gel run under identical conditions.

Technology) were used to check the transcriptional specificities of the extracts. The rates of transcription using extract B with three DNA templates, namely, SV40 form I DNA, the Pst I-cleaved SV40 DNA fragments and their NPCs - all purified on sucrose gradients - are shown in Fig. 9. The NPC of neither form I DNA containing the promoter region, nor the Pst I-generated small fragment (L_t) containing the termination region, showed appreciable synthesis without additional extract (Fig. 9A and 9C). The NPC of the Pst I-generated large fragment (L_p) containing the promoter region, showed a small amount of synthesis without added extract (Fig. 9B). In the presence of extract, naked L_p served efficiently as a template, whereas form I and L_t were poor templates (Fig. 9). When the NPCs were tested with additional extract, the best transcription was seen for L_p , followed by a significant improvement for form I and no improvement for L_t (Fig. 9). Thus, promoter-containing NPCs appear to serve as better templates than their DNA counterparts. The RNA transcripts from these various templates are being analyzed for specificity of initiation and termination (results to be published elsewhere).

Fig. 9. Rates of transcription with extract B and various DNAs and their NPCs as templates. (A) SV40 form I DNA, (B) *Pst* I-generated large fragment having the SV40 promoter region, and (C) *Pst* I-generated small fragment having the SV40 termination region. a. $\bigcirc-\bigcirc$, NPC template without extract; b. $\bullet-\bullet$, NPC template with 10 μ l of extract B; c. $\blacktriangle-\blacktriangle$, Extract B (10 μ l) without template; d. $\triangle-\triangle$, Naked DNA with 10 μ l of extract B.

DISCUSSION

We have made the surprising observation that SV40 DNA assumes an ordered nucleoprotein structure when incubated with cell-free extracts capable of RNA synthesis mimicking *in vivo* transcription by eukaryotic RNA polymerase II. Cleaner (as revealed by electron microscopy) preparations of these NPCs could be obtained when purified by gel filtration. This was probably due to proteins co-migrating with the complexes in the sucrose gradients, but not during gel filtration. Proteolytic digestion of the purified complexes allows for recovery of naked, largely undegraded SV40 DNA, indicating the proteinaceous nature of the NPCs. Ordered NPCs can be obtained with either

supercoiled or relaxed circular SV40 DNA, and also with linear DNA.

In preliminary studies, the NPCs formed during active transcription of either form I or linear SV40 DNA were examined. The ternary (DNA-nascent RNA-protein) complexes, conveniently purified on Bio-Gel-A-15m columns, also possess ordered structures similar to those described in the present studies.

Nucleosomal structures are by far the most thoroughly characterized organized structures of eukaryotic DNA. The "beads-on-a-string" structure (44, 45) of eukaryotic chromatin can be reproduced in vitro in the presence of histones, DNA, and certain assembly factors (reviewed in 46, 14). However, certain proteins, other than histones and from both eukaryotic and prokaryotic sources, can convert DNA into ordered structures resembling nucleosomes (47-49). Nucleosome-like structures with SV40 DNA can be formed with the HU protein from E. coli (47), the HMG proteins 1 and 2 from calf thymus (48), and the S-25 protein from rat liver (49). The NPCs isolated by us and HU-DNA complexes are similar to SV40 minichromosomes with respect to compaction ratio and contour length. However, compared to nucleosomes, the bead diameters of our NPCs are smaller (by about 20%) whereas that for HU-DNA complexes is greater (by about 50%). Furthermore, the beads are less uniform in size for the NPCs and HU-DNA complexes in comparison to nucleosomes. For HMG-SV40 DNA complexes, micrococcal nuclease and DNase I digestion patterns were not suggestive of a truly nucleosome-like structure (48). The nuclease digestion patterns of our NPCs appear to reflect structures similar to a nucleosomal structure. Although there is a DNA-nicking activity in the extracts, we have been able to investigate the constraints put on the superhelical SV40 DNA within the ordered NPCs. Under certain conditions, we observe electrophoretic band patterns characteristic of superhelical SV40 DNA. This suggests that the DNA is wrapped around the beads, as also suggested by the condensation of the DNA fiber and the DNase digestion patterns.

It is unlikely that the beads seen under the electron microscope are due solely to the binding of the endogenous RNA polymerase to SV40 DNA. The RNA polymerase contents of the three extracts differ widely (1-3), yet equal amounts of the extract protein produce similar numbers of beads. Under the electron microscope, the purified RNA polymerase-DNA complexes appear quite different from the nucleosome-like structures seen in these studies. Furthermore, only 1 to 3 beads per DNA molecule could be seen when 3 and 6 μ g of purified RNA polymerase II were incubated with 5 μ g of SV40 DNA.

Because of the methods of preparation (1-3), none of the transcription extracts used in these studies is expected to contain appreciable amounts of

histones, and therefore, the ordered NPCs do not contain histones. Nor do these contain HMG proteins. The complexes are apparently formed with high m.w. NHC proteins of the low mobility group (LMG). Tsai *et al* (6) found that transcription factors from HeLa cells and chick oviduct are similar and interchangeable, and showed that the most important fraction (P1000) for correct initiation of transcription was also highly enriched in high m.w. DNA-binding proteins. Our results (Fig. 8) suggest that some of the NHC proteins responsible for ordered nucleoprotein formation may be similar, if not identical, to the HeLa DNA-binding proteins of Tsai *et al* (6). Furthermore, incubation of the HeLa P1000 fraction with SV40 DNA also leads to the formation of ordered NPCs. These results reinforce our suggestion that the different transcription extracts (1-3), may have factors in common that interact with the template such that the resulting nucleoprotein template is suitable for accurate initiation of transcription.

Our results (Fig. 9), showing that promoter-containing NPCs are better templates than their DNA counterparts for synthesis with additional extract, suggest that certain DNA-binding protein factors are needed for efficient transcription. The results also suggest that these factors may interact at or near the promoter region. We are also investigating the possibility that the more efficient transcription with L_p , as compared to that with form I DNA, may be due to nicks in the former. Lewis and Burgess (50) found that such nicks can act as primers with SV40 DNA and purified RNA polymerase II.

The important question remains as to whether the systems currently employed for specific transcription *in vitro* (1-3) are truly comparable to those occurring *in vivo*. Although the *in vitro* transcription systems function correctly, this may not truly reflect the *in vivo* situation. This is particularly true for SV40 transcription, since it is well-established (reviewed in ref. 14) that such transcription occurs in the form of its minichromosome which is quite similar to eukaryotic chromatin in physical properties and protein composition. Our results indicate that the SV40 DNA-protein complexes formed in the *in vitro* systems mimic (in physical properties) the SV40 minichromosomes but differ from the latter in their protein composition. The RNA polymerase II "sees" nucleoprotein structures that resemble the *in vivo* chromatin templates but these are far from being identical.

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*Abbreviations used are: SV, simian virus; NPC, nucleoprotein complex; NPC-A, NPC-B and NPC-C, nucleoprotein complexes obtained using extract A, B and C, respectively; EDTA, ethylenediaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl) amino-methane; NHC, nonhistone chromosomal; SDS, sodium dodecylsulfate; bp, base pairs; HMG, high mobility group

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