THE TRANSCRIPTION OF THE SV40 GENOME IN PRODUCTIVELY INFECTED AND TRANSFORMED CELLS

By G. Sauer and J. R. Kidwai*

INSTITUT FÜR VIRUSFORSCHUNG AM DEUTSCHEN KREBSFORSCHUNGSZENTRUM, HEIDELBERG, GERMANY

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A particular feature of cells which have been transformed by DNA-containing tumor viruses is the restricted expression of the viral genome. These cells do not display any of the viral functions which occur late during a productive cycle of infection—such as extensive replication of the viral DNA, production of viral coat protein, and maturation of infectious viral progeny. They do contain, however, tumor antigen which is synthesized early after infection. Hence, it appears that in transformed cells only "early" but no "late" products are being synthesized.

With regard to the mechanism of viral oncogenesis, it is of particular importance to determine whether the block, which prevents the expression of "late" viral functions in transformed cells, occurs at the level of transcription or at the level of translation.

It has been established by various experimental approaches[1-4] that the viral DNA, in some form, persists in transformed cells. These results, however, do not indicate the extent to which the viral genome is actually transcribed.

SV40 specific messenger RNA (mRNA) sequences synthesized in SV40-transformed cells were first reported by Aloni *et al.*[5] According to their results, about one third of the viral genome is transcribed in SV40-transformed 3T3 cells. In a recent publication, Oda and Dulbecco[6] reported results similar to those of Aloni *et al.*[5] and to those presented in this paper.

It was not known, however, whether the same sequences of the viral genome were being copied in SV40-transformed cells of different origins. Using the DNA-RNA hybridization technique, we have examined the characteristics of SV40 mRNA in virus-free 3T3 and African green monkey kidney (GMK) cells transformed by SV40. It will be shown that in transformed 3T3 cells only a section of the viral genome is transcribed (40% homology with late mRNA). On the other hand, in SV40-transformed GMK cells a far larger portion of the SV40 DNA is being copied (80% homology with late mRNA). This difference may be related to particular properties of the host cell system, the 3T3 cells being nonpermissive for the productive cycle of infection, whereas GMK cells permit the growth of SV40. In the latter cells transformation can be achieved only under conditions of low input multiplicities.[7, 8]

Thus, the common denominator for various SV40-transformed cell species is the regulation of viral functions at the level of transcription, even though the sections of the SV40 genome transcribed may be variable.

Materials and Methods.—Virus and cells: The SV40 strain Rh 911,[9] which had been plaque-purified twice, was used in these experiments. The SV40 pool titered 5×10^7 plaque-forming units (PFU) per ml in primary GMK cells.

CV-1 cells, a permanent line of monkey kidney origin,[10] were productively infected

with 20 PFU per cell. Only confluent cultures were used. The adsorption period was 2 hr.

The following SV40-transformed cells were investigated: (1) 3T3 cells transformed by SV40;[11] (2) SV40-transformed GMK cells, designated GMK-EVa,[3] which yield infectious SV40 upon fusion with indicator cells; and (3) a clone derived from these cells called GMK EVa-Cl 2A-1, which upon fusion proved to be non-virus-yielding.[3] The above-mentioned cells could not be successfully superinfected with SV40, and all were free of infectious SV40. This was demonstrated by freezing and thawing 2×10^6 cells and assaying the cell lysates for plaque production on GMK cells. All plaque assays proved to be negative.

The cultures were maintained in Earle's balanced salt solution, with twice the concentration of amino acids and vitamins, and in Eagle's basal medium supplemented with 10% calf serum.

Preparation of SV40 DNA: The virus was purified in saturated KBr according to the method described by Yoshiike and Axelrod.[12] For extraction of the DNA the suspension of purified virus was made 1 *M* with respect to NaCl and shaken at room temperature (15 min) with an equal volume of 1 part of 2 *M* sodium trichloroacetate containing 0.01 *M* ethylenediaminetetraacetate (EDTA), pH 7.1, and 9 parts of 80% phenol. The aqueous phase was extracted once more with 80% phenol. After removal of the remaining phenol from the aqueous phase with ether (the ether was removed by bubbling N_2 through the solution), the DNA was precipitated with ethanol (2.5 × the volume). The precipitate was suspended in 0.1 × SSC (SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate), denatured (5 min at 100°C, then cooled rapidly in ice water), and layered on a methylalbumin-kieselguhr column.[13] The viral DNA was then eluted by increasing concentrations of NaCl. The fractions containing the viral DNA were pooled and the DNA was precipitated with ethanol.

Preparation of ^{3}H-labeled RNA: The cells were labeled for different periods of time (see *Results*) with [^{3}H]-uridine (spec. act., 27 c/m*M;* The Radiochemical Centre, Amersham). At the end of the labeling period, the cells were washed extensively with buffered salt solution, harvested by scraping, and suspended in 0.13 *M* NaCl, 0.05 tris (hydroxymethyl)aminomethane (Tris-HCl), pH 7.8. The procedure of extraction was similar to that described by Benjamin.[2] The cell suspension was adjusted to 1% sodium dodecyl sulfate (SDS) and 4×10^{-3} *M* $MgCl_2$ and shaken at 60°C for 5 min with an equal volume of 80% phenol which contained 0.1% 8-hydroxyquinoline. The mixture was then chilled in ice water and shaken again for 3 min at 60°C. After being cooled, the mixture was centrifuged and the aqueous phase was extracted twice with phenol at room temperature. Traces of phenol were removed by three successive extractions with ether, and the ether was removed by bubbling N_2 through the solution. The final aqueous phase was made 1 *M* for NaCl, and the RNA was precipitated by adding 2.5 times the volume of ethanol. The RNA was then dissolved in 0.1 × SSC containing 0.005 *M* $MgCl_2$ and treated with 20 μg/ml of DNase (RNase-free) for 45 min at 37°C. The mixture was then extracted again with phenol at room temperature. The aqueous phase was made 1 *M* for NaCl and mixed with 2.5 times the volume of ethanol. The precipitated RNA was resuspended in 0.1 × SSC and passed through a Sephadex G-100 column. The leading fractions which contained the bulk of the material (as determined by spectrophotometry) were pooled, precipitated, and redissolved in 2 × SSC. This material, after determination of the specific activity, was used in the hybridization experiments.

DNA-RNA hybridization: The DNA-RNA hybridization was carried out according to Benjamin's modification[2] of the technique described by Gillespie and Spiegelman.[14] The SV40 DNA in 0.1 × SSC was denatured (10 min at 100°C, followed by rapid cooling in an ice bath), adjusted to 0.5 *M* KCl, 0.01 Tris-HCl, pH 7.25, and passed slowly under suction on nitrocellulose membrane filters which had been presoaked in the same buffer. The filters containing the DNA were dried at room temperature and then heated at 80°C for 4 hr. The hybridization assay was carried out in Tricarb scintillation vials. The labeled RNA (in 2 × SSC) was added to the membrane filters and incubated for 24

hr at 60°C, the total volume of the reaction mixture being 2 ml. After this period of time the hybrid formation had reached a plateau. The filters were washed extensively with 2 × SSC, incubated with 20 μg/ml RNase (which had been heated for 15 min at 85°C) in 1 × SSC for 1 hr at 37°C. The filters were then rinsed in 0.5 *M* KCl, washed from each side with the same buffer, and dried. The radioactivity was then determined in the Tricarb liquid scintillation spectrometer.

Results.—SV40 mRNA in productively infected cells: The experiments described here were concerned with the functional status of the SV40 genome during the productive cycle of infection in CV-1 cells. The growth cycle of SV40 can be divided into two major periods with regard to the onset of viral DNA replication. Prior to the onset of viral DNA replication, T-antigen is formed, whereas late functions (viral coat protein synthesis and virus maturation) can be detected not earlier than 24–27 hours postinfection, i.e., after the start of viral DNA synthesis. Thus, SV40-specific RNA that is synthesized prior to 24 hours after infection will be referred to as "early." The SV40 mRNA that is transcribed later will be referred to as "late."

To demonstrate the specificity of the hybridization reaction, SV40 DNA was incubated with increasing amounts of RNA extracted 48 hours postinfection from productively infected CV-1 cells or with RNA from noninfected CV-1 cells (Table 1). It can be seen from the results that no homology exists between the viral DNA and the cellular RNA.

TABLE 1. *Hybridization of SV40 DNA with SV40-specific RNA and with RNA from uninfected CV-1 cells.*

[^{3}H]RNA from	Cpm assayed (× 10^4)	Cpm bound to filter
SV40-infected CV-1 cells	2.25	212
	4.5	396
	9.0	627
Uninfected CV-1 cells	1.75	24
	3.5	23
	9.0	26

CV-1 cells were infected with SV40 at a multiplicity of 20 PFU/ml. The cells were labeled with 20 μc/ml [^{3}H]-uridine for 30 min at 47 hr postinfection. Uninfected CV-1 cells were labeled similarly and the RNA extracted from infected and uninfected cells was tested for its ability to form a hybrid complex with 0.6 μg of SV40 DNA immobilized on filter pads. The specific activity of late and CV-1 RNA was 1850 cpm/μg RNA and 1500 cpm/μg RNA, respectively. The values indicated in the table are corrected for the nonspecific background bound to the blank filters.

The relative amounts of SV40 mRNA present in RNA preparations obtained early and late after infection were determined by exhausting the extraneous material from the RNA which is hybridizable with immobilized SV40 DNA. The results presented in Table 2 indicate that the amount of SV40-specific RNA which is produced 48 hours postinfection is about 25 times greater than the amount synthesized early after infection.

The increasing rate of SV40 mRNA synthesis after the onset of viral DNA replication is also reflected in the slopes of the saturation curves presented in Figure 1. The level of saturation is attained more rapidly with late than with early RNA preparations. Assuming that all RNA species synthesized early and late postinfection have the same specific activities, it appears that about four

TABLE 2. *Relative amounts of SV40-specific RNA in productively infected CV-1 cells at different times after infection.*

Type of [^{3}H]RNA	Cpm assayed	Cpm Bound to Filter: 1st hybridization	2nd hybridization	3rd hybridization	mRNA (% of input)
Early	16.74×10^5	950	54	20	0.06
Late	1.76×10^5	2470	36	21	1.42

CV-1 cells were infected with SV40 at a multiplicity of 20 PFU per cell. The cells were labeled with 20 μc/ml [^{3}H]-uridine for 30 min at 17 and 47 hr postinfection, respectively. The RNA was extracted as described under *Materials and Methods* and hybridized for 24 hr at 60°C with 0.5 μg of SV40 DNA immobilized on a filter. In order to exhaust the RNA from material hybridizable with SV40 DNA, the filter was replaced by a new filter also containing 0.5 μg of immobilized SV40 DNA. This procedure was repeated once more. The values indicated in the table are corrected for the nonspecific background bound to the blank filters.

times more late SV40 mRNA than early SV40 is bound to the SV40 DNA at saturation level.

To detect whether early SV40 mRNA shares homologous sequences with late SV40 mRNA, a competition experiment was completed (Fig. 2). Two controls were included in this experiment. Since large amounts of unlabeled "cold" RNA extracted early after infection are required for the competition, it might be argued that high concentrations of RNA interfere nonspecifically with the hybridization reaction. Therefore, heterologous "cold" HeLa RNA was added in increasing amounts to the hybridization reaction between late [^{3}H]SV40 RNA and SV40 DNA (the late SV40 RNA being added in all cases at saturation level). It can be seen, however, that even large amounts of HeLa RNA do not interfere with the hybridization reaction between late [^{3}H]SV40 RNA and SV40 DNA. It is also shown that unlabeled late SV40 RNA successfully competes with late [^{3}H]SV40 RNA, because the binding of late [^{3}H]SV40 mRNA to SV40 DNA is completely inhibited by the addition of increasing amounts of late cold SV40 mRNA.

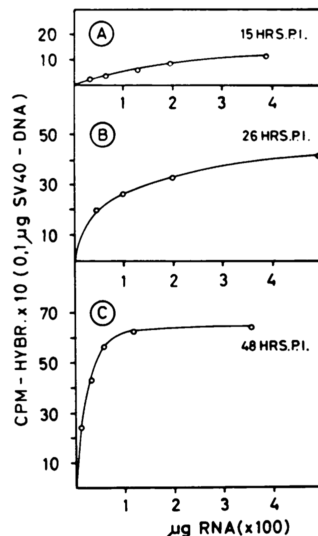

FIG. 1.—Saturation of SV40 DNA with early and late [^{3}H]SV40 mRNA from productively infected CV-1 cells. CV-1 cells were infected with SV40 at a multiplicity of 20 PFU/ml. At 15, 26 and 48 hr postinfection, respectively, the cells were labeled for 30 min with 20 μc/ml of [^{3}H]-uridine. The RNA was extracted and hybridized, in increasing amounts, with 0.1 μg of SV40 DNA immobilized on filters. The specific activities were: 15 hr postinfection, 8500 cpm μg; 26 hr postinfection, 7300 cpm/μg; and 48 hr postinfection, 7800 cpm/μg.

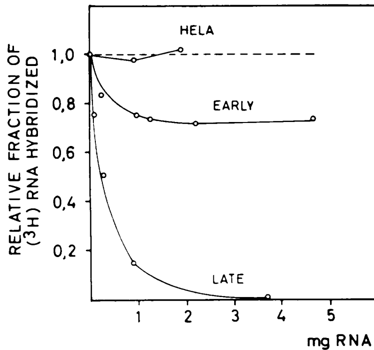

FIG. 2.—Hybridization competition between ^{3}H-labeled late, unlabeled late, and unlabeled early SV40 mRNA. Late [^{3}H]RNA was extracted 51 hr postinfection from CV-1 cells infected with 20 PFU/ml of SV40. The cells had been labeled with 20 μc/ml [^{3}H]-uridine from 48 to 51 hr. The specific activity of this preparation was 5440 cpm/μg RNA.

Filters containing 0.05 μg of immobilized SV40 DNA were incubated with 240 μg of late RNA (this is a saturating amount), and with increasing amounts of (1) unlabeled HeLa RNA, (2) unlabeled early RNA which had been extracted 17 hr postinfection from SV40-infected CV-1 cells, and (3) unlabeled late RNA which had been extracted 51 hr postinfection. The broken line indicates the level of hybridization of late [^{3}H]RNA without any cold RNA.

The competition between cold early and late [^{3}H]SV40 mRNA revealed that 28 per cent of the sequences present in late SV40 mRNA are transcribed prior to the onset of viral DNA replication. Also, it is evident from the results of the competition experiment that sequences of the viral genome, other than early, are being copied late. However, early SV40 mRNA is present also in preparations of late SV40 RNA. Thus, the data clearly demonstrate the existence of at least two different species of SV40 mRNA.

SV40 mRNA in transformed cells: To examine the homologies shared between RNA extracted from transformed cells and late SV40 mRNA, competition experiments were performed. First, saturation plateaus were established for late [^{3}H]SV40 mRNA obtained from productively infected cells. Late [^{3}H]SV40 mRNA was then added to each hybridization experiment at saturation level, and unlabeled RNA from transformed cells was added in increasing amounts to the hybridization reaction mixture.

As shown in Figure 3(*A*), the RNA from SV40-transformed 3T3 cells inhibits the hybridization of late [^{3}H]RNA by 40 per cent. Thus, the sequences of the viral genome transcribed in transformed 3T3 cells are smaller than those transcribed late during a productive cycle of infection. 3T3 cells are nonpermissive for the growth of SV40, even after infection with high multiplicities.[15] Therefore, the transcription of the viral genome must be curtailed by deficiencies of the host cell.

If host cell factors were playing a prominent role in the regulation of the transcription of the viral DNA, then SV40 mRNA sequences other than those detected in 3T3 cells might be present in transformed GMK cells which are originally permissive. The RNA extracted from SV40-transformed GMK cells (GMK-EVa) which did not contain any infectious virus was subjected to a hybridization competition with late [^{3}H]SV40 mRNA. As can be seen in Figure 3(*B*), the hybridization of late SV40 mRNA is inhibited by 80 per cent. This experiment shows that considerably more nucleotide sequences are represented in SV40-transformed GMK cells than in transformed 3T3 cells.

Previous reports[3, 16] demonstrated that, upon fusion with GMK cells, both GMK-EVa and SV40-transformed 3T3 cells revealed infectious SV40 although a

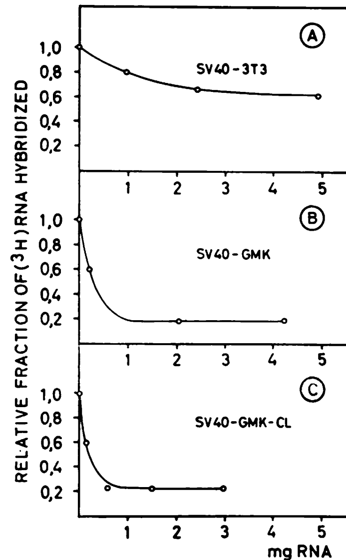

FIG. 3.—Hybridization competition between late [^{3}H]SV40 mRNA and unlabeled RNA from 3T3 and African green monkey kidney cells transformed by SV40. Filters containing 0.05 μg of immobilized SV40 DNA were incubated with 240 μg of late [^{3}H]RNA (for details see legend to Fig. 2) and with increasing amounts of (*A*) unlabeled RNA from SV40-transformed 3T3 cells, (*B*) unlabeled RNA from SV40-transformed GMK-EVa cells, and (*C*) unlabeled RNA from SV40-transformed EVaCl 2A-1 cells.

cell clone derived from GMK-EVa proved to be non-virus-yielding in fusion experiments.[3] In the latter case, mutations of the SV40 genome might account for the failure to initiate virus production after fusion. Such mutations or deletions could conceivably be reflected in the base sequences transcribed in the non-yielding cell line. That this is not the case is revealed by a comparison of the curves shown in Figure 3(*B*) (GMK-EVa) and Figure 3(*C*) (clone 2A-1 derived from GMK-EVa). In either case the hybridization of late SV40 mRNA is inhibited by 80 per cent.

So far, homologies between SV40 mRNA from transformed cells and late SV40 mRNA have been studied, but the results of these studies do not indicate the extent to which early sequences are transcribed in transformed cells.

Since only small quantities of SV40 mRNA are produced early postinfection (Table 2), it was difficult to obtain the amounts of early [^{3}H]SV40 mRNA required for saturation of the SV40 DNA. In preliminary hybridization competition experiments (unpublished), the early [^{3}H]RNA had to be used below saturation level (about 50% of the amount required for saturation). In these experiments the binding of early [^{3}H]RNA to the SV40 DNA appears to be almost completely inhibited by unlabeled RNA extracted from the SV40-transformed GMK-EVa Cl 2A-1 cells. Therefore, most, if not all, early sequences seem to be present in these transformed cells.

Discussion.—The aim of these experiments was to determine whether the late functions of the SV40 genome are blocked in transformed cells at the level of transcription and, if so, whether the same sections of the viral DNA are copied in various cell species transformed by SV40.

Generally, in all SV40-transformed cell lines fewer SV40 mRNA sequences are represented than during a productive cycle of infection. This is true for

both SV40-transformed 3T3 and GMK cells. Thus, the regulation of the viral genome functions apparently takes place at the level of transcription.

The data presented here have shown that the sections of the viral genome copied in SV40-transformed GMK cells are twice as large as in 3T3 cells. This observation may shed some light on the mechanisms which control the transcription of the SV40 genome. With regard to the 3T3 cells, it is clear that particular properties of the host cells must be responsible for the limited transcription of the SV40 DNA, since virus multiplication does not occur even after infection with high multiplicities.

The problems posed by GMK cells are much more difficult to explain. In this case the permissiveness is multiplicity-dependent. Large numbers of virus particles per cell cause lysis, whereas transformation of at least fractions of the cell culture can be achieved by very low multiplicities of infection.[7, 8]

The implications of these facts may be discussed in the light of two different hypotheses: (*a*) For reasons presently unknown, certain sections of the SV40 genome cannot be transcribed in GMK cells when only one or very few complete genomes are present in one cell. Such sequences would be transcribed, however, upon infection with high multiplicities. (*b*) The presence of mutant SV40 genomes in SV40 pools which are able to code for early but not for all late viral functions has been demonstrated previously.[17] Assuming that all base sequences of the SV40 DNA are transcribed in GMK cells (regardless of the number of viral genomes initially present per cell), it seems likely that only those cells which have been infected with mutant viral genomes would be transformed. If this hypothesis were correct, no infectious SV40 should be induced by fusion experiments. This applies for the cell clone derived from GMK-EVa in which no SV40 is inducible by fusion experiments.[3] Conversely, the parental cultures (GMK-EVa) which had not been subjected to any cloning procedure yielded infectious virus upon fusion.[3] This, however, may be the result of complementation processes between different mutant SV40 genomes which, although present in different cells, participated after fusion in the formation of one syncytium. Further experiments will be required to decide the correct hypothesis.

With regard to the mechanism of viral oncogenesis, the restricted transcription of the SV40 genome appears to be the rule, since the complete transcription would kill the host cell. Furthermore, the sections of the SV40 DNA copied in transformed cells are larger than those transcribed early during a productive cycle, although no late functions become apparent. The biological significance of this fact remains to be elucidated. It can be concluded from these observations that there are several different SV40 mRNA species. Further studies would be greatly facilitated by the characterization of their biophysical properties.

Summary.—SV40 mRNA has been studied by means of the DNA–RNA hybridization technique in productively infected monkey cells and in several transformed (virus-free) cell species of monkey and mouse origin.

Prior to the onset of viral DNA replication, during a productive cycle, 28 per cent of the viral genome is transcribed (early mRNA). This is followed by the synthesis of late SV40 mRNA which also includes the early mRNA species. In competition experiments, RNA from transformed 3T3 cells inhibits the hy-

bridization of late [^{3}H] SV40 mRNA by 40 per cent. Thus, more sequences of the SV40 genome are transcribed than early during a productive cycle of infection. The SV40 mRNA in transformed monkey kidney cells has also been examined for homologies with late [^{3}H]SV40 mRNA, with results indicating that about 80 per cent of the SV40 DNA sequences present in late SV40 mRNA are transcribed in transformed monkey kidney cells. Similar results have been obtained in the case of a clone derived from SV40-transformed GMK cells, which proved to be non-virus-yielding upon fusion.

Therefore, depending on the cell species, different portions of the viral genome are copied in the transformed cells. The common feature shared by all transformed cell species is that fewer sections of the genome are transcribed than those which are operative late during a productive cycle of infection.

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* Scholar of the German Academic Exchange Service (Deutscher Akademischer Austauschdienst). Permanent address: Central Drug Research Institute, Lucknow, India.

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