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Polyoma-induced stimulation of cellular RNA synthesis is paralleled by changed expression of the viral genome*

Consuelo Salomon, Hans Türler and Roger Weil

Department of Molecular Biology, University of Geneva, 1211 Geneva 4, Switzerland

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ABSTRACT

We studied synthesis of viral and cellular RNA in the presence and absence of 5-fluorodeoxyuridine (FdU, an inhibitor of DNA synthesis) during lytic infection with polyoma virus in confluent, primary mouse kidney cell cultures. In the presence of FdU, synthesis of early 19S polyoma mRNA and of polyoma tumor (T)-antigen, i.e. expression of the early viral gene, is rapidly followed by a mitogenic reaction of the host cell; it leads to an increase of $30^{\pm}5\%$ in cellular, mainly 28S and 18S rRNA, followed by activation of the cellular DNA-synthesizing apparatus. Polyoma-induced cellular RNA synthesis is paralleled by increased production of early 19S mRNA and begin of expression of the late viral genes, leading to synthesis of small amounts of late 19S and 16S mRNAs. Changed expression of the viral genome occurs in the absence of detectable synthesis of polyoma DNA I. Infection in the absence of FdU induces the same sequence of events; it is followed, however, by duplication of the mouse cell chromatin (S-phase) and production of progeny virus.

INTRODUCTION

Polyoma virus is a small oncogenic virus (1) that contains as genome a molecule of circular, double-stranded DNA with a molecular weight of about $3x10^6$ (2). In confluent, primary mouse kidney cell cultures it induces a lytic infection that leads in virtually all cells to the production of progeny virus (3). The lytic infection has been subdivided into an early and a late phase corresponding to the events occurring before and after the onset of cellular and viral DNA replication (4). Infection begins with the expression of the early viral gene, i.e. synthesis of early 19S polyoma mRNA (5,6) and polyoma-specific tumor (T)-antigen (7); this is followed by duplication of mouse chromatin (8,9), replication of viral DNA as a nucleohistone complex (10,11,12), synthesis of increasing amounts of late 16S and 19S mRNAs (5,6,13) and of

viral capsid proteins (14), assembly of progeny virus and cell death.

Studies on the transition from early to late viral gene expression were rendered difficult because of the high asynchrony of infection (14, and Fig. 1). To overcome this difficulty we used two methods: (i) infection in the presence of 5-fluorodeoxyuridine (FdU), an inhibitor of cellular and viral DNA replication (15) and,(ii) infection at 27°C which markedly slows down the time course of the lytic infection (16 and Fig.1).

In this paper we will show that appearance of T-antigen is rapidly followed by stimulation of cellular RNA synthesis, leading to a marked increase in total RNA, and that this is paralleled by changed expression of the viral genome.

MATERIALS AND METHODS

Preparation and infection of cell cultures. Primary mouse kidney cell cultures (3) were prepared as described earlier and plated in plastic petri dishes (10 cm diameter) (7). Unless indicated otherwise the cultures were infected 2 or 3 days after confluence with 0.4 ml of a suspension of plaque-purified polyoma virus containing about 10^9 pfu/ml. After adsorption of the virus (2 h at 37°C) the cultures were covered with 10 ml reinforced Eagle's medium (without serum), prewarmed to 37°C or 27°C respectively. For experiments taking place in the presence of FdU, 15 µg per ml of the inhibitor (provided by Dr. R. Duschinsky, Hoffmann-LaRoche) were present in the medium. In experiments at 27°C extending for more than 30 h, the culture medium ($\pm$ FdU) was replaced daily. Mock-infected cultures were treated in the same way as infected cultures.

Radioisotopic labeling and extraction of RNA. To obtain ^{3}H-labeled RNA, cultures were labeled with ^{3}H-uridine (New England Nuclear Corp., about 25 Ci/mmol, at 100-500 µCi/ml medium $\pm$ FdU) at the times and for the lengths indicated in results. RNA from total cultures was extracted by the hot (65°C) phenol procedure described earlier (17, 18). Cells were separated into a cytoplasmic and nuclear fraction using 1% Nonidet-P 40 (BDH Chemicals, Ltd)(13). Cytoplasmic RNA was extracted by phenol-chloroform-isoamylalcohol (50/50/1) at room temperature (13) and nuclear RNA by the hot phenol procedure.

RNA-DNA hybridizations. ^{3}H-labeled polyoma RNA was detected by hybridization to single-stranded polyoma DNA (18) fixed on membrane filters (0.2 µg DNA per 3.5 mm square filter) and present in excess. Unless indicated, hybridizations were done in 4xSSC (SSC is 0.15 M NaCl, 0.015 M Na-citrate pH 7.2) at 65°C for about 48 h (18) with 60 µg of RNA. Filters were washed and treated with RNase as described earlier (18). "Hybridizable RNA" is defined as RNase resistant radioactivity (cpm) present on 2 polyoma DNA containing filters minus cpm on 2 blank filters (no DNA) incubated in the same hybridization mixture. RNA extracted from mock-infected cultures gave an unspecific background hybridization in 4xSSC of about 0.001% of total acid-precipitable radioactivity in the hybridization mixture. We consider as polyoma RNA, "hybridizable RNA" after substraction of unspecific background hybridization (19). Hybridization of ^{3}H-labeled RNA to individual restriction enzyme fragments of polyoma DNA was done as described (6).

Other techniques. Cellular RNA and DNA were fractionated with a modified Schneider procedure (20) using duplicate cultures for each experimental point: cultures were covered with 5 ml 5% TCA and kept on ice for 20 min. TCA was removed and the cells were collected in 1 ml 5% TCA with a silicone rubber. The cells were pelleted by centrifugation at 1500xg for 15 min (4°C), washed twice with 5 ml cold 5% TCA and then resuspended in 2 ml 1 M KOH. After incubation for about 20 h at 37°C, 0.4 ml HCl (25%) and 0.25 ml 50% TCA were added and the tubes were kept on ice for 30 min. After centrifugation (as above) RNA was determined in the supernatant by the orcinol color reaction using d-ribose as standard (20). The pellets were washed once with 2 ml cold 10% $HClO_4$ and incubated in 2 ml 10% $HClO_4$ for 20 min at 70°C. After centrifugation (as above) DNA was determined in the supernatant by the diphenylamine color reaction using a calibrated calf thymus DNA solution as standard (21). Experimental variations of RNA and DNA determinations were $\leq$ 5%.

For assays of polyoma T-antigen by the immunofluorescence reaction (16) and autoradiography after pulse-labeling (1 h) with ^{3}H-thymidine (15) we used parallel cultures (grown in 10 cm diameter dishes) containing two glass cover slips per dish.

Sedimentation analyses of cellular and viral RNA in sucrose gradients were done as described previously (7, 13, 22).

RESULTS

1. Polyoma-induced stimulation of cellular RNA synthesis

For all experiments reported here we used primary mouse kidney cell cultures which consist mainly of epitheloid cells arrested in phase G_o of the mitotic cycle (7). In Fig. 1 we summarize previously published results on the lytic infection with polyoma virus

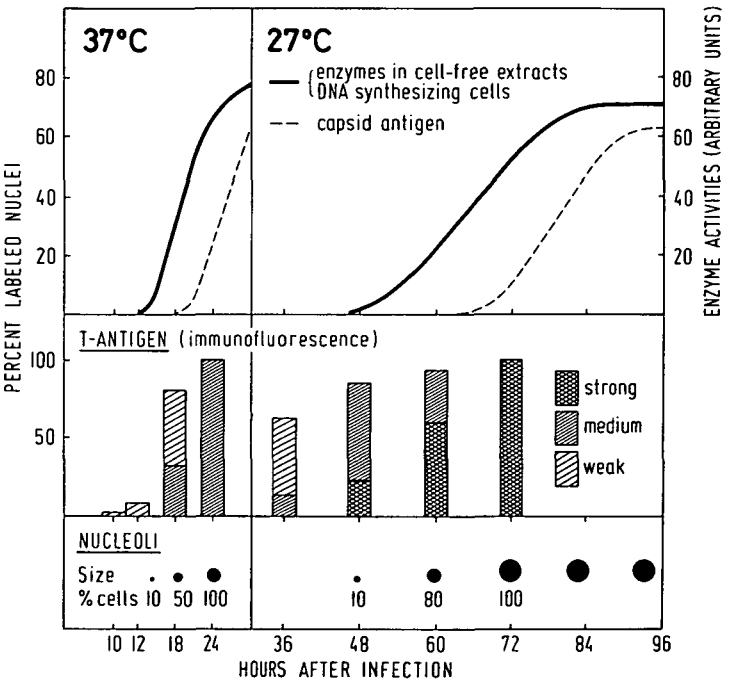


Fig. 1: Scheme of the time course of lytic infection at 37°C and at 27°C with polyoma virus in confluent, primary mouse kidney cell cultures (10^5 cells/cm^2). Cultures were infected one day after confluence under conditions described in Methods (ca 10^9 pfu/ml; 25-50 pfu/cell). In cultures infected three days (or longer) after confluence, the time course of appearance of T-antigen is virtually unchanged while increase in enzymatic activities and onset of DNA synthesis are delayed (for details see text and compare Fig. 4). Increase in infective titers to 10^{10} or 10^{11} pfu/ml does not detectably influence the time course of infection. The effects of lower input multiplicities have been described (24).

(7,16,23), as far as they are relevant to the present study. If infection takes place in the presence of FdU, synthesis of T-antigen and activation of the cellular DNA-synthesizing apparatus (i.e. of the enzymes involved in biosynthesis of pyrimidine deoxyribonucleotides and DNA) (23) take place as in normal infection, while no detectable amounts of viral DNA and little if any capsid proteins are synthesized (14,15,16).

At different times after infection total RNA was extracted with hot phenol from polyoma-infected and mock-infected cultures (± FdU) and quantitated by UV-spectrophotometry and the orcinol color reaction. Polyoma-infected cultures yielded by 24-30 h after infection (p.i.) at 37°C and around 70 h p.i. at 27°C about 30% more RNA than mock-infected cultures as determined in more than 10 independent experiments. The same results were obtained when RNA was extracted by the Schneider procedure which was more reproducible and yielded both for infected and mock-infected cultures 20-25% more RNA than hot phenol extraction; this was probably due to lower losses of RNA during extraction according to Schneider. Using the Schneider procedure the same increase in RNA content (30±5%) was observed in cultures infected at 37°C or 27°C in the presence or absence of FdU, in cultures infected in medium containing 10% calf serum, and also in cultures infected either 1,3 or 5 days after confluence. In some early experiments, instead of FdU, we used cytosine arabinoside (ara-C, 20 µg/ml) which inhibits DNA synthesis efficiently (> 98%) in uninfected, growing mouse kidney cells. If ara-C was added immediately after virus adsorption, nucleolar increase and polyoma-induced stimulation of cellular RNA synthesis occurred as in presence or absence of FdU, whereas polyoma-induced cellular and viral DNA synthesis was inhibited by only 80%, as judged from incorporation of ^{3}H-thymidine and autoradiography (unpublished results). For this reason we subsequently abandoned the use of ara-C.

The time course of the increase in total RNA in cultures infected in the presence of FdU at 37°C and 27°C is shown in Fig. 2. The increase becomes noticeable shortly after appearance of T-antigen and coincides in time with the increase in size of the

nucleoli (compare Fig. 1). As expected (8), neither at 27°C (Fig. 2) nor at 37°C (not shown) DNA content of the cultures increased.

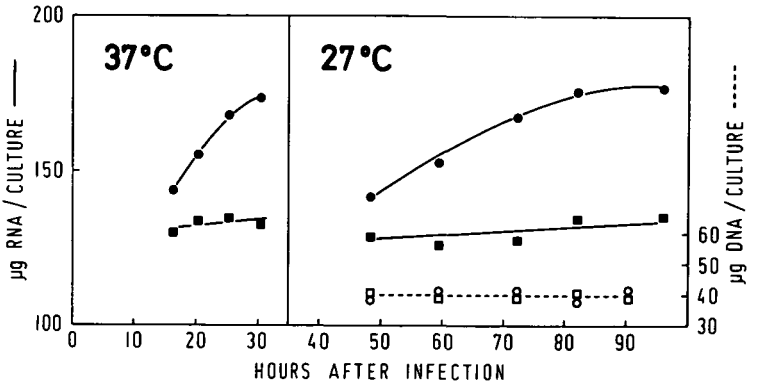


Fig. 2: Polyoma-induced stimulation of cellular RNA synthesis. Mouse kidney cell cultures were infected two days after confluence in the presence of FdU at 37°C or 27°C. At the times indicated infected (●) and mock-infected (■) cultures were fractionated by the Schneider procedure and RNA was quantitated by the orcinol color reaction. In the experiment done at 27°C DNA was determined by the diphenylamine color reaction (o infected; □ mock-infected).

In several experiments infected and mock-infected cultures (37°C, ± FdU) were pulse-labeled with ^{3}H-uridine at 24 h p.i. for various times between 10 min and 3 h. After the pulse RNA was extracted either from total cultures or from isolated cytoplasm. RNA determinations by UV absorbance or the orcinol color reaction showed that the increase in RNA in infected cultures was mainly due to increase in cytoplasmic RNAs. Aliquots of RNA from total cultures or from cytoplasm were sedimented through sucrose gradients. Under all experimental conditions tested the sedimentation profiles of UV absorption and radioactivity were closely similar for infected and mock-infected cultures, whereas the specific radioactivity of RNA extracted from infected cultures was 1.5-2 times higher throughout the gradient. Analogous results were obtained with cultures infected or mock-infected at 27°C and pulse-labeled around 70 h p.i. These results indicate that the net increase of 30% in total RNA is mainly due to increase in cytoplasmic 28S and 18S rRNA. Results obtained by oligo dT-cellulose

chromatography of nuclear and cytoplasmic RNA suggest that infection also stimulates synthesis of cellular "nuclear heterogeneous", RNA, mRNAs and 4-7S RNAs (25 and unpublished results).

2. Time course of synthesis of polyoma RNA

Cultures infected and mock-infected in the presence of FdU were pulse-labeled with ^{3}H-uridine; after extraction with hot phenol, total RNA was hybridized to polyoma DNA present in excess. Operationally, we assume that the percentage of hybridizable polyoma RNA reflects the rate of synthesis of polyoma RNA during the period of labeling. Two typical experiments done at 37°C and at 27°C are shown in Fig. 4. The results of all time course experiments can be summarized as follows. In cultures infected at 37°C (10 experiments) small amounts of polyoma RNA (0.002-0.003%) could first be detected by 6-7 h p.i., while by 12-13 h p.i. polyoma RNA accounted for 0.02-0.05%. Around 15-16 h p.i., coincident with increase in nucleolar size and stimulation of cellular RNA synthesis, the rate of synthesis of polyoma RNA rapidly increased; it tended to level off after 24 h p.i. when polyoma RNA accounted for 0.5-1.5% of newly synthesized, radioactive RNA. In cultures infected at 27°C (5 experiments), small amounts of polyoma RNA could first be detected by 17-20 h p.i. (0.002-0.005%). Thereafter, the rate of synthesis of polyoma RNA slowly increased. By 30-40 h p.i. polyoma RNA accounted for 0.01-0.05%. Between 40-50 h p.i. rate of synthesis of polyoma RNA increased, coinciding in time with the increase in nucleolar size and polyoma-induced cellular RNA synthesis. By 70-80 h p.i., i.e. at about the time when increase in cellular RNA had reached its maximum, the percentage of polyoma RNA tended to reach a plateau (0.3-0.5%). No evidence for synthesis of polyoma DNA I and of capsid proteins was obtained (see legend to Fig. 3 and ref. 14).

In some experiments parallel cultures were infected in the absence of FdU. The time course of synthesis of polyoma RNA was essentially the same until onset of viral DNA synthesis, i.e. until about 13 h p.i. at 37°C and 40-50 h p.i. at 27°C. In the experiment shown in Fig. 4 (27°C) rate of synthesis of polyoma RNA was by 48 and 60 h p.i. only about 2 fold higher than in

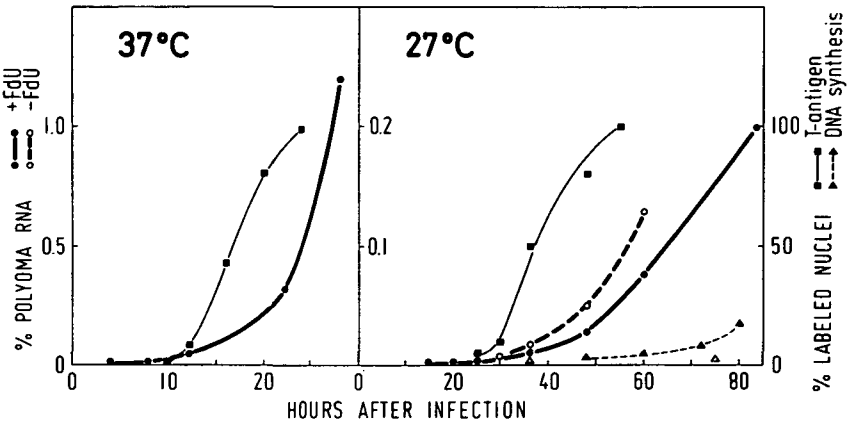


Fig. 3: Time course of synthesis of polyoma RNA at 27°C and at 37°C

At 27°C: Cultures were infected or mock-infected in the presence or absence of FdU 4 days after confluence. At the times indicated, T-antigen was determined (in cultures + FdU) by the immunofluorescence reaction; 2 infected and 2 mock-infected cultures (+ or -FdU) were labeled with ^{3}H-uridine (200 µCi/ml) for 1 h. RNA was extracted with hot phenol and 60 µg per assay were hybridized to polyoma DNA (4xSSC, 65°C). Parallel cultures (± FdU) were labeled with ^{3}H-thymidine (New England Nuclear Corp., 20 Ci/mmol, 5 µCi/ml) for 1 h and the number of DNA synthesizing cells was determined by autoradiography; the results ± FdU being closely similar, only those obtained in the absence of FdU are indicated: (▲) infected, (Δ) mock-infected. Increase in nucleolar size could be observed in about 10% of the cells around 50 h p.i., while by 84 h p.i. all cells contained markedly increased nucleoli. In cultures infected in the absence of FdU intranuclear polyoma capsid antigen could first be detected in about 0.01% of the cells by 48 h p.i.; by 60, 72 and 84 h p.i. the values increased to 0.6%, 2% and 4% respectively. No capsid antigen could be detected in cultures infected in the presence of FdU for 96 h. Synthesis of polyoma DNA I was monitored by T. Seebeck as follows: 2 infected and 2 mock-infected cultures (± FdU) were labeled with ^{3}H-cytidine (New England Nuclear Corp., about 25 Ci/mmol, 8 µCi/ml, ± FdU) from 36-48 h p.i. (sample A), 48-60 h p.i. (sample B) and 60-72 h p.i. (sample C). Viral DNA was selectively extracted (26) and subjected to equilibrium centrifugation in ethidium bromide-CsCl density gradients (27). In cultures infected without FdU a small but distinct band of polyoma DNA I was observed in sample A(650 cpm) which markedly increased in sample B (4900 cpm) and C (15500 cpm). In extracts from mock-infected cultures (± FdU) and from cultures infected in the presence of FdU no band corresponding to radioactive polyoma DNA I was found.

At 37°C: Cultures were infected or mock-infected in the presence of FdU 2 days after confluence. Cultures were labeled with ^{3}H-uridine (250 µCi/ml, + FdU) for 30 min and assayed for polyoma RNA and for T-antigen as described above. For details on the time course of lytic infection with polyoma virus in the presence of FdU see ref. 15.

parallel cultures infected in the presence of FdU; however, rate of synthesis of polyoma DNA I increased about 7 fold between 48 and 60 h p.i. (for details see figure legend). As determined by autoradiography, a significant increase in the number of DNA synthesizing cells could only be detected after 60 h p.i. Synthesis of small amounts of polyoma DNA I and the presence of a very small fraction of cells (about 0.1%) containing intranuclear capsid protein by 48 h p.i. indicate that polyoma-induced DNA synthesis started in a few cells considerably earlier, though it could not be detected by autoradiography above the background of DNA synthesizing cells (1-2%). These observations make it appear unlikely that the marked increase in synthesis of polyoma RNA observed around 40-50 h p.i. in cultures infected at 27°C in the presence of FdU was due to incomplete inhibition of synthesis of polyoma DNA I.

Synthesis of early polyoma RNA and of T-antigen do not detectably depend on the age of the cultures at the time of infection. In contrast, onset of virus-induced cellular RNA synthesis and, apparently as a consequence, polyoma-induced DNA synthesis are delayed if the cultures are infected later than 2-3 days after confluence. This is particularly striking during infection at 27°C where this delay amounts to 10-12 h (16 and unpublished observations).

3. Stability of newly synthesized polyoma RNA before and after onset of virus-induced cellular RNA synthesis.

Cultures infected in the presence of FdU were labeled with ^{3}H-uridine for various lengths of time before and after onset of virus-induced cellular RNA synthesis. Total RNA was extracted with hot phenol, specific radioactivity (cpm/µg) determined and polyoma RNA assayed by hybridization to polyoma DNA. The results of typical experiments done at 27°C and at 37°C are listed in Table 1.

Before induction of cellular RNA synthesis, around 30 h p.i. at 27°C, polyoma RNA and specific radioactivity of total RNA increased linearly with length of labeling, resulting in an unchanged

Table 1: Stability of newly synthesized polyoma RNA before and after onset of virus-induced cellular RNA synthesis [1)]

temperature	labeling with ^{3}H-uridine	specific activity of total RNA (cpm/µg)	total RNA hybridized (µg)	hybridized polyoma RNA cpm	hybridized polyoma RNA %
27°C	30 h p.i.:				
	1.5 h	9040	60	112	0.021
	5 h	36120	60	428	0.020
	65 h p.i.:				
	1 h	14520	60	1701	0.2
	3 h	39880	60	4324	0.18
	8 h	57470	60	6268	0.11
37°C	24 h p.i.:				
	15 min	1226	55	1238	1.9
	30 min	2985	55	2234	1.3
	60 min	6214	50	3058	0.9

1) FdU was present throughout infection. Cultures were labeled at 27°C with 100µCi/ml, at 37°C with 50 µCi/ml. Hybridization was done in 4xSSC at 65°C with polyoma DNA present in excess.

percentage of hybridizable polyoma RNA. The same results were obtained with cultures infected in the absence of FdU (not shown). During infection at 37°C rate of synthesis of polyoma RNA rapidly increases after 7 h p.i. Labeling with ^{3}H-uridine for different lengths of time between 7 and 12 h p.i. showed that newly synthesized polyoma RNA was stable and accumulated during the pulses (not shown). In contrast, polyoma RNA synthesized after induction of cellular RNA synthesis (later than 50 h p.i. at 27°C and 15 h p.i. at 37°C) apparently turns over rather rapidly (see Table 1). This is particularly striking at 37°C (+FdU) where the turnover is comparable to that of polyoma RNA synthesized late in normal lytic infection (18 and unpublished results). In both instances it is mainly due to rapid intranuclear turnover of newly

synthesized polydisperse, "giant" polyoma RNA (unpublished observations).

4. Synthesis of early polyoma RNA seems not to require de novo synthesis of proteins

Cycloheximide (25 µg/ml) decreased in polyoma- and mock-infected cultures (37°C) protein synthesis by 80-90% within 30 min, as determined by incorporation of radioactive amino acids into acid-precipitable material. Under the same conditions incorporation of ^{3}H-uridine was reduced by about 20% after 3-4 h and by 60-70% after 8-9 h. In several experiments cycloheximide was added to infected cultures (37°C, +FdU) either immediately after virus adsorption, shortly before or at the time of onset of polyoma RNA synthesis. Total RNA was extracted with hot phenol and hybridized to polyoma DNA.

Table 2: Synthesis of early polyoma RNA in the presence of cycloheximide (37°C, + FdU)

experiment	cycloheximide (25 µg/ml) present h p.i.	^{3}H-uridine labeling (250µCi/ml) h p.i.	specific activity of total RNA (cpm/µg)	total RNA hybridized (µg) 1)	%hybridized polyoma RNA 2)
(i)	none	8 - 11	84600	60	0.009
	7 - 11	8 - 11	66800	60	0.008
(ii)	none	7 - 10	77500	60	0.014
	2 - 10	7 - 10	21400	60	0.014
(iii)	8 - 10.5	8 - 10.5	85000	60	0.035
	8 - 13	10.5- 13	44600	15	0.089

1) Hybridization was done in 4xSSC at 65°C;
2) the results were the same in the absence of FdU.

The results in Table 2 show that the percentage of polyoma RNA remained essentially unchanged if protein synthesis was inhibited (experiments i and ii) and that in the presence of cycloheximide the percentage of polyoma RNA showed the expected increase between

10 and 13 h p.i. (experiment iii; compare time course experiments reported above). In sucrose gradients polyoma RNA synthesized in the presence or absence of cycloheximide exhibited essentially the same sedimentation patterns (not shown). However, in infected and mock-infected cultures exposed to cycloheximide for 30 min or longer, then labeled with ^{3}H-uridine, little if any radioactive 18S rRNA was found. A similar effect of cycloheximide was observed in lymphocytes (28).

5. Molecular properties of polyoma RNA synthesized before onset of virus-induced cellular RNA synthesis

(i) Polyoma RNA from total cultures. In 10 independent experiments polyoma-infected cultures (+FdU) were labeled with ^{3}H-uridine for 1,2,3 or 4 h between 7 and 14 h p.i. at 37°C, or for 1,3 and 5 h at different times between 25 and 35 h p.i. at 27°C. RNA was extracted with hot phenol and sedimented through sucrose gradients. Each fraction of the gradients was hybridized to polyoma DNA: in all experiments a fraction of polyoma RNA sedimented as a relatively uniform band with a sedimentation coefficient of 19S, while the remainder sedimented faster and exhibited a polydisperse sedimentation pattern (24 and unpublished results). With increasing labeling times the proportion of 19S polyoma RNA increased. Sedimentation in denaturing DMSO-chloralhydrate gradients did not remove polyoma RNA sedimenting with sedimentation coefficients >19S, while 19S polyoma RNA sedimented at 18S with respect to 18S mouse rRNA and 16S *E. coli* rRNA (24).

(ii) Nuclear and cytoplasmic RNA. Polyoma-infected cultures (+FdU) were labeled as described above. Polyoma RNA extracted from isolated nuclei exhibited in sucrose gradients sedimentation patterns similar to those of polyoma RNA from total cultures. However, in different experiments the relative amounts of intranuclear polydisperse and 19S polyoma RNA varied somewhat. Although the nuclei seemed not to be contaminated with cytoplasm (as judged from microscopic observation and from the presence of only small amounts of 18S rRNA in nuclear RNA preparations), we can not exclude that presence of 19S polyoma RNA was partially due to contamination with

perinuclear cytoplasm (unpublished results).

In most experiments the bulk of cytoplasmic polyoma RNA sedimented as a rather uniform band with a sedimentation coefficient of 19S (7,22); it is polyadenylated (22), polyribosome-associated (E. Buetti, unpublished results), early 19S mRNA corresponding to the transcript of the early gene (effector gene) of polyoma DNA (5,6). If labeling with ^{3}H-uridine was extended to about 14 h p.i. (37°C, +FdU), a small, second band of polyoma RNA appeared (7,22). We will show below that it is late 16S mRNA synthesized after onset of polyoma-induced cellular RNA synthesis.

The sedimentation patterns of nuclear and cytoplasmic polyoma RNA synthesized before onset of virus-induced cellular RNA synthesis (37°C or 27°C) in the absence of FdU were indistinguishable.

6. All three species of polyoma mRNAs are synthesized in the presence of FdU after onset of virus-induced cellular RNA synthesis

Results reported earlier showed (i) that polyoma RNA synthesized in the presence of FdU after onset of virus-induced cellular RNA synthesis and extracted either from total cultures or from isolated nuclei exhibited in sucrose gradients a polydisperse sedimentation pattern (24,25) similar to that of polyoma RNA synthesized late in normal lytic infection (13,18) and, (ii) that the bulk of cytoplasmic polyoma RNA was present in two bands with sedimentation coefficients of 19S and 16S (7,25). During infection at 37°C (+FdU) small amounts of 16S polyoma RNA could first be detected in the cytoplasm around 14 h p.i., while by 20 h p.i. or later (tested until 30 h p.i.) equal amounts of 19S and 16S polyoma RNA were present (7). In cultures infected at 27°C small amounts of 16S polyoma RNA became first detectable around 50-60 h p.i.; however, at all times tested (until 96 h p.i.) the amounts of 16S polyoma RNA remained always below those present in the 19S band (unpublished results). By 25-30 h p.i. at 37°C (+FdU) and around 70 h p.i. at 27°C (+FdU) polyoma RNA accounted for 0.1-0.2% of newly synthesized, radioactive cytoplasmic RNA.

An endonuclease of Hemophilus parainfluenzae (HpaII) cleaves polyoma DNA into 8 fragments (30). Hybridization of the two size

classes (19S and 16S) of cytoplasmic polyoma RNA synthesized early and late in lytic infection (13,22) to individual fragments of polyoma DNA revealed three species of polyoma mRNA (5,6): early 19S mRNA which is transcribed from the early strand, late 19S mRNA and late 16S mRNA, both transcribed from the late strand, whereby the sequence of late 16S mRNA is comprised within late 19S mRNA (5,6,29).

Fragments 2 and 4 of the early region represent almost the entire stretch from which early 19S mRNA is transcribed (5,6). Fragments 1 and 3 constitute the late region from which late 19S and 16S mRNAs are transcribed (5,6).

Polyoma mRNAs synthesized in the presence of FdU after onset of virus-induced cellular RNA synthesis were characterized by the same approach used earlier to map polyoma mRNAs synthesized in normal lytic infection (6). Cultures infected with polyoma virus (37°C, +FdU) were labeled with ^{3}H-uridine from 27-30 h p.i.

Cytoplasmic RNA was extracted and sedimented through sucrose gradients. Aliquots of the fractions containing polyoma RNA were hybridized to Hpa II fragments 1,2,3,4, which had been denatured and fixed on membrane filters. Fig. 4 A shows polyoma RNA hybridizing to the early region (i.e. sum of RNase resistant cpm bound to fragments 2 and 4) and to the late region respectively (sum of cpm bound to fragments 1 and 3). In individual experiments 30-40% of 19S polyoma RNA hybridized to fragments 2 + 4, while the remainder hybridized to fragments 1 + 3. 19S polyoma RNA thus contained both early and late 19S mRNA. About 80% of 16S polyoma RNA hybridized to fragments 1 and 3, indicating that it is mainly late 16S mRNA while the remainder hybridized to fragment 2 and 4; the presence of early RNA sequences is probably the result of overlap with the band of early 19S mRNA and of contamination with fragments of (metabolized) early mRNA. Fig. 4 B shows RNase resistant cpm bound to individual Hpa II fragments 1 or 3 respectively and Fig. 4 C the cpm bound to fragments 2 or 4. The hybridization profiles are thus similar to those obtained with polyoma RNA synthesized late in normal lytic infection (6)

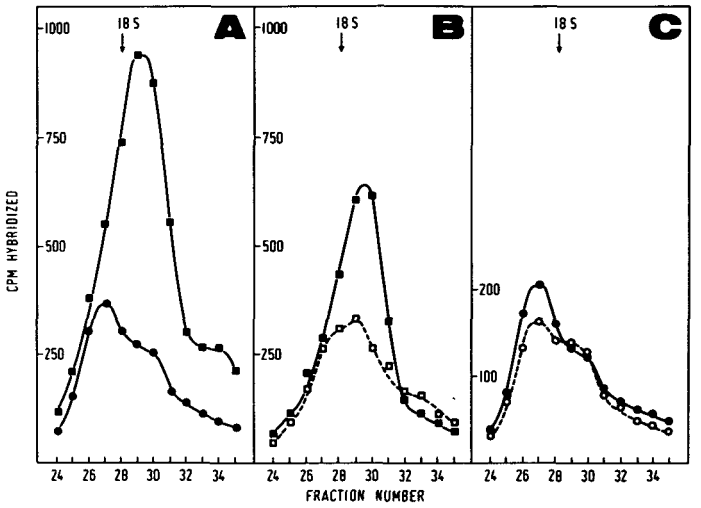


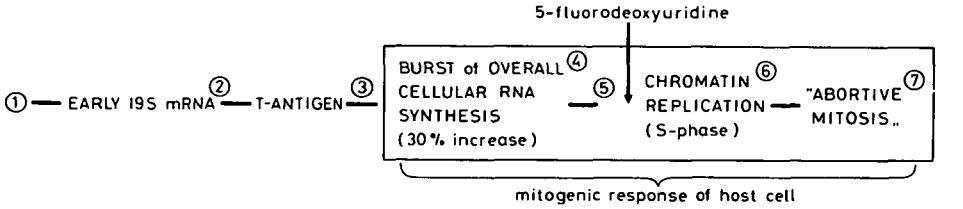
Fig. 4: Hybridization to individual Hpa II DNA fragments of polyoma RNA synthesized in the presence of FdU after onset of virus-induced cellular RNA synthesis.

Polyoma-infected cultures (37°C, +FdU) were labeled with ^{3}H-uridine (200 µCi/ml, +FdU) from 27-30 h p.i. Cytoplasmic RNA was sedimented through 3 parallel sucrose gradients: 120 µg RNA per gradient (23100 cpm/µg) in 100 µl Tris-HCl, pH 7.2 were layered on a 10-25% (w/w) linear sucrose gradient and centrifuged in a Spinco SW56 rotor at 50000 rpm for 2.5 h at 10°C. To detect polyoma RNA in the gradients, aliquots of each fraction were first hybridized to total polyoma DNA. Since the sedimentation pattern of 19S and 16S polyoma RNA in the three gradients was virtually identical, the corresponding fractions of the gradients containing polyoma RNA were pooled. Aliquots of the pooled fractions were then hybridized in 4xSSC at 65°C to filters containing either Hpa II fragment 1,2,3 or 4, DNA being present in excess. Thereafter the filters were treated with RNase.
A: hybridization to the late region, i.e. sum of cpm hybridized to fragments 1 and 3 (■) and to the early region, i.e. sum of cpm hybridized to fragments 2 and 4 (●).
B: cpm bound to fragment 1 (■) and to fragment 3 (□)
C: cpm bound to fragment 2 (●) and to fragment 4 (o).

although the proportion of early polyoma RNA sequences is higher both in 19S and 16S polyoma RNA.

DISCUSSION

Viral particles can be detected in the nucleus of mouse kidney cells by 15 min after addition of the virus. Viral DNA is rapidly released from the particles and intranuclear parental polyoma DNA



1 intact virus penetrates into nucleus where viral DNA is released

2 synthesized throughout infection

3 lag depends on physiological state of the cells

4 increase in nucleolar size; increased synthesis of early 19S mRNA and begin of synthesis of small amounts of late 19S and 16S mRNAs; integration of polyoma DNA.

5 activation of cellular DNA-synthesizing apparatus

6 polyoma DNA replicates as nucleohistone; synthesis of increased amounts of late 19S and 16S mRNAs followed by synthesis of capsid proteins, assembly of progeny virus and cell death

7 "abortive mitosis": no prophase, but apparently (pre-)mitotic cell surface alterations.

Fig. 5: Lytic infection with polyoma virus in mouse kidney cells arrested in phase G_0 of the mitotic cycle
For details and references see text

continuously increases until about 10 h p.i. (31 and R. Consigli, personal communication).

In the experiments reported here synthesis of early polyoma RNA could first be detected by 6-7 h at 37°C and by 17-20 h p.i. at 27°C. In all experiments a fraction of newly synthesized, intranuclear early polyoma RNA exhibited in sucrose and in denaturing dimethyl-sulfoxide-chloralhydrate density gradients a polydisperse sedimentation pattern and sedimentation coefficients equal to or higher than that of a complete transcript of polyoma DNA (26 S) (24). Kamen et al. showed that intranuclear early polyoma RNA synthesized before onset of viral DNA synthesis contains sequences complementary to most or all sequences present in the early strand of polyoma DNA (29). It therefore

appears likely that intranuclear, rapidly sedimenting, polydisperse polyoma RNA molecules are the result of at least one round of transcription of the early strand. In contrast, Beard et al. detected intranuclear polyoma RNA sequences complementary to only 40-45% of the early strand of polyoma DNA (32). The reason for this discrepancy remains unknown. Results from pulse-chase experiments with ^{3}H-uridine ($\pm$ Actinomycin D) suggest that at least a fraction of newly synthesized, polydisperse intranuclear polyoma RNA is processed to rather stable early 19S mRNA (unpublished results). However, direct proof for such a precursor-product relationship remains to be obtained. Synthesis of early 19S polyoma mRNA seems not to require de novo synthesis of proteins, a conclusion also reached with respect to synthesis of early 19S viral mRNA in abortive infection with SV40 (7; E. and P. May, personal communication). Taking into account the results on integration of polyoma DNA into mouse chromosomal DNA during lytic infection, it appears very likely that expression of the early viral gene does not require integration of parental viral DNA and that synthesis of early 19S mRNA and of T-antigen is carried out by host cell enzymes present before infection (Türler, in preparation).

Synthesis of early 19S polyoma mRNA is followed within a few hours by appearance of intranuclear polyoma T-antigen, detectable by the immunofluorescence reaction. The close temporal and quantitative relation between synthesis of early polyoma and SV40 mRNAs, appearance of virus-specific T-antigens and the subsequent events of lytic and abortive infection led to the hypothesis that polyoma and SV40 T-antigens are virus coded pleiotropic regulator proteins which act, among other, as mitogens (7,33). Recently it was shown that SV40 T-antigen is actually a large polypeptide (86000 (34) or 100000 (35) Daltons) specified by the early gene of SV40 (36).

In those cells where polyoma T-antigen has reached a threshold concentration, a rather sudden darkening of the nucleoli and an increase in nucleolar size can be observed (16) which seems to be the morphological expression of polyoma-induced stimulation of

cellular RNA synthesis; the latter is the earliest presently known event of the virus-induced mitogenic reaction (Fig. 5) and corresponds to (or comprises) the "psychrosensitive event" described earlier (16): infection in the presence or absence of FdU leads to a net increase of 30±5% in total RNA by 24-30 h at 37°C and around 70 h p.i. at 27°C. Although the increase in total RNA is mainly due to cytoplasmic 28S and 18S rRNAs, virus-induced stimulation seems also to comprise cellular mRNAs and 4-7 S RNAs. Stimulation of cellular RNA synthesis is paralleled by increased synthesis of early 19S polyoma mRNA and by onset of synthesis of late 19S and 16S viral mRNAs, albeit at rather low levels; these changes precede detectable onset of polyoma-induced (cellular and viral) DNA synthesis and also take place if the latter is inhibited by FdU. From these and earlier observations we conclude that in the presence of FdU synthesis of early 19S polyoma mRNA and of T-antigen (16), stimulation of cellular RNA synthesis, changed expression of the viral genome and activation of the cellular DNA-synthesizing apparatus (15) occur just as they do in parallel cultures infected in the absence of the inhibitor (Fig. 5).

Comparative studies of the lytic infection with SV40 in confluent, primary monkey kidney cell cultures revealed a sequence of events closely similar to that depicted in Fig. 5 (manuscript in preparation). However, in accordance with the results reported by others (37), no late 19S and 16S SV40 mRNAs could be detected before onset of SV40-induced DNA synthesis or if the latter was inhibited with ara-C.

The mechanism responsible for the change in expression of the polyoma viral genome after onset of polyoma-induced stimulation of cellular RNA synthesis remains unknown. We presently consider the following possibilities which do not exclude each other: a) T-antigen-induced activation of the cellular RNA-synthesizing system may provide conditions that lead to increased transcription of the early strand of parental polyoma DNA and allow transcription of the late strand, resulting in synthesis of small amounts of late 19S and 16S polyoma mRNAs. These changes might be the result of virus-induced modification(s) of a DNA-dependent

host cell RNA polymerase(s); b) before onset of polyoma-induced DNA synthesis, parental polyoma DNA may undergo structural changes that render it more efficient as template for transcription of the early strand and allow transcription of the late strand; c) before detectable replication of polyoma DNA I, small amounts of viral DNA may be synthesized in a form which could not be detected by the methods used in the present work (e.g. rolling circle, viral DNA attached to cell chromatin) but which serves as template for synthesis of early and late mRNAs.

The results now available show that polyoma virus induces in confluent, primary mouse kidney cell cultures a highly reproducible lytic infection which triggers in virtually all cells a mitogenic response analogous to that induced in abortive ("transforming") infection both by polyoma virus and SV40 (7,38). This cell-virus system is therefore particularly useful to study the molecular basis of the mitogenic action of oncogenic DNA viruses (39) and to investigate the host factors that render mouse cells permissive for polyoma virus.

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