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Adenovirus early region 4 stimulates mRNA accumulation via 5′ introns

(pre-mRNA splicing/chloramphenicol acetyltransferase genes/rabbit β-globin)

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ABSTRACT **The adenovirus major late transcription unit accounts for most virus-specific transcription late after infection. All mRNAs expressed from this unit carry a short spliced leader, the so-called tripartite leader, attached to their 5′ ends. Here we describe a function for an adenovirus gene product in the control of major late mRNA abundance. We show that early region 4 (E4) stimulates mRNA accumulation from tripartite leader intron-containing transcription units ≈10-fold in short-term transfection assays. The effect was already detectable in nuclear RNA and was not due to a transcriptional activation through any of the major late promoter elements or through an effect at nuclear to cytoplasmic mRNA transport. A surprising positional effect of the intron was noted. To be E4 responsive, the intron had to be placed close to the pre-mRNA 5′ end. The same intron located far downstream in the 3′ untranslated region of the mRNA was not E4 responsive. The E4 enhancement was not dependent on specific virus exon or intron sequences. These results suggest that E4 modulates a general pathway in mammalian mRNA formation.**

The adenovirus type 2 (Ad2) early region 4 (E4) covers ≈10% of the viral genome and encodes at least five proteins (1) that could potentially have a regulatory role during the virus life cycle. The function of these hypothetical proteins is poorly understood. Deletion mutants lacking most of E4 show a very complex phenotype, which includes defects in virus assembly, viral DNA accumulation, late viral mRNA accumulation, and protein synthesis as well as a failure to shut off host-cell macromolecule synthesis (2–5). These mutants also overexpress the E2A 72-kDa DNA binding protein (4) even though E4 is required for maximal E2A promoter activity (6–10). Studies of E4 mutants have shown that E4 controls late mRNA abundance posttranscriptionally by increasing the nuclear stability of pre-mRNAs transcribed from the major late transcription unit (MLTU) (11, 12).

Most structural polypeptides of the virion are translated from mRNAs originating from the MLTU, which extends in the rightward direction from a promoter at coordinate 16.8 to a position near the right end of the viral genome. More than 20 cytoplasmic mRNAs are generated by differential processing of this ≈28,000-nucleotide precursor RNA. These mRNAs can be grouped into five families, each consisting of species with coterminal 3′ ends and having a common tripartite leader attached to the 5′ end (reviewed in ref. 13).

Since most mRNAs expressed late after infection originate from the MLTU, one would expect to find early viral gene products that facilitate major late mRNA expression. Here we describe one such mechanism. We show that E4 augments major late mRNA accumulation posttranscriptionally, possibly through a direct effect at the level of RNA splicing. This latter conclusion comes from the observation that an intron is required for E4 responsiveness.

MATERIALS AND METHODS

Plasmids. pβCAT-3 was generated from pβCAT-2 (14) by substituting the β-globin 3′ sequences with the simian virus 40 (SV40) 3′-flanking sequences. pL1CAT contains Ad2 sequences (numbers refer to the sequence presented in ref. 1) from position 5362 (*Bal* I) to 6071 (*Pvu* II) fused to the *Hin*dIII site upstream of chloramphenicol acetyltransferase (CAT) in pTripCAT. The *Bal* I and *Pvu* II sites were converted to *Bam*HI and *Hin*dIII sites, respectively, by linker addition. pL1+190CAT differs from pL1CAT in that the Ad2 sequences extend to position 6231. pL1-int-2CAT contains Ad2 sequences down to position 7167 [recloned from plasmid pAdSVII (15)]. preTripCAT extends further to the *Xho* I site at position 9686. A 16-base-pair SV40 fragment [*Avr* II position 5187 (converted to *Xho* I) to *Hin*dIII position 5171] separates Ad2 sequences and the CAT gene. pL1,2-int-3CAT is identical to preTripCAT except that the first leader intron has been replaced by a leader 1, 2 cDNA. pTripβCAT contains the 720-base-pair *Bam*HI (position 480) to *Bgl* II (position 1200) fragment encoding the large intron from the rabbit β-globin gene (16) cloned as a *Hin*dIII fragment in plasmid pTripCAT. Plasmids pTripCATβ3 and pTripCATβ6.5 are identical to plasmids pβCAT, pβCAT3, and pβCAT6.5 (17) except that the β-globin promoters present in the original constructs were replaced by the major late promoter/tripartite leader cDNA from plasmid pTripCAT.

DNA Transfection and RNA Preparation. 293 monolayer cells were transfected by the calcium phosphate coprecipitation technique as described (18, 19) with 0.5 or 2.5 μg of CAT plasmid and 5 μg of E4-expressing plasmid. Carrier DNA was added to give a total of 15 μg of DNA per 6-cm Petri dish. Approximately 50 hr posttransfection cells were lysed by IsoB Nonidet P-40 treatment and the cytoplasmic RNA was isolated by phenol extraction (20). In some experiments, total cell RNA or nuclear RNA was prepared by the guanidium isothiocyanate method (21).

S1 Endonuclease Analysis. The protocol described by Weaver and Weissmann (22) was essentially followed. Total cytoplasmic RNA (10 μg) was hybridized overnight at 45°C to the 5′- or 3′-end-labeled DNA fragments depicted in the figure legends. S1 nuclease cleavage and electrophoretic separation were as described (19).

RESULTS

Stimulation of Major Late mRNA Accumulation by E4. To determine the effect of E4 on mRNA accumulation from the MLTU, plasmid preTripCAT was cotransfected together

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Abbreviations: Ad2, adenovirus type 2; MLTU, major late transcription unit; E4, early region 4; SV40, simian virus 40; CAT, chloramphenicol acetyltransferase; ORF, open reading frame.

with plasmid pKGO-895 (encoding the Ad2 E4 transcription unit; ref. 20) into 293 cells. These cells are transformed by Ad5 and constitutively express mRNAs and proteins from regions E1A and E1B (23). Approximately 50 hr posttransfection, cytoplasmic RNA was prepared and the effect of E4 on reporter mRNA accumulation was quantitated by the S1 nuclease protection assay (22). Although all reporter plasmids encode a functional CAT protein, we used the S1 nuclease protection assay to quantitate gene expression. This was done to ensure that any effect of E4 that we were detecting was due to an increase in RNA levels. As shown in Fig. 1*B* (lanes 1 and 2) pKGO-895 cotransfection resulted in a significant increase in CAT mRNA expression. We estimate from a number of experiments that the E4 enhancement of preTripCAT expression is ≈10-fold. To investigate the promoter specificity of E4 we also tested plasmids pβCAT-3 and pSVECAT (Fig. 1*A*) in our cotransfection assay. As shown in Fig. 1*B* (lanes 3–6), pKGO-895 cotransfection had no effect on CAT mRNA accumulation from either of these plasmids.

E4 Regulates mRNA Accumulation Posttranscriptionally. The transcriptional activity of the major late promoter is known to be regulated through sequences located both upstream (−66 to +33; ref. 24) and downstream (+33 to +190; refs. 15, 25, and 26) of the transcription initiation site. Furthermore, the spliced tripartite leader has been shown to increase the cytoplasmic half-life of mRNA during a virus infection (27). To investigate whether any of these sequences were required for the E4-mediated enhancement of major late mRNA accumulation, we tested plasmids pL1CAT, pL1+190CAT, and pTripCAT (Fig. 2*A*) in our transient expression assay. Plasmid pL1CAT contains all of the major late upstream regulatory sequences (−400 to +33) and pL1+190CAT extends further downstream of the initiation site and includes the regulatory elements located between positions +33 and +190. Plasmid pTripCAT contains a complete cDNA copy of the tripartite leader (14). As shown in Fig. 2*B*, none of these plasmids gave rise to a significant increase in CAT mRNA accumulation after pKGO-895 cotransfection. We usually observe a small, but reproducible, increase in reporter RNA accumulation in pTripCAT and pKGO-895 cotransfected cells (<2-fold; see below). This is probably due to the effect of E4 proteins with transcription activation capacity (7–10).

To pinpoint the mechanism of E4 action, we prepared nuclear and cytoplasmic RNA from 293 cells cotransfected with preTripCAT (Fig. 1*A*) and pKGO-895. As shown in Fig. 3, the stimulatory effect of E4 was detectable already in nuclear RNA preparations. Taken together these results suggest that E4 enhances major late mRNA accumulation by

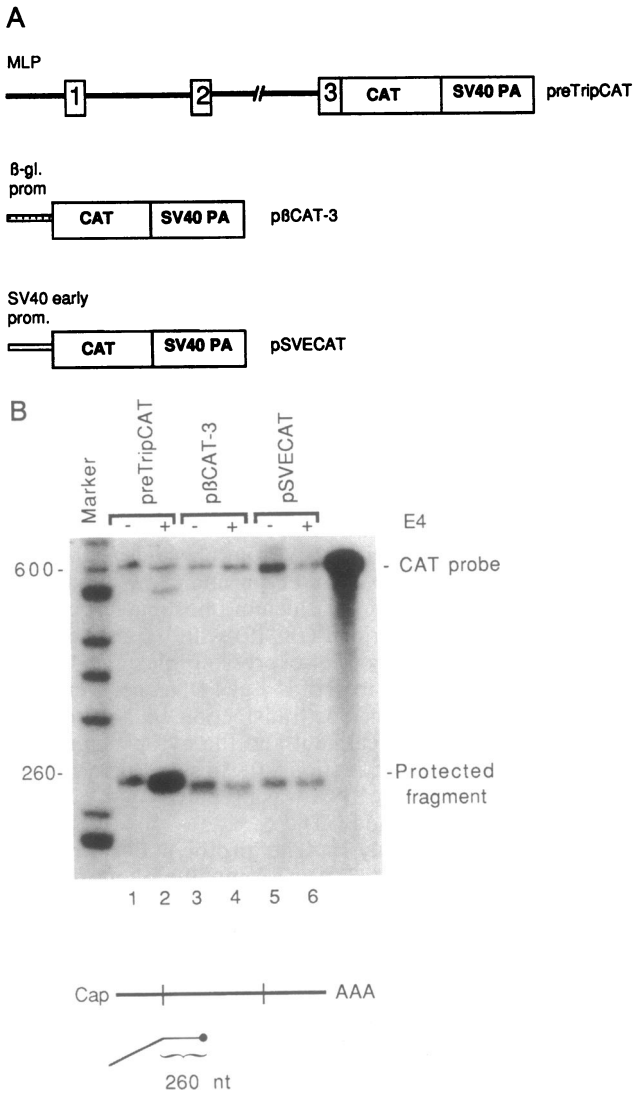

FIG. 1. Effect of E4 on cytoplasmic mRNA accumulation. (*A*) Schematic drawing of plasmids used in the experiment. MLP, major late promoter; β-gl., β-globin. (*B*) S1 endonuclease analysis of CAT mRNA accumulation in 293 cells transfected with CAT plasmids and carrier DNA (lanes −) or pKGO-895 (lanes +) encoding Ad2 E4. Numbers on left are nucleotides (nt).

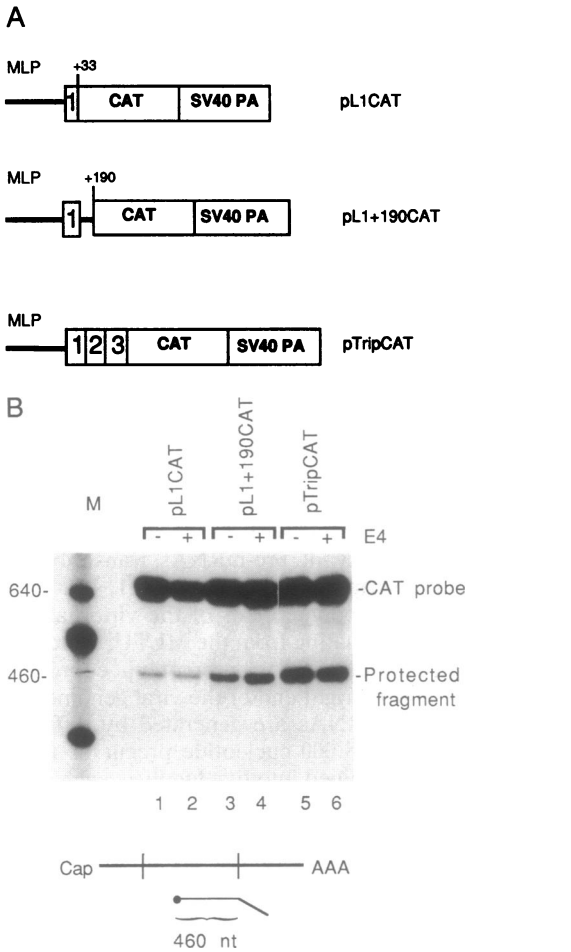

FIG. 2. Effect of E4 on CAT mRNA accumulation from major late promoter (MLP)/tripartite leader sequence variants. (*A*) Schematic drawing of plasmids used in the experiment. (*B*) S1 endonuclease analysis of CAT mRNA levels in 293 cells transfected with CAT plasmids and carrier DNA (lanes −) or pKGO-895 (lanes +) encoding Ad2 E4. Numbers on left are nucleotides (nt).

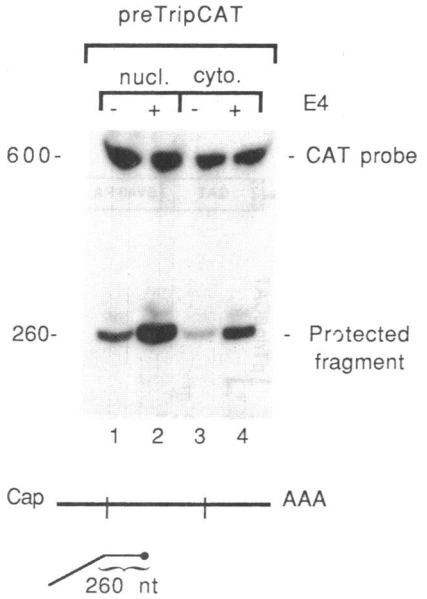

FIG. 3. Effect of E4 on nuclear and cytoplasmic RNA accumulation. Plasmid preTripCAT was transfected together with carrier DNA (lanes −) or pKGO-895 (lanes +) encoding the Ad2 E4 into subconfluent monolayers of 293 cells. Approximately 50 hr post-transfection nuclear and cytoplasmic RNA were prepared and the level of CAT mRNA was quantitated by S1 endonuclease analysis. Numbers on left are nucleotides (nt).

a posttranscriptional mechanism other than transport or cytoplasmic mRNA stability.

E4 Does Not Enhance mRNA Expression from Long Transcription Units. To explain the preferential effect of E4 on cytoplasmic mRNA accumulation in preTripCAT transfected cells (Figs. 1 and 2), we considered the possibility that the length of the transcription unit was an important factor. If E4 has an effect on nuclear RNA stability (ref. 11; Fig. 3) or on the frequency of premature transcription termination, one might expect that the pre-mRNA encoded by preTripCAT (5200 nucleotides) would be stimulated more efficiently than, for example, pTripCAT, which is considerably shorter (1500 nucleotides).

This possibility was tested with plasmids pTripCATβ3 (4800 nucleotides) and pTripCATβ6.5 (8300 nucleotides) (Fig. 4*A*). These plasmids were transfected into 293 cells with or without pKGO-895. As shown in Fig. 4*B*, increasing the pre-mRNA length to a similar or a greater length than preTripCAT did not result in a significantly E4 responsive pre-mRNA. Thus, E4 does not selectively stimulate transcript levels from long transcription units.

Intron Sequences Are Required for the E4-Mediated Enhancement of mRNA Accumulation. To test whether the presence of introns in the leader region were important, we constructed deletion mutants of preTripCAT that precisely lack either the first (pL1,2-int-3CAT) or the second (pL1-int-2CAT) tripartite leader intron (Fig. 5*A*). Both plasmids gave rise to an ≈10-fold increase in CAT mRNA levels when cotransfected with pKGO-895 (Fig. 5*B*). This was in contrast to pTripCAT, which was only marginally stimulated by pKGO-895 cotransfection (Fig. 5*B*). The absence of an effect of E4 on pTripCAT was not due to a saturation of mRNA expression since transfection of decreasing amounts of pTripCAT still did not yield any significant stimulation by pKGO-895 cotransfection (data not shown; see also Fig. 6*B*).

These experiments demonstrated that an intron was required and that both the first and the second tripartite leader introns were responsive to E4. This raised the possibility that E4, in a sequence-specific manner, recognized the tripartite

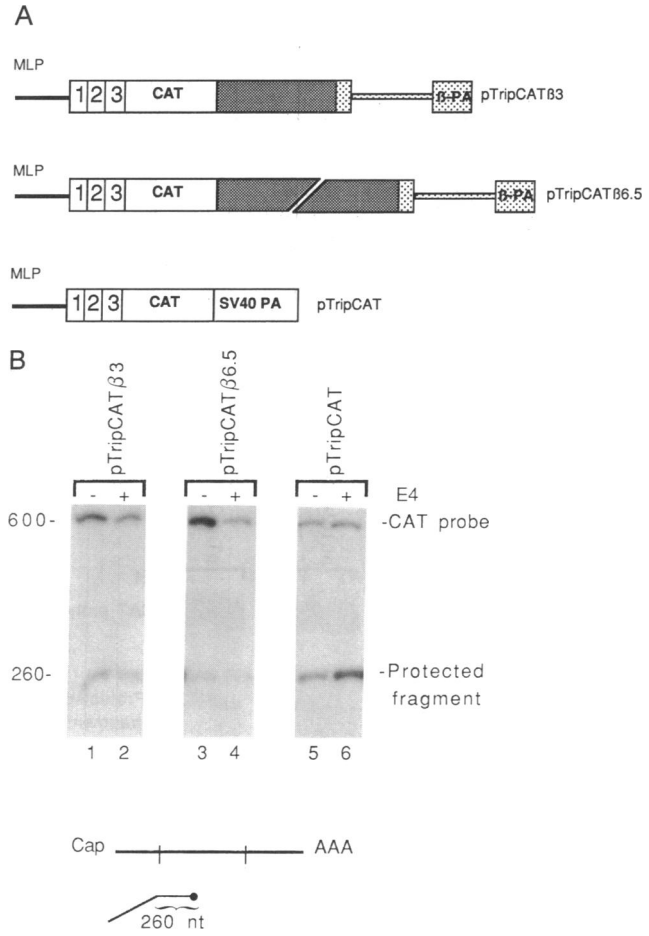

FIG. 4. Effect of E4 on mRNA accumulation from long transcription units. (*A*) Schematic drawing of plasmids used in the experiment. MLP, major late promoter. (*B*) S1 endonuclease analysis of CAT mRNA accumulation in 293 cells transfected with CAT plasmids and carrier DNA (lanes −) or pKGO-895 (lanes +) encoding Ad2 E4. Numbers on left are nucleotides (nt).

leader introns. Alternatively, the results may indicate that the E4 effect requires the presence of an intron close to the pre-mRNA 5′ end; as shown above, an intron located downstream of CAT, in the 3′ untranslated region, was not E4 responsive (Figs. 1 and 3, SV40 small-t; Fig. 4, β-globin). To discriminate between these possibilities, the rabbit β-globin large intron was moved from a position close to the pre-mRNA 3′ end (Fig. 4) to the 5′ untranslated region of pTripCAT, thus generating plasmid pTripβCAT (Fig. 6*A*). As shown in Fig. 6*B*, basal CAT mRNA expression was similar in pTripβCAT and pTripCAT transfected cells. However, pTripβCAT was stimulated 4-fold by pKGO-895 cotransfection (lanes 3 and 4), whereas mRNA accumulation from the parental pTripCAT plasmid was not (lanes 1 and 2). Thus, a heterologous intron placed close to the pre-mRNA 5′ end could substitute for the natural leader introns. In fact, the wild-type rabbit β-globin gene was also stimulated by E4 (data not shown), demonstrating that no viral sequences were required for E4 responsiveness.

DISCUSSION

Most eukaryotic genes contain introns. The precise role of these introns in mRNA biogenesis remains undefined. It is known that certain transcription units fail to produce stable cytoplasmic mRNA if they lack introns (see ref. 28 for a review). However, it has not been clearly demonstrated that introns facilitate mRNA accumulation through an effect at

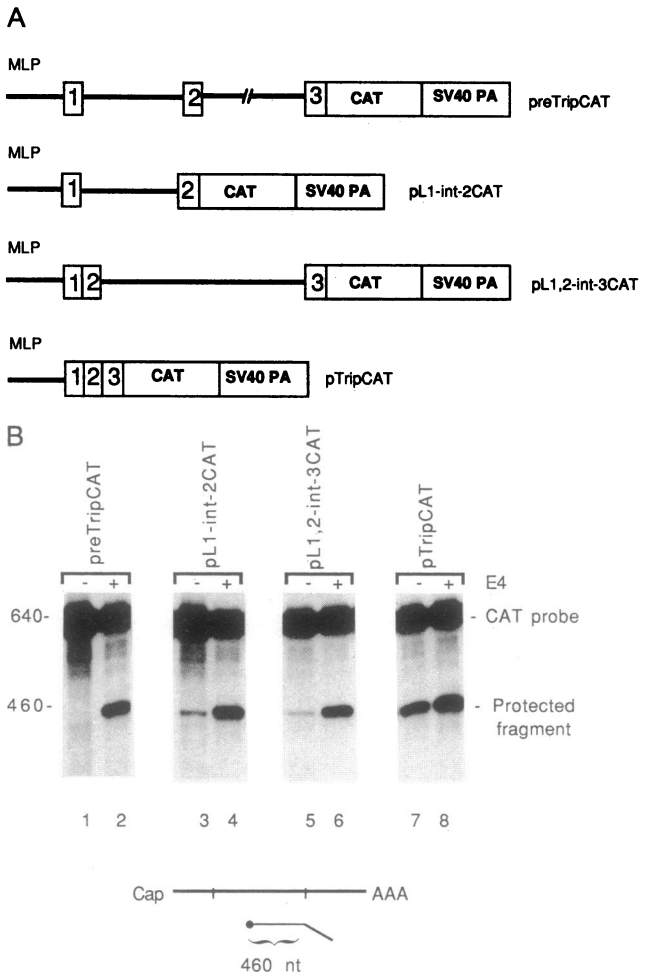

FIG. 5. Requirement of an intron for E4 responsiveness. (*A*) Schematic drawing of plasmids used in the experiments. MLP, major late promoter. (*B*) S1 endonuclease analysis of CAT mRNA levels in 293 cells transfected with CAT plasmids and carrier DNA (lanes −) or pKGO-895 (lanes +) encoding Ad2 E4. Numbers on left are nucleotides (nt).

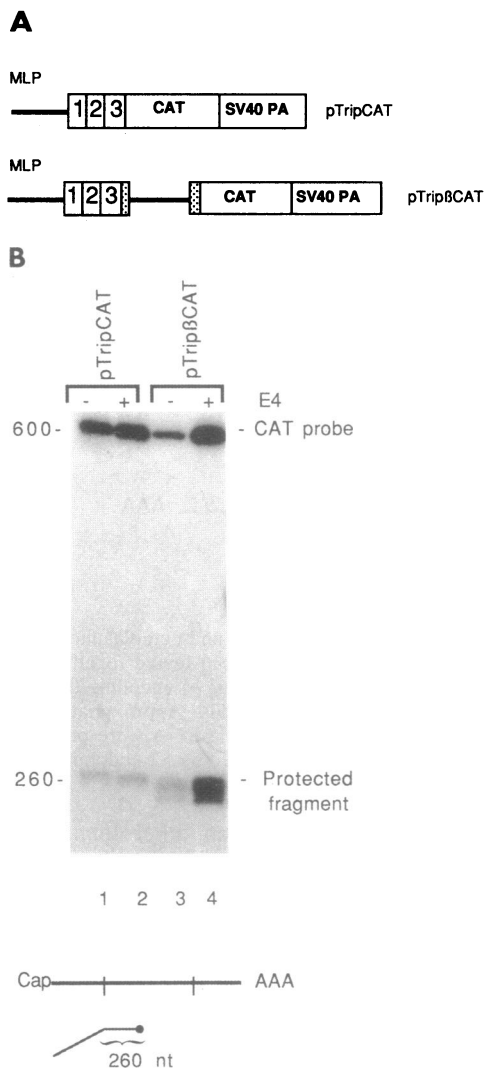

FIG. 6. Positional effect of an intron for E4 responsiveness. (*A*) Schematic drawing of plasmids used in the experiment. MLP, major late promoter. (*B*) S1 endonuclease analysis of mRNA levels in 293 cells transfected with marker plasmids and carrier DNA (lanes −) or pKGO-895 (lanes +) encoding Ad2 E4. Numbers on left are nucleotides (nt).

the level of pre-mRNA splicing. In fact, recent data suggest that introns increase cytoplasmic transport of mRNA through an effect on the efficiency of polyadenylylation (29). The position of an intron in a transcription unit does not seem to be of critical importance for stable cytoplasmic mRNA accumulation in animal cells (28). In contrast, a positional dependence of introns in yeast and maize have been observed; 5′ introns work better than 3′ introns (30, 31). Our experiments extend this scenario by demonstrating that adenovirus E4 facilitates mRNA accumulation in an intron-dependent fashion. Virus-specific intron or exon sequences (Fig. 6) were shown not to be essential for E4 responsiveness. Thus, E4 appears to enhance mRNA expression by modulating a general mechanism in mammalian mRNA formation. Much to our surprise, we found that the position of the intron was critical for E4 responsiveness; it had to be located close to the pre-mRNA 5′ end. The rabbit β-globin large intron positioned in the 5′ untranslated region of the CAT mRNA was enhanced by E4 (Fig. 6*B*), whereas the same intron positioned downstream of CAT, close to the pre-mRNA 3′ end, was not (Fig. 4*B*). The lack of an effect of E4 on downstream introns was not restricted to the β-globin intron. All transcription units examined contain the SV40 small t intron in the 3′ untranslated region. These were all inactive in our E4 coexpression assay except in those cases in which an intron in the 5′ region was also present. A computer search for sequence homology between the tripartite leader introns and the rabbit β-globin large intron has not revealed any obvious nucleotide sequence motifs except for the splice signals. The precise sequence requirement for the E4 effect has to await a detailed mutational analysis.

The effect of E4 on major late mRNA expression was manifested already in the nucleus (Fig. 3), a result that is both compatible with a regulation at the level of transcription initiation and nuclear RNA stability. Our experiments show that the effect is not mediated through any of the major late promoter elements (Fig. 2) or through stabilization of transcription elongation (Fig. 4) and, thus, should represent a type of posttranscriptional regulation. Although we have not rigorously excluded the presence of an E4-regulated enhancer far downstream of the major late transcription start site, this possibility appears unlikely; it would require an enhancer in both tripartite leader introns and the β-globin intron.

The results presented here are consistent with previous results suggesting that a product encoded by E4 enhances major late mRNA accumulation posttranscriptionally by increasing nuclear RNA stability (10, 11). Our results extend this model by suggesting that an intron in the 5′ untranslated

region is required for this enhancement. We do not know, at the present time, whether E4 directly participates in pre-mRNA splicing or whether it acts at other steps in mRNA formation. Theoretically, several mechanisms could be envisaged. For example, an E4 product may directly bind RNA and facilitate RNA folding or initiate heterogeneous nuclear ribonucleoprotein assembly on nascent RNA chains. Both mechanisms would lead to a protection of growing transcripts against nuclease degradation. Alternatively, an E4 product may enhance RNA abundance by facilitating spliceosome assembly. It is known that spliceosomes form rapidly on nascent heterogeneous nuclear RNA transcripts (32) in a cap-dependent fashion (33). Formation of such complexes appears, at least *in vitro*, to stabilize RNA against unspecific nuclease degradation. All the mechanisms presented could explain our observation of a requirement for a 5′ intron for E4 responsiveness. With an intron located far away from the 5′ end, nascent transcripts would be vulnerable to nuclease attack before a protective E4 signal is synthesized. Such a cleavage would separate the cap structure from the elongation complex and would be expected to result in a rapid degradation of the nascent chains. With a 5′ intron, the growing transcript would rapidly be protected against nuclease degradation.

Our current efforts are also focused on the identification of the E4 protein(s), which is responsible for the 5′ intron-dependent enhancement of mRNA accumulation. E4 encodes a minimum of five translational open reading frames (ORFs) (1), which theoretically could be involved in this regulation. Among them, the ORF6 protein is a very likely candidate gene since several studies have shown that expression of this protein from E4 is sufficient for establishment of an essentially wild-type infection (12, 34, 35). The E4-ORF6 protein is also of interest since it has been shown to form a stable complex with the E1B-55K protein during a productive infection (36). This complex represents a functional unit that is necessary for normal transport and accumulation of late viral mRNA (2, 4, 37).

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