SV40 GENE ACTIVITY DURING LYTIC INFECTION AND IN A SERIES OF SV40 TRANSFORMED MOUSE CELLS

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Abstract.—Transcription of SV40 DNA was measured during lytic infection and in five SV40 transformed mouse cell lines. During productive infection, 50 per cent of viral DNA reacted with saturating amounts of lytic RNA. Varying portions of the SV40 genome were transcribed in transformed mouse cells, ranging from 15 per cent in a line which failed to yield virus, to 50 per cent in one line from which high titers of virus were obtained following cell fusion. The RNA from the SV40 transformed cell line, which saturated 50 per cent of the viral DNA, could not be distinguished from SV40 lytic RNA in reciprocal competition-hybridization experiments. These results suggest that some block or defect, subsequent to the transcription of SV40 DNA, prevents the appearance of progeny virus in this transformed 3T3 cell.

The regulation of simian virus 40 (SV40) gene activity in transformed cells is currently under study in several laboratories.[1-3] Aloni *et al.*,[1] using the technique of nucleic acid hybridization, first reported the presence of SV40 specific RNA in transformed mouse cells. Their findings suggested that about 20 per cent of the SV40 genome is transcribed in transformed 3T3 cells. Similar results have been reported by Oda and Dulbecco[2] and Sauer and Kidwai.[3]

The examination of SV40 DNA expression during productive infection or in transformed cells is most difficult because of the small amount of SV40 specific mRNA present in cells undergoing lytic infection or transformation, compared to the various cellular RNA species. For this reason, transcription of SV40 DNA has been examined only *indirectly* through the use of DNA-RNA competition-hybridization techniques. Labeled RNA of unknown specific activity, prepared from cells undergoing lytic infection, has served as the indicator of polynucleotide sequence homologies between virus-specific RNA present during transformation and productive infection. These competition-hybridization experiments all indicate that unlabeled SV40 transformed cell RNA can partially inhibit the reaction of labeled SV40 "lytic" RNA with SV40 DNA. However, the virus-specific RNA not labeled during some of the shorter "pulsing" periods would not be available to be "competed" by an excess of unlabeled RNA. We have recently described a method which overcomes some of these difficulties and permits the examination of the RNA spectrum produced during transformation and lytic infection with polyoma virus.[4] This technique, using randomly labeled RNA and saturation hybridization, has been used to study SV40 transcription in several mouse transformed cell lines.

The factors controlling viral DNA expression in cells transformed by oncogenic viruses are not fully understood. In fact, the actual level of such control (tran-

scription versus translation) is not clear. Polyoma transformed cells, for example, exhibit only 40 per cent of the virus gene activity measured during lytic infection.[4] Assuming the presence of the entire polyoma genome in these transformed cells, this observation suggests that a block exists at the transcriptional level. However, unlike the SV40 system, polyoma virus has not been recovered from transformed cells following cell fusion which suggests that something less than the entire polyoma genome is present. Consequently, uncertainty about the proportion of polyoma DNA actually present in these transformed cells makes the interpretation of these results most difficult.

The rescue of SV40 virus from several different transformed cell lines following cell fusion[5, 6] indicates that the *entire* viral genome is probably present. Experiments can therefore be designed to determine whether the level of control of viral gene activity in transformed cells is at transcription or translation. We have examined SV40 DNA transcription during productive infection and in a series of mouse transformed cells. Our results indicate that: (1) 50 per cent of the DNA is transcribed during lytic infection; (2) 30–50 per cent of the SV40 DNA is transcribed in a series of mouse transformed cells, each capable of yielding virus following cell fusion; and (3) only 15-20 per cent of SV40 DNA is transcribed in mouse transformed lines which do not yield virus following cell fusion.

Materials and Methods.—SV40 transformed cell lines: SV40 transformed Balb/C (SV-T2) and 3T3 (SV-PY-11; 935-C; SV-UV-15-5; SV-UV-30-1) cells were kindly supplied by Dr. George Todaro. Properties of each transformed cell type are summarized in Table 1.

Preparation of randomly labeled lytic RNA: A continuous line of African green monkey kidney cells (VERO),[7] in 1-gal-sized roller bottles, were exposed to carrier-free ^{32}P-orthophosphate (10 μCi/ml) in the presence of phosphate-free Eagle's no. 2 minimal essential medium at a time when the cells had achieved approximately 20% of their final growth. When the cells became confluent (3×10^8 cells/bottle), the medium with ^{32}P was removed and small-plaque SV40 virus, previously dialyzed against phosphate-free Eagle's medium, was added at a multiplicity of 30 to 50 plaque-forming units/cell. Following a 2-hr adsorption period, the same medium with ^{32}P, used during the preceding 4–5 days, was added back to the infected monolayers. At 6, 12, 19, 24, 30, and 37 hr following infection, one roller bottle was removed and the cells lysed in the presence of 0.35% SDS, 0.1 *M* NaCl, 0.01 *M* sodium acetate pH 5.3, and Bentonite 2 mg/ml. RNA from each time period was separately extracted twice with phenol at 60°C and precipitated with 2 vol of cold 95% ethanol. Each preparation was treated with electrophoretically purified DNase (Worthington Biochemical), 50 μg/ml, for 3 hr at room temperature in the presence of 0.002 *M* $MgCl_2$ and then exposed to Pronase (Calbiochem), 50 μg/ml, previously self-digested for 2 hr at 37°C. The RNA from each time

TABLE 1. *Properties of SV40 transformed mouse cells.*

SV40 mouse transformed cell line	Mouse line transformed	Ease of recovery of SV40 following cell fusion*
SV-T2	Balb C	High titers of virus readily "rescued"
SV-PY-11	3T3	High titers of virus readily rescued
SV-935C	3T3	Low titers of virus occasionally rescued
SV-UV-15-5	3T3	No virus rescued
SV-UV-30-1	3T3	No virus rescued

* The recovery of SV40 virus from transformed cells was done by Drs. K. Takemoto and G. Todaro.

period was extracted a final time with phenol and applied to a G-100 Sephadex column equilibrated with 0.05 *M* NaCl. The radioactive RNA appearing in the excluded volume was collected, shaken with 0.01% acid washed Norit, and centrifuged at 12000 × *g* for 30 min. Specific activities of the RNA's prepared over the 37-hr infection period ranged from 87 to 90 × 10^3 cpm/μg. The RNA's from each of the six time periods during productive infection were pooled and used in the experiments to be described below.

Preparation of randomly labeled SV40 transformed cell RNA: SV 40 transformed mouse cells were labeled in tissue culture with carrier free ^{32}P-orthophosphate over a 96 to 108-hr period in phosphate-free Eagle's medium supplemented with 2% fetal bovine serum. RNA was extracted as described in the preceding paragraph.

Preparation of labeled SV40 DNA: Confluent monolayers of (VERO) cells, growing in 1-gal roller bottles, were infected with small plaque SV40 virus at a multiplicity of 0.5 to 1.0 PFU/cell. Twenty-four hours following infection, 0.1 mCi ^{14}C-thymidine in 100 ml of Eagle's medium supplemented with 2% fetal bovine serum was added. Infected cells were harvested six days postinfection and radio-labeled virus was purified by sedimentation onto a CsCl cushion followed by a cycle of CsCl equilibrium density centrifugation.[8] SV40 DNA I was quantitatively prepared as follows:[9] The DNA was released from the virus particle by heating in 1% sodium dodecyl sulfate (SDS) at 50°C for 30 min. The SDS was removed by precipitation with CsCl (1 gm/c^3) and the double-stranded twisted circular DNA (DNA-I) was separated from nicked circular (DNA-II) and linear forms by equilibrium density centrifugation in CsCl containing ethidium bromide (130 μg/ml).[10] Ethidium bromide was removed from both DNA forms by chromatography on Dowex-50 equilibrated with one-tenth standard saline citrate (SSC).[10] Viral DNA was further purified by gel filtration on G-100 Sephadex. The specific activity of the ^{14}C-thymidine DNA was 14,800 cpm/μg.

DNA-RNA hybridization: Randomly ^{14}C-labeled SV40 DNA-II or heat-treated (100° for 15 min in 1X SSC) DNA-I was immobilized on 50-mm nitrocellulose filters (type B6, Schleicher & Schuell Co., Keene, New Hampshire) as described by Gillespie and Spiegelman.[11] Saturation-hybridization reactions were carried out with 7-mm filters containing 0.006–0.008 μg immobilized SV40 DNA labeled with ^{14}C-thymidine as described in our earlier publication.[4] Increasing amounts of randomly labeled ^{32}P RNA were added to a series of reaction mixtures containing 0.8 *M* NaCl, 0.001 *M* TES, pH 7.5, and 0.1% SLS in a final volume of 0.25 ml. Following a 16-hr incubation at 67°, the filters were extensively washed in 4X SSC at 67°C, dried, and counted. Less than 0.004% of the input ^{32}P-labeled RNA reacted with nitrocellulose filters containing no DNA. The saturation values obtained when RNase stability was used as the criterion of hybrid formation were indistinguishable from those obtained with 67°C-4X SSC treatment.

Results.—Saturation of SV40 DNA with SV40 "lytic" RNA: SV40 DNA transcription during productive infection was examined by adding increasing amounts of randomly labeled "lytic" RNA to nitrocellulose filters containing C^{14}–SV40 DNA. At the end of the experiment, the *amount* of RNA reacting and the *amount* of immobilized SV40 DNA present was determined and the ratio of RNA/DNA (percentage of DNA saturated) was calculated. Figure 1 shows that 50 per cent of SV40 DNA (or the equivalent of one DNA strand) was saturated with an excess of lytic RNA. If one assumes that one-half of the polynucleotide sequences present in double-stranded DNA have the potential of being transcribed, this result indicates that such maximal gene activity is measurable during productive infection.

Saturation of SV40-DNA with SV-40 transformed cell RNA: SV40 gene activity in transformed animal cells was examined directly in a series of saturation-hybridization experiments using ^{32}P-RNA labeled over 4 to 5 cell generations. Virus-specific RNA, prepared from a transformed mouse cell line which occa-

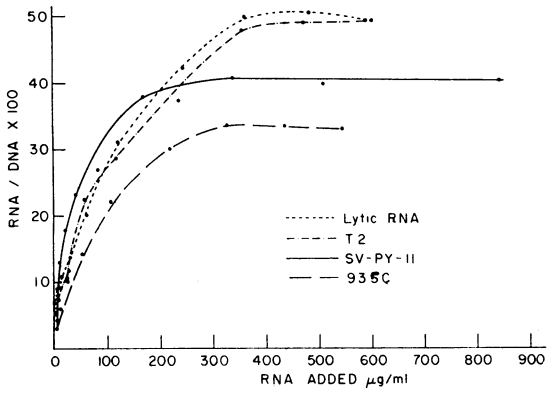

FIG. 1.—Saturation of ^{14}C-labeled SV40 DNA (15×10^3 cpm/μg) with ^{32}P-labeled lytic or SV40-transformed cell RNAs. Increasing amounts of ^{32}P-lytic RNA (112×10^3 cpm/μg), SV-T2 (98×10^3 cpm/μg), SV-PY-11 (135×10^3 cpm/μg), or 935C (208×10^3 cpm/μg) were added to a reaction mixture (0.25 ml) containing 4X SSC, 0.1% SDS and two 7-mm diameter nitrocellulose filters, one containing 0.006–0.008 μg ^{14}C-SV-40 DNA and the other containing no DNA. The filters were incubated, washed, and assayed as described in *Materials and Methods*.

sionally yields small amounts of SV40 virus following cell fusion (935C), saturated 33 per cent of the available SV40 DNA (Fig. 1). RNA prepared from a second mouse line, doubly transformed with both SV40 and polyoma (SV-PY-11), saturated 41 per cent of the SV40 DNA sites (Fig. 1). As noted in Table 1, SV-PY-11 transformed 3T3 cells readily yield large amounts of SV40 virus subsequent to cell fusion. Figure 1 also shows that one SV40 transformed mouse line (SV-T2), saturates the equivalent of one DNA strand. This 50 per cent saturation value is numerically similar to the result reported with the SV40 lytic RNA described in the preceding paragraph.

Two SV40 transformed mouse cell lines (SV-UV-15-5 and SV-UV-30-1) did *not* yield virus after cell fusion (Table 1). These lines were also a gift of Dr. George Todaro, who transformed 3T3 cells with SV40 virus previously exposed to ultraviolet irradiation for 15 or 30 minutes. Figure 2 shows that RNA prepared from these two transformed cell lines saturated 15 and 19 per cent of the SV40 DNA. This value represents about one-half the gene activity observed with 935C (Fig. 1), a mouse transformed cell which only *occasionally yields* small amounts of SV40 virus.

Identification of DNA sequences transcribed in SV40 transformed mouse cells: The ability to saturate the equivalent of one strand of SV40 DNA with either SV40 lytic or one SV40 transformed mouse cell (SV-T2) RNA provides a means for comparing virus gene activity in lytic and transformed cells. For example, are the regions of the viral DNA, which are transcribed during lytic infection the same polynucleotide sequences which are expressed in the mouse transformed line showing 50 per cent saturation? Do the viral DNA sites, which are functional in any of the mouse transformed cells, overlap with those active in green monkey cells undergoing productive infection with SV40? The combination of saturation and competition-hybridization reactions can provide answers to these questions.

In the first series of experiments, saturating amounts of *unlabeled* RNA from SV40 transformed cells were incubated for 16 hours with filters containing ^{14}C-labeled SV40 DNA in the standard reaction mixture. At this point, saturating amounts of ^{32}P-labeled lytic RNA were added to the reaction mixture and al-

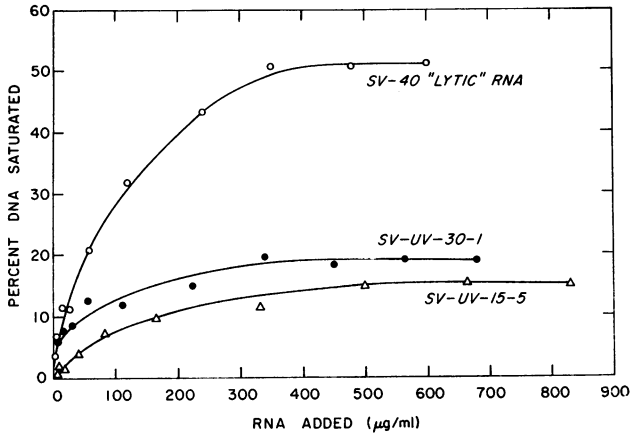

FIG. 2.—Saturation of ^{14}C-labeled SV40 DNA (15×10^3 cpm/μg) with ^{32}P-labeled lytic or transformed cell RNA's. Conditions are similar to those described for Fig. 1 except that the specific activities were: lytic RNA (98×10^3 cpm/μg); SV-UV-30-1 (98×10^3 cpm/μg); SV-UV-15-5 (82×10^3 cpm/μg).

lowed to incubate with the immobilized DNA for an additional 16 hours. The nitrocellulose filters were removed from the reaction mixture, washed, and assayed for radioactivity. Since SV40 lytic RNA reacted with 50 per cent of the SV40 DNA, any reduction in its saturation value would indicate an overlap of gene activity. If, on the other hand, regions of the SV40 genome transcribed in transformed cells were different from those functioning during productive infection, no reduction in the saturation value should be observed when ^{32}P-lytic RNA is used to monitor gene activity.

Table 2 shows the results obtained from such a series of saturation-competition experiments. It can be seen that RNA prepared from the SV-935C mouse transformed line reduces the DNA sites available for reaction with labeled lytic RNA from 50 to 20 per cent. This reduction of the DNA sites reacting with the ^{32}P-lytic RNA is numerically similar to the 30 per cent saturation value obtained when the ^{32}P-SV-935C transformed cell RNA was assayed directly (Fig. 1). Probably the most striking finding is the virtual absence of any SV40 DNA still

TABLE 2. *Reduction of SV40 DNA sites available for reaction with ^{32}P lytic RNA after exposure to an excess of unlabeled SV40 transformed cell RNA.*

Unlabeled RNA added	Amount (μg)	SV40 DNA sites saturated by ^{32}P lytic RNA
None	—	49.8
935C	290	19.6
SV-PY-11	360	16.1
SV-T2	260	1.2

The indicated amounts of unlabeled RNA were added to a standard reaction mixture (0.25 ml) which contained one nitrocellulose filter with immobilized C^{14} SV40 DNA and one blank filter. After a 16-hr incubation, the two filters were removed, gently blotted, and added to a second reaction mixture which contained saturating amounts of ^{32}P-lytic RNA. Following a second 16-hr incubation, filters were removed, washed, and assayed for radioactivity.

TABLE 3. *Reduction of SV40 DNA sites available for reaction with ^{32}P-SV-T2 transformed cell RNA after exposure to an excess of unlabeled lytic RNA.*

Unlabeled RNA added	Amount (μg)	SV40 DNA sites saturated by ^{32}P SV-T2 RNA
None	—	51.8
Lytic	410	0.7
SV-T2	260	0.8

Similar to the conditions described for Table 2 except that ^{32}P SV-T2 RNA was added in the second phase of this experiment.

available for reaction with the labeled lytic RNA following the addition of saturating amounts of SV-T2 transformed cell RNA. When tested directly (Fig. 1) the SV-T2 saturated the equivalent of one strand of SV40 DNA, numerically indistinguishable from the results obtained with the ^{32}P-lytic RNA.

The results of the reciprocal of the last saturation-competition reaction are shown in Table 3. In these experiments, the proportion of SV40 DNA still available for reaction with ^{32}P randomly labeled SV-T2 transformed cell RNA after the addition of saturating amounts of unlabeled SV40 lytic RNA is measured. It can be seen that the unlabeled SV40 lytic RNA molecules occupy nearly all of the SV40 DNA sites which were previously available to the ^{32}P SV T2 RNA. When an excess of the homologous unlabeled (SV-T2) RNA was added to this system, similar results were observed.

Several experiments were performed to rule out the possibility that the SV-T2 cell line might contain free SV40 virions. SV-T2 cells were tested and found to be negative for the SV40 capsid antigen. These cells were also subjected to three cycles of freezing and thawing and then were added to monolayers of (VERO) cells. At no time during the next five weeks was there cytopathic evidence of any SV40 in this permissive cell line. To detect the presence of any free SV40 DNA, SV-T2 cells were labeled in tissue culture over a 72-hour period with ^{3}H-thymidine. Cells were harvested and the low-molecular weight DNA was extracted as described by Hirt[12] and was centrifuged to equilibrium in CsCl in the presence of ethidium bromide. No SV40 DNA-I could be detected. Finally, SV-T2 DNA was added to primary African green monkey cells in the presence of DEAE-dextran.[13] No SV40 plaques appeared when 8 μg of SV-T2 DNA was added to this system. Under similar assay conditions, SV40 (small plaque) DNA-I had a titer of 2×10^5 PFU/μg DNA.

Discussion.—The results described above indicate that SV40 gene activity can be *directly* measured during productive infection or in several virus-transformed mouse cells. The saturation-hybridization reactions performed with randomly labeled lytic RNA provide a means of assessing the expression of SV40 DNA during lytic infection where maximal gene activity should be occurring. It is apparent from Figure 1 that 50 per cent of SV40 DNA reacts with saturating amounts of lytic RNA suggesting that *all* regions of the viral genome were being transcribed.

Indirect measurements of SV40 gene activity in transformed cells have suggested that a smaller portion of viral DNA is expressed.[1-3] Our results indicate that varying amounts of SV40 DNA are transcribed in animal cells transformed by this agent. The fraction of SV40 DNA reacting with an excess of randomly

labeled RNA prepared from five SV40 mouse transformed lines ranged from 15 to 50 per cent (Figs. 1 and 2). That is to say, these virus-free transformed mouse lines exhibited 30 to 100 per cent of the SV40 gene activity observed during the lytic cyle.

The saturation values obtained with the RNA's prepared from several SV40 transformed cell lines can also be correlated with the phenomenon of virus rescue following cell fusion. RNA's from two cell lines (SV-T2, SV-PY-11), from which SV40 was readily rescued, saturated greater than 40 per cent of the SV40 genome. The one transformed cell line (935C), from which only low titers of virus were occasionally recovered, showed intermediate levels of SV40 DNA transcription (33%). The two transformed cell lines that failed to yield virus (SV-UV-15-5 and SV-UV-30-1) exhibited the lowest level of SV40 DNA transcription (15–20%). In an entirely different oncogenic DNA virus system, it has been shown that only 20 per cent of the polyoma genome is transcribed.[4] It is interesting to note that no virus has been recovered from these polyoma-transformed cells or the SV40 transformed cell lines in which only 15–20 per cent viral DNA transcription is measured.

Transcription of SV40 DNA during lytic infection in African green monkey cells and in one virus-free transformed mouse line (SV-T2) appear to be indistinguishable as measured by DNA-RNA hybridization techniques (Tables 2 and 3). Why, then, is replication of SV40 so efficient in monkey cells, and not measurable in SV-T2 cells? It should be mentioned at the outset that, while hybridization techniques permit one to compare polynucleotide sequences, minor differences would be extremely difficult to detect. That is to say, there is the possibility that the virus-specific RNA's prepared from SV-T2 cells and monkey cells supporting productive SV40 infection, are not identical even though they were indistinguishable under the conditions of our assay. For example, a few essential polynucleotide sequences, necessary for the initiation of virus-directed protein synthesis, may never be transcribed in the SV-T2 cells, thus preventing a successful lytic infection.

Alternatively, the results obtained with lytic and SV-T2 RNA's may indicate that *similar* regions of the SV40 genome are transcribed. Why then does SV40 fail to replicate in the transformed mouse cell? Several possible explanations can be offered. First, while all of the RNA necessary for virus multiplication appears to be present in SV-T2 cells, our data sheds no light on either the relative amounts or the sequential appearance of different species of virus-specific RNA which may be necessary to modulate successful replication. Second, both chemical and biological attempts to detect *free* SV40 DNA in SV-T2 cells were unsuccessful. In SV-T2 cells, the integrated SV40 genome may not be excised from the host chromosome or function as template for progeny DNA molecules. The absence of any free SV40 DNA would therefore preclude the appearance of mature virions despite the presence of the complete complement of viral messenger RNA. A third possibility is that a block at a *translational* level prevents the efficient utilization of SV40 specific RNA by the protein synthesizing apparatus of the mouse cell. Experiments are currently in progress to determine which of these possibilities could prevent the appearance of SV40 virus.

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