*REGULATION OF TRANSCRIPTION OF THE SV40 DNA IN PRODUCTIVELY INFECTED AND IN TRANSFORMED CELLS**

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Communicated April 11, 1968

The expression of genes of the oncogenic virus SV40 is regulated. In productive infection, some genes are expressed before viral DNA replication begins ("early" genes); others are expressed after it has begun ("late" genes).[1] In transformed cells, which contain the complete viral genome,[2] some genes are expressed; others are not. Whether either type of regulation is at the level of transcription of viral genes is the question raised in the work to be reported here. The basic technique was hybridization of RNA synthesized in infected or transformed cells to viral DNA, and hybridization competition experiments. Results partly similar to those reported here were recently published by Aloni *et al.*[3]

Materials and Methods.—Cells, media, and viruses: The following cells were used: African green monkey kidney cells (AGMK) and human embryonic kidney (HEK) purchased from Flow Laboratories; the permanent BSC-1 line of AGMK cells; and the SV3T3 line (SV40-transformed 3T3) obtained from Dr. Howard Green. Medium was reinforced Eagle's medium with 10% fetal bovine serum for AGMK cells, 10% fetal bovine serum and 10% tryptose phosphate for BSC-1 cells, and 10% calf serum for SV3T3 and HEK cells. SV40 virus was propagated in BSC-1 cells. $E46^+$ virus (no. 1237), obtained from Dr. W. P. Rowe, was propagated in AGMK cells. SV40 was titrated by plaque formation in BSC-1 cells; $E46^+$ on HEK cells.

Labeling of DNA was carried out with H^3-thymidine (1.9 c/mmole); that of RNA with H^3-uridine (24.7 c/mmole, together with 2×10^{-5} *M* of the unlabeled thymidine).

Cell fractionation into nuclei, membranes, and cytoplasmic fraction was carried out essentially according to Attardi and Attardi.[4]

Preparation of viral and cellular DNA: SV40 DNA was extracted from virus with water-saturated phenol, pH 8.0, containing 0.2 *M* Na trichloroacetate and 0.01 *M* ethylenediaminetetraacetate (EDTA), and then with chloroform-isoamyl alcohol (24:1). It was sedimented through a self-forming gradient of CsCl (initial $\rho = 1.5$) in 0.01 *M* tris-(hydroxymethyl)aminomethane-HCl (Tris-HCl), pH 8.0, and 0.002 *M* EDTA. Component I was collected, dialyzed, and concentrated by evaporation at room temperature in a dialysis tubing to *ca.* 100 μg/ml, and stored at 4°C.

$E46^+$ virus was incubated for 5 hr at 37°C with 500 μg papain, 0.002 *M* EDTA, and 0.3 *M* 2-mercaptoethanol; 1% sodium dodecyl sulfate (SDS) was then added at 37°; after 30 min, DNA was extracted as above.

Cellular DNA was prepared from nuclei according to Marmur,[5] omitting RNase treatment.

Preparation of RNA: Cells or nuclei were suspended in 0.05 *M* Na acetate, pH 5.1 (*ca.* 2×10^7 cells/ml), and made 1% with SDS. The mixture was heated at 60° for 10 min and, after the addition of $^1/_5$ vol 5 *M* Na perchlorate, was extracted two or three times with 2 vol of chloroform-isoamyl alcohol. Nucleic acids were precipitated with 2 vol ethanol, resuspended in 0.05 *M* Tris, pH 8.0, and 0.01 *M* $MgCl_2$ at the initial concentration. After 20 min at 37° with 20 μg/ml of electrophoretically purified pancreatic DNase I (Worthington), RNA was re-extracted two to three times with chloroform-isoamyl alcohol, precipitated with ethanol, and stored at −20°.

RNA was extracted from cytoplasm or polysome fraction according to Oda and Joklik.[6]

DNA-RNA hybridization was carried out essentially according to Gillespie and Spiegelman.[7] SV40 DNA was converted to Component II by controlled DNase treatment, and

brought to pH 12.5 with 1 *N* NaOH at 0°. After 10 min the pH was adjusted to 7.0 with 1 *N* HCl. The DNA solution was heated at 100°C for 5 min, rapidly cooled, and diluted with 6 × standard saline citrate (SSC) to 0.1–1 μg/ml; it was slowly filtered through presoaked Millipore membrane filters. When a membrane had less than 1 μg DNA, 5 μg of denatured *E. coli* DNA were added. Hybridization was carried out in 1 ml of 6 × SSC, 0.1% SDS at 66°C for 18–24 hr. To some mixtures with more than 10^6 cpm input RNA, 1 mg of yeast RNA was added in order to decrease background, and the edge of the membrane filter where DNA was not immobilized was cut off after it was washed with 6 × SSC. The addition of yeast RNA and SDS did not alter the efficiency of hybridization. Counts fixed by labeled RNA with the same amount of *E. coli* DNA as SV40 were 0.003–0.001% of input RNA. This background was subtracted from the experimental counts. In competition experiments, labeled RNA was always below saturation.

Sucrose density gradient centrifugation: Polyribosomes were separated by centrifuging the cytoplasmic fraction into 15–30% w/w sucrose gradient in RSB (RSB = 10^{-2} *M* tris pH 7.4, 10^{-2} *M* KCl, and 1.5 × 10^{-3} *M* $MgCl_2$). RNA's were separated in 15–30% w/w sucrose gradient in 0.5% SDS, 0.005 *M* Tris, pH 7.4, and 0.1 *M* NaCl. The gradients were collected through a Gilford automatic absorbance recorder.

Results.—Time course of synthesis and localization of virus-specific RNA in productively infected BSC-1 cells: Figure 1 shows that most of the virus-specific RNA was synthesized after the onset of DNA synthesis, which in these cells is known to be entirely viral.[8] It appeared last in the membranes. Although the membranes had the lowest total counts (Fig. 1*a*), they had the highest proportion of counts (Fig. 1*b*), probably owing to the slow labeling of membrane-associated ribosomes.[9]

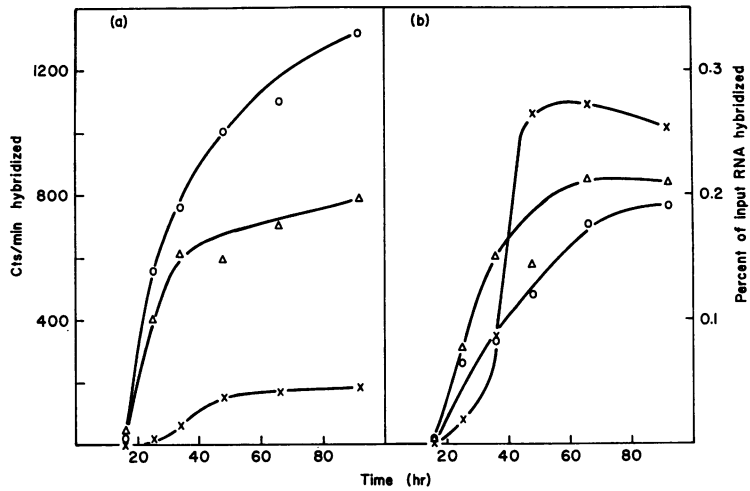

FIG. 1.—Time course of synthesis of SV40-specific RNA in infected BSC-1 cells. Confluent monolayers of BSC-1 cells were infected with SV40 at a multiplicity of 100. Other parallel cultures were mock-infected (controls). At the times indicated, H^3-uridine (10 μc/ml) was added to ten 100-mm cultures. After 3 hr, the cells were washed and fractionated into nuclei, membrane, and cytoplasm, and the RNA was extracted from each fraction. The RNA of the nuclear and cytoplasmic fractions were each dissolved in 2.2 ml of SSC/10; that of the membrane fraction in 1.3 ml SSC/10. Aliquots of 0.4 ml were hybridized with 1 μg SV40 DNA. In parallel cultures labeled with H^3-thymidine, viral DNA synthesis was found to begin between 16 and 20 hr.

O, Nuclear fraction; Δ, cytoplasmic fraction; ×, membrane fraction.

Virus-specific RNA in the membrane fraction is not an accidental contamination from either nuclei or free polysomes. In fact, cytoplasmic fractions from cells labeled with H^3-thymidine before infection, followed by excess cold thymidine at infection, contained 1–3 per cent of all counts; and centrifugation of a labeled membrane fraction in a sucrose gradient yielded no counts at the level of free polysomes.

To see whether the virus-specific RNA's of membranes and of polysomes are different, they were compared in hybridization competition experiments. Labeled membrane RNA (Fig. 2*a*) and labeled free polysome RNA (Fig. 2*b*) were each competed by either cold membrane or free polysome RNA. Although in no case was there enough cold RNA to reach a plateau of hybridized counts, the identity of the points obtained suggests that membrane-associated and free polysome RNA are identical.

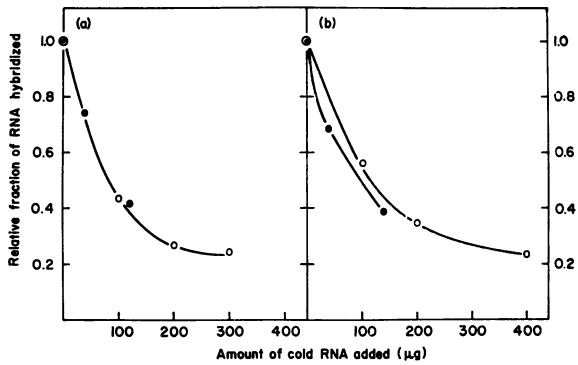

FIG. 2.—Hybridization competition between free-polysome and membrane-associated SV40-specific RNA. BSC-1 cultures were infected, labeled, and fractionated as indicated in Fig. 1. Label was added 70 hr after infection. The polysome fraction was obtained after centrifuging the cytoplasmic fraction in a 15–30% sucrose density gradient. Labeled RNA's were derived from forty 100-mm cultures; cold RNA's from 90 cultures. RNA's used for each hybridization mixture were in (*a*) 36 μg of labeled membrane RNA (2.8×10^3 cpm/μg); in (*b*) 85 μg of labeled free-polysome RNA (1.1×10^3 cpm/μg). Competing cold RNA was added in increasing amounts to the hybridization mixtures. O, Cold free polysome RNA; ●, cold membrane RNA. Cpm hybridized without cold RNA were 602 in (*a*) and 1445 in (*b*).

Characteristics of virus-specific RNA synthesized in productive infection: The distribution of sedimentation rates of virus-specific RNA is given in Figure 3. Early RNA, synthesized within 20 hours of infection, was much less than late RNA, synthesized after 90 hours; both were heterogeneous. The heterogeneity was not caused by breakdown during fractionation, since there was no breakdown of 28*S* or 16*S* ribosomal RNA in the same gradient. The heterogeneity may therefore reflect the size heterogeneity of the messengers.

Competition experiments between early and late RNA, given in Figures 4 and 5, show that cold late RNA competes about 95 per cent against labeled late RNA (Fig. 4*b*) and about 90 per cent against labeled early RNA (Fig. 4*a*). Early unlabeled RNA, however, competes only about 40 per cent against late labeled RNA (Fig. 5*c*). In the competition of cold late toward labeled early RNA, a definite plateau was not reached, and this is attributed to the low concentration of both labeled and competing RNA. It seems likely that the curve would reach essentially complete competition if larger amounts of competing RNA were employed. We conclude that, although late RNA contains all sequences present in

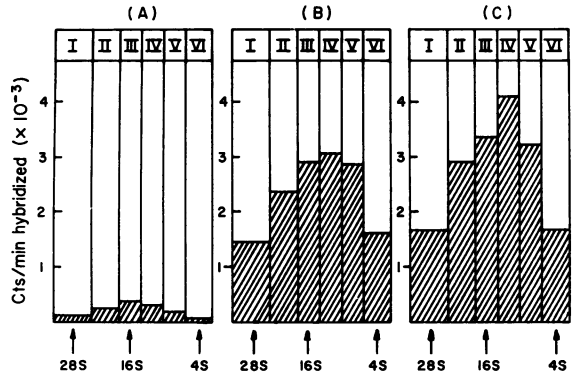

FIG. 3.—Size distribution of SV40-specific RNA in infected BSC-1 cells. BSC-1 cultures were infected as in Fig. 1. Three batches, 20 cultures (100 mm) each, were labeled with H^3-uridine as follows: (*A*) 30 μc/ml, between 10 and 22 hr after infection; (*B*) 25 μc/ml, between 50 and 56 hr; and (*C*) 25 μc/ml, between 93 and 99 hr. The RNA extracted from each batch was sedimented in a SDS-sucrose gradient. Six fraction pools were collected as follows: I = 7 fractions; II = 5 fractions; III, IV, and V = 4 fractions; VI = 5 fractions. Each fraction represents a constant cut of the gradient. The RNA in each fraction was precipitated with ethanol after adding 0.5 μg of yeast RNA as carrier.

After dissolving each pool in 1.4 ml of SSC/10, 0.3 ml of each pool for the early RNA (*A*), or 0.2 ml for the middle (*B*) and late (*C*) RNA were hybridized to 2 μg of SV40 DNA. The cpm shown are those hybridized by each pool divided by the number of fractions in the pool. The arrows indicate the positions of the peak of the two rRNA's and of tRNA.

early RNA, 90 per cent is the maximal degree of competition observable in practice under the conditions employed.

Characteristics of SV40-specific RNA in cells infected by E46⁺ virus: E46⁺ virus is a mixture of hybrid virus, which contains recombinant adeno-SV40 DNA enclosed in adeno-7 capsids,[10] and adenovirus-7. The proportion of SV40 DNA incorporated in the hybrid molecule is about 0.40.[11] The SV40 component of the hybrid DNA expresses early SV40 functions, both in productively infected and in transformed cells.[12] Cold E46⁺ RNA competes about 90 per cent with the hybridization of labeled early RNA (Fig. 5*a*). This is probably maximal competition, as discussed above. Thus, the early SV40 genes are all contained in the SV40 DNA expressed in E46⁺ infection. Since, as shown below, early SV40 RNA and E46⁺ RNA with SV40 specificity behave identically toward SV3T3 RNA, the two virus-specific RNA's may be identical.

There is somewhat more viral RNA in E46⁺-infected AGMK cells than in early

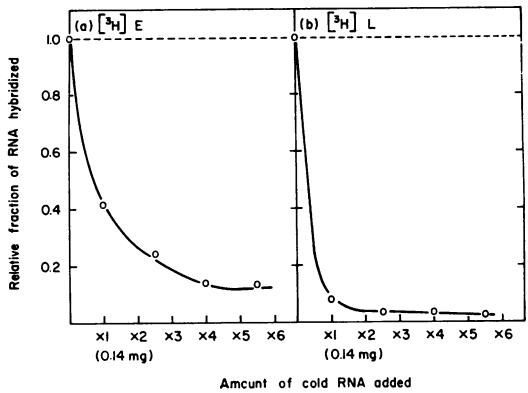

FIG. 4.—Hybridization competipetition of labeled early and late RNA with cold late RNA. BSC-1 cultures were infected as in Fig. 1, and labeled with H^3-uridine as follows: (*a*) 30 μc/ml, between 6 and 18 hr; (b) 20 μc/ml, between 93 and 99 hr. Hybridization competition was carried out in two steps: increasing amounts of cold late RNA were first hybridized to 0.1 μg of SV40 DNA; then the labeled RNA was added ((*a*) 93 μg, 6.4 $\times$ 10^4 cpm/μg; (*b*) 8 μg, 9 $\times$ 10^3 cpm/μg). Incubation under hybridization conditions was continued for an additional 8 hr. Cpm hybridized without competing RNA were 259 in (*a*) and 1366 in (*b*).

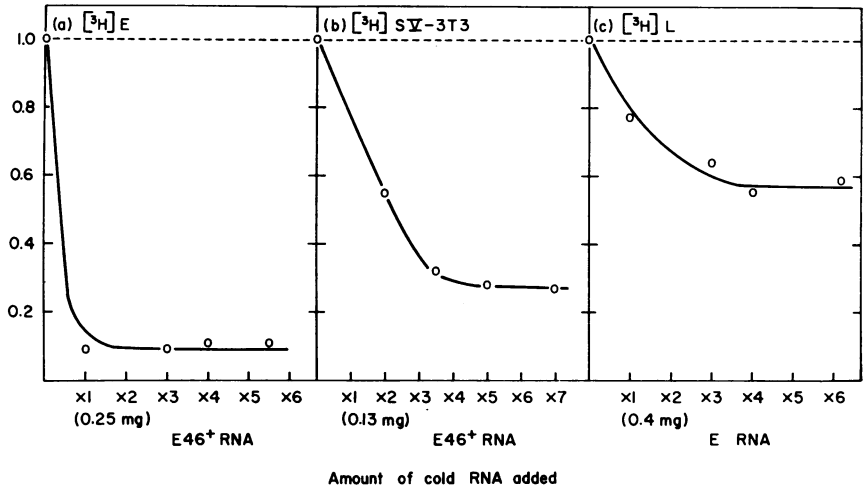

FIG. 5.—Hybridization competition of labeled early, late, and SV3T3 RNA with cold E46$^+$ or early RNA. Cold E46$^+$ RNA was prepared from African green monkey kidney cells primary cultures 60–72 hr after infection with E46$^+$ virus at a multiplicity of 4. Cold early RNA was prepared from BSC-1 cells, 18 hr after infection, as in Fig. 1. Labeled RNA's were employed as follows: (*a*) early RNA, 93 μg per hybridization mixture (6.4 $\times$ 10^4 cpm/μg); (*b*) SV3T3 RNA, 153 μg (4.6 $\times$ 10^4 cpm/μg); (*c*) late RNA (9 $\times$ 10^3 cpm/μg). Experimental procedure as in Fig. 4, with 0.1 μg SV40 DNA per filter. Cpm hybridized without competing RNA's: (*a*) 307, (*b*) 267, and (*c*) 314.

SV40 infection. Therefore, E46$^+$ is a convenient source of early SV40 RNA. In E46$^+$ infection, contrary to SV40 infection, there is a slight increase in the rate of synthesis of SV40 RNA after the viral DNA replicates. The characteristics of SV40 RNA in E46$^+$ infection will be described more fully elsewhere.

Characteristics of virus-specific RNA in transformed cells: Hybridization competition experiments of this RNA with the various RNA's defined above are reported in Figures 5 and 6. Unlabeled E46$^+$ competes less efficiently against labeled SV3T3 cell RNA (Fig. 5*b*) than against early RNA (Fig. 5*a*), thus showing that viral DNA sequences not expressed in early lytic infection are expressed in the transformed cells. Cold SV3T3 RNA competes about 90 per cent against labeled SV3T3 RNA (maximal competition) (Fig. 6*d*), but only about 60 per cent against labeled early SV40 or E46$^+$ RNA (Fig. 6*a* and *b*), and about 40 per cent against labeled late RNA (Fig. 6*c*). Thus both early SV40 and E46$^+$ RNA contain sequences of approximately the same length, not present in the virus-specific RNA of SV3T3 cells. Late RNA contains an even longer length of sequences not present in the RNA of SV3T3 cells.

Discussion.—The competition hybridization experiments gave the following results: (*a*) Early viral RNA transcribes about 40 per cent of the late genes; in contrast, late RNA transcribes early genes. (*b*) Early RNA is essentially identical to the SV40 RNA synthesized in AGMK cells infected by E46$^+$ virus. (*c*) The SV40 sequences transcribed in SV3T3 cells are about one-third those of late RNA. (*d*) Although early and SV3T3 RNA's transcribe approximately the same length of viral DNA, they are not identical. They overlap in part, but each

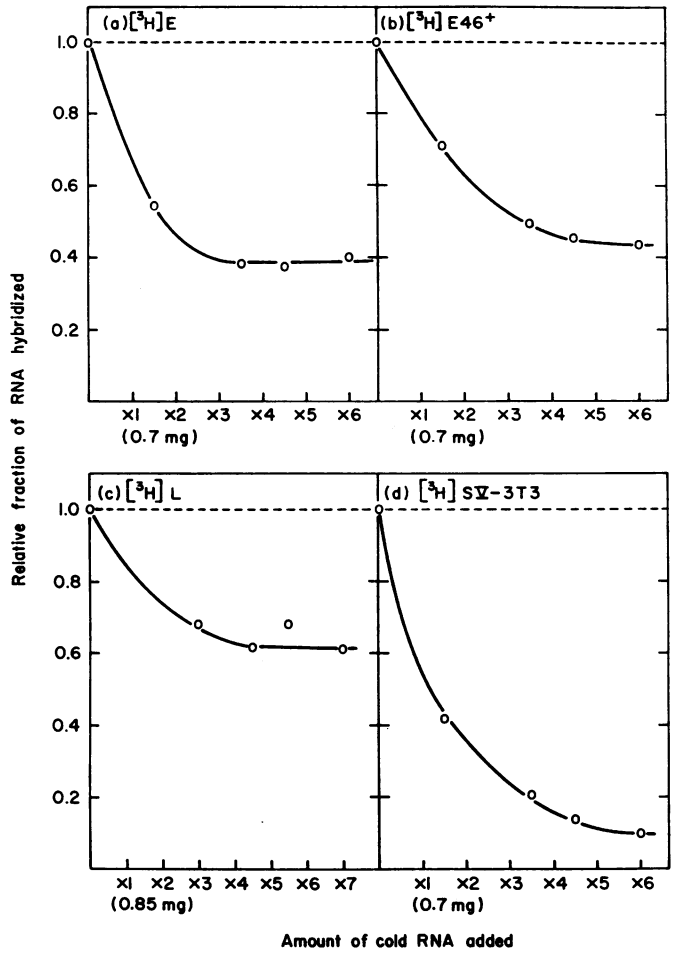

FIG. 6.—Hybridization competition of labeled early, E46⁺, late, and SV3T3 RNA with cold SV3T3 RNA. The various RNA's were prepared as in Figs. 4 and 5. Labeled RNA's were employed as follows: (*a*) early RNA, 93 μg per hybridization mixture (6.4 × 10^4 cpm/μg); (*b*) E46⁺ RNA, 10 μg (2.8 × 10^4 cpm/μg); (*c*) late RNA, 12 μg (9 × 10^3 cpm/μg); (*d*) SV3T3 RNA, 218 μg (5 × 10 cpm/μg). Experimental procedure as in Fig. 4 with 0.1 μg SV40 DNA per filter. Cpm hybridized without competing RNA (*a*) 389, (*b*) 631, (*c*) 2128, and (*d*) 390.

mRNA contains sequences absent in the other RNA. Results (*a*) and (*c*) are similar to those reported by Aloni *et al.*[3]

For a quantitative analysis, the data must be corrected for maximal degree of competition obtainable in practice, which is 95 per cent for labeled late RNA and 90 per cent for labeled early or SV3T3 RNA's. Another correction is for unspecific competition (e.g., with 3T3 or BSC-1 RNA); this amounts to about 15 per cent. The corrected data are given in Table 1 and represented diagrammatically in Figure 7.

The main conclusion from these results is that lack of expression of some viral

TABLE 1. *Results of hybridization competition experiments.*

Labeled RNA	Cold RNA	Maximal inhibition of hybridization observed	Corrected competition
Late	SV3T3	0.38	0.287
E46$^+$	SV3T3	0.60	0.600
Early	SV3T3	0.60	0.600
SV3T3	E46$^+$	0.70	0.735
Late	Early	0.42	0.337

Corrected competition was obtained by subtracting 0.15 from column 3 (unspecific competition) and normalizing the figures to either 0.80 (lines 1 and 5) or 0.75 (lines 2–4), as discussed in the text. The length of the overlap region between early RNA and SV3T3 RNA is calculated as 0.202 of the total genome from lines 3 and 5 (0.600 × 0.337), and as 0.210 from lines 4 and 1 (0.735 × 0.287).

genes, either in the early period of productive infection or in transformed cells, is caused by failure of transcription. The sequences transcribed in the two cases are not identical, pointing to two mechanisms by which transcription is blocked. The early block of the transcription of late genes may be a property of the virus, since it is tied up to the replication of the viral DNA; in contrast, the block of transcription in transformed cells is likely to be a property of the host cells. It may be the main factor making the 3T3 cells nonpermissive for SV40 multiplication. Since infectious virus is released after the SV3T3 cells are fused to BSC-1 cells,[2] the fusion must activate transcription.

The differences in the viral DNA sequences transcribed in early productive infection and in transformed cells are intriguing. On the basis of previous results, one difference could be an early gene that participates in the specification of the virus-specific thymidine kinase and is not expressed in SV3T3 cells.[13] The inference that a late gene is transcribed in the transformed cells is of special interest, but there is no hint as to the nature of this gene.

The study of the viral RNA bound to membranes has not supported the hypothesis that it may represent a special class devoted to the synthesis of a membrane protein.[4] Since viral RNA appears late in membranes (see Fig. 1) and competes well with free-polysome RNA, it may be a sample of free-polysome RNA secondarily associated with membrane-bound ribosomes. Some viral RNA could also be demonstrated in the membrane fraction of transformed SV3T3 cells. The function of this RNA is still an open question.

The identity of the early SV40 RNA with the RNA synthesized in cells productively infected by the E46$^+$ virus could arise because, by accident, the hybrid virus contains exactly the early SV40 genes. Or late genes are present but are

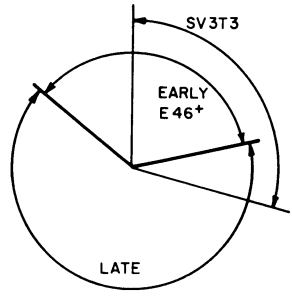

FIG. 7.—Diagram representing the proportions of SV40 DNA transcribed under the different conditions examined in the text.

not transcribed in these cells, owing to a control mechanism similar to that limiting the early transcription in productive SV40 infection; in $E46^+$ infection, however, transcription would remain limited, even after the hybrid viral DNA has replicated.

* This work was supported by a grant from the National Institutes of Health (grant no. CA 07592).

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