JOURNAL OF VIROLOGY, Dec. 1972, p. 1236-1239
Vol. 10, No. 6

Density Heterogeneity of Simian Virus 40 Ribonucleic Acid Late After Infection of Permissive Cells

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Received for publication 7 August 1972

Ribonucleic acid (RNA) was isolated from CV-1 cells 44 hr after simian virus 40 (SV40) infection. The molecules containing SV40 base sequences were characterized with respect to their buoyant density distribution. The density of these molecules was compared to that of single- and double-stranded RNA synthesized by using SV40 DNA and *Escherichia coli* RNA polymerase. The results suggest that about 20% of the SV40 RNA in the infected cells consisted of partially double-stranded molecules.

In cells infected by simian virus 40 (SV40), some features about the structure and transcriptional control of the SV40 ribonucleic acid (RNA) molecules have been established. The size of these molecules has been characterized (9, 12, 13). In addition, it has been shown that only one of the two SV40 deoxyribonucleic acid (DNA) strands is transcribed early in infection, whereas both strands are partially transcribed late in infection (5–7). Results are described in this report that were obtained from buoyant density analysis of SV40 RNA molecules isolated from permissive monkey CV-1 cells late after infection by SV40.

CV-1 cells were grown on 60-mm plastic petri dishes and infected by SV40 at a multiplicity of infection of 20 plaque-forming units per cell (11). Forty-four hours after infection, the cells were labeled with ^{3}H-uridine (150 μCi per dish) for 1 hr at 37 C. The RNA was extracted by a hot-phenol method essentially as described elsewhere (1), except that magnesium, 5×10^{-3} M, was substituted for manganese during the deoxyribonuclease treatment. The RNA was then analyzed by equilibrium density centrifugation (Fig. 1). The bulk of the RNA, which is predominantly 18*S* and 28*S* ribosomal RNA, banded at a density of 1.865 g/cm^3 as was evidenced by the distribution of optical density (Fig. 1a). Because more than 10 times as much ^{3}H-uridine goes into cellular RNA as into SV40 RNA (13), the pattern of radioactivity on the gradient essentially reflects the density distribution of newly synthesized cellular RNA. This distribution is relatively homogeneous with a peak at about 1.865 g/cm^3 (Fig. 1b).

SV40 RNA molecules were detected by hybridizing RNA in a given gradient fraction with SV40 DNA that had been immobilized on nitrocellulose filters (Fig. 1b). The main peak (marked A) of SV40 RNA is at a density of 1.865 g/cm^3 and is almost at the identical density as the peak of labeled cellular RNA. The distribution of SV40 RNA also has a shoulder at density of 1.85 g/cm^3 (marked B) and a broad second shoulder at a density of 1.835 g/cm^5 (marked C). The spreading of the RNA over these regions of the gradient must reflect true heterogeneity of density of SV40 RNA and cannot be explained by mixing of the gradient during its collection. The profile of total radioactivity, which is much more homogeneous with respect to density, provides an internal control in this respect.

In a control experiment, uninfected CV-1 cells were grown in parallel with the infected cells. The labeling of the RNA with ^{3}H-uridine, its extraction, and its analysis on a density gradient were done in the same manner as for the RNA extracted from infected cells (Fig. 1c). Only a small amount of RNA was found to be hybridizable to SV40 DNA, about 10% as much as in infected cells, and this occurred predominantly at densities corresponding to those of the SV40 RNA in peak A in Fig. 1b (Fig. 1c).

To help interpret the density heterogeneity of the SV40 RNA in infected cells, the buoyant density of single- and double-stranded SV40 RNA was determined by using SV40 synthesized in vitro (SV40 cRNA). The cRNA was synthesized by using purified SV40 DNA and *Escherichia coli* RNA polymerase. When the cRNA product was dissociated into single-stranded RNA, sedimentation analysis revealed that 25 to 50% of the RNA was larger than 1.5×10^6 daltons. The cRNA transcribed under the conditions used (legend, Fig. 2) therefore represents

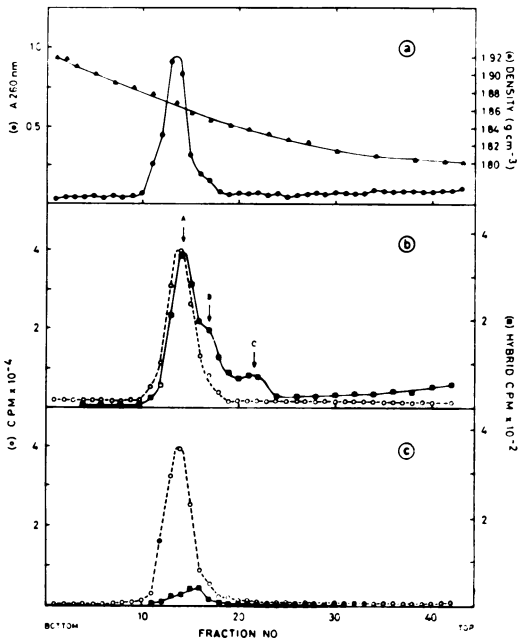

FIG. 1. *Equilibrium density analysis of RNA from CV-1 cells infected by SV40 (a and b) and from uninfected CV-1 cells (c). A 20-μg amount of ^{3}H-labeled RNA (specific activity 90,000 counts per min per μg) was isolated from CV-1 cells infected by SV40 and dissolved in 440 μliters of NaE buffer (2.3×10^{-2} M Na_2HPO_4, 2.5×10^{-3} M NaH_2PO_4, 5×10^{-3} M ethylenediaminetetraacetic acid. This RNA solution was mixed with 3,300 μliters of saturated (at 20 C) CsCl solution and 660 μliters of saturated Cs_2SO_4 solution (8). This solution was centrifuged in a Spinco 65 rotor at 44,000 rev/min for 42 hr at 20 C. Fractions of 100 μliters were collected from the bottom of the tube. a and b, Results of analyzing the gradient. The counts per minute refer to the number of ^{3}H counts per minute per 10 μliters of each fraction. The hybridization assay for SV40 RNA was done for 16 hr at 66 C. The post-hybridization ribonuclease treatment is described elsewhere (14). In the hybridization assay, 10 μliters of a given gradient fraction was added to 990 μliters of 5.5 × SSC solution (5.5 × the concentration of SSC solution) (SSC solution: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.7) plus 0.1% sodium dodecyl sulfate. The solution was incubated with 1 μg of single-stranded SV40 DNA immobilized on a Millipore filter. An additional 10 μliters of the same gradient fraction was assayed in a similar manner by using Millipore filters without immobilized DNA. The amount of counts per minute bound to these blank filters never exceeded 17 counts per min. The hybrid counts per minute on the graph is equal to the number of counts bound to the filter with the DNA less the counts per minute bound to the blank filter. The arrows marked A, B, and C are drawn for purposes of discussion of the graph in the text. c, A 16-μg amount of ^{3}H-labeled RNA (specific activity 90,000 counts per min per μg) was isolated from uninfected CV-1 cells and centrifuged in the same manner as the RNA from infected cells discussed above. The RNA in each gradient fraction was also analyzed in the same manner except that 12 μliters of each fraction was added to the hybridization mixture. Both the counts per minute and the hybrid counts per minute represent the values per 12 μliters of a given fraction. A_{260nm} is the optical density at 260 nm.*

a complete transcript of at least one of the two complementary strands of SV40 DNA (A. H. Fried and F. Sokol, J. Gen. Virol., *in press*). This cRNA was analyzed with respect to its buoyant density distribution, and the ribonuclease sensitivity of the cRNA in each gradient fraction was determined (Fig. 2). The ribonuclease sensitivity of the cRNA in a given fraction can be calculated by comparing the total trichloroacetic acid-precipitable ^{3}H counts per minute in that fraction with the acid-precipitable ^{3}H counts per minute after exposure of that cRNA to ribonuclease. A peak of ribonuclease-sensitive material can be seen at a density of 1.86 g/cm^3. This peak was more pronounced in some other preparations of cRNA. From the high ribonuclease sensitivity of this material, it was assumed that it is single-stranded cRNA, and its position on the gradient is denoted by the arrow marked SS RNA. The RNA molecules with maximum resistance to ribonuclease are in fraction 21 of the gradient and have a buoyant density of 1.84 g/cm^3. In this fraction, about 90% of the acid-precipitable ^{3}H counts per minute remains acid-precipitable after ribonuclease treatment. It was assumed that the cRNA in this fraction was about 90% double stranded, and its position on the gradient, at a density of 1.84 g/cm^3, is denoted by the arrow marked DS RNA. The double-stranded cRNA indicates that to some extent the transcription was symmetrical. Symmetrical transcription in this system has been reported previously, although to a lesser extent (7, 14). At densities less than 1.84 g/cm^3, a significant portion of the cRNA in each fraction consists of a species of cRNA that is spread out in small quantities over the entire gradient and has a ribonuclease resistance of about 30%.

RNA from the peak of labeled cellular RNA in Fig. 1b (gradient fractions 14 and 15) was also treated with ribonuclease under the conditions described in the legend to Fig. 2. Only 5% of the ^{3}H counts per minute in these fractions was resistant to ribonuclease treatment. As a result, it can be concluded that the density of single-stranded cellular RNA and single-stranded SV40 cRNA are almost identical, both having a buoyant density of about 1.86 g/cm^3.

From the above results it can be concluded

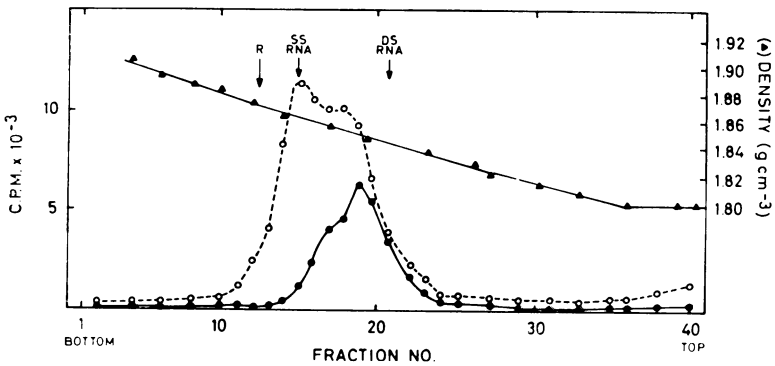

FIG. 2. *Buoyant density analysis of SV40 RNA synthesized in vitro (SV40 cRNA). SV40 cRNA was synthesized for a period of 80 min by using 1.2 μg of E. coli RNA polymerase and 1.5 μg of SV40 component I DNA in a total volume of 200 μliters. ^{3}H-cytidine triphosphate (14Ci/mmole) and ^{3}H-guanosine triphosphate (6 Ci/mmole) were the radioactive triphosphates in the reaction mixture. Other details of the reaction were identical to those described elsewhere (14). The cRNA was purified from the reaction mixture by adding 70 μg of CV-1 RNA (isolated from uninfected cells and not labeled with tritium) in 800 μliters of 0.05 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (pH 7.5), 2 mM ethylenediaminetetraacetic acid plus 1/10 volume of 5% (w/w) sodium dodecyl sulfate and an equal volume of phenol containing 0.01% 8-hydroxy quinoline. After two phenol extractions, the RNA was precipitated with ethanol and redissolved in 300 μliters of 0.01 M sodium acetate (pH 5.1), 5 × 10^{-3} M $MgCl_2$. Deoxyribonuclease (Schwarz/Mann) was added to a final concentration of 10 μg/ml, and the mixture was incubated at 20 C for 10 min. The cRNA was phenol extracted and precipitated with ethanol before its subsequent analysis by gradient centrifugation. The SV40 component I DNA was extracted from purified SV40 virus with phenol (3) and subsequently purified by CsCl equilibrium centrifugation in the presence of ethidium bromide (10). The RNA polymerase was purified first by a procedure previously described (4) and subsequently isolated from the 21S peak after sedimentation through a glycerol gradient at low ionic strength (2). A 0.12-μg amount of cRNA (150,000 counts per min), with 10 μg of unlabeled CV-1 RNA added as optical density marker, was centrifuged in the same manner as the RNA in Fig. 1. The arrow denoted by the letter R indicates the position of the peak of optical density at 260 nm; ○, total number of trichloroacetic acid-precipitable counts per minute per gradient fraction; ●, number of acid-precipitable counts per minute per gradient fraction after subjecting the RNA in that fraction to ribonuclease treatment. The ribonuclease treatment was performed by taking a 40-μliter sample of a given gradient fraction, adding it to 960 μliters of 1 × SSC buffer containing 20 μg of pancreatic ribonuclease, and incubating this mixture at 37 C for 15 min. The RNA was then precipitated in the presence of 100 μg of bovine serum albumin and trichloroacetic acid (5% w/v), and the precipitate was collected on Millipore filters.*

that most of the SV40 RNA synthesized in infected cells is structurally similar to cellular RNA synthesized during the same time interval in that both are single stranded. There also exists, however, a population of SV40 RNA molecules which have a density less than that of the single-stranded molecules. These molecules span a relatively narrow range with respect to buoyant density, with the lightest ones being only about 0.03 g/cm^3 lighter than the single-stranded RNA. In addition, it can be seen from the experiments with SV40 cRNA that these molecules fall into the same narrow density range characteristic of partially double-stranded SV40 RNA molecules with the lightest molecules having the buoyant density of almost fully double-stranded SV40 RNA. This argues strongly against DNA-RNA hybrids being involved, as SV40 DNA was found to have a density of 1.65 g/cm^3 in gradients containing the same ratio of Cs_2SO_4 to CsCl as the gradients in Fig. 1 and 2. This density is 0.21 g/cm^3 less than that of single-stranded SV40 RNA in these gradients, and so the probability is small that such DNA-RNA hybrids would be clustered in the narrow density range of the observed SV40 molecules. In addition, the RNA preparation from infected cells was treated with deoxyribonuclease during its extraction, which further reduces the possibility of RNA-DNA hybrids.

The observation that there are apparently double-stranded regions on some SV40 RNA molecules, late after infection, is interesting in view of the recent observations that at least part of both strands of SV40 DNA are transcribed late in infection (5–7). The double-stranded RNA regions may be the result of hybrids formed between two RNA molecules and reflect the fact that, on at least one region of the SV40 DNA molecules, the complementary base sequences of each DNA strand are transcribed. Further char-

acterization of the structure of the SV40 molecules will be required to answer that question.

ADDENDUM IN PROOF

Additional evidence supporting the conclusions made in this paper have recently been published by Yosef Aloni (Proc. Nat. Acad. Sci. U.S.A. **69**:2404–2409, 1972).

I thank Gerhard Sauer for helpful discussions and Helmut Bannert for excellent technical assistance. This research was supported by the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. Acheson, N. H., E. Buetti, K. Scherrer, and R. Weil. 1971. Transcription of the polyoma virus genome: synthesis and cleavage of giant late polyoma-specific RNA. Proc. Nat. Acad. Sci. U.S.A. **68**:2231–2235.
2. Burgess, R. R. 1969. A new method for the large scale purification of *Escherichia coli* deoxyribonucleic acid-dependent ribonucleic acid polymerase J. Biol. Chem. **244**:6160–6167.
3. Carp, R. I., G. Sauer, and F. Sokol. 1969. The effect of actinomycin D on the transcription and replication of simian virus 40 deoxyribonucleic acid. Virology **37**:214–226.
4. Chamberlin, M., and P. Berg. 1961. Deoxyribonucleic acid directed synthesis of ribonucleic acid by an enzyme isolated from *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **48**:81–94.
5. Khoury, G., J. C. Byrne, and M. A. Martin. 1972. Patterns of simian virus 40 DNA transcription after acute infection of permissive and nonpermissive cells. Proc. Nat. Acad. Sci. U.S.A. **69**:1925–1928.
6. Khoury, G., and M. A. Martin. 1972. Comparison of SV40 DNA transcription *in vivo* and *in vitro*. Nature N. Biol. **238**:4–6.
7. 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.
8. Lozeron, H. A., and W. Szybalski. 1966. Suppression of RNA precipitation during Cs_2SO_4 density gradient centrifugation. Biochem. Biophys. Res. Commun. **23**:612–618.
9. Martin, M. A., and J. C. Byrne. 1970. Sedimentation properties of simian virus 40-specific ribonucleic acid present in green monkey cells during productive infection and in mouse cells undergoing abortive infection. J. Virol. **6**: 463–469.
10. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Nat. Acad. Sci. U.S.A. **57**:1514–1521.
11. Sauer, G., and J. R. Kidwai. 1968. The transcription of the SV40 genome in productively infected and transformed cells. Proc. Nat. Acad. Sci. U.S.A. **61**:1256–1263.
12. Sokol, F., and R. I. Carp. 1971. Molecular size of simian virus 40-specific RNA synthesized in productively infected cells. J. Gen. Virol. **11**:171–188.
13. Tonegawa, S., G. Walter, A. Bernardini, and R. Dulbecco. 1970. Transcription of the SV40 genome in transformed cells and during lytic infection. Cold Spring Harbor Symp. Quant. Biol. **35**:823–831.
14. Westphal, H. 1970. SV40 strand selection by *Escherichia coli* RNA polymerase. J. Mol. Biol. **50**:407–420.
15. Westphal, H., and R. Dulbecco. 1968. Viral DNA in polyoma and SV40-transformed cell lines. Proc. Nat. Acad. Sci. U.S.A. **59**:1158–1165.