Proc. Nat. Acad. Sci. USA
Vol. 69, No. 6, pp. 1517–1520, June 1972

Strand Orientation of Simian Virus 40 Transcription in Productively Infected Cells

(strand switch/mRNA/DNA virus)

DONA M. LINDSTROM AND RENATO DULBECCO

The Salk Institute for Biological Studies, San Diego, California 92112

Contributed by Renato Dulbecco, April 10, 1972

ABSTRACT **Strand orientation of transcription in BSC-1 cells infected by simian virus 40 (SV40) was investigated by annealing RNAs extracted from infected cells with asymmetric complementary RNA from SV40 DNA synthesized *in vitro* by RNA polymerase from *Escherichia coli*. The results suggest that the early viral RNA sequences (those made before replication of viral DNA) and the late RNA sequences (those synthesized concurrently with replication of viral DNA) are transcribed from opposite strands of the SV40 DNA. The RNA synthesized *in vitro* is of the same polarity as the early *in vivo* RNA and is complementary to the late *in vivo* RNA. Knowledge of strand selection in lytic infection is a step towards understanding the regulation of transcription of this viral DNA.**

The small DNA virus, simian virus 40 (SV40), grows lytically in permissive African green monkey cells and usually transforms nonpermissive cells, such as mouse or hamster, in which a complete replicative cycle is absent. This difference of interaction with host cells often correlates with a difference in transcription of the viral genome. In the lytic cycle about one-third to one-half of the genome is transcribed early in infection, before replication of viral DNA begins, and comprises the class of early RNA(s) (1–5). After DNA replication has begun, the remainder of the genome (1, 6) is transcribed such that late in infection both early and late RNA sequences are synthesized. In certain transformed cells, transcription is limited to about 40% of the genome (1, 2). Variable amounts of the genome are transcribed in other transformed lines (6). Most of the sequences transcribed in transformed cells are also transcribed early in lytic infection (2, 4).

To understand how the various modes of transcription might be regulated, we have investigated which strand of viral DNA serves as template for RNA synthesis *in vivo*. This was made possible by the observation that, *in vitro*, *Escherichia coli* RNA polymerase preferentially transcribes only one DNA strand, often in its entirety (7). We compared viral RNA made early and late in infection with that made *in vitro*. The results show that early sequences are of the same polarity as the *in vitro* RNA, whereas the late sequences are complementary to the *in vitro* RNA. We conclude that early and late viral RNA species are transcribed from different strands of the SV40 DNA.

MATERIALS AND METHODS

Cells and Virus. BSC-1 cells from E. Winocour were grown in Dulbecco's modification of Eagle's medium (8) containing 10% fetal bovine serum (Microbiological Associates), 500 units/ml of penicillin (Charles Pfizer & Co.), and 100 μg/ml of streptomycin (Charles Pfizer & Co.). Small plaque SV40 was propagated at low multiplicity (<0.01 plaque forming units/cell) in BSC-1 cells as described by Hatanaka and Dulbecco (9).

Preparation of SV40 DNA. Confluent cultures of BSC-1 cells in 100-mm plastic petri dishes were infected with 0.4 ml of a stock preparation (2.3×10^8 plaque forming units/ml; input multiplicity of infection = 0.5 plaque forming units/cell). After 8 days, SV40 DNA was selectively extracted by the procedure of Hirt (10). The supernatant was extracted three times with redistilled phenol [saturated with 1 M Tris (pH 7.4)], once with $CHCl_3$–isoamyl alcohol (24:1 v/v), and precipitated with two volumes of 95% ethanol at −20°. The precipitate was dissolved in 0.1 M Tris (pH 7.4)–1 mM EDTA, and the DNA was centrifuged in an ethidium bromide (400 μg/ml)–CsCl (density = 1.580 g/cm³) gradient at 43,000 rpm for 48 hr in a Spinco type 50 rotor. The lower band containing the closed twisted circular form I DNA was extracted three times with an equal volume of isopropanol: H_2O (9:1 v/v), and extensively dialyzed against 0.01 M Tris (pH 7.4)–1 mM EDTA. Form I DNA was then sedimented in a 5–20% (w/v) neutral sucrose gradient containing standard saline citrate–0.1 M Tris (pH 7.4)–1 mM EDTA for 17.5 hr at 25,000 rpm in a Spinco SW27 rotor at 4°. The 21S fraction was precipitated overnight with two volumes of ethanol at −20°. DNA concentration was determined by the procedure of Burton (11) or by absorbence at 260 nm.

Preparation of In Vivo RNA. Late RNA was prepared by infection of confluent monolayers of BSC-1 cells with 0.4 ml of a stock preparation (2×10^5 plaque forming units/ml; input multiplicity of infection = 20 plaque forming units/cell). 48 hr after infection, the cell layers were washed and RNA was extracted by one of two alternate procedures. (*a*) Cells were lysed with 0.6% sodium dodecyl sulfate, 0.01 M Tris (pH 7.4), 1 mM EDTA, and NaCl added to 0.5 M. The lysate was allowed to stand at 4° overnight and was then centrifuged at 12,000 × *g* for 1 hr. The supernatant was extracted three times with redistilled phenol [saturated with 1 M Tris (pH 7.4)], once with $CHCl_3$–isoamyl alcohol, and precipitated with ethanol. The precipitate was dissolved in 0.01 M NaAc (pH 5.3)–0.01 M $MgCl_2$, and electrophoretically purified DNase (Worthington Biochemical), which had been previously treated with iodoacetic acid (12), was added at a concentration of 20 μg/ml for 1 hr at 37°. DNase

Abbreviations: cRNA, complementary RNA; SSC, standard saline citrate, 0.15 M sodium chloride–0.015 M sodium citrate; SV40, simian virus 40.

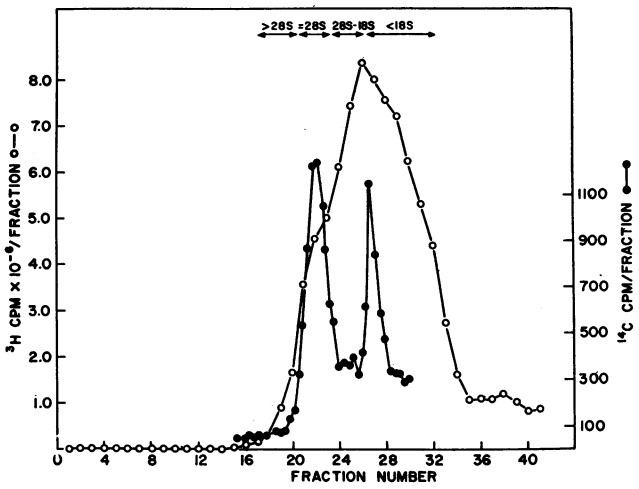

FIG. 1. Size fractionation of cRNA. 330 μl containing 4.5 μg cRNA in 45 μl of H_2O–180 μl of dimethylsulfoxide (Me_2SO) (Matheson, Coleman & Bell, spectroquality)–105 μl of dimethylformamide (Mallinckrodt Chemical) was layered on a 11.0 ml 0–10% sucrose gradient in 99% Me_2SO, containing 1% (v/v) 0.1 M EDTA in H_2O. The cRNA (O——O) was centrifuged for 19 hr at 34,000 rpm. in a Spinco SW41 rotor at 29°. Fractions were collected, and 1-μl aliquots were dried on 24-mm Whatman GF/C glass fiber discs and counted in a toluene-based scintillation fluid. Fractions greater than 28 S, equal to 28 S, 28 S–18 S, and less than 18 S, were pooled as indicated in the figure, adjusted to 0.1 M NaCl and precipitated with ethanol with 50 μg/ml of carrier yeast RNA. Fractions were treated once with DNase as described in *Methods*, precipitated with ethanol, and stored at −20°. Marker ribosomal RNA (●——●), prepared from BSC-1 cells labeled for 72 hr with media containing 2 μCi/ml [^{14}C]uridine was extracted by procedure (*a*) of *Methods*, except DNase treatment was omitted. Marker RNA was sedimented in a parallel gradient as above. Fractions were collected on glass fiber discs and counted.

was removed by phenol and $CHCl_3$ extraction and the RNA was precipitated with ethanol. (*b*) Cells were lysed in 1% sodium dodecyl sulfate–0.01 M NaAc (pH 5.3) and extracted three times with redistilled phenol [saturated with 1.0 M NaAc (pH 5.3)], once with $CHCl_3$–isoamyl alcohol and precipitated with ethanol. The precipitate was treated with DNase as above. After removal of DNase, the RNA was selectively precipitated in 2 M NaCl for 18 hr at 4°, and again treated with DNase. The RNA was precipitated with ethanol and stored at −20° after removal of the DNase. RNA concentration was determined by orcinol reaction (13) or by absorbence at 260 nm. In final RNA preparations, less than 0.5 μg DNA/100 μg RNA could be detected by the Burton assay for DNA.

RNA from uninfected BSC-1 cells was extracted by procedure (*a*).

Early RNA was prepared from infected BSC-1 cells as above, except 12.5 μg/ml of cytosine arabinoside hydrochloride (Sigma) was added to the media at the time of infection. 25 hr after infection, RNA was isolated as described in procedure (*a*).

Preparation of In Vitro Complementary RNA (cRNA). RNA complementary to SV40 DNA was synthesized in a volume of 200 μl containing 5.2 μg of SV40 form I DNA, 15 μg *E. coli* RNA polymerase [purified according to Burgess (14) on glycerol gradients], 0.04 M Tris (pH 7.9), 0.15 M KCl, 0.01 M $MgCl_2$, 0.1 mM dithiothreitol, 0.06 mM [8-^{3}H]ATP (20.5 Ci/mmol; Schwartz BioResearch), 0.055 mM [5-^{3}H]CTP (22 Ci/mmol; New England Nuclear), 0.067 mM [5,6-^{3}H]UTP (36.5 Ci/mmol; New England Nuclear), 0.125 mM GTP (P. L. Biochemicals), 1 mg/ml phosphoenolpyruvate (Sigma), and 9 μg/ml pyruvate kinase (Calbiochem). After incubation for 90 min at 37°, the reaction mixture was applied to a 2.0-ml Sephadex G-50 column equilibrated with SSC–0.1% sodium dodecyl sulfate–0.01 M Tris (pH 7.4)–1 mM EDTA. The cRNA was pooled, extracted twice with phenol and once with $CHCl_3$–isoamyl alcohol, and precipitated with ethanol with 50 μg of yeast RNA. The yield was 9.0 μg of cRNA having a specific radioactivity of 1.2×10^8 dpm/μg, as calculated from the specific radioactivity of the incorporated nucleotides.

RESULTS AND DISCUSSION

Analysis of complementary RNA (cRNA)

Using SV40 form I DNA as template, *E. coli* RNA polymerase synthesizes cRNA that is highly asymmetric and of heterogenous size. When this cRNA is self-annealed, between 80 and 95% remains RNase sensitive depending on the particular cRNA preparation. In agreement with previous data (7), this suggests that between 90 and 97.5% of the cRNA is transcribed from one DNA strand. The size heterogeneity of the cRNA is shown by centrifugation in a dimethylsulfoxide gradient (15) in which cRNA forms a broad band with a median sedimentation constant about 21 S. Fractions consisting of different sizes of cRNA were pooled as indicated in Fig. 1. These fractions retained their original S values when resedimented. As determined by self-annealing (Fig. 2), the different size fractions were of approximately equal asymmetry.

28S cRNA is the length of a complete single strand of SV40 DNA (4). If the RNA polymerase transcribes se-

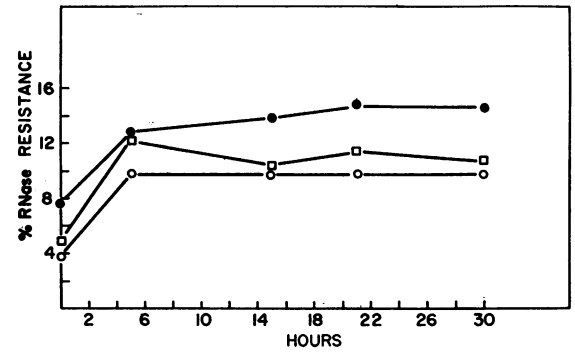

FIG. 2. Self-annealing of different size classes of cRNA. cRNA was synthesized and fractionated as described in *Methods* and the legend to Fig. 1. Fractions of different sizes were self-annealed at 0.88 μg/ml in 2 × SSC at 65°. At the indicated times, samples were removed, incubated in 2 × SSC with 20 μg/ml pancreatic RNase (Worthington Biochemical) and 15 units/ml T1 RNase (Worthington Biochemical) for 1 hr at 37°. Samples were then dried on glass fiber discs, precipitated with cold 5% trichloroacetic acid, washed with ethanol, and counted. At each time-point, parallel samples were dried and precipitated as above without RNase treatment. The percentage of RNase resistance was calculated from each pair of count values. ●, 18 S–28 S; □, >28 S; O, = 28 S.

quentially, 28S cRNA should contain all the sequences in the template DNA strand in nearly equal quantities. For this reason, the 28S size fraction of cRNA was used in the experiments described below.

Annealing of late *in vivo* RNA with asymmetric cRNA

Unlabeled late RNA was extracted from BSC-1 cells infected with SV40 48 hr after infection. When asymmetric 28S cRNA was annealed with this unlabeled late RNA, 55–60% of the input cRNA became resistant to RNase digestion, whereas the RNase resistance of the cRNA was not increased by RNA from uninfected BSC-1 cells (Fig. 3). That the RNase-resistant material consists of double-stranded RNA is shown by two results. First, previous treatment of the late RNA with 0.3 N KOH for 18 hr at 25° abolishes its ability to protect [^{3}H]cRNA from RNase digestion; second, the RNase-resistant RNA, formed by the annealing of late RNA to [^{3}H]cRNA, bands at the density predicted for double-stranded RNA in Cs_2SO_4 equilibrium gradients. The incomplete protection of cRNA against RNase digestion by late RNA is not due to the unavailability of some cRNA sequences. In fact, the addition of denatured SV40 DNA to the annealing mixture after the late RNA has annealed to 55–60% of the [^{3}H]cRNA, protected more than 90% of the [^{3}H]cRNA.

These results suggest that sequences in late RNA are transcribed from the DNA strand opposite to that serving as template for cRNA. Since the true late sequences (those which are synthesized after viral DNA replication begins) comprise 60% of the genome (1–5), the amount of protection of [^{3}H]cRNA by late RNA is consistent with annealing of only the true late sequences with cRNA.

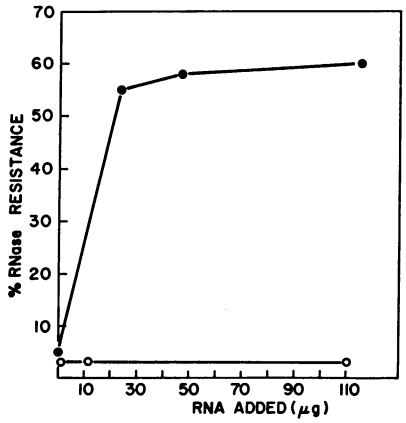

FIG. 3. Annealing of late RNA with asymmetric 28S cRNA. [^{3}H]cRNA and RNA from late infected and uninfected cells was prepared as described in *Methods*. 0.5 ng of 28S [^{3}H]cRNA and unlabeled RNA from uninfected (O——O) and late infected (●——●) cells were annealed in 500 μl of 50% formamide–0.9 M NaCl–0.1% sodium dodecyl sulfate–0.01 M Tris (pH 7.4) for 72 hr at 37°. Samples were precipitated with ethanol with carrier yeast RNA, dissolved in 200 μl of 2 × SSC, digested with 20 μg/ml pancreatic RNase and 15 units/ml T1 RNase for 1 hr at 37°. Samples were applied to glass fiber discs, dried, precipitated with cold 5% trichloroacetic acid, washed with ethanol, and counted. Aliquots of each sample were removed before RNase treatment and precipitated as above to determine total counts present before RNase treatment. RNase resistance was calculated from each pair of count values. 100% of the cRNA remained precipitable by ethanol after the 72-hr annealing.

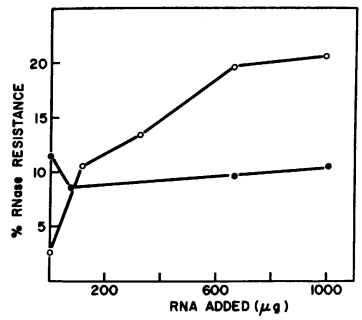

FIG. 4. Annealing of early RNA with asymmetric 28S cRNA and with symmetric 28S cRNA. (●——●) 0.5 ng of 28S asymmetric cRNA and the indicated amount of early RNA were annealed and analyzed as in the legend to Fig. 3. (O——O) 0.5 ng of symmetric 28S cRNA, which had been denatured by boiling for 8 min in water, and the indicated amount of early RNA were annealed and analyzed as above. Symmetric cRNA self-annealed to the extent of 3% in the absence of added early RNA as shown in the figure. Symmetric 28S cRNA was prepared by RNase digestion of self-annealed 28S cRNA. The cRNA used for the preparation of symmetric cRNA was synthesized and fractionated according to size as described in *Methods* and the legend to Fig. 1, except the reaction volume was 112 μl containing 4.1 μg of SV40 form I DNA, 7.9 μg of *E. coli* RNA polymerase, 0.114 mM [8-^{3}H]ATP (20.5 Ci/mmol), 0.083 mM [5-^{3}H]CTP (28.5 Ci/mmol), 0.128 mM [5,6-^{3}H] UTP (36.5 Ci/mmol), and 0.24 mM GTP. 28S RNA was pooled, treated with DNase, and self-annealed for 21 hr at 1.7 μg/ml in 6 × SSC at 65°. RNase digestion was performed as in the legend to Fig. 2, and the RNases were removed by two phenol extractions and one $CHCl_3$–isoamyl alcohol extraction.

Other size classes of [^{3}H]cRNA were annealed to late RNA and the results were the same as those obtained with 28S cRNA.

Annealing of early RNA with asymmetric and symmetric cRNA

Early RNA was prepared from BSC-1 cells infected with SV40 that were treated from the time of infection with 12.5 μg/ml of cytosine arabinoside to inhibit replication of SV40 DNA. Under these conditions, over 99% of tritiated thymidine incorporation into DNA is inhibited and only early RNA sequences should be transcribed (4).

Annealing of unlabeled early RNA with 28S asymmetric [^{3}H]cRNA (Fig. 4) did not increase the RNase resistance of the [^{3}H]cRNA above the 10% value produced by self-annealing cRNA. However, the same early RNA protected 20% of the sequences in *symmetric* 28S cRNA, which should contain sequences from both strands of the DNA in equal proportion. Symmetric 28S was prepared by RNase digestion of self-annealed 28S cRNA and was denatured by boiling before being annealed with early RNA. The annealing with symmetric cRNA suggests that the failure of early RNA to anneal with asymmetric cRNA was not due to the absence of viral sequences in the *in vivo* RNA, but because early RNA and asymmetric cRNA are *not* complementary and are, therefore, transcribed from the same DNA strand. Protection of 20% of the symmetric 28S cRNA is equivalent to 40% protection of one strand. According to independent evidence (1–5), this is the fraction of the genome transcribed early in infection.

These results suggest that the early and late viral RNAs are transcribed *in vivo* from opposite strands of the SV40 DNA and that the cRNA, synthesized *in vitro* by *E. coli* RNA polymerase, is transcribed from the same strand as the early RNA and is complementary to the late RNA. This model of transcription is in agreement with the data of G. Khoury and M. Martin (16), who independently demonstrated transcription from both strands of the SV40 DNA in late lytic infection (when both early and true late sequences are synthesized) and from one strand during *in vitro* cRNA synthesis with the *E. coli* RNA polymerase.

Experiments in which lytic RNA is hybridized to the separated strands of the SV40 DNA are necessary to confirm the present model of strand orientation of transcription.

This work was supported by a grant from the National Institutes of Health (no. 5 RO 1 CA07592).

1. Aloni, Y., Winocour, E. & Sachs, L. (1968) "Characterization of the simian virus 40-specific RNA in virus-yielding and transformed cells," *J. Mol. Biol.* **31**, 415–429.
2. Oda, K. & Dulbecco, R. (1968) "Regulation of transcription of the SV40 DNA in productively infected and in transformed cells," *Proc. Nat. Acad. Sci. USA* **60**, 525–532.
3. Sauer, G. & Kidwai, J. R. (1968) "The transcription of the SV40 genome in productively infected and transformed cells," *Proc. Nat. Acad. Sci. USA* **61**, 1256–1263.
4. Sauer, G. (1971) "Apparent differences in transcriptional control in cells productively infected and transformed by SV40," *Nature* **231**, 135–138.
5. Oxman, M. N., Levine, A. S., Crumpacker, C. S., Levin, M. J., Henry, P. H. & Lewis, A. M., Jr. (1971) "Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. IV. Characterization of the simian virus 40 ribonucleic acid species induced by wild-type simian virus 40 and by the nondefective hybrid virus, $Ad2^{+}ND_{1}$," *J. Virol.* **8**, 215–224.
6. Martin, M. A. & Axelrod, D. (1969) "SV40 gene activity during lytic infection and in a series of SV40 transformed mouse cells," *Proc. Nat. Acad. Sci. USA* **64**, 1203–1210.
7. Westphal, H. (1970) "SV40 DNA strand selection by *Escherichia coli* RNA polymerase," *J. Mol. Biol.* **50**, 407–420.
8. Vogt, M. & Dulbecco, R. (1963) "Steps in the neoplastic transformation of hamster embryo cells by polyoma virus," *Proc. Nat. Acad. Sci. USA* **49**, 171–179.
9. Hatanaka, M. & Dulbecco, R. (1966) "Induction of DNA synthesis by SV40," *Proc. Nat. Acad. Sci. USA* **56**, 736–740.
10. Hirt, B. (1967) "Selective extraction of polyoma DNA from infected mouse cell cultures," *J. Mol. Biol.* **26**, 365–369.
11. Burton, K. (1956) "A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of the deoxyribonucleic acid," *Biochem. J.* **62**, 315–323.
12. Zimmerman, S. B. & Sandeen, G. (1966) "The ribonuclease activity of crystallized pancreatic deoxyribonuclease," *Anal. Biochem.* **14**, 269–277.
13. Schneider, W. C. (1957) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, Inc., New York), pp. 680–684.
14. 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.
15. Strauss, J. H., Jr., Kelly, R. B. & Sinsheimer, R. L. (1968) "Denaturation of RNA with dimethyl sulfoxide," *Biopolymers* **6**, 793–807.
16. Khoury, G. & Martin, M. (1972) "A comparison of SV40 DNA transcription *in vivo* and *in vitro*," *Nature*, in press.