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DNA template effect on RNA splicing: two copies of the same gene in the same nucleus are processed differently

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Many cellular and viral genes are parts of complex transcription units containing multiple splicing choices. During the course of an adenoviral replicative cycle, different spliced versions of a single gene predominate, depending on the stage of infection. This is true for several adenoviral genes. In this paper we show for the viral E1B transcription unit that splice site usage regulates this process. The change in alternative splicing in this system does not depend on the sequence of the transcribed genes. Non-adenoviral genes, such as the SV40 early region and the polyoma early region, which normally show little or no regulation of spliced RNA product formation, become regulated for mRNA production after insertion into the adenoviral genome. Additional studies show that E1B splicing regulation in adenovirus is a *cis* effect. Staggered infections using two discernable viral genomes resulted in a situation where both early and late genomes exist in the same nucleus. Neither genome was able to impose its regulated splicing pattern on the other, indicating that the cue for the switch in viral gene splicing is not directly dependent on global changes in *trans*-acting splicing factors. This suggests a model where the signal for changes in RNA processing for the E1B gene is linked to the state of the DNA template or its localization within nuclear subcompartments.

Key words: adenovirus 5/alternative mRNA splicing/E1B

Introduction

Regulated formation of alternatively spliced mRNA products is one way in which gene expression is controlled in eukaryotic cells (Smith *et al.*, 1989, for review). Like cellular genes, many viral genes can also exhibit regulated RNA processing. This is indicated when different spliced versions of a single gene predominate depending on the stage of infection. In addition to changes in the accumulation of various processed RNA products during the adenovirus replicative cycle in human cells, differences also occur in the relative rate of transcription of certain viral genes (Ginsberg, 1984). Both of these changes in transcription and RNA processing occur following the initiation of viral DNA replication, the event which divides the early and late phases.

The adenovirus E1B gene was chosen as a model to study changes in RNA products at early versus late times following adenovirus infection of human HeLa cells. The E1B precursor RNA is spliced to produce primarily two mRNA products (Anders and Pettersson, 1985), a 22S product which predominates early after infection and a 13S product which predominates later. The identification of the level of regulation of this switch has been controversial. Initial studies suggested a role for differences in RNA stability in the cytoplasm, but subsequent analyses indicated that much of the switch is due to regulated splicing (Wilson and Darnell, 1981; Montell *et al.*, 1984b).

Several cellular gene transcription units have been shown to produce differentially spliced mRNAs in a manner that suggests the existence of cell specific *trans*-acting splice regulatory factors (Breitbart and Nadal-Ginard, 1987; Leff *et al.*, 1987; Barone *et al.*, 1989; Zacher *et al.*, 1987; Sosnowski *et al.*, 1989; Smith *et al.*, 1989). As an example, when the troponin gene is transfected into muscle and non-muscle cell types, both the endogenous and transfected version of the genes are spliced in a cell-specific manner. Therefore, regulated formation of the different spliced products occurs for these genes regardless of the state of the DNA template or the site of the integrated gene. Based on these results it has been suggested that differences in the levels of *trans*-acting factors regulate this splice choice event, although definitive proof by demonstrating cell specific regulation of splicing *in vitro* using a non-chimeric system (Siebel and Rio, 1990) has not been reported (Maniatis, 1991).

Our studies invoke a new model where the relative levels of the spliced E1B RNA products are not dependent on changes in *trans*-acting factors in the cell, but instead are dependent on changes in the viral DNA template that occur following its replication. The unexpected result that the non-regulated splice site choices in the SV40 and polyoma early transcription units become regulated once inserted into the adenoviral genome show the effect is not peculiar to the E1B gene. This raises the possibility of a general linkage of chromosome context and RNA processing.

Results

The E1B 22S mRNA predominates during the pre-replicative phase of the viral infection of HeLa cells, while the 13S predominates following viral DNA replication

Following a wild-type adenovirus 5 (Ad5) infection in human HeLa cells, almost all stable cytoplasmic E1B RNA is spliced using either the 13S or 22S splice sites (Figure 1). As previously shown, there is a large change in the composition of stable E1B mRNAs from early after infection to late (post-viral DNA replication) (Spector *et al.*, 1978). Cytoplasmic RNA was isolated from In340 virus infected HeLa cells (Hearing and Shenk, 1983) and the E1B RNA species were identified using a T2 RNase protection assay (Adami and Babiss, 1990). In340 virus is phenotypically wild-type and contains an intact E1B transcription unit. Results with a probe

spanning the 22S 5′ splice site (ss) and the 22S/13S 3′ ss are shown (Figure 2). There are two protected RNA species of interest; one is 183 nt in size, representing RNA containing the 22S splice and the other is 195 nt and is derived from the 3′ exon shared by both the 13S and 22S spliced RNA. This 195 nt fragment is indicative of total spliced E1B RNA. At 7 h post-In340 virus infection, the band indicative of 22S spliced RNA is about half the intensity of that of total spliced RNA. At 23 h post-infection very little of the 22S specific band is seen. This corroborates the 50:1 change in levels of 22S versus 13S RNA reported earlier (Spector *et al.*, 1978). For the 23 h time point it should be noted that one-tenth the amount of RNA was assayed when compared with the early time point. At this late time in the viral replicative cycle many viral DNA templates contribute to the increase in transcription and accumulation of the E1B RNAs (Spector *et al.*, 1978). Actual rates of synthesis of the 22S form of E1B RNA are about the same as or higher than early after infection (Spector *et al.*, 1978).

Levels of E1B mRNAs are controlled by splice site selection

Several models for the change in the relative levels of the two major processed forms of E1B RNA have been formulated. A splicing model suggests that changes occur in splice site usage during infection, with the 22S 5′ ss preferred early after infection and the 13S 5′ ss chosen late. A stability model proposes that the 22S and 13S RNAs are produced at a fixed relative level, but that the two RNAs have variable relative stabilities, the 22S form being stable early and the 13S form being stable later. A third type of control would be regulated transport of E1B RNA to the cytoplasm. Montell *et al.* (1984b) have shown that throughout the viral replicative cycle, the relative levels of 13S and 22S E1B RNAs are nearly identical in the nucleus and the cytoplasm of the infected cells, suggesting that differential E1B RNA transport does not contribute to the regulation of the relative levels of these RNAs.

To differentiate between splicing and RNA stability models, we constructed a recombinant virus (Bla3) that contained a substitution of the E1B 22S 5′ ss with a mutated 5′ ss (see Materials and methods). Analysis of RNA from early and late Bla3 infected HeLa cells allowed us to evaluate the stability of the 13S RNA when it is the only E1B mRNA expressed.

Cytoplasmic RNA from late and early In340 and Bla3 virus infections were compared by T2 RNase protection assays. Using a wild-type E1B probe that hybridizes across the mutated 22S 5′ exon and the downstream 13S and 22S 3′ ss and 3′ exon, we show that the mutated 22S 5′ ss in Bla3 was not used efficiently early after infection (lack of a band at 183 nt, see Figure 2). A similar assay, using probes which span the E1B gene region, showed that cryptic splice sites were not used and that all stable Bla3 E1B RNA was in the 13S form (data not shown). E1B mRNA levels were measured during this pre-replicative phase of the infection to control for variations in virus input, which would have an impact on the E1B mRNA levels. The results indicate that at 7 h post-infection, E1B RNA accumulated to similar levels for both viruses (7 h, Bla3 and In340, 195 nt bands are at same intensity). Therefore, when the virus is forced to make 13S mRNA, it accumulates to the same levels as total 22S E1B RNA does in In340 virus infected cells. The

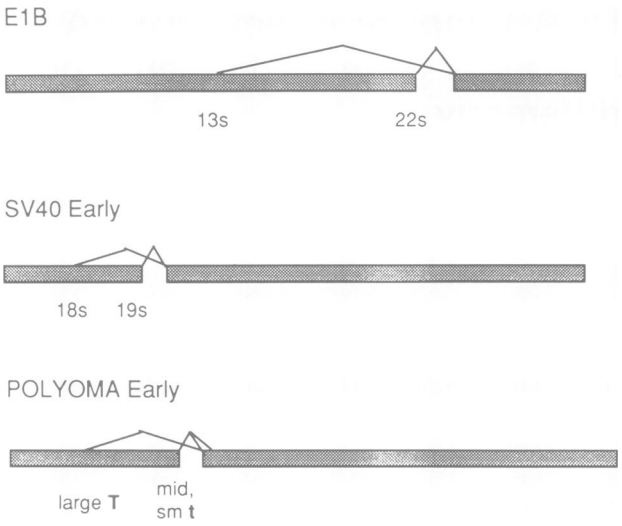

Fig. 1. Diagram of the transcription units studied. The major splicing signals and mRNA products are indicated for the adenovirus E1B gene, and the SV40 and polyoma early regions. Alternative 5′ splice sites and the mRNAs to which they correspond are identified. Angled lines represent the alternative splices that are made. The shaded boxes represent sequences that can act as exons, while the breaks are introns.

simplest conclusion is that early after infection there is no difference in stability of the two RNAs. The experimental finding that inactivation of one splice site results in efficient usage of an alternative site has been seen in other systems (Montell *et al.*, 1982; Kedes and Steitz, 1988).

According to a differential stability model, we would expect to see an increase in E1B RNA levels at late times following Bla3 virus infection. Late gene mRNA levels were measured using a probe that hybridizes across the third leader of all major late transcripts (Figure 2). This allowed us to score for differences in the kinetics of the replicative cycles for both viruses (Bla3 virus replication is delayed compared with In340 virus). Two exposures of the same autoradiogram are shown to facilitate comparisons of the assays from the In340 and Bla3 virus infected cells. Following normalization to the third leader RNA signals, both viruses express equal amounts of E1B RNA late after infection (compare E1B 195 nt band with late leader 89 nt band for Bla3 and In340 in short exposure). This is not what one would expect in a model where the 13S RNA is unstable early and stable late. In summary, when 22S splice site selection is inhibited, relative levels of the two RNA species change, but total amounts of E1B RNA are unchanged. This is the opposite of what would be predicted by a regulated differential stability model and thus indicates that much if not all of the regulation of the relative levels of the major E1B RNAs seems to occur by differential synthesis.

Superinfection experiments to look for trans-acting, splicing regulatory factors

Adenovirus infected HeLa cells show marked changes in viral gene transcription once replication of the viral DNA takes place. It has been determined that this change in transcription is a *cis*-acting (or DNA template) effect (Thomas and Mathews, 1980). Superinfection of distinguishable adenovirus genomes into the same cells revealed that the replicating virus does not influence the rate of early gene expression from the superinfecting virus. The superinfecting

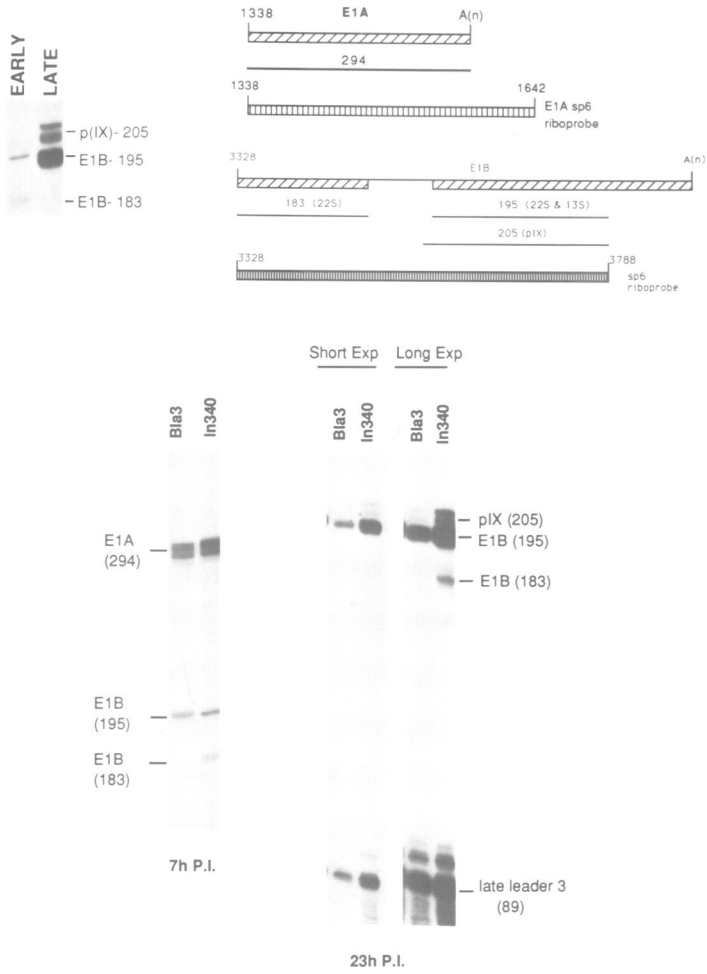

Fig. 2. The change in the cytoplasmic concentrations of the E1B mRNAs at early and late times after infection are due to alternative splicing and not to differential mRNA stability. Monolayer cultures of HeLa cells were infected with 20 p.f.u. of the viruses shown per cell for the times indicated. Total cytoplasmic RNA was isolated and analyzed using a T2 RNase protection assay at the times indicated. Different amounts of RNA were analyzed depending on the length of time of infection. 1/15 and 1/150 of the RNA from a 100 mm plate of cells was used for the early and late time points respectively. Protected RNA bands are identified by size. The transcript for protein IX is from its own promoter, is unspliced and is made only late after infection (Ginsberg, 1984). In the diagram, the E1B exons are depicted as hatched boxes, while introns are shown as lines. Expected protected probe sizes are indicated and identified according to the mRNAs they represent. The riboprobes are shown as vertically hatched boxes. Markers (not shown) are *Hpa*II digested pBR322 with the ends filled in with [^{32}P]CTP and DNA polymerase. The autoradiogram in the upper left shows a comparison of T2 RNase protection assays of E1B RNA from early and late wild-type (In340) infected cells. The lower autoradiogram show results from a similar T2 RNase protection assay, this time comparing RNA from wild-type and Bla3 infected cells. In this assay controls for RNA levels are included. At 7 h.p.i. E1A mRNA was scored to show similar viral inputs. At 23 h.p.i., the late leader 3 RNA was scored, and revealed a lag in the kinetics of Bla3 virus replication when compared with In340 virus. The long exposure was 10 times longer than the short exposure.

virus shows a similar lag in the initiation of viral DNA replication (Crossland and Raskas, 1983). Using complementation of mutant viruses and other experimental approaches it was established that in the superinfection assay, the second viral infection is efficient (Thomas and Mathews, 1980; Gaynor and Berk, 1983). This allowed the conclusion that two distinct viral genomes can simultaneously exist in the same nucleus, one transcribing in the early mode and the other in the late mode.

Superinfection studies were done to examine if *trans*-acting factors play a dominant role in regulating E1B mRNA splicing. These studies required viruses which were distinguishable in areas of interest, while still retaining the wild-type E1B splice regulation phenotypes. Two E1B deletion mutants (dl110 and dl112) (Babiss and Ginsberg, 1984; Babiss *et al.*, 1984) were tested for E1B RNA expression early and late after HeLa cell infection. Like In340 virus, the E1B deletion mutant viruses contain intact 13S and 22S 5′ splice sites. All these viruses were found to show the dominance of the 22S splice early and the 13S splice late (Figure 3). Early after HeLa cell infection, the 22S 5′ exon produces a band of nearly equal intensity to that of the shared 13S and 22S 3′ exon, while late after infection the band indicative of the 22S 5′ exon becomes minor. These results have been corroborated by assays using probes spanning the 13S 5′ ss and the minor splice sites (Figure 4 and data not shown). There were variabilities in total RNA levels among these viruses, most likely due to the non-functional E1B proteins they encode. These proteins have been shown to play roles in DNA stability, RNA synthesis and RNA transport and are necessary for an efficient infectious cycle (Babiss and Ginsberg, 1984; White *et al.*, 1984; Leppard and Shenk, 1988). However, our results using both E1B mutant viruses suggest that the E1B

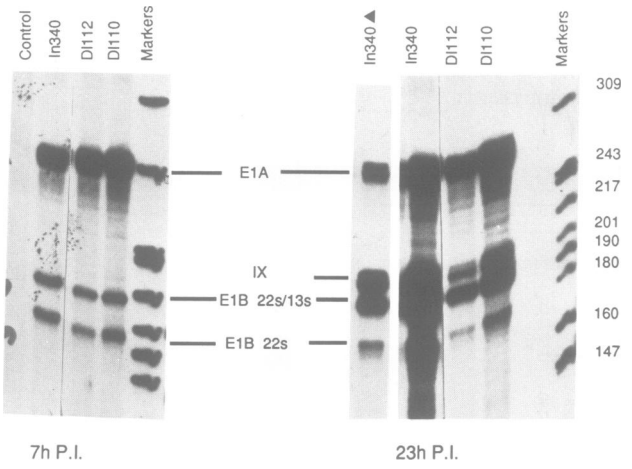

Fig. 3. E1B deletion mutants that contain the 13S and 22S 5′ splice sites show the same switch in their usage early versus late after infection as wild-type virus. Lanes are labeled according to the virus used to infect the HeLa cells, or were mock-infected. The lane labeled In340D is a shorter exposure of the wild-type, In340 lane. Riboprobes used, virus infections and cytoplasmic RNA isolation are as described in Figure 2.

primary transcript can contain extensive deletions, and the E1B encoded 21 kDa or the 55 kDa proteins can be mutated, and still the viruses will behave like wild-type with regard to the E1B RNA splicing switch.

No dominant positive 13S splicing factor is present late after infection

A superinfection protocol similar to that of Thomas and Mathews (1980) was done using the two E1B mutant viruses (dl110 and dl112) which show the early to late switch in E1B splicing (Figure 3), make distinguishable E1B RNAs (Figure 4) and have well characterized viral life cycles (Babiss *et al.*, 1984, data not shown). HeLa cells were infected with dl110 virus at 20 p.f.u. per cell. At 15 h post-infection, well after dl110 virus DNA replication had begun (Babiss and Ginsberg, 1984; Babiss *et al.*, 1984, and data not shown), a second virus, dl112 was introduced into these cells. Examination of cytoplasmic RNA from these super-infected cells was done using a T2 RNase protection assay. The probe used was homologous to the 5′ end of the dl112 E1B gene and can distinguish the E1B RNAs transcribed by other viruses (see Figure 4). The chart in Figure 4A

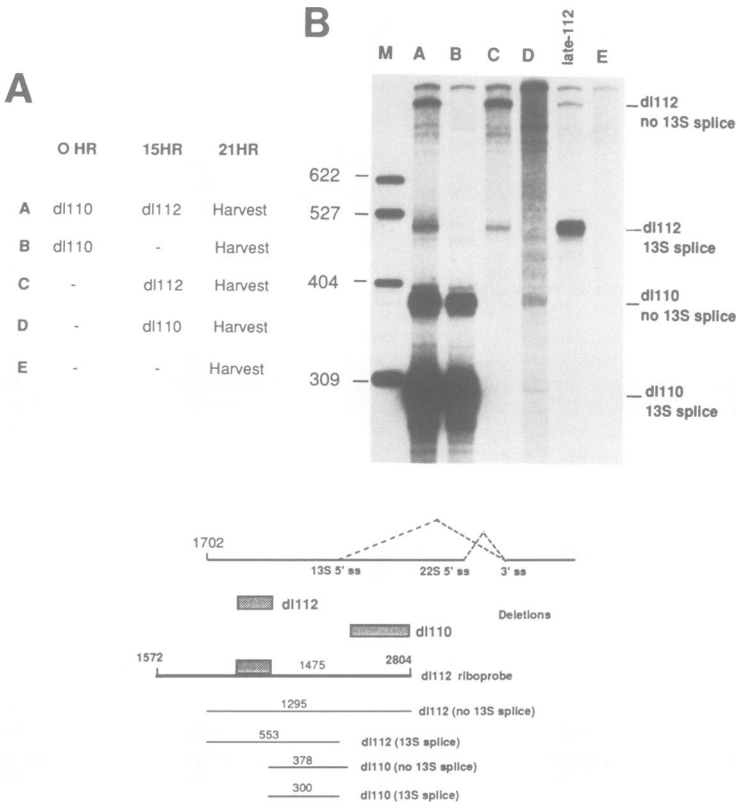

Fig. 4. There is no dominant *trans*-acting factor that is induced late after viral infection that can regulate E1B alternative RNA splicing. (**A**) The chart shows the scheme of the experiment and identifies the lanes in the autoradiogram according to viruses used and time of infection of the HeLa cells. The primary infection was at 0 h at an m.o.i. of 20 p.f.u./cell. At 15 h, the second virus was introduced into the same cells (when applicable) at an m.o.i. of 100 p.f.u./cell. Cytoplasmic RNA was harvested at 21 h after the first infection and oligo(dT) was selected (**B**) RNase T2 analysis of the RNA is described in Materials and methods using the probe diagrammed below. The dark line represents the E1B gene with the 13S and 22S splices shown as stippled lines. The two viruses used in the experiment contain deletions in their E1B genes (represented as shaded boxes). The probe used is homologous to the superinfecting virus dl112 through the 5′ end of the E1B gene and the 13S 5′ ss. The sizes of the expected protected fragments for the two viruses used in this experiment are distinguishable and are as shown. The identity of the largest band at 1475 nt was verified using a shorter probe in a similar assay (data not shown). 1/15 of the RNA from a 100 mm plate was used in each assay A–E. The late-112 infection is from HeLa cells infected for 24 h with dl112 virus, and using ~10-fold less RNA.

identifies each lane as an assay of the RNA from the various infections.

Examination of the early dl112 infected cells revealed the surprising result that only the 22S form of the dl112 mRNA was present regardless of whether or not the cells contained another virus, in the later stages of infection. In Figure 4B, dl112 virus-encoded 13S mRNA produces a band of 553 nt, while mRNA without this splice (22S mRNA) gives a band of 1295 nt. The ratios of these bands are related to the ratio of dl112 13S and 22S mRNA. dl112 virus was introduced into late stage infected cells and mock infected cells. Cytoplasmic RNA was harvested during the early phase of the dl112 virus infection. Comparing the ratios of 13S and 22S mRNA expressed by the dl112 virus, we find that within a factor of 2 there is no change in the 13S to 22S mRNA ratio (compare lanes A and C, Figure 4B). This slight difference is probably due to some dl112 DNA replication in the preinfected cells and was not observed when AraC was included to block replication of the second virus (data not shown). When we compare lane C (early dl112 infection) with an assay of RNA from a dl112 late infection (22 h, lane late-112) we see the expected 50-fold change in relative 13S versus 22S mRNAs (as described earlier). It should be noted that much less total RNA was assayed for the dl112 late control lane. To reiterate, the fact that the dl110 was in the late stages of infection had very little impact on splice site utilization by the superinfecting dl112 virus.

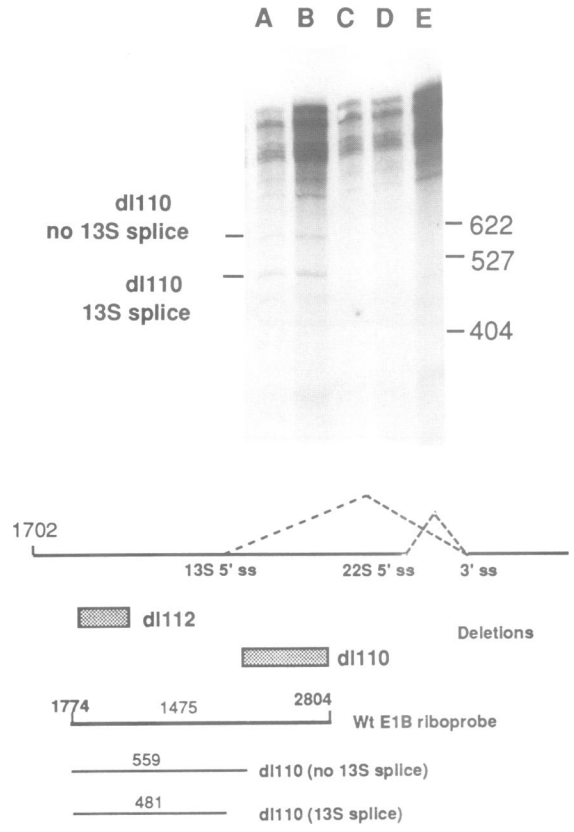

Fig. 5. Processing of E1B RNA from two functionally identical templates in the same nucleus depends on template identity. The chart in Figure 4A shows the scheme of the experiment and identifies the lanes in the autoradiogram according to the virus used and the time of infection. Cells were labeled continuously with [^{32}P]orthophosphate (0.8 MCi/100 mm dish) beginning at 1 h after the second infection. Infections for the dl110 and dl112 viruses were at an m.o.i. of 20 p.f.u./cell. Cytoplasmic RNA was isolated and analyzed by a T2 RNase protection assay using an unlabeled E1B 13S 5′ ss probe, diagrammed in the figure. RNA was oligo(dT) purified twice and 1/15 of the RNA isolated from a 100 mm plate was used for each lane. Specific protected *in vivo* labeled RNA fragments are identified. Background bands were reduced with each oligo(dT) purification and are probably not mRNA.

To confirm that all cells were dually infected and that both sets of viral genes were expressed in the same cells, we introduced an E1A mutant virus (dl1500, Montell *et al.*, 1984a) into uninfected or late stage virus infected cells. In late stage virus infected cells a 5 to 10-fold increase in dl1500 E1B RNA was seen, indicating that the E1A 13S-encoded 289R protein from the first virus is indeed complementing the transcriptional defect of the second virus (data not shown).

Two viral templates in the same cells produce E1B RNA that is processed in a template specific manner

There was a possibility that early after infection a factor transiently exists that is responsible for the 22S mRNA product's dominance. To examine this idea it was necessary to look at how superinfection effects E1B mRNA processing from a virus already in the late stages of infection. In the superinfection experiment shown in Figure 4, we showed that the stable E1B mRNA expressed from the first virus (which is in the late phase when assayed), favors the 13S 5′ ss regardless of the presence of the superinfecting virus (compare lanes B and C). However, it is difficult to draw definitive conclusions about RNA processing from this experiment since 13S E1B mRNA is quite stable (half life of 70 min, Wilson and Darnell, 1981).

A parallel experiment was done where [^{32}P]orthophosphate was added to the cells 1 h.p.i. of the second virus (Figure 5). Using this approach only RNA synthesized and processed following infection by the second virus would be labeled. The protocol is similar to that outlined in Figure 4 except that ^{32}P was added at 16 h.p.i. T2 RNase protection assays were done using an unlabeled probe to detect *in vivo* labeled RNA. No changes in the relative 22S:13S E1B RNA levels were seen late versus early (Figure 5, lanes A and B). The experiment was done with two different amounts of cold RNA probe to verify the condition of probe excess (data not shown). Since the labeling was inefficient, only late viral RNA was detectable. The lower band at 481 nt is representative of 13S mRNA while the band at 559 nt represents E1B mRNA without the 13S splice (predominantly 22S RNA) (data not shown). The ratio of the intensity of the bands representing the 13S and 22S mRNAs is 2:1. It should be noted that this is different from the 8:1 ratio seen when comparing the 13S versus 22S mRNA levels as measured using the probe outlined in Figure 4B. These assays use different probes and are of a different nature (with *in vivo* labeling the mRNA is radioactive while in the earlier experiment the probe is labeled) which probably explains the discrepancy. The important conclusion is that there is no change in the ratio of labeled 13S versus 22S RNA produced by the dl110 viral template whether the cells are superinfected with dl112 or not.

All of these findings support the conclusion that DNA template specific RNA processing occurs. Although it does not address the mechanism, this proves that two essentially

identical DNA templates can produce RNA processed in a specific manner and that somehow this signalling can be maintained within the nucleus of a single cell.

The switch in RNA processing is not restricted to the E1B gene sequences

Recent work from other laboratories has shown the existence of a general factor that favors splicing of small introns (Ge and Manley, 1990; Krainer *et al.*, 1990). In our laboratory, mutational mapping of the E1B gene has failed to reveal sequences responsible for the switch in RNA processing of the primary transcript seen early versus late after infection (data not shown). These two pieces of information suggested the possibility that whatever the template effect on RNA processing was, it might be able to exert its effect over other similarly spliced but normally unregulated genes once they were inserted in the adenoviral genome. An adenovirus recombinant with the SV40 early region replacing the E1A and E1B early region was obtained (Ad-SVR112; Gluzman *et al.*, 1982). This virus produces both SV40 18S (large T) and 19S (small t) RNAs when introduced into HeLa cells. The SV40 early primary transcript contains two 5′ splice sites and one 3′ ss (see Figure 1). This arrangement is very similar to that of the adenoviral E1B gene, although in contrast to E1B, during a wild-type SV40 virus infection of monkey cells, little change in the relative levels of the SV40 RNAs was observed (Khoury *et al.*, 1979; Mark and Berg, 1979).

HeLa cells were infected with the Ad-SVR112 virus, and cytoplasmic RNA was isolated at 10 h.p.i. (in the presence of AraC to block viral DNA replication, Gaynor and Berk, 1983) or 24 h.p.i., following virus replication. T2 RNase protection analysis of oligo(dT) selected RNA was done using a probe spanning all three splice sites (see Figure 6). The 10 h time point shows protected fragments corresponding to usage of various splices. Protected probe fragments of 533 nt are indicative of 19S 5′ ss usage. A smaller band of ~400 nt is probably contributed by similarly spliced RNA and is an artefact due to the AT richness of that region of the RNA and DNA probe. It is minimized when lower levels of T2 enzyme are used in the assay. The band at 253 nt is indicative of RNA spliced at the 18S 5′ ss. Finally the 3′ exon of both these messages is predicted to give a band of 162 nt in length, which is seen as a broad band due to the AT richness of this sequence.

We see that early after infection the intensities of the 18S and 19S mRNAs are roughly equal (Figure 6). At this stage of infection, relatively high levels of the mRNA with the smaller intron (19S) are seen; this is similar to what was observed for the E1B gene. Late after infection, mRNA with the larger intron dominates with a 20-fold relative increase in 18S mRNA. The same types of experiments were done with an adenovirus recombinant containing the early region from polyoma virus linked to the adenovirus major later promoter (Massie *et al.*, 1986). The early transcription unit of polyoma contains two alternative 5′ splice sites and two alternative 3′ splice sites (see Figure 1). Intact polyoma virus shows no change in splicing choices in stable early RNA early versus late after infection of 3T6 cells (data not shown). In contrast the recombinant adenovirus which contains the early polyoma gene shows a >5-fold change in splice site preference early versus late after infection (data not shown). This indicates that the adenovirus template effect may involve changing accessibility to, or function of a general splicing factor early versus late after infection (a result implied by earlier studies) (Spector *et al.*, 1980; Falck-Pedersen and Logan, 1989).

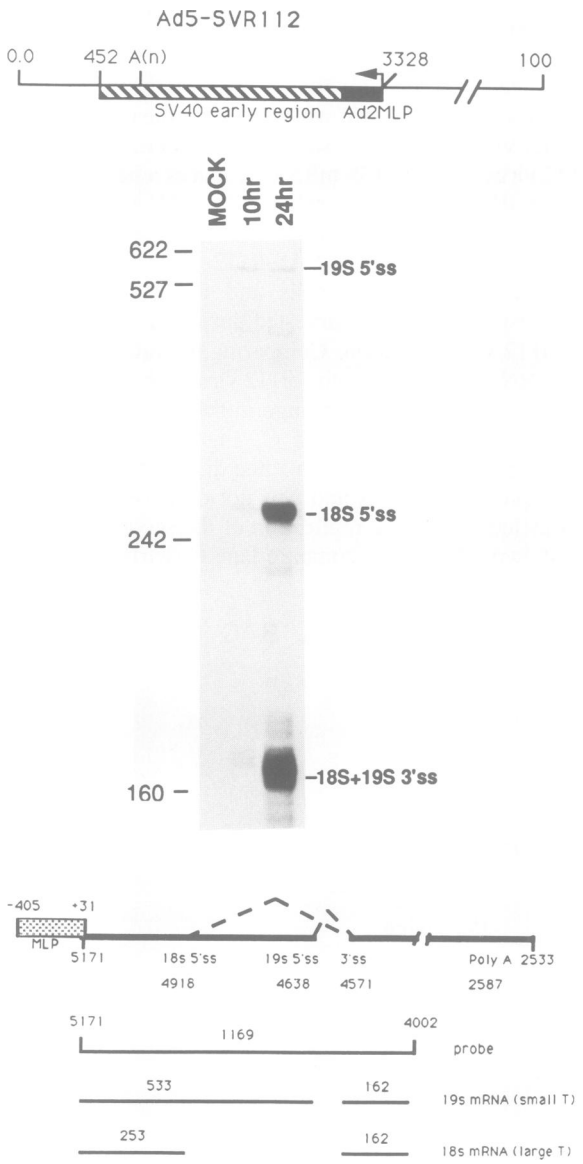

Fig. 6. Alternative splicing of SV40 early gene transcripts becomes regulated when these sequences are inserted into the adenoviral genome. The adenoviral recombinant Ad-SVR112 (Gluzman *et al.*, 1982) is diagrammed at the top of the figure. The hatched box represents the SV40 early region nt 5171–2533 which includes the entire coding region of this transcription unit through to the poly(A) site. The black box represents the Ad2 MLP fused to the SV40 early region at +33 in relation to the MLP capsite. Note that transcription of this chimeric gene is in the opposite orientation to that of the native E1A and E1B genes. The recombinant virus was used to infect monolayers of 293 cells at 20 p.f.u. per cell. For the early infection, 50 μg/ml of AraC was added to the media to block viral DNA replication (Gaynor and Berk, 1983). At this concentration, the switch in E1B mRNA processing in wild-type adenovirus-infected cells has been shown to be blocked (data not shown). The 24 h time point contains no AraC. Cytoplasmic RNA was isolated at the times indicated and oligo(dT) purified. 1/30 of the RNA from the early time point and 1/150 from the late time point were subjected to T2 RNase protection analysis using the probe diagrammed below. Lanes are identified according to the time of harvesting post-infection.

The switch in SV40 splicing is a cis effect

We have shown that introducing the SV40 early region into the adenovirus genome results in a 20-fold change in the ratios of SV40 18S and 19S RNAs, and that this change depends on the stage of adenovirus infection. However, it was unclear whether this effect was dependent on the SV40 transcription unit being localized on the viral genome (as is the E1B gene). It was possible that during the course of infection a *trans* factor was produced that caused the regulated change in accumulation of SV40 early gene mRNAs. Therefore it was crucial to determine whether adenovirus infection could affect SV40 early region RNA processing when this gene was not localized on the adenoviral genome.

The SV80 and COS cells cell lines contain the SV40 early region integrated into the cellular genome (Flint *et al.*, 1983; Gluzman, 1981). The 18S and 19S forms of SV40 RNA are produced in both cell lines. Previous studies have shown that adenovirus infection of SV80 cells has no effect on the relative production of these two mRNAs (Flint *et al.*, 1983). This was determined using pulse labeling to look at newly synthesized nuclear RNA during the early and late phases of adenovirus infection. A decrease in SV40 RNA levels was detected, but no relative change in synthesis of the two species was seen. Looking at cytoplasmic RNA in adenovirus infected COS cells, we similarly found no changes in ratios of the mRNAs produced from the endogenous SV40 early region (data not shown). However, virus infection of COS cells resulted in the characteristic change in adenoviral gene splicing early versus late after infection (data not shown). We conclude that for the SV40 early region, like the E1B gene, the adenovirus induced regulation of splicing is a *cis* effect.

Discussion

Much of the switch in the cytoplasmic levels of the E1B 22S and 13S mRNAs during a viral replicative cycle in human HeLa cells is based on splicing choices (Montell *et al.*, 1984b). We have established that the signal that regulates this dramatic change in splice site utilization late versus early after adenovirus infection is based on the state of the viral DNA template. Superinfections studies showed that the splicing switch is not the result of dominant changes in the expression and/or modifications of those cellular factors that regulate both E1B 22S and 13S splice site usage. If splicing choices for this gene were based on global changes of a *trans*-acting factor(s) in the nucleus, two templates in the same nucleus should be processed similarly. Our data indicate that for the adenovirus E1B gene and the adenovirus recombinant SV40 early region the processing event is regulated by the state of the DNA template. Somehow, the way these primary transcripts interact with a splicing regulation factor changes during infection.

The SV40 early transcription unit contains two alternative 5′ splice sites and a single 3′ ss. Ge and Manley (1990) have demonstrated in an *in vitro* splicing system, that splice site choice for the SV40 early primary transcript depends on the level of a general splicing factor, ASF. For complex transcription units, ASF appears to favor the most proximal 5′ ss when alternative sites are available (Krainer *et al.*, 1990; Ge and Manley, 1990). One might consider a hypothetical model for the control of the E1B switch in splice site choice where ASF, or some other splice choice factor, is at high levels early after infection and at low levels late. In agreement with this model we found that when the adenovirus/SV40 recombinant is introduced into cells, there was an ~20-fold change in the relative levels of the stable SV40 RNAs made late versus early. The SV40 transcription unit was regulated like the E1B transcription unit; early after infection, the small intron was favored and late after infection the large intron was spliced. This change in SV40 processing was not seen or was minimal (Khoury *et al.*, 1979; Mark and Berg, 1979) during wild-type SV40 virus infection of CV1 or primary AGMK cells. However there is evidence that adenovirus infection has no effect on levels of a splice choice factor involved in SV40 early region or E1B gene splicing. For example, adenovirus infection has no effect on SV40 early splicing in SV80 or COS cells. If the virus changes the interaction of ASF or some other splicing factor with nascent RNA it would have to be a highly specific effect. The effect depends on the DNA template from which the RNA is transcribed; global changes in a splicing factor are not indicated.

An attractive explanation for the template directed change in splicing is that some aspect of nuclear localization of the adenovirus template changes during the course of infection. Thus, the E1B transcription unit is exposed to a different population of *trans*-regulatory factors early versus late after infection. Localization studies of viral RNA have been confusing, but since it is generally accepted that transcription and processing occur simultaneously (Beyer and Osheim, 1988), DNA template localization may be informative. Several groups have shown that adenovirus DNA is linked tightly to the nuclear matrix throughout infection and that this linkage is important for viral transcription and replication (Bodnar *et al.*, 1989; Schaack *et al.*, 1990). Recent electron microscope work has localized adenovirus DNA in the late phase to discrete foci or 'replication factories' in the nucleus (Moen *et al.*, 1990) and thus one could postulate that two templates in the same cell can be in separate nuclear compartments. Additionally there is evidence that not only transcription but also processing of mRNA is believed to occur on the nuclear matrix and in discrete places (Lawrence *et al.*, 1989). There is no direct evidence on how the microenvironment around an early template versus a late template changes to cause the broad modifications in processing of the E1B transcripts. It is possible that in the regions of high levels of adenovirus transcription, splice factors are limiting, so changes in splice site choice occur (as seen *in vitro*)—(Krainer *et al.*, 1990; Ge and Manley, 1990). This type of model would require that within the nucleus there is variability in concentrations of factors that regulate splicing. Certainly not contradicting this model, it has already been shown that spliceosomes are attached to the nuclear matrix and in discrete places (Zeitlin *et al.*, 1989; Fu and Maniatis, 1990).

Adenovirus transcription control is template dependent (Thomas and Mathews, 1980) as is the control of E1B mRNA splicing. It is possible that changes in transcription early versus late after infection have a direct effect on the type of RNA processing observed. Besides the physical link between transcription and RNA processing cited earlier (Beyer and Osheim, 1988), there are examples of functional links (Neuberger and Williams, 1988; Sisodia *et al.*, 1987). Maybe newly replicated DNA is transcribed in a different

way from virion DNA. One would then have to speculate further that possible changes in regard to elongation rates, transcriptional pausing or changes in the transcriptional machinery could somehow affect splice site choice. This type of model would suggest that it was not nascent RNA localization that determined processing but that somehow the mode of transcription had a direct effect on how the RNA was processed.

Cellular genes like adenoviral genes show changes in transcription and nucleosome association. Like the viral chromosome, cellular chromatin is fixed to the nuclear matrix at discrete sites (reviewed by Gasser and Laemmli, 1987). It is known that there are many precedents for genes in transgenic mice and transformed cell lines showing changes in transcription control due to changes in an inserted gene's localization within the chromosome (Grosveld *et al.*, 1987; Phi-Van *et al.*, 1990). Our studies show that within the same cell, a gene (E1B) can have its RNA spliced in different ways without there being global changes in RNA processing factors. If DNA template specific control of mRNA processing is applicable to cellular genes it predicts that it is possible that not all cellular genes will show regulated RNA processing when inserted at new locations in the cellular genome or when introduced into cells by transient transfection. It is proposed that cellular gene RNA synthesis may be potentially regulated by the state of the chromatin (or DNA template), although whether cells actually use this mechanism remains to be shown.

Materials and methods

Cells and viruses

Monolayer cultures of human HeLa and 293 (a human embryo kidney cell line which constitutively expresses the Ad5 E1A and E1B gene products (Graham *et al.*, 1977) cells were grown in Dulbecco modified Eagle medium supplemented with 10% defined calf serum. The In340 (Hearing and Shenk, 1983), dl110, dl112 and dl118 viruses have been described (Babiss and Ginsberg, 1984; Babiss *et al.*, 1984). The Ad-SVR112 adenovirus SV40 recombinant contains the SV40 early region replacing the adenovirus E1A and E1B regions. It was constructed by Gluzman *et al.* (1982) and was a gift from Alan Wildeman. An analogous virus Ad5-PyR39 containing the polyoma virus early genes substituted for much of the E1A and E1B genes was obtained from John Hassell (Massie *et al.*, 1986). Viral stocks were obtained as described earlier by using the 293 cell line (Babiss and Ginsberg, 1984).

Virus construction

The plasmid EAB8 contains adenovirus genome nucleotides 1–4623 (Adami and Babiss, 1990). The sequence for the E1B 22S 5′ ss in this plasmid (nt 3517–3520) was deleted and converted to an *Xho*I site by site directed oligonucleotide mutagenesis (Kunkel, 1985). This *Xho*I site was converted to an *Apa*I site following *Xho*I cleavage, filling in with Klenow polymerase and ligation of an *Apa*I linker. An oligonucleotide duplex containing nt 954–983 of Ad5 and *Apa*I sticky ends was inserted into the *Apa*I site of this plasmid. The resulting construct contains the left end of the adenovirus genome with the E1B 22S 5′ ss substituted by a duplication of the E1A 12S 5′ ss. It is probably not a functional 5′ ss due to the 10 base GC rich hairpin surrounding the splice site (Eperon *et al.*, 1988). This plasmid DNA was reconstructed into the virus Bla3 by *in vivo* overlap recombination in human 293 cells as previously described (Babiss and Ginsberg, 1984), followed by two rounds of plaque purification on 293 cells.

RNA analysis

Cytoplasmic RNA isolation was described earlier (Adami and Nevins, 1988). When indicated, poly(A)$^+$ RNA was isolated by oligo(dT) chromatography. The production of stable mRNAs was scored using internally ^{32}P-labeled antisense RNA probes (Adami and Nevins, 1988). These probes were synthesized *in vitro* from linearized templates using T3 or Sp6 polymerase when appropriate. The wild-type E1B probe contains nt 1769–2804 of adenovirus cloned into the *Sac*I and *Hin*dIII sites of pGem1 and was a gift from Lynne Vales. The dl112 E1B 13S 5′ ss probe contains nt 1572–2804 cloned into the *Hin*cII and *Hin*dIII sites of Bluescript (BS) KS+. The SV40 HS probe includes nt 5171–4409 of the SV40 genome subcloned from pSVR and inserted into the *Hin*dIII site of BS KS+. Probes contain additional sequences from that shown in the figures (usually 10–40 bases from the cloning region). Other probes have been described earlier (Adami and Babiss, 1990). The RNase protection assays include hybridization to RNA at 60°C, followed by RNase T2 digestion at various concentrations (6–33 U/ml) and temperatures (27–30°C) depending on the AT nucleotide distribution in the probe. The products were sized on 6% denaturing polyacrylamide gels.

In vivo labeling was done using 0.8 mCi of [^{32}P]orthophosphate (Amersham) per 100 mm culture dish. Phosphate-free Dulbecco modified Eagle medium was obtained from Sigma. Labeling was allowed to continue for 4.5 h as indicated. All RNA was oligo(dT) selected twice to minimize the level of non-poly(A)-containing RNA, some of which was resistant to T2 digestion. The RNase protection assays were done with the wild-type E1B 13S 5′ ss probe. Five- to twenty-fold more probe was used than when assays with internally labeled probes were done. This was done because the *in vivo* labeling was not efficient so large amounts of RNA were assayed at one time. Two levels of probe were used for all assays and gave the same signal showing that there was an excess of probe and that relative ratios of species seen were correct.

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