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Inhibition by α-Amanitin of Simian Virus 40-Specific Ribonucleic Acid Synthesis in Nuclei of Infected Monkey Cells

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Conditions affecting the synthesis of simian virus 40-specific ribonucleic acid (RNA) in nuclei isolated from lytically infected cells were investigated. Deoxyribonucleic acid-RNA hybridization results show that an α-amanitin-sensitive RNA polymerase activity is responsible for transcription of simian virus 40 deoxyribonucleic acid.

Simian virus 40 (SV40) grows lytically in monkey cells. Throughout the entire infection, ribonucleic acid (RNA) can be detected which is complementary to about 30% of the sequences of one of the strands of viral deoxyribonucleic acid (DNA): transcripts corresponding to about 70% of the sequences of the other strand appear only at late times after infection (5; G. Khoury and M. Martin, Nature, *in press*; J. Sambrook, P. A. Sharp, and W. Keller, J. Mol. Biol., *in press*). Whether this early-late shift in SV40-specific RNA species reflects a change in the DNA template, in RNA processing, or in the enzymes transcribing the DNA is not known. The experiments described in this paper are designed to determine which of the two major DNA-dependent RNA polymerases found in mammalian cells (1, 8) is responsible for transcribing SV40 DNA.

The principal forms of RNA polymerase, I and II, differ in their chromatographic properties, cation requirements, salt optima, intranuclear location, and sensitivity to α-amanitin (9, 11). α-Amanitin is a toxin extracted from the poisonous mushroom *Amanita phalloides* which inhibits the activity of enzyme II, whereas that of polymerase I is unaffected. This differential sensitivity provides a simple way to distinguish the two polymerase activities both in vivo and in vitro.

The work of Ledinko (4) suggests that some types of primary cells in culture are permeable to α-amanitin. However, several experiments in this laboratory indicated that different batches of primary monkey kidney cells showed variable sensitivity to α-amanitin, and we therefore chose to use isolated nuclei from a continuous line of green monkey kidney cells in which α-amanitin consistently shows an inhibitory effect. Isolated nuclei have been employed successfully by Price and Penman (7) and Wallace and Kates (13) to investigate transcription of adenovirus 2 in HeLa cells.

MA 134 cells (obtained from J. Pagano) were grown at 37 C in 5% CO_2 on 100-mm plastic tissue culture dishes in Dulbecco modified Eagle medium (2) (Gibco) supplemented with 10% fetal bovine serum (Gibco). Semi-confluent monolayers of the cells were infected at a multiplicity of 20 plaque-forming units per cell with a stock of SV40 grown at low multiplicity. Thirty hours later, nuclei were isolated from infected and mock-infected cells as follows. The cells were removed from the dishes with trypsin, washed in ice-cold phosphate-buffered saline (3), and collected by centrifugation for 7 min at 2,000 rev/min in a Sorvall SS34 rotor at 0 C. The cells were washed once with ice-cold swelling buffer (SB-0.01 M potassium phosphate, *p*H 7.9, 0.002 M $MgCl_2$) and were resuspended at a concentration of 1.5×10^6 cells/ml. After about 1 min at 0 C, Triton-X was added to a final concentration of 0.5% and dithiothreitol to 0.001 M, and the cells were lysed by gentle pipetting for 3 min at 0 C. The nuclei were sedimented for 3 min at $800 \times g$ and resuspended in SB at 10^7 to 4×10^7 nuclei/ml; 0.05-ml samples of nuclear suspension were used per assay.

RNA polymerase activity was measured by following incorporation of ^{3}H-uridine triphosphate (UTP) into trichloroacetic acid-precipitable material. Standard assays consisted of 4% glycerol, 0.001 M dithiothreitol, 0.01 M $MgCl_2$, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydro-

chloride, *p*H 7.9, 0.05 M adenosine triphosphate (Calbiochem), 0.001 M cytidine triphosphate and guanosine triphosphate (Calbiochem), 0.033 mCi of ^{3}H-UTP (26 Ci/mmole; New England Nuclear Corp.), 0.5 $\times$ 10^6 to 3.0 $\times$ 10^6 nuclei, and salt concentrations ranging from 0.0 to 0.4 M NH_4Cl in a total volume of 0.15 ml. After the incubation period (30 min at 37 C) the reaction was stopped by chilling and by addition of 5 ml of a solution of ice-cold 5% trichloroacetic acid containing 0.01 M $Na_4P_2O_7$. The precipitates were collected on Whatman GF/C 24-mm glass-fiber filters and counted in toluene-based scintillation fluid.

Synthesis of RNA in uninfected nuclei in 0.3 M NH_4Cl was linear for about 10 min and reached a plateau after 30 min. The isolated (see below for method) labeled products of these syntheses were greater than 99% sensitive to ribonuclease as shown by treatment with 20 μg of pancreatic ribonuclease (Worthington) per ml at 37 C for 60 min in 0.01 M sodium acetate, *p*H 5.5, 0.001 M ethylenediaminetetraacetic acid (EDTA).

When the effects of different salt and cation concentrations on RNA synthesis in uninfected cell nuclei were compared, it was found that RNA synthesis occurred at all salt concentrations tested (0.01 to 0.4 M NH_4Cl) and that generally more RNA was made at high ionic strength than low. The use of either Mg^{2+} or Mn^{2+} as a cation stimulated total RNA synthesis approximately equally.

To determine the dosage effect of α-amanitin (Boehringer Sohn-Germany), various concentrations were included in assays containing uninfected MA 134 nuclei at 0.3 M NH_4Cl. Maximal inhibition of RNA synthesis (70%) was obtained with 0.1 μg of α-amanitin per ml, and 0.9 μg/ml was used in all subsequent experiments.

The effect of α-amanitin on total RNA synthesis in SV40-infected nuclei at different salt concentrations is shown in Fig. 1. α-Amanitin inhibited RNA synthesis at all salt concentrations tested, and the amount of inhibition was greatest at high salt concentrations, ranging from 55 to 80% in different experiments.

To test the sensitivity of SV40-specific RNA synthesis to α-amanitin, RNA synthesized by SV40-infected nuclei in the presence or absence of α-amanitin was extracted and hybridized to SV40 DNA. RNA was synthesized in the reaction mixture described previously (except that it contained 0.1 mCi of ^{3}H-UTP per ml) at six NH_4Cl concentrations, with and without α-amanitin. RNA was extracted by the hot phenol-sodium dodecyl sulfate (SDS) method of Scherrer (10), precipitated with ethanol at -20 C, digested for 30 min at 37 C with 25 μg of pancreatic deoxyribonuclease (Worthington) per ml, extracted once with phenol and once with chloroform-isoamyl alcohol at room temperature, and reprecipitated. The RNA was resuspended in 0.001 M EDTA, dialyzed against 0.01% SDS, 0.01 M Tris-hydrochloride (*p*H 7.9), 0.001 M EDTA, and used directly in the hybridization reaction. Filters containing SV40 DNA were prepared essentially as described by Martin (6). Eight micrograms of SV40 component I DNA, prepared as described elsewhere (12), was denatured by boiling for 20 min in 0.01 M NaOH, then rapidly diluted with 250 ml of cold 4 $\times$ SSC (SSC = 0.15 M NaCl plus 0.015 M sodium-citrate) and passed through washed squares (70 by 70 mm) of B-6 nitrocellulose membrane filters (Schleicher & Schuell). After vacuum drying at 80 C, the filters were cut into 6-mm circles containing approximately 0.25 μg of SV40 DNA. Hybridization was carried out for 36 hr at 68 C in 0.8 M NaCl, 0.1% SDS, 0.02 M Tris-hydrochloride (*p*H 7.9) in a volume of 0.3 ml. Each hybridization vial contained one filter bound with SV40 DNA and one blank. After hybridization, the filters were washed with 50 ml of 2 $\times$ SSC and treated with 20 μg of pancreatic ribonuclease (Worthington) per ml in 0.5 ml of 2 $\times$ SSC for 60 min at room temperature. They were then washed again with 50 ml of 2 $\times$ SSC, dried, and counted. The validity of any conclusion to be drawn from the RNA-DNA hybridization experiments rests on our hybridization conditions being exhaustive. To test these conditions 0.005 μg of SV40 RNA (specific activity = 1.6 $\times$ 10^6 counts per min per μg) made in vitro with *Escherichia coli* RNA polymerase as described by Westphal (14) was hybridized in the presence of yeast transfer RNA (500 μg/ml) to SV40 DNA-containing filters under our standard conditions

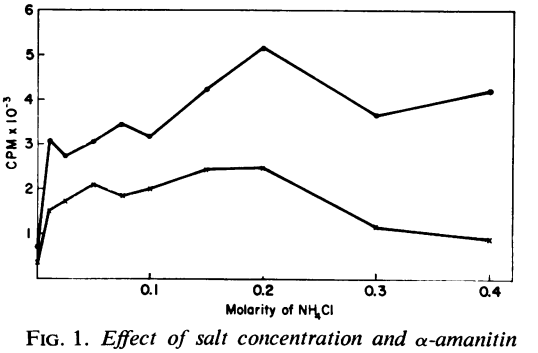

FIG. 1. *Effect of salt concentration and α-amanitin on RNA polymerase activity of SV40-infected MA 134 nuclei. Nuclei were prepared, and the enzyme activity was assayed as described in the text. Each assay contained 0.5 $\times$ 10^6 nuclei. Each point represents the average of duplicate assays. Symbols: RNA polymerase activity in the absence of α-amanitin (●); RNA polymerase activity in the presence of 0.9 μg of α-amanitin per ml* (×).

described above. After 24 hr at 68 C, 57% of the labeled RNA had bound to the filter; after 36 hr, 55% had bound; and after 48 hr, 46% had bound. Because the percentage of RNA bound does not increase with incubation times beyond our 36-hr standard, we consider our hybridization conditions to be exhaustive.

RNA synthesized by nuclei isolated from SV40-infected MA 134 cells in the absence of α-amanitin hybridized to SV40 DNA (Fig. 2). Control experiments demonstrated that the quantity of RNA bound to the filters was proportional to the amount of RNA added indicating that the DNA remained in excess during the hybridization reaction. The specific activity of the RNA ranged from 200 to 500 counts per min per μg. The vast majority of the material is unlabeled RNA present in the nuclei before the in vitro synthesis as well as contaminating cytoplasmic species. Because the SV40 DNA immobilized on the filters remained in excess, the detection of labeled RNA bound to it is a direct measure of the extent of in vitro synthesis of SV40-specific sequences. The fraction of the input RNA that hybridized to SV40 was dependent upon the conditions of synthesis. When corrected for counts per minute bound to blank filters, the SV40 DNA filters, incubated with RNA synthesized at 0.3 M NH_4Cl, hybridized eightfold more SV40 sequences than those incubated with RNA synthesized at 0.01 M NH_4Cl. Results obtained above 0.3 M NH_4Cl were inconsistent, probably because of the extensive aggregation and lysis of the nuclei.

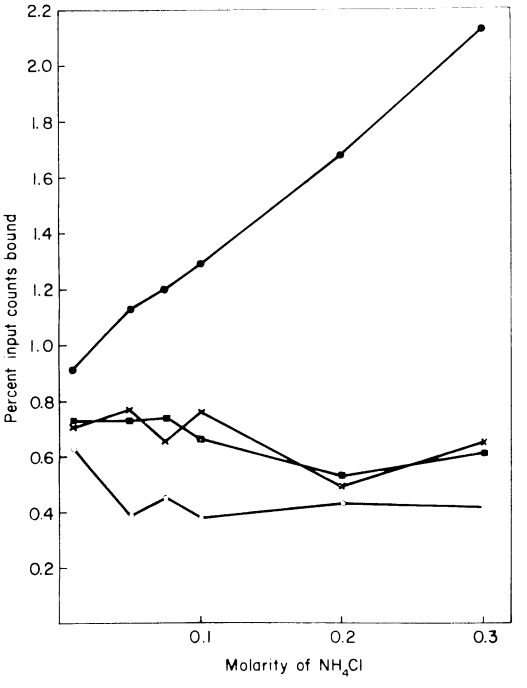

FIG. 2. *Hybridization to SV40 DNA of RNA synthesized by nuclei from SV40-infected MA 134 cells in the presence and absence of α-amanitin. RNA was isolated and hybridized as described in the text. Each point represents an average of the percent of input counts bound for two different RNA concentrations. Backgrounds have not been subtracted. The counts per minute of RNA synthesized in the absence of α-amanitin that bound to SV40 DNA filters increased from 130 to 510 counts/min over the salt range tested. Symbols: percent input counts synthesized without α-amanitin bound to SV40 DNA filters (●); percent input counts synthesized without α-amanitin bound to blank filters (○); percent input counts synthesized with α-amanitin bound to SV40 DNA filters (×); percent input counts synthesized with α-amanitin bound to blank filters (■).*

The RNA synthesized by nuclei from SV40-infected MA 134 cells in the presence of α-amanitin showed no detectable hybridization to SV40. These results mean that an α-amanitin-sensitive enzyme is responsible for transcription of SV40 DNA. The data, therefore, demonstrate that RNA polymerase I does not transcribe SV40 DNA under our experimental conditions.

The sequences transcribed from SV40 early in infection are also transcribed late, and it seems likely the same enzyme transcribes these "early" sequences both early and late in infection. Were these sequences to be transcribed by an α-amanitin-resistant RNA polymerase they would probably be detected as an α-amanitin-resistant subset of "late" RNA. No such class was observed.

The simplest interpretation of these experiments is that, in MA 134 cells late in SV40 infection, host polymerase II transcribes the viral DNA, and that the same enzyme is responsible for the synthesis of both early and late sequences of viral RNA. This conclusion is consistent with the postulated role of enzyme II in uninfected cells (4, 9) and agrees with the results of Price and Penman (7) and Wallace and Kates (13) for transcription of adenovirus 2 DNA in infected cells.

To rule out more complicated explanations such as a viral-coded polymerase or a viral-modified host polymerase, RNA polymerase II from uninfected and infected permissive cells must be purified and shown to transcribe SV40 DNA in vitro and to synthesize the RNA species found in vivo. Experiments to characterize such in vitro products are in progress.

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LITERATURE CITED

1. Chambon, P., F. Gissinger, J. L. Mandel Jr., C. Kedinger, M. Gniazdowski, and M. Meihlac. 1970. Purification and properties of calf thymus DNA-dependent RNA polymerases A and B. Cold Spring Harbor Symp. Quant. Biol. 35:693–707.
2. Dulbecco, R., and G. Freeman. 1959. Plaque production by the polyoma virus. Virology 8:396–397.
3. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167–182.
4. Ledinko, N. 1971. Inhibition by α-amanitin of adenovirus 12 replication in human embryonic kidney cells and of adenovirus 12 transformation of hamster cells. Nature N. Biol. 233:247–248.
5. Lindstrom, D. M., and R. Dulbecco. 1972. Strand orientation of simian virus 40 transcription in productively infected cells. Proc. Nat. Acad. Sci. U.S.A. 69:1517–1520.
6. Martin, Malcolm A. 1969. Characteristics of the Syrian hamster ribonucleic acid present in cells transformed by polyoma, simian virus 40, or adenovirus 12. J. Virol. 3:119–125.
7. Price, R., and S. Penman. 1972. Transcription of the adenovirus genome by an α-amanitin-sensitive ribonucleic acid polymerase in HeLa cells. J. Virol. 9:621–626.
8. Roeder, R. G., and W. J. Rutter. 1969. Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. Nature (London) 224:234–237.
9. Roeder, R. G., and W. J. Rutter. 1970. Specific nucleolar and nucleoplasmic RNA polymerases. Proc. Nat. Acad. Sci. U.S.A. 65:675–682.
10. Sherrer, K. 1969. Isolation and sucrose gradient analysis of RNA, p. 413–432. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
11. Stirpe, F., and L. Fiume. 1967. Studies on the pathogenesis of liver necrosis by α-amanitin: effect of α-Amanitin on ribonucleic acid synthesis and on ribonucleic acid polymerase in mouse liver nuclei. Biochem. J. 105:779–782.
12. Trilling, D. M., and D. Axelrod. 1970. Encapsidation of free host DNA by simian virus 40: a simian virus 40 pseudovirus. Science 168:268–271.
13. Wallace, R. D., and J. Kates. 1972. State of the adenovirus deoxyribonucleic acid in the nucleus and its mode of transcription: studies with isolated viral deoxyribonucleic acid-protein complexes and isolated nuclei. J. Virol. 9:627–635.
14. Westphal, H. 1970. SV40 DNA strand selection by *Escherichia coli* RNA polymerase. J. Mol. Biol. 50:407–420.