JOURNAL OF VIROLOGY, Feb. 1989, p. 532–541
0022-538X/89/020532-10$02.00/0

Vol. 63, No. 2

Regulation of Poly(A) Site Selection in Adenovirus

ERIK FALCK-PEDERSEN[1]* AND JOHN LOGAN[2]

Hearst Research Foundation, Department of Microbiology, Cornell University Medical College, 1300 York Avenue, New York, New York 10021,[1] and Agricultural Research Division, American Cyanamid Company, Princeton, New Jersey 08540[2]

Received 8 August 1988/Accepted 24 October 1988

We have investigated the mechanisms involved in the early-to-late RNA-processing switch which regulates the mRNA species generated from the adenovirus major late transcription unit (MLTU). In particular, polyadenylation choice mechanisms were characterized by using a reconstructed adenovirus E1A gene as a site for insertion of MLTU poly(A) regulation signals (L1 and L3). Adenovirus constructs containing the variant poly(A) recognition elements were used to compare E1A poly(A) signal utilization with wild-type MLTU (L1 to L5) utilization. In both early and late stages of infection, either polyadenylation site (L1 or L3) is capable of being utilized when presented as the only operational poly(A) site. In an early infection, a virus which contains multiple elements presented in tandem (L13) uses the first poly(A) site, L1, preferentially (ratio of L1 to L3, 8:1) in both E1A and MLTU loci. Transcription termination is not involved in restricting the utilization of the downstream L3 site. In a late infection, when each of the five MLTU poly(A) sites is used, a switch also occurs for the E1AL13 construct, with utilization of both the L1 and L3 poly(A) sites. The switch from early to late was not the result of altered processing factors in the late infection, as demonstrated by superinfecting the E1AL13 construct into cells which had already entered a late stage of infection. The superinfecting virus gave an L1-only phenotype; therefore, a *cis* mechanism is involved in adenovirus poly(A) regulation.

Regulation of mRNA biosynthesis is a complex and poorly understood event in eucaryotic cells. Transcription initiation, RNA elongation, RNA processing, and transcription termination are nuclear events leading to the synthesis of mRNA (18). Following processing, the mRNA is transported from the nucleus to the cytoplasm, where translation occurs. Within the cytoplasm, mRNA turnover (half-life) is an additional type of posttranscriptional regulation which influences the mRNA population of each cell (18). Complex transcription units are distinguished from simple transcription units by the RNA processing alternatives available in splice donor sites, splice acceptor sites, and polyadenylation sites (12, 34, 45, 47). Because of this diversity, they are capable of producing a variety of gene products depending on the final processing choices. These processing choices can be regulated by tissue-specific (3, 4, 31), developmental (2, 6), or temporal (44, 50) means. The molecular mechanisms involved in selecting splice sites and polyadenylation sites have been technically difficult to study and remain largely undefined.

Two cellular transcription units, the calcitonin–calcitonin gene-related peptide tissue-specific transcription unit and the developmentally controlled immunoglobulin M (IgM) heavy-chain μ-s (secreted) and μ-m (membrane-associated) mRNAs, have been used as models to characterize processing control mechanisms (3, 4, 17, 21, 33, 40, 48). In both of these transcription units the processing choice actually involves two choices, a polyadenylation site choice and a splice acceptor choice. Regulation of mRNA processing requires a *cis* linkage of processing elements and an appropriate cellular environment; this implies a coupling of processing reactions to *cis* RNA structure and/or coupling to transcription complexes. For the IgM processing switch, transcription termination is occurring in a tissue-specific manner (21, 40), which has a direct effect on the use of downstream polyadenylation sites. It has also been shown that the length of spacing between recognition elements can influence their use (21, 48). In the heavy-chain IgM studies it is still not known whether poly(A) site choice or splicing is acting as the major regulating force. Control of the calcitonin–calcitonin gene-related peptide processing choice is interpreted as being directed more by splice commitment than by poly(A) site choice (33). Regardless of which choice is considered the primary mode of regulation, the processing elements can function in both of these systems independently. A choice between potential processing sites is made only when the elements are coupled in a *cis* orientation.

Another example of a regulated complex transcription unit is the adenovirus major late transcription unit (MLTU), where a temporal switch in processing affects the mRNA population (1, 46, 50). This transcription unit dominates a late adenovirus infection, producing as much as 10% of total steady-state poly(A)$^+$ mRNA, which codes for the bulk of viral structural proteins (38). The MLTU contains five mRNA families distinguished by their polyadenylation elements (L1 to L5), all of which are used in the late viral infection (20, 46, 50). Each family is then subdivided into three to seven different mRNA species on the basis of alternative use of multiple splice acceptor sites (7, 14, 15, 46). The MLTU is also expressed in an early infection before DNA replication has occurred. The diversity of MLTU mRNAs produced in the early infection is greatly reduced, with only the first (L1) of the poly(A) families being substantially represented (46, 50). Development of an understanding of the regulatory mechanisms controlling the temporal early-to-late switch in RNA processing should provide insight into similar types of RNA processing control acting on cellular genes. We have taken advantage of the viral system to ask some basic questions about the mechanisms involved in poly(A) choice.

In the work described in this report we have used the adenovirus E1A transcription unit to examine in a simple and stepwise fashion the elements contributing to the regu-

* Corresponding author.

lation of poly(A) site selection. We chose adenovirus sub-360 (37) as a parent virus because it expresses high levels of E1A both early and late in the viral infection as a result of insertion of the major late transcription control region (MLTCR) substituted directly 5′ to the E1A cap site. Using the E1A poly(A) constructs, we examined the operation of specific sequence elements in a new locus (the E1A transcription unit) expressed in the same genomic context early and late in a virus infection. We also compared regulation of the E1A poly(A) transcription unit constructs with the regulation of processing signals from the major late transcription unit as an internal control. The work presented here illustrates several key events in choice of poly(A) sites. The data support a mechanism for poly(A) site selection operating on a first-come first-served basis in an early infection, with any functional polyadenylation element and regardless of the number of polyadenylation elements present. This restricted use of the first poly(A) site is not due to a transcription termination event as has been shown for the IgM locus, nor is there a sequence-specific factor operating early in the virus infection. From superinfection experiments, we demonstrate that the processing switch is mediated by *cis* interactions, not by the presence or absence of *trans*-acting processing factors. This implies a direct interaction of processing choice with either the DNA template or the character of the elongating transcription complex.

MATERIALS AND METHODS

Virus and cell culture. Human 293 monolayer cells which express the E1A and E1B regions of adenovirus (23) were used for all virus constructions and propagation of virus stocks. They were maintained in Eagle medium containing 10% fetal bovine serum. Both 293 and HeLa Spinner cells were used in experimental studies and were maintained in Joklik modified Spinner media plus 5% fetal bovine serum. The virus was purified as previously described (43).

Plasmid and virus constructions. The parent plasmid, dlpMLP6, was constructed by deleting adenovirus sequences from positions 1571 to 2045 present in pMLP6 (29, 36). Polyadenylation elements were taken from existing adenovirus type 2 genomic subclones (46). The L1 element spans nucleotides 13710 to 14281 (571 base pairs [bp]), with AAUAAA at 14092 and the poly(A) addition site at 14113. The L3 sequence starts at nucleotide 22233 and ends at 22790 (557 bp), with AAUAAA at 22418 and a poly(A) addition site 22443. The lengths of the inserted exon sequences for the L1 and L3 fragments are 403 and 210 nucleotides, respectively. Following insertion of L1, L3, or tandemly linked L13, into dlpMLP6, each plasmid was used for virus construction by the method of overlap recombination (13). Plasmids were linearized by *Eco*RI digestion and cotransfected into 293 cells with the large *Xba*I fragment (nucleotides 1336 to 35937) of *dl*309.

The virus nomenclature follows the parent virus sub-360 as 1200, 1200-L1, 1200-L3, and 1200-L13. The 1200 series are deleted in the normal E1A polyadenylation site as well as the E1B promoter; therefore, expression of the E1B sequence is the result of E1A readthrough. E1 region genomic subclones contain inserts which map to positions 0 to 1010 (1A1), 1010 to 1336 (1A2), 1336 to 1674 (1A3), 2055 to 2510 (1B1), 2810 to 3328 (1B2), 3328 to 3827 (1B3), and 3806 to 4235 (1B4). M13mp18 E1AL13 was subcloned from dlpMLP6-L13 and contains sequence from 1105 to 2420 (1,950 bp).

Preparation of RNA. HeLa cells were harvested after a 4-h infection with 25 PFU per cell, and nuclei were isolated by Dounce homogenization in reticulocyte standard buffer (RSB). Nuclei were washed with transcription buffer and resuspended in transcription buffer (100 μl/10^8 cells) with 0.6 mM ATP, CTP, and GTP. [^{32}P]UTP was used at a final concentration of 2.5 μM at 40 μCi/nmol (750 μCi/ml). Transcription elongation was done for 10 min at 30°C and was followed by DNase I digestion and hot-phenol extraction (43, 49). RNA was then purified by 2 M LiCl precipitation. Processing of RNA for hybridization included alkali cleavage (with 0.1 M NaOH for 10 min), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) neutralization, and hybridization to DNA dots in 2× TESS (18, 30, 46). In vivo labeling required 2 × 10^8 293 cells infected as above and concentrated to 10^7 cells/ml in the presence of 200 μCi of [^{3}H]uridine per ml for 15 min at 37°C. Total RNA was purified as described above. Poly(A)$^+$ RNA was isolated by passage over oligo(dT)-cellulose and eluted as described previously (39).

Northern (RNA) blot, S1 and T_1-S1 analysis of RNA or RNA-DNA duplex. Oligo(dT)-cellulose-selected poly(A)$^+$ RNA was loaded onto a 50-mM borate–3% formaldehyde–1.4% agarose gel and, following electrophoresis, transferred to nitrocellulose and probed with nick-translated DNA probes.

S1 endonuclease analysis of mRNA was performed as follows. An *Xba*I digest of dlpMLP6-L13 released the 1,152-bp L13 insert, which was purified in a low-melting-temperature agarose gel. The Klenow fragment of DNA polymerase I was used to fill in the 3′ end of each strand in the presence of [^{32}P]dCTP, and the 3′ end of the L3 termini was then removed by *Sau*3A digestion. The DNA fragment was then phenol extracted and ethanol precipitated. The labeled fragment was hybridized to 2 μg of poly(A)$^+$ RNA at 55°C in 80% formamide–20 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (PIPES)–0.4 M NaCl–1 mM EDTA for 3 h. Samples were immediately diluted with buffer containing 50 mM sodium acetate (pH 4.5), 200 mM NaCl, and S1 endonuclease at 100 U/ml (Boehringer Mannheim Biochemicals). Following digestion at 37°C for 30 min, samples were phenol extracted, ethanol precipitated, and loaded onto a denaturing formaldehyde gel as described above.

For the T_1-S1 nuclease experiment (54), [^{3}H]uridine-labeled poly(A)$^+$ RNA (as described above) from 10^8 293 cells infected at 50 PFU per cell for 4 or 18 h was coprecipitated with 100 μg of single-stranded M13mp18 E1AL13 and suspended in 200 μl of H_2O. After 10 min at 80°C, 50 μl of 5× hybridization buffer was added (5× hybridization buffer is 3.7 M NaCl, 0.25 M HEPES [pH 7.0], and 5 mM EDTA), and the mixture was hybridized at 55°C overnight. Samples were diluted with sterile H_2O to 0.3 M NaCl, and 150 U of RNase T_1 was added. RNase digestion was carried out at 30°C for 2 h and was followed by treatment with proteinase K (200 μg/ml) at 37°C for 1 h. NaCl was added to 0.75 M, and the sample was filtered through a nitrocellulose filter (25 mM; pore size, 0.45 μm) which had been boiled and equilibrated in hybridization buffer. Filters were washed with the same buffer and then transferred to a glass vial and exposed for 1 h at 37°C to 1.0 ml of S1 buffer containing 1,000 U of S1. The supernatant was collected (filters rinsed with S1 buffer), phenol-$CHCl_3$ (1:1) extracted, and ethanol precipitated in the presence of 30 μg of carrier yeast RNA. Precipitated RNA-DNA duplex was loaded onto nondenaturing 1.4% agarose gels. Following electrophoresis, the gels were treated with liquid En^{3}Hance (Du Pont, NEN Research Products) for 1 h and then rinsed with H_2O and dried. Dried gels were exposed to Kodak XAR-7 film for designated times

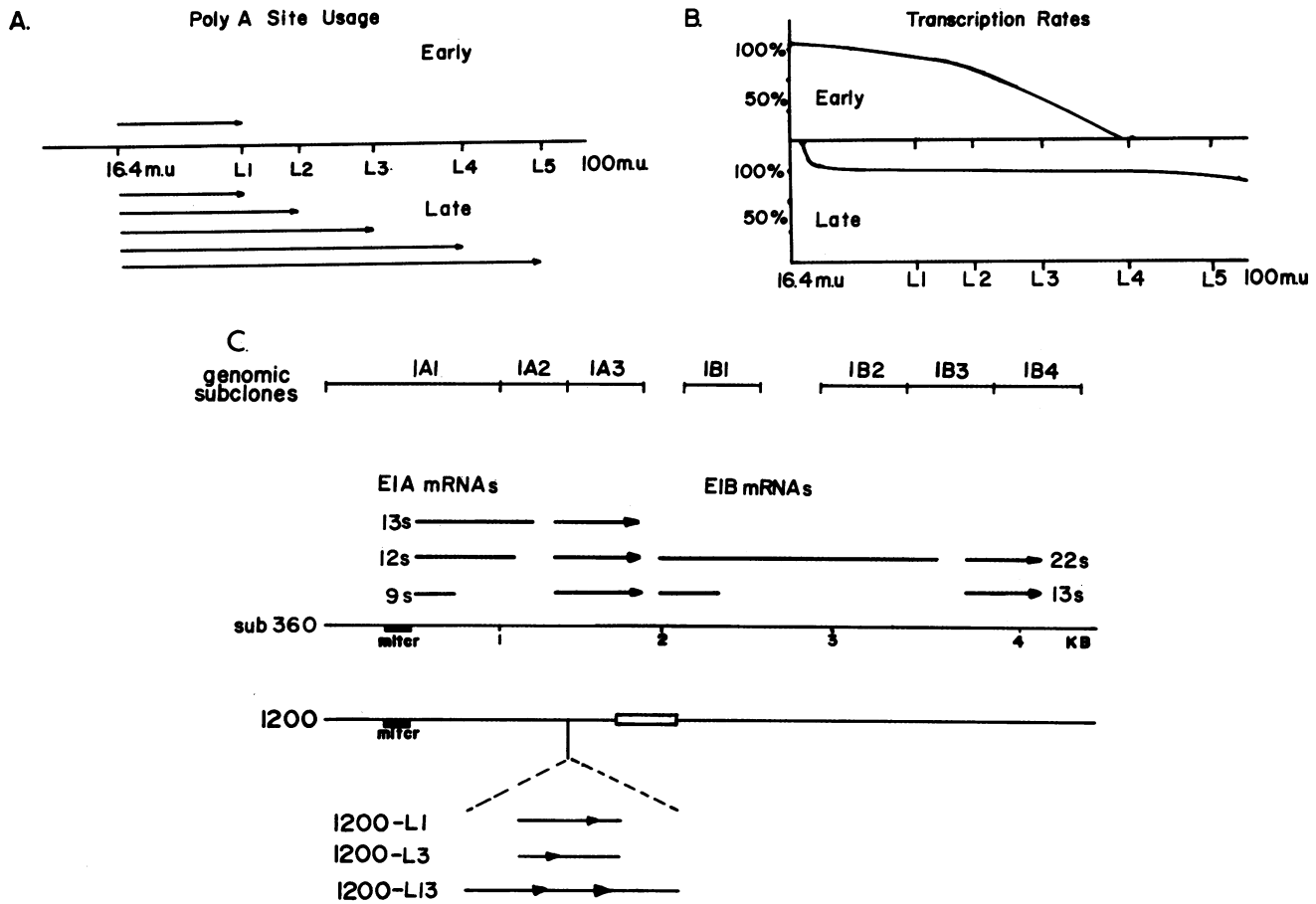

FIG. 1. Genomic organization of MLTU poly(A) sites and construction of E1A poly(A) recombinants. (A) Schematic representation of MLTU poly(A) site utilization in an early and a late infection. The five poly(A) families, L1 to L5, show temporal regulation, with L1 dominating the early infection and L1 to L5 being utilized in the late infection. (B) Transcription rate analysis across the 30.5-kilobase MLTU (12, 13, 40) indicates equimolar transcription across the entire MLTU unit in the late adenovirus infection, but early, transcription begins to decline 3′ to the L1 poly(A) site and rapidly declines 3′ to the L3 site with no transcription proceeding into the L4 coding region. (C) Diagram of plasmids used to construct poly(A) virus constructs. Parental sub-360 contains wt E1A and E1B sequences, differing from wt adenovirus by substitution of the MLTCR upstream of the E1A cap site. In 1200, sequences containing the E1A polyadenylation site and the E1B promoter (nucleotides 1571 to 2045) were deleted. The L1, L3, or L13 fragments isolated from genomic subclones were inserted at the *Xba*I site (nucleotide 1366) by blunt-end ligation (L1 and L3) or with *Xba*I linkers (L13). Genomic subclones 1A1, 1A2, 1A3, 1B1, 1B2, 1B3, and 1B4 used as probes were constructed into plasmid vectors as described previously (19). E1A splice products (9S, 12S, and 13S mRNAs) represent wt E1A, and 13S and 22S represent the major E1B mRNA products. m.u., Map units.

without intensifying screens. After fluorography the dried gels were peeled from the support paper, treated with 0.5 M NaOH–1.5 M NaCl, and neutralized with 0.5 M Tris–1.5 M NaCl. These slightly hydrated gels were hybridized to nick-translated L13 DNA probe for 15 h and then washed and dried. They were then exposed to film in the presence of a Du Pont Cronex Lightning-Plus intensifying screen.

RESULTS

Characterization of steady-state mRNA production from E1A poly(A) constructs. In an early adenovirus infection, the MLTU expresses mRNA products derived predominantly from the L1 family, whereas in a late adenovirus infection, all five poly(A) recognition sites are used (46, 50) (Fig. 1A). Our approach to characterizing how this processing switch is effected has been to construct an artificial and simplified MLTU at the E1A locus. In the E1A poly(A) constructs (Fig. 1C), we placed the L1, L3, or tandemly arranged L13 polyadenylation elements into the E1A second exon present within the dlpMLP6 plasmid (the plasmid counterpart to the 1200 virus [Fig. 1C]) where the E1A polyadenylation site and the E1B promoter were removed. By using homologous recombination, these plasmids were then built into viable adenovirus constructs which carry our altered E1A sequences.

The use of the E1A poly(A) viruses allows us to ask whether under similar nuclear conditions we can detect sequence specificity in the mechanism associated with the restricted use of the L1 poly(A) site. With the 1200 poly(A) viruses and the wild-type (wt) sub-360 virus as a control, HeLa cells were infected for 4 h, and poly(A)$^+$ mRNA was isolated by oligo(dT) selection. The mRNA was applied to a formaldehyde-agarose gel and probed with a nick-translated 5′ (1A1) DNA probe. Depending on the polyadenylation site used, each virus should produce a characteristic size of mRNA which corresponds to the amount of E1A exon 1 (12S and 13S exons for early infections; 9S, 12S, and 13S exons for late infections [Fig. 1C]) plus exon 2 sequences from the

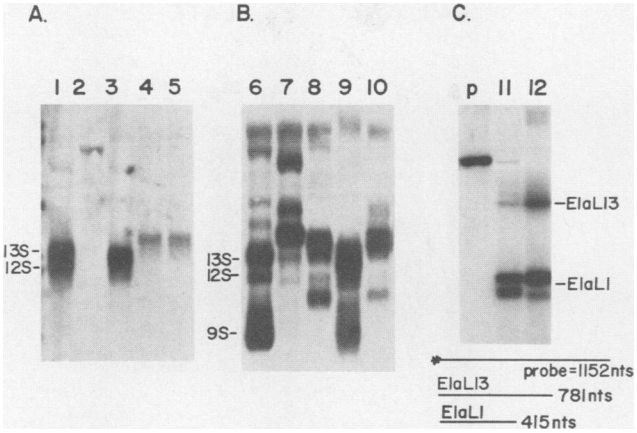

FIG. 2. Analysis of steady-state poly(A)$^+$ mRNA from 1200 poly(A) infections. (A) Early HeLa cell poly(A)$^+$ RNA (2 μg) isolated from HeLa cells infected with 25 PFU of sub-360 (lane 1), 1200 (lane 2), 1200-L3 (lane 3), 1200-L1 (lane 4), or 1200-L13 (lane 5) per cell was electrophoresed in a 1.4% agarose–formaldehyde gel and transferred to a nitrocellulose filter. Following transfer, the filter was hybridized to nick-translated 1A1 genomic subclone (Fig. 1C). The arrows correspond to the expected 13S and 12S mRNA species, which predominate in an early adenovirus infection. (B) Poly(A)$^+$ mRNA (0.5 μg) prepared from late-infected HeLa cells (18 h) and electrophoresed as described above: sub-360 (lane 6), 1200 (lane 7), 1200-L3 (lane 8), 1200-L1 (lane 9), or *dl*362-L13 (lane 10). (C) S1 analysis of poly(A)$^+$ RNA (2 or 0.5 μg) isolated from an early (lane 11) or late (lane 12) infection of 293 cells with 25 PFU of 1200-L13. Probe (lane p) was end-labeled *Xba*I fragment (1,152 bp) isolated from dlpMLP6-L13 plasmid DNA. The expected products for utilization of the L1 or L13 polyadenylation site are 415 and 781 nucleotides, respectively. Following S1 digestion (see Materials and Methods), samples were electrophoresed in a 1.4% agarose–formaldehyde gel. nts, Nucleotides. *, L13 polyadenylation site.

common splice acceptor site to the specific poly(A) site of each virus. The control virus sub-360 is presented as a marker for E1A transcripts, with 9S, 12S, and 13S RNA species being 516, 879, and 1,017 nucleotides, respectively. The distance from the site of insertion in E1A to the L1 poly(A) site results in mRNA species larger by approximately 100 nucleotides, whereas use of the L3 polyadenylation site in 1200-L3 produces an mRNA which is 100 nucleotides shorter than wt E1A. The wild-type poly(A) site used in the parent sub-360 virus has been removed in the 1200 viral construct, so it is not a surprising result that the 1200 virus did not produce characteristic E1A mRNA (Fig. 2A, compare lanes 1 and 2). In 1200 there is a small amount of large transcript, which is consistent with the use of the downstream E1B poly(A) site.

The 1200-L3 and 1200-L1 E1A early transcripts (Fig. 2A, lanes 3 and 4) show that both sequence elements, when inserted into E1A exon 2, are capable of being recognized by the poly(A) processing machinery that is present in the early infection. It is interesting that the L3 transcript is present at higher levels than the L1 transcript when they are presented as separate elements. This indicates that the early virus infection does not create a factor or environment which acts to suppress L3 utilization, and there does not seem to be a sequence-specific component which favors the L1 element over the L3 element.

The virus 1200-L13, which contains the L1 element immediately 5′ to the L3 element (Fig. 1C), produces early E1A transcripts which by size correspond to utilization of only the L1 polyadenylation signal sequence (Fig. 2A, compare lane 5 with lane 4). In 1200-L13, the distance between the two elements is roughly 500 bp, compared with the 8.5 kbp which separate them in the MLTU. We have eliminated a considerable amount of spacer sequence and potential RNA secondary structure in these constructs, yet the preference for the first poly(A) site, L1, dominates in the E1A locus, just as it does in the MLTU locus. When the plasmid for 1200-L13 is transfected into either HeLa or 293 cells, the L1 utilization still dominates (data not shown); therefore, this phenotype is not restricted to a virus function.

The late mRNAs produced from these constructs are represented by the expected 9S, 12S, and 13S RNA species with the sub-360 virus (Fig. 2B, lane 6) as a control for the normal E1A transcripts. Although sub-360 is used here as a control, it represents abnormally high levels of E1A expression owing to the presence of the MLTCR. This high level of expression enhances the visualization of a small amount of aberrantly processed transcript which utilizes a combination of E1A and E1B processing signals. In the late infection, the 1200 E1A transcripts are present at levels which are comparable to those of the normal sub-360 E1A mRNA (Fig. 2B, compare lanes 6 and 7) and, by their size, imply the use of the downstream E1B poly(A) site and a combination of E1B and E1A splicing sites. This is in contrast to the early expression, in which 1200 E1A was barely detectable (Fig. 2A, lane 2, versus Fig. 2B, lane 7). Both the 1200-L1 and the 1200-L3 poly(A) sites (Fig. 2B, lanes 8 and 9, respectively) are utilized in the late infection, with all three spliced products being present for each virus [although in different concentrations depending on the poly(A) site]. The sequences present in these elements are capable of functioning in both types of cellular environments, arguing against negative or positive sequence-specific regulation of poly(A) site utilization.

When measured in a late infection, the poly(A) site utilization occurring in the third viral construct, 1200-L13, gave a result which is difficult to interpret. The estimated sizes of the RNA products are L1 transcripts which after splicing should be 614, 977, and 1,115 nucleotides; mRNAs which utilize the L3 polyadenylation sites will be equal to the size of the 1200-L3 mRNA plus the 571 nucleotides which are contributed by the L1 insert 5′ to the L3 sequence; thus, transcripts which utilize the L3 poly(A) site will be 1,064, 1,517, and 1,665 nucleotides. From the Northern blot analysis (Fig. 2B, lane 10), we detected species which correspond to E1AL1 utilization (compare L1 with L13, lanes 8 and 10), but we also detected mRNA of increased size as well as a mobility shift in the 12S-13S band at ca. 1,100 nucleotides. Remembering that in a late infection the 9S splice product becomes a major species, the band at 1,100 nucleotides could be a composite of 12S and 13S species using the L1 poly(A) site and 9S species using the L3 poly(A) site. To determine the contribution of each poly(A) site to this population of mRNA, S1 analysis of adenylated mRNA from an early and late 1200-L13 infection was carried out (Fig. 2C, lanes 11 and 12, respectively). The RNA protection provided by the early virus represents a population of adenylated RNA which is produced almost exclusively by cleavage at the L1 poly(A) site. There are two S1 bands generated from the L1 poly(A) site which differ by approximately 35 nucleotides. This doublet was not seen in the Northern analysis or when run on a nondenaturing gel (Fig. 3, lane EtBr +) and is considered the result of an S1 artifact. The protected species from the late 1200-L13-infected mRNA shows that in addition to bands representing L1

poly(A) site utilization, a band is produced which corresponds to use of the downstream L3 site. Thus, at the level of steady-state mRNA, the early-to-late processing switch which operates on the wild-type major late transcription locus is also acting on our E1AL13 transcription unit. Using the reconstructed E1A transcription unit, we have been able to approximate the early-to-late processing switch. This implies that the regulatory components involved in the switch are in fact operative in our simplified transcription unit.

Comparison of wt and E1AL13 poly(A) sites by rapid pulse labeling and steady-state RNA comparisons. The Northern and S1 experiments suggest that a processing switch is occurring at the E1A locus of the 1200-L13 construct when it progresses to the late phase of a viral infection. The steady-state mRNA measurements present quantitatively reliable information about the overall concentration of a given transcript, but these data might be misleading in terms of the actual event of poly(A) site selection, since it occurs in the nucleus because of differential half-lives of mRNA species. A technique which has successfully characterized the nuclear polyadenylation event involves a rapid [^{3}H]uridine pulse-labeling of cells. Analysis of the newly labeled nascent transcripts allows characterization of RNA before half-life effects take place.

An examination of the 1200-L13 virus RNA has been made in this manner, by simultaneously measuring utilization of E1A poly(A) sites and poly(A)$^+$ RNA produced from the parent MLTU (Fig. 3). An M13 single-stranded bacteriophage was constructed which contains an insert of the E1AL13 region including sequences 5′ and 3′ to the site of the L13 insertion. [^{3}H]uridine pulse-labeled poly(A)$^+$ mRNA isolated from an early or a late 1200-L13 infection of 293 cells was hybridized to the complementary M13 E1AL13 clone followed by RNase T_1 and nuclease S1 treatment. The protected bands were analyzed by gel electrophoresis and detected by fluorography. The dried gels were further characterized by a Southern analysis with nick-translated L13 probe to detect steady state-levels of individual RNA species. The expected sizes of the wt and E1AL13 mRNA products were calculated in terms of the use of the poly(A) site and splice acceptor site where appropriate (Fig. 3). Comparison of the nascently labeled [^{3}H]RNA-[^{3}H]DNA hybrid with the steady-state ^{32}P-labeled hybrid gives us an indication of the comparative turnover of each RNA species.

Early 1200-L13-infected [^{3}H]mRNA clearly demonstrates the dominant use of the wt L1 poly(A) site over the wt L3 site which, after compensating for band size, results in an 8- to 10-fold difference in utilization (Fig. 3, lane EARLY ^{3}H, lower two bands). From the E1A locus we see in the early infection, the [^{3}H]E1AL1 duplex is also preferred over the duplex produced from the E1AL13 site which, when the band size is taken into account, indicates a ratio of L1 to L3 very close to the wt ratio (8:1). Comparison of steady-state early transcripts of E1AL1 with wt L1 (Fig. 3, lane EARLY ^{32}P) indicates that the half-life of the wt L1 species is short compared with that of the E1AL1. The [^{3}H]E1AL1-to-[^{3}H]E1AL13 ratio is slightly higher than the ^{32}P ratio, indicating a relatively small potential for stability differences between these early mRNAs.

The analysis of the late RNA-DNA duplex is more complex than that of the early products because elevated levels of nascent transcripts are produced from both the MLTU and the E1A transcription unit (both driven by the MLTCR) and processing intermediates from both transcription units are more abundant, resulting in a more complex RNA-DNA duplex band pattern. For that reason we will first examine the products of steady-state analysis. Two forms of steady-state analysis are presented. Ethidium bromide staining of the poly(A)$^+$ DNA-RNA duplex (Fig. 3, lane EtBr +) indicates that four major species are produced in the late infection (corresponding to E1AL13, E1AL1, wt L1, and wt L3) and one major species is present in the poly(A)$^-$ fraction. The nonadenylated duplex corresponds to RNA generated from transcripts which read through the wt L1 and L3 regions but have not yet been processed. From this analysis we get a clear sense of the wt MLTU switch. We see that the ratio of L1 to L3 has now shifted to a point at which L3 is actually present at two- to threefold higher concentrations than L1. A similar shift can be detected in the E1AL1-to-E1AL13 ratio, which, although not at quite the level of the wt L1-to-L3 ratio, is very close to equimolar. The [^{32}P]poly(A)$^+$ lane corroborates the results from the ethidium bromide analysis. Because of the small size of the wt L3 band, this species was not quantitatively retained when the gel was subjected to solution hybridization.

When analyzed by the RNA-DNA duplex method, the ^{3}H-labeled nascent adenylated mRNA generated by the late

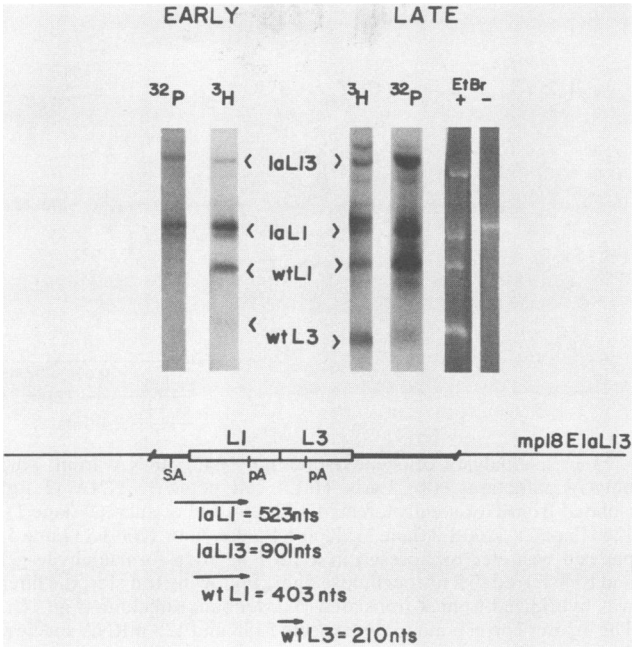

FIG. 3. Characterization of in vivo pulse-labeled 1200-L13 poly(A)$^+$ RNA. 293 Spinner cells (2.5×10^8) infected for 4 (early) or 16 (late) h with 2,000 particles (50 PFU) of 1200-L13 virus were concentrated and labeled for 15 min with [^{3}H]uridine (200 μCi/10^7 cells per ml). Total RNA was purified, and poly(A)$^+$ was selected by oligo(dT)-cellulose chromatography. Adenylated RNA from 10^8 cells was coprecipitated with 100 μg of M13mp18 E1AL13 single-stranded DNA. Each sample was resuspended, and hybridization, RNase T_1, filter selection, and S1 treatment were performed as described in Materials and Methods. Samples were resolved in a 1.4% nondenaturing agarose gel, En^{3}Hance treated, and exposed to film for 14 days (lane EARLY ^{3}H) or 4 days (lane LATE ^{3}H). Following exposure, each gel was partially hydrated and hybridized to [^{32}P]L13 nick-translated DNA (lane ^{32}P EARLY or ^{32}P LATE) for steady-state levels of each RNA species. The late RNA-DNA duplex was also visualized by ethidium bromide staining of the agarose gel (lanes LATE EtBr + and −) showing the products generated from adenylated and nonadenylated RNA-DNA duplex. nts, Nucleotides.

1200-L13 infection of 293 cells showed bands which comigrated with the fully processed steady-state products (Fig. 3 lanes LATE, compare ^{32}P with ^{3}H), as well as several bands which represent processing intermediates from both the E1A transcription unit and MLTU. When we focused on bands whose migration is identical to that of the steady-state bands, we saw wt L1 and wt L3 products, indicating that L3 transcripts are present in higher concentration than L1 transcripts; this clearly demonstrates the switch of poly(A) utilization. For the E1AL13 transcripts, the bands which comigrate with the steady-state transcripts also show a switch from sole utilization of L1 to an approximately equal use of the downstream L3 polyadenylation signal. This result indicates that at both the nascent RNA level and the steady-state level, the heterologous E1A transcription unit of 1200-L13 is able to reproduce the early-to-late switch associated with the complex MLTU.

Transcription termination is not involved in regulating poly(A) site selection. Experiments which have characterized poly(A) site utilization and the rate of transcription through the MLTU (27, 46, 50) (Fig. 1A and B) demonstrate transcription termination as an obvious restriction to utilizing the poly(A) sites L4 and L5 in early viral infection. The effect of termination on the recognition of the L2 and L3 polyadenylation sites is not as clear, since, although diminished, transcription through these polyadenylation signals does occur. By using the poly(A) virus constructs, it is a simple matter to examine the effect of each element on transcription termination. Transcription rates through the E1 region were examined by using the same assay previously applied to transcription termination studies of the mouse major β-globin transcription unit (19, 36). In the 1200 series of constructs, when HeLa cells were infected with virus for 4 h, expression of the E1 region was dependent solely on the E1A promoter (Fig. 1C). In the nuclear runon assays, transcription termination was measured by comparing the amount of nascently labeled RNA hybridizing to the genomic subclones which are located 5′ and 3′ to the poly(A) element. Because the 1A3 sequence is partially deleted in the 1200 virus, we are comparing 1A2 and 1B1 genomic subclones (Fig. 1C) by using sub-360 and 1200 as control measurements. When this assay was used with wt sub-360 virus, expression through the E1A and E1B sequences was equimolar with that of the wt sub-360 virus (Fig. 4A and B). Expression of the E1 region in HeLa cells infected for 4 h with the 1200 parental virus resulted in comparatively weak expression throughout the entire E1 region. This is expected since 1200 lacks both the E1A polyadenylation sequence and the E1B promoter; therefore, the *trans*-activating E1A polypeptide is weakly expressed (8, 28, 44). The poly(A) constructs 1200-L1 and 1200-L3 showed a stronger level of E1 region transcription when assayed by the nuclear runon technique extending through the 1A and 1B dots. The higher level of E1 region expression in these constructs was attributed to use of the inserted MLTU polyadenylation sites. Translation of the E1A poly(A) transcript resulted in *trans*-activation by the truncated E1A proteins. From the runon experiment, neither the L1 nor the L3 polyadenylation signals have a significant effect on downstream transcription, and therefore complexes initiated at the E1A cap site were not affected by passage through the poly(A) sites contained in viruses 1200-L1 and 1200-L3. Similarly, when two polyadenylation elements, L1 and L3, were tandemly linked as in the virus 1200-L13 (Fig. 1C), transcription rate measurements showed that transcription complexes successfully transcribed through both poly(A) sites and continued into the E1B coding sequence (Fig. 4). That a poly(A) site by itself does not induce transcription termination of RNA polymerase II complexes is in agreement with previous studies (36), and the apparent lack of any effect of these sequences means that a mechanism other than termination is involved in poly(A) site selection.

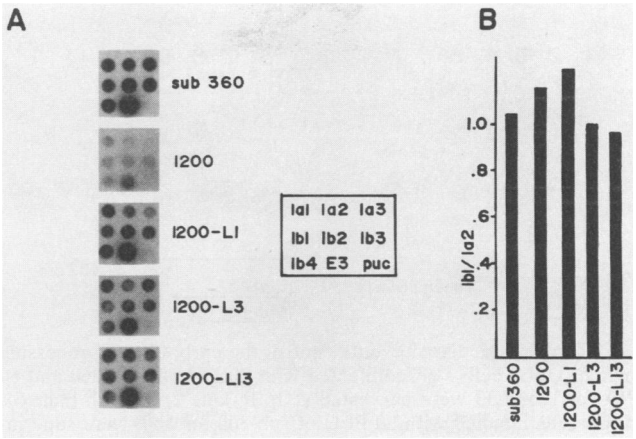

FIG. 4. Early in vitro nuclear runons of HeLa cells infected with the 1200 poly(A) viruses. Spinner HeLa cells (10^8 cells per sample) were infected with 1,000 particles (25 PFU) of virus (sub-360, 1200, 1200-L1, *dl*362-L3, and 1200-L13) per cell, and nuclei were isolated after 4 h. Following nascent RNA chain elongation in the presence of [^{32}P]UTP, 10^7 cpm of labeled RNA was hybridized to nitrocellulose grids containing adenovirus genomic plasmid subclones (5 μg per dot) as indicated by the key.

***trans*-Acting factors do not mediate the early-to-late processing switch.** The data indicate that a switch of poly(A) choice in the E1AL13 virus is mediated by an undefined event occurring when the virus passes from the early infection to the late infection. The late phase of an adenovirus infection is characterized by a number of key events in the biology of the cell. A block of host mRNA translation results in a decline of host proteins with a concomitant rise in the expression of late virus mRNAs and gene products (38). In theory, this change in protein synthesis may affect levels of critical processing proteins which are linked to processing control. A second feature of the late viral infection is the initiation of viral DNA replication. Approximately 6 to 8 h into the infectious cycle, DNA replication begins and continues through the late stages of virus infection, producing between 10^4 and 10^5 viral DNA molecules (only a fraction of which eventually become packaged into functional virions). The early-to-late transition is also associated with increased expression from the MLTU, the protein IX promoter, and the IVa2 promoter (16, 53). The activation of these promoters is directly associated with a *cis*-mediated regulation which is dependent on DNA replication.

One of our reasons for using the adenovirus model to study in vivo processing events lies in the infectious nature of the virus particle. By varying the multiplicity of infection we can quantitatively titrate the number of templates which are introduced into a given cell population. In addition, virus superinfection experiments can be carried out which allow introduction of phenotypically distinct viruses into cells which have been previously infected with wt virus. For our purposes, this provides an ideal format for expressing a newly introduced virus, 1200-L13, into a cell which has already entered the late phase of a sub-360 virus infection. If *trans* factors are present or lost in the late infection which

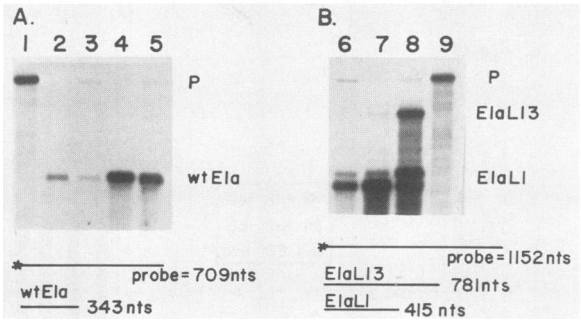

FIG. 5. *cis*-Mediated events control the early-to-late processing switch. HeLa cells (10^8 coinfected with 30 PFU of sub-360 and 60 PFU of 1200-L13 were harvested at 16 h (lane 1) and 4 h (lane 3). HeLa cells infected with 30 PFU of sub-360 for 12 h were superinfected with 60 PFU of 1200-L13, and after an additional 4-h period (total incubation period, 16 h for sub-360 and 4 h for *dl*362-L13), cells were harvested and RNA was processed as previously described. (A) Measurement of E1A mRNA by nuclease S1 analysis. E1A DNA was 3′ end labeled at the *Xba*I site and hybridized to poly(A)$^+$ mRNA and then treated with nuclease S1 as previously described. Expected RNA-protected DNAs are 343 nucleotides for appropriate usage of the E1A poly(A) site. Lanes: 2, RNA from sub-360 alone; 3, sub-360 plus 1200-L13 4-h coinfection; 4, sub-360 plus superinfection by 1200-L13 at 12 h; 5, late coinfection. (B) Measurement of E1AL13 mRNA from an early coinfection (lane 6), the sub-360 plus 1200-L13 superinfection (lane 7), and the late coinfection (lane 8). The DNA probe (P) and expected RNA protection products were described in the legend to Fig. 2. nts, Nucleotides.

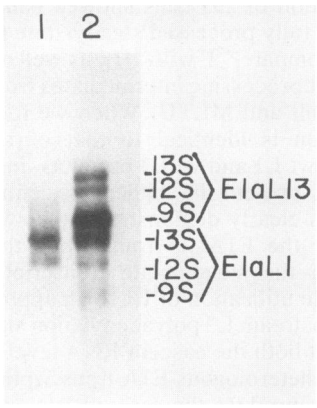

FIG. 6. Northern analysis of E1A transcripts generated from *dl*309 and 1200-L13 late superinfection and coinfection. The superinfection and coinfection was carried out as previously described by using Spinner HeLa cells (10^8 PFU per infection), and poly(A)$^+$ mRNA was selected by passage over oligo(dT) as described in Materials and Methods. Adenylated poly(A)$^+$ RNA (1 μg) was loaded and run in a formaldehyde denaturing agarose gel. After transfer to nitrocellulose, the filter was probed with a nick-translated plasmid containing E1A sequences from nucleotides 500 to 1336. Lane 1 contains RNA from *dl*309–1200-L13 superinfection; lane 2 contains RNA from the *dl*309–1200-L13 late coinfection.

mediate the early-to-late processing switch, they should freely interact with the transcript produced from superinfecting virus. A superinfecting 1200-L13 virus would then have an unrestricted processing phenotype, which is capable of using both L1 and L3 polyadenylation sites.

Such a superinfection experiment was carried out by using the parent sub-360 virus and the 1200-L13 virus. As control experiments we carried out an early and a late coinfection of these viruses to demonstrate the proportion of E1AL13 poly(A) site utilization in the presence of wt sub-360 virus (Fig. 5). The wt sub-360 virus produced levels of E1A mRNA in an early infection or in the early coinfection which represented normal levels for an early virus infection (Fig. 5A, lanes 2 and 3). When the sub-360 E1A mRNA was assayed in the superinfection or late coinfection, levels were present which were elevated as a result of enhancement by the MLTCR. Since both of these infections showed comparable levels of wt E1A, we conclude they were at the same stage of a late infection (Fig. 3A, lanes 4 and 5). The mRNA from superinfecting virus 1200-L13 added to a 12-h infection of sub-360 and allowed an additional 4-h incubation was compared with the mRNA from an early or late coinfection by an endonuclease S1 assay (Fig. 5B, lanes 6 to 8). The processing phenotype of 1200-L13 was the same in the superinfection as it was in the early coinfection in which poly(A) site usage was restricted to the first polyadenylation signal L1 (Fig. 5B, compare lanes 6 and 7). From this result, we conclude that the nuclear milieu created by a late sub-360 virus infection does not contribute or remove factors which act in *trans* on the pre-RNA to mediate the RNA-processing switch. This implies a *cis*-mediated mechanism of poly(A) choice in the early-to-late processing transition. A second superinfection was done with *dl*309 as the primary infecting virus followed by 1200-L13 superinfection; this was compared with a late *dl*309 and 1200-L13 coinfection and analyzed by probing a Northern blot with an E1A-specific nick-translated probe. The *dl*309 wt E1A mRNAs were not expressed in appreciable quantities in a late infection, allowing clear visualization of the restricted use of the L1 polyadenylation site in the superinfection when compared with the late coinfection in which both the L1 and L3 polyadenylation sites were utilized (Fig. 6, compare lanes 1 and 2).

cis-Regulatory mechanisms have been previously implicated in the early-to-late switch at the level of transcription control and splicing controls of E1B mRNA synthesis (16, 53). These general control mechanisms might be responsible at least in part for the poly(A) processing switch we have identified in this work.

DISCUSSION

In eucaryotes, there are several levels of regulation which can influence the mRNA population of the cell. In this report we are interested in the molecular mechanisms involved in one of those steps, poly(A) site choice in complex transcription units. We have constructed several recombinant adenoviruses to create a simplified experimental model. The model involves the use of the promoter element (MLTCR) of the complex MLTU and the normal E1A splicing elements (a single splice acceptor site which can accept one of three splice donors). Inserted within exon 2 of the E1A transcription unit are DNA elements containing poly(A) sites separated from splice acceptor elements. We are simply addressing the issue of sequence specificity and poly(A) site choice. The conclusions we draw from this study are that a first-come first-served mechanism primarily determines poly(A) choice and that overriding this mechanism in the late adenovirus infection requires at the very least a *cis* interaction and is not simply the result of an altered population of *trans*-acting processing factors.

In the E1A constructs, any polyadenylation signal can function in both an early or late infection in this transcription unit; therefore, sequence specificity is not the factor determining poly(A) site usage as long as that poly(A) site is the

first one generated in a given transcript. This observation is true with adenovirus polyadenylation sequences, for cellular sequences such as the globin poly(A) site (19) or the simian virus 40 T-antigen poly(A) site (9, 10). When multiple polyadenylation elements are tandemly arranged (1200-L13), the situation is different. In an early virus infection, only the first poly(A) site is utilized. At late times, both sites are equally utilized. This is consistent with wt MLTU regulation, as is the poly(A) switch which occurs when the virus enters the late phase of an infection. Using ^{3}H-pulse-labeling of nascent transcripts, we have shown that the change in transcript distribution is made at the level of poly(A) choice and not differential mRNA stability. Therefore, the simple location of a poly(A) recognition site within the transcription unit dominates in a first-come first-served manner (for the early or uninfected environment), whereas at late times this dominance is lost, allowing downstream poly(A) sites to be used with nearly equal efficiency.

In the E1A poly(A) constructs, nuclear runon experiments demonstrated that termination is not responsible for the inability to use downstream poly(A) sites in the E1A locus in the early infection. Direct studies of transcription termination induced by the mouse major β-globin termination element concluded that two elements are required for 3′ termination: the AATAAA polyadenylation signal sequence and a downstream termination element (36). Polyadenylation by itself is not sufficient to induce termination, but in the absence of a functional polyadenylation signal, termination at the 3′ end of the mouse major β-globin gene does not occur. We conclude that the E1A poly(A) constructs (and the MLTU) do not contain a termination element 3′ to the L1 poly(A) site but show restricted poly(A) site usage; therefore, termination is not involved in this example of poly(A) site regulation.

The tandem poly(A) site construct we have examined involves considerable reduction of spacing between the polyadenylation elements in question. wt L1 and L3 are separated by 8.5 kbp, whereas the E1AL13 construct has approximately 0.5 kbp between elements. In these experiments we did not set out to intentionally vary the distance between elements, but we note that spacing is not exceptionally critical when dealing with poly(A) choice. This is different from the observations made in the IgM heavy-chain studies, in which varying the space in the intervening sequence has considerable effect on processing decisions. The regulation in the IgM case involves two selections, poly(A) choice and splice acceptor choices. In our studies of the adenovirus poly(A) constructs we have tried to separate the issue of splicing from poly(A) choice. By focusing on polyadenylation elements, we conclude that spacing does not necessarily play a primary role in choosing a polyadenylation site.

The transition from early- to late-phase processing obviously involves an alteration in the first-come first-served mechanism which allows complexes to read through 30 kbp (the size of the MLTU) by using one of several available poly(A) sites scattered throughout the transcription unit. We do not believe that the character of the early infecting viral template or early specific compartmentalization is responsible for the restricted processing phenotype, because the transiently transfected dlpMLP6-L13 plasmid gave the same phenotype as the early virus infection (data not shown). This implies that the first-come first-served phenotype is the normal or simplest pathway for processing. Deviation from this pathway takes place when control of poly(A) choice is occurring. A simplified explanation for the processing switch would invoke a factor which interacts with the RNA or transcription complex and suppresses poly(A) recognition associated with the elongation complex. Selection of the poly(A) site would depend on the kinetics of each poly(A) site associating with the processing machinery. The order of poly(A) site presentation may still contribute to the final distribution but not in such a restrictive fashion.

To demonstrate the presence of such a *trans*-acting factor, we carried out the experiment in which the 1200-L13 virus was superinfected over a wt late infection (with sub-360 virus). If a late infection produces or eliminates a factor required in *trans*, it should act equally well on both superinfecting and primary infecting viral transcripts. The result of this experiment indicates that the superinfecting virus processes the E1AL13 transcript as if it were an early infection, producing predominantly the E1AL1 poly(A)$^+$ mRNA. Altered processing factors present in a late infection cannot mediate an effect in a simple *trans* fashion. This does not mean that altered factors are not a part of the processing switch, but it does mean that they are not able to interact with the superinfecting virus transcript directly. Instead, there is a requirement for some undefined modification before the switch can occur. Previous experiments characterizing *cis* regulation of late adenovirus transcripts have noted promoters that require a *cis* modification and also the possibility that *cis* mechanisms are involved in processing controls (16, 22, 41, 53). In those studies, DNA replication was required for the late switch to take place. Our observations suggest a definite *cis* effect on processing at the level of poly(A) choice. Although it is relatively simple to appreciate how *cis* mechanisms can affect transcription control in which the interaction of transcription factors can influence promoter utilization, the way in which *cis* interactions influence processing choices is a more abstract concept, especially since it is well established that an RNA substrate and appropriate processing complexes are all that is required for polyadenylation or splicing to take place in vitro (47).

Although *cis*-processing control is difficult to envision, there is some precedent. One *cis* interaction which is independent of DNA sequence is the requirement of transcription by RNA polymerase II in executing processing in vivo. This requirement is still under considerable debate (35, 51, 52), but in all cases it appears that normal high-efficiency processing occurs only in the presence of RNA polymerase II (and the 5′ cap structure which is characteristic of RNA polymerase II transcripts). It has also been shown, for the U1 and U2 small nuclear RNAs, that appropriate 3′-end processing requires an appropriate 3′ processing box and the presence of the compatible small nuclear RNA promoter and enhancer element, implying that a specific transcription complex is established by the promoter which dictates processing activities (25, 42). Other candidates for *cis*-processing control include site-specific protein DNA interactions which inhibit or allow utilization of downstream polyadenylation elements; altered promoter loading which affects the RNA polymerase II interaction with a given poly(A) signal sequence; or an altered transcription complex which has lost the ability to respond to the first-come first-served control mechanism, yet can still transcribe unimpeded through considerable amounts of DNA sequence (11, 25, 32, 55). Since the termination of transcription complexes has been associated with the recognition of the AATAAA polyadenylation signal sequence (36), the inability to recognize and act on the first polyadenylation sequence present within a given transcript could be mediated through an antipolyadenylation function of the elongating transcrip-

tion complex. A paradigm for the poly(A) switch control might be found in the N-nut or Q-qut antitermination mechanism that has been studied in phage lambda (5, 24, 26). The first-come first-served phenotype characteristic of an early adenovirus infection substitutes poly(A) site selection for the bacterial termination function [since true termination or polymerase displacement is not required for the poly(A) choice]. Loss of the obligatory first poly(A) site usage as it occurs in the late virus infection (antipolyadenylation) could be seen as an antitermination activity, comparable to the phage lambda N-nut or Q-qut *cis* and *trans* functions which allow RNA polymerase to transcribe through pause-termination sites without undergoing displacement. Regardless of how the *cis* mechanism is acting, the potential for a similar type of control for cellular transcription units is apparent.

ACKNOWLEDGMENTS

This work was supported in part by American Cancer Society grant NP571. Erik Falck-Pedersen is the recipient of a Teacher Scientist Award from the Andrew W. Mellon Foundation.

LITERATURE CITED

1. **Akusjarvi, G., and M. Persson.** 1980. Controls of RNA splicing and termination in the major late adenovirus transcription unit. Nature (London) **292**:420–426.
2. **Alt, F. W., A. L. M. Bothwell, M. Knapp, 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.
3. **Amara, S. G., R. M. Evans, and M. G. Rosenfeld.** 1984. Calcitonin/calcitonin gene-related peptide transcription unit: tissue specific expression involves selective use of alternative polyadenylation sites. Mol. Cell. Biol. **4**:2151–2160.
4. **Amara, S. G., V. Jones, M. G. Rosenfeld, E. S. Ong, and R. M. Evans.** 1982. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature (London) **298**:240–244.
5. **Barik, S., B. Ghosh, W. Whalen, D. Lazinski, and A. Das.** 1987. An antitermination protein engages the elongating transcription apparatus at a promoter proximal recognition site. Cell **50**: 485–494.
6. **Basi, G. S., M. Boardman, and R. V. Storti.** 1984. Alternative splicing of a *Drosophila* tropomyosin gene generates muscle isoforms with different carboxy-terminal ends. Mol. Cell. Biol. **4**:2828–2836.
7. **Berget, S. M., C. Moore, and P. A. Sharp.** 1977. Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. Proc. Natl. Acad. Sci. USA **74**:3171–3175.
8. **Berk, A. J.** 1987. Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. **20**:45–80.
9. **Berkner, K. L., and P. A. Sharp.** 1984. Expression of dihydrofolate reductase and of adjacent E1b region, in an Ad5 dihydrofolate reductase recombinant virus. Nucleic Acids Res. **12**: 1925–1941.
10. **Berkner, K. L., and P. A. Sharp.** 1985. Effect of the tripartite leader on synthesis of a nonviral protein in an adenovirus 5 recombinant. Nucleic Acids Res. **13**:841–857.
11. **Beyer, A. L., A. H. Bouton, L. D. Hodge, and O. L. Miller, Jr.** 1981. Visualization of the major late R strand transcription unit of adenovirus serotype 2. J. Mol. Biol. **147**:269–295.
12. **Breitbart, R. E., A. A. Andreadis, and B. Nadal Ginard.** 1987. Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes. Annu. Rev. Biochem. **56**:467–496.
13. **Chinnadurai, G., S. Chinnadurai, and J. Brusca.** 1979. Physical mapping of a large plaque mutation of adenovirus type 2. J. Virol. **32**:623–628.
14. **Chow, L., R. Gelinas, T. Broker, and R. Roberts.** 1977. An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNAs. Cell **1**:1–8.
15. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of adenovirus-2. J. Mol. Biol. **134**:265–279.
16. **Crossland, L. D., and H. J. Raskas.** 1983. Identification of adenovirus genes that require template replication for expression. J. Virol. **46**:737–748.
17. **Danner, D., and P. Leder.** 1985. Role of an RNA cleavage/polyadenylation site in the production of membrane-bound and secreted IgM mRNA. Proc. Natl. Acad. Sci. USA **82**:3949–3953.
18. **Darnell, J. E., Jr.** 1982. Variety in the level of gene control in eucaryotic cells. Nature (London) **297**:365–371.
19. **Falck-Pedersen, E., J. Logan, T. Shenk, and J. E. Darnell, Jr.** 1985. Transcription termination within the E1A gene of adenovirus induced by insertion of the mouse β-major globin terminator element. Cell **40**:897–905.
20. **Fraser, N. W., and E. Ziff.** 1978. RNA structures near poly (A) of adenovirus-2 late messenger RNAs. J. Mol. Biol. **124**:27–51.
21. **Galli, G., J. W. Guise, M. A. McDevitt, P. W. Tucker, and J. R. Nevins.** 1987. Relative position and strengths of poly (A) sites as well as transcription termination are critical to membranes versus secreted μ-chain expression during B-cell development. Genes Dev. **1**:471–481.
22. **Gaynor, R. B., and A. J. Berk.** 1983. Cis acting induction of adenovirus transcription. Cell **33**:683–693.
23. **Graham, F., J. Smiley, W. Russell, and R. Niam.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
24. **Grayhack, E. J., X. Yang, L. F. Lau, and J. W. Roberts.** 1985. Phage lambda gene Q antiterminator recognizes RNA polymerase near the promoter and accelerates it through a pause site. Cell **42**:259–269.
25. **Hernandez, N., and A. M. Weiner.** 1986. Formation of the 3′ end of U1 snRNA requires compatible snRNA promoter elements. Cell **47**:249–258.
26. **Horwitz, R. J., J. Li, and J. Greenblatt.** 1987. An elongation control particle containing the N gene transcriptional antitermination protein of bacteriophage lambda. Cell **51**:631–641.
27. **Iwamoto, S., F. Eggerding, E. Falck-Pedersen, and J. E. Darnell, Jr.** 1986. Transcription unit mapping in adenovirus: regions of termination. J. Virol. **59**:112–119.
28. **Jochemson, A. G., L. T. C. Peltenburg, M. F. W. te Pas, C. M. de Wit, J. L. Bos, and A. J. van der Eb.** 1987. Activation of adenovirus 5 E1A transcription by region E1b in transformed primary rat cells. EMBO J. **6**:3399–3405.
29. **Jones, N., and T. Shenk.** 1979. Isolation of an adenovirus type 5 host range deletion mutant defective for transformation of rat embryo cells. Cell **17**:683–689.
30. **Kafotis, F. C., and A. Efstradiadis.** 1979. Determination of nucleic acid sequence homologues by a dot hybridization procedure. Nucleic Acids Res. **7**:1541–1551.
31. **Laski, F. A., D. C. Rio, and G. M. Rubin.** 1986. Tissue specificity of Drosophila P element transposition is regulated at the level of mRNA splicing. Cell **44**:7–19.
32. **Law, R., M. D. Kuwabara, M. Briskin, N. Fasel, G. Hermansen, D. S. Sigman, and R. Wall.** 1987. Protein-binding site at the immunoglobulin M-m polyadenylation signal: possible role in transcription termination. Proc. Natl. Acad. Sci. USA **84**: 9160–9165.
33. **Leff, S. E., R. M. Evans, and M. G. Rosenfeld.** 1987. Splice commitment dictates neuron-specific alternative RNA processing in calcitonin/CGRP gene expression. Cell **48**:517–524.
34. **Leff, S. E., M. G. Rosenfeld, and R. M. Evans.** 1986. Complex transcriptional units: diversity in gene expression by alternative RNA splicing. Annu. Rev. Biochem. **55**:1091–1118.
35. **Lewis, E. D., and J. L. Manley.** 1986. Polyadenylation of an mRNA precursor occurs independently of transcription by RNA polymerase II *in vivo*. Proc. Natl. Acad. Sci. USA **83**:8555–8559.
36. **Logan, J., E. Falck-Pedersen, J. E. Darnell, Jr., and T. Shenk.** 1987. A poly (A) addition site and a downstream termination region are required for efficient association of transcription by RNA polymerase II in the mouse β maj-globin gene. Proc. Natl.

Acad. Sci. USA **84**:8306–8310.
37. **Logan, J., and T. Shenk.** 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. USA **81**:3655–3659.
38. **Lucas, J. J., and H. S. Ginsberg.** 1971. Synthesis of virus specific RNA in KB cells infected with type 2 adenovirus. J. Virol. **8**:203–212.
39. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Mather, E. L., K. J. Nelson, J. Haimovich, and R. P. Perry.** 1984. Mode of regulation of immunoglobulin mu and delta chain expression varies during B lymphocyte maturation. Cell **36**: 329–338.
41. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1984. Control of adenovirus E1B mRNA synthesis by a shift in the activities of RNA splice sites. Mol. Cell. Biol. **4**:966–972.
42. **Neuman de Vegvar, H. E., E. Lund, and J. Dahlberg.** 1986. 3′ end formation of UI snRNA precursors is coupled to transcription from snRNA promoters. Cell **47**:259–266.
43. **Nevins, J. R.** 1980. Definition and mapping of adenovirus-2 nuclear transcription. Methods Enzymol. **65**:765–785.
44. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**: 213–220.
45. **Nevins, J. R.** 1983. The pathway of eucaryotic mRNA formation. Annu. Rev. Biochem. **52**:441–466.
46. **Nevins, J. R., and M. J. Wilson.** 1981. Regulation of adenovirus-2 gene expression at the level of the transcriptional termination and RNA processing. Nature (London) **290**:113–118.
47. **Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp.** 1986. Splicing of messenger RNA precursers. Annu. Rev. Biochem. **55**:1119–1150.
48. **Petersen, M. L., and R. P. Perry.** 1986. Regulated production of μm and μs mRNA requires linkage of the poly (A) sites and is dependent on the length of the μm-μs intron. Proc. Natl. Acad. Sci. USA **83**:8883–8887.
49. **Salditt-Georgieff, M., M. Harpold, S. Chen Kiang, and J. E. Darnell.** 1980. The addition of 5′ cap structures occurs early in hnRNA synthesis and prematurely terminated molecules are capped. Cell **19**:69–78.
50. **Shaw, A., and E. B. Ziff.** 1980. Transcripts from the adenovirus major late promoter yield a single early family of 3′ coterminal mRNAs and five late families. Cell **22**:905–916.
51. **Sisodria, S. S., B. Sollner-Webb, and D. W. Cleveland.** 1987. Specificity of RNA maturation pathways: RNAs transcribed by RNA polymerase III are not substrates for splicing or polyadenylation. Mol. Cell. Biol. **7**:3602–3612.
52. **Smale, S. S., and R. Tijan.** 1985. Transcription of herpes simplex virus TK sequences under the control of wild-type and mutant human RNA polymerase I promoters. Mol. Cell. Biol. **5**:352–362.
53. **Thomas, G. P., and M. B. Mathews.** 1980. DNA replication and the early to late transition in adenovirus infection. Cell **22**: 523–533.
54. **Tseng, R. W., and N. H. Acheson.** 1986. Use of a novel S1 nuclease RNA-mapping technique to measure efficiency of transcription termination on polyomavirus DNA. Mol. Cell. Biol. **6**:1624–1632.
55. **Wogelmuth, D. J., and M. T. Hsu.** 1981. Visualization of nascent RNA transcripts and simultaneous transcription and replication of viral nucleoprotein complexes from adeno virus-2 infected Hela cells. J. Mol. Biol. **147**:247–268.