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Control of Adenovirus E1B mRNA Synthesis by a Shift in the Activities of RNA Splice Sites

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The primary transcript from adenovirus 2 early region 1B (E1B) is processed by differential RNA splicing into two overlapping mRNAs, 13S and 22S. The 22S mRNA is the major E1B mRNA during the early phase of infection, whereas the 13S mRNA predominates during the late phase. In previous work, it has been shown that this shift in proportions of the E1B mRNAs is influenced by increased cytoplasmic stability of the 13S mRNA at late times in infection. Two observations presented here demonstrate that the increase in proportion of the 13S mRNA at late times is also regulated by a change in the specificity of RNA splicing. First, the relative concentrations of the 13S to 22S nuclear RNAs were not constant throughout infection but increased at late times. Secondly, studies with the mutant, adenovirus 2 pm2250, provided evidence that there was an increased propensity to utilize a 5′ splice in the region of the 13S 5′ splice site at late times in infection. Adenovirus 2 pm2250 has a G→C transversion in the first base of the E1B 13S mRNA intron preventing splicing of the 13S mRNA but not of the 22S mRNA. During the early phase of a pm2250 infection, the E1B primary transcripts were processed into the 22S mRNA only. However, during the late phase, when the 13S mRNA normally predominates, E1B primary transcripts were also processed by RNA splicing at two formerly unused or cryptic 5′ splice sites. Both cryptic splice sites were located much closer to the disrupted 13S 5′ splice site than to the 22S 5′ splice site. Thus, the temporal increase in proportion of the 13S mRNA to the 22S mRNA is regulated by two processes, an increase in cytoplasmic stability of the 13S mRNA and an increased propensity to utilize the 13S 5′ splice site during the late phase of infection. Adenovirus 2 pm2250 was not defective for productive infection of HeLa cells or for transformation of rat cells.

The primary transcripts from many viral and some cellular transcription units are processed by alternative RNA splicing into two or more overlapping mRNAs (3, 4, 13, 23, 27, 30, 33, 40, 44). Many of the alternatively spliced viral mRNAs share common 5′ and 3′ termini but differ due to varying sizes of internal sequences removed by RNA splicing (44). During the course of infection by adenovirus 2 (Ad2), changes in the relative concentrations of such differentially spliced mRNAs occur for many of the viral transcription units (44). In most cases, the factors that control the temporal change in proportions of the various mRNA forms have not been described.

Early region 1B (E1B) of Ad2 is an example of a viral transcription unit coding for overlapping mRNAs which is under temporal control. The primary transcript from E1B is processed by alternative RNA splicing into either a 13S or 22S mRNA sharing common 5′ and 3′ termini (7, 10, 28, 38) (Fig. 1). The 13S mRNA is translated from the first AUG in the message into a protein of 21 kilodaltons, whereas the 22S mRNA is translated into this same protein from the first AUG and a 55-kilodalton protein from the second AUG (8). During the early phase of infection, the 22S mRNA is the major E1B species; however, at late times in infection, when the overall level of E1B expression is elevated, the 13S mRNA becomes the major E1B RNA form (42). In this report, we investigate whether the change in proportions of the E1B mRNAs is influenced by a change in the frequency of splicing the primary E1B transcript at one or the other alternative 5′ splice site. Although it seems likely that shifts in the relative concentrations of differentially spliced mRNAs are controlled by changes in the activities of different splice sites, this has not yet been shown to be a general phenomenon. Currently, the best evidence that a change in the specificity of RNA splicing is responsible for altering the pattern of RNAs produced during the course of infection is provided by studies of the L1 family of adenovirus mRNAs (2, 31, 48).

In earlier studies of the regulation of E1B expression, it has been found that an increase in cytoplasmic stability of the 13S mRNA during the late phase of infection influences the relative abundance of the two E1B mRNAs (47). In this paper, we provide evidence demonstrating that RNA splicing is also a major factor in controlling the change in proportions of the 13S and 22S mRNAs.

MATERIALS AND METHODS

Construction of Ad2pm2250. The dodecanucleotide 5′-GTACAGCTGGCT-3′ was synthesized by the method of Fisher and Caruthers (15), and oligonucleotide-directed mutagenesis (25, 39) was performed as previously described (35) with the M13 clone M131B13S. M131B13S was constructed by cloning an *Sst*I-*Pvu*II fragment (nucleotides 1768 to 2480 of the R strand of Ad2 [19]) from pBE5 (43) between the *Sst*I and *Pvu*II sites of M13G*ori*1 (24). After one cycle of enrichment for the desired mutant, M131B13Spm2250, plaques were screened by dideoxynucleotide DNA sequencing (41) and for the creation of a new *Pvu*II site. The mutation was transferred from M131B13Spm2250 into the Ad2 genome as described previously (12, 35). The mutant adenovirus, Ad2pm2250, was plaque purified twice on 293 cells (20) before a large virus stock was prepared. The mutation in Ad2pm2250 was verified by identification of the

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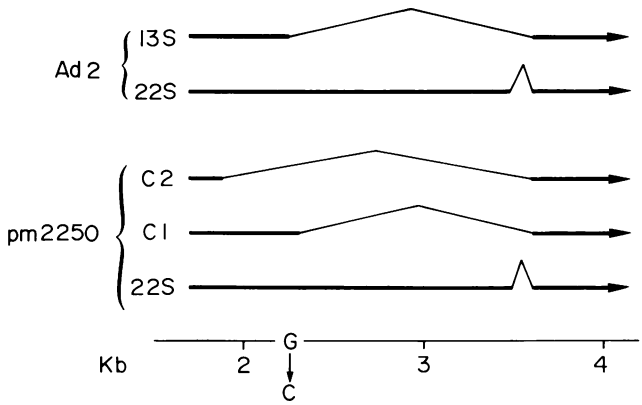

FIG. 1. Structures of the spliced Ad2 and pm2250 RNAs at late times in infection. The bottom line represents the 36-kilobase Ad2 genome demarcated in kilobase pairs. The G→C transversion disrupting the 13S 5′ splice site of pm2250 is shown. The exons of the RNAs are represented by the horizontal lines joined by caret symbols. Arrowheads indicate the 3′ ends of the RNAs. C1 and C2 refer to the aberrantly spliced pm2250 RNA observed specifically during the late phase of infection.

new *Pvu*II site at position 2247 in DNA isolated from mutant virions.

Hybridization–S1 nuclease analyses. Nuclear and cytoplasmic RNAs were prepared from suspension culture HeLa cells 6 and 20 h postinfection (p.i.) with Ad2 or Ad2pm2250 at a multiplicity of infection of 10. Nuclear and cytoplasmic fractions were isolated as described previously (29). Briefly, infected cells were washed with phosphate-buffered saline, lysed with Nonidet P-40 in an isotonic buffer, and separated from cytoplasmic components by differential centrifugation. The pelleted nuclei were washed twice by suspension in five volumes of Nonidet P-40 lysis buffer followed by centrifugation. In some experiments (see Fig. 3), half the nuclei were further purified by sedimentation through a sucrose gradient as described previously (32). Cytoplasmic and nuclear RNAs were prepared as described previously (5, 34). S1 analyses were performed with 100 μg of nuclear or cytoplasmic RNA. Probe A (see Fig. 2) was prepared by digesting 2 μg of *Xho*I fragment D (nucleotides 1 to 5779) from Ad2 or Ad2pm2250 with *Bst*EII and 3′ end labeling at nucleotide 1914 in total volume containing 50 μCi of [α-^{32}P]dATP (3,000 Ci/mmol), 12 U of reverse transcriptase (Life Sciences, Inc.), 40 μM dGTP, dTTP, and the recommended reverse transcriptase buffer for 45 min at 42°C. The ^{32}P-labeled DNA probe B (see Fig. 4) was prepared by digesting 2 μg of M131B13S or M131B13Spm2250 with *Bst*EII and 3′ end labeled as described above. Hybridizations were as described previously (6) for 3 h with the homologous probe A or for 12 h with the homologous probe B at 55°C. Probe C (see Fig. 5) was prepared by uniformly labeling M13 E1A single-stranded DNA with ^{32}P as described previously (37). S1 analyses with probe C were as described previously (34). The S1-protected fragments were fractionated on 8 M urea–5% polyacrylamide gels (16 cm) and visualized by autoradiography.

RESULTS

The proportion of 13S to 22S E1B nuclear RNA increases during the late phase of infection. If a change in the specificity of RNA splicing were a factor controlling the increased proportion of 13S to 22S E1B mRNA during the late phase of infection, then the proportion of 13S to 22S nuclear RNA would be expected to increase late in infection since RNA splicing occurs in the nucleus. Alternatively, if an increase in the stability of 13S mRNA in the cytoplasm were the sole mechanism controlling the change in ratio of 13S to 22S mRNA concentrations and there were no change in the frequency of splicing at the 13S 5′ splice site, then the proportion of nuclear 13S and 22S RNAs would remain constant throughout infection. If the ratio of the E1B mRNAs were controlled by a combination of both cytoplasmic and nuclear processes, then some shift in the relative nuclear RNA levels would be observed between the early and late phases, although a less dramatic one than that observed at corresponding times in the cytoplasm.

To discriminate between these possibilities, nuclear and cytoplasmic RNAs were prepared from HeLa cells during the early phase (6 h p.i.) and during the late phase of infection (20 h p.i.) with Ad2 and analyzed by the hybridization–S1 nuclease method (6) (Fig. 2). Since the level of E1B

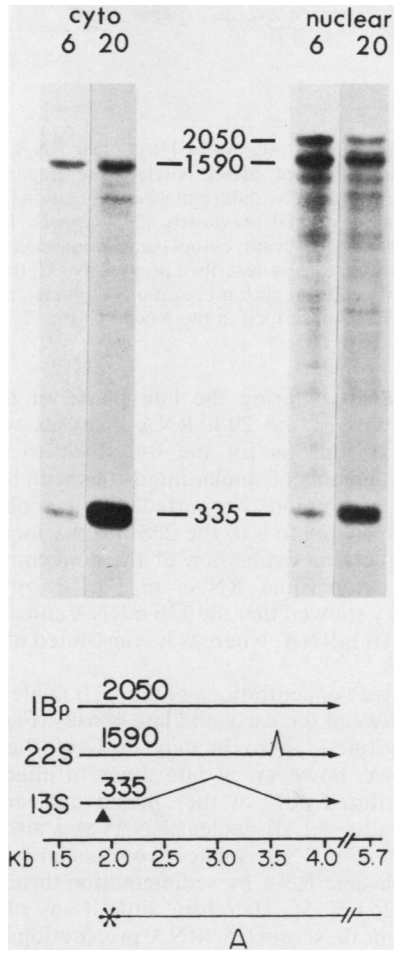

FIG. 2. Analysis of the nuclear and cytoplasmic Ad2 E1B RNAs during the early and late phases of infection. Nuclear or cytoplasmic Ad2 RNA (100 μg), harvested from HeLa cells 6 or 20 h p.i., was analyzed by the hybridization–S1 nuclease technique (6), using probe A. Probe A is 3′ end labeled with ^{32}P at nucleotide 1914 and extends beyond the 3′ end of E1B (represented by the bottom line). The S1-protected DNA fragments were fractionated by electrophoresis and visualized by autoradiography. The autoradiograms for the 6-h RNA analyses were exposed 10 times longer than the analyses of the 20-h RNA. The asterisk and solid triangle represent the position of the 3′ end label on the DNA probe A. 1Bp refers to the unspliced nuclear E1B RNA.

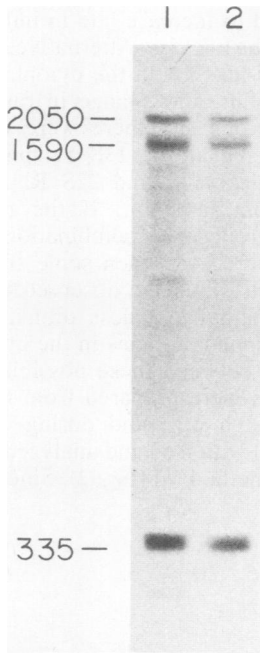

FIG. 3. Analysis of late Ad2 E1B nuclear RNA prepared from sucrose gradient-purified nuclei. Nuclei were prepared from HeLa cells 20 h p.i. with Ad2 by differential centrifugation in Nonidet P-40 lysis buffer as described previously (29) (lane 1). Half the nuclei were further purified from cytoplasmic components by sucrose gradient centrifugation as described previously (32) (lane 2). Nuclear RNA was isolated from both preparations of nuclei, and S1 analysis was performed as described in the legend to Fig. 2.

RNA is elevated during the late phase of infection, the autoradiograms of the 20-h RNA analyses were exposed only 10% as long as for the 6-h RNA to generate S1-protected fragments of similar intensities with both early and late RNAs. As previously reported (42), the concentration of the 13S mRNA relative to the 22S mRNA increases at late times in infection. Estimation of the concentrations of the Ad2 E1B cytoplasmic RNAs in Fig. 2 by quantitative densitometry showed that the 13S mRNA constituted 35% of the early E1B mRNA, whereas it constituted 85% of the late E1B mRNA.

The relative concentrations of the E1B nuclear RNAs also changed between the early and late phases (Fig. 2). The 13S RNA constituted approximately 20% of the spliced 6-h nuclear RNA; however, at late times in infection the 13S RNA constituted 60% of the spliced nuclear RNA. This change in ratio of E1B nuclear RNAs was also observed in S1 analyses with RNA isolated from nuclei further purified from cytoplasmic RNA by sedimentation through a sucrose gradient (32; Fig. 3). Therefore, little if any of the 13S and 22S RNAs in these nuclear RNA preparations were due to contaminating cytoplasmic RNA. The 2,050-nucleotide band found exclusively in the analyses of nuclear RNA was protected by the unspliced E1B RNA (28, 35) (1Bp, Fig. 3). The observed increase in the ratio of 13S to 22S nuclear RNA during the late phase demonstrates that the change in ratio of these E1B RNAs is controlled predominantly in the nucleus. In the next section, evidence is provided that the nuclear control is an increase in the frequency of splicing the initial transcript into the 13S rather than the 22S species.

The array of faint S1-protected fragments observed between the 335 and 1590 fragments (Fig. 2) may represent infrequently used 5′ splice sites in E1B or S1-sensitive sites in the RNA-DNA hybrids. Indeed, indirect evidence for the existence of at least one minor E1B mRNA with an alternative splicing pattern from the major 13S and 22S mRNAs has been provided by the observation of a minor E1B primary translation product of approximately 17 kilodaltons which shares amino and carboxy terminal sequences with the E1B 55-kilodalton protein (21; J. Lewis and C. Anderson, personal communication).

Cryptic splice sites utilized only at late times by an Ad2

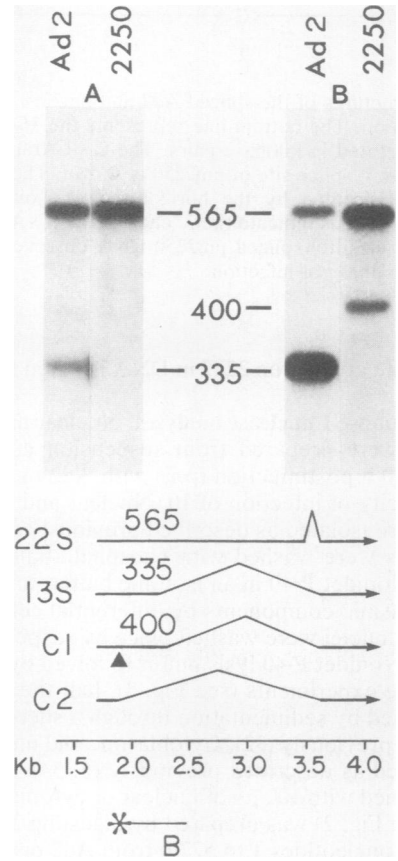

FIG. 4. Analysis of E1B mRNAs from pm2250. Cytoplasmic RNA was prepared from HeLa cells 6 h (early RNA) or 20 h (late RNA) p.i. with Ad2 or pm2250 and analyzed by the hybridization–S1 nuclease technique (6). RNA (100 μg) was hybridized to a DNA probe 3′ end labeled with ^{32}P at nucleotide 1914 (probe B). The DNA sequences in probe B homologous to E1B RNA are represented by the bottom line below panels A and B. This Ad2 sequence was continuous with a sequence from M13*Gori*1 (24) represented by the dashed line. The end label is represented by the asterisk. The hybridizations were followed by S1 digestions, and the S1-protected fragments were fractionated by electrophoresis and visualized by autoradiography. The autoradiograms from the 6-h RNA analyses were exposed 10 times longer than the analyses of the 20-h RNA to generate bands of similar intensities. The sizes of the S1-protected fragments are given in nucleotides. The structures of the RNAs which gave rise to the S1-protected fragments are represented by the lines joined by caret symbols below the corresponding panels. A portion of the Ad2 genome is indicated by the line demarcated in kilobase pairs. C1 refers to the aberrantly spliced pm2250 RNAs that utilized 5′ splice sites near nucleotide 2318. C2 refers to a second aberrantly spliced mRNA described in the text and is mapped in Fig. 5. The solid triangle below C1 indicates the position of the 3′ end label in the DNA probe B, also indicated by the asterisk. A, S1 analysis of 6-h RNA; B, S1 analysis of 20-h RNA.

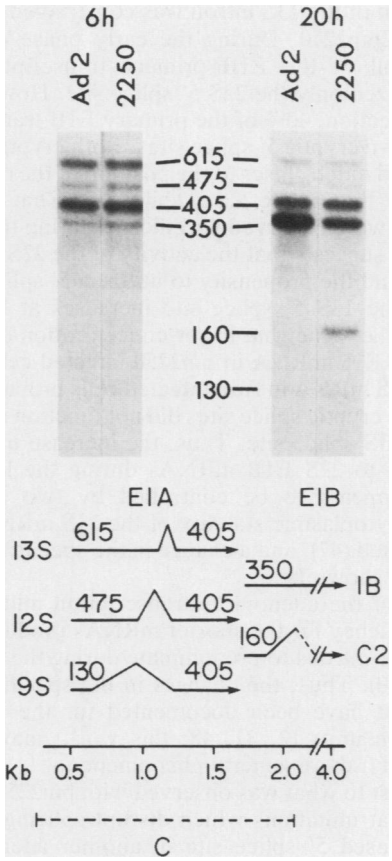

FIG. 5. Analysis of the 5′ portion of the E1B pm2250 RNAs. S1 analyses of cytoplasmic RNAs were performed as described in the legend to Fig. 3 with a DNA probe uniformly labeled with ^{32}P (probe C, represented by a broken line). C2 refers to the aberrantly spliced pm2250 RNAs that utilize a 5′ splice site near nucleotide 1853. 1B refers collectively to all of the E1B pm2250 RNAs except for C2.

mutant which fails to synthesize the 13S mRNA. To facilitate our studies of the regulation of E1B gene expression, an Ad2 mutant was constructed that was defective in splicing the 13S mRNA. Comparison of sequences encompassing 5′ splice sites has shown that nearly all introns begin with the dinucleotide GU (9, 36). Mutations of either the G (45, 46) or U (34) disrupt mRNA splicing. Therefore, to prevent splicing of the E1B 13S mRNA, we constructed a G→C transversion in the first base of the 13S mRNA intron, creating the mutant Ad2pm2250 (pm2250 is the point mutation at nucleotide 2250 in the Ad2 sequence [19]). Studies of pm2250 RNA processing described below provided evidence for an increased splicing activity at the 13S 5′ splice site during the late phase of infection. During the early phase of infection with pm2250, the E1B initial transcripts were processed into 22S mRNA only. However, during the late phase of infection, E1B RNAs were observed which were spliced at normally unused or cryptic splice sites near the disrupted 13S 5′ splice site. The observation of these cryptic splice sites in the vicinity of 13S 5′ splice site during the late phase of infection specifically is further evidence that there is an increased tendency to form the 13S splice during the late phase.

S1 analyses were performed with cytoplasmic RNA prepared from HeLa cells infected with Ad2 or pm2250 at 6 and 20 h p.i. The results with the early RNA sample shown in Fig. 4A demonstrate that although the total concentration of pm2250 cytoplasmic RNA is similar to that of Ad2, within the sensitivity of the S1 analysis, the 13S mRNA was eliminated. The 335-nucleotide band protected by the 13S mRNA in wild-type Ad2 was not detected in pm2250-infected cells. Synthesis of the 22S mRNA, which protects the 565-nucleotide band, was not affected by the point mutation. Therefore, the G that begins all mRNA introns is required for splicing of the E1B 13S mRNA.

In contrast to the results with early pm2250 RNA, approximately 40% of the late pm2250 RNA was aberrantly spliced at two cryptic 5′ splice sites near the disrupted 13S splice site. A 400-nucleotide band was present in the S1 analysis of late pm2250 RNA (Fig. 4B) which was not seen in analyses of late Ad2 RNA (Fig. 4B), early pm2250 RNA (Fig. 4A), or in longer exposures of these gels (data not shown). We interpret this 400-nucleotide band to be created by a cryptic 5′ splice site (C1). Based on the size of the S1-protected fragment and homologies to known consensus splice site sequences (9, 36), this cryptic splice site probably occurs at nucleotide 2318 in the Ad2 sequence. Additional S1 analyses of early and late Ad2 and pm2250 RNA with other ^{32}P-labeled DNA probes indicated that one other late-specific cryptic splice site was utilized in pm2250-infected cells, again only during the late phase of infection. The 160-nucleotide band seen in an S1 analysis with the uniformly ^{32}P-labeled DNA probe C (Fig. 5) and late pm2250 RNA indicated that the second cryptic 5′ splice site (C2) mapped near nucleotide 1853 (Fig. 5). The distance between these cryptic splice sites and the disrupted 13S 5′ splice site is similar to the distance between cryptic splice sites and disrupted splice sites in mutant globin transcripts (14, 45, 46). The aberrantly spliced pm2250 RNAs were also observed in S1 analyses of pm2250 nuclear RNA isolated during the late phase, but not the early phase, of infection (data not shown). We estimated by quantitative densitometry that the concentrations of the two aberrantly spliced mRNAs together constituted approximately 40% of the total pm2250 cytoplasmic RNA at 20 h p.i.; 30% was spliced at C2 and 10% was spliced at C1.

No cryptic 3′ splice sites were detected at late times in pm2250-infected cells (data not shown). Therefore, the two cryptic 5′ splice sites are most likely spliced to the same 3′ splice site used to form the 13S and 22S mRNAs. Since pm2250 RNA was aberrantly spliced only during the late phase of infection and near the disrupted 13S 5′ splice site, we conclude that there is an increased propensity to utilize a 5′ splice site in this region during the late phase of infection. Furthermore, this increased propensity to splice shorter E1B mRNAs during the late phase of infection is not simply a consequence of the increased E1B transcription during the late phase. In infected cells treated with the DNA synthesis inhibitor cytosine arabinoside and incubated for 45 h, the rate of E1B transcription increases 30 times (17, 18). Yet under these conditions the cryptic E1B splices were not observed in pm2250-infected cells (data not shown).

Replication and transforming activity of Ad2pm2250. Ad2pm2250 was propogated in 293 cells (20) which complement essential functions encoded in E1A and E1B (22) due to the constitutive expression of an integrated copy of Ad5 E1A and E1B (1, 5). To determine whether any essential viral functions were encoded by the E1B 13S mRNA, we assayed the ability of pm2250 to form plaques on HeLa cells as compared with 293 cells. The mutant yielded virtually equal numbers of plaques on the two cell lines, indicating that no essential viral functions were defective.

We also tested the ability of pm2250 to transform rat

primary embryo cells and CREF cells, a line of rat cells particularly susceptible to transformation by group C adenoviruses (16). The frequency of focus formation by pm2250 was similar to that of Ad2 on primary cells and reduced approximately twofold on CREF cells. We conclude that the E1B 13S mRNA does not uniquely encode any viral functions that are essential for a productive infection of HeLa cells or for transformation of primary or immortalized rat cells.

DISCUSSION

Although gene expression in eucaryotic cells appears to be regulated most commonly at the transcriptional level, other regulatory processes occur (11). In this report, a posttranscriptional process that temporally modulated expression of the Ad2 E1B mRNAs was investigated. The results given here indicate that a major factor controlling changes in the proportions of the 13S and 22S mRNAs during the course of infection is an increase in the frequency of splicing at the 13S compared with the 22S 5′ splice site during the late phase. Although shifts in the activities of RNA splice sites may influence changes in the relative levels of the individual mRNAs from many complex transcription units, in most cases this has not been examined. Only in the case of the adenovirus L1 family of RNAs is there evidence that mRNA splicing is involved in controlling the temporal change in the proportions of the mRNAs (2, 31, 48).

Evidence that the relative concentrations of the individual E1B mRNAs are strongly influenced by a change in the specificity of mRNA splicing was provided in two types of analyses. In the first approach, the ratios of the nuclear Ad2 E1B RNAs were compared at early and late times since RNA splicing occurs in the nucleus. It was found that the relative concentrations of the Ad2 E1B nuclear RNAs changed between the early and late phases. If increased cytoplasmic stability of the 13S mRNA accounted completely for the higher proportion of the 13S mRNA at late times (47), then the relative levels of the 13S and 22S nuclear RNAs would have been constant throughout infection. Analysis of the Ad2 nuclear RNAs demonstrated that the 13S nuclear RNA, which was the minor species at early times, became the major E1B nuclear RNA at late times. This observation demonstrated that the temporal change in the relative proportions of the E1B mRNAs was controlled largely by nuclear processes. The result is consistent with a change in the specificity of RNA splicing favoring the 13S 5′ splice site during the late phase of infection. Alternatively, the increased proportions of 13S nuclear RNA, from 20% of the total spliced E1B nuclear RNA during the early phase to 60% during the late phase, could be due to increased nuclear stability of the 13S RNA during the late phase. Evidence for nuclear control at the level of RNA splicing is presented in the second type of analysis.

Previous results suggest that there is a requirement for many primary transcripts to be processed by RNA splicing. In globin genes, mutations that interfere with splicing at a 5′ splice site result in RNA splicing at formerly unused or cryptic 5′ splice sites (14, 45, 46). We reasoned that if the high proportion of E1B 13S mRNA observed during the late phase were due in part to increased splicing activity at the 13S 5′ splice site, then disruption of the 13S 5′ splice site might lead to the appearance of cryptic splice sites during the late phase of infection specifically. Therefore, in a second approach to determine whether there was a shift in the activities of the E1B splice sites, a G → C transversion at the first position of the 13S intron was constructed, creating the mutant Ad2pm2250. During the early phase of a pm2250 infection, all of the E1B primary transcripts that were spliced utilized only the 22S 5′ splice site. However, at late times in infection, 40% of the primary E1B transcripts were spliced at two cryptic 5′ splice sites. Both cryptic splice sites were located much closer to the position of the disrupted 13S 5′ splice site than to the 22S 5′ splice site. That these cryptic splice sites were observed specifically during the late phase of infection suggests that the activity of the 22S 5′ splice site decreases and the propensity to utilize a 5′ splice site in the region of the 13S 5′ splice site increases at late times in infection. The somewhat lower concentration of cryptically spliced late E1B mRNA in pm2250-infected cells compared with late 13S mRNA in Ad2-infected cells probably occurred because the cryptic splice sites did not function as well as the wild-type 13S splice site. Thus, the increase in the proportion of 13S to 22S E1B mRNAs during the late phase of infection appears to be controlled by two processes: a change in cytoplasmic stability of the 13S mRNA, as previously reported (47), and a change in the specificity of mRNA splicing, as shown here.

In each of the adenovirus transcription units, there is a general tendency for the shorter mRNAs produced by splicing out large introns to predominate during the late phase of infection (10). Thus, the changes in the specificity of RNA splicing that have been documented for the L1 and E1B transcription units (2, 31, 48; this work) may be specific examples of a more general phenomenon.

In contrast to what was observed with pm2250, we recently found that mutations which disrupt splicing of the most frequently used 5′ splice site in another adenovirus transcription unit, early region 1A (E1A), did not result in aberrant splicing (C. Montell and A. J. Berk, Cell, in press). The primary E1A transcript is processed at two alternative 5′ splice sites, 12S and 13S, during the early phase of infection (7, 10, 28). Throughout the course of infection, the 13S mRNA predominates over the 12S mRNA (34). Mutations that prevent splicing of the 13S mRNA, which constitutes 70% of the early nuclear and cytoplasmic E1A RNA (7; Montell and Berk, unpublished data), do not lead to cryptic splicing at any time during the lytic cycle (Montell and Berk, in press). Instead, all of the early primary E1A transcripts are spliced at the 12S 5′ splice site.

The observation that in the E1A mutants no cryptic E1A splice sites were used when splicing of the major mRNA was prevented, whereas in pm2250 aberrant E1B splicing did occur at late times, could be accounted for as follows. The splicing apparatus might initially be directed to a specific region of the primary transcript, for example, the left end of E1B during the late phase of infection. Khoury et al. (26) have presented findings suggesting that secondary or tertiary RNA structure influences the location in which a splicing event occurs. Once the splicing apparatus is directed to the preferred region, the region might then be scanned for the best 5′ splice site based on primary sequence. When the E1B 13S 5′ splice site is disrupted, other nearby cryptic 5′ splice sites are selected since no natural 5′ splice site occurs within some critical distance. On the other hand, the alternative E1A 5′ splice sites are only 138 nucleotides apart. Disruption of the major E1A 5′ splice site may not cause aberrant splicing because of the close proximity of the alternative natural 5′ splice site. The 12S and 13S E1A 5′ splice sites are situated closer together than the two cryptic 5′ splice sites in pm2250 and may both occur within a region of the E1A primary transcript most accessible to the splicing apparatus.

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