JOURNAL OF VIROLOGY, Apr. 1996, p. 2286–2295 Vol. 70, No. 4
0022-538X/96/$04.00+0

Possible Role of Splice Acceptor Site in Expression of Unspliced *gag*-Containing Message of Moloney Murine Leukemia Virus

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Received 11 September 1995/Accepted 6 January 1996

Moloney murine leukemia virus (MLV) having the *gag* coding region alone, $G^{3.6}$, produced a low level of mRNA (1/10 of the wild-type level). Ligation of 441 nucleotides (nt) containing a splice acceptor (SA) site to the downstream portion of the remaining *gag* region restored the level of the unspliced message, simultaneously activating a cryptic splice donor (SD) site in the middle of the p30 coding region (between nt 1596 and 1597). Ligation of the 441 nt in the same site in the inverted orientation also increased the level of the unspliced message, activating the same SD site (between nt 1596 and 1597) and a new SA site just in front of the inserted 441 nt (between nt 4770 and 4771). Deletion or inversion of the 441-nt SA sequence from the wild-type MLV or from *int* in-frame deletion or *int* frameshift mutant MLVs of nearly full size resulted in the loss of spliced mRNA and concomitantly in a severe reduction of the unspliced mRNA, particularly at 37°C. Deletion of the 5′ SD site did not result in reduction of the unspliced-mRNA level. When the *gag* region in $G^{3.6}$ was replaced with a Neo^r coding region, the level of expression was high. The data taken together suggest that the presence of an SA signal is necessary for high-level expression of unspliced mRNA encoding Gag or Gag-Pol.

In Moloney murine leukemia virus (MLV), unspliced mRNA is packaged into virions as genomic RNA and used also as a template for translation of Gag-Pol fusion and Gag precursor proteins. The spliced mRNA is used for translation of Env protein. For replication, MLV has to simultaneously produce both spliced and unspliced mRNAs at a proper ratio. Though *cis* elements affecting the efficiency of splicing have been reported (2, 3, 11, 19, 20, 23, 24, 28), how both unspliced and spliced mRNAs are produced in MLV is unknown. It could be a stochastic phenomenon or a regulated process requiring cellular or viral proteins or a viral *cis* element(s). In this paper, we show that production of the proper amount of unspliced message required the presence of a functional splice acceptor (SA). The splice signal probably affects the stability in the nucleus and/or the stability during the nuclear-cytoplasmic transport of the message. The dependence on the SA signal appeared to reside in the MLV *gag* sequence. The phenomenon observed in this study could be one of the important mechanisms securing the simultaneous production of unspliced and spliced messages.

MATERIALS AND METHODS

Plasmids. Plasmid constructs are shown in Fig. 1. $pGE^{6.4}$hmB was constructed by inserting a simian virus 40 (SV40) promoter-driven hygromycin resistance (Hyg^r) gene (cut out from pSVKhmB, a derivative of pCHD2L [17]) into the *Sal*I site 0.1 kb downstream of the 3′ long terminal repeat (LTR) of $pGE^{6.4}$ (27). pGE^{invSA}hmB was obtained by inverting the 441-nucleotide (441-nt)-long *Xba*I-*Xba*I fragment containing the SA site (nt 5325 to 5766 [XAX]) in $pGE^{6.4}$hmB, and pGE^{dSA}hmB was made by deleting the XAX fragment from $pGE^{6.4}$hmB. $pG^{3.6}$hmB was obtained by deleting the *Hin*dIII-*Cla*I region in $pGE^{6.4}$hmB. To tag the SA site in the downstream region of *gag* in $G^{3.6}$, the XAX fragment was cloned into the *Xba*I site in the multiple cloning site of Bluescript SK+ (Stratagene, La Jolla, Calif.). The *Hin*dIII site (nt 4894) in $pGE^{6.4}$hmB was replaced with a *Not*I site by polymerization with Klenow fragment and ligation with a *Not*I linker (Toyobo, Tokyo, Japan). The *Not*I-*Cla*I fragment of this plasmid was replaced by the XAX-containing *Not*I-*Cla*I fragment of the constructed Bluescript plasmid described above. The plasmid obtained with the SA fragment inserted in the sense orientation was designated pG^{SA}hmB, and the plasmid with the SA fragment in the antisense orientation was designated pG^{invSA}hmB. To substitute the *gag* gene with the Neo^r coding region, the *Pst*I site (nt 563) of $pGE^{6.4}$hmB or that of $pG^{3.6}$hmB was replaced with an *Mlu*I site by polymerization with Klenow fragment and replaced with a *Mlu*I site by polymerization with Klenow fragment and ligation with a *Mlu*I linker (Toyobo). The *Mlu*I-*Hin*dIII regions of these plasmids were replaced with an *Mlu*I-*Hin*dIII fragment containing the Neo^r gene of pMC1neo (26) to obtain pneoEnv hmB and pneo-dE hmB, respectively. The transcription of the Neo^r gene was driven by the 5′ LTR.

The full-length provirus with a Hyg^r marker gene in its downstream portion was designated pwthmB. pΔwthmB, an in-frame deletion mutant, was constructed by deleting the *Bam*HI-*Bam*HI fragment (nt 3229 to 3535) from pwthmB. pfswthmB, a frameshift mutant (TCGA insertion at nt 3705 to produce a 2-base frameshift), was made by polymerization at the *Sal*I site (nt 3705) of pwthmB and ligation of the blunt ends. pwt^{dSA}hmB, $p\Delta wt^{dSA}$hmB, and $pfswt^{dSA}$hmB were constructed by deleting the XAX fragment from the respective parental constructs, pwthmB, pΔwthmB, and pfswthmB. $p\Delta wt^{invSA}$hmB and $pfswt^{invSA}$hmB were constructed by inverting the XAX fragment containing SA from the respective parental constructs, pΔwthmB and pfswthmB.

pGE^{dSD}neo was derived from $pGE^{6.4}$ by replacing the 5′ LTR and the flanking splice donor (SD)-containing region (down to 363 nt) with the 3′ LTR and the contiguous 3′-side cellular flanking sequence (80 nt), thus deleting the SD sequence to replace it with the cellular sequence.

Cell transfection. Subconfluent cultures of NIH 3T3 cells (2×10^5/6-cm-diameter dish) were transfected with 10 μg of plasmid DNA per dish by the standard calcium phosphate precipitation method (18). Selection with 200 μg of hygromycin per ml was started 48 h after transfection and continued for 3 weeks. A total of 50 to 100 colonies appeared in each dish.

DNA analysis. Genomic DNA from each transfectant was prepared by treatment with proteinase K (10 μg/ml) (Merck Laboratory, Darmstadt, Germany) in 100 mM Tris (pH 8.0)–50 mM EDTA for 24 h at 55°C followed by extraction with phenol-chloroform and ethanol precipitation. Purified DNA was subjected to *Cla*I digestion, electrophoresed, and blotted onto Hybond-N^+ filters (Amersham International plc, Bucks, United Kingdom). The *Xba*I-*Bam*HI fragment (nt 5766 to 6537; EXB0.8 in Fig. 1) was used to probe the proviral copies.

RNA extraction. RNAs of the transfected cells were isolated by the acid-guanidinium thiocyanate-phenol-chloroform method as described previously (10). Cytoplasmic and nuclear RNAs were fractionated and prepared by a modification of the method of Greenberg and Ziff (15). In brief, the cells were treated with Nonidet P-40 (NP-40) lysis buffer (10 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.6% NP-40), and the lysates were centrifuged at $1,500 \times g$ for

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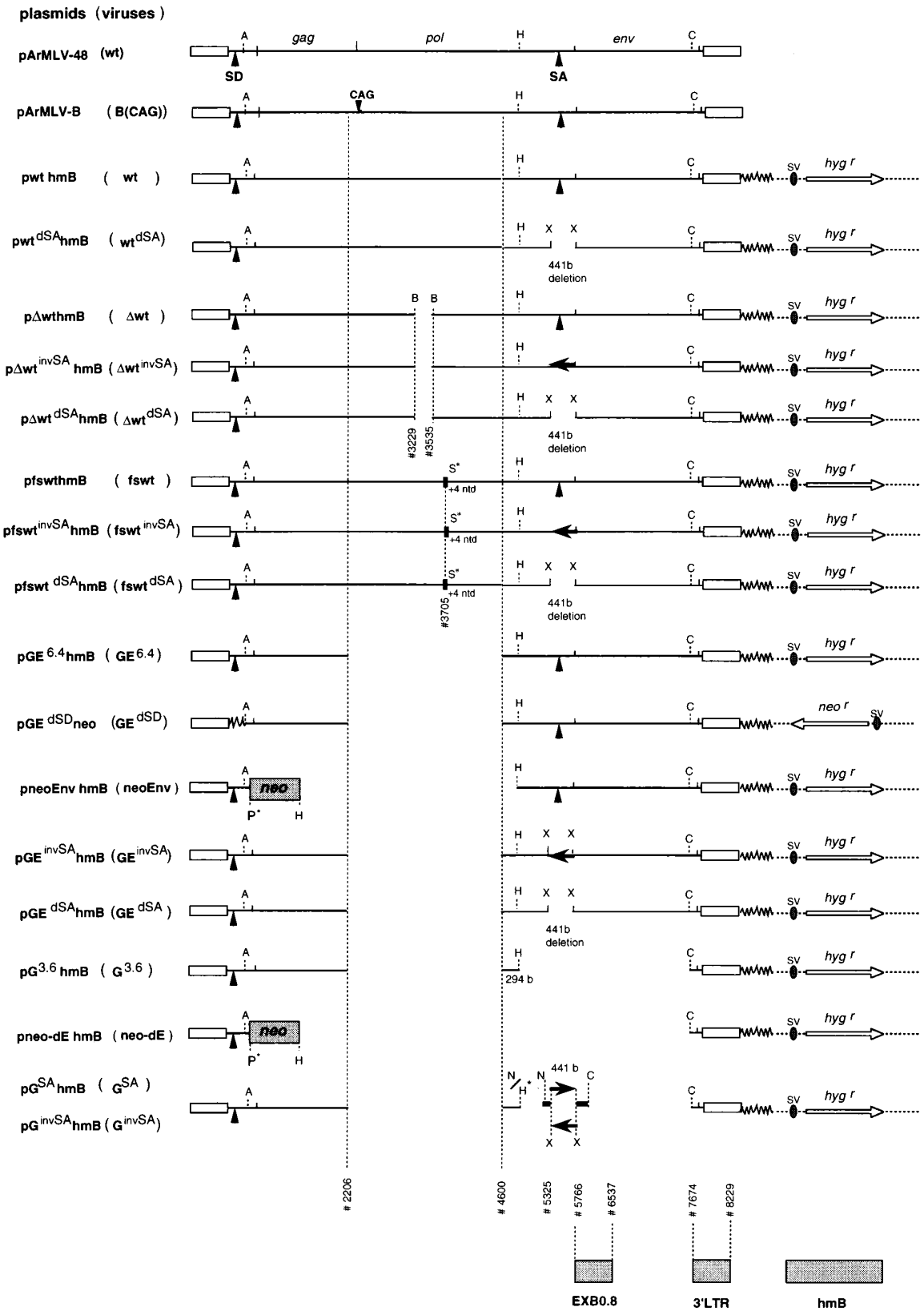

FIG. 1. Structures of the plasmids. All the constructs listed were inserted in Bluescript. The hygromycin resistance gene driven by the SV40 promoter was inserted 0.1 kb downstream from the end of the 3′ LTR. pGE^{dSD}neo, pGE$^{6.4}$neo, and pG$^{3.6}$neo, carrying the Neo^r gene cut out from pSV2neo (CLONTECH Laboratories, Inc., Palo Alto, Calif.) instead of the Hyg^r gene, were also constructed. The name of the virus encoded by each plasmid is shown in parentheses. A, *Aat*II; B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; N, *Not*I; P, *Pst*I; S, *Sal*I; X, *Xba*I. Restriction sites lost after ligation (asterisks) are indicated. hyg^r, hygromycin resistance coding region (the arrow indicates the direction of transcription); neo^r, neomycin resistance coding region; SV, SV40 promoter; 441b, 441-base. The probes for Northern blotting are shown at the bottom. Nucleotide numbers are according to Shinnick et al. (32). Plasmid-derived sequences (dotted lines) and cellular sequences (wavy lines) are indicated.

10 min. The pellet and the supernatant were used for preparation of nuclear and cytoplasmic RNAs, respectively. The pellet containing the nuclear fraction was washed once with 3 ml of NP-40 lysis buffer, and RNA was extracted by the guanidinium thiocyanate-acid-phenol method. To avoid contamination of the nuclear fraction, the upper one-third of the supernatant containing the cytoplasmic fraction was mixed with an equal volume of 7 M urea mixture (10 mM Tris-HCl [pH 7.5], 0.35 M NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate [SDS], 7 M urea). RNA was extracted with phenol-chloroform, ethanol precipitated, and purified by guanidinium thiocyanate dissociation and isopropanol precipitation.

poly(A)$^+$ RNAs were selected from the total RNAs by using Oligotex-dT super (Roche Japan, Tsukuba, Japan). The amount of poly(A)$^+$ RNAs recovered from the total RNA was 3.5%, so the recovery of poly(A)$^+$ was near 100% (mRNA constitutes 3 to 5% of the total RNA [1]).

RNA analysis. RNA was electrophoresed in a formalin–1% agarose gel and blotted onto a Hybond-N$^+$ filter (Amersham). The *Cla*I-*Sac*I fragment (nt 7674 to 8229; 3′ LTR probe in Fig. 1) of a cloned MLV, p8.2 (33), was used to detect the viral messages, and the *Sma*I-*Hpa*I fragment of pSVKhmB containing the Hyg^r coding region (hmB probe in Fig. 1) was used for detecting the Hyg^r gene message. The *Eco*RI fragment of the human 18S-5.8S-28S rRNA gene, pHr14E3 (35), was used to evaluate the separation of cytoplasmic and nuclear fractions.

For quantitation of mRNA transcripts, the radioactivity of each band on the hybridized filter was measured with a Bas 2000 bioimaging analyzer (Fuji Photo Co., Ltd., Tokyo, Japan). Levels of transcripts expressed by various MLV constructs were compared with reference to that of the SV40 promoter-driven hygromycin or neomycin resistance gene ligated in the downstream region of the construct and/or to that of the cellular β-actin gene (the β-actin clone was kindly provided by T. Kakunaga, Research Institute for Microbial Diseases, Osaka University).

Immunoblotting. Protein was extracted with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 0.5% sodium deoxycholate, 1% Triton X-100, 0.05% SDS) from the confluent cultures grown in 6-cm-diameter dishes. An aliquot of 8 μg of protein extract was electrophoresed through an SDS–10% polyacrylamide gel and blotted onto a Nitro-plus 2000 filter (Micron Separations Inc., Westboro, Mass.). Pr65^{gag} was detected with anti-p30 monoclonal antibody R18-7 (9). Antibody bound to the filter was detected with an enhanced chemiluminescence Western immunoblotting detection system (Amersham) and exposure to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.). The polyacrylamide gels were stained with Coomassie brilliant blue to check the amounts of loaded proteins.

RT-PCR and sequencing. Oligonucleotide primers were synthesized with a 380 DNA synthesizer (Applied Biosystems, Foster City, Calif.). Reverse transcriptase PCR (RT-PCR) was performed with whole RNA extracts (30). The amplified fragments were subcloned into the *Sma*I site of Bluescript SK+ and were sequenced with a Sequenase kit (United States Biochemicals, Cleveland, Ohio).

RESULTS

Role of 441-base sequence containing *env* SA in the expression of *gag* message. A mutant MLV, designated MLV-B(CAG), which was obtained by converting the suppressible UAG stop codon at the *gag-pol* junction to CAG, a glutamine codon, was unable to replicate because it lacked the translation termination at the *gag-pol* junction and produced only the Gag-Pol fusion protein; its replication required Gag precursor Pr65^{gag} supplied in *trans* (14, 27).

MLV GE$^{6.4}$ (Fig. 1), which had a 2.4-kb deletion in the 5′ half of the *pol* region, produced unspliced and spliced messages in amounts and a ratio comparable to those of the wild-type (wt) MLV and produced enough Pr65^{gag} to complement MLV-B(CAG) (27). MLV G$^{3.6}$ (Fig. 1), which was derived from MLV GE$^{6.4}$ by deleting a 2.8-kb fragment containing the *env* SA and most of *env*, could not complement MLV-B(CAG), although MLV G$^{3.6}$ and MLV GE$^{6.4}$ had the same Gag coding frame; in both, the C-terminal six amino acids were replaced by four unrelated amino acids (27). MLV G$^{3.6}$ expressed the unspliced message nearly 10-fold less than MLV GE$^{6.4}$ (Fig. 2A, lanes 4 and 5) and consequently produced less Pr65^{gag} protein (Fig. 2B). The above observation suggested that the 2.8-kb region removed from MLV GE$^{6.4}$ to obtain MLV G$^{3.6}$ was necessary for production of sufficient *gag* mRNA. Frameshift mutations in the *env* region in the 2.8-kb fragment of MLV GE$^{6.4}$ did not affect the Pr65^{gag} level, and such a mutant could complement MLV-B(CAG) (data not shown). Therefore, the 2.8-kb nucleotide sequence but not Env product was considered necessary for maintaining high-level expression of the unspliced message.

The 2.8-kb sequence contained the SA for producing the *env* spliced message. The 441-nt *Xba*I-*Xba*I region containing the SA (XAX sequence) of MLV GE$^{6.4}$ was inverted or deleted to obtain MLV GE^{invSA} or MLV GE^{dSA}, respectively. These mutations resulted in severe reductions of the unspliced message (Fig. 2A, lanes 7 and 8) and of translation product Pr65^{gag} (Fig. 2B, lanes 3 and 4) to the level of G$^{3.6}$, indicating that the XAX sequence was required for the high-level expression of the unspliced message. Deletion from MLV GE$^{6.4}$ of a sequence containing the authentic SD, on the other hand, did not result in any reduction of the unspliced message (Fig. 2A, lane 10) or of its translation product (Fig. 2B, lane 6). (As expected, the spliced message was missing in all the SD or SA deletion mutants.)

SD and SA sites activated by insertion of SA sequence in the downstream region of *gag*. In order to confirm the enhancing effect of the XAX sequence on the level of unspliced mRNA, we added XAX sequence back to the downstream region of *gag* of MLV G$^{3.6}$ (G^{SA}, Fig. 1). The insertion of XAX restored the level of the unspliced mRNA (Fig. 3B, lane 2) and that of the translation product (Fig. 3C, lane 2) close to those of MLV GE$^{6.4}$ (Fig. 3B and C, lanes 4). We also inserted XAX in the inverted orientation in the same site of MLV G$^{3.6}$ (G^{invSA}, Fig. 1). It also restored the level of the unspliced message (Fig. 3B, lane 3) and that of the product (Fig. 3C, lane 3) close to those of MLV GE$^{6.4}$.

In both constructs, a new class of spliced mRNA appeared (Fig. 3B, stars). The new splice sites were determined by sequencing the RT-PCR-amplified DNA containing the junction (30). The same SD site was used in MLV G^{SA} and G^{invSA}; it was in the middle of the p30 coding region, between nt 1596 and 1597 from the 5′ end of the genome (Fig. 3A). This position corresponded exactly to the SD site of the temperature-sensitive (*ts*) spliced message of murine sarcoma virus *ts*110 (12, 13, 16, 34). As a consequence, MLV G^{SA} encoded three mRNA species, large amounts of the unspliced RNA (3.6 kb), a spliced RNA (2.6 kb) generated by using the newly activated SD and the normal SA, and a small amount of spliced species (0.8 kb) produced by the normal SD and SA. MLV G^{invSA} encoded two mRNA species, the unspliced mRNA (3.6 kb) and a spliced mRNA (2.9 kb) generated by using the newly activated SD and a newly activated SA between nt 4770 and 4771 just upstream of the introduced XAX sequence (Fig. 3A). The consensus sequence of the branching point (25) was present 28 bases upstream from the new SA site (underlined in Fig. 3A) in the newly activated SA.

***ts* splicing.** As the newly activated SD was identical to the one used by murine sarcoma virus *ts*110 (12, 13, 16, 34), the effect of temperature on the transcripts in MLV G^{SA} and MLV G^{invSA} constructs was examined. RNAs were extracted from cells cultured for 5 days at 29, 37, or 39°C. The spliced mRNA generated by using the new SD site was barely detectable at 39°C in both MLV G^{SA} and MLV G^{invSA} (Fig. 4), while it was abundant at 29°C; the splicing was thus *ts*. The unspliced mRNAs in MLV G^{SA} and MLV G^{invSA} were not *ts*; rather, they tended to increase as the temperature was raised (Fig. 4, lanes 4 to 9). In MLV GE$^{6.4}$, the levels of unspliced and spliced mRNAs were not detectably affected by the temperature (Fig. 4, lanes 1 to 3).

Effect of substitution of the *gag* gene with the Neo^r gene. To see whether the dependence of the expression of the unspliced RNA on SA-containing sequence was specific to *gag*-containing constructs, we replaced the *gag* gene of MLV G$^{3.6}$ and that of MLV GE$^{6.4}$ with the Neo^r coding region to obtain MLV

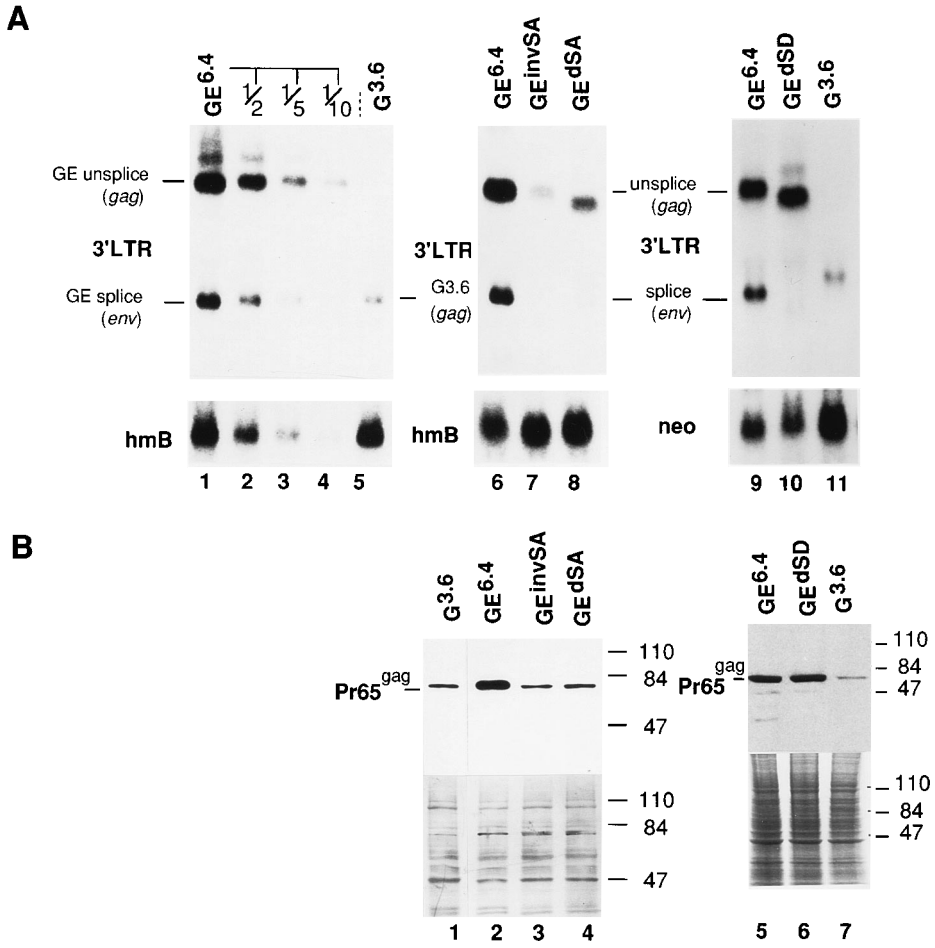

FIG. 2. Northern blot (A) and Western blot (B) analyses of viral messages in NIH 3T3 cells. NIH 3T3 cells harboring $GE^{6.4}$, $G^{3.6}$, GE^{dSA}, GE^{invSA}, and GE^{dSD} were cultured in 10-cm-diameter dishes to confluency at 37°C. Cells were harvested for extraction of RNA or protein. Ten micrograms of total RNA was applied to each lane (for $GE^{6.4}$, 1/2, 1/5, and 1/10 volumes were applied also [lanes 2 to 4]) and probed with the 3′-LTR probe (*Cla*I-*Sac*I fragment of p8.2 MLV [Fig. 1]), the Neo^r probe (*Bam*HI-*Sfi*I fragment from pSV2neo [CLONTECH]), or the hmB probe (*Sma*I-*Hpa*I fragment of pSVKhmB [Fig. 1]). Western blot analysis was performed by using anti-MLV p30 monoclonal antibody (panel B, top); the filter was stained with amido black for evaluating the amount of proteins transferred to the filter (panel B, bottom). Molecular weights (in thousands) are indicated on the right.

neo-dE and MLV neoEnv, respectively (Fig. 1). MLV neo-dE, deficient in producing the spliced mRNA, expressed as much unspliced message as MLV $GE^{6.4}$ or MLV neoEnv, which produced the spliced mRNA (Fig. 5). Therefore, the phenomenon appeared specific to the *gag*-containing constructs.

Effects of SA deletion or inversion in full-length or nearly full-length MLV genomes. The experiments described above suggested that expression of unspliced message encoding Gag depended on the presence of SA, which has been considered necessary for expression of only the spliced RNA. If this hypothesis is correct, the mechanism secures the simultaneous production of the spliced and unspliced messages, both of which are essential for proper viral replication.

In order to explore this possibility further, we derived mutants with deletions or inversions of the SA-containing XAX sequence from the wt or replication-deficient, nearly full-length MLV genomes and compared the levels of expression from these mutants with those from the parental types. One replication-deficient parental construct, MLV Δwt, had an in-frame 306-nt deletion (nt 3229 to 3535) in *int*, and another mutant, MLV fswt, had an insertion of 4 nt at the *Sal*I site (nt 3705) in the *int* gene. MLV wt^{dSA}, MLV Δwt^{dSA}, and MLV $fswt^{dSA}$ were obtained by deleting the 441-nt XAX sequence from MLV wt, MLV Δwt, and MLV fswt, respectively. MLV Δwt^{invSA} and MLV $fswt^{invSA}$ were obtained by inverting the 441-nt XAX sequence in MLV Δwt and MLV fswt, respectively.

Figure 6 compares the amounts of copies of MLV wt, MLV wt^{dSA}, MLV Δwt, MLV Δwt^{dSA}, MLV fswt, and MLV $fswt^{dSA}$ in the chromosomes of the respective NIH 3T3 transfectants. Except for MLV wt-infected cells, which contained about 16-fold more MLV copies than the rest, similar numbers of copies were present in the transfectants (the high copy number in the MLV wt-infected cells was probably due to multicycle infections in the cells). The levels of expression from these proviruses were compared on the basis of either the same provirus copy number (for MLV wt and MLV wt^{dSA}) or the same level of expression of mRNA of the SV40 promoter-driven Hyg^r gene inserted in the downstream region of the provirus (for MLV Δwt, MLV Δwt^{dSA}, and MLV Δwt^{invSA} and MLV fswt, MLV $fswt^{dSA}$, and MLV$fswt^{invSA}$). To determine the effect of temperature, the cells were cultured at 30 or 37°C for 5 days and were harvested for RNA extraction. For MLV wt, serial twofold dilutions of RNA were made so that determination of the mRNA level per proviral copy was possible. As shown in Fig. 7B, the levels of expression of MLV wt, MLV Δwt, and

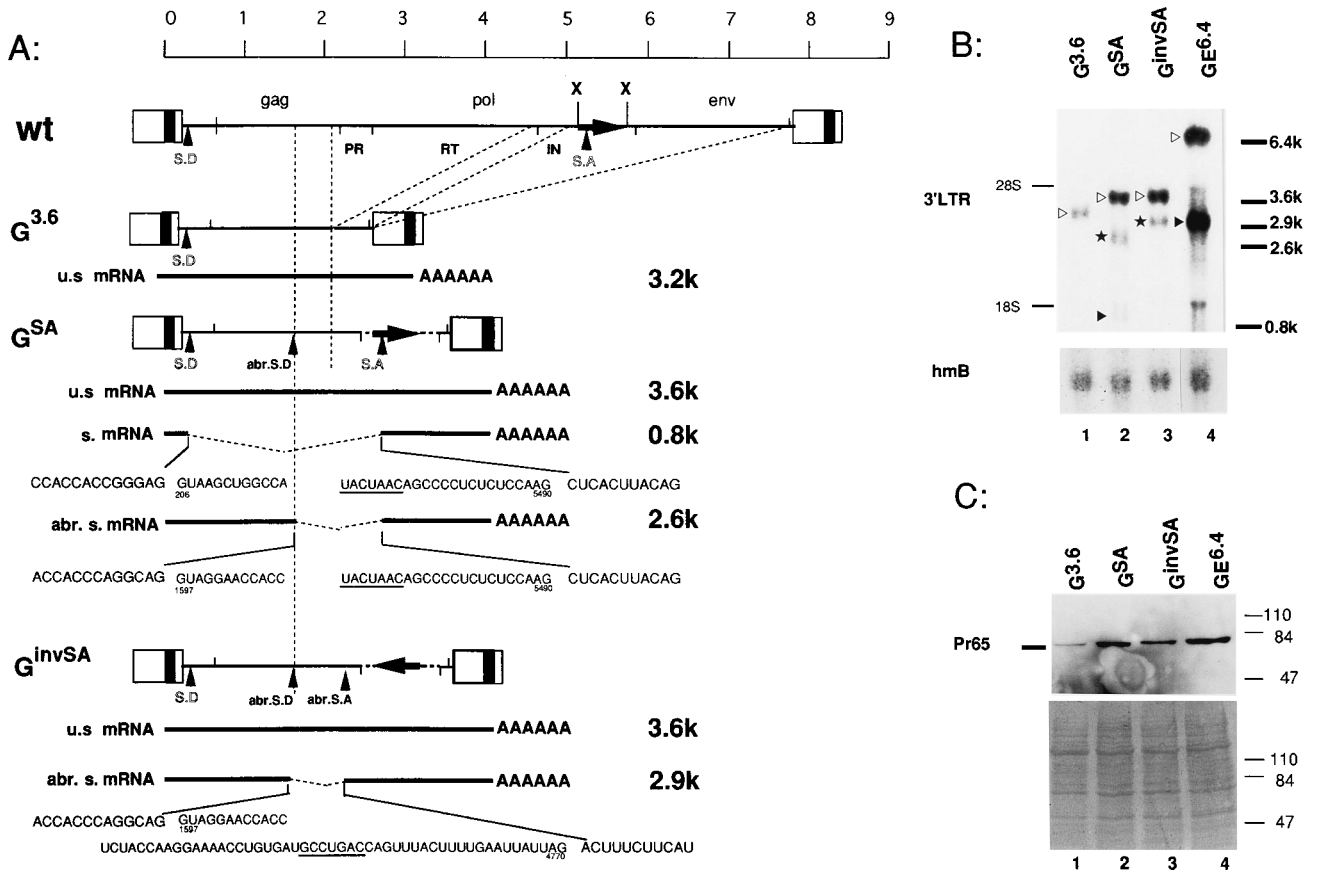

FIG. 3. Viral messages of $G^{3.6}$, G^{SA}, and G^{invSA}. (A) Diagrams of transcripts. The 441-bp XAX fragment and its direction of transcription (arrow) are indicated. The sizes of mRNAs are shown on the right. Aberrant splice sites (abr. s. mRNA) were determined by sequencing the PCR products. The nucleotide numbering is according to Shinnick et al. (32). The underlined sequence is a potential branching point sequence (25). Approximate sizes (in kilobases) are shown at the top. u.s., unspliced; s., spliced; X, *Xba*I restriction site. (B) Northern blot analysis. Ten micrograms of total RNA was applied to each lane and probed with the 3′-LTR probe. The unspliced RNAs (open arrowheads), the aberrantly spliced RNAs (stars), and the spliced RNAs produced by the normal SD and SA (closed arrowheads) are indicated. mRNA sizes (in kilobases) are shown on the right. (C) Western blot analysis using anti-MLV p30 monoclonal antibody (top) and an amido black-stained gel (bottom). Molecular weights (in thousands) are shown on the right.

MLV fswt per provirus were similar (compare lanes 9 to 12 with lanes 5 and 6, which were loaded with 1/16 of the RNA applied to lanes 9 to 12). The deletion of the SA-containing 441 nt in MLV wt resulted in a >10-fold reduction of transcript level at 30 and 37°C (Fig. 7B, compare lanes 7 and 8 with lanes 5 and 6). For MLV Δwt, deletion of the 441 nt reduced the transcript level severely at 37°C (Fig. 7A, compare lanes 2 and 4) but hardly at all at 30°C (compare lanes 1 and 3); inversion of the 441 nt reduced the transcript levels at both temperatures, but the reduction was more severe at 37°C (compare lanes 5 and 6 with lanes 1 and 2). For MLV fswt, the deletion resulted in a strong reduction of the transcript levels at both temperatures (Fig. 7A, compare lanes 9 and 10 with lanes 7 and 8). The inversion of the XAX region in the same construct reduced the transcript strongly at 37°C but only slightly at 30°C (compare lanes 11 and 12 with lanes 7 and 8).

We examined whether the differences in transcript levels were due to different levels of poly(A) tailing. We selected poly(A)$^+$ RNAs from the total RNAs of the cells cultured at 37°C by using Oligotex-dT super. The Oligotex-dT super-selected RNAs were about 3.5% of the total RNA and free of rRNAs. As mRNA constitutes about 3 to 5% of the total RNA (1), the recovery of poly(A)$^+$ RNA was near 100%. The total RNAs and the poly(A)$^+$ RNAs were electrophoresed and hybridized with the 3′-LTR probe or with hmB probe. The hybridization patterns were identical for the total RNAs and the poly(A)$^+$ RNAs (Fig. 7C, compare lanes 1 to 7 with lanes 8 to 14), indicating that the levels of poly(A)$^+$ tailing were similar for all the constructs.

The transcript levels in the nuclear and cytoplasmic fractions were compared pairwise for MLV Δwt and MLV Δwt^{dSA} and for MLV fswt and MLV $fswt^{dSA}$. The separation of nuclear and cytoplasmic fractions was monitored by rRNA; the unprocessed rRNAs were detected only in the nuclear fractions (Fig. 8, bottom). Though the mRNA levels of MLV Δwt^{dSA} in the nucleus at both 30 and 37°C and in the cytoplasm at 30°C were almost as high as those of MLV Δwt (Fig. 8, lanes 1 to 8), the level of mRNA in the cytosol was severely reduced at 37°C (Fig. 8, compare lane 8 with lane 6). For the frameshift-type MLV, the 441-nt deletion resulted in severe reductions of the transcripts in the nuclear fraction at both temperatures (Fig. 8, compare lanes 11 and 12 with lanes 9 and 10) and also reduction of the transcripts in the cytoplasm, particularly at 37°C (Fig. 8, compare lanes 15 and 16 with lanes 13 and 14). In the parental mutant constructs, i.e., MLV Δwt and MLV fswt, culture at the higher temperature (37°C) tended to result in a slight accumulation of mRNA in the nucleus (Fig. 8, compare lane 2 with lane 1 and lane 10 with lane 9) and, as a conse-

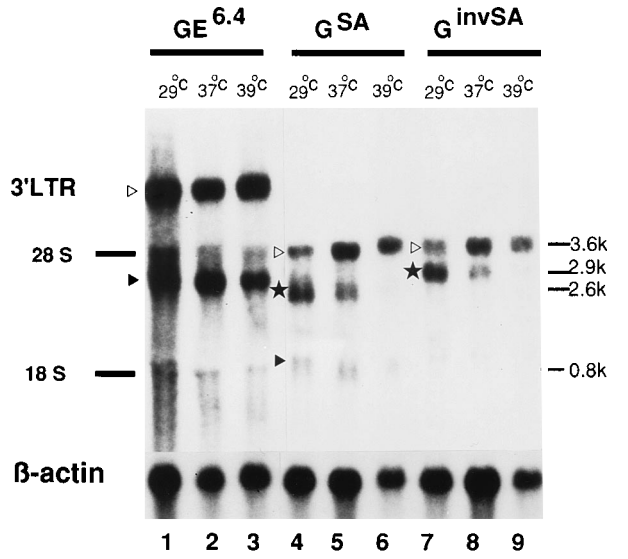

FIG. 4. Viral transcripts of NIH 3T3 cells at different temperatures. NIH3T3 cells infected with the molecularly cloned MLV (pArMLV-48) (5) and stable transfectants of $GE^{6.4}$, G^{SA}, and G^{invSA} were cultured in 10-cm-diameter dishes at 29, 37, or 39°C for 5 days. Cells were harvested, and total RNAs were extracted. Ten μg of total RNA was applied to each lane, electrophoresed, and hybridized with the 3′-LTR probe and a human β-actin-specific probe. Unspliced mRNAs (open arrowheads), spliced mRNAs produced by the normal SD and SA (closed arrowheads), and aberrantly spliced mRNAs (stars) are indicated. The sizes of mRNAs (in kilobases) are shown on the right.

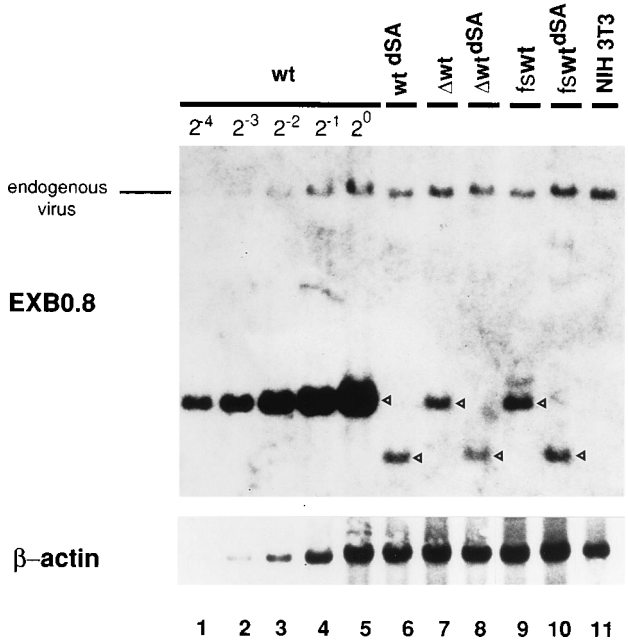

FIG. 6. Southern blot analysis of chromosomal DNAs prepared from wt-MLV-infected NIH 3T3 cells or cells transfected with mutant proviruses with nearly full-length genomes (pwt^{dSA}hmB, pΔwthmB, $pΔwt^{dSA}$hmB, pfswthmB, and $pfswt^{dSA}$hmB) and digested with *Cla*I enzyme. Ten micrograms of DNA from each culture (and serial twofold dilutions for MLV wt-infected cells [lanes 1 to 4]) was electrophoresed and hybridized with MLV-specific *env* probe (EXB0.8) (Fig. 1) or β-actin probe.

quence, a lesser expression of the mRNA in the cytoplasm (Fig. 8, compare lane 6 with lane 5 and lane 14 with lane 13, respectively), or expression of mRNA as a whole (Fig. 7A, lanes 1, 2, 7, and 8).

The stability of mRNA transcribed from these nearly full-length proviruses at high and low temperatures was examined. The transfectants were cultured at 29°C for 7 days to confluency. After addition of actinomycin D (10 μg/ml) to stop de novo RNA synthesis, cells were cultured either at 29 or 37°C and harvested at intervals for RNA extraction. RNA was electrophoresed, Northern blotted, and hybridized with the 3′-LTR or β-actin probes. From our rough calculation, at least 90% of the RNA was derived from the cytoplasm. As shown in Fig. 9, the stabilities of mRNAs of MLV Δwt, MLV $Δwt^{dSA}$, MLV fswt, MLV $fswt^{dSA}$, and β-actin were not significantly different, although all the mRNAs were less stable at 37 than at 30°C, i.e., the 441-nt deletion from their parental proviruses appeared not to affect the stability of the total RNA severely.

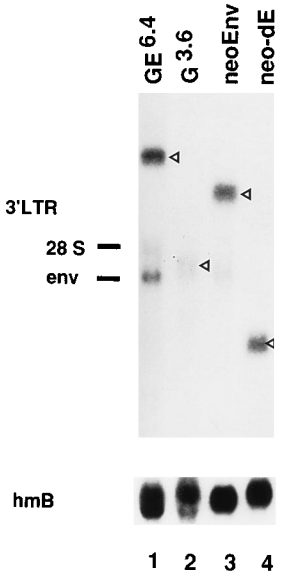

FIG. 5. Transcripts of $GE^{6.4}$- and $G^{3.6}$-type constructs whose *gag* gene was replaced by the Neo^r gene. Cells transfected with $pGE^{6.4}$hmB, $pG^{3.6}$hmB, pneo-Env hmB, or pneo-dE hmB were selected for hygromycin B (200 μg/ml) resistance. The hygromycin B-resistant cells were grown for 3 days in 10-cm-diameter dishes at 37°C to confluency and were harvested for RNA extraction. Ten micrograms of total RNA was electrophoresed and probed with the 3′-LTR or hmB probe. Unspliced message (arrowheads) is indicated.

We examined whether the difference in transcript levels in the constructs described above was reflected in the levels of translation products. Cells cultured at 30 or 37°C were harvested for Western blot analysis. For MLV wt (Fig. 10, lanes 2 and 3), 1/16 the amount of protein used with the other constructs was loaded (since the MLV wt-infected cells [Fig. 10, lane 1] contained 16-fold more copies). The deletion of SA from these constructs resulted in lower levels of translation products (Fig. 10, compare lanes 4 and 5 with lanes 2 and 3, lane 9 with lane 7, and lanes 12 and 13 with lanes 10 and 11). The processed p30 was detected in MLV wt and MLV fswt. In the SA deletion mutants, the processing of Gag precursor was incorrect (Fig. 10, lanes 4, 5, 12, and 13). This is probably due to the lack of normal virion assembly, which requires Env translated from the spliced mRNA. Shields et al. (31) reported that MLV lacking in Env expression failed to process $Pr65^{gag}$ properly. For MLV Δwt and MLV $Δwt^{dSA}$, the major product was detected as p40, and no p30 was detected. The 306-base in-frame deletion in the middle of *pol* appears to have resulted in the altered processing pattern.

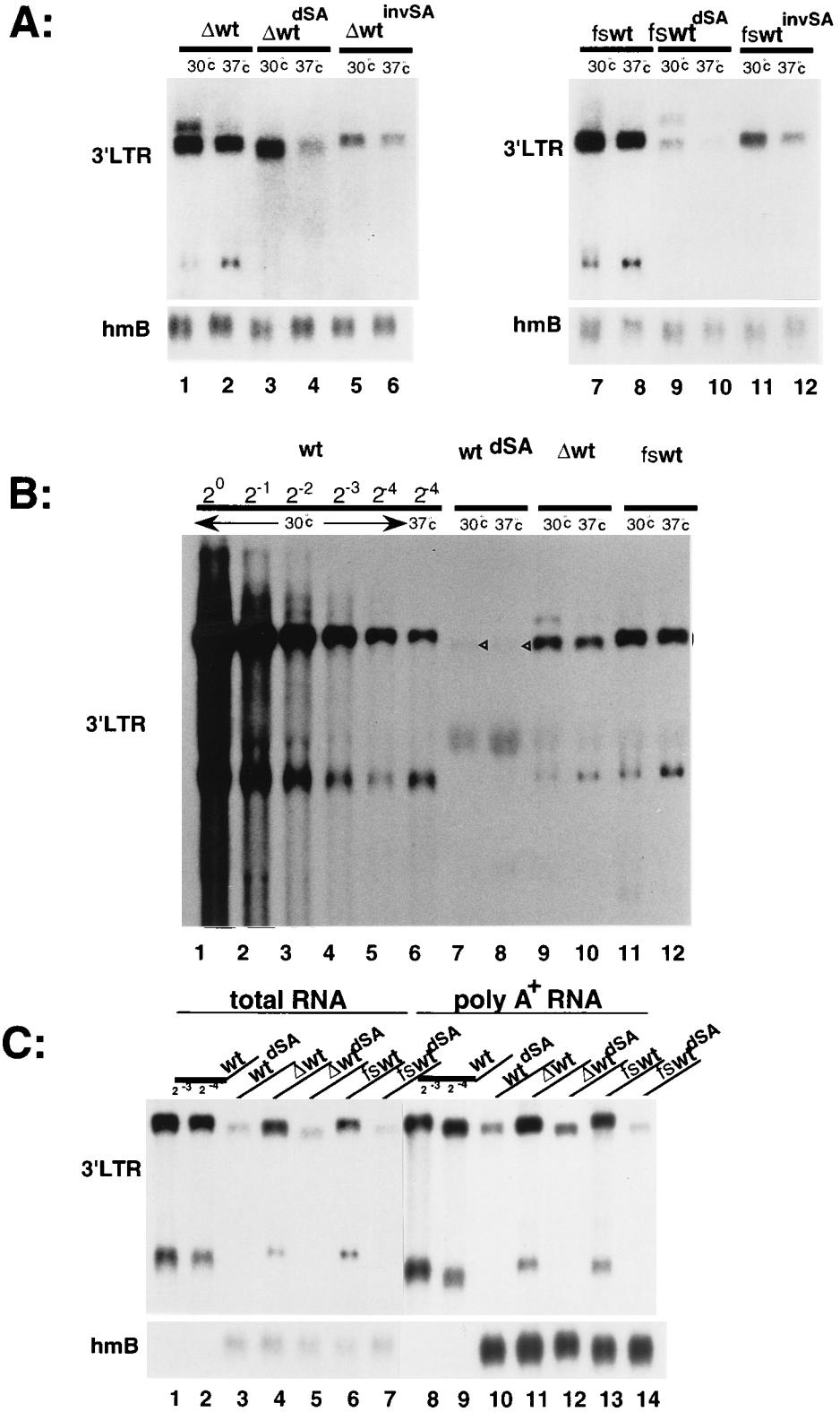

FIG. 7. Effect of SA deletion or inversion from wt MLV or MLV mutants with nearly full-length genomes. Cells were cultured in 10-cm-diameter dishes at 30 or 37°C for 4 days, and the total RNA was extracted for Northern blot analysis. (A) Five micrograms of the total RNA was electrophoresed and hybridized with the 3′-LTR or hygromycin B (hmB) probe. (B) Five micrograms of total RNA from each transfectant (and serial twofold dilutions for wt-MLV-infected cells [lanes 2 to 6]) was electrophoresed and hybridized with the 3′-LTR probe. (C) Five micrograms of total RNA (lanes 3 to 7) and 1 μg of poly(A)$^+$ RNA (lanes 10 to 14) from each transfectant (and 2^3- and 2^4-fold dilutions for wt-MLV-infected cells) were electrophoresed and hybridized with the 3′-LTR probe or with hmB probe.

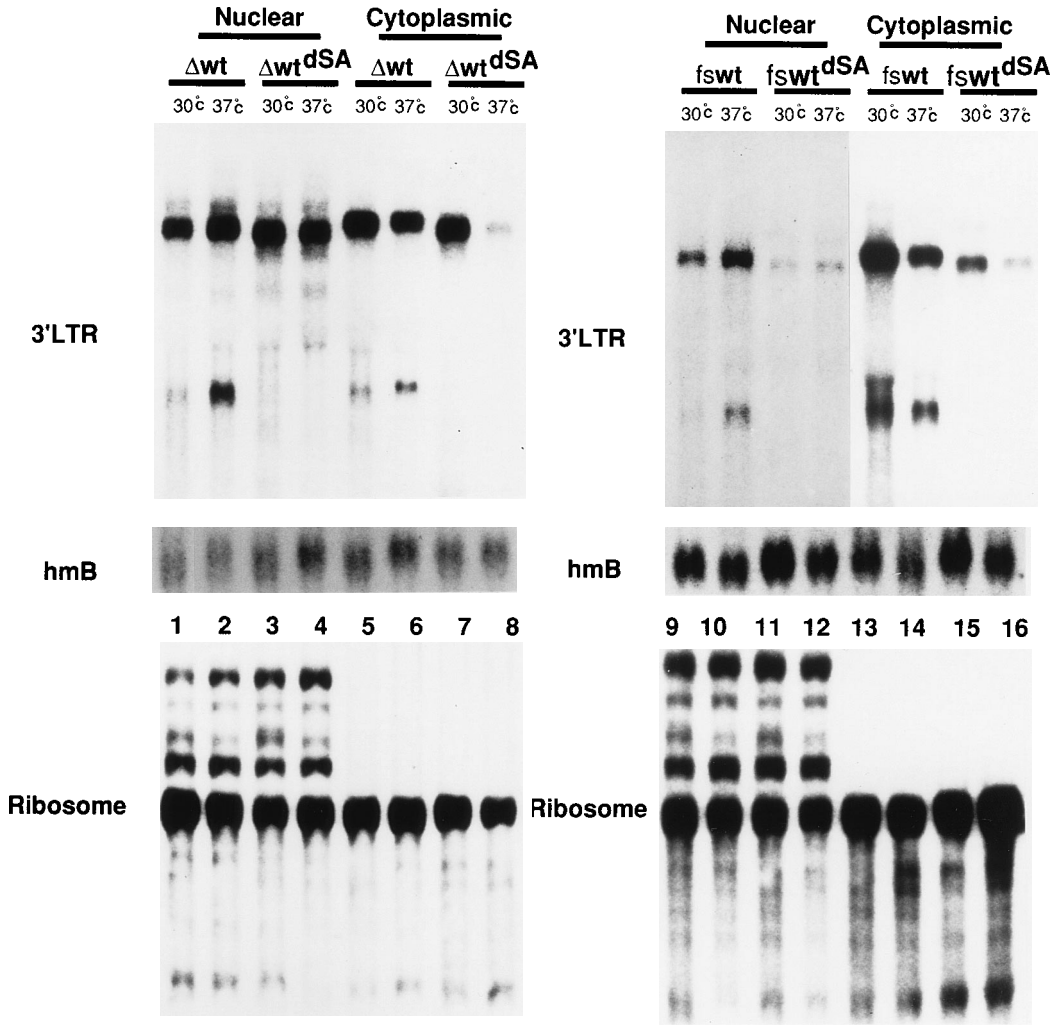

FIG. 8. Subcellular localization of transcripts of MLV mutants with nearly full-length genomes and their SA deletion mutants at different temperatures. Cells were grown to semi-confluency in 10-cm-diameter dishes for 4 days at 30 or 37°C, and nuclear and cytoplasmic RNAs were fractionated. Five micrograms of nuclear or cytoplasmic RNA was electrophoresed and hybridized with the 3′-LTR or hmB probe. For evaluating fractionation, 1 μg of each fraction was applied to the gel and hybridized with an rRNA-specific probe, an *Eco*RI fragment of pHr14E3. Though the cytoplasmic RNA was free from nuclear contamination, the nuclear RNA may not have been entirely free from the cytoplasmic fraction, but the extent of contamination, if it existed, was similar among the samples.

DISCUSSION

MLV $GE^{6.4}$, which has a 2.4-kb deletion in the 5′ half of the *pol* region, produced enough $Pr65^{gag}$ to complement the *gag-pol* read through mutant MLV-B(CAG), but MLV $G^{3.6}$, retaining the same *gag* coding region but with a further 2.8-kb deletion covering the SA site, did not. When the 441-nt sequence containing the SA (XAX) was deleted (MLV GE^{dSA}) or inverted (MLV GE^{invSA}), the *gag* message transcribed from MLV $GE^{6.4}$ was reduced to the level of MLV $G^{3.6}$. Inversely, insertion of XAX in the downstream region of *gag* of MLV $G^{3.6}$ (MLV G^{SA}) restored the transcript level to that of MLV $GE^{6.4}$. In MLV wt and also in the replication-defective MLV mutants with nearly full-length genomes (MLV Δwt and MLV fswt), deletion or inversion of the 441 nt resulted in a severe reduction of the transcript, particularly at 37°C. It was therefore suggested that the SA sequence was necessary for high-level expression of *gag* message.

Insertion of the XAX fragment in the inverted orientation in the downstream region of *gag* of MLV $G^{3.6}$ (MLV G^{invSA}), activating new SD and SA sites, restored the *gag* message to the level of MLV $GE^{6.4}$. Thus, no particular sequence in the XAX fragment in the transcript was responsible for the high-level expression of the unspliced RNA. It was possible that the XAX fragment was a transcription enhancer (4, 7, 29), but, as the whole sequence of high-level-expression construct MLV G^{invSA} was present in the low-level-expression construct MLV GE^{invSA} (which produced no spliced RNA), that possibility was unlikely. Then what function donated by the XAX fragment was responsible for elevating the level of unspliced RNA? The constructs expressing the unspliced RNA at a high level, MLV $GE^{6.4}$, MLV G^{SA}, and MLV G^{invSA}, MLV Δwt, and MLV fswt, all had active splice sites, while those expressing the unspliced RNA at a low level, MLV $G^{3.6}$, MLV GE^{dSA} and MLV GE^{invSA}, MLV wt^{dSA}, MLV Δwt^{dSA}, MLV Δwt^{invSA}, MLV $fswt^{dSA}$, and MLV $fswt^{invSA}$, had no active splice sites. The sequence used for the SA in producing an aberrantly spliced RNA in the high-level-expression construct MLV G^{invSA} was present in all the low-level-expression constructs but was not functional. Thus, it was not a specific sequence but an active SA that was necessary for high-level expression of unspliced

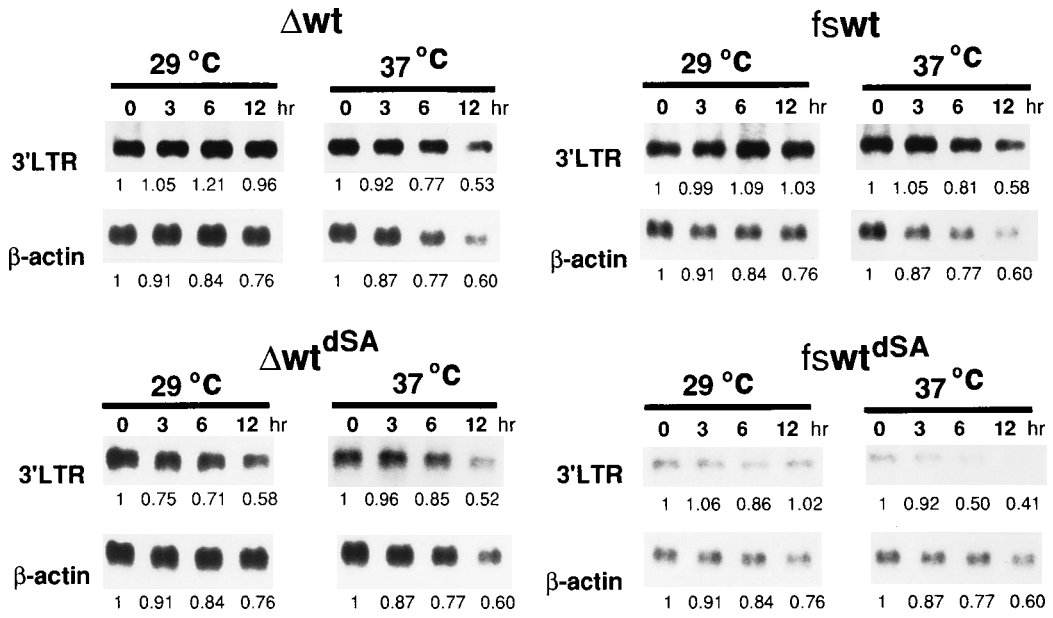

FIG. 9. The stability of message from constructs with nearly full-length genomes at high or low temperature. Stable transfectants with nearly full-size genomes, pΔwthmB, pΔw^{dSA}hmB, pfswthmB, and pfswt^{dSA}hmB, were grown in 10-cm-diameter dishes at 29°C for 7 days. The medium was changed, and actinomycin D (10 μg/ml) was added to each dish. The temperature was shifted to 37°C or kept at 29°C. Cells were harvested at intervals for extraction of total RNA. Five micrograms of total RNA was electrophoresed and hybridized with 3′-LTR or β-actin probe. The radioactivity of each band was measured with a Bas 2000 (Fuji Photo Co.), and the ratio of the activity to that at time zero was calculated.

gag mRNA. As the deletion of the SD from high-level-expression construct MLV GE$^{6.4}$ did not result in the reduction of the transcript, the SD was considered dispensable. Thus, the active SA may be a sequence which can be used as an SA site if the SD is intact. This implies that although the presence of an active SA was necessary, the concomitant splicing event itself was unnecessary for high-level expression of the unspliced RNA. This statement is supported by the finding that, in MLV G^{SA} and MLV G^{invSA}, though the splicing using the activated *ts* SD (12, 13, 16) was negligible at 37°C, the unspliced message was abundantly produced at 37°C. It is likely that a local secondary structure of RNA supporting as a functional SA may be required for efficient expression of the unspliced mRNA at 37°C.

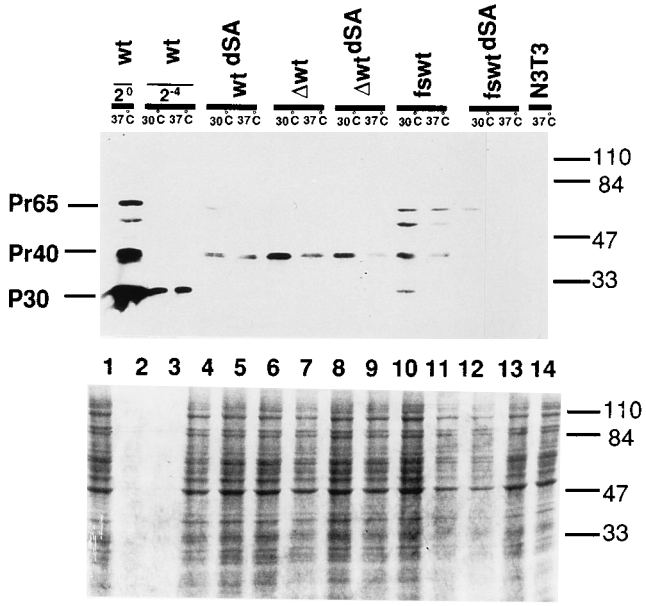

FIG. 10. Western blot analysis of stable transfectants of proviruses with nearly full-length genomes. Stable transfectants of mutants with nearly full-length genomes and MLV wt-infected cells were cultured in 10-cm-diameter dishes to confluency at 30 and 37°C. Cells were scraped off, and protein was extracted. An aliquot of 8 μg of the protein (except for MLV wt-infected cells, for which 0.2 μg was loaded [lanes 3 to 6] because the cells contained 16-fold more proviral copies than the rest) was electrophoresed and reacted with anti-p30 antibody R18-7. The same gel stained with Coomassie brilliant blue is shown bottom. Molecular weights (in thousands) are shown on the right.

When the *gag* sequence in MLV G$^{3.6}$ was replaced with the Neo^r coding region (MLV neo-dE), the Neo^r gene was expressed as efficiently as in MLV GE$^{6.4}$ (Fig. 7), i.e., the dependence of the transcript level on SA signal was not universal. We found that deletion from MLV G$^{3.6}$ of 457 nt between the *Bst*XI site (nt 1663) and the *Nru*I site (nt 2120) in the *gag* gene elevated the transcript level to that of MLV GE$^{6.4}$. This sequence appears to have a signal which makes the RNA expression dependent on the SA. (This topic will be dealt with elsewhere in more detail.) The presence of such a sequence will make the production of the unspliced mRNA dependent upon the splice signal, which is required for production of the spliced mRNA. Our observations, taken together, appear to suggest a close relation between the SA signal and stable expression of the *gag*-containing unspliced mRNA.

It remains to be elucidated how the SA signal affects the expression of the unspliced RNA. As the same promoter-enhancer in the MLV LTR was used for transcription in all the constructs, the difference was not brought about at the transcription initiation level. The levels of poly(A)$^+$ tailing appeared similar in all the constructs (Fig. 7C). From the data in Fig. 9, it was clear that the SA signal did not significantly affect the stability of RNA already transported to the cytoplasm. The observed distribution of the transcripts in the nucleus and in the cytoplasm (Fig. 8) did not support the idea that the transcripts lacking in SA are retained and accumulated in the

nucleus. For example, when the SA-containing XAX sequence was deleted from an MLV frameshift mutant (MLV fswt^{dSA}), the amounts of RNAs in the nucleus and the cytoplasm were equally reduced, and in MLV Δwt, the XAX deletion (Δwt^{dSA}) did not affect the amount of RNA in the nucleus but resulted in a severe reduction of the RNA in the cytoplasm at 37°C (Fig. 8). One possible explanation, among several, is that the stability of the RNA was reduced in the nucleus (for MLV fswt^{dSA} transcripts) or during the nuclear-cytoplasmic transport at 37°C (for MLV Δwt^{dSA}).

Although the detailed mechanism is unknown, the involvement of the SA in the expression of unspliced RNA provides a mechanism which secures the simultaneous production of spliced and unspliced mRNAs. An analogous observation has been made for human retroviruses. Rev- or Rex-dependent nuclear-cytoplasmic transport or stabilization of intron-containing message of human immunodeficiency virus or human T-cell leukemia virus type 1 requires splice sites (8, 18). The small nuclear RNAs involved in the splicing events appears to play a role in Rev function (21, 22). A cellular factor(s) involved in splicing may be required for nuclear-cytoplasmic transport of mRNA (6).

It was interesting that the adverse effect of the 441-nt deletion on the transcript level was nearly abolished at the low temperature (30°C) in some constructs, such as MLV Δwt^{dSA}, but not in other constructs, such as MLV fswt^{dSA}. The temperature sensitivity appears to be controlled by an as yet unknown factor. Finally, the amount of Pr65^{gag} expressed by MLV G$^{3.6}$, which failed to complement MLV-B(CAG), was about 1/10 of that produced by MLV GE$^{6.4}$ (Fig. 2). This may suggest the presence of a threshold level for proper function of Pr65^{gag}. As the viral capsid is a large multimer of subunit proteins, even a 10-fold reduction in the concentration of the subunit may result in a severe reduction of the functional multimers.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for the Comprehensive 10-Year Strategy for Cancer Control and a grant for AIDS Research from the Ministry of Health and Welfare (H.Y.), by grants from the Ministry of Education (A.I. and T.O.), and by a donation from Banyu Pharmaceutical Corporation.

pCHP2L was kindly provided by I. Saito, Institute of Medical Science, University of Tokyo, and pMCIneo was provided by J. Miyazaki, Faculty of Medicine, University of Tokyo. Anti-p30 monoclonal antibody R18-7 was kindly supplied by B. Chesbro, National Institute of Allergy and Infectious Diseases, and by M. Miyazawa, Tohoku University.

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