A Premature Termination Codon in Either Exon of Minute Virus of Mice P4 Promoter-generated Pre-mRNA Can Inhibit Nuclear Splicing of the Intervening Intron in an Open Reading Frame-dependent Manner*

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How premature translation termination codons (PTCs) mediate effects on nuclear RNA processing is unclear. Here we show that a PTC at nucleotide (nt) 385 in the NS1/2 shared exon of P4-generated pre-mRNAs of the autonomous parvovirus minute virus of mice caused a decrease in the accumulated levels of doubly spliced R2 relative to singly spliced R1, although the total accumulated levels of R1 plus R2 remained the same. The effect of this PTC was evident within nuclear RNA, was mediated by a PTC and not a missense transversion mutation at this position, and could be suppressed by improvement of the large intron splice sites and by mutation of the AUG that initiated translation of R1 and R2. In contrast to the PTC at nt 385, the reading frame-dependent effect of the PTC at nt 2018 depended neither on the initiating AUG nor the normal termination codon for NS2; however, it could be suppressed by a single nucleotide deletion mutation in the upstream NS1/2 common exon that shifted the 2018 PTC out of the NS2 open reading frame. This suggested that there was recognition and communication of reading frame between exons on a pre-mRNA in the nucleus prior to or concomitant with splicing.

Premature termination codons (PTCs)† have been shown to result in decreased levels of PTC-containing mRNAs in many organisms, and there is an increasing appreciation of the effect of PTCs on RNA levels in mammalian cells. In some of these cases, PTCs have been shown to affect nuclear-associated mRNA abundance by a process termed nonsense-mediated decay, which has been suggested to degrade fully spliced mRNAs in the nucleus, possibly during mRNA export (reviewed in Refs. 1 and 2).

PTCs have also been implicated in altering nuclear RNA processing events other than decay in mammalian cells, which results in both intron retention and exon skipping, suggesting that PTCs may influence splice site selection (1). In two cases, exon skipping of the 66-nucleotide exon 51 of fibrilllin FBN1 RNA (3) and exon skipping and intron retention for the P4-generated pre-mRNAs that generate the nonstructural proteins of the autonomous parvovirus minute virus of mice (MVM) (4, 5), the effects of PTCs on RNA processing have been shown to be reading frame-dependent.

MVM is an autonomous parvovirus that is organized into two overlapping transcription units that produce three major classes of RNA (6–8) (see Fig. 1). Transcripts R1 and R2 are generated from a promoter (P4) at map unit 4 and encode the viral nonstructural proteins NS1 and NS2, respectively, whereas the R3 transcripts are generated from a promoter at map unit 38 (P38) and encode the viral capsid proteins (6, 9). Both NS1 and NS2 play essential roles in viral replication and cytopathicity (10), and so maintenance of their relative steady state levels, which is controlled at least in part by alternative splicing, is critical to the viral life cycle (Refs. 11–13; reviewed in Ref. 14). All MVM mRNAs generated during infection or following transfection are very stable (15), and no viral proteins are known to participate in the alternative splicing of MVM pre-mRNAs (reviewed in Ref. 14).

There are two types of introns in MVM P4-generated transcripts (14) (see Fig. 1). An overlapping downstream small intron, which undergoes an unusual pattern of overlapping alternative splicing using two donors (D1 and D2) and two acceptors (A1 and A2) (16), is located between nt 2280 and 2399 and is common to both P4- and P38-generated transcripts. An upstream large intron, located between nt 514 and 1989, is additionally excised from a subset of P4-generated pre-mRNAs to generate R2 mRNA. This upstream intron utilizes a nonconsensus donor at nt 514 and has a weak polypyrimidine tract at its 3’ splice site (13).

The NS2-specific exon is a 290-nt alternatively spliced exon that is translated in two open reading frames (ORFs). In singly spliced R1, this region utilizes ORF3 to encode NS1; in doubly spliced R2, this exon utilizes ORF2 to encode NS2 (Fig. 1). We have previously shown that PTCs in the NS2-specific exon caused a decrease in the accumulated levels of R2 relative to R1, although the total accumulated levels of R1 plus R2 remained the same. This decrease was a consequence of the artificially introduced translation termination signal acting in cis rather than the absence of a functional viral gene product and was shown to be evident in nuclear RNA and independent of RNA stability, suggesting an effect on nuclear splicing (4, 5). Although perhaps not directly comparable with PTCs that cause genetic diseases in higher organisms, analyses of PTCs introduced into viral genes such as MVM have proven to be informative models for the effects of PTCs on RNA accumulation (4, 5).

Efficient inclusion of the NS2-specific exon as an internal exon in vivo and consequent excision of the upstream large intron from P4-generated pre-mRNA to generate R2 require an internally redundant, bipartite exon splicing enhancer (ESE)
Fig. 1. Genetic map of MVM. The three major transcript classes and protein-encoding open reading frames are shown. The two promoters (P4 and P38) are indicated by arrows. The large intron, the small intron, and the NS2-specific exon are indicated. The nonconsensus donor (ncD) and the poor polypyrimidine tract (poor (Py)n) of the large intron are also shown. The bottom diagram shows nucleotide locations, and the probe B (nts 1854–2378) used for RNase protection assays as was as described fully under “Experimental Procedures.”

**FIG. 2. Effects of nonsense and missense mutations in either the first or second exons of NS2 pre-mRNA.**

A, sequences of the codons at nt 385 (UTT indicates the insertion of the universal translation termination cassette at nt 385; see text and “Experimental Procedures”) and 2018 in wild type MVM and mutants are shown underneath their appropriate map positions (deviations from the wild type sequence are underlined), together with quantitations of the R2/R1 ratio obtained by RNase protection analysis with probe B for each mutant. All the values are the average of at least three separate experiments. Standard deviations are indicated in parentheses. B and C, RNase protection analysis of total RNA using probe B (see Fig. 1), of RNA generated by wild type MVM (WT), mutants (as described in text), or mock-transfected, as designated at the top of each lane. The identities of the protected bands are shown on the left and explained under “Experimental Procedures.” *, undigested probe B. D, RNase protection analysis of nuclear RNA using probe B (Fig. 1) of RNA generated by wild type MVM (WT) or p385UTT as designated at the top of each lane. The identities of the protected bands are shown on the left as explained under “Experimental Procedures.” Nuclear fractions were determined to be >95% pure as explained under “Experimental Procedures.” Nuclear RNA from equivalent cell amounts were loaded for each sample.

comprised of 5' and 3' elements within the NS2-specific exon (17). The function of this ESE is sensitive to the presence of PTCs (5). A nonsense but not a missense mutation in the NS2 open reading frame at nt 2018 within the 5' element of the ESE affected definition of the NS2-specific exon by interfering with the ability of the bipartite ESE to strengthen interactions at the upstream large intron polypyrimidine tract. A PTC at nt 2018 alone resulted in retention of the upstream intron without a net decrease in the total accumulated P4-generated product. When the PTC at nt 2018 was combined with a mutation in the 3' element of the bipartite enhancer, the NS2-specific exon was skipped. These effects were independent of RNA stability and were shown to be reading frame-dependent; single nucleotide deletions in front of the 2018 mutation, which removed the PTC from the NS2 open reading frame, fully suppressed its effect (5).

Although the NS1/NS2 shared exon is a 5' terminal exon, PTCs at nt 385 within this exon have also been shown to result
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**Fig. 3.** Improvement of either the weak donor (5′ splice site) or the weak polypyrimidine tract (3′ splice site) of the upstream intron can suppress the effects of nonsense mutations in either the first or second exons of NS2 pre-mRNA. A, sequences of the codons at nt 385 (UTT indicates the insertion of the universal translation termination cassette at nt 385, see text and “Experimental Procedures”) and 2018, as well as sequences of the large intron donor and polypyrimidine tract, in wild type MVM and mutants are shown underneath their appropriate map positions (deviations from the wild type sequence are underlined), together with quantitations of the R2/R1 ratio obtained by RNase protection analysis with probe B (see Fig. 1) of RNA generated by wild type MVM (WT), mutants (as described in text), or mock-transfected as designated at the top of each lane. The identities of the protected bands are shown on the left and explained under “Experimental Procedures.” *, undigested probe B. A doublet is occasion-

in a decrease in the accumulated level of doubly spliced R2 relative to singly spliced R1, in a manner independent of the stability of R2 or R1 (4). In this report we show that the effect of the PTC at nt 385 was evident within nuclear RNA, was dependent on a PTC and not a missense transversion mutation at this position, and could be suppressed by improvement of the large intron splice sites. The effects of the 385 PTC and a PTC at nt 2018 were at least partially additive, suggesting that they operated by at least partially independent mechanisms. This was further underscored by the observation that the effect of the PTC at nt 385 depended upon the AUG that initiated translation of R1 and R2, whereas the reading frame-dep-...
FIG. 4. The nonsense-mediated reduction of R2 mRNA depends on the initiating AUG for mutations in the first exon but not the second exon of NS2 pre-mRNA. A, sequences of the codons at nt 385 (UTT) indicate the insertion of the universal translation termination cassette at nt 385; see text and “Experimental Procedures”) and 2018, as well as the presence (wt) or mutation (−) of AUG codons at nt 260, 474, and 504 in wild type MVM and mutants are shown underneath their appropriate map positions (deviations from the wild type sequence are underlined), together with quantitations of the R2/R1 ratio obtained by RNase protection analysis with probe B for each mutant. All the values are the average of at least three separate experiments. Standard deviations are indicated in parentheses, wt, wild type sequence.

Because of Effects on Excision of the Downstream Large Intron—Introduction of an ochre (TAA) PTC at nt 385 in the NS1/NS2 common exon of P4-generated pre-mRNA, either by point mutagenesis or by insertion of a 15-nt linker with overlapping PTCs in all three reading frames, led to a significant inversion in the accumulated levels of doubly spliced R2 relative to singly spliced R1 in total RNA, compared with that generated by wild type, although the total P4-generated product (R1 + R2) was unchanged (Fig. 2, A and B). When the PTCs at nt 385 and 2018 were combined, an even greater decrease in the accumulated level of R2 relative to R1 was seen (Fig. 2, A and B). That the effects of the two mutations were at least somewhat additive suggested that the mechanisms behind their effects were at least partially independent, a possibility that is further supported by the observations described below. The effect of the PTC at nt 385 was also evident in pure preparations of nuclear RNA, suggesting that the effect of this PTC was manifest within the nucleus (Fig. 2D), as was previously reported for the PTC at 2018 (5). Subtle improvements of the large intron splice sites could suppress the effect of the PTC at nt 385, consistent with the suggestion that this effect was nuclear. As shown in Fig. 3 (A and B), improvement of either the large intron 5′ splice site to consensus or the large intron 3′ splice site polypyrimidine tract

RESULTS

A Nonsense but Not a Missense Mutation at nt 385 in the NS1/NS2 Common Exon of P4-generated Pre-mRNAs Resulted in a Reduced Accumulated Level of R2 Relative to R1, Likely
by as few as two additional pyrimidines, restored the accumulated levels of R2 relative to R1 to near wild type levels. Improvement of the large intron 5' splice site to consensus also suppressed the phenotype of the PTC at nt 2018 (Fig. 3, A and C); we have previously reported that improvements of the 3' poly-pyrimidine tract also suppresses the effect of the PTC at nt 2018 (5). Improvement of the large intron 3' splice site poly-pyrimidine tract by four pyrimidines resulted in splicing of the P4 product almost exclusively to R2 (Fig. 3B, sixth lane).

Thus the effects of the PTCs can be overcome by improvements of the large intron splice sites, which do not appear in the final spliced R2 mRNA (Fig. 3), further suggests that the effects of the PTCs are independent of effects on RNA stability, as has been shown previously (4, 5). Taken together, these results suggested that PTCs in either exon of R2 can interfere with the nuclear excision of the upstream large intron from P4-generated pre-mRNAs.

The Effect of the PTC at nt 385, but Not That of the PTC at nt 2018, Could Be Suppressed by Mutations in the Initiating AUG—The accumulated level of R2 relative to R1 was affected by a PTC but not by a missense transversion at nt 385, and we have previously shown that the effect of a PTC at nt 2018 was reading frame-dependent, i.e. it was suppressed by a frameshift mutation in front of nt 2018 (5). Therefore, we chose to ask whether the effects of the PTCs were linked to translation in the cytoplasm by determining whether their effect was dependent on the initiating AUG that begins the reading frame for NS1 and NS2. Although such mutations may conceivably have other effects on RNA processing, similar mutations have been used to suppress the effects of PTCs in the triosephosphate isomerase gene RNA in order to link the PTC effects to cytoplasmic translation (19). As can be seen in Fig. 4, destruction by point mutagenesis of the initiating AUG at nt 260 suppressed the effect of the PTC at nt 385 but not the effect of the...
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A PTC transversion mutation but not a missense transversion mutation at nucleotide 385 in the 5′-terminal NS1/2 common exon of P4-generated pre-mRNAs of the autonomous parvovirus MVM resulted in an increased level of the singly spliced R1 mRNA relative to doubly spliced R2 mRNA in the nucleus of transfected cells. This effect could be suppressed by improvement of the splice signals of the large intron, suggesting that the effect on MVM RNA processing was at the level of excision of the large intron. Mutation of the initiating AUG at nt 260 also suppressed the effect of the 385 PTC, suggesting a link to the establishment of the reading frame of this 5′-terminal exon. These results help place the MVM P4-generated pre-mRNAs in a growing list of examples in which PTCs can either directly or indirectly affect nuclear RNA processing in an open reading frame-dependent manner (1, 22).

We have previously shown that a PTC at nt 2018 in the downstream internal NS2-specific exon of MVM P4-generated pre-mRNA also inhibited excision of this large intron, likely by virtue of interfering with the function of an ESE within the NS2 specific exon (5). In that case, the effect of the PTC on splicing was shown to be dependent upon an intact open reading frame. Here we show further that the effect of the PTC at nt 2018, although reading frame-dependent, was not affected by alterations of either the protein translation initiating AUG or terminating TAA signals, which have been shown to be important for PTC effects in other systems (1, 19, 20, 22). Surprisingly, the effect could be suppressed by a frameshift mutation in the 5′ terminal NS1/2 common exon of R2 message, suggesting that the effect of the 2018 PTC was dependent upon communication of reading frame between the two exons in the nucleus, prior to the completion of the splicing process.

PTCs have been shown to have significant effects on RNA processing associated with the nucleus of mammalian cells (1, 20). In some cases PTCs have been shown to trigger nucleus-associated nonsense-mediated decay; in other cases PTCs have been shown to affect other nuclear processes that lead to altered steady state RNA levels (1). We have recently shown that the PTC at nt 2018 likely affects splicing of MVM P4-generated pre-mRNA by interfering with the nuclear function of an ESE within the internal NS2-specific exon of MVM RNA (5). These observations are most consistent with “nuclear scanning”-type models of reading frame recognition (1, 23). Because our results suggest that recognition of the PTC at nt 2018 is not influenced by the initiating AUG and yet can be suppressed by a frameshift mutation in an upstream exon, such a model would have to accommodate recognition of frame between the upstream NS1/2 common exon and the NS2-specific exon prior to splicing. On the other hand, because the PTC at nt 385 is suppressible by mutation of the initiating AUG, it is formally possible that the effect of this PTC could be linked to cytoplasmic translation. This seems unlikely, however, because the large intron begins only 129 nt downstream of the PTC at nt 385. The observation that the effects of the two PTC mutations together are additive suggests that they may function somewhat differently.

Although the mechanisms of PTC action in the nucleus are not well understood, their effect has provoked a growing appreciation that the recognition of reading frame in the nucleus of mammalian cells can influence RNA processing events (1, 3). How such recognition can occur is not known; however, there have recently been some interesting observations that suggest that an appropriate apparatus may be in place. Ribosomal proteins and RNAs, elongation factor subunits eIF2A (20) and eIF4E (24), and aminoacyl tRNAs have recently been detected in the nucleus of mammalian cells (25). The U5 snRNP, which binds to exon sequences adjacent to 5′ and 3′ splice sites and has a role in juxtaposing exons together during splicing (26, 27), contains a 116-kDa protein that is both essential for splicing and closely related to the ribosomal translocase EF-2 (28). In addition, a 200 S ribonuclear protein particle that contains pre-mRNA and splicing factors but is much larger than the 60 S spliceosome identified in extracts competent for splicing in vitro has been identified in mammalian cells (29).
observations (4, 5) and those in this manuscript taken together provide strong evidence for the existence of such an exon-scanning reading frame recognition mechanism in the nucleus of mammalian cells that can interfere with the processing of PTC-containing RNAs.

REFERENCES
1. Maquat, L. E. (1995) RNA 1, 453–465
2. Peltz, S. W., Peng, H., Welch, E., and Jacobson, A. (1994) Prog. Nucleic Acid Res. Mol. Biol. 47, 271–298
3. Dietz, H. C., and Kendzior, R. J., Jr. (1994) Nat. Genet. 8, 183–188
4. Naeger, L. K., Schoborg, R. V., Zhao, Q., Tullis, G. E., and Pintel, D. J. (1992) Genes Dev. 6, 1107–1111
5. Gersappe, A., and Pintel, D. (1999) Mol. Cell. Biol. 19, 1640–1650
6. Pintel, D. J., Doshi, J. B., Astell, C. R., and Ward, D. C. (1983) Nucleic Acids Res. 11, 1019–1035
7. Clemens, K. E., and Pintel, D. J. (1984) Virology 160, 511–514
8. Astell, C. R., Gardiner, E. M., and Tattersall, P. (1986) J. Virol. 57, 656–669
9. Cotmore, S. F., and Tattersall, P. (1986) J. Virol. 58, 724–732
10. Clemens, K. E., Cerutis, D. R., Burger, L. R., Yang, C. Q., and Pintel, D. J. (1996) J. Virol. 64, 3967–3973
11. Jongeneel, C. V., Sahli, R., McMaster, G. K., and Hirt, B. (1986) J. Virol. 59, 564–573
12. Zhao, Q., Gersappe, A., and Pintel, D. J. (1995) J. Virol. 69, 6170–6179
13. Pintel, D. J., Gersappe, A., Haut, D., and Pearson, J. (1995) Semin. Virol. 6, 283–290
14. Schoborg, R. V., and Pintel, D. J. (1991) Virology 181, 22–34
15. Haut, D. D., and Pintel, D. J. (1998) J. Virol. 72, 1834–1843
16. Gersappe, A., and Pintel, D. J. (1999) Mol. Cell. Biol. 19, 364–375
17. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148
18. Zhang, J., and Maquat, L. E. (1997) EMBO J. 16, 826–833
19. Carter, M. S., Li, S., and Wilkinson, M. F. (1996) EMBO J. 15, 5865–5975
20. Padgett, R. A., Grabowski, P. J., Kenarska, M. M., Seiler, S. R., and Sharp, P. A. (1986) Annu. Rev. Biochem. 55, 1119–1150
21. Maquat, L. E. (1996) An. J. Hum. Genet. 58, 727–786
22. Urlaub, G., Mitchell, P. J., Giudad, C. J., and Chasin, L. A. (1989) Mol. Cell. Biol. 9, 2688–2880
23. Lejbiakowicz, F., Goyer, C., Darveau, A., Neron, S., Lemieux, R., and Sonenberg, N. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9612–9616
24. Lund, E., and Dahlberg, J. E. (1995) Science 222, 2082–2085
25. Liu, F., and Dahan, D. (1995) Science 262, 2082–2085
26. Nilsen, T. W. (1994) Cell 78, 1–4
27. Teigelkamp, S., Newman, A. J., and Beggs, J. D. (1995) EMBO J. 14, 2602–2612
28. Fabriczio, P., Laggerbauer, B., Lauber, J., Lane, W. S., and Luhrmann, R. (1997) EMBO J. 16, 4092–4106
29. Mirianni, E., Sperling, J., and Sperling, R. (1994) Nucleic Acids Res. 22, 3084–3091