Expression of Adenovirus Type 2 Late Genes in HeLa Cells Infected with Adenovirus Type 2 or Adenovirus Type 2-Simian Virus 40 Hybrid Viruses

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The relative levels of expression of the five 3'-coterminal mRNA families encoded in the 16.4 to 99 map unit transcription unit of adenovirus type 2 (Ad2) are determined in part by the frequency of utilization of five possible polyadenylation sites. The possibility that polyadenylation frequencies at these sites may be regulated was tested by examining the expression of the fiber (polypeptide IV) gene in cells infected with nondefective (ND) Ad2-simian virus 40 hybrid viruses which express a sixth late mRNA family. Both the relative rate of fiber protein synthesis and the relative abundance of fiber mRNA were normal in cells infected with the hybrid viruses Ad2⁺ND1, Ad2⁺ND2, or Ad2⁺ND4.

The generation of 3' termini for eucaryotic mRNA's is a possible point of regulation of gene expression. Present evidence suggests that transcription by RNA polymerase II through mRNA polyadenylation [poly(A)] sites followed by endonucleolytic cleavage and poly(A) addition at these sites is a common, if not general, mechanism for the generation of 3' termini of polyadenylated mRNA's. Such a mechanism has been demonstrated for the major late transcription unit of adenovirus type 2 (Ad2; 15), two early Ad2 transcription units (14), the simian virus 40 (SV40) late transcription unit (3), and the mouse β -major globin gene (4).

This process can potentially be regulated in two different ways. First, the utilization of different possible poly(A) sites within one transcription unit can specify the synthesis of mRNA's encoding different polypeptides. The switch in the synthesis of immunoglobulin M (IgM) from the membrane-bound form to the secreted form in B lymphocytes is most likely due to differential usage of two potential poly(A) sites in the heavy-chain gene (1, 2). The synthesis of five late mRNA families and two early mRNA families of Ad2 results from the utilization of five and two poly(A) sites, respectively, within single transcription units (15, 21). The multiple forms of dihydrofolate reductase mRNA in murine cells may also be due to the usage of different poly(A) sites; however, in this case all mRNA forms probably encode the same protein (19).

The efficiency with which a potential poly(A)

site in a primary transcript is used can also determine the abundance of the mature mRNA resulting from the processing of that transcript. This type of regulation may explain the inefficient expression of certain Ad2 late mRNA families early in infection (16, 20).

The major late transcription unit of Ad2 is an important model for the study of the polyadenylation mechanism and its regulation because it includes poly(A) sites whose use may be regulated, at least early in infection (see above), and because the large quantities of late mRNA's synthesized late in infection will facilitate the study of these processes at the biochemical level. Nevins and Darnell (15) have found that a portion of possibly every transcript of this transcription unit is conserved and transported to the cytoplasm as mRNA and that the portion of the transcript which is conserved is determined by which of the five possible poly(A) sites in each transcript is utilized for cleavage and poly(A) addition. Because each of the mRNA's encoded in this transcription unit have the same 5'-three-segment leader sequence encoded only once per transcript, it is clear that each transcript can only be processed to a single mRNA and that utilization of the various poly(A) sites for cleavage must be limited to allow the expression of mRNA's encoded downstream from them. The mechanism by which poly(A) sites are chosen and whether this choice is regulated are of considerable interest.

We approached the question of whether the choice of poly(A) sites may be regulated by using the nondefective (ND) Ad2-SV40 hybrid viruses isolated by Lewis et al. (9). The genome of these

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adenoviruses has a deletion from approximately 81 to 86 map units and an insertion of a portion of the early region of the SV40 genome in the same polarity of transcription as the Ad2 late genes (5, 6, 11-13). The different viruses in this series have different amounts of the SV40 early region.

The SV40 sequences in these viruses are expressed late in infection as a series of proteins related to SV40 T-antigen but not normally observed in cells infected or transformed with SV40 (10, 22). These proteins are specified by a family of SV40-specific mRNA's which are 3'-coterminous at the SV40 early poly(A) site and have the late three-segment Ad2 leader spliced to their 5' termini (Fig. 1). These features demonstrate that the SV40 mRNA's are likely to be transcribed and processed in a manner similar to the late Ad2 mRNA's; that is, they are transcribed as part of a primary transcript initiated at 16.4 on the Ad2 map followed by cleavage and polyadenylation of the transcript at the SV40-coded site and splicing of Ad2 leader sequences to SV40-coded sequences (8, 17, 23). Thus, the family of SV40-specific mRNA's form a sixth 3'-coterminous late mRNA family in the nondefective Ad2-SV40 hybrid viruses.

SV40-specific sequences are expressed at a high level in cells infected with the hybrid viruses. In cells infected with Ad2⁺ND4, 18% of the late cytoplasmic RNA is SV40 specific (17), indicating efficient use of the SV40-coded poly(A) site. This is an underestimate of the molar fraction of SV40 mRNA's conserved from the 16.4 to 99 transcript because the SV40-spe-

cific RNAs are, on the average, of lower molecular weight than the abundant Ad2 mRNA's and because transcription from the other transcription units which are active late in infection is not taken into account.

The efficient expression of the sixth mRNA family (SV40 specific) in the 16 to 99 transcription unit of the hybrid viruses is particularly interesting in light of the observations of Nevins and Darnell (15) which demonstrate that the relative rates of synthesis of the various Ad2 mRNA families in this transcription unit are determined by the relative frequencies with which the five possible poly(A) sites are utilized. The observed level of expression of SV40 sequences in the hybrid viruses would be expected to result in a severe polar effect on the expression of fiber mRNA because the SV40 insertion in the Ad2 genome is just to the 5' side of the region which specifies the fiber mRNA main body. It can be estimated from the frequencies of conservation of sequences adjacent to poly(A) in the five Ad2 mRNA families measured by Nevins and Darnell (15) that approximately 80% of the nascent transcripts of the 16.4 to 99 transcription unit will be cleaved at the four Ad2-specified sites coded upstream from the SV40 insertion. To produce the observed level of expression of SV40 sequences in Ad2⁺ND4, virtually all of the remaining unprocessed transcripts would have to be cleaved at the SV40-coded poly(A) site, resulting in very few unprocessed transcripts which could become fiber mRNA. Consequently, a strong polar effect on the expression of that gene would be expected

FIG. 1. Late mRNA families of Ad2 and Ad2⁺ND4. The SV40-specific mRNA's of Ad2⁺ND4 represent a sixth late mRNA family in the 16.4 to 99 transcription unit of this virus. The structure of the Ad2 late mRNA's present in cells infected with either Ad2 or Ad2⁺ND4 is indicated in the upper portion of the figure. The additional SV40-specific mRNA family present in cells infected with Ad2⁺ND4 is indicated in the lower portion of the figure. Bars represent the spliced structure of the mature mRNA's; arrows indicate the 5' → 3' direction of transcription. The position of poly(A) sites in the genome of each virus is also indicated.

in hybrid virus-infected cells. On the other hand, if the relative expression of the late mRNA families is actively regulated at a post-transcriptional level, possibly by modulating the poly(A) frequency at the Ad2-coded sites, then a reduced polar effect or no polar effect at all on the expression of the fiber gene might be expected in hybrid virus-infected cells. Thus, the synthesis of the fiber polypeptide and its mRNA in hybrid virus-infected cells should give some indication as to whether the poly(A) frequencies at the Ad2-specified sites are regulated.

We examined the possibility of such a polar effect on the expression of the fiber gene by measuring the relative rate of fiber protein synthesis in HeLa cells infected with Ad2 or the hybrid viruses Ad2⁺ND1, Ad2⁺ND2, and Ad2⁺ND4. These were chosen because they contain different fractions of the 3' portion of the early region of SV40 and express the SV40 sequences efficiently late in infection (22). Infected cells were pulse-labeled with [³⁵S]methionine (Amersham Corp.), whole cells were collected and lysed, and total labeled proteins were analyzed on a polyacrylamide gel (Fig. 2). Radioactivity in the major late viral proteins was quantified. The comparison of the rates of synthesis of viral proteins is illustrated in the bar graph in Fig. 3B. All late viral proteins were synthesized in about the same relative level in cells infected with wild-type Ad2 or the three hybrid viruses. In particular, there was no significant difference in the amount of the fiber polypeptide being synthesized in the hybrid virus-infected cells.

Since the presence of reduced levels of fiber mRNA in hybrid virus-infected cells might be overcome by varying the efficiency of translation of the late mRNA's, the levels of late mRNA's actually present in the infected cells were assayed by cell-free translation in the messenger-dependent reticulocyte lysate system (18) of mRNA isolated from cells infected with the four different viruses. Before the experiment we determined that under the conditions used the amount of all viral polypeptides synthesized *in vitro* was directly proportional to the amount of their mRNA's added to the cell-free system (data not shown). Figure 2 shows the translation products labeled *in vitro* with [³⁵S]methionine and analyzed on a polyacrylamide gel. Radioactivity in the major proteins was quantified and compared in Fig. 3C. Again, no significant differences were evident in the relative abundance of the various mRNA's among the four viruses, including the mRNA for fiber.

We conclude that the presence of the sixth mRNA family (in the hybrid virus genome) just upstream from the fiber gene has little or no

FIG. 2. Fluorographs of polyacrylamide gel analysis of Ad2 proteins synthesized *in vivo* and *in vitro*. HeLa cells grown in suspension culture were infected with 50 PFU of Ad2, Ad2⁺ND1, Ad2⁺ND2, or Ad2⁺ND4 per cell as described previously (7). At 24 h after infection, a portion of each culture was used for *in vivo* pulse-labeling or isolation of RNA. In *in vivo* lanes: 1 ml of each culture was labeled with 120 μ Ci of [³⁵S]methionine at 37°C for 30 min. Ice-cold saline (10 ml) was added, and cells were collected by centrifugation and suspended in 200 μ l of gel sample buffer. A 15- μ l amount was analyzed on a 12.5% polyacrylamide gel (7). In *in vitro* lanes: poly(A)-containing cytoplasmic RNA from 70 ml of each culture was isolated as previously described (7) and dissolved in a final volume of 25 μ l of H₂O. A 0.1- μ l amount of each was translated in 10 μ l of messenger-dependent reticulocyte lysate in the presence of [³⁵S]methionine and analyzed on a 12.5% polyacrylamide gel. Polypeptide IX comigrates with unlabeled globin present in the lysate and appears as a wide band in the gel. Quantification of radioactivity in the viral proteins in this figure is presented in Fig. 3B and C.

effect on the relative expression of that gene. There are two possible interpretations of this result. The first is that normally only a fraction of the transcripts of the late transcription unit of Ad2 are processed to become mature mRNA so that an insertion of a sixth poly(A) site would not have a significant effect on the expression of fiber mRNA. This would, however, contradict the results of Nevins and Darnell (15) and seems

FIG. 3. (A) Radioactivity of ^{35}S -labeled viral proteins present in purified virions. Purified Ad2 virions were isolated from infected cells labeled with [^{35}S]methionine, viral proteins were resolved on a polyacrylamide gel (data not shown), and the radioactivity present in excised bands was quantified. The radioactivity in each polypeptide is expressed as a fraction of the radioactivity present in polypeptide II (hexon) and plotted above. (B) Relative rate of synthesis of viral proteins. Radioactivity in viral proteins pulse-labeled in vivo and analyzed in Fig. 2 was quantified from excised gel bands. Radioactivity incorporated into each protein is a measure of its rate of synthesis because degradation of viral protein is insignificant during the labeling period. Incorporation is expressed as a fraction of the radioactivity present in polypeptide II. The group of four values for each polypeptide represents the incorporation into that protein in cells infected with (from left to right): Ad2, Ad2⁺ND1, Ad2⁺ND2, Ad2⁺ND4. (C) Messenger RNA activities for viral proteins. Proteins synthesized in vitro and analyzed in Fig. 2 were quantified and expressed as in (B). Incorporation into each protein is a measure of the abundance of its mRNA in the infected cell.

likely not to be the case. The second possibility is that fiber mRNA levels are regulated in such a way as to produce a particular ratio of Ad2 late mRNA's.

Three possible mechanisms by which the relative abundance of fiber mRNA might be regulated are: (i) altering the poly(A) frequency at

the Ad2-specified sites, (ii) altering the rates of mRNA turnover, and (iii) altering the efficiency of mRNA transport to the cytoplasm. Since polyadenylated Ad2 mRNA's are transported quantitatively to the cytoplasm (15), transport does not seem to be a likely point of control in this system. The two remaining possibilities can

be distinguished by direct measurement of poly(A) frequencies at the six possible sites and by determination of mRNA stabilities in the hybrid virus-infected cells. We favor the idea that the potential polar effect is overcome by modulating the poly(A) frequency at Ad2-specific sites because the expected magnitude of the polar effect is so large that stabilization of the few transcripts which would be processed to fiber mRNA would not be sufficient to produce normal fiber mRNA levels in the hybrid virus-infected cells.

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LITERATURE CITED

- Alt, F. W., A. L. M. Bothwell, M. Knapp, E. Siden, E. Mather, M. Koshland, and D. Baltimore. 1980. Synthesis of secreted and membrane bound immunoglobulin mu heavy chains is directed by mRNAs that differ at their 3' ends. *Cell* 20:293-301.
- Early, P., J. Rogers, M. Davis, K. Calame, M. Bound, R. Wall, and L. Hood. 1980. Two mRNAs can be produced from a single immunoglobulin gene by alternative RNA processing pathways. *Cell* 20:313-319.
- Ford, J., and M.-T. Hsu. 1978. Transcription pattern of in vivo labeled late simian virus 40 RNA: equimolar transcription beyond the mRNA 3' terminus. *J. Virol.* 28:795-801.
- Hofer, E., and J. E. Darnell, Jr. 1981. The primary transcription unit of the mouse β -major globin gene. *Cell* 23:585-593.
- Kelly, T. J., Jr., and A. M. Lewis, Jr. 1973. Use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. *J. Virol.* 12:643-652.
- Khoury, G., A. M. Lewis, Jr., M. N. Oxman, and A. S. Levine. 1973. Strand orientation of SV40 transcription in cells infected by nondefective adenovirus 2-SV-40 hybrid viruses. *Nature (London) New Biol.* 246:202-205.
- Lawrence, C. B. 1980. Multiple mRNA species for adenovirus type 2 polypeptides III and pVII. *J. Virol.* 35:306-313.
- Lawrence, C., T. Hunter, and G. Walter. 1979. Characterization of the simian virus 40-specific messenger RNAs isolated from HeLa cells infected with the nondefective adenovirus 2-simian virus 40 hybrid viruses Ad2*ND2 and Ad2*ND4. *J. Mol. Biol.* 134:95-108.
- Lewis, A. M., Jr., M. J. Levin, W. H. Wiese, C. S. Crumpacker, and P. H. Henry. 1969. A nondefective (competent) adenovirus-SV40 hybrid isolated from the Ad2-SV40 hybrid population. *Proc. Natl. Acad. Sci. U.S.A.* 63:1128-1135.
- Mann, K., T. Hunter, G. Walter, and H. Linke. 1977. Evidence for simian virus 40 (SV40) coding of SV40 T-antigen and the SV40 specific proteins in HeLa cells infected with nondefective adenovirus type 2-SV40 hybrid viruses. *J. Virol.* 24:151-169.
- Meyer, J., P. D. Neuwald, S.-P. Lai, J. V. Maizel, Jr., and H. Westphal. 1977. Electron microscope of late adenovirus type 2 mRNA hybridized to double-stranded viral DNA. *J. Virol.* 21:1010-1018.
- Morrow, J. F., P. Berg, T. J. Kelley, Jr., and A. M. Lewis, Jr. 73. Mapping of simian virus 40 early functions on the viral chromosome. *J. Virol.* 12:653-658.
- Mulder, C., P. A. Sharp, H. Delius, and U. Pettersson. 1974. Specific fragmentation of DNA of adenovirus serotypes 3, 5, 7, and 12, and adeno-simian virus 40 hybrid virus Ad2*ND1 by restriction endonuclease R-EcoRI. *J. Virol.* 14:68-77.
- Nevins, J. R., J.-M. Blanchard, and J. E. Darnell, Jr. 1980. Transcription units of adenovirus type 2: termination of transcription beyond the poly(A) addition site in early regions 2 and 4. *J. Mol. Biol.* 144:377-386.
- Nevins, J. R., and J. E. Darnell, Jr. 1980. Steps in processing of Ad2 mRNA: poly(A)⁺ nuclear sequences are conserved and poly(A) addition precedes splicing. *Cell* 15:1477-1493.
- Nevins, J. R., and M. C. Wilson. 1981. Regulation of adenovirus-2 gene expression at the level of transcriptional termination and RNA processing. *Nature (London)* 290:113-118.
- Oxman, M. N., M. J. Levin, and A. M. Lewis, Jr. 1974. Control of simian virus 40 gene expression in adenovirus-simian virus 40 hybrid viruses. Synthesis of hybrid adenovirus 2-simian virus 40 RNA molecules in cells infected with a nondefective adenovirus 2-simian virus 40 hybrid virus. *J. Virol.* 13:322-330.
- Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.
- Setzer, D. R., M. McGrogan, J. H. Nunberg, and R. T. Schimke. 1980. Size heterogeneity in the 3' end of dihydrofolate reductase messenger RNAs in mouse cells. *Cell* 22:361-370.
- Shaw, A. R., and E. B. Ziff. 1980. Transcripts from the adenovirus-2 major late promoter yield a single early family of 3' coterminal mRNAs and five late families. *Cell* 22:905-916.
- Stilman, B. W., J. B. Lewis, L. T. Chow, M. B. Mathews, and J. E. Smart. 1981. Identification of the gene and mRNA for the adenovirus terminal protein precursor. *Cell* 23:497-508.
- Walter, G., and H. Martin. 1975. Simian virus 40 specific proteins in HeLa cells infected with nondefective adenovirus 2-simian virus 40 hybrid viruses. *J. Virol.* 16:1236-1247.
- Westphal, H., S.-P. Lai, C. Lawrence, T. Hunter, and G. Walter. 1979. Mosaic adenovirus-SV40 RNA specified by the nondefective hybrid virus Ad2*ND4. *J. Mol. Biol.* 130:337-351.