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Elimination of mRNA splicing by a point mutation outside the conserved GU at 5' splice sites

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ABSTRACT

Nearly all mRNA introns begin with the dinucleotide GU. Mutations in either of these virtually invariant bases have been found to inactivate the corresponding 5' splice site. Until now single base changes in neighboring bases have not been found to completely inactivate a 5' splice site. Here we show that a single A → U transversion in the third position of the adenovirus 2 E1A 13S mRNA intron does prevent RNA splicing at the corresponding 5' splice site.

INTRODUCTION

Nearly all mRNA introns begin with the dinucleotide GU flanked by less highly conserved bases (1). The sequence 5'-(exon)-AAG/GUGAGU-(intron)-3' is found to be a consensus of the many 5' splice sites examined (2). Although exceptions to the GU have been reported (3-7) they are rare and appear to represent only about 1% of naturally utilized 5' splice sites. Mutations of the G (8) or U (9) prevent splicing of adenovirus primary transcripts at the corresponding 5' splice sites. Similarly, mutation of the 5' G disrupts normal splicing of globin mRNAs (7,10). However, all single point mutations adjacent to the GU examined previously do not eliminate RNA splicing (7,11). Here we show that a single A → U transversion at the third position of an adenovirus mRNA intron does inactivate the corresponding 5' splice site.

The question as to whether a single point mutation outside the nearly invariant GU dinucleotide can eliminate RNA splicing is of interest for at least two reasons. Firstly, we would like to define the nucleotide sequences required for mRNA splicing. Since all four bases have been observed at every position adjacent to the GU (2), substitutions at these positions might be expected to decrease, but not eliminate mRNA splicing. Secondly, we are interested in expanding the applicability of an approach we described previously for resolving the functions of overlapping viral genes by site-specific mutagenesis at a mRNA splice site (9). Distinct viral mRNAs are

frequently processed by splicing a single transcript in alternative ways. This results in mRNAs which share extensively overlapping sequences as in adenovirus 2 (Ad2) early region 1A (E1A) (Fig. 1). It is often not possible to introduce a mutation affecting one viral protein without altering the protein encoded by an overlapping mRNA. To circumvent this problem, we used a strategy whereby mRNA splicing of one overlapping mRNA is prevented by changing a base in the GU which begins the corresponding intron. A nucleotide change is chosen which does not alter any amino acid in the protein encoded in the overlapping mRNA due to degeneracy in the genetic code. Unfortunately, it is not always possible to change either the G or U beginning the intron of one RNA, without causing an amino acid change in the protein encoded by the overlapping mRNA. Thus, it is useful to define other nucleotide changes which will inhibit RNA splicing.

Wieringa et al. recently addressed the question as to whether a single nucleotide change outside the nearly invariant GU can eliminate mRNA splicing by N^4-hydroxy-dCTP mutagenesis of the nucleotides surrounding a 5' splice site of the rabbit β-globin gene (7). In this approach only purine-purine transitions were generated and no mutation outside the GU affected RNA splicing. In the current work we substituted a U for an A in the third position of the Ad2 E1A 13S mRNA intron creating the mutant designated Ad2pm1114 (Fig. 1). A pyrimidine substitution was selected because over 90% of all bases occurring at this position are purines (2).

The primary E1A transcript is processed by alternative RNA splicing into two major early mRNAS, 12S and 13S (12-14). The mRNAs encode highly related proteins of 243 and 289 amino acids which differ only by the 46 amino acids encoded by the unique region of the 13S mRNA. The 13S mRNA was chosen for this study because the 289 protein is required for viral replication (9,15,16), a biological activity which can be easily and very sensitively assayed.

METHODS

Construction of Ad2 pm1114: The point mutation was constructed by oligonucleotide-directed mutagenesis using the 20-mer 5'-CATAATTTTCACTAACTGTA-3' and a bacteriophage M13 clone of E1A, $M13GG_1$ as described (9,28,29). The underlined nucleotide in the 20-mer corresponds to the base pair mismatch at nucleotide 1114. The mutation was identified by DNA sequencing and transferred into the 36 kb adenovirus genome by hybridizing a restriction fragment containing the mutation to exonuclease III digested Ad2 DNA-protein complex and

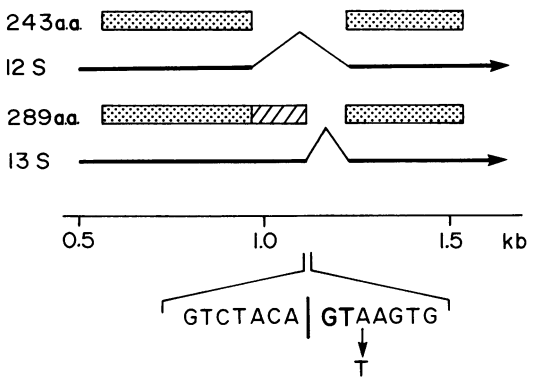

Fig. 1. Structure of the major E1A mRNAs during early times of infection. The line in the center represents the left end of the Ad2 genome marked in kilobase pairs. The horizontal lines joined by carat symbols represent exons of the 12S and 13S mRNAs with arrowheads indicating the 3' ends. The stippled portions of the boxes represent the common regions of the E1A proteins. The unique region of the 289 protein is indicated by the box with hatches. The DNA sequence of the L strand of Ad2 spanning the 13S 5' splice junction is shown with the exon-intron boundary indicated by the vertical line. The GT which begins the intron is highlighted and the A → T transversion at nucleotide 1114 is indicated.

transfecting the hybrid into 293 cells all as described (30,31). The adenovirus mutant with the transversion at the third position of the 13S intron is referred to as pm1114 [pm1114 = point mutation at nucleotide 1114 in the Ad2 sequence] (32).

Analysis of E1A mRNA: Suspension culture HeLa cells were infected at an moi = 10 with either Ad2 or pm1114 and harvested 6 or 22 h post-infection as described (17). 200 µg cytoplasmic RNA was annealled at 56°C for 12 hours with 0.25 µg Ad2 genome equivalent of a ^{32}P-labeled DNA probe in 80% formamide buffer as described (12). Hybridizations were performed in DNA excess so that the intensities of the bands in the autoradiogram were proportional to the concentration of RNA in the analysis. The DNA probe was prepared by 3' end-labeling a HinfI (BRL) digest of M13·GG$_1$ (9) with α^{32}-P-dATP and reverse transcriptase (Life Sciences, Inc.). The DNA fragment (nucleotides 763-1372 on the R strand of the Ad2 sequence) (32) was eluted from a 6% polyacrylamide gel as described (33). The hybridization products were digested at 25°C with 200 U S1 nuclease (BRL) and fractionated on a 16 cm, 8 M urea-5% polyacrylamide gel which was subjected to autoradiography with an intensifying screen.

RESULTS AND DISCUSSION

To determine whether the pm1114 mutation affected synthesis of the 13S mRNA we prepared cytoplasmic RNA from HeLa cells (17) infected with Ad2 or pm1114 6 or 22 h post-infection and analyzed the RNA by the hybridization/S1 nuclease method (18). Within the sensitivity of the S1 analysis the 13S mRNA was eliminated (Fig. 2). We estimate that if any 13S mRNA was synthesized it was present at less than 2% of the wild-type level of the 13S mRNA. As previously observed with other E1A mutants which affect the 289 amino acid protein (17), the overall level of 6h E1A mRNA was reduced five-fold.

Disruption of the 13S mRNA 5' splice site in pm1114 or the 12S E1A mRNA 5' splice in mutants previously analyzed did not lead to utilization of formerly unused or cryptic splice sites (9,19). However, mutations preventing splicing at normally used globin 5' splice sites do result in the utilization of cryptic 5' splice sites in the primary globin transcript (7,10,20). This difference in the consequence of splice site mutations between globin and the adenovirus E1A gene probably occurs because alternative adenovirus 5' splice sites already exist in E1A.

Additional evidence that the pm1114 mutation dramatically reduced the 13S mRNA was obtained by comparing the abilities of pm1114 and Ad2dl1500 to replicate in HeLa cells. Ad2dl1500 has a nine base deletion spanning the exon-intron boundary of the Ad2 13S mRNA removing the most highly conserved nucleotides at 5' splice sites (34). The 289 amino acid protein specified by the 13S mRNA is required for Ad2 to form plaques on HeLa cells (9,15,16). However, a ten-fold reduction in the 13S mRNA level has no affect on the ability of adenovirus mutants to form plaques on HeLa cells (21). Therefore, a diminished capacity to replicate in HeLa cells is suggestive of very low levels of the 13S mRNA. As with dl1500, the capacity of pm1114 to replicate in HeLa cells was greatly reduced compared to its ability to replicate in the complementing 293 cells (22) (Table 1). 293 cells complement defects in E1A and E1B due to constitutive expression of integrated copies of this portion of the adenovirus genome (17,22-24). The small difference in the HeLa/293 ratio between pm1114 and dl1500 may indicate production of a very low level of 13S mRNA in pm1114 infected cells.

Since a U occurs in the third position of 6% of introns (2), and previous results show that single point mutations outside the GU have little or no effect on the activity of RNA splicing, the results with pm1114 were surprising. In analyses of cloned β-globin genes from patients with β-thalassemia, Treisman et al. (11) found that single nucleotide substitutions

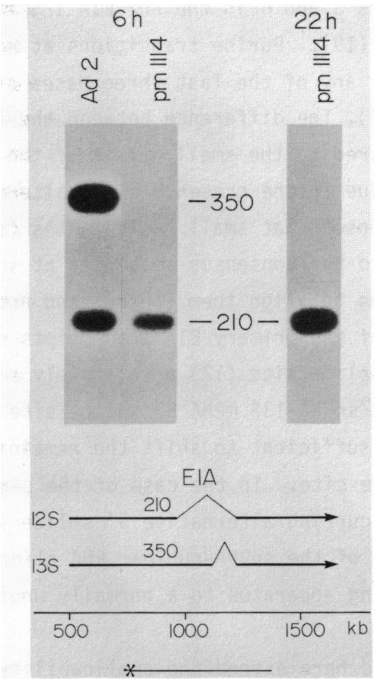

Fig. 2. Hybridization/S1 nuclease analysis of pm1114 cytoplasmic RNA. The sizes of the S1 protected fragments are indicated in nucleotides. The bottom line in the diagram below the autoradiogram represents the ^{32}P-labeled DNA probe. The asterisk on the left represents the 3' end-label at nucleotide 763 on the DNA strand complementary to the RNA.

at positions 5 or 6 of IVS 1 cause at most a 50% reduction in the concentration of the correctly spliced globin mRNA. These mutations also resulted in the utilization of three nearby cryptic splice sites. However,

Table I. Host Range Analysis of pm1114.

Virus	HeLa	293	HeLa/293
Ad2	5.5×10^{10}	5.0×10^{10}	1.1
d11500	4.0×10^{5}	9.3×10^{9}	4.3×10^{-5}
pm1114	2.5×10^{6}	9.8×10^{9}	2.5×10^{-4}

Stocks of Ad2, d11500 and pm1114 were serially diluted and assayed in duplicate for plaque forming units (PFU) on cell monolayers. Titers listed are PFU/ml based on the average of duplicate plates yielding 20-200 plaques.

mutation of both positions 5 and 6 in the Ad2 E1A 12S mRNA intron prevented splicing of the 12S mRNA (19). Purine transitions at positions 3 or 4 of the rabbit β-globin intron or any of the last three bases of the exon had no effect on RNA splicing (7). The difference between the large effect exerted by the pm1114 mutation compared to the small effect of the point mutations in the β-globin genes might be due to the presence of an alternative 5'-splice site in E1A. It has been proposed that small nuclear RNAs (snRNAs), which contain sequences complementary to the consensus sequences at splice sites, base pair with mRNA precursors so as to align them for cutting and splicing (25-27). Since approximately 30% of the primary E1A transcripts normally select the alternative 12S mRNA 5' splice site (12) a relatively small decrease in the stability of the presumed snRNA-13S mRNA 5' splice site alignment by the pm1114 mutation could be sufficient to shift the remaining splicing activity to this alternative splice site. In the case of the β-globin genes where there are no naturally occurring alternative 5' splice sites, a similar decrease in the stability of the snRNA-nuclear RNA alignment might be less prone to shift the splicing apparatus to a normally unused or cryptic splice site.

The results presented here extend the applicability of our approach for resolving the functions of overlapping viral genes. When a base substitution within the GU would change an amino acid in the protein encoded in the overlapping mRNA, a point mutation in the more degenerate portion of the 5' splice site may, as illustrated here, eliminate the mRNA.

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REFERENCES

1. Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. and Chambon, P. (1978) Proc. Natl. Acad. Sci. USA 75, 4853-4857
2. Mount, S.M. (1982) Nucleic Acids Res. 10, 459-472.
3. Avvedimento, V.E., Vogeli, G., Yamada, Y., Maizel, J.V. Jr., Pastan, I. and de Crombrugghe, B. (1980) Cell 21, 689-696.
4. Dodgson, J.B. and Engel, J.D. (1983) J. Biol. Chem. 258, 4623-4629.

5. Erbil, C. and Neissing, J. (1983) EMBO J. 2, 1339-1343.
6. King, C.R. and Piatigorsky, J. (1983) Cell 32, 707-712.
7. Wieringa, B., Meyer, F. Reiser, J. and Weissman, C. (1983) Nature 301, 38-43.
8. Montell, C., Fisher, E.F., Caruthers, M.H. and Berk, A.J. (1984) Cell. and Mol. Biol., in press.
9. Montell, C., Fisher, E.F., Caruthers, M.H. and Berk, A.J. (1982) Nature 295, 380-384.
10. Treisman, R., Proudfoot, N.J., Shander, M. and Maniatis, T. (1982) Cell 29, 903-911.
11. Treisman, R., Orkin, S.H. and Maniatis, T. (1983) Nature 302, 591-596.
12. Berk, A.J. and Sharp, P.A. (1978) Cell 14, 695-711.
13. Chow, L.T., Broker, T.R. and Lewis, J.B. (1979) J. Mol. Biol. 134, 265-303.
14. Kitchingman, G.R. and Westphal, H. (1980) J. Mol. Biol. 137, 23-48.
15. Harrison, T., Graham, F. and Williams, J. (1977) Virology 77, 319-329.
16. Ricciardi, R.P., Jones, R.L. Cepko, C.L., Sharp, P.A. and Roberts, B.E. (1981) Proc. Natl. Acad. Sci. USA 78, 6121-6125.
17. Berk, A.J., Lee, F., Harrison, T., Williams, J. and Sharp, P.A. (1979) Cell 17, 935-944.
18. Berk, A.J. and Sharp, P.A. (1977) Cell 12, 721-732.
19. Solnick, D. (1981) Nature 291, 508-510.
20. Felber, B.K., Orkin, S.H. and Hamer, D.H. (1982) Cell 29, 685-902.
21. Osborne, T.F., Gaynor, R.B. and Berk, A.J. (1982) Cell 29, 139-148.
22. Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977) J. Gen. Virol. 36, 59-72.
23. Aiello, L., Guilfoyle, R., Huebner, K. and Weinmann, R. (1979) Virology 94, 460-469.
24. Lassam, N.J., Bayley, S.T. and Graham, F.L. (1979) Cold Spring Harbor Symp. Quant. Biol. 44, 477-491.
25. Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L. and Steitz, J.A. (1980) Nature 283, 220-224.
26. Rogers, J. and Wall, R. (1980) Proc. Natl. Acad. Sci. USA 77, 1877-1879.
27. Ohshima, Y., Itoh, M., Okada, N. and Miyata, T. (1980) Proc. Natl. Acad. Sci. USA 78:4471-4474.
28. Razin, A., Hirose, T., Itakura, K. and Riggs, A. (1978) Proc. Natl. Acad. Sci. USA 75, 4268-4270.
29. Hutchison, C.A., Phillips, S., Edgell, M.H., Gillam, S., Jahnke, P. and Smith, M. (1978) J. Biol. Chem. 253, 6551-6560.
30. Dunsworth-Browne, M., Schell, R.E. and Berk, A.J. (1980) Nucleic Acids Res. 8, 543-554.
31. Montell, C., Fisher, E.F., Caruthers, M.H. and Berk, A.J. (1983) Nature 305, 600-605.
32. Gingeras, T.R., Sciakly, D., Gelinas, R.E., Bing-Dong, J., Yen, C.E., Kelley, M.M., Bullock, P.A., Parsons, B.L., O'Neill, K.E. and Roberts, R.J. (1982) J. Biol. Chem. 257, 13475-13491.
33. Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564.
34. Montell, C., Courtois, G., Eng, C. and Berk, A.J. (1984) Cell 36, 951-961.