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Control of Adenovirus Early Gene Expression: Accumulation of Viral mRNA After Infection of Transformed Cells

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We studied the accumulation of viral mRNA in the presence of inhibitors of protein synthesis in an adenovirus type 5-transformed cell line (line 293 cells). An analysis of the endogenous viral mRNA's and proteins revealed that only early regions 1A and 1B were expressed in uninfected 293 cells. However, viral mRNA's from early regions 2, 3, and 4, as well as mRNA's from early regions 1A and 1B, accumulated in 293 cells after infection with adenovirus type 2. Cells treated with anisomycin before infection showed a drastic enhancement of mRNA from early region 4 compared with drug-free controls. This increase in viral mRNA was detected by using filter hybridization, S1 endonuclease mapping, and in vitro translation. The rate of transcription of early region 4 nuclear RNA also increased significantly in the presence of anisomycin. In contrast to the results with early region 4, the levels of cytoplasmic mRNA's from early regions 2 and 3 did not increase in cells treated with inhibitors. These results suggested that multiple virus encoded controls operate on the early regions of the adenovirus genome.

Early after adenovirus infection, before the onset of DNA replication, cytoplasmic RNA is derived from five regions of the viral genome (3, 24). Since the early proteins encoded in these regions are expressed at different rates and at different times after infection, it has been suggested that there is a temporal regulation of expression from the viral genome (23, 26). Nevins et al. (19) have shown that the time course for the appearance of transcripts is different for each of the five genomic regions.

Studies with adenovirus host range and deletion mutants have demonstrated that a major element of genetic control lies within early region 1A (2, 14). In addition, the accumulation of cytoplasmic mRNA species encoded by early regions 1A and 1B appears to be controlled by complex regulatory patterns (29). Recently, the control of adenovirus gene expression in infected HeLa cells was examined by using inhibitors of protein synthesis from the start of infection (16, 22). The results with the mutants and the inhibitors led to the hypothesis that viral gene products encoded by early region 1 may control the accumulation of viral mRNA since decreased levels of virus-specific mRNA were observed in cells treated with inhibitors before virus infection (22).

Early region 1 is also of interest since it contains the DNA sequences necessary for transformation (9, 30). Graham et al. (12) have described a human embryonic kidney cell line that is transformed by sheared fragments of adenovirus type 5 (Ad5) DNA. These cells, designated 293 cells, contain the left 12% and right 9% of the viral genome (1). The transformed cells express both RNAs and viral proteins from early regions 1A and 1B. Accordingly, infection of 293 cells with mutants defective in early region 1A results in a productive infection (2, 14).

In this study, we analyzed the gene expression of wild-type Ad2 in 293 cells to determine the effects of early region 1A and 1B products on viral RNA accumulation. Using inhibitors of protein synthesis, we found that early region 1 gene products regulate both transcription and accumulation of early viral mRNA from region E4. Our results suggested that the different early regions of the viral genome may be under separate controls.

MATERIALS AND METHODS

Cells and virus. The Ad5-transformed human embryonic kidney cell line 293 was grown in monolayers in Eagle minimal essential medium containing 10% fetal calf serum (12). Early-passage, untransformed human embryonic kidney cells were purchased from Flow Laboratories and were propagated in the same way as the 293 cells. Wild-type Ad2 and Ad5 were purified from infected HeLa cells as described previously (24).

Labeling conditions and virus infection. Uninfected 293 cells were labeled with [^{35}S]methionine (10 μCi/ml) for 6 h in medium containing 1/40 the normal concentration of methionine. After the cells were labeled for 3 h with [^{3}H]uridine (50 μCi/ml), cytoplasmic RNA was prepared from uninfected 293 cells by the

method of Brawermann et al. (5). Both 293 cells and untransformed human embryonic kidney cells were infected with purified Ad2 at a multiplicity of 100 PFU/cell. Cytoplasmic RNA was prepared from infected cells that were labeled from 2 to 5 h postinfection with [^{3}H]uridine (50 μCi/ml). Nuclear RNA was extracted by the method of Smith et al. (27) from infected 293 cells labeled for 5 min with [^{3}H]uridine (500 μCi/ml) at 5 h postinfection.

Immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and in vitro translation. The procedures used for immunoprecipitation and translation in a rabbit reticulocyte cell-free system have been described previously (23). Virus-specific RNA was selected on Ad2 DNA or purified restriction enzyme fragments (24) bound to nitrocellulose filters, as described by McGrogan et al. (18). Eluted RNA was translated in the mRNA-dependent reticulocyte cell-free system. Samples were analyzed on 13% sodium dodecyl sulfate–polyacrylamide slab gels as described previously (21, 23). The gels were analyzed by fluorography (4).

S1 endonuclease mapping of mRNA. We used the method originally described by Berk and Sharp (3) and modified by Persson et al. (22). Briefly, 20 μg of cytoplasmic RNA was hybridized in a solution containing a high concentration of formamide (6) to 1 μg of Ad2 DNA. Each sample was treated with S1 endonuclease (225 U) and separated on a 2.0% alkaline agarose gel. The gels were blotted onto nitrocellulose sheets (28) and hybridized to ^{32}P-labeled nick-translated probes (25).

Filter hybridization. Equal amounts of RNA from the different samples were hybridized for 16 h at 65°C to nitrocellulose filters containing *Eco*RI fragments of Ad2 derived from 10 μg of viral DNA. After hybridization, the filters were washed extensively and then treated with RNase A (20 μg/ml). The amounts of hybridized RNA were measured with a Beckman liquid scintillation counter.

RESULTS

Viral mRNA and proteins expressed in uninfected 293 cells. Each human embryonic kidney cell line (293 cells) transformed with sheared Ad5 DNA contains four to five copies of the region which includes the leftmost 12% of the Ad5 genome and one copy of the region which includes the rightmost 9% of the viral genome (1). We analyzed the viral proteins synthesized in this cell line by immunoprecipitation from [^{35}S]methionine-labeled cells with different anti-T sera. The anti-T sera used were prepared against either virus-transformed rat cells or virus-transformed hamster cells. These antisera have been shown to react with a variety of early proteins encoded in early regions 1A and 1B (11, 15). Although we observed nonspecific immunoprecipitation, all of the antisera which we used immunoprecipitated a 58,000-dalton protein and a set of proteins with molecular weights of approximately 42,000 to 50,000. A 15,000-dalton protein was also immunoprecipitated by two of the anti-T sera (Fig. 1A). Separation of the 293 cells into a cytoplasmic fraction and a nuclear fraction showed that the 15,000-dalton protein predominated in the cytoplasm, whereas the 58,000-dalton protein was mostly confined to the nuclear fraction. The 42,000- to 50,000-dalton proteins were equally distributed between the cytoplasm and the nuclear fraction. None of these proteins was immunoprecipitated from [^{35}S]methionine-labeled untransformed human embryonic kidney cells (data not shown).

The viral mRNA present in 293 cells was analyzed by using hybridization selection of 293 cell cytoplasmic RNA to Ad5 DNA, followed by in vitro translation. The selected RNA directed the synthesis in vitro of the same set of proteins (molecular weights, 58,000, 42,000 to 50,000, and 15,000) that were observed after labeling in vivo. In some experiments a 17,000-dalton protein was also synthesized from selected viral mRNA (Fig. 1B). The cytoplasmic RNA from 293 cells was also selected by hybridization to DNA fragments specific for early regions 1A or 1B. The early region 1A selected viral mRNA synthesized four proteins (molecular weights, 50,000, 48,000, 44,000, and 42,000), whereas two proteins (molecular weights, 58,000 and 15,000) were synthesized when early region mRNA was translated in vitro (Fig. 1B). These proteins corresponded in size and map location to the early proteins previously identified in lytically infected HeLa cells from early regions 1A and 1B of Ad5 DNA (7, 17). Figure 1B also shows that no functional viral mRNA from early region 4 of the viral genome was detected, indicating that the viral DNA from the rightmost end of the genome is not transcribed into mature mRNA. A more sensitive assay for the presence of early region 4 mRNA, which involved hybridization of labeled viral RNA to a specific DNA probe, confirmed that early region 4 was not expressed in 293 cells. Expression of early region 4 mRNA in uninfected 293 cells was also not detected in the presence of the protein synthesis inhibitor anisomycin (see below).

Infection of 293 cells with Ad2. The experiments described above showed that human embryonic kidney cell line 293 transformed by Ad5 DNA only expressed functional viral mRNA and proteins from early regions 1A and 1B. In addition, these gene products appeared to be identical to the products synthesized during lytic infection with Ad5 in human cells (7). Studies with host range mutants have suggested that early region 1A of the viral genome plays an important role in the accumulation of functional viral mRNA from other early regions (2, 14). The addition of anisomycin, a stringent inhibitor of

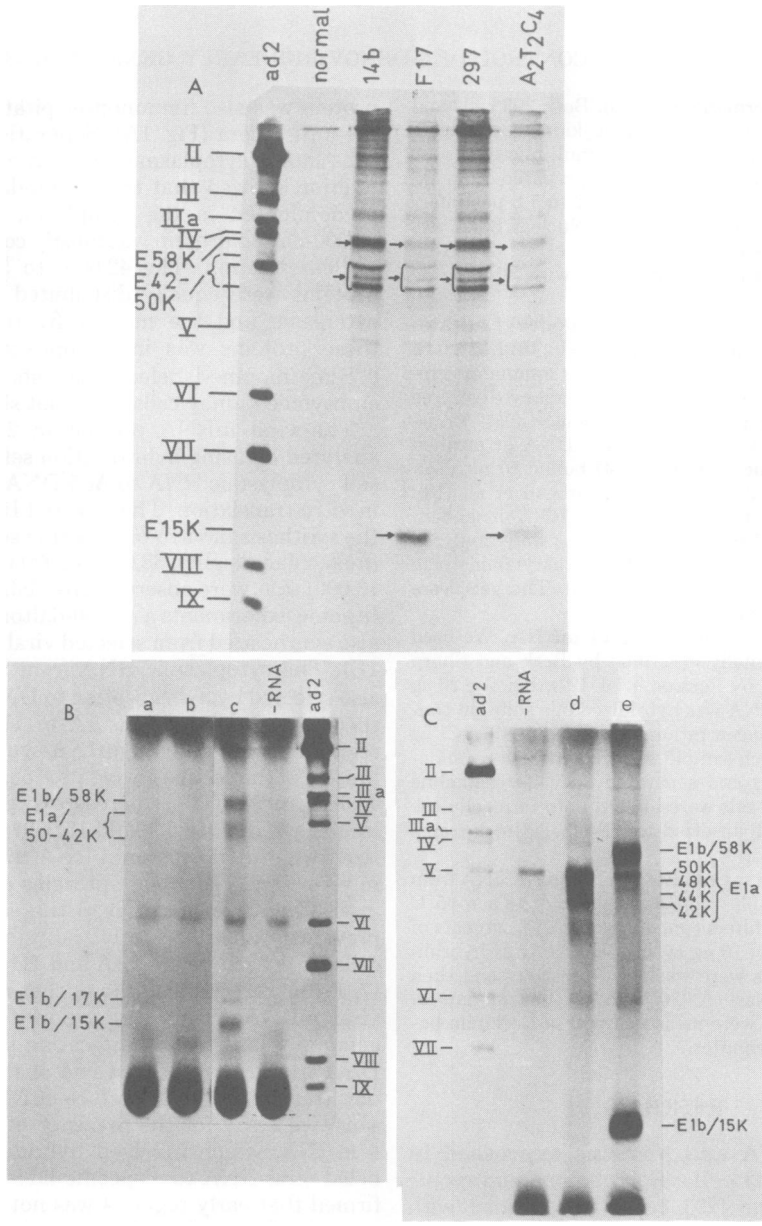

FIG. 1. *(A) Immunoprecipitation of [^{35}S]methionine-labeled 293 cell extracts with different anti-T sera. Monolayers of 293 cells were labeled with [^{35}S]methionine for 6 h, and cell extracts were prepared by disrupting the cells in RIPA buffer (0.02 M potassium phosphate, pH 7.5, 0.1 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride). The antisera used for immunoprecipitation are indicated at the top, as follows: normal, preimmune serum from hamsters; 14b, serum from hamsters bearing tumors induced by the Ad5-transformed cell line 14b; F17, serum from rats injected with the Ad2-transformed rat cell line F17; 297, serum from hamsters bearing tumors induced by the Ad5-transformed cell line 297-C43; $A_2T_2C_4$, serum from rats injected with the Ad2-transformed rat cell line $A_2T_2C_4$. Cell line 14b cells contain the left 40% of the Ad5 genome and transcribe early region 1 (8). The 297-C43 cell line contains sequences from 0 to 23 map units, as well as sequences from 32 to 52 map units (31). The F17 cell line contains only the left 14% of the Ad2 genome, whereas the $A_2T_2C_4$ cell line contains sequences from all early viral regions (9). The immunoprecipitates were analyzed on a 13% sodium dodecyl sulfate–polyacrylamide gel, and the gel was analyzed by fluorography. In this and subsequent figures, Ad2 denotes a [^{35}S]methionine-labeled Ad2 marker virus. (B and C) Cytoplasmic RNA was prepared from 293 cells and selected by hybridization to filters containing Ad5 DNA or restriction enzyme fragments of Ad2 DNA. The selected RNA was translated in vitro, and the translational products were analyzed on 13% sodium dodecyl sulfate–polyacrylamide gels. The DNA probes used for hybridization selection were as follows: lanes a and b, EcoRI fragment B of Ad5 DNA (84.0 to 100.0 map units); lane c, total viral DNA from Ad5. The RNA used for hybridization selection in lane b was prepared from 293 cells maintained in the presence of anisomycin (100 μM) for 5 h. Lane-RNA, no RNA added to the cell-free system, lane d, SmaI fragment J of Ad2 DNA (0 to 2.8 map units); lane e, HindIII fragment C of Ad2 DNA (7.9 to 17.0 map units).*

protein synthesis, before viral infection in HeLa cells depresses the accumulation of early viral mRNA (16, 22). These observations suggested that a viral protein is involved in the accumulation of early viral mRNA. Since 293 cells expressed early region 1A and 1B proteins, we infected these cells with Ad2 in the absence and in the presence of anisomycin and analyzed the accumulation of early viral mRNA. The early region 1A and 1B proteins should have been present in 293 cells even after anisomycin was added, provided that they were stable, since they were synthesized before the addition of the drug. To study the stability of these proteins, [^{35}S]-methionine-labeled 293 cells were treated with anisomycin for 2 h. Cell extracts were prepared before and after this treatment. Immunoprecipitation with anti-T sera revealed no substantial reduction in the amounts of the early region 1A 42,000- to 50,000-dalton proteins during drug treatment (data not shown). This suggested that the early region 1A proteins were stable in the 293 cells for at least 2 h. Therefore, if the early region 1A proteins are involved in the accumulation of early viral mRNA, the addition of anisomycin before viral infection should not depress the accumulation of early viral RNA.

Cytoplasmic RNA was prepared from 293 cells infected with Ad2 in the absence and in the presence of anisomycin. The RNA was hybridized to nitrocellulose filters containing DNA fragments specific for the different early regions. Table 1 shows that addition of anisomycin 30 min before viral infection did not depress the accumulation of early viral RNA from early region 2, 3, or 4. Instead, filter hybridization showed that the amount of viral RNA from early region 2 was increased about 2-fold and that the amount of viral RNA from early region 4 was increased at least 10-fold by the drug treatment. Identical results were obtained in several independent experiments. The same results were obtained when we used puromycin, another potent inhibitor of protein synthesis (data not shown). To obtain further evidence that the enhanced accumulation of early region 4 RNA was controlled by viral proteins, cytoplasmic RNA was prepared from Ad2-infected untransformed human embryonic kidney cells. The addition of anisomycin before viral infection in untransformed human embryonic kidney cells did cause a slight decrease in the accumulation of viral RNAs from early regions 2, 3, and 4 (Table 2), as previously demonstrated in HeLa cells (22).

Rate of transcription. The enhanced accumulation of viral early region 4 RNA could have been at the level of transcription or at a post-transcriptional level. To distinguish between these two possibilities, 293 cells infected with Ad2 in the absence and in the presence of anisomycin were pulse-labeled with [^{3}H]uridine for 5 min at 5 h postinfection. The nuclear RNAs were extracted and hybridized to filter-bound Ad2 DNA fragments representing the early viral transcription units. No increase in the rate of transcription was observed for early region 2 or 3 in the presence of the drug (Table 3); indeed, a twofold decrease was observed, which may have been due to the prolonged exposure to the drug. However, the rate of transcription from

TABLE 1. *Effect of anisomycin on the accumulation of cytoplasmic early viral RNA in Ad2-infected 293 cells*

Prepn	Amt of RNA hybridized (cpm)[a]		
	Early region 2	Early region 3	Early region 4
Ad2	459	1,325	652
Anisomycin-treated Ad2[b]	981	1,220	10,466

[a] Cytoplasmic RNA was prepared 5 h after infection with Ad2 from 293 cells maintained in the presence or absence of anisomycin. The cells were labeled with [^{3}H]uridine (50 μCi/ml) from 2 to 5 h postinfection. Equivalent amounts of early viral RNA were hybridized to filters containing viral DNA fragments from different early regions. The Ad2 DNA probes used were as follows: early region 2, *Eco*RI-B (58.5 to 70.7 map units); early region 3, *Eco*RI-D (75.9 to 84.0 map units); early region 4, *Eco*RI-C (89.7 to 100.0 map units). Background radioactivity (approximately 40 cpm, as determined by using filters without DNA) was subtracted from each value.

[b] Ad2-infected 293 cells were maintained in the presence of 100 μM anisomycin (Pfizer Laboratories) from 30 min before infection until 5 h after infection.

TABLE 2. *Effect of anisomycin on the accumulation of cytoplasmic early viral RNA in Ad2-infected human embryonic kidney cells*

Prepn	Amt of RNA hybridized (cpm)[a]		
	Early region 2	Early region 3	Early region 4
Ad2	166	205	371
Anisomycin-treated Ad2[b]	105	92	240

[a] Cytoplasmic RNA was prepared from Ad2-infected human embryonic kidney cells labeled with [^{3}H]uridine (50 μCi/ml) from 2 to 5 h postinfection. The RNA was hybridized to Ad2 DNA probes representing early regions 2, 3, and 4; the DNA probes used were the ones described in Table 1, footnote *a*. Background radioactivity (40 cpm as determined by using filters without DNA) was substracted from each value.

[b] The cells were treated with 100 μM anisomycin from 30 min before infection until 5 h after infection.

TABLE 3. *Rate of transcription in Ad2-infected 293 cells*

Prepn	Amt of nuclear RNA hybridized (cpm)[a]		
	Early region 2	Early region 3	Early region 4
Ad2	728	727	221
Anisomycin-treated Ad2[b]	362	385	1,119

[a] Ad2-infected 293 cells were pulse-labeled for 5 min with 500 μCi of [^{3}H]uridine per ml at 5 h postinfection. Equivalent amounts of nuclear RNA were hybridized to DNA filters as described in the text. The Ad2 DNA probes used were the ones described in Table 1, footnote *a*. Background radioactivity (approximately 40 cpm, as determined by using filters without DNA) was substracted from each value.

[b] Ad2-infected 293 cells were maintained in the presence of 100 μM anisomycin from 30 min before infection until 5 h after infection.

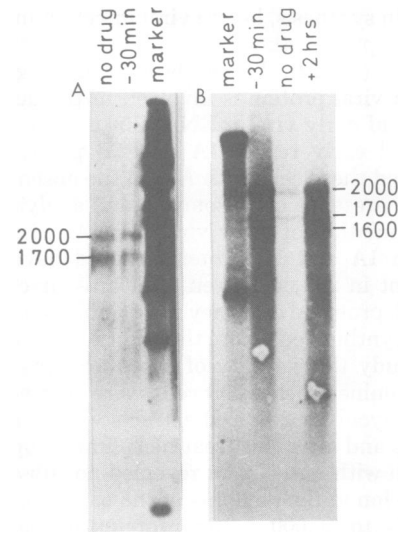

FIG. 2. *S1 endonuclease analysis of early region 2 and 4 RNAs accumulated in 293 cells infected with Ad2. Cytoplasmic RNA was prepared from Ad2-infected 293 cells maintained in the absence or presence of anisomycin (100 μM). The RNA was hybridized to Ad2 DNA in a solution containing a high formamide concentration, treated with S1 endonuclease, and separated on a 2.0% alkaline agarose gel. The gel was blotted onto a nitrocellulose sheet and hybridized to EcoRI restriction endonuclease fragment B of Ad2 DNA (58.5 to 70.7 map units) (A) or to EcoRI fragment C (89.7 to 100.0 map units) (B). The filter was washed extensively and then analyzed by autoradiography. −30 min, Anisomycin added 30 min before infection and maintained throughout until harvest of the cells at 5 h postinfection; no drug, cells not treated with anisomycin; +2 hrs, anisomycin added 2 h postinfection and maintained until harvest at 5 h postinfection; marker, HindIII digest of ^{32}P-labeled Ad2 DNA.*

early region 4 was significantly increased if the cells were treated with anisomycin 30 min before viral infection. Therefore, the enhanced accumulation of early region 4 RNA may have been caused at least in part by an increased rate of transcription.

Processing of RNA. The structure of the early region 4 RNA which accumulated in 293 cells infected with Ad2 was studied by the S1 endonuclease technique (3). Cytoplasmic RNAs prepared from 293 cells infected with Ad2 in the absence and in the presence of anisomycin were hybridized to Ad2 DNA, treated with S1 endonuclease, separated on alkaline agarose gels, and blotted onto nitrocellulose sheets. The filters were then hybridized to a ^{32}P-labeled restriction fragment representing early region 4. Figure 2B shows that addition of anisomycin 30 min before virus infection greatly increased the amount of early region 4 mRNA. We observed no qualitative difference between the RNA pattern obtained from cells which were treated and the pattern obtained from cells which were not treated with anisomycin. Thus, the increased level of early region 4 RNA obtained after treatment with anisomycin represented correctly spliced RNA species. Early region 4 mRNA prepared from Ad2-infected 293 cells treated with anisomycin at 30 min before infection and at 2 h after infection accumulated to the same extent. Viral RNA accumulated from early region 2 was also studied by this technique. Addition of anisomycin 30 min before infection did not increase the amount of viral RNA compared with cells not treated with the drug (Fig. 2A).

In vitro translation. To determine whether the viral RNA which accumulated in Ad2-infected 293 cells in the presence of anisomycin was functional, this RNA was isolated by hybridization to various viral DNA fragments, and the selected RNAs were translated in vitro. Figure 3 shows that treatment with anisomycin 30 min before infection or 2 h after infection caused substantial increases in viral early region 4 mRNA's. All early region 4 proteins identified from drug-treated cells were also present in cells that were not treated with the drug, as shown by a longer exposure of the film (Fig. 4). In addition, all proteins were enhanced to the same extent. In contrast, both early region 2 mRNA and early region 3 viral mRNA were depressed if the drug was added 30 min before infection. If the drug was added 2 h postinfection, these mRNA species accumulated to the same extent as cytoplasmic RNA prepared from drug-free controls.

Addition of anisomycin at 2 h before viral

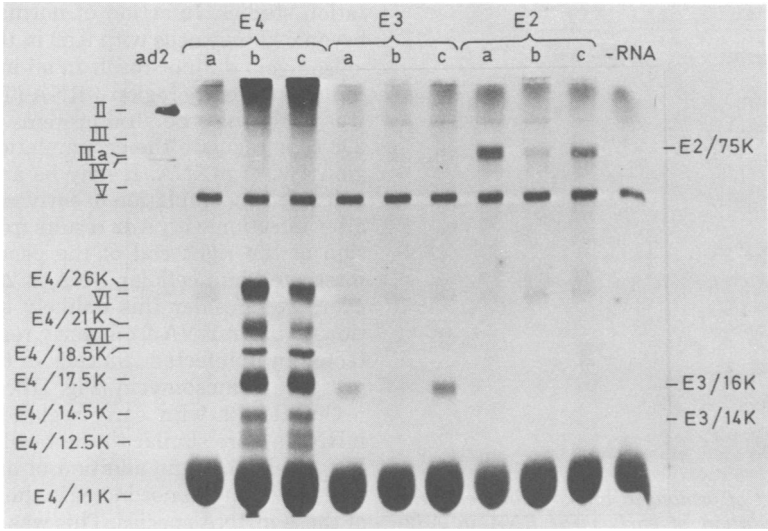

FIG. 3. *In vitro translation with hybridization-selected RNA from Ad2-infected 293 cells. At 5 h postinfection cytoplasmic RNA was prepared from Ad2-infected 293 cells maintained in the absence or presence of anisomycin. The RNA was hybridized to filters containing Ad2 DNA fragments representing different early regions. The hybridized RNA was then translated in the mRNA-dependent reticulocyte cell-free system. The translational products were separated on a 13% sodium dodecyl sulfate-polyacrylamide gel, and the gel was analyzed by fluorography. The Ad2 DNA restriction enzyme fragments used as probes were as follows: early region 4 (E4), EcoRI-C (89.7 to 100 map units); early region 3 (E3), EcoRI-D (75.9 to 84.0 map units); early region 2 (E2), EcoRI-B (58.5 to 70.7 map units). Lanes a, Infection without drug treatment; lanes b, anisomycin (100 μM) added 30 min before infection; lanes c, anisomycin (100 μM) added 2 h after infection. -RNA, No RNA added to the cell-free system.*

infection also caused an enhanced accumulation of early region 4 viral mRNA's compared with untreated cells (Fig. 4). Under these conditions early viral mRNA's from regions 2 and 3 were depressed dramatically. In summary, early region 4 mRNA's were enhanced irrespective of when the anisomycin was added, whereas the levels of early region 2 and 3 mRNA's were depressed unless the drug was added at least 1 h after infection.

DISCUSSION

Early region 1A of the adenovirus genome is located between 1.5 and 4.5 map units and is important for the accumulation of viral RNA from other early regions of the genome. This conclusion is based on results with host range mutants having deletions or point mutations in this region of the genome (2, 14). The deletion mutants are defective in more than one viral gene product since they lack all of early region 1A. The host range mutants in complementation group I (10) express at least some viral gene products from early region 1A (7, 15). Elsewhere, we have shown that the viral gene products that are defective in these mutants are responsible in part for the accumulation of early viral mRNA (M. G. Katze, H. Persson, and L. Philipson, Mol. Cell. Biol., in press).

Provided that the viral gene product involved in the accumulation of early viral RNA is a protein, the addition of inhibitors of protein synthesis before viral infection should mimic the situation with the early region 1A mutants; i.e., this treatment should prevent the accumulation of early viral RNAs from regions 2, 3, and 4. Experiments have suggested that this hypothesis is correct (16, 22) and that the early region 1A gene products controlling mRNA accumulation act at a posttranscriptional level.

The human embryonic kidney cell line 293 transformed by Ad5 DNA contains viral DNA representing early regions 1A and 1B and expresses early viral RNAs from these two regions of the genome (1). By using immunoprecipitation with anti-T sera of [^{35}S]methionine-labeled 293 cells and hybridization selection of cytoplasmic RNA followed by in vitro translation, we demonstrated that viral mRNA's from these regions are translated into proteins (Fig. 1A and B). Furthermore, all proteins which we detected corresponded in size and map location to the proteins expressed during lytic infection of human cells with Ad5. Recently similar results have been described by Jochemsen et al. (13).

To study the effects of the early region 1A and 1B gene products on viral mRNA accumulation, 293 cells were infected with Ad2, and the

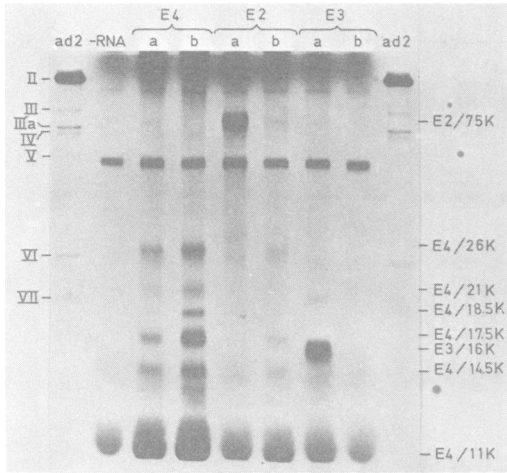

FIG. 4. *Effect of prolonged anisomycin treatment on the accumulation of early viral RNA in Ad2-infected 293 cells. At 5 h postinfection cytoplasmic RNA was prepared from Ad2-infected 293 cells maintained either in the absence of anisomycin (lanes a) or in the presence of anisomycin (100 μM) from 2 h before infection (lanes b). This RNA was hybridized to Ad2 DNA restriction enzyme fragments representing early regions 2, 3, and 4 (E2, E3, and E4, respectively), as described in the legend to Fig. 3. The selected RNA was then translated in vitro, and the samples were analyzed on a 13% sodium dodecyl sulfate-polyacrylamide gel, followed by fluorography of the dried gel.*

amounts of Ad2 early viral RNAs from regions 2, 3, and 4 were determined. If a viral protein encoded in early region 1 regulates the accumulation of early viral RNA, the addition of anisomycin before Ad2 infection of 293 cells should not depress the accumulation of mRNA's from early regions 2, 3, and 4 since early region 1 proteins are synthesized endogenously in these cells. We tested this hypothesis.

An increase in viral RNA from early region 4 of more than 10-fold was observed if anisomycin was added 30 min before infection (Table 1 and Fig. 2) Early region 4 RNA also accumulated at an enhanced level if anisomycin was added 2 h before infection. In addition, the mRNA which accumulated was functional, as assayed by in vitro translation (Fig. 3 and 4). The enhanced accumulation of early region 4 mRNA was more pronounced if anisomycin was added 30 min before infection than if it was added 2 h before infection (Fig. 3 and 4). This may be an alternative way to measure qualitatively the turnover of the early region 1 proteins that affect mRNA accumulation. Our results may be interpreted to indicate that the functional half-life of these proteins is at least 2 to 3 h, which is in agreement with the results obtained from immunoprecipitation studies. Infection of normal human embryonic kidney cells with Ad2 in the presence of anisomycin did not result in an increased accumulation of early region 4 RNA (Table 2). Thus, it is likely that the viral proteins present in the 293 cells regulate the accumulation of early region 4 viral mRNA. It may be argued that the enhanced accumulation of early region 4 mRNA after infection with Ad2 results from the expression of the right end of the genome, which is integrated into cellular DNA in 293 cells. However, we consider this unlikely since no functional viral mRNA from early region 4 was detected in uninfected 293 cells in the absence or presence of anisomycin (Fig. 1B).

Our results with early region 2 and 3 viral mRNA's were similar to the results obtained in HeLa cells; i.e., the addition of anisomycin before infection did not increase the accumulation of these mRNA species. This was demonstrated both by S1 endonuclease analysis and in vitro translation studies. Therefore, the viral proteins encoded in early region 1 exhibit a differential control on the expression of other early regions. Early regions 2 and 3 require either more of the early region 1 proteins or some viral protein not present in 293 cells to accumulate early viral mRNA. On the other hand, early region 4 is directly controlled by the early region 1 proteins.

The rate of transcription from early region 4 was also increased in Ad2-infected 293 cells in the presence of anisomycin (Table 3). This increased rate of transcription may explain the enhanced accumulation of early region 4 mRNA, but since no such rate increase was observed in HeLa cells infected with Ad2 or its host range mutants in the presence of anisomycin (22; Katze et al., in press), amplified early region 1 proteins in 293 cells may exert controlling effects at two levels (at transcription and at mRNA accumulation). The effect on transcription cannot be detected in HeLa cells since the presence of anisomycin prevents expression of the early region 1A proteins. Since the DNA-binding protein encoded in early region 2 (E2/75K) appears to repress early region 4 transcription specifically (20), the enhanced transcription of early region 4 which we observed probably would not have been detected in studies with defined mutants in early region 1. Such mutants would probably show a reduced level of transcription from early region 4 but since the early region 2 also would be expressed, it would be impossible to differentiate between an early region 1 effect and an early region 2 effect. Therefore, studies with a protein synthesis inhibitor in 293 cells should produce less biased effect of the early region 1 proteins since all other products are not formed.

It is interesting that in 293 cells treated with anisomycin, preformed early region 1 proteins appear to have an effect only on early region 4, producing both increased transcription and increased accumulation of mRNA from this region. Early regions 2 and 3 transcribe and express mRNA in a pattern similar to that observed previously in drug-treated HeLa cells (22; Katze et al., in press). These results suggest that proteins from early region 1 primarily control the early region 4 transcription unit in the temporal sequence of adenovirus early gene expression (19, 23, 26). Whether the early region 4 proteins in turn affect the next transcription unit, early region 3 in the adenovirus early cascade, can only be revealed with early region 4 mutants.

Our results with protein synthesis inhibitors (22; Katze et al., in press) suggest that early adenovirus gene regulation is complex and involves several cellular and viral elements. Cells probably contain a system which is capable of preventing viral mRNA accumulation and is counteracted by a virus-encoded protein defective in the hr-1 host range mutant from early region 1A (Katze et al., in press). In addition, our results suggest that the early region 1 proteins have a selective effect on early region 4 at the level of transcription and probably also on mRNA accumulation. An early region 1B product may finally control translation of the adenovirus mRNA (22). Next, since some evidence now has been obtained for the sites of the controls, it will be necessary to develop in vitro systems to assay for the proposed control mechanisms specifically.

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