JOURNAL OF VIROLOGY, Mar. 1978, p. 719-729
0022-538X/78/0025-0719$02.00/0

Vol. 25, No. 3
Printed in U.S.A.

Polyoma Virus-Specific RNA Synthesis in an Inducible Line of Polyoma Virus-Transformed Rat Cells

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Received for publication 7 April 1977

Viral RNA present in the inducible LPT clone 1A of polyoma virus-transformed rat cells was characterized before and after mitomycin C induction by hybridization with ^{32}P-labeled separated E and L strands of polyoma viral DNA restriction endonuclease fragments. In clone 1A cells maintained under normal growth conditions, the cytoplasm contained a transcript of the E-strand DNA from the "early" region similar to that previously identified in lytically infected cells, as well as minor quantities of RNA complementary to less than one-half of the L- and the E-strand DNA from the "late" region. Nuclei of normally growing cells contained the same species found in the cytoplasm, as well as an additional abundant RNA complementary to one-half of the L-strand DNA of the late region. No significant changes occurred in the cytoplasmic viral RNA after mitomycin C treatment before the onset of viral DNA replication, but the concentration of the nuclear L-strand DNA transcript diminished. After the onset of viral DNA replication after mitomycin C treatment, transcripts of virtually the entire L-strand DNA were found in the nuclei, and a 10-fold increase was observed in the abundance of RNA transcribed from the E strand of the early region. In the cyotplasm, the abundance of the early RNA increased about 25-fold, and late RNA complementary to the L-strand DNA of the late region was found in a similar quantity. The synthesis of both the early and the late RNA species was inhibited if viral DNA replication was blocked with 5-fluorodeoxyuridine. We conclude that the induction of viral DNA replication in LPT cells is not determined at the level of mRNA synthesis.

The LPT line of polyoma virus (Py)-transformed rat cells (6) is unusual among Py-transformed cell lines. A relatively high proportion of the LPT cells (0.01 to 0.2%) spontaneously produce infectious virus. Moreover, the proportion of virus-producing cells can be increased to approximately 50% by treatment with various physical and chemical agents (5-7).

The average yield of Py DNA in normally growing LPT cultures is 30 to 40 genome equivalents per cell. Only about 20% of this viral DNA remains associated with the chromosomal DNA after alkaline glycerol gradient centrifugation (17); most of the DNA presumably exists in a nonintegrated form (21). The viral DNA in LPT cultures is unequally distributed among the cells. A small proportion of the cells ($\leq$0.23%) contain about 24,000 genome equivalents of viral DNA per cell (19), and thus the vast majority of the cells in each culture must contain far less than the average number of viral DNA molecules.

The most effective inducing agent in the LPT system is mitomycin C (MMC). Py DNA replication in MMC-treated cells is enhanced after a lag period of about 9 h and then continues at an enhanced rate for at least 24 h (18, 19). The onset of viral DNA replication after MMC treatment is blocked by exposure to inhibitors of protein synthesis (18).

In this paper we compare the Py-specific RNA present in normally growing LPT cells with the viral RNA found in MMC-treated cells before and after the onset of viral DNA replication. This study is a part of an effort to elucidate the mechanism of Py induction in the LPT line. The reason for examining viral transcription is the long-recognized analogy between Py induction in LPT cells and the induction of temperate bacteriophages in lysogenic bacteria (5, 6); the same physical and chemical agents act as inducers in these systems. In the case of the bacteriophages, induction is mediated by changes in the transcription of the phage DNA before the onset of phage DNA replication (22).

We employed in this study the same methods that have been used previously for mapping Py-

specific RNA sequences in productively infected cells (12, 14). Restriction endonucleases were used to cleave ^{32}P-labeled Py DNA into specific fragments. The complementary strands of each fragment were separated and hybridized with unlabeled cytoplasmic and nuclear RNA from normally growing and MMC-treated LPT cells. These assays allowed qualitative and quantitative evaluations of the virus-specific sequences in the LPT RNA preparations.

MATERIALS AND METHODS

Cells and growth media. Cells of the LPT clone 1A were grown in Dulbecco-modified Eagle medium supplemented with 5% fetal calf serum. To induce the virus, MMC was added to the medium at a final concentration of 1 μg/ml. After 1 h, the medium was replaced and the cells were incubated in the absence of the drug (see reference 18 for further details).

Separation of nuclei from cytoplasm. The nuclei were separated from the cytoplasm by the method of Lindberg and Darnell (16). Briefly, each plate was washed once with 5 ml of phosphate-buffered saline (4); the cultures were then cooled to 4°C, and to each plate was added 3 ml of a buffer containing 0.01 M Tris (pH 8.4), 0.14 M NaCl, and 0.0015 M $MgCl_2$ (isoHipH). The cells were scraped from the plates with a rubber policeman and centrifuged for 3 min at 1,000 rpm. The precipitated cells were suspended in isoHipH at a density of 2×10^6 to 3×10^6 cells per ml, and the detergent Nonidet P-40 was added to a final concentration of 0.50%. The suspension was centrifuged as described above; the supernatant containing the cytoplasm and the precipitate containing the nuclei were then collected. The nuclei were suspended once more in isoHipH + 0.50% Nonidet P-40 and collected by centrifugation.

RNA extraction. The method of Penman (20) for nuclear RNA extraction was modified as follows. The nuclear pellet was suspended in a buffer containing 0.05 M Tris-hydrochloride (pH 7.4), 0.50 M NaCl, and 0.02 M $MgCl_2$ at a density of 10×10^6 to 15×10^6 nuclei per ml. DNase (Worthington Biochemicals Corp.; electrophoretically purified) was added at a concentration of 100 μg/ml. After a 5-min incubation at 25°C, the following additions were made: sodium dodecyl sulfate (SDS), polyvinyl sulfate, and Pronase to final concentrations of 0.50%, 5 μg/ml, and 0.50 mg/ml, respectively. The mixture was incubated 30 min at 37°C, after which time it was diluted fivefold with 0.01 M Tris (pH 7.4) and extracted once at 60°C with an equal volume of a solution containing phenol, chloroform, and isoamyl alcohol at a ratio of 50:49.5:0.50 (vol/vol/vol). After two additional extractions at 25°C, the RNA was precipitated by adding 2 volumes of ethanol to the aqueous phase and leaving the mixture for at least 5 h at −20°C. The preciptiate was collected by centrifugation (10,000 rpm for 20 min at 4°C) and then washed twice with a solution containing two-thirds ethanol and one-third 0.01 M Tris (pH 7.5). The ethanol was evaporated, and the RNA was dissolved in a buffer containing 0.01 M Tris-hydrochloride (pH 7.5) and 0.01 M $MgCl_2$. DNase was added again at a concentration of 50 μg/ml, and the solution was incubated for 30 min at 37°C. At the end of the incubaton period, the mixture was diluted fivefold with 0.01 M Tris (pH 7.5), brought to 0.50% SDS, and then extracted three times at 25°C with phenol-chloroform-isoamyl alcohol, as described above. The RNA was precipitated with ethanol, and the precipitate was washed as described above. It was later dissolved in 0.01 M Tris (pH 7.5) containing 0.10% SDS and used for hybridization.

Cytoplasmic RNA was extracted by the same procedure, except that the first DNase treatment was omitted.

Preparation of specific fragments of Py DNA and DNA strand separation. The preparation of the ^{32}P-labeled separated strands of the restriction endonuclease *Eco*RI/*Hha* I fragments was as described by Kamen and Shure (14). The initial specific radioactivity of this preparation was estimated as 5×10^6 to 10×10^6 cpm/μg. The Pst I/*Bam* I fragments were similarly prepared from DNA that had been labeled with ^{32}P by nick translation with *Escherichia coli* DNA polymerase (15). The initial specific radioactivity of this preparation was estimated as 8.8×10^7 cpm/μg.

RNA-DNA hybridization. Hybridization and S1 digestion were carried out as described by Kamen and Shure (14). The ^{32}P-labeled single-stranded DNA was incubated with unlabeled RNA for 5 days at 72°C in 0.10 ml of a solution containing 1 M NaCl, 50 mM Tris (pH 7.5), 1 mM EDTA, and 0.10% SDS. At the end of the incubation period, the samples were cooled to 4°C until further processing. To determine the percentage of ^{32}P radioactivity in hybrid, the mixtures were diluted with 2 ml of S1 buffer (0.05 M KCH_3CO_3 [pH 4.5]–0.2 M NaCl–0.0035 M $ZnSO_4$–25 μg of native calf thymus or salmon sperm DNA per ml–7 mM 2-mercaptoethanol) and incubated for 1 h at 50°C with sufficient S1 endonuclease to digest at least 95% of the single-stranded DNA. The acid-insoluble radioactivity was determined by precipitating the samples in trichloroacetic acid, collecting the precipitate on GFC filters, and counting with a scintillator, as described previously (14, 18).

RESULTS

Figure 1 shows a physical map of the Py genome oriented in the conventional manner (10) and indicating the positions of the DNA fragments produced by cleavage with each of the two pairs of restriction endonucleases, *Hha* I + *Eco*RI and *Pst* I + *Bam* I, used for the present study. Also shown on the map are the chemical polarities of the E and L complementary DNA strands (13), the two regions of the genome designated as "early" and "late" (12), and the locations of the different mRNA's found in the cytoplasm late during lytic infection of mouse cells (14). We shall employ the symbols L_1, L_2 ... E_1, E_2 ..., preceded by the names of the enzymes used to cleave the DNA, to denote the L and the E strands of the fragments produced by these enzymes.

We used for most of the work to be reported

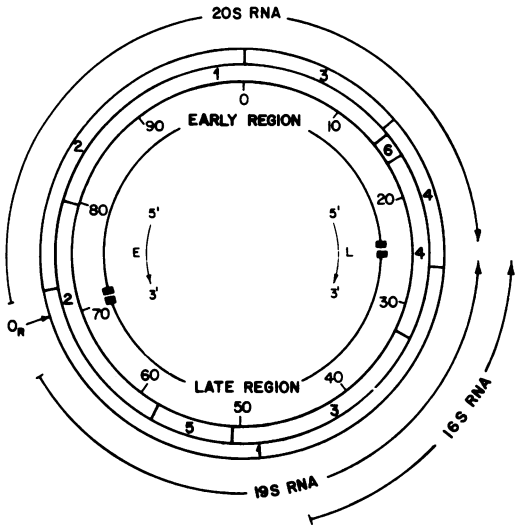

FIG. 1. *Physical map of the Py genome. The circular map of the Py genome is divided into 100 units, 0 point on the map being the site of cleavage by the restriction endonuclease EcoRI (10). The outer ring shows the fragments produced by a successive cleavage of the DNA with the restriction endonucleases EcoRI and Hha I (8). The inner ring shows the fragments produced by a successive cleavage of the DNA with the restriction endonucleases Pst I (3) and Bam I (9). The arrows outside the rings show the map positions of the early and late cytoplasmic mRNA species synthesized in mouse cells lytically infected with Py, as well as the direction of transcription of these RNA species (14). The arrows inside the ring show the chemical polarities of the L and E strands of the DNA (13). Also indicated in this figure are the two parts of the genome designated as the early and late regions (12).*

below the ^{32}P-labeled separated strands of the four fragments produced by a successive cleavage of the Py DNA with the enzymes *Eco*RI and *Hha* I. As Fig. 1 shows, the single-stranded fragment *Eco*RI/*Hha* I L_1, which spans most of the late region of the virus genome, is the template for most of the cytoplasmic 19S mRNA that accumulates late in infection. A small portion at the 3′ terminus of this RNA molecule is transcribed from the fragment *Eco*RI/*Hha* I L_4. The 16S mRNA corresponds to the 3′-terminal half of the 19S mRNA. The 20S RNA synthesized both early and late in infection is transcribed from the fragments *Eco*RI/*Hha* I E_2, E_3, and most of E_4, which together span the early region of the genome. Nuclear RNA isolated late in infection contains transcripts of the entire L and E strands of the Py genome (12). Thus, transcripts of the fragments *Eco*RI/*Hha* I E_1, L_2, L_3, and most of L_4, which are found in nuclei, are not represented in cytoplasmic viral mRNA.

We used the methods described by Kamen and Shure (14) to prepare the ^{32}P-labeled fragments *Eco*RI/*Hha* I E_1, E_2, E_3, E_4, L_1, L_2, L_3, and L_4 (see above). Self-annealing of the L fragments, followed by digestion with the single-strand-specific nuclease S1, showed that these fragments contain less than 10% of the corresponding E-strand complements. A similar analysis of the E-strand fragments revealed the presence of less than 2% of the corresponding L complements.

In all of the experiments reported in this article, a constant amount of each ^{32}P-labeled single-stranded fragment was annealed with varying concentrations of unlabeled RNA. The fraction of DNA that formed an RNA-RNA hybrid was determined as the fraction of ^{32}P radioactivity that became resistant to S1 digestion. Figure 2 shows the results of one experiment of this type in which Py complementary RNA (cRNA), which had been asymmetrically transcribed from the L strand of the virus DNA by the *E. coli* RNA polymerase (12), was annealed with the four *Eco*RI/*Hha* I L fragments. It can be seen that the cRNA hybridized with 75 to 85% of each of these fragments. These numbers should be considered as minimal estimates, because hybridization was carried out for 5 days

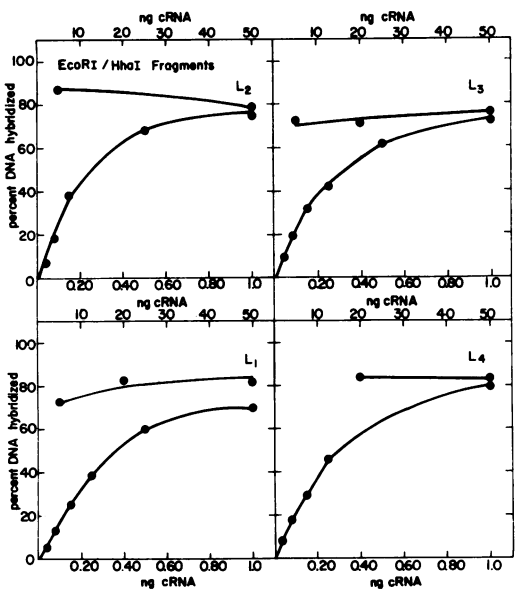

FIG. 2. *Hybridization of the EcoRI/Hha I L DNA fragments with Py cRNA. Hybridization was carried out as described in the text. The percent DNA in the hybrid was calculated as the percentage of the ^{32}P radioactivity rendered resistant to digestion with the S1 endonuclease. The abscissa at the bottom of each square refers to the lower curve; the abscissa at the top refers to the upper curve. Both curves were obtained in the same experiment.*

at 72°C, during which time some degradation of the DNA and/or the RNA could have occurred. (These conditions were chosen to detect minor species of virus-specific RNA in LPT cells.) As expected, hybridization of the cRNA with the fragments *Eco*RI/*Hha* I E_1, E_2, E_3, and E_4 was found to be negligible even at the highest concentration of RNA shown in Fig. 2 (data not shown). The data shown in Fig. 2 were employed as calibration curves to estimate the abundance of virus-specific transcripts in the LPT cell RNA preparations. We assumed that the hybridization reaction between Py-specific sequences in RNA extracted from LPT cells and each of the single-stranded DNA fragments proceeded with the same efficiency as did the reaction of the cRNA with the corresponding L fragments and that the cRNA contained sequences complementary to the entire L strand of Py DNA. On the basis of these two assumptions, we used the saturation levels and the slopes of the hybridization curves obtained with LPT cell RNA samples to calculate the extent of transcription from different DNA fragments and the abundance of the transcripts (see footnote *a* to Table 1 for the details of such calculations). The assumption concerning the efficiency of hybridization may not hold when transcripts of both strands of a particular DNA fragment were present in the same RNA sample. Such transcripts could self-anneal, and the RNA-RNA reaction could compete with the RNA-DNA hybridization. Therefore, saturation levels calculated in these situations may underestimate the true extent of transcription. Calculation of the abundance of self-complementary transcripts would yield even less reliable values, because the results depend on the estimations of the true plateau levels and on additional assumptions concerning the possible extent of RNA-RNA self-annealing at each point on the curves. We therefore have not calculated transcript abundances when it was possible that self-complementary sequences were present.

Py-specific RNA in the cytoplasm of untreated and MMC-treated clone 1A cells. We used a clonal derivative of the LPT line designated as clone 1A for the present studies. Cells of this clone can be induced to synthesize infectious virus at a somewhat higher efficiency than cells of other LPT clones (19). We first characterized the RNA accumulated within the cytoplasm of normally growing clone 1A cells by hybridizing cytoplasmic RNA prepared from these cells with each of the single-stranded *Eco*RI/*Hha* I fragments. Figure 3 shows the hybridization saturation curves, and Table 1 (line 1) presents the quantitative analysis of these data. It can be seen that this RNA preparation contained transcripts of virtually all of *Eco*RI/*Hha* I E_2 and E_3 and about 63% of fragment E_4. It should be noted that the transcripts of E_3 and E_4 were detected at the level of approximately 88 molecules per cell, whereas those of E_2 were nearly twice as abundant. In addition, molecules that were apparently complementary to about 30 and 24% of the fragments *Eco*RI/*Hha* I E_1 and L_1, respectively, were detected.

We next characterized the virus-specific RNA accumulated in the cytoplasm of MMC-treated clone 1A cells during the early period of induction before the onset of viral DNA replication. In this experiment, RNA was prepared from cells harvested 6.5 h after MMC treatment and hybridized with the *Eco*RI/*Hha* I fragments, as described above. Figure 4 and Table 1 (line 2) show the results of these assays. It can be seen that the pattern of virus-specific RNA in these cells is similar in both the extent of hybridization and the abundance to the pattern found in the normally growing cells, except that the extent of hybridization of the *Eco*RI/*Hha* I E_1 transcript has increased from about 30 to 49% and that of L_1 has decreased from 24 to 13% (corrected values in Table 1).

A similar analysis of cytoplasmic virus-specific RNA from cells that had been treated with MMC and harvested after 24 h, that is, 15 h after the onset of viral DNA replication, is shown in Fig. 5 and Table 1 (line 3). It can be seen that both qualitative and quantitative changes in the virus-specific RNA occurred in these cells. First, an abundant transcript of the fragment *Eco*RI/*Hha* I L_1 appeared. Second, the concentrations of the *Eco*RI/*Hha* I E_2, E_3, and E_4 transcripts increased. The abundance of the L- and E-strand transcripts is similar and is 20 to 25 times greater than is the abundance of the E-strand transcripts found in the cytoplasm of untreated cells. The increase in abundance of the E transcripts was not due to a decrease in the amount of total RNA per cell (the latter parameter is not substantially affected by MMC treatment), but reflects an absolute increase in the number of viral RNA molecules per cell.

Py-specific RNA in the nuclei of untreated and MMC-treated clone 1A cells. Nuclear RNA preparations from normally growing clone 1A cells and from cells harvested 6.5 and 24 h, respectively, after MMC treatment, were hybridized with the *Eco*RI/*Hha* I fragments, as described above. Figures 6 through 8 show the results of these hybridization assays. It can be seen (Fig. 6) that the nuclei in the normally growing cells contain transcripts complementary to more than 60% of the fragments *Eco*RI/*Hha* I E_2, E_3, and E_4. The abundance of

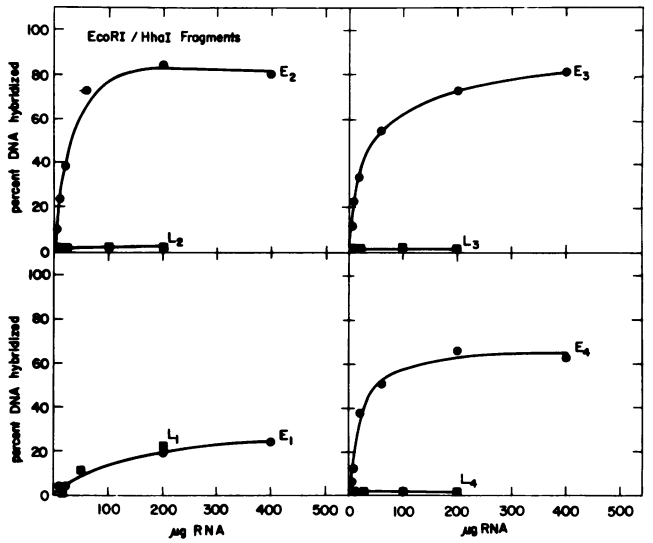

FIG. 3. *Hybridization of cytoplasmic RNA from normally growing clone 1A cultures with the EcoRI/Hha I L and E DNA fragments. Hybridization was carried out as described in the legend to Fig. 2.*

TABLE 1. *Abundance of RNA transcripts complementary to the EcoRI/Hha I fragments of the Py genome in the cytoplasm of clone 1A cells*[a]

Type of RNA	E_1		E_2		E_3		E_4		L_1		L_2		L_3		L_4	
	P	*N*	*P*	*N*	*P*	*N*	*P*	*N*	*P*	*N*	*P*	*N*	*P*	*N*	*P*	*N*
Untreated	30	ND	86	154	100	88	63	88	24	ND						
MMC (6.5 h)	49	ND	86	132	100	88	73	88	13	ND						
MMC (24 h)			76	3,300	80	2,288	65	1,892	76	2,948						

[a] The data presented in Fig. 2 through 5 were used to calculate the parameters *P* and *N* shown in this table. These parameters are defined as follows: *P*, percent saturation of a particular E or L fragment of Py DNA with clone 1A RNA, relative to the saturation level of the L fragment with cRNA; *N*, number of molecules per cell of Py-specific RNA complementary to the DNA fragment. The calculations are based on the assumption that the cRNA contains equimolar amounts of sequences complementary to the entire L strand of the Py genome and are carried out as follows. Let: P_R = plateau level of the hybridization curve of clone 1A RNA with an E or L fragment of Py DNA; P_C = plateau level of cRNA with the L fragment; S_R = concentration of RNA required to attain 40% of the plateau level; S_C = concentration of cRNA required to attain 40% of the plateau level; F = fraction of the Py genome in the fragment; r = ratio of Py-specific RNA complementary to the fragment and total RNA in a sample, calculated in terms of micrograms of Py-specific RNA per microgram of clone 1A RNA; M = number of molecules in 1 μg of virus-specific RNA complementary to the fragment. (i) The values of *P*, presented in the table, are calculated as $P = P_R/P_C \times 100$. (ii) M is calculated as: $M = \frac{6 \times 10^{23}}{330 \times 10^6 \times 5{,}500 \times F \times P_R/P_C}$. (iii) r is calculated as: $r = \frac{S_C \times P_R \times F}{S_R \times P_C}$. (iv) The values of N are calculated as $N = M \times r \times 44 \times 10^{-6}$, where 44×10^{-6} μg is the amount of cytoplasmic RNA per cell based on the value of 51×10^{-6} μg of RNA per cell determined for clone 1A cells (Manor, unpublished data) and the assumption that 86% of the total cellular RNA is found in the cytoplasm (2). ND, Not determined. Empty spaces indicate RNA species indetectable under our assay conditions or whose concentration was too small to allow any quantitative evaluation.

the E_3 and E_4 transcripts is several times smaller than the abundance of the E_2 transcript, as revealed by comparing the slopes of the hybridization curves. In addition, the nuclei in the normally growing cells contain RNA molecules complementary to about 30% of the fragment *Eco*RI/*Hha* I E_1, as well as a rather abundant RNA species transcribed from about 45% of the fragment *Eco*RI/*Hha* I L_1 and a minor RNA species complementary to about 15% of the fragment *Eco*RI/*Hha* I L_2. These numbers are minimal estimates of the extent of transcription from the fragments E_1, L_1, and L_2, because of the potential for RNA-RNA self-annealing in these assays. A comparison of Fig. 3 with Fig. 6 also indicates that only a small fraction of the abundant L_1 transcript found in the nucleus may be exported to the cytoplasm.

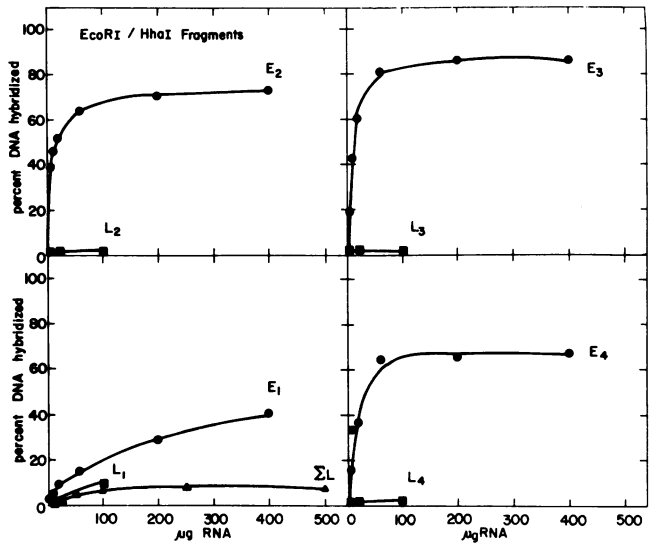

FIG. 4. *Hybridization of the EcoRI/Hha I L and E DNA fragments with cytoplasmic RNA from clone 1A cells treated with MMC and harvested after 6.5 h. Cytoplasmic RNA was prepared and hybridized with the EcoRI/Hha I fragments as described in the legend to Fig. 2. ΣL is a mixture of all EcoRI/Hha I L DNA fragments in equimolar proportions.*

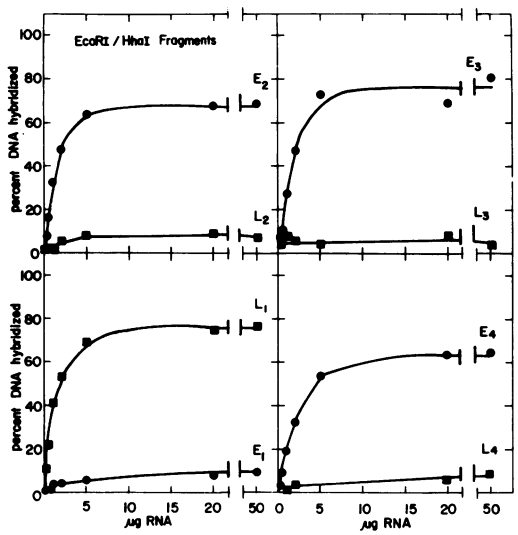

FIG. 5. *Hybridization of the EcoRI/Hha I L and E DNA fragments with cytoplasmic RNA from clone 1A cells treated with MMC and harvested after 24 h. Clone 1A cells were treated with MMC, as described in the text, and harvested after 24 h. Cytoplasmic RNA was prepared and hybridized with the EcoRI/Hha I fragments, as described in the legend to Fig. 2.*

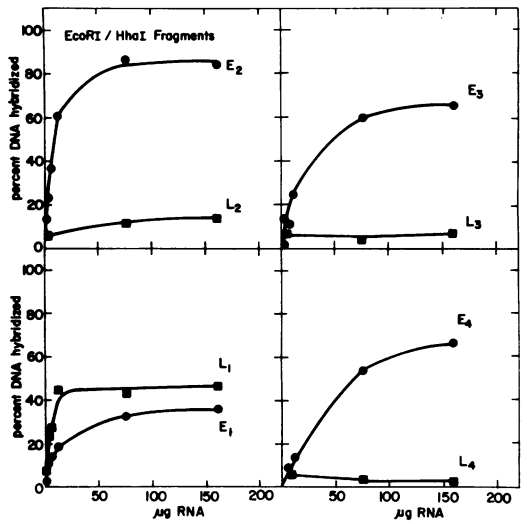

FIG. 6. *Hybridization of nuclear RNA from normally growing clone 1A cultures with the EcoRI/Hha I L and E DNA fragments. Hybridization was carried out as described in the legend to Fig. 2.*

Figure 7 shows that the E DNA strand transcripts found in the nuclei of the cells harvested 6.5 h after MMC treatment are qualitatively similar to the corresponding transcripts found in the nuclei of the normally growing cells, except that the highest extent of hybridization with the fragment *Eco*RI/*Hha* I E_4 drops from 70% (Fig. 6) to about 45% (Fig. 7). It should be noted, however, that both values may not represent the final plateau levels and that the difference between the two curves may simply result from a decrease in the concentration of the E_4 transcript in the MMC-treated cells. Another quantitative difference between these two nuclear RNA preparations is the apparent drop in the abundance of the *Eco*RI/*Hha* I E_1 tran-

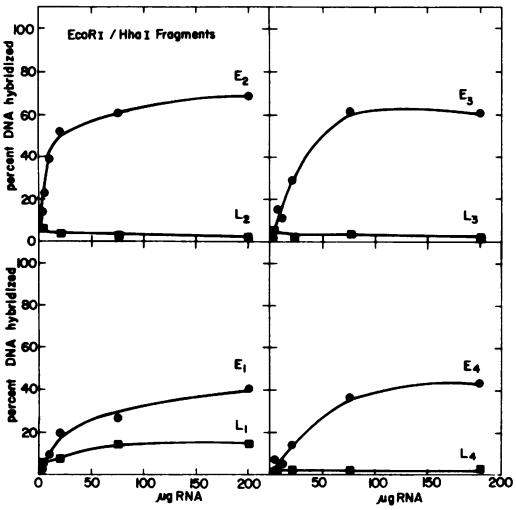

FIG. 7. *Hybridization of the EcoRI/Hha I L and E DNA fragments with a nuclear RNA from clone 1A cells treated with MMC and harvested after 6.5 h. Clone 1A cells were treated with MMC, as described in the text, and harvested after 6.5 h. Nuclear RNA was prepared and hybridized with the EcoRI/Hha I fragments, as described in the legend to Fig. 2.*

script. However, the most dramatic change in the nuclear RNA occurring during the 6.5-h period after MMC treatment is the decrease in the concentration of the *Eco*RI/*Hha* I L_1 transcript. We repeated these experiments with different RNA preparations and found that even though the variations in the extent of hybridization and the slopes amounted to 10 to 15% and 10 to 30%, respectively, the changes that occur in the patterns of the virus-specific nuclear RNA after MMC treatment are reproducible.

Figure 8 shows the results obtained in a similar analysis of nuclear RNA from cells harvested 24 h after MMC treatment. It can be seen that these cells have accumulated transcripts that are complementary to all four L DNA strand fragments. However, a comparison of the slopes of the four curves indicates that the concentration of the *Eco*RI/*Hha* I L_1 transcript is five times higher than the concentrations of the other transcripts. Figure 8 also shows the presence of nuclear RNA species complementary to about 60% of the fragment *Eco*RI/*Hha* I E_2 and to about 40% of the fragment *Eco*RI/*Hha* I E_3. Sequences complementary to the fragment *Eco*RI/*Hha* I E_4 are barely detectable. Since transcripts of these fragments accumulate in the cytoplasm (Fig. 5), it is probable that these transcripts exist in the nuclear RNA, but are not available for RNA-DNA hybridization, because they form RNA-RNA duplexes with the corresponding L-strand complements that are present in excess (12).

In view of the uncertainties in evaluating the plateau levels and the slopes of many of the curves shown in Fig. 6 through 8, we have been unable to obtain reliable estimations of the concentrations of the viral RNA transcripts examined in all of these assays. In some cases, however, such estimations can be made. We found that the nuclei in normally growing cultures contain about 60 molecules of *Eco*RI/*Hha* I E_2 transcripts per nucleus. About 50 molecules of the E_2 transcripts per nucleus are found in the nuclei of cells harvested 6.5 h after MMC treatment, whereas 24 h after MMC treatment the number of E_2 transcripts increases to about 600 molecules per nucleus. The more extensive L-strand transcription that occurs in MMC-treated cells after the onset of viral DNA replication leads to an accumulation of about 950 molecules of *Eco*RI/*Hha* I L_1 transcripts per nucleus and about 200 molecules of *Eco*RI/*Hha* I L_2, L_3, and L_4 transcripts per nucleus.

Py-specific RNA in cells treated with MMC and FUdR. In mouse cells lytically infected with Py, transcription from the L DNA strand does not occur if the initiation of viral DNA replication is inhibited (11). The following experiment was designed to determine whether in clone 1A cells treated with MMC the onset of transcription from the L strand and the accumulation of RNA complementary to the E strand depend on viral DNA replication. In this experiment, clone 1A cultures were treated with

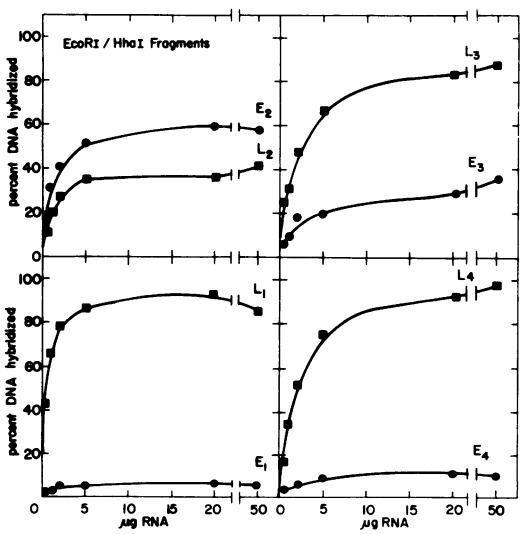

FIG. 8. *Hybridization of the EcoRI/Hha I L and E DNA fragments with nuclear RNA from clone 1A cells treated with MMC and harvested after 24 h. Clone 1A cells were treated with MMC, as described in the text, and harvested after 24 h. Nuclear RNA was prepared and hybridized with the EcoRI/Hha I fragments as described in the legend to Fig. 2.*

MMC, as previously described. Half of the plates were harvested after 3.5 h. The rest were incubated in a medium containing 15 μg of fluorodeoxyuridine (FUdR) per ml for an additional 21.5 h. We have shown that at this concentration FUdR inhibits completely any increase in the amount of Py DNA, whereas in the absence of FUdR the amount of virus DNA increases at least 150-fold during the same time period (19; H. Manor, unpublished data).

Cytoplasmic RNA extracted from the FUdR-treated cells and from the cells that had been harvested before the addition of FUdR to the growth medium were hybridized with the ^{32}P-labeled separated strands of the *Pst* I/*Bam* I fragments 1 and 5 derived from the early and late regions of the Py genome, respectively (Fig. 1). Figure 9 shows that, as expected from the results obtained in experiments with the *Eco*RI/*Hha* I fragments, the cytoplasm of the cells harvested 3.5 h after treatment with MMC contains a predominant species of RNA complementary to the fragment *Pst* I/*Bam* I E_1 and a smaller amount of RNA complementary to the fragment *Pst* I/*Bam* I E_5. A minor RNA species was found to be transcribed from the fragment *Pst* I/*Bam* I L_5, and no transcript of the fragment *Pst* I/*Bam* I L_2 could be detected.

In the cells that were further incubated in the presence of FUdR, there was no detectable increase in RNA complementary to the fragment *Pst* I/*Bam* I L_1. There was only a slight increase in RNA complementary to the fragment *Pst* I/*Bam* I E_1 and a two- to threefold increase in RNA complementary to the fragment *Pst* I/*Bam* I L_5. It should be noted that both fragments *Pst* I/*Bam* I L_1 and L_5 hybridized with Py cRNA to an extent of 75 to 80%, whereas the corresponding E fragments hybridized with the cRNA to an extent of less than 1.2%. These results show that the accumulation of transcripts of both the E and the L DNA strands is inhibited in MMC-treated cells if the onset of viral DNA replication is blocked.

DISCUSSION

The primary motivation for the experiments presented here was to determine whether alterations in the pattern of viral mRNA synthesis occur in LPT cells after MMC treatment but before the onset of viral DNA replication. We found that the viral RNA present in the cytoplasm of MMC-treated cultures that had not yet begun Py DNA replication was virtually indistinguishable in sequence composition and amount from that found in untreated cultures. In both cases, most of the cytoplasmic RNA was complementary to the E strand of the early region of Py DNA. After the onset of Py DNA replication in the MMC-treated cultures, the abundance of the early mRNA increased 20- to 25-fold, and transcripts of the L strand of the late region of Py DNA, similar to the mRNA found late during productive infection of permissive cells, appeared. We tentatively conclude from these observations that induction by Py in LPT cells is not determined at the level of mRNA synthesis. However, since the induction of viral DNA replication in LPT cells is asynchronous (19), it is possible that in individual cells a change in mRNA synthesis occurs immediately before DNA replication. This change could be undetectable when studying the entire cell population at 6.5 h after MMC treatment because only a few cells in the culture at this time are ready to begin Py DNA replication. We attempted to test this possibility by blocking Py DNA replication with FUdR and measuring

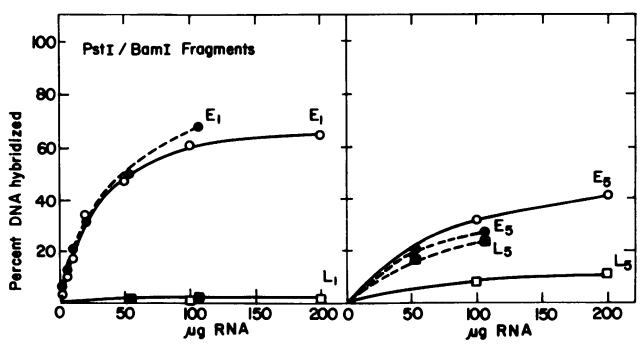

FIG. 9. *Effects of FUdR on Py-specific transcription in MMC-treated clone 1A cells. Clone 1A cultures were treated with MMC, as described in the text. Half of the plates were harvested after 3.5 h. FUdR (15 μg/ml) was then added to the rest of the plates, and these were further incubated for 21.5 h. Cytoplasmic RNA was extracted from each of these two groups of cultures and hybridized with the EcoRI/Pst I fragments L_1, L_5, E_1, and E_5 shown in Fig. 1. Hybridization was carried out, as described in the text, except that the temperature of incubation was 68 instead of 72°C. Symbols: ○, □, RNA from MMC-treated cells harvested after 3.5 h; ●, ■, RNA from MMC-treated cells exposed to FUdR from 3.5 until 24 h.*

the amount of cytoplasmic viral RNA at a time (24 h) after MMC treatment when most cells in an unblocked culture would have begun Py DNA replication. We still found that the Py-specific cytoplasmic RNA was the same as that present in uninduced cultures. Although any conclusion based on experiments using inhibitors such as FUdR must be considered tentative, we feel that it is unlikely that induction of Py DNA replication by MMC is preceded by changes in cytoplasmic mRNA.

A secondary motivation for the experiments presented was to characterize in some detail the viral RNA present in the nucleus and cytoplasm of uninduced and induced LPT clone 1A rat cells and to compare these results with previous analyses of Py RNA in productively infected or transformed mouse cells (1, 12). The study of the Py-specific RNA in the LPT line is complicated by the fact that both the normally growing and the MMC-treated LPT cultures contain uninduced transformants as well as induced cells in which the virus is replicating. Clearly, each of the hybridization assays presented in this paper yielded an average value reflecting the contributions of these two populations of cells. However, in the cultures harvested 24 h after MMC treatment, at which time about one-half of the cells have been induced to replicate Py DNA, most of the E and L DNA strand transcripts must be derived from induced cells, because blocking DNA replication in these cells by FUdR treatment prevented both the appearance of L DNA strand transcripts and the increase in the abundance of E DNA strand transcripts. In the normally growing cultures, 0.23% of the cells were previously found to be spontaneous virus producers (19). Spontaneous induction seems to be equivalent to MMC induction, because the spontaneously induced and the MMC-induced cells accumulate the same amount of viral DNA (about 24,000 genome equivalents per cell [19]) and produce similar yields of infectious virus (7; M. Fogel, unpublished data). We thus assume that the pattern of viral transcription is also similar in the spontaneously induced and the MMC-induced cells. Hence, the observation that the ratio of L DNA strand transcripts to E DNA strand transcripts in the cytoplasmic RNA from the normally growing cultures is considerably smaller than the ratio found in the cultures harvested 24 h after MMC treatment (compare, for example, the L_1 and the E_1 curves in Fig. 3 and 5) indicates that most of the viral RNA in the normally growing cultures is derived from the uninduced transformants. It should also be noted that the fraction of induced cells increases at least 130-fold 24 h after MMC treatment (19), whereas the amount of early RNA increases only 20- to 25-fold (Table 1). Therefore, if one assumes that a spontaneously induced cell contains the same amount of early RNA as does an MMC-induced cell, it follows that less than one-fifth of the early RNA in the normally growing cultures is found in the spontaneously induced cells. Similar arguments can be advanced to show that most of the viral RNA in the cultures harvested 6.5 h after MMC treatment is not derived from the spontaneously induced cells, whose proportion remains the same as in the normally growing cultures (19). Thus, unless the spontaneously induced cells contain at least five times as much early RNA as the MMC-induced cells and a considerably smaller ratio of L transcripts to E transcripts, the assays of the RNA prepared from normally growing cultures yield essentially the pattern of viral transcripts in the uninduced transformants.

We have shown that the predominant viral cytoplasmic RNA in normally growing clone 1A cells is complementary to the E DNA strand of virtually the entire early region of the Py genome. The mapping data obtained for this RNA are indistinguishable from those reported for the early mRNA found in productively infected mouse cells (12, 14). A transcript of the E strand of the entire early region was also found in a Py-transformed rat cell line that does not shed virus and in a 3T3 mouse cell line transformed by the *ts-a* mutant of polyoma virus (1, 12). The *ts-a* mutant has a temperature-sensitive lesion in the *A* gene whose product, presumably the T-antigen, is required for viral DNA replication. The *ts-a* 3T3 cell line yields infectious virus upon transfer to a low temperature (1). However, noninducible derivatives of the *ts-a* 3T3 cell line, which do not yield virus even at a low temperature, and other mouse cell lines transformed by a wild-type Py or by late Py mutants produce early RNA transcripts that lack about one-fourth of the sequences found in the early mRNA of productively infected cells. The missing portion is near the 3′ end of the molecules (1, 12). A plausible interpretation of these results is that the permissive Py-transformed mouse cells can survive only if virus growth is prevented either by the absence of complete early mRNA(s) or by thermal inactivation of the *A* gene product. Whether the remaining portion of the early mRNA encodes a viral gene product necessary for the maintenance of transformation remains an open issue. On the other hand, the semipermissive rat cell lines can survive even though these cells are transformed by a wild-type Py and produce all of the early mRNA sequences found in productively infected cells.

Our analysis of the nuclear RNA preparations has shown that the nuclei of normally growing clone 1A cells contain RNA molecules transcribed from fragments *Eco*RI/*Hha* I E_2, E_3, and E_4, as well as molecules complementary to at least 40% of the E_1 fragment. We also found that the abundance of the E_2 transcript is several times larger than the abundance of the E_3 and the E_4 transcripts. Such quantitative relationships would be expected if there were two, or possibly three, classes of molecules complementary to the E DNA strand. One or two of these classes would be complementary to fragments *Eco*RI/*Hha* I E_1 and E_2, and a less abundant class would contain sequences complementary to fragments *Eco*RI/*Hha* I E_2, E_3, and E_4. Clearly, only transcripts of the E strand of the entire early region reach the cytoplasm, and hence the most abundant RNA complementary to fragments E_1 and E_2 alone must be retained and/or degraded in the nuclei. The nuclei of normally growing cells contain, in addition, an abundant RNA species that is complementary to at least 45% of the L strand of the late region. This RNA species is apparently not exported to the cytoplasm.

Whereas concentrations of the E DNA strand transcripts found within the nuclei of normally growing cells are not significantly altered during the 6.5-h period after MMC induction, the concentration of the L_1 DNA transcript diminishes considerably. It is possible that this RNA species is transcribed from viral DNA that is integrated into chromosomal DNA and that the decrease observed in its concentration after the cells are treated with MMC is a consequence of excision of the virus DNA template from the chromosomal site of integration.

The nuclei of cells harvested 24 h after MMC treatment contain RNA transcribed from virtually the entire L DNA strand of the Py genome. However, the RNA complementary to the L DNA strand of the late region, corresponding to the cytoplasmic late mRNA, is four times more abundant than is the RNA complementary to the L DNA strand of early region fragments. Since the less abundant nuclear RNA is not exported to the cytoplasm, its reduced concentration is probably the result of selective degradation. A similar situation occurs in productively infected mouse cells (F. Birg, J. Favaloro, and R. Kamen, Proc. Natl. Acad. Sci. U.S.A., in press), but the ratio of true messenger to "anti-messenger" sequences in the nuclear RNA is somewhat lower. A complete analysis of E-strand transcripts found in the nuclei 24 h after MMC treatment was not feasible, because RNA-RNA self-annealing may have affected all of the hybridization reactions of these transcripts with the corresponding DNA fragments. Nevertheless, it is evident that the abundance of RNA complementary to at least a part of the E strand of the early region increases substantially after the onset of viral DNA replication (Fig. 8).

The MMC-induced clone 1A cells differ from productively infected mouse cells in that the transcripts of the E and L DNA strands are similar in abundance. By contrast, E DNA strand transcripts constitute less than 10% of the viral RNA present late during productive infection of mouse cells (12). One explanation of this difference is that the transcriptional DNA template strand selection ratio is determined by the rate of initiation of DNA replication per available template DNA molecule. In the semipermissive rat cells, viral DNA replication is far less efficient than it is in permissive mouse cells, and thus the induced LPT cells may synthesize relatively more early RNA. However, this explanation cannot account for the difference mentioned above between the LPT cells and the *ts-a*-transformed 3T3 mouse cells, namely, the fact that virus activation leads to a 20- to 25-fold increase in the abundance of early RNA in the LPT cells, but does not cause the appearance of additional E DNA strand transcripts in the *ts-a* 3T3 cells (1). Another explanation that could account for both differences is that the reported transcriptional control activity of the viral T-antigen (at least in simian virus 40-infected and -transformed cells [23, 24]), which results in repression of E-strand transcription, may be less effective in the induced rat cells.

ACKNOWLEDGMENTS

This work was supported by grants from the Leukemia Research Foundation and the U.S.A.-Israel Binational Science Foundation and by a short-term EMBO fellowship (to H.M.).

We thank Yaffa Bot for able technical assistance.

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