Regulation of RNA Splicing in *gag*-Deficient Mutants of Moloney Murine Sarcoma Virus MuSVts110

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We investigated whether the MuSVts110 *gag* gene product (P58^{gag}) can regulate the novel growth temperature dependence of MuSVts110 RNA splicing. MuSVts110 mutants with either frameshifts or deletions in the *gag* gene were tested for their ability to maintain the MuSVts110 splicing phenotype. Only small decreases in splicing efficiency and no changes in the thermosensitivity of viral RNA splicing were observed in MuSVts110 *gag* gene frameshift mutants. Deletions within the *gag* gene, however, variably decreased MuSVts110 splicing efficiency but had no effect on its thermosensitivity. Another class of MuSVts110 splicing mutants generated by treatment of MuSVts110-infected cells with NiCl₂ was also examined. In these "nickel revertants," P58^{gag} is made, but splicing of the viral transcript is nearly complete at all growth temperatures. The splicing of "tagged" viral RNA transcribed from a modified MuSVts110 DNA introduced into nickel revertant cells remained thermosensitive, arguing against *trans* effects of viral gene products on splicing efficiency. These experiments indicated that neither the MuSVts110 P58^{gag} protein nor any other viral gene product acts in *trans* to regulate MuSVts110 splicing.

In eucaryotic cells, most intron-containing pre-mRNAs are spliced to completion (17, 34). Retroviral pre-mRNAs, however, must be spliced incompletely to provide unspliced viral RNA used in the packaging of infectious particles and the translation of the *gag* and *pol* gene products, as well as spliced viral RNA from which the *env* gene products are translated (37). Thus, unless viral gene products can modify the activity of the cellular splicing apparatus, the splicing machinery must distinguish between viral and cellular pre-mRNAs on the basis of transcript structure.

Deletions in various murine and avian retroviral *env* introns either inhibit or enhance splicing of the retroviral *env* mRNA (1, 2, 8, 10, 22, 24, 30, 31). The effects of these structural changes on splicing efficiency might be due either to the disruption of *cis*-acting sequences or the absence of essential viral gene products since in retroviruses the *env* intron is at the same time the structural gene for the viral *gag* and *pol* gene products. There is precedence among retroviruses for both these mechanisms. In Rous sarcoma virus pre-mRNA, there is a *cis*-acting, translation-independent negative regulatory sequence (*nrs*) in the *gag* gene that acts to downregulate *env* mRNA splicing (2). On the other hand, the splicing of human immunodeficiency virus RNA is indirectly regulated in *trans* by the human immunodeficiency virus *rev* gene product. The *rev* protein recognizes a sequence in the human immunodeficiency virus *env* intron and transports incompletely spliced RNAs out of the nucleus (13, 28). Moreover, the results of studies in a variety of systems argue that *trans*-acting factors are involved in the regulation of differential splicing (3, 7, 11, 14, 27), although, for the most part, these factors and the signals with which they interact are ill-defined.

Moloney murine sarcoma virus (MuSV) MuSVts110 is a retrovirus whose RNA undergoes novel, growth tempera-

ture-dependent splicing between splice sites in the *gag* and *mos* genes (12, 32, 33). The full-length MuSVts110 transcript, which remains unspliced if infected cells are grown at 39°C, is translated as a truncated "gag-x" gene product, P58^{gag}, x denoting a chimeric C terminus composed of 14 amino acids from an alternate v-*mos* frame (33). However, if MuSVts110-infected cells are grown at 28°C, about 50% of the MuSVts110 pre-mRNA is spliced (9). In this respect, the incomplete nature of MuSVts110 RNA splicing at the permissive temperature resembles that of other retroviral RNAs. The product of the spliced MuSVts110 RNA, P85^{gag-mos}, a serine kinase (25, 26), is responsible for cell transformation (15). This unusual thermosensitive splicing phenotype could be mediated either by *cis*-acting RNA sequences or in *trans* by viral gene products. In the current study, we asked whether translation of P58^{gag} was required to regulate either the efficiency or thermosensitivity of MuSVts110 RNA splicing. Using three classes of MuSVts 110 mutants—those with frameshifts or deletions in the *gag* gene and revertants selected after treating MuSVts110-infected cells with a carcinogen—we conclude that neither P58^{gag} nor any other viral gene product is likely to act in *trans* to regulate MuSVts110 RNA splicing.

MATERIALS AND METHODS

Cell cultures and transfection. NRK cells infected with the MuSVts110 retrovirus (6m2 cells) (6) cells were maintained in complete McCoy medium containing 10% bovine serum supplemented with transferrin (Hyclone). NIH 3T3 cells transfected with the various viral DNAs (*ts32*, *Xho19*, *BstE5*, *XBs.300*, and *XBst.835*) were established in complete McCoy medium containing 10% fetal bovine serum (Hyclone) and then transferred to the same medium containing 10% bovine serum. The various NiCl₂-induced revertants of the 6m2 cell line were established as described previously (4, 5) and maintained in the same medium as the parental 6m2 cells. Psi-2 (29) and NIH 3T3 cells (a gift from Donald

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FIG. 1. MuSVts110 *gag* exon frameshift and deletion mutants. The structure of wild-type MuSVts110 DNA is shown at the top of the figure. In this diagram, the *gag* and *mos* genes are designated E1 and E2, respectively. LTR, Long terminal repeat. (A) MuSVts110 *gag* frameshift mutants. The Xho19 mutant contains a frameshift at the boxed *XhoI* site and can be translated into a *gag* gene product about 40 kilodaltons in length. The BstE5 mutant contains a frameshift at the boxed *BstEII* site and can be translated into a *gag* gene product about 8 kDa in length. (B) MuSVts110 *gag* deletion mutants. The XBs.300 and XBst.835 mutants contain 336 and 871 bases, respectively, of the *gag* exon. Abbreviations used are as follows: Bs, *BsmI*; Bst, *BstEII*; Hg, *HgiAI*; Sp, *SpeI*; X, *XhoI*.

Blair) were maintained in Dulbecco high-glucose minimal essential medium containing 10% bovine serum.

NIH 3T3 or Psi-2 cells were cotransfected with linearized viral DNA and pSV2neo DNA by a standard calcium phosphate protocol (16). G418-resistant cells were selected by growth in 400 μ g of G418 (geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml.

Construction of viral DNAs. Construction of the MuSVts110 "equivalent" DNA, *ts32* DNA, has been described previously. *ts32* DNA was derived from MuSV-124 DNA (36) by the introduction of a 1,487-base deletion between nucleotides 2404 and 3892 (12).

(i) ***gag* gene frameshift mutants.** The Xho19 mutant (Fig. 1A) was derived by digestion of *ts32* DNA with *XhoI*, filling in with the Klenow fragment of DNA polymerase I, and religation, generating a frameshift at nucleotide 1981 that results in the termination of translation at nucleotide 2020 instead of nucleotide 2448.

The BstE5 mutant (Fig. 1A) was derived by digestion of *ts32* DNA with *BstEII*, filling in with the Klenow fragment, and ligation, thus shifting translation out of the *gag* frame at nucleotide 1152. Translation of BstE5 RNA is not termi-

nated, however, until nucleotide 1296. The sequence of both the Xho19 and BstE5 mutants was confirmed by dideoxy sequencing.

(ii) ***gag* gene deletion mutants.** XBs.300 (Fig. 1B) contains 336 bases of the *gag* exon between the *BsmI* site (nucleotide 1681) and the 5' splice site (nucleotide 2017). XBs.300 was constructed by removal of the *SpeI-XhoI* fragment (nucleotides 727 to 1981) from *ts32* DNA, digestion of this fragment with *BsmI*, and insertion of the resulting 300-nucleotide *BsmI-XhoI* fragment into *SpeI-XhoI*-digested *ts32* DNA by ligation, blunting, and then religation.

XBst.835 (Fig. 1B) contains 871 bases of the *gag* exon between the *BstEII* site (nucleotide 1146) and the 5' splice site. XBst.835 was constructed by digestion of the *ts32* DNA *SpeI-XhoI* fragment with *BstEII*. The resulting 835-nucleotide *BstEII-XhoI* fragment was ligated to *SpeI-XhoI*-digested *ts32* DNA as described for the XBs.300 mutant.

Construction of an oligonucleotide-"tagged" MuSVts110 DNA (*ts32udo*). To construct a DNA whose transcripts would be distinct from *ts32* RNA in S1 analyses, we modified *ts32* DNA by insertion of a 14-base oligonucleotide at the *XhoI* site located at -36 relative to the 5' splice site and a

FIG. 2. Structure of the tagged MuSVts110 DNA, *ts32udo*, and S1 analysis of its transcripts. To construct *ts32udo*, a 14-base synthetic oligonucleotide was inserted at the *Xho*I site (X) in MuSVts110 E1 (the MuSVts110 DNA is designated *ts32* DNA [12]) and a 46-base oligonucleotide (containing several cloning sites including *Hind*III [H]) was inserted at the *Acc*I site (A) in E2. S1 analysis of *ts32udo* RNA was done by using the pBA.36 (lower left) and XH.570 (lower right) probes. While the native unspliced and spliced MuSVts110 RNAs will protect 175 and 122 bases, respectively, of pBA.36, *ts32udo* RNA, whether unspliced or spliced, will protect only 34 bases of pBA.36 owing to the interruption of the native MuSVts110 sequence by the synthetic oligonucleotide. The unspliced and spliced *ts32* RNAs will protect 570 and 107 bases, respectively, of the XH.570 probe. Native MuSVts110 RNA will not protect any of the 5'-end-labeled XH.570 DNA since the 5' end is at the *Hind*III site in the *ts32udo*-specific synthetic oligonucleotide. LTR, Long terminal repeat.

46-base oligonucleotide at the *Acc*I site located at +88 relative to the 3' splice site (Fig. 2).

Cellular RNA isolation. Total cellular RNA was isolated by guanidine thiocyanate extraction and centrifugation through a cesium chloride cushion as described previously (4).

S1 analysis. S1 analyses with 5'- or 3'-end-labeled probes capable of measuring cleavage at the MuSVts110 5' splice site or ligation at the 3' splice site were done as described previously (32, 33). To estimate splicing of the tagged *ts32udo* RNA, a *ts32udo*-specific probe, designated XH.570, was derived by digestion of *ts32udo* DNA with *Hind*III-*Xho*I (Fig. 2). S1 analysis with 5'-end-labeled XH.570 will detect only *ts32udo* RNA since native MuSVts110 RNA does not contain the oligonucleotide-derived sequences at the *v-mos* gene terminus of this DNA fragment (Fig. 2). Unspliced and spliced *ts32udo* RNA will protect 570 and 107 bases, respectively, of XH.570.

Quantitation of viral gene expression. The relative levels of unspliced and spliced viral RNA in a given amount (usually 10 to 50 μ g) of cellular RNA were quantitated by scanning the S1 analysis gel profiles on a Beckman DU-8 spectrophotometer equipped with a scanner.

Measurement of RNA turnover. Cells were seeded in T-25 or T-75 flasks, grown to approximately 75% confluency at 33°C, and then shifted to 28°C overnight. Actinomycin D was added to 5 μ g/ml, and the cells were shifted to 39°C for various times. RNA was prepared from individual cultures, and all the RNA isolated from each flask was subjected to S1 analysis. The S1 autoradiographs were scanned, and the half-lives were calculated as the $\log_2$ of the fraction of the total viral RNA represented by each RNA species (9).

RESULTS

Gag gene frameshifts and MuSVts110 splicing. We first asked whether translation-terminating frameshifts in the

MuSVts110 *gag* gene would affect the efficiency of splicing. In previous work, we reported that MuSVts110 and *ts32* RNA splicing efficiency in infected NRK cells varies from 45 to 55% at 28°C to 0 to 3% at 39 to 41°C (9, 12). However, we have noted that the splicing phenotype in NIH 3T3 cells transfected with *ts32* DNA (*ts32*/3T3 cells), while qualitatively thermosensitive, shows some quantitative differences from the phenotype observed in infected cells (12). Relative to infected cells, a slightly lower proportion of the *ts32* RNA in the *ts32*/3T3 transfectants is spliced at 28°C (approximately 45%), and at 39°C, a slightly higher proportion of the viral RNA (approximately 5%) is spliced. The reason for this small reduction in the magnitude of thermosensitive splicing is not known, but because of these differences, the splicing efficiency of RNA transcribed from all the *gag*-deficient mutant DNAs transfected into NIH 3T3 cells was always compared with *ts32* RNA splicing efficiency in similarly transfected NIH 3T3 cells.

To assess the effect of *gag* gene frameshift mutations, we measured viral RNA splicing efficiency in NIH 3T3 cells transfected with either the *Xho*19 or *Bst*E5 frameshift mutants (*Xho*19/3T3 cells and *Bst*E5/3T3 cells, respectively) as a function of growth temperature. In the *Xho*19 mutant, *gag* gene translation is predicted to terminate at nucleotide 2020. Under these conditions, the P58^{gag} gene product will lack its chimeric C terminus. At 28°C, about 40% of the *Xho*19 RNA was spliced (Fig. 3, lane 4; Table 1), compared with a 45% splicing efficiency for wild-type *ts32* RNA (Fig. 3, lane 1; Table 1). This small decrease in splicing efficiency suggested that the P58^{gag} C-terminal region does not significantly influence the efficiency of MuSVts110 splicing. In the *Bst*E5 mutant, translation of the *gag* frame stops at nucleotide 1152, allowing the translation of only 37 amino acids at the extreme N terminus of P58^{gag}. In *Bst*E5/3T3 cells, there was a reduction in *Bst*E5 RNA splicing efficiency somewhat

FIG. 3. Splicing of MuSVts110 *gag* frameshift RNA transcripts. Cellular RNA was isolated from *ts32/3T3*, *Xho19/3T3*, or *BstE5/3T3* cells grown at 33°C and shifted to either 28 or 39°C for 24 h, and 10 to 50 μ g was hybridized to the pBacc probe (shown at the bottom of the figure). The hybrids were digested with S1 nuclease, and the protected probe fragments were analyzed on a 5% polyacrylamide gel containing 8 M urea. Lanes: 1 to 3, *ts32/3T3* RNA at 28, 33, and 39°C, respectively; 4 to 6, *Xho19 3T3* RNA at 28, 33, and 39°C, respectively; 7 to 9, *BstE5 3T3* RNA at 28, 33, and 39°C, respectively.

more pronounced than that observed in the *Xho19* RNA. At 28°C, about 37% of the *BstE5* RNA was spliced (Fig. 3, lane 7). This moderate inhibition of splicing efficiency observed in the presence of a nearly complete blockade to *gag* gene product translation reinforced the suggestion that *P58^{gag}* is not involved in the regulation of splicing efficiency.

The characteristic thermosensitivity of MuSVts110 splicing was not substantially altered by the *Xho19* or *BstE5* frameshifts in the *gag* gene. At 28, 33, and 39°C, 37, 16, and <5%, respectively, of the *BstE5* transcript was spliced (Fig. 3, lanes 7 to 9; Table 1), comparing favorably to the 45, 25, and <5% efficiencies, respectively, observed for *ts32* RNA (Fig. 3, lanes 1 to 3; Table 1). In the *Xho19* mutant, a slightly different, although still distinctly thermosensitive, pattern of splicing efficiency was observed. In contrast to either the *ts32* or *BstE5* RNAs, *Xho19* RNA was spliced with approximately the same efficiency at 33°C (38%) as at 28°C (40%) and was more efficiently spliced than either the *ts32* or *BstE5* RNAs at 39°C (13%) (Fig. 3, lanes 4 to 6; Table 1). This slight alteration of the splicing phenotype might be due to that fact that the frameshift mutation in *Xho19* is only 36 bases upstream of the 5' splice site in a region containing *cis*-acting

TABLE 1. RNA splicing efficiency as a function of growth temperature in the MuSVts110 *gag* gene frameshift mutants^a

Cell line	Splicing efficiency (%) at:		
	28°C	33°C	39°C
<i>ts32/3T3</i>	45	25	<5
<i>Xho19/3T3</i>	40	38	13
<i>BstE5/3T3</i>	37	16	<5

^a RNA splicing efficiency was determined by averaging the fraction of the total MuSVts110 RNA represented by the 89-base protection fragment in several S1 analyses of the type shown in Fig. 3.

sequences that strongly affect splicing efficiency (D. A. Sterner and E. C. Murphy, Jr., unpublished data). Considered together, however, these data suggest strongly that translation of *P58^{gag}* is not required either to limit MuSVts110 splicing efficiency or to regulate the thermosensitivity of the splicing event.

Stability of *gag* frameshift mutant RNA. It has been shown that the stability of unspliced Rous sarcoma virus RNA is diminished by frameshifts in the *gag* gene (2). Since a decrease in the stability of MuSVts110 pre-mRNA would result in an overestimate of splicing efficiency, we compared the stability of *ts32*, *Xho19*, and *BstE5* RNA in NIH 3T3 cells in which both transcription and splicing were halted by a shift from 28 to 39°C in the presence of actinomycin D. In these experiments, the *ts32*, *Xho19*, and *BstE5* unspliced RNAs had half-lives of 4.5, 10, and 9.0 h, respectively, while the corresponding spliced RNAs had half-lives of 4.0, 10.5, and 13.5 h. Thus, rather than being destabilized, the frameshifted RNAs were somewhat more stable than the unmutated *ts32* RNA. However, since the relative half-lives of the unspliced and spliced RNAs remained nearly constant within *ts32*-, *Xho19*-, and *BstE5*-transfected cells, our comparative estimates of splicing efficiency were considered valid.

Splicing in 6m2 nickel revertants. We have previously described 6m2 revertants that can be selected as transformed foci at 39°C after treatment with a variety of mutagens, including NiCl_2 (4). In these nickel revertants, MuSVts110 RNA splicing is stably altered such that it proceeds nearly to completion at all growth temperatures (4, 5). These changes in the splicing phenotype are a result of *cis*-acting mutations induced in the MuSVts110 viral DNA. For example, in one of the nickel revertants, the change to constitutive splicing is caused by a head-to-tail duplication of 70 bases surrounding the 3' splice site (S. M. Chiocca, D. A. Sterner, N. W. Biggart, and E. C. Murphy, Jr., submitted for publication). An analysis of viral proteins (e.g., mobility in gels, immunoprecipitability by anti-*gag* sera, and serine kinase activity) in these nickel revertants showed that *P58^{gag}* and *P85^{gag-mos}* were unaltered as defined by these criteria (4, 5), arguing that the presence of functional *gag* protein does not ensure maintenance of the MuSVts110 splicing phenotype.

We used these nickel revertant cells to address a concern that the MuSVts110 splicing phenotype might be regulated in *trans* by the unidentified products of alternate reading frames or by mutations in the *gag* proteins not detectable by the criteria used. To examine this possibility, we infected the N1 nickel revertant cell line with oligonucleotide-tagged *ts32udo* virions (Fig. 2) harvested from transfected Psi-2 cells, our rationale being that if *ts32udo* RNA maintained its thermosensitive splicing phenotype in the N1 environment, this would argue against *trans* regulation of splicing by viral gene products. To estimate *ts32udo* splicing efficiency, we performed S1 analysis with the XH.570 probe (Fig. 2). Unspliced and spliced *ts32udo* RNA is expected to protect 570 and 107 bases, respectively, of XH.570 DNA. As is clear from the growth temperature-dependent protection of the 107-base fragment of XH.570 DNA, *ts32udo* RNA splicing remained thermosensitive in *ts32udo*-infected N1 cells, being efficiently spliced in N1 cells grown at 28°C (Fig. 4B, lane 5) but progressively more poorly spliced if these cells were shifted to 33, 37, or 39°C (Fig. 4B, lanes 6 to 8), while, simultaneously, the splicing of the revertant MuSVts110 RNA in these same cells remained independent of growth temperature (Fig. 4A, lanes 3 to 5). The stability of both the nickel revertant MuSVts110 and *ts32udo* splicing pheno-

FIG. 4. Viral RNA splicing in *ts32udo*-infected N1 cells. S1 analysis with the pBA.36 or XH.570 probe (refer to Fig. 2) was performed on cellular RNA isolated from control 6m2 cells, N1 nickel revertant cells (N1 cells), and *ts32udo*-infected N1 cells (N1-udo cells). In each case, the cells were grown at 33°C and shifted to 28, 37, or 39°C. (A) S1 analysis with the pBA.36 probe on RNA from N1 cells at 28 and 33°C (lanes 1 and 2) or N1-udo cells at 28, 33, and 39°C (lanes 3 to 5). Lanes 6 and 7 contain a probe-only control and a pBR3220 *Hinf*I marker, respectively. (B) S1 analysis with the XH.570 probe on RNA from 6m2 cells at 28 and 39°C (lanes 1 and 2), N1 cells at 28 and 39°C (lanes 3 and 4), and N1-udo cells at 28, 33, 37, and 39°C (lanes 5 to 8, respectively). Lanes 9 and 10 contain a probe-only control and a pBR322-*Hinf*I marker, respectively.

types in the presence of each other's gene products argues against *trans* regulation of MuSVts110 RNA splicing by viral gene products.

Splicing of MuSVts110 *gag* gene deletion mutant RNAs. To provide a further test of whether alternate reading frames in the *gag* gene region could yield proteins that might regulate MuSVts110 splicing in *trans*, we tested the efficiency of RNA splicing in two MuSVts110 *gag* region deletion mutants (Fig. 1B). These mutants were constructed to disrupt a relatively long open region in frame 2 of the *gag* gene (nucleotides 1127 to 1790) and four shorter open regions between nucleotides 1000 and 2000 in frame 3, while taking into consideration the fact that about 300 nucleotides of the MuSVts110 *gag* exon just upstream of the 5' splice site are required in *cis* for splicing (D. A. Sterner and E. C. Murphy, manuscript in preparation).

In a MuSVts110 mutant (XBs.300) containing these 300 nucleotides but lacking nucleotides 727 to 1681 (i.e., virtually all the open region in frame 2 and the first three of the frame 3 open regions), while splicing efficiency was reduced to about 30% of the control *ts32* RNA (Fig. 5, lane 5; Table

FIG. 5. Splicing of MuSVts110 *gag* deletion mutant RNA transcripts. S1 analysis with the pBacc probe was performed on cellular RNA isolated from *ts32*/3T3 cells or NIH 3T3 cells transfected with either the XBs.300 or XBst.835 deletion mutant. In each case, the cells were grown at 33°C and shifted to either 28 or 39°C for 24 h. Lanes: 1 to 3, pBR322-*Hinf*I markers, probe-only control, and RNA from untransfected NIH 3T3 cells, respectively; 4, RNA from *ts32*/3T3 cells at 28°C; 5 and 6, RNA from XBs.300/3T3 cells at 28 and 39°C, respectively; 7 and 8, RNA from XBst.835/3T3 cells at 28 and 39°C, respectively.

2), the thermosensitivity of splicing was not affected (Fig. 5, compare lanes 5 and 6; Table 2). In XBst.835 RNA, lacking the *gag* gene region (nucleotides 727 to 1146) which included the 5' end of the open region in frame 2 as well as the longest open region in frame 3 (nucleotides 853 to 1179), splicing was at 67% of the original efficiency (Fig. 5, lanes 7 and 8; Table 2). As was observed with the XBs.300 mutant, XBst.835 RNA splicing remained thermosensitive (Fig. 5, compare lanes 7 and 8; Table 2). As was done with the *gag* frameshift mutants, the half-lives of XBs.300 and XBst.835 RNA species were estimated to provide assurance that splicing efficiencies were calculated correctly. No differential stabilities were noted; the XBs.300 and XBst.835 unspliced RNAs had half-lives of 14 and 13h, respectively, while the corresponding spliced RNAs had half-lives of 12 and 11 h, respectively.

These observations, considered together with the genetic data obtained from the infection of N1 cells with *ts32udo* virions, suggest that neither the *gag* gene product nor any as yet unidentified product of an alternate reading frame serves to regulate the MuSVts110 RNA thermosensitive splicing phenotype.

TABLE 2. RNA splicing efficiency as a function of growth temperature in the MuSVts110 *gag* gene deletion mutants^a

Cell line	Splicing efficiency (%) at:	
	28°C	39°C
<i>ts32</i> /3T3	45	<5
XBs.300/3T3	14	<5
XBst.835/3T3	29	<5

^a RNA splicing efficiency was determined by averaging the fraction of the total MuSVts110 RNA represented by the 89-base protection fragment in several S1 analyses of the type shown in Fig. 5.

DISCUSSION

MuSVts110 RNA splicing efficiency appears to be regulated at several levels. At one level, common to all retroviruses, splicing is limited to about 50% of the viral transcripts to maintain a balance between the unspliced and spliced RNA species, both of which are required for a complete infectious cycle in replication-competent retroviruses (37). While MuSVts110 splicing efficiency is also limited to approximately the same fraction, MuSVts110 RNA splicing occurs between cryptic splice sites in the *gag* and *v-mos* genes used by no other murine retrovirus instead of the *env* gene 5' and 3' splice sites used by replication-competent murine leukemia virus (MuLV) (9, 33). Thus, regulatory mechanisms operating to limit RNA splicing in MuSVts110 may not be directly comparable to those at work in MuLV. Interestingly, however, the *gag* gene region of the MuSVts110 intron (*gag* gene nucleotides 2017 to 2404 fused to *v-mos* gene nucleotides 3892 to 3936) maps very closely to an *XhoI-PvuI* region of MuLV DNA (MuLV nucleotides 1560 to 2120 [35], corresponding to nucleotides 1981 to 2541 in murine sarcoma virus) determined to be essential for efficient MuLV *env* mRNA splicing (22). Consequently, MuSVts110 and MuLV *env* RNA splicing may be regulated by similar mechanisms. At another level, unique to MuSVts110, growth temperature regulates the splicing competence of MuSVts110 RNA (9, 12, 18, 32, 33). Previously, we have shown that RNA transcribed from MuSVts110 DNA (*ts32* DNA) transfected into Psi-2, NRK, or NIH 3T3 cells is spliced as a function of growth temperature, establishing that thermosensitive splicing is a viral property (12).

However, these experiments left open the question of whether splicing was regulated at the level of pre-mRNA structure or was mediated by viral gene products. Arguments could be made in favor of either possibility. MuSVts110 is a deletion mutant of MuSV-124, having lost 1,487 bases between the *gag* and *mos* genes (32, 33). This deletion is sufficient to activate thermosensitive splicing between cryptic splice sites at nucleotides 2017 in the *gag* gene and 2448 (nucleotide 3936 in wild type MuSV-124 [36]) in the *mos* gene, suggesting that the change in intron structure was a critical determinant and that splicing was regulated by *cis*-acting RNA sequences (12). On the other hand, it could be argued that since in MuSVts110 the fused *gag* and *mos* genes allow the translation of a chimeric *gag-x* protein (P58^{gag}) with a 14-amino-acid C terminus derived from an alternate *v-mos* frame (32, 33), P58^{gag} might have acquired properties that mediate thermosensitive splicing as a function of truncation and/or the addition of a novel C terminus. While possible, the acquisition by P58^{gag} of splicing regulatory properties as a function of these structural alterations was considered unlikely in view of the fact that thermosensitive splicing can be activated by alterations to the MuSV-124 intervening sequence which do not truncate the normal MuSV-124 Pr63^{gag} gene product (M. de Mars, P. E. Cizdziel, and E. C. Murphy, Jr., submitted for publication).

It is not clear how P58^{gag} would act to regulate splicing. Necessarily, the regulatory property would have to be thermolabile, since P58^{gag} is made at all growth temperatures while splicing is restricted to 28 to 33°C (9, 21, 23, 32, 33). Moreover, since P58^{gag} is translated in the cytoplasm from the unspliced 3.8-kilobase MuSVts110 RNA, it would have to be transported intact to the nucleus. Unlike MuSV-124 Pr63^{gag}, P58^{gag} is not processed to mature viral proteins (20, 38), probably because MuSVts110 lacks the *pol* domain

encoding the MuLV protease (23, 33). Thus, it is conceivable that P58^{gag} might serve another function. To regulate splicing efficiency, it seems likely that P58^{gag} would interact with either MuSVts110 pre-mRNA or a spliceosome component, but the results of Hamelin et al. (19) argue against either of these possibilities. In these experiments, MuSVts110-infected cell extracts were exposed to both anti-*gag* and anti-small nuclear ribonucleoprotein particle sera. However, neither the unspliced or spliced MuSVts110 RNAs were coprecipitated with P58^{gag} with an anti-*gag* serum, while under identical conditions, anti-small nuclear ribonucleoprotein particle sera precipitated both these MuSVts110 RNA species but not P58^{gag} (19). The lack of demonstrable P58^{gag} interaction with MuSVts110 pre-mRNA or with spliceosome components suggests that it is unlikely to act as a regulator of MuSVts110 RNA splicing.

Despite these objections to a role for *gag* protein in the regulation of splicing, direct evidence has been lacking. Moreover, there is evidence in other systems for the regulation of splicing by *trans*-acting, presumably nonspliceosome component factors. For example, simian virus 40 T antigen has been reported to facilitate alternate splicing of adenovirus L1 RNA (11). Our observations on the regulation of viral RNA splicing in cells transfected with the three classes of MuSVts110 mutants studied here present a good case against regulation of MuSVts110 RNA splicing in *trans* by the chimeric P58^{gag} protein or, as is possible but less likely, by unidentified products of alternate reading frames in the *gag* region. RNA splicing in the MuSVts110 *gag* gene frameshift mutants was only minimally inhibited, and the characteristic thermosensitivity of splicing was essentially unaffected. These are not the results predicted if P58^{gag} were a *trans*-acting regulator of splicing, regardless of the mechanism that might be proposed for the intervention of P58^{gag} in splicing. For example, if P58^{gag} were a positive regulator of splicing active at 33°C but unable to function at 39°C, it would be predicted that in the absence of P58^{gag}, splicing might not occur or would be inefficient at splicing-permissive temperatures. Alternately, if P58^{gag} were a negative regulator of splicing active at 39°C but inefficient or inactive at 33°C, its absence should result in increased levels of spliced RNA at both permissive and formerly nonpermissive temperatures for splicing. Since neither of these possible outcomes occurred in cells transfected with the P58^{gag}-defective Xho19 and BstE5 mutants, we conclude that P58^{gag} is not involved in the regulation of the MuSVts110 splicing phenotype. Our observations with the 6m2 nickel revertants take this argument one step further, suggesting that even in the presence of normal P58^{gag}, the MuSVts110 splicing phenotype can be perturbed. In these revertant cells, MuSVts110 RNA splicing is efficient and independent of growth temperature even though unaltered (according to the criteria employed) P58^{gag} is made (4, 5), strongly suggesting that the production of *gag* protein does not guarantee the maintenance of the characteristic MuSVts110 splicing phenotype. However, these results did not exclude the possibility that P58^{gag} or the unidentified product of an alternate reading frame in MuSVts110 DNA might be a negative regulator of splicing and that an undetected mutation had occurred as a result of nickel treatment that inactivated this regulatory function, thus resulting in efficient splicing regardless of growth temperature. This interpretation is now thought unlikely for two reasons. First, the simultaneous maintenance in 6m2 nickel revertant cells of a thermosensitive splicing phenotype by *ts32* RNA and the nonthermosensitive phenotype by revertant MuSVts110 RNA in the

presence of each other's polypeptide products constitutes strong genetic evidence against *trans* regulation by any viral gene product. Second, recent experiments have identified the nickel-induced mutation in MuSVts110 in N1 cells to be a head-to-tail duplication of 70 nucleotides surrounding the 3' splice site that has no effect on P58^{gag} structure or synthesis (S. M. Chiocca and E. C. Murphy, Jr., submitted for publication).

The results obtained with the *gag* deletion mutants reinforce the conclusion that it is very unlikely that thermosensitive splicing is regulated in *trans* by the products of alternate reading frames. Removal of large portions of the *gag* gene, thus preventing polypeptide expression from any of the *gag* frames, while complicated by the fact that some diminution of splicing efficiency is observed due to *cis* effects exerted by *gag* exon sequences (Sternier and Murphy, in preparation), does not affect the thermosensitivity of splicing.

The results presented here are consistent with the concept that the novel MuSVts110 splicing phenotype is mediated in *cis* by pre-mRNA structure rather than by any known viral gene products. It seems most probable that these sequence elements affect the higher-order structure of pre-mRNA and its interaction with spliceosome components and that the thermosensitivity of MuSVts110 RNA splicing is an unusual manifestation of those *cis*-acting controls in a mutant virus.

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