Coordinate Regulation of Two Cytoplasmic RNA Species Transcribed from Early Region 2 of the Adenovirus 2 Genome

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Early region 2 (E2) of the adenovirus 2 genome specifies a 72,000-dalton DNA-binding protein that is required for viral DNA replication. Electron microscopy studies have detected two major forms of 20S E2 mRNA, one species with a 5' leader from map position 75 and a second form having a leader from position 72 (Chow et al., *J. Mol. Biol.* **134**:265-303, 1979). Only the species with a leader from position 75 was detected at early times; however, both forms were found at late times. We have analyzed the temporal regulation of E2 expression by documenting mRNA accumulation in the cytoplasm. Kinetic studies of pulse-labeled RNAs demonstrated a peak of E2 cytoplasmic RNA synthesis at 10 to 12 h, coinciding with the time of maximal synthesis of the 72,000-dalton DNA binding protein and viral DNA. To estimate the relative abundances of the two major E2 RNA species at various times during infection, total E2 cytoplasmic and polysomal 20S RNAs were isolated by hybridization-selection with specific DNA probes. The leader sequences in the selected RNAs were then quantitated by further RNA-DNA hybridization. We found that the elevated accumulation rate for E2 cytoplasmic RNA at late times reflected an increase in formation of both major species. Moreover, for all time points examined 66% of the mRNA species had a 5' end from map position 75, and 33% had a 5' terminus from position 72. Continuous labeling experiments provided evidence that both RNA forms have comparable half-lives. The results suggest that the two major species encoded by E2 are regulated in a coordinate fashion late in infection.

Five regions of the adenovirus 2 (Ad2) genome are expressed at early times in productive infection (11, 35). One of these regions, early region 2 (E2) codes for a 72,000-dalton (72K) protein which binds to single-stranded DNA (13, 16, 21). This DNA-binding protein (DBP) is required for the initiation and maintenance of viral DNA synthesis (18, 33, 34). E2 mRNA is specified by DNA sequences within map positions 61 to 76 (12, 27, 31) and is copied from the strand transcribed in the leftward direction (30). The early mRNA species consists of 70 nucleotides from positions 75 to 74.6 spliced to 170 nucleotides from positions 68.8 to 68.3 and 1,700 nucleotides from positions 66.3 to 61.6 (Fig. 1) (3, 6, 19). Recently, Chow et al. (7) reported that the E2 mRNA present at late times includes multiple species. One form is identical to the early species; a second major form differs only in its leader sequences, containing a 5' end from map position 72 instead of position 75. Rare forms of E2 transcripts containing other 5' leaders were also described.

The expression of multiple forms of E2 transcripts offers an opportunity to study several regulatory features of these viral sequences. In

the present study we have quantitated the appearance of E2 cytoplasmic RNAs during the course of infection. Isolating E2 RNA molecules by hybridization-selection made it possible for us to estimate the relative abundances of the two major species of DBP mRNA's as they were synthesized during the infection. The results suggest that the early RNA species having a 5' leader from map position 75 continues to be synthesized during the late phase of infection. Moreover, both major species encoded by E2 are regulated in a coordinate fashion late in infection.

MATERIALS AND METHODS

Cell culture, virus infection, and labeling of infected cells. Maintenance of KB cell suspension cultures, procedures for Ad2 infections, and labeling conditions with [³H]uridine were performed as described previously (10). KB monolayer cultures were maintained in Joklik minimal essential medium containing 10% fetal calf serum; 150-mm plates at 50 to 80% of confluence were infected at a multiplicity of 50 or 100 PFU/cell and labeled with 167 μ Ci of [³H]uridine (40 Ci/mmol; New England Nuclear Corp.) per ml.

RNA purification and preparation of adenovirus DNA. Procedures for purification of polyadenylic acid-containing RNA from cytoplasmic fractions, preparative hybridization of RNAs to DNA fragments bound to nitrocellulose membranes, thermal elution of specific RNAs, fractionation by electrophoresis in polyacrylamide-formamide gels, and RNA-DNA hybridization conditions have been described elsewhere (1, 8, 9, 14, 23). Conditions for propagation of Ad2 in KB cells, DNA extraction from purified virions, restriction enzyme digestion and purification of DNA fragments by agarose gel electrophoresis were as described previously (10, 22, 32).

Rabbit reticulocyte cell-free translation system. Nuclease-treated reticulocyte lysates were prepared by established procedures (26). For translation, preparative amounts of selected RNAs were pelleted at 25,000 rpm and suspended in 5 to 10 μ l of sterile distilled water. Translation assays and gel electrophoresis were carried out as described previously (17).

Cloning of E2 DNA fragments. The Ad2 DNA fragment with termini at map positions 58.5 and 70.7 (58.5–70.7 Ad2 DNA) fragment was prepared by restriction endonuclease *EcoRI* digestion (Fig. 1) and then cleaved with restriction endonuclease *BamHI* (cleavage site at map position 59). The 59–70.7 fragment was then ligated to the pBR 322 DNA, which had been digested first with *BamHI* and then with *EcoRI* (4). Similarly, the 70.7–75.9 *EcoRI*-F fragment was cleaved with restriction endonuclease *HindIII* (Fig. 1). This fragment was then ligated to the pBR 322 DNA that had been sequentially digested with *HindIII* and *EcoRI*. Calcium chloride-treated cells of *Escherichia coli* strain SB 2201 (Δ *pro-lac* Δ *his-3153* thiamine⁻) were transformed with these two sets of ligated DNAs. Since the inserted DNA interrupts the tetracycline resistance gene, bacteria containing recombinant plasmids with an insert at the *BamHI* or *HindIII* sites were characterized by ampicillin resistance and tetracycline sensitivity. We examined 55 colonies of *E. coli* SB 2201 transformed to ampicillin resistance by pBR 322 ligated to the 59–70.7 Ad2 fragment; 39 were found to be tetracycline sensitive. Each isolate contained the

Ad2 DNA fragment as verified by hybridization to ³²P-labeled Ad2 DNA and restriction endonuclease digestion with restriction endonuclease *BalI* (data not shown). Similarly the 70.7–72.9 and 72.0–75.9 Ad2 DNAs were inserted at the *HindIII* site of pBR 322; 56 colonies of 167 screened were tetracycline sensitive. The hybrid plasmids were identified by restriction enzyme digestion followed by agarose gel electrophoresis (not shown). The leftward strand of the *EcoRI*-B (58.5–70.7) fragment was cloned into the single-stranded bacteriophage M13hol (Bhaduri et al., manuscript in preparation) (2, 4). This phage contains histidine genes, thereby facilitating selection. The cloning and growth of the recombinant plasmid and phage DNAs were conducted under P1 containment according to the National Institutes of Health guidelines.

RESULTS

The 20S mRNA transcribed from E2 has been characterized by S1 nuclease mapping (3), electron microscopy of RNA-DNA hybrids (6, 19), and structural studies of pulse-labeled RNAs (14). These analyses have delineated one early E2 mRNA that has the structure shown in Fig. 1: 70 nucleotides from coordinates 75 to 74.6 spliced to 170 nucleotides from 68.8 to 68.3 and 1,700 nucleotides from coordinates 66.3 to 61.6. Electron microscopy has revealed a late form of the mRNA having 5' leader sequences derived from coordinates 72.0 to 71.9 (Fig. 1). The late form was discerned after the onset of viral DNA replication. This form of E2 RNA was identified previously only by electron microscopy, a technique which utilizes steady-state RNA populations. With this approach it was not possible to analyze the metabolism of the various species. We have quantitated and analyzed in more detail several properties of the major pulse-labeled E2 RNAs synthesized during the course of infection.

Time course of appearance of E2 cytoplasmic RNA. Before analyzing the different forms of E2 mRNA, we examined the rates of appearance in the cytoplasm of total 20S E2 RNA during the infectious cycle. Infected monolayer cultures were labeled with [³H]uridine for 2-h intervals, and polyadenylic acid-containing cytoplasmic RNA was purified. The [³H]RNAs were subjected to electrophoresis on polyacrylamide gels cast in formamide. Eluted RNAs from each gel slice were hybridized to the 62.8–65.9 DNA fragment immobilized on nitrocellulose membranes. This fragment annealed only E2 mRNA's (Fig. 1), even at late times in infection when the genome is fully expressed. After 24 h of hybridization the filters were removed, washed, and counted. To assure that RNA hybridization was performed in DNA excess, a second set of DNA filters was incubated with the RNA for an additional 24 h. In all cases a

FIG. 1. Major E2 RNA classes present late in infection. Solid bars represent the nucleotides present in the two major forms of late cytoplasmic 20S mRNA (7). The arrows indicate direction of transcription, and the caret-shaped symbols indicate splice points. The map positions show the relative distances from the left end of the genome. The cleavage sites for restriction enzymes used in this study have been reported previously (14).

single sharp peak was obtained (14), and the hybridization reaction was greater than 75% complete after the first annealing. The results of one such time course experiment are shown in Fig. 2. At 12 h after infection there was a significant increase in the labeling of 20S RNA. By 22 h, however, we observed markedly decreased amounts of cytoplasmic region 2 RNAs. The data from four different experiments were normalized to the amount of total cytoplasmic RNA and expressed as counts per minute per microgram of RNA. The cumulative data illustrate the rates of labeling of cytoplasmic 20S RNA during productive infection (Fig. 3). The peak rate of synthesis occurred approximately 12 h after infection, near the time when maximal viral DNA synthesis is achieved (15, 25).

A similar time course was obtained for E2 RNAs synthesized in infections performed with suspension cultures. However, with suspension cultures the amount of labeled 20S mRNA reached a peak of appearance between 8 and 10 h (data not shown). This result is expected since the course of productive infection is more rapid in suspension cultures.

Accumulation of different forms of DNA-binding protein RNA. The accelerated production of region 2 mRNA at the time of maximal viral DNA synthesis (Fig. 2 and 3) might reflect preferential synthesis of one of the forms of E2 mRNA. For this reason we investigated the relative accumulation rates of the two major E2 mRNA's during the course of infection.

Cultures were labeled with [^{3} H]uridine for two-h intervals at different times during infection. Cytoplasmic RNA was purified, and the polyadenylated RNA was selected by oligodeoxythymidylic acid chromatography. E2 RNAs were then purified by preparative RNA-DNA hybridization and elution. The DNA probe was the 62.8–65.9 fragment as noted above. The 62.8–65.9 DNA anneals only E2 mRNA's, therefore allowing purification even in the presence of late mRNA's.

After purification by hybridization-selection to 62.8–65.9 DNA, the eluted RNA was fractionated by size. Greater than 95% of the eluted RNA migrated as a single peak, similar to the result obtained by direct hybridization (Fig. 2). Eluted [^{3} H]RNA in peak gel slices was then hybridized to DNA fragments which discriminate between the two major forms of E2 RNA present at late times (Fig. 1). The 70.7–72.9 probe anneals [^{3} H]RNA only from the late form, the species having leader sequences from position 72. A second probe, 72.9–75.9, anneals only the early form with its leader sequences from position 75. The counts per minute hybridized to each probe were summed and compared in

FIG. 2. E2 cytoplasmic RNA synthesis during the course of Ad2 infection. Infected KB monolayer cultures were labeled with [^{3} H]uridine (167 μ Ci/ml) at various times postinfection. At 2 h after the addition of label, cultures were harvested, and cytoplasmic RNA was purified. The total yield of RNA was determined before oligodeoxythymidylic acid selection. Polyadenylic acid-containing RNA was subjected to electrophoresis through 3.75% formamide acrylamide gels at 2 mA/gel for 12 h. Gels were sliced into 2-mm fractions, and the eluted RNA from each slice was hybridized to approximately 1- μ g equivalents (the amount of DNA fragment that would be derived from 1 μ g of whole genome DNA) of the 62.8–65.9 DNA fragment. After hybridization and removal of the membrane, the supernatants were rehybridized with an additional membrane to verify that the hybridization was performed in DNA excess. The times shown in each panel represent the time of harvesting. The 22-, 12-, and 5-h polyadenylic acid-containing RNAs loaded on the gels were derived from 280, 170, and 150 μ g of total cytoplasmic RNA, respectively.

Table 1. The selectivity of the E2 purification scheme was confirmed by the absence of hybridization to DNA fragments to the left (58.5–60.2) and right (75.9–83.4) of region 2 (see Fig. 5). When we compared RNA samples purified from total cytoplasmic RNA at 12, 14, or 16 h after infection, the relative labeling of the early form (5' end at map position 75) to the major late form (5' end at map position 72) was approximately the same. For all time points, 66% of the

FIG. 3. Rates of accumulation of cytoplasmic E2 RNAs during the course of infection. This figure is a compilation of four separate experiments (○, ●, △, and □) of the type present in Fig. 2. The 20S [^3H]-RNA hybridized for each time point was summed, the background was subtracted, and the counts per minute were normalized to the total amount of cytoplasmic RNA from which the analyzed polyadenylic acid-containing RNA was derived.

TABLE 1. Relative abundance of region 2 RNAs during adenovirus 2 infection^a

Expt	Labeling time (h) for cytoplasmic RNA	RNA with leader at 75 (cpm hybridized to 75.9-72.9 DNA)	RNA with leader at 72 (cpm hybridized to 70.7-72.9 DNA)	A/B ^b
I	3-6	420	0	0
II	12-14	240	120	2
	14-16	365	185	1.9
III	10-12	235	120	1.9
IV (polysomal RNA)	10-12	1,100	640	1.7

^a Infected monolayer cultures were labeled with [^3H]uridine as described in the text for the indicated times. Cytoplasmic polyadenylic acid-containing RNA was purified and selected with a 62.8-65.9 DNA fragment. Eluted RNAs (100×10^3 cpm for experiment I, 55×10^3 cpm for both time points in experiment II, and 63×10^3 cpm in experiment III) were fractionated by size on formamide gels and hybridized to the indicated DNA fragments. The hybridized counts per minute of the 20S RNA were summed, and the background was subtracted. For experiment IV, cells in suspension were labeled with [^3H]uridine as described previously (18), and mRNA was isolated from polysomes (29). Polyadenylated RNAs were purified and selected with the 58.5-70.7 leftward DNA strand. Eluted RNAs (25×10^4 cpm) were analyzed as above.

^b Ratio of RNA with leader at map position 75 to RNA with leader at position 72.

newly labeled cytoplasmic RNA species had a 5' end at map position 75, and 33% had a 5' end at map position at 72. At early times only the species with the 5' leader at 75 was detected (14).

In addition to comparing the leader sequences of cytoplasmic E2 RNAs, we analyzed polysomal RNAs. For the experiments with polysomal RNAs, RNA selection was performed with a single-strand DNA probe, *EcoRI*-B leftward single-strand sequence cloned in the phage M13 (see above). The results of these experiments are presented in Table 1. The selected 20S RNA hybridized to the probes for both leaders; the ratio of [^3H]uridine incorporation into the two leader sequences was essentially the same as that for total 20S cytoplasmic RNA. Since mRNA's containing both leader sequences were present in polysomal RNA labeled 10 to 12 h after infection, we conclude that both forms of E2 mRNA are being translated actively at the time of maximal DNA synthesis.

Having found both major forms of E2 mRNA on polysomes isolated at late times, we examined the polypeptides encoded by these mRNA's. Cytoplasmic RNA selected by the 62.8-65.9 DNA fragment was used to program in vitro protein-synthesizing systems (Fig. 4). At least

FIG. 4. *In vitro* translation of late E2 RNAs selected by the 62.8-65.9 DNA fragment. Late polyadenylated cytoplasmic RNA from 3×10^6 infected cells was selected by the 62.8-65.9 DNA fragment, and a sample was translated in reticulocyte lysates. Lanes: A, ^{35}S -labeled virion markers; B, RNA isolated at 13 h after infection; C, RNA isolated at 22 h after infection.

95% of the *in vitro* product migrated as 72K, as expected for the E2 polypeptide product. Assuming that there are no great differences in the translational efficiencies of the two major E2 mRNA's, we conclude that both forms encode a 72K product.

Relative stabilities of the E2 mRNA's as determined by continuous labeling experiments. The data in Table 1 demonstrate that both major forms of E2 mRNA are labeled in constant proportions after the onset of viral DNA synthesis. This result suggested that the two forms may have similar metabolic properties. We have examined one particular parameter, the stability of the cytoplasmic mRNA's in a continuous labeling experiment.

Beginning at 10 h after infection, cultures were labeled for 1, 2, and 4 h with [^{3} H]uridine. The polyadenylated E2 cytoplasmic RNAs were purified by hybridization-selection to the 62.8–65.9 DNA fragment. Eluted RNAs were then fractionated by size (Fig. 5, top panel) and rehybridized to DNA probes containing the 72 or 75 leader sequences (Fig. 5, middle and bottom panels). The hybridized counts per minute in each peak were summed, and the background was subtracted; the results are shown in Fig. 6. The curves were extrapolated to 30 min, the time when E2 RNAs are first detected in the cytoplasm (14, 28). For the time period examined, the rate of labeling of the two major RNA species remained constant. Approximately 66% of the 20S RNA population contained a 5' leader from map position 75, and 33% contained a 5' leader from position 72. We conclude that in the first 4 h of a continuous [^{3} H]uridine label both RNA species have similar stabilities in the cytoplasm, and that the steady-state ratio of the two RNAs made at late times is approximately 2:1.

DISCUSSION

Initial studies of E2 suggested a simple genetic unit. The DNA sequence appeared to specify a single mRNA, 20S in size (10, 14), and a single 72K polypeptide product that bound to single-stranded DNA and had a functional role in DNA replication (13, 16, 18, 21, 33, 34). Since these first studies, several observations have revealed an increasingly complex pattern of E2 expression. We now know that E2 specifies two major cytoplasmic RNAs and several minor species. All of the cytoplasmic RNAs are spliced, and, based on the nucleotides at the 5' termini, it may be that several different promoters function in transcription of the region (7). In addition to the structural complexity of E2 RNAs, a functional complexity has also been revealed for the 72K protein. Apparently E2 has an autoregulatory

FIG. 5. Relative abundance of the two classes of 20S E2 RNAs accumulated in different labeling times. Suspension cultures were infected and labeled with [^{3} H]uridine (50 μ Ci/ml) for the indicated times. The polyadenylic acid-containing cytoplasmic RNA was isolated and purified with the 62.8–65.9 DNA fragment as described in footnote a of Table 1. Then the RNAs were subjected to electrophoresis in formamide gels. A sample of the RNAs eluted from each gel fraction was counted directly (top three panels). The remainder of the eluted RNAs was hybridized to 1- μ g equivalents of various DNA fragments. The middle three panels represent hybridizations to the 70.7–72.9 DNA probe. The bottom three panels represent the hybridizations to the 72.9–75.9 (○) and 75.9–83.4 (□) DNA probes. The samples were also annealed to the 58.5–60.2 DNA, with results identical to those shown for 75.9–83.4.

role (5) and is also involved in regulating the stability of early mRNA's (J. R. Nevins, personal communication). These findings indicate that

FIG. 6. Continuous labeling kinetics of the two major E2 RNA species. The data of Fig. 5 were used to obtain the kinetics shown here. The total counts per minute that hybridized to each 5' leader specific probe (70.7-72.9 [●], 72.9-75.9 [○]) were summed, the background was subtracted, and the resulting values were plotted as a function of labeling time. The values were extrapolated to 30 min, the time at which cytoplasmic RNA from E2 is first detected (14, 28).

information about the regulation of E2 expression should aid in our understanding of many aspects of adenovirus lytic infection.

The temporal expression of E2. As measured by accumulation of newly synthesized cytoplasmic RNA (Fig. 3), E2 expression reaches its maximal level at the time when the highest level of viral DNA synthesis is reported to occur (25). The accumulation rate of E2 RNA is maximal at 12 h postinfection and then declines. This time course also parallels (i) the intracellular concentration of E2 transcripts as measured by RNA-DNA hybridizations in liquid and (ii) the *in vivo* rate of synthesis of 72K protein (25). Different kinetics of E2 expression were obtained when cultures were infected in the presence of cytosine arabinoside (24). In these experiments the synthesis of E2 transcripts was monitored by quantitation of pulse-labeled nuclear RNA. In the absence of DNA synthesis the synthetic rate reached a maximal level in 6 h and then declined. By comparison (Fig. 3 this paper; Fig. 2 of reference 24) it appears that the 6-h rate in the presence of cytosine arabinoside is about five times less than the maximal rate we found at 12 h. We have confirmed this observation by analyzing cytoplasmic RNA synthesis in the presence of cytosine arabinoside (data not shown). Thus, the prevention of viral DNA replication appears to preclude the accelerated production of E2 mRNA after 6 h and to lead prematurely to a decrease in the level of expression.

Expression of the major E2 species. As first noted by Chow et al. (7), E2 expression at early times is restricted to the formation of one species with a 5' terminus at map position 75. A second major species, with a leader at map position 72, is detected at late times. Based on

electron microscopy studies of RNA-DNA hybrids in region 2, Chow et al. (7) concluded that the RNA species with a leader from coordinate 72 constituted nearly 70% of the E2 RNA at both 13 and 22 h after infection. From these results we considered the possibility that the increased expression of E2 during viral DNA synthesis might be effected solely by the appearance of the new species with a leader at 72. Our results do not support this model. In contrast, we find that the elevated accumulation rate for E2 cytoplasmic and polysomal RNA reflects an increase in formation of both major species. Thus, the onset of viral DNA synthesis triggers an increase in formation of mRNA with the structure found exclusively at early times and also initiates formation of mRNA having a different 5' terminus.

We found different ratios of the major E2 mRNA's as compared with the results of Chow et al. (7). Since our analysis utilized pulse-labeled RNAs, whereas that of Chow et al. (7) relied on steady-state RNA populations, these apparent differences in the species with leaders from positions 72 and 75 might reflect differential stabilities of the mRNA species. Therefore, we performed continuous labeling experiments to estimate the relative half-lives of the two species (Fig. 6). No difference was detected between the labeling pattern of the species with leaders at map positions 72 and 75. We conclude that the different ratios are likely to reflect the different methods used to obtain the data. The quantitation based on electron microscopy utilizes a very small sample and therefore is subject to uncertainty. Moreover it is not clear whether preparation of RNA-DNA hybrids for microscopy introduces a bias in favor of one mRNA. For example, the species with a larger intervening sequence (75 leader) might give a less stable hybrid.

Reasons for multiple forms of E2 mRNA.

The reasons for multiple mRNA's from E2 are not clear at present. It is important to recall the multifunctional role of E2 (5, 18, 20, 33, 34) and to consider that the region may specify more than one polypeptide product. The nucleotide sequence of the E2 mRNA's has not yet been determined, and it is possible that minor splice changes allow the translation of the RNA in different reading frames. The *in vitro* translation studies we performed did not reveal any major proteins in addition to the 72K product (Fig. 4). However, it is possible that the 72K product may have a heterogeneous primary structure (20). In addition, we have observed multiple minor peptides that merit further investigation. It should be possible to purify E2 mRNA's on the basis of their leader structures rather than the mRNA body. This approach would allow a

further investigation of the products of each mRNA form.

Our study does not point to any obvious distinctions in the metabolic properties of the E2 mRNA's. It is tempting to suggest that the termini of the E2 species reflect the promoter sites and that the two major forms arise from different transcriptional start points. The nucleotide sequence of the 5' ends of both E2 mRNA's is consistent with the existence of two different promoter sites (C. Baker and E. Ziff, personal communication). The second promoter might be recognized only at late times and provide enhanced amounts of mRNA to coincide with DNA replication.

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