Regulation of the Primary Expression of the Early Adenovirus Transcription Units

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Received for publication 13 June 1979

The time course of appearance of transcriptional activity from five early adenovirus type 2 transcription units has been determined. RNA complementary to region 1A (1-4.4 map units), the first region to be transcribed, was detectable at 45 min after infection; a maximal rate of RNA synthesis was reached at 3 h after infection and was maintained thereafter for at least 6 h. RNA from region 2 (75-56 map units), which encodes the mRNA for the 72,000-dalton DNA-binding protein, was the last to be synthesized; transcription commenced at about 2 h postinfection, reached a maximum at 7 h, and then declined. Transcription of regions 3 (76-86 map units) and 4 (99-91 map units) reached a maximal value at 3 h postinfection. The rates of RNA synthesis from these regions then declined over the next 6 h. The decline of transcription from regions 2 and 4 appeared to be a specific repression of these transcription units. The repression did not occur in the absence of protein synthesis, suggesting that a viral protein might be involved. Transcription of all early regions was initiated and continued for at least 2 to 3 h in cells that were treated with cycloheximide or emetine before and during infection, suggesting that at least the initiation of RNA synthesis from the five early adenovirus type 2 transcription units does not depend on the formation of a viral protein. Moreover, mRNA was formed in the absence of protein synthesis that hybridized to DNA fragments representing each of the five early transcription units. The increase in mRNA accumulation in the presence of cycloheximide (or emetine) does not appear to be due to increased RNA synthesis; thus, either increased mRNA stability or increased efficiency of nuclear RNA processing must occur.

After infection of HeLa cells by adenovirus type 2 (Ad2), four separate regions of the viral genome are expressed into mRNA's that code for the early viral proteins (24, 26). Recently, it was shown that early region 1 actually consists of two distinct transcription units (25, 33); therefore, at least five transcription units are active in the production of mRNA before the onset of viral DNA replication (1, 11). At approximately 6 h postinfection, viral DNA replication begins, followed by the onset of the late stage of the replication cycle in which a large 30-kilobase transcription unit is activated with a single promoter at genome position 16.45 (11, 35).

The mechanism of control of expression of genes in eucaryotic cells is of paramount importance to a wide range of problems in molecular biology. The control of the amount of functional mRNA's in the cell could be brought about by

regulating: (i) primary transcription, (ii) processing and transport of the nuclear transcripts, or (iii) messenger stability in the cytoplasm, or possibly a combination of all three. Clearly, the shift from the early to the late phase of viral replication represents a critical control of gene expression; i.e., the late 16-99 transcription unit is not expressed until DNA replication occurs. It is not known, however, whether controls are exerted on the expression of the early transcription units. This possibility was suggested by studies in which the time course of early viral polypeptide synthesis was analyzed (18; L. Philipson, personal communication). The syntheses of early 19,000- and 11,000-dalton proteins, which are now known to be encoded by mRNA from region 3, and of an 18,500-dalton protein encoded by mRNA from region 4 are detectable before the synthesis of the early 72,000-dalton protein, which is encoded in region 2 (12, 17, 29-31). This differential appearance of early proteins could be due to either (i) regulated expression of the various early transcription

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units or (ii) constant expression of the transcription units with variation in either processing efficiency or stability of mRNA or both. To distinguish between these possibilities, the rates of RNA synthesis for the early transcription units were measured as a function of time after infection. The results indicate that there is differential expression of the five early transcriptional units with regard to both the onset and continuation of transcription.

MATERIALS AND METHODS

Cells and virus. HeLa cells were maintained in suspension culture in minimal essential medium-Joklik containing 5% fetal calf serum. Virus was grown and purified as described previously (19a, 32).

RNA labeling. Purified Ad2 (5,000 particles per cell) was added to HeLa cells concentrated to 10^7 cells per ml in minimal essential medium with no serum. After a 20-min adsorption period, the cells were diluted to 5×10^5 cells per ml with minimal essential medium containing 5% fetal calf serum. At the appropriate time, a sample of cells was concentrated to 5×10^6 cells per ml in fresh prewarmed medium, and [3 H]-uridine was added at 200 μ Ci/ml for 5 min. Incorporation of label was halted by pouring the cells onto an equal volume of crushed, frozen phosphate-buffered saline (0.14 M NaCl, 0.01 M NaPO₄, pH 7.0). Cells were washed twice with cold phosphate-buffered saline and lysed with 0.5% Nonidet P-40. RNA was extracted from detergent-washed nuclei (23) as described elsewhere (27). After the addition of sodium dodecyl sulfate to 0.5% (wt/vol) and EDTA to 10 mM, RNA was obtained from the cytoplasmic fraction by two extractions with phenol-chloroform (1:1) and one extraction with chloroform.

RNA-DNA hybridization. RNA samples were precipitated once from 2 M LiCl to remove DNA and then partially degraded with alkali (15). Exhaustive hybridization to adenovirus restriction DNA fragments immobilized on membrane filters (Millipore Corp.) was carried out as previously described (20). The complete removal of hybridizable RNA sequences was proved by demonstrating that a fresh filter added after the initial reaction hybridized less than 20% of the RNA hybridized during the initial reaction.

DNA procedures. The purification of viral DNA and the procedures for restriction endonuclease digestion and the purification of resulting fragments are described elsewhere (19a). *EcoRI* was obtained from Miles Laboratories, Inc., *HpaI* was obtained from Bethesda Research Laboratories, and *SmaI* was purified by the procedure of Carel Mulder (personal communication).

RESULTS

Time course of expression of the early Ad2 transcription units. The major aim of this study was to determine the kinetics of the primary expression of the early Ad2 transcription units. Therefore, samples of HeLa cells at selected intervals after infection in the presence

of cytosine arabinoside were concentrated and labeled for 5 min with [3 H]uridine. A labeling time of 5 min is considerably less than either the half-life of the late Ad2 nuclear RNA (21) or the time required for processing the nuclear RNA, either late or early Ad2, into mature mRNA (3, 13, 19); therefore, incorporation of [3 H]uridine for 5 min into nuclear RNA specific for each of the Ad2 early transcription units should provide an accurate measure of rates of RNA synthesis rather than a composite of transcription, decay, and processing. The labeled RNA was then extracted and hybridized to the DNA fragments specific to each of the early regions of the Ad2 genome (Fig. 1). The results of a representative experiment are shown in Fig. 2. Clearly, the kinetics of expression of the early Ad2 transcription units differed. Regions 1A, 3, and 4 were expressed first and reached a maximal rate of transcription at about 3 h postinfection. Region 1A continued to be expressed at about 90% of its maximal rate through 9 h of infection. However, region 3 transcription decreased steadily to about 55% of its maximal rate, and the expression of region 4 dropped sharply to about 25% of its maximal rate by 9 h after infection. Thus, it seemed that transcription of regions 3 and 4 was actively turned off, in comparison with region 1A, rather than simply declining owing to the course of infection.

The kinetics of expression of regions 1B and 2 were in sharp contrast to the other regions: they did not reach their maximal rates until approximately 7 h after infection, and the expression of region 1B continued at the same rate through 9 h postinfection. This latter result is not surprising since it was previously shown that the mRNA's from this region continue to be produced at late times (28) and, in fact, an additional transcription unit, with a promoter between 8.0 and 11.2, is activated (33). The results shown in Fig. 2 were also obtained in cells incubated without cytosine arabinoside (data not shown), using separated DNA strands for hybridization to distinguish early and late transcripts (regions 2 and 4). Therefore, the differential expression was not simply due to an effect of inhibition of DNA synthesis.

An additional experiment was carried out to define more clearly the initial appearance of transcriptional activity from the five early regions (Fig. 3). As suggested in Fig. 2, region 1A was the first to be activated; RNA synthesis from this region began soon (~45 min) after the infection was established. In contrast, the expression of region 2 was not initiated until ~2 h after the beginning of the infection. Regions 1B, 3, and 4 were activated by about 60 to 90

FIG. 1. Map of the adenovirus genome depicting the early transcription units (heavy arrows) and the DNA restriction fractions employed in this study.

min postinfection.

Relative rates of expression of the early transcription units. From the measurements shown in Fig. 2, estimates can be made of the relative maximal rates of expression of the early Ad2 transcription units. The hybridized counts per minute obtained at the time of maximal expression was divided by the number of nucleotides of DNA represented in the hybrid to give the relative rates of transcription (Table 1). Of the five early transcription units, region 3 appeared to be the most active, transcribing RNA at about 1.5 to 2.3 times the rates of the other early regions. The rates of transcription from the other early regions, however, were nearly identical; therefore, the frequency of initiation at the early promoters appears to be quite similar, with the exception of region 3.

Effect of inhibition of protein synthesis on the expression of the early Ad2 transcription units. Several reports have demonstrated a marked increase in the levels of early adenovirus RNA in the presence of inhibitors of protein synthesis such as cycloheximide (8-10, 22, 34). This finding could be the result of one or more factors, such as (i) an increased rate of transcription, (ii) an inability to shut off early transcription, (iii) a more efficient nuclear RNA processing, or (iv) an increased mRNA stability. To ascertain whether the effect produced by the inhibition of protein synthesis was simply due to an altered rate of transcription of the various early genes, we measured the rates of transcription of the early adenovirus transcription units in the presence of cycloheximide (Fig. 4). Cycloheximide had different effects on the expression of the early transcriptional units. The initial rates of transcription of regions 2 and 4 were the same despite the presence of cycloheximide; however, unlike the normal situation, there was no turnover of the transcriptional activity. In contrast, the expression of region 3 appeared to be diminished by cycloheximide, never attaining the normal maximal rate of expression. However, this result could be due in part to a general inhibition of RNA synthesis by cycloheximide. (None of the effects is likely to be due to any changes in UTP pool labeling, since transcrip-

tion of regions 2 and 4 remained high despite the presence of the drug, whereas transcription of region 3 declined.) The initial rates of transcription of regions 1A and 1B were unaffected by the action of cycloheximide; in each case though, the full maximal expression of the transcription unit was not attained.

Even though cycloheximide produced a >95% inhibition of protein synthesis at these concentrations, it still remained a possibility that a sufficient quantity of a viral protein was produced to allow expression of the remaining transcription units. This possibility was tested with the drug emetine, which at a concentration of 10^{-4} M results in >99% inhibition of protein synthesis within minutes after addition of the drug (14). A drawback of this experiment results from the fact that emetine also caused an almost complete inhibition of LiCl-precipitable RNA labeling (~95%) after 3 h of incubation with the drug. However, as shown in Table 2, if viral RNA synthesis was assayed before emetine caused the drastic inhibition of RNA labeling, there was still transcriptional activity from all five early regions. Even though protein synthesis was completely inhibited throughout viral infection, some RNA synthesis from each of the early transcription units was possible. It thus seems highly unlikely that the protein product of one of the early genes was absolutely required for the primary expression of any of the other early genes.

The above results with cycloheximide and emetine treatments of infected cells indicate that: (i) the expression of the 1A transcription unit did not require continuous protein synthesis, (ii) protein synthesis may be required for the maximum expression of early transcription units 1B and 3 and (iii) protein synthesis was required for the active repression of two early adenovirus transcription units (regions 2 and 4). Finally, protein synthesis was not required for the processing of the early nuclear transcripts and their transport to the cytoplasm (Table 3). Although the data shown in Table 3 were obtained with total cytoplasmic RNA, it has also been found that polyadenylic acid-terminated RNA complementary to regions 3 and 4 was the same size in

FIG. 2. Rates of adenovirus RNA synthesis from the five early transcription units at selected intervals postinfection. At the indicated time postinfection, samples of 2×10^7 cells (which had been maintained in the presence of cytosine arabinoside at $25 \mu\text{g/ml}$) were concentrated to 4×10^6 cells per ml in fresh, warm minimal essential medium and labeled for 5 min with [^3H]uridine ($200 \mu\text{Ci/ml}$). The labeled nuclear RNA was hybridized to filters containing DNA fragments of genome coordinates 0-4.4 (region 1A), 4.4-11.1 (region 1B), 58.5-70.7 (region 2), 75.9-83.4 (region 3), and 91.9-98.2 (region 4).

FIG. 3. Sequence of activation of the five early adenovirus transcription units. Nuclear RNA from cells labeled for 5 min with [^3H]uridine at the indicated times postinfection was hybridized to filters containing the DNA fragments described in the legend to Fig. 2, with the exception that region 4 RNA was detected with the 89.7-100 fragment. (A) ●, Region 1A; ○, region 2. (B) ●, Region 1B; ○, region 3; △, region 4. The data are plotted as counts per minute per kilobase of hybrid, therefore representing relative molar amounts of transcription.

cells treated with emetine as that from control cells. The previously reported results demonstrating an increase in early mRNA in the presence of cycloheximide (8-10, 22, 34) compared with control cells were confirmed with emetine. For instance, the amount of mRNA from region 1A was increased 1.4-fold in the presence of emetine despite a 2.5-fold reduction of labeled nuclear RNA, indicating an actual 3.5-fold increase in mRNA accumulation. Similar increases were also found for the mRNA from regions 3 and 4.

DISCUSSION

The results presented in this report allow several conclusions to be made concerning early adenovirus transcription. First, each of the five early adenovirus transcription units exhibits a distinct time course of expression. In addition, in two instances there is a correlation between previous data on the time course of the appearance of the protein products (18; Philipson, personal communication) and the present data on the primary expression of the transcription units. Thus, the delayed appearance of the early 72,000-dalton DNA-binding protein is most likely due to a delayed expression of the transcription unit responsible for the production of

TABLE 1. *Relative maximal rate of expression of the early adenovirus transcription units*

Transcription unit	Time of maximal transcription (h) ^a	Hybridized cpm	DNA fragment	Nucleotides hybridized ^b	cpm/kilobase	Relative rate of transcription
1A	3-5	400	0-4.4	1.05	380	0.5
1B	7-9	970	4.4-11.1	2.21	438	0.57
2	7	1,430	58.5-70.7	4.27	335	0.44
3	3	1,850	75.9-83.4	2.42	764	1.00
4	3	1,100	91.9-98.2	2.21	498	0.65

^a See Fig. 2.

^b Represents the number of nucleotides (expressed as kilobases) of the DNA fragment expected to be in the RNA hybrid. The genome coordinates of the transcription units were taken as 1.4-4.4 (region 1A), 4.8-11.4 (region 1B), 75.0-56.0 (region 2), 76.5-86.0 (region 3), and 99.1-91.5 (region 4). The coordinates are based on map positions for the various early caps (C. Baker and E. Ziff, personal communication) as well as polyadenylic acid sites (2, 7, 16). Although the polyadenylic acid site for region 2 is located at 61.5, termination of the primary transcript occurs at about 56 (J.-M. Blanchard, J. Nevins, and J. E. Darnell, manuscript in preparation).

FIG. 4. Effect of cycloheximide on the rates of RNA synthesis from the five early Ad2 transcription units. A portion of the infected culture from the experiment described in the legend to Fig. 2 was maintained in the presence of 25 μ g of cycloheximide (along with cytosine arabinoside) per ml from the beginning of the experiment (including virus adsorption). Samples were prepared, and the nuclear RNA was hybridized as described in the legend to Fig. 2. Symbols: ●, cytosine arabinoside; ○, cycloheximide plus cytosine arabinoside. Regions 1A and 2, left-hand ordinate; regions 1B, 3, and 4, right-hand ordinate.

the mRNA (region 2). Furthermore, the various early transcription units demonstrated differences in the overall kinetics of expression: transcription of region 4 declined rapidly after the initial maximal rate, whereas transcription of regions 1A and 1B continued at approximately the same maximal rates, indicating that the primary expression of the various early transcription units is regulated differentially.

Second, the onset of expression of each of the early transcription units does not appear to de-

TABLE 2. *Effect of emetine on the expression of the early Ad2 transcription units*

Transcription unit	Time after infection (min) ^a	Hybridized cpm ^b		
		Control	Emetine	Control (%)
1A	90	392	159	41
	135	2,129	256	12
	180	2,660	301	11
1B	90	123	73	59
	135	592	128	22
	180	1,003	117	12
2	90	168	36	21
	135	974	50	5
	180	3,345	29	1
3	90	411	64	16
	135	4,584	89	2
	180	5,811	49	1
4	90	182	159	87
	135	3,478	230	7
	180	5,916	306	5

^a At the indicated time postinfection, samples of 2×10^7 cells that had been incubated either without emetine or with emetine (10^{-4} M) since 10 min before virus addition were labeled for 5 min with [3 H]uridine.

^b Nuclear RNA samples were hybridized to DNA fragments 0-4.4 (region 1A), 4.4-11.1 (region 1B), 58.5-70.7 (region 2), 75.9-83.4 (region 3), and 89.7-100 (region 4). Hybridization with no DNA on filters was fewer than 15 cpm.

pend on the synthesis of a critical viral protein. All early transcription units were expressed in the presence of cycloheximide, although the rate of transcription of region 3 was somewhat diminished. Even in the presence of emetine, which almost completely inhibits protein synthesis at

TABLE 3. *Effect of emetine on early Ad2 mRNA transport*^a

Region	Nuclear RNA hybridized cpm			Cytoplasmic RNA hybridized cpm		
	Control	Emetine	Control (%)	Control	Emetine	Control (%)
1A	736	290	39	2,487	3,572	144
1B	242	151	62	339	348	103
2	344	87	25	520	167	32
3	981	82	8	354	223	63
4	1,548	492	32	1,939	1,846	95

^a At 2 h postinfection, samples of 2×10^7 cells that had been incubated either without emetine or with emetine (10^{-4} M) since 5 min before virus addition were labeled for either 5 or 45 min with [3 H]uridine. Nuclear RNA was prepared from the samples labeled for 5 min, and cytoplasmic RNA was prepared from the samples labeled for 45 min. Each sample was then hybridized to the DNA fragments described in Table 2.

concentrations of 10^{-4} M, each of the early transcription units was activated. Thus, there does not appear to be a "pre-early" viral protein which is required for the expression of the remaining viral genes.

Third, the results shown in Table 3 indicate that a viral protein is not required for the processing and transport of the various early RNAs. Furthermore, these results, together with those shown in Fig. 4, suggest that the large increase in mRNA production observed in the presence of cycloheximide cannot be accounted for by an increased rate of transcription. The increase in mRNA must therefore depend on either an increased mRNA stability in the absence of protein synthesis or an increased efficiency of processing of the primary nuclear transcripts.

Finally, the normal decline in RNA synthesis from transcription units 3 and 4 did not occur in the presence of cycloheximide, suggesting a possible role for a viral protein in actively repressing RNA synthesis from these regions. Perhaps this "repression" is a property of the 72,000-dalton DNA-binding protein specified by mRNA from region 2. Early transcription continues, at the nonpermissive temperature, in cells infected with a temperature-sensitive mutant of Ad5 (*ts125*) (4-6) that has been mapped to the region coding for the 72,000-dalton protein. After infection with this mutant under permissive conditions, total Ad2-specific RNA synthesis declines after about 6 h postinfection. Therefore, our results showing that cycloheximide prevents the repression of transcription of regions 2 and 4 may also be due to a prevention of formation of the 72,000-dalton DNA-binding protein.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA 16006-05 from the National Institutes of Health and by grant VC 295H from the American Cancer Society. H.S.G. was a faculty scholar of the American Cancer Society (no. SG 96). M.C.W. was a training grant (CA 09256) fellow.

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