JOURNAL OF VIROLOGY, May 1982, p. 748–754
0022-538X/82/050748-07$02.00/0

Vol. 42, No. 2

# Synthesis of Human Adenovirus Early RNA Species Is Similar in Productive and Abortive Infections of Monkey and Human Cells

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Received 2 November 1981/Accepted 21 January 1982

Northern (RNA) blot analysis has been used to show that synthesis of early mRNA species is similar in monkey cells productively or abortively infected with human adenovirus. mRNA species from all five major early regions (1A, 1B, 2, 3, 4) are identical in size and comparable in abundance whether isolated from monkey cells infected with adenovirus type 2 or with the host range mutant Ad2*hr*400 or coinfected with adenovirus type 2 plus simian virus 40. The mRNA species isolated from monkey cells are identical in size to those isolated from human cells. Production of virus-associated RNA is also identical in productive and abortive infections of monkey cells. Synthesis of virus-associated RNA is, however, significantly greater in HeLa cells than in CV1 cells at late times after infection regardless of which virus is used in the infection.

Human adenovirus is a nuclear replicating virus with a linear double-stranded DNA genome encoding approximately 30 proteins. The virus replicates efficiently on cells derived from its normal human host, but grows poorly (~1,000-fold reduction of virus yield) in monkey cells. The block to replication of human adenovirus in monkey cells can be overcome by coinfection with the simian virus 40 (SV40) (31) or infection with adenovirus type 2 (Ad2)-SV40 hybrid viruses (e.g., Ad2$^+$ND1), which contain only a segment of the SV40 genome encoding the carboxyl terminus of T antigen (19, 25, 26). In addition, mutants of adenovirus have been isolated (Ad2*hr*400 to 403 and Ad5*hr*404) which have alterations in the gene encoding the 72,000-dalton DNA-binding protein (DBP) that enable the virus to replicate efficiently in monkey cells (22, 24).

The block to replication of human adenovirus in monkey cells has been assumed to be a late event since the synthesis of several late viral proteins (especially fiber) is severely reduced (4, 14, 16, 23), even though viral DNA synthesis appears to be normal in abortive infections (14, 18, 32). An alternate explanation for this phenomenon, however, is that an early gene product(s) required for proper expression of late genes (but not necessarily DNA synthesis) may not be present in sufficient quantity to allow efficient synthesis of late proteins. The production of such an early gene product could be controlled by the DBP. This would explain how a mutation in the gene encoding DBP (e.g., Ad2*hr*400) can relieve the block to replication of adenovirus in monkey cells. Temperature-sensitive lesions in the DBP of Ad5 (e.g., Ad5*ts*125) have already been shown to enhance the expression of several early genes at the nonpermissive temperature (2a, 7–9, 29a), and it is not unreasonable to expect that other alterations in the DBP (e.g., Ad2*hr*400) might also affect the synthesis of early gene products. The carboxyl-terminal end of SV40 T antigen could provide an alternate control function in monkey cells infected with adenovirus plus SV40 or nondefective adenovirus-SV40 hybrid viruses.

To investigate the possibility that a reduction in the amount of some early gene product might be responsible for the block to replication of adenovirus in monkey cells, a systematic examination of early mRNAs synthesized in productive and abortive infections was undertaken. In these experiments, cytoplasmic polyadenylated RNA was prepared from productively or abortively infected monolayer cultures of HeLa (human) cells or CV1 cells (an established line of African green monkey kidney cells). Equal amounts of RNA from each infection were electrophoresed in parallel tracks on a denaturing methyl mercury agarose slab gel (3), blotted onto activated diazobenzyloxymethyl paper (2), and hybridized with cloned probes specific for each of the major early regions of adenovirus (1A, 1B, 2, 3, and 4).

Figure 1 shows the results of an experiment in which RNA was isolated at 20 h postinfection (hpi) from Ad2-, Ad2*hr*400-, or Ad2-plus-SV40-infected CV1 cells treated with cytosine arabinoside (araC) after adsorption. AraC inhibits viral

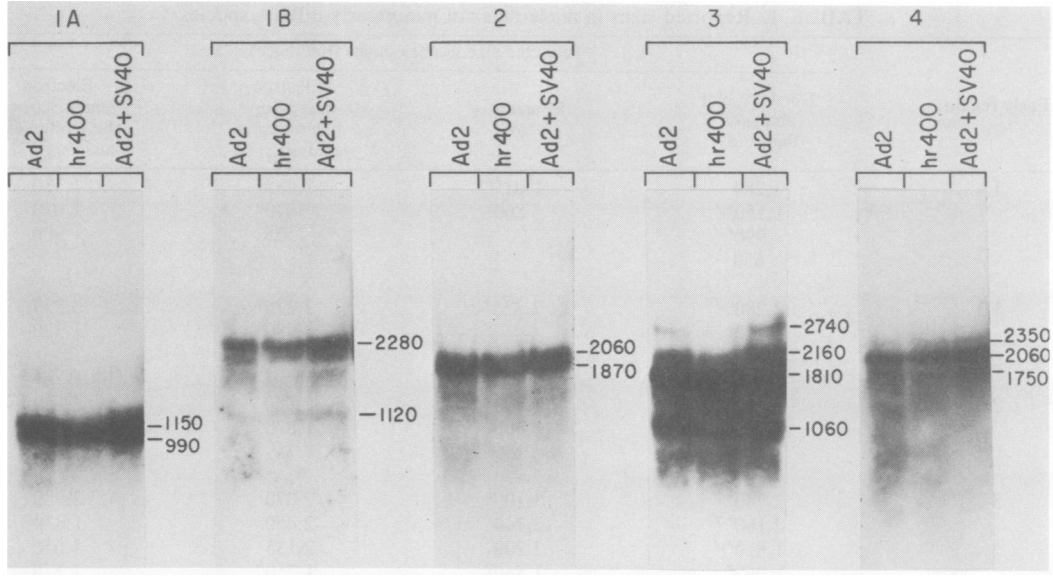


FIG. 1. Northern blot analysis of early mRNA species isolated from CV1 cells productively or abortively infected in the presence of araC. Cytoplasmic RNA was isolated at 20 hpi from infected CV1 cells which were treated with 25 μg of araC per ml after adsorption. Polyadenylated RNA, selected by oligodeoxyribosylthymine cellulose chromatography from 100 μg of cytoplasmic RNA, was fractionated on 1.2% agarose gels containing 10 mM methylmercury hydroxide (3), transferred to diazobenzyloxymethyl paper (2), and hybridized with nick-translated pBR322-cloned fragments specific for each major early region (1A, 0.8 to 4.5%; 1B, 6.0 to 7.7%; 2, 58.5 to 70.7%; 3, 75.9 to 84.0%; and 4, 89.5 to 97.1%). The brackets at the top of each panel indicate the hybridization probe, and the lettering above each track denotes the infecting virus. The approximate size in nucleotides of major mRNA species based on migration of single-stranded DNA restriction fragments in the same gel is indicated by the numbers to the right of each panel. The apparent mRNA species just below 2,280 nucleotides in early region 1B are caused by tailing of the 2,280 species and saturation of the diazobenzyloxymethyl paper in this area by contaminating 18S rRNA.

DNA synthesis and maintains infected cells at the early stage of infection, thereby allowing the accumulation of early mRNA (5, 6, 10, 36). mRNA species complementary to each of the major early regions were identical in size and comparable in abundance for each of the three infections. In addition, the sizes of the major messages corresponded well with the published sizes of mRNA species transcribed from these regions in HeLa cells (6, 10, 20, 21). Table 1 lists the sizes of the major mRNA species in nucleotides and compares these to previously published sizes of early mRNA species in HeLa cells.

The results of a similar experiment are shown in Fig. 2. The same protocol was followed in this experiment except that mRNA was isolated from both HeLa and CV1 cells at several times after infection in the absence of any drug treatment. Using this protocol, we could monitor the steady-state levels of individual early mRNA species throughout the infectious cycle of the virus. At the same time, this protocol eliminated the possibility of any artifacts caused by the drug treatment. The results of this experiment are summarized below.

At early and intermediate times after infection (8 and 15 hpi), the mRNA species complementary to each early region were identical in size and comparable in abundance regardless of the virus used for infection. The major bands observed in RNA isolated at these times after infection corresponded with the major mRNA species observed in cells infected in the presence of araC. The early mRNA species isolated from infected CV1 cells comigrated with those isolated from infected HeLa cells, but slightly elevated steady-state levels of mRNA species were observed in monkey cells compared with human cells. With the possible exception of early region 4 RNA, early mRNA species isolated at 15 hpi were more abundant than those isolated at 8 hpi.

By 28 hpi (well into the late phase of the lytic cycle), the pattern of mRNA species complementary to most early regions varied somewhat from the pattern seen at earlier times in infection. However, the altered pattern observed for

TABLE 1. Reported sizes in nucleotides of major early mRNA species

| Early region | Reported size in nucleotides (reference) | | | |
|---|---|---|---|---|
| | Northern blot analysis[a] (this study) | S1 analysis[b] (6) | Electron microscope heteroduplex analysis[c] (10) | Electron microscope heteroduplex analysis[d] (21) |
| 1A | 1,280<br>1,150[e,f]<br>990[e,f]<br>650 | 1,035[g]<br>860[g] | 1,085[h]<br>840[h]<br>525 | 1,180<br>1,070<br>900 |
| 1B | 2,280[e,f]<br>1,120[e] | 2,335[g] | 2,275[h]<br>980[h]<br>455 | 2,290<br>1,320 |
| 2 | 2,060[e]<br>1,980[e] | 1,900[g]<br>1,800[g] | 1,960<br>1,820[h] | 2,180<br>1,890<br>1,800[g] |
| 3 | 2,740[e]<br>2,160[e,f]<br>1,810[e,f]<br>1,060[e] | 3,100<br>2,740<br>2,200<br>1,840[g] | 2,870<br>2,450<br>2,135<br>1,750[h]<br>1,715[h]<br>980[h] | 2,500<br>1,820[g]<br>1,650<br>1,570<br>1,130<br>1,040<br>990 |
| 4 | 2,350[e,f]<br>2,060[e,f]<br>1,750[e,f] | 2,330<br>1,930[g]<br>1,630 | 2,100<br>1,960[h]<br>1,575<br>1,260<br>875<br>560 | 1,940<br>1,210 |

[a] Only mRNA species observed at or before 15 hpi or in the presence of araC are listed here. In most cases these sizes are 100 to 200 nucleotides larger than previously reported sizes due to the presence of polyadenylate tails which were not included in size determinations using S1 or electron microscope analysis.
[b] RNA isolated at 8 hpi in the presence of araC.
[c] RNA isolated at various times after infection without drug treatment and in the presence of cycloheximide or araC.
[d] RNA isolated at 5.5 hpi in the absence of any drug treatment.
[e] Abundant mRNA seen at 20 hpi in the presence of araC.
[f] Abundant RNA at 8 hpi.
[g] Reported as an abundant mRNA by the authors.
[h] Major early mRNA as deduced from reported frequencies of occurrence.

each early region was the same in both productive and abortive infections. An example of this type of early-to-late switch can be seen for mRNA complementary to early region 1A. The relative concentrations of the two major early messages (1,150 and 990 nucleotides) as well as the less abundant (1,280-nucleotide) message are reduced at late times, whereas a new message 650 nucleotides in length was observed in all infections at 28 hpi. These changes, as well as changes in other early regions, are consistent with previously published reports from other labs on early adenovirus mRNA in human cells (10, 12, 33, 35).

The pattern alterations observed for early regions 2, 3, and 4 were somewhat less definitive than those observed for early regions 1A and 1B since the probes used for these early regions also contain sequences which are complementary to the major late transcription unit of adenovirus. For all three of these early regions the pattern of bands observed remained identical in productive and abortive infections, but the amount of mRNA from Ad2*hr*400-infected CV1 cells hybridizing to probes for early regions 2, 3, and 4 appears to be greater than that from other infections (Ad2 infection of CV1 or HeLa cells or Ad2*hr*400-infected HeLa cells). This result was confirmed in a separate experiment (not shown here) in which 28-hpi mRNA, complementary to the same early region probes, was found to be more abundant in Ad2*hr*400- or Ad2-plus-SV40-

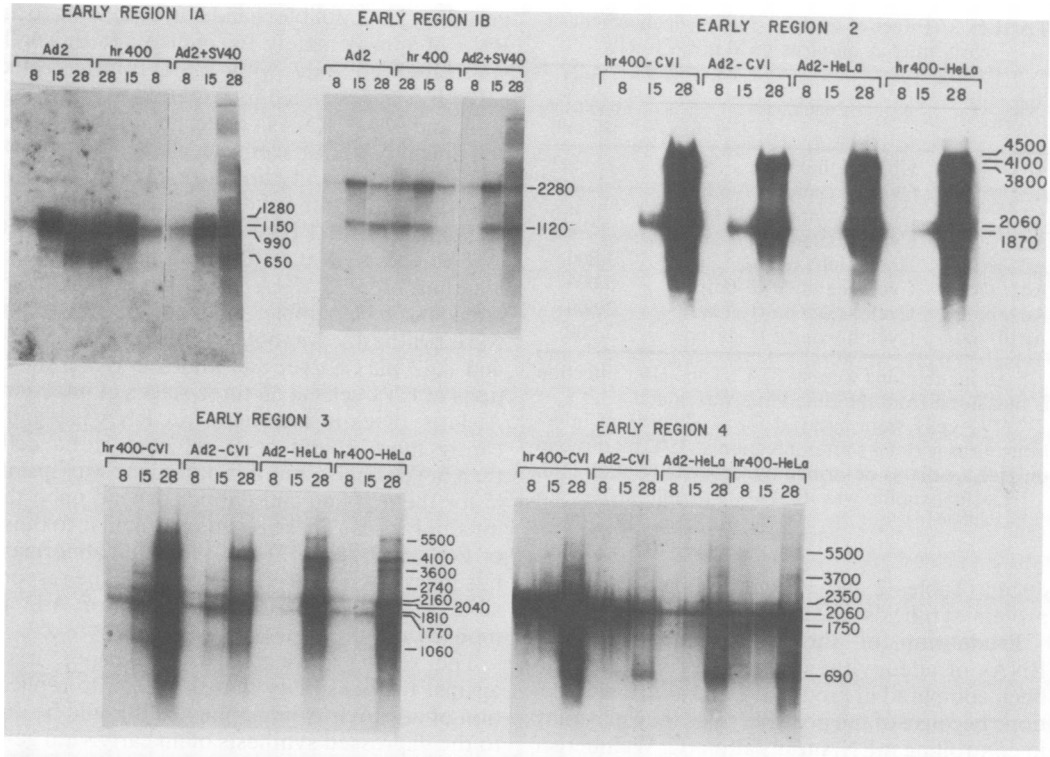


FIG. 2. Northern blot analysis of early mRNA species at different stages of infection. Experimental details are as in Fig. 1, except that RNA was isolated from cells at several times after infection in the absence of any drug treatment. This figure represents a compilation of data from two experiments. In the first experiment, RNA from Ad2-, Ad2*hr*400-, or Ad2-plus-SV40-infected CV1 cells was isolated at 8, 15, and 28 hpi. In the second experiment, RNA was isolated at the same times after infection from both HeLa and CV1 cells infected with either Ad2 or Ad2*hr*400. In each experiment, all five early regions were examined, and in both cases the results were the same. However, only the best autoradiograph from each early region is shown here. For early regions 1A and 1B, RNA was isolated from CV1 cells, and the brackets above the panels indicate the infecting virus. RNA used for early regions 2, 3, and 4 was isolated from both infected HeLa and CV1 cells; the brackets indicate the infecting virus and cell line used. The numbers above each track indicate the hours after infection that the RNA was isolated. The numbers at the right of each panel indicate the approximate size in nucleotides of mRNA species.

infected CV1 cells than in CV1 cells infected with Ad2 alone. Neither experiment showed any variation between infections in the amount of 28-hpi mRNA hybridizing to probes for early regions 1A and 1B. Perhaps this phenomenon reflects the increased viral mRNA levels present in productively versus abortively infected monkey cells at this time after infection (11, 23).

Some experiments (not shown here), in which only 8-hpi mRNA was examined, showed a significantly increased level of early mRNA in Ad2*hr*400-infected cells. The difference in abundance was variable (2- to 3-fold to greater than 10-fold) and appeared to differ from experiment to experiment. This difference was the same for all early regions in a given experiment and occurred in both CV1 and HeLa cells. It is considered unlikely that this variable increase in abundance of early mRNA in Ad2*hr*400-infected cells could be responsible for alleviating the block to replication of adenovirus in CV1 cells since no increase in abundance of early mRNA was ever observed in cells coinfected with Ad2 and SV40. In addition, no enhancement of infectious virus yield was observed in cells which were manipulated to increase the steady-state concentrations of early mRNA. In these experiments, Ad2-infected CV1 cells were treated with cycloheximide to inhibit protein synthesis, or Ad5*ts*125-infected CV1 cells were incubated at the nonpermissive temperature after infection. After being treated for various lengths of time, the cells were returned to permissive growth conditions (cycloheximide removed or cells incubated at the permissive temperature). The artificial accumulation of early mRNA with these protocols (2a, 8, 9, 17, 36) resulted in no change in the amount of infectious virus pro-

TABLE 2. Effect of cycloheximide enhancement of early mRNA on virus yield in CV1 cells

| Infecting virus | Conditions[a] | PFU produced per cell[b] |
|---|---|---|
| Ad2 | No cycloheximide | 9.3 |
| Ad2 | Cycloheximide[c] (1–6 hpi) | 6.6 |
| Ad2 | Cycloheximide (1–9 hpi) | 4.6 |
| Ad2 | Cycloheximide (1–12 hpi) | 5.4 |
| Ad2*hr*400 | No cycloheximide | 3,800 |
| Ad2*hr*400 | Cycloheximide (1–6 hpi) | 5,000 |
| Ad2*hr*400 | Cycloheximide (1–9 hpi) | 2,600 |
| Ad2*hr*400 | Cycloheximide (1–12 hpi) | 3,200 |

[a] All incubations were at 37°C. Time after infection is measured from the time virus was added.

[b] CV1 cells were assayed for production of infectious virus at 3 days after infection at 37°C by titration on HeLa cells as described by Grodzicker et al. (16).

[c] Cycloheximide was used at 25 μg/ml.

duced (Tables 2 and 3) or in the pattern of late protein synthesis (data not shown).

Production of the virus-associated (VA) RNAs of adenovirus (VAI and VAII) has also been compared in productive and abortive infections because of the possible roles they may play in controlling mRNA translation (C. Weinberger and T. Shenk, personal communication) or processing (28) or both. These small (~160 nucleotides), uncapped, and non-polyadenylated RNAs have been sequenced and mapped on the adenovirus genome (1, 27, 29, 30), but their roles in the expression of viral genes is largely speculative. Cytoplasmic RNA was extracted from cells immediately after labeling with [$^{32}$P]orthophosphate at three different intervals after infection and fractionated on denaturing acrylamide gels (Fig. 3). A doublet band corresponding to an RNA of approximately 160 nucleotides in length was observed only with RNA from infected cells. It was presumed to represent a mixture of VAI and VAII RNAs, since no other infected cell-specific RNAs were seen even after prolonged exposure of the autoradiograph. RNA extracted from this band hybridized specifically to *Bam*HI-*Hin*dIII restriction fragments at 17.0 to 29.0 and at 29.0 to 31.5, consistent with the location of VA RNA encoding genomic sequences. Comparable amounts of VA RNA were synthesized in both productive (Ad2*hr*400 and Ad2 plus SV40) and abortive (Ad2) infections of CV1 cells at all three stages of infection. At later times in infection, VA RNA is significantly more abundant in infected HeLa cells than in CV1 cells, but this difference was specific for the cell line and did not depend upon the infecting virus. These results are similar to those of Fox and Baum (13) and show that abnormalities in production of VA RNA cannot be responsible for the block to replication of Ad2 in monkey cells.

The results presented in this paper argue against the possibility that the block to replication of adenovirus in monkey cells could be due to the depressed synthesis of an early viral gene product necessary for proper expression of late genes. Early mRNA species complementary to each of the five major early regions of adenovirus, as well as the VA RNA species, were identical in size and comparable in abundance whether isolated from productively or abortively infected monkey cells. Furthermore, shifts in expression of certain early mRNA species during the replication cycle of the virus were the same in productive and abortive infections, pro-

TABLE 3. Effect of temperature shift on virus yield in CV1 cells

| Infecting virus | Conditions[a] | Infectious virus produced per CV1 cell[b] | |
|---|---|---|---|
| | | Expt 1 | Expt 2 |
| Ad5 | 39.5°C | 93 | 62 |
| Ad5 | 39.5°C, shift to 32.5°C at 24 hpi | 5.2 | |
| Ad5 | 39.5°C, shift to 32.5°C at 13 hpi | 2.1 | 0.9 |
| Ad5 | 32.5°C | 1.5 | 0.5 |
| Ad5*hr*404 | 39.5°C | 3,500 | 2,300 |
| Ad5*hr*404 | 39.5°C, shift to 32.5°C at 24 hpi | 2,700 | |
| Ad5*hr*404 | 39.5°C, shift to 32.5°C at 13 hpi | 127 | 293[c] |
| Ad5*hr*404 | 32.5°C | 197 | 61 |
| Ad5*ts*125 | 39.5°C | 0.2 | 0.1 |
| Ad5*ts*125 | 39.5°C, shift to 32.5°C at 24 hpi | 1.1 | |
| Ad5*ts*125 | 39.5°C, shift to 32.5°C at 13 hpi | 1.1 | 1.0 |
| Ad5*ts*125 | 32.5°C | 1.6 | 1.2 |

[a] Time after infection is measured from the time virus was added. Adsorption was at 37°C for 1 h.

[b] Progeny virus were harvested after 4 or 5 days of incubation at 39.5°C and 32.5°C, respectively, and then titrated on HeLa cells.

[c] Ad5*hr*404 is cold sensitive and grows very slowly at 32.5°C (D. F. Klessig, unpublished data).

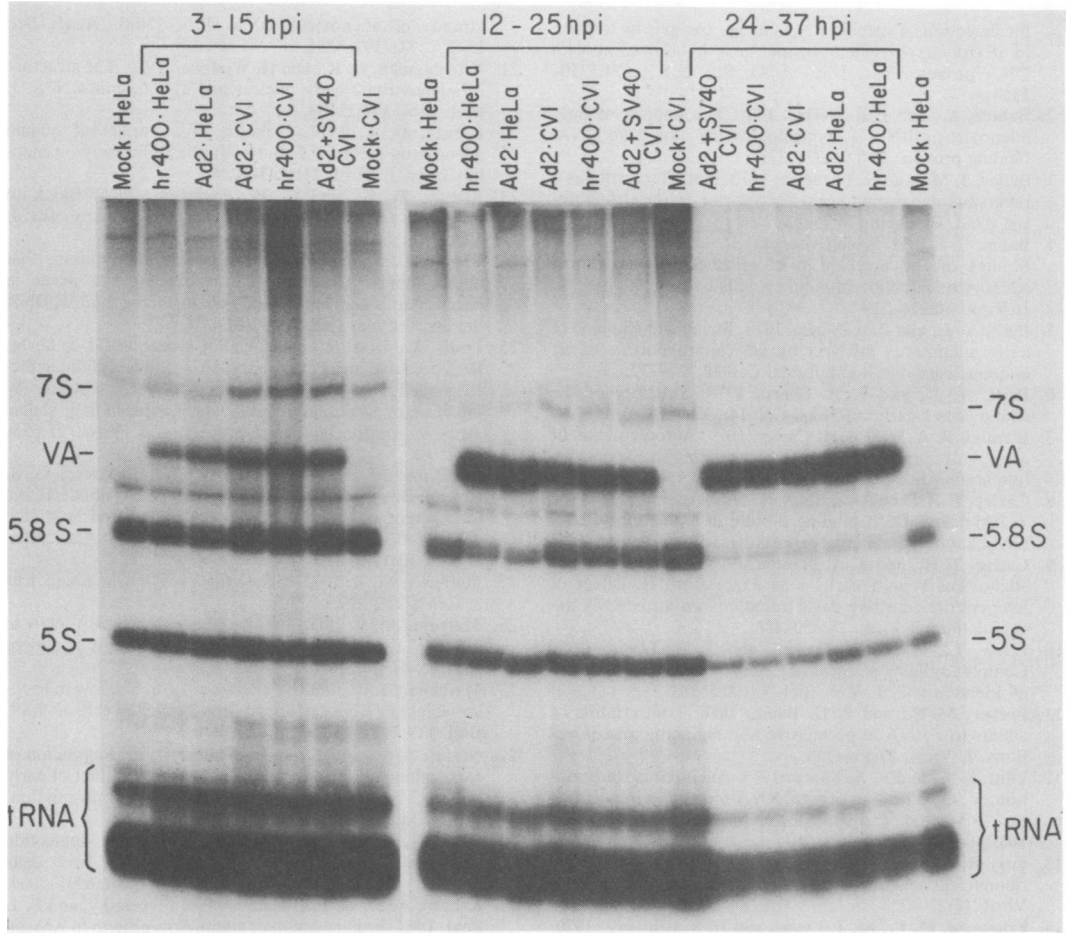


FIG. 3. Synthesis of VA RNA in productive and abortive infections. Monolayer cultures of infected cells were labeled with [$^{32}$P]orthophosphate (100 μCi/ml in phosphate-free media plus 2% dialyzed calf serum) at different intervals after infection. Total cytoplasmic RNA was extracted from the cells, and 20-μg portions were analyzed on 12% polyacrylamide (acrylamide:bis = 75:1) slab gels (14 by 18 by 0.15 cm) containing 7 M urea in 90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3. The top 2 mm of the gels was removed before autoradiography. The numbers at the top of the panel indicate the time interval after infection in which the cells were labeled. The lettering above each track indicates the virus and the cell line used in each infection. The position of VA RNA as well as other cellular RNA components as discussed by Mathews and Pettersson (29) is indicated at the side of each panel.

viding additional evidence that early mRNA metabolism is normal in abortive infections of monkey cells.

The data presented here are not unequivocal, however, since expression of early genes as mRNA is not conclusive evidence that synthesis of polypeptide products is normal. Also, only the expression of the major early regions of Ad2 has been examined. Minor messages, such as those originating from the recently elucidated early region 2B (15, 34), or expression of other regions of the Ad2 genome early in infection has not been examined. However, in the absence of any evidence that early gene expression is altered in abortive infections of monkey cells, it is now clear that efforts must be focused on determining why the expression of certain late genes is depressed in abortive infections and how control of their expression is altered in different cellular environments.

This work was supported by American Cancer Society grant no. MV-93. K.P.A. was supported by American Cancer Society postdoctoral fellowship no. PF-1901.

We would like to thank V. Cleghon for excellent technical assistance.

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