TRANSCRIPTION AND REISOLATION OF THE SIMIAN VIRUS 40 NUCLEOPROTEIN COMPLEX

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SUMMARY: Nucleoprotein complexes, containing cellular histones associated with Simian Virus 40 DNA, were isolated from virus infected cells. These complexes supported RNA synthesis when incubated with E. coli DNA-dependent RNA polymerase. Under the conditions of assay, transcripts synthesized by E. coli polymerase were shown to be highly specific for viral DNA by hybridization assay. Of particular importance is the demonstration that the viral nucleoprotein complexes isolated from the transcription mixture have not been physically altered.

INTRODUCTION

Viral nucleoprotein complexes (NPC), isolated from Simian Virus 40 (SV40) or polyoma virus virions (1,2) and from virus infected cells (3-6), have been reported to contain four cellular histones (7,8). These histones, H2A, H2B, H3, and H4, are also found in the nucleosomes characteristic of cellular chromatin (9). Electron microscopic studies have shown that the viral NPCs contain approximately 20 nucleosomes and are similar in structure to cellular chromatin (10,11).

The similarity between viral NPC and cellular chromatin suggests the possibility that NPCs might be ideal models for studying the effects of chromosomal proteins on transcription of a defined genome. Indeed, recent studies have shown that polyoma and SV40 NPCs can be transcribed by exogenous and endogenous polymerases (12,13). The present report supports these studies and in addition provides evidence that the viral NPCs can be utilized as model chromosomes (mini-chromosomes) in a transcription assay without alteration of their structure.

MATERIALS AND METHODS

African green monkey kidney cells (CV-1) were infected with strain RH911 SV40 virus at a multiplicity of 10-20 pfu/cell. At 40 h postinfection, cultures were pulsed with [^{3}H]TdR (15 Ci/mM) for 120 min at 14 μCi/ml,

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5.0 ml/culture. Nucleoprotein complexes containing SV40 DNA were extracted as previously described (6). The crude lysates were cleared of nuclear aggregates and large cellular debris by centrifugation at 800 x g for 10 min at 4°. The resulting NPC containing supernatant was divided into three fractions for transcription studies. One fraction was deproteinized and closed circular supercoiled viral DNA isolated from propidium diiodide-CsCl gradients. The second fraction was concentrated by pressure dialysis in order to produce a "crude NPC fraction" containing viral DNA at a concentration of 2.0 µg/ml. Finally, the third fraction was fractionated by a combination of hydroxyapatite (HA) chromatography and velocity centrifugation previously described by Meinke et al. (8) to produce "purified NPCs".

Velocity centrifugation of complexes in linear 5 to 20% (w/w) sucrose gradients, and buoyant density centrifugation of glutaraldehyde fixed complexes as well as collection, processing and scintillation counting of centrifuged samples have all been previously described (5,6).

In vitro transcription of SV40 NPCs and viral DNA was carried out by a modification of Astrins (14) assay system. Except where indicated, RNA was synthesized from NPC templates or purified viral DNA at 1.0 µg of DNA per assay. Each reaction also contained, in a total volume of 1.0 ml, 20 units of E. coli DNA-dependent RNA polymerase (Miles Laboratories), 15 mM KCl, 40 mM TRIS-HCl (pH 7.9), 0.1 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% Triton X-100, 40 mM NaCl, 2 mM EDTA, and 1 mM each, ATP, GTP, CTP, and UTP ([^{3}H]UTP 10 µCi). The reaction was terminated after 15 min incubation at 37°C, by addition of 5.0 ml of cold 10% TCA. The acid-precipitable material was collected on glass fiber filters (Whatman GF/C), dried and counted in a liquid scintillation counter.

To prepare RNA for filter hybridization, the preceeding reaction mixtures were incubated for 3 hr at 37°C. The reaction was terminated by addition of SDS to a final concentration of 1.0%. RNA was extracted with phenol saturated with acetate buffer pH 5.2 (13) and collected by ethanol precipitation.

For DNA-RNA hybridization, SV40 DNA I, purified through propidium diiodide-CsCl gradients, was heat denatured and immobilized onto 25 mm nitrocellulose filters (Schleicher & Schuell Co., type B6) by the methods of Aloni et al. (15). DNA-RNA hybrid analyses were performed in scintillation vials containing one blank filter and one filter containing either 2.5 or 5.0 µg of viral DNA. Incubations were for 24 hrs at 65°C with viral RNA in 2.0 ml 2 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate). The filters were then chilled, washed in 2 x SSC and exposed to 20 µg/ml pancreatic ribonuclease in 2 x SSC for one hr at room temperature. Filters were then rinsed in 2 x SSC, dried and counted in a scintillation counter.

RESULTS

In Vitro Transcription of SV40 DNA, Crude NPCs, and Purified NPCs

Prior to use in the transcription assay, aliquots of the purified NPCs were analyzed to assure that they maintained the sedimentation velocity (61S) and density (1.437 g/cm^3) previously reported for SV40 NPCs (6). The purified NPC preparation utilized in these studies had sedimentation coefficients of between 58 and 61S and buoyant densities between 1.435 and 1.445 g/cm^3.

Table 1. In Vitro Transcription of SV40 DNA and Nucleoprotein Complexes by E. coli DNA-Dependent RNA Polymerase.

Template	RNA Synthesis *(cpm[^{3}H]UTP Incorporated)
Crude NPC	†180
Purified NPC	970
SV40 DNA	4100

* RNA was synthesized from NPC templates containing 1.0 μg of DNA per assay or from 1.0 μg of purified Form I SV40 DNA as described in Materials and Methods. These values are corrected for approximately 85 cpm of incorporation in the absence of template.

† Corrected for approximately 120 cpm of endogenous polymerase activity.

Purified SV40 DNA and NPCs as well as crude NPCs were prepared and utilized as templates for E. coli RNA polymerase as described in Materials and Methods. The results presented in Table 1 show that all three templates supported incorporation of [^{3}H]UTP into RNA catalyzed by E. coli DNA dependent RNA polymerase. However, it is evident that the crude complex is a relatively poor template which supports incorporation of only approximately 20% as much [^{3}H]UTP as purified complex. It is also apparent that purified viral DNA was at least 4 times as efficient a template as purified complex and 20 to 22 times as effective as crude complex.

The best evidence that transcription of NPCs by E. coli RNA polymerase is accurate, was obtained by DNA-RNA hybridization experiments. RNA synthesized from either purified viral DNA or purified NPCs was hybridized to viral DNA immobilized on nitrocellulose filters (see Materials and Methods). Unfortunately, we could not recover sufficient RNA from crude NPC reaction mixtures to allow filter hybridization experiments. As shown in Table 2, RNA synthesized from purified NPCs hybridized to viral DNA to approximately the same extent as RNA synthesized from purified viral DNA. This suggests

Table 2. Hybridization of [^{3}H]-Labeled RNA Synthesized In Vitro from Purified SV40 Nucleoprotein Complexes or Purified Supercoiled SV40 DNA.

Template for RNA Synthesis	[^{3}H]RNA Input (CPM)	[^{3}H]RNA Hybridized (CPM) 2.5 µg DNA	5.0 µg DNA	(% of Input)
Purified NPC	3,200	2,685	2,750	84.9
SV40 DNA	11,000	9,690	9,800	88.6

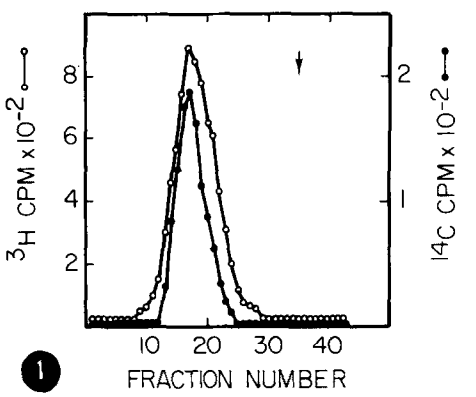
RNA was synthesized from each template by the procedure described in Materials and Methods except that the incubation time was increased from 15 min to 3 hr. At the end of the incubation period, RNA was extracted and purified and DNA-RNA hybridization performed as described in Materials and Methods. All values have been corrected for background counts (1-2% of input) of [^{3}H]RNA bound to blank filters.

that viral DNA sequences in NPCs are transcriptionally active and are transcribed faithfully.

Posttranscriptional Recovery of Nucleoprotein Complex Templates

Since one of the main objectives of these studies is to determine the suitability of NPCs for use as model chromosomes, it was essential that the physical integrity of the complex be retained following transcription. Therefore, several experiments were performed to physically characterize posttranscriptional complexes (PT-NPCs) and compare them with pretranscriptional purified NPCs. As shown in Figure 1, the sedimentation velocity of [^{3}H]-thymidine labelled PT-NPCs is identical to that of [^{14}C]-thymidine labelled pretranscriptional NPCs. A [^{14}C]-thymidine labelled purified viral DNA marker (21 S) was also present in this gradient (arrow over fraction 35) to assist in determination of sedimentation coefficients for the complexes. The position of the NPCs relative to viral DNA allows calculation of their sedimentation coefficients as 60 S.

The average density reported for glutaraldehyde fixed SV40 NPCs is 1.437 ± .008 g/cm^3 (6). Density analyses of glutaraldehyde fixed



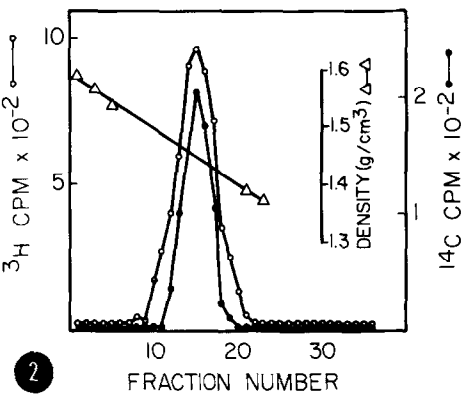


Figure 1. Velocity sedimentation, in a linear sucrose gradient, of SV40 nucleoprotein complexes. After 15 min incubation in the transcription assay mixture (see Materials and Methods), an aliquot of [^{3}H]-thymidine labeled NPC (0.15 ml) was removed from the assay mixture, mixed with 0.05 ml of [^{14}C]-thymidine labeled marker SV40 NPC and [^{14}C]-thymidine labeled viral DNA (10 lambda), and centrifuged as described (5,6). An arrow marks the position of [^{14}C]-thymidine labeled, purified viral DNA included as a marker.

Figure 2. Equilibrium centrifugation of glutaraldehyde-fixed SV40 nucleoprotein complexes in linear CsCl gradients. After 15 min incubation in the transcription assay mixture (see Materials and Methods), aliquots (0.25 ml) of [^{3}H]-thymidine labeled NPC were removed and centrifuged on 5% to 20% sucrose gradients. Peak fractions from each gradient were pooled, mixed with [^{14}C]-thymidine labeled marker NPCs, and treated with glutaraldehyde (5,6). Aliquots (0.2 ml) of the glutaraldehyde fixed complexes were layered on preformed linear CsCl gradients and centrifuged to equilibrium as previously described (5,6).

pretranscriptional and posttranscriptional NPCs were conducted on preformed linear CsCl gradients as previously described (5,6). It is evident (Figure 2) that these two NPC preparations had identical buoyant densities. In addition, their apparent density of 1.444 is within the normal range for SV40 NPCs.

Although not shown, standard propidium diiodide-CsCl equilibrium gradients of the DNA extracted from pretranscriptional and posttranscriptional NPCs exhibit similar patterns of banding of viral DNA with approximately 90% of the DNA being in the closed circular supercoiled form.

DISCUSSION

Nucleoprotein complexes isolated from polyoma and SV40 virus infected cells have been shown to contain cellular histones bound to viral DNA in approximately the same ratios as are found in eukaryotic chromatin (11). The

similarity of viral NPCs and chromatin has led to the proposal that NPCs might be ideal models for studying the effects of chromosomal proteins on gene expression (16).

The results presented here indicate that SV40 NPCs are transcriptionally active although at a reduced rate as compared to viral DNA. This is in agreement with a previous report (12) which demonstrated that the SV40 viral core complex was only 30% as effective a template for E. coli polymerase as was deproteinized viral DNA. Similarly, in a study of polyoma NPCs (13), crude NPCs were almost totally invactive as templates for RNA synthesis. It was suggested that, in addition to restriction of transcription by histones present in the polyoma NPCs, the crude preparations also contained cellular inhibitors of RNA synthesis or ribonucleases (13). Our results, presented in Table 1, indicate that similar inhibitory substances might be present in crude extracts of SV40 NPCs. There is an approximately five fold increase in RNA synthesis when NPCs are purified free of soluble host cell proteins.

Recent work has shown that both SV40 subviral cores (12) and viral DNA sequences in chromatin of transformed cells (SV3T3) (14) can be transcribed accurately by E. coli RNA polymerase. Significantly, the sequences transcribed in vitro from SV3T3 chromatin were the same as those present in transformed cells (14). Our present work supports these studies and indicates (Table 2) that E. coli polymerase can accurately copy SV40 NPCs in vitro. However, whether these transcripts represent true messages remains to be determined.

Most important, relative to the utility of these viral DNA-histone complexes for studies on control of gene expression, is the evidence that complexes may be isolated intact from the transcription solutions. This is strong indirect evidence that transcription is actually occurring on the DNA-protein template rather than some deproteinized form and is in marked contrast to recent reports in which sarcosyl was incorporated into the assay mixture (17,18). It is likely that sarcosyl removed all DNA bound proteins, except the polymerase, in these assays. The recovery of

unaltered NPCs after in vitro transcription offers the possibility of transcribing unaltered NPCs, reisolating the NPC template, modifying the template-complex (DNA or Protein), transcribing it again and comparing transcripts.

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REFERENCES

1. Huang, E-S., M.K. Estes, and J.S. Pagano. (1972) J. Virol. 9: 923-929.
2. McMillen, J., and R.A. Consigli. (1974) J. Virol. 14: 1326-1336.
3. Green, M.H., H.J. Miller, and S. Hendler. (1971) Proc. Nat. Acad. Sci., U.S.A. 68: 1032-1036.
4. White, M., and R. Eason. (1971) J. Virol. 8: 363-371.
5. Goldstein, D., M.R. Hall, and W. Meinke. (1973) J. Virol. 12: 887-900.
6. Hall, M.R., W. Meinke, and D.A. Goldstein. (1973) J. Virol. 12: 901-908.
7. Pett, D.M., M.K. Estes, and J.S. Pagano. (1975) J. Virol. 15: 379-385.
8. Meinke, W., M.R. Hall, and D.A. Goldstein. (1975) J. Virol. 15: 439-448.
9. Kornberg, R.D. (1974) Science 184: 868-871.
10. Griffith, J.D. (1975) Science 187: 1202-1203.
11. Cremisi, C., P.F. Pignatti, O. Croissant, and M. Yaniv. (1976) J. Virol. 17: 204-211.
12. Huang, E-S., M. Nonoyama, and J.S. Pagano. (1972) J. Virol. 9: 930-937.
13. Shmookler, R.J., J. Buss, and M.H. Green. (1974) Virology 57: 122-127.
14. Astrin, S.M. (1973) Proc. Nat. Acad, Sci., U.S.A. 70: 2304-2308.
15. Aloni, Y., E. Winocour, and L. Sacks. (1968) J. Mol. Biol. 31: 415-429.
16. Germond, J.E., B. Hirt, P. Oudet, M. Gross-Bellard, and P. Chambon. (1975) Proc. Nat. Acad. Sci., U.S.A. 72: 1843-1847.
17. Gariglio, P., and S. Mousset. (1975) FEBS Letters 56: 149-155.
18. Green, M.H., and T.L. Brooks. (1976) Virology 72: 110-120.