Molecular Basis Underlying Phenotypic Revertants of Moloney Murine Sarcoma Virus MuSVts110

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We examined the molecular basis for phenotypic reversion in cells infected with a transformation mutant of murine sarcoma virus, MuSVts110. In MuSVts110-infected NRK cells (6m2 cells), the manifestation of the transformed phenotype at 33°C and the normal phenotype at 39°C is governed by thermosensitive splicing of the MuSVts110 primary transcript, a 4.0-kilobase (kb) RNA which contains the *gag* and *mos* genes joined out of frame. At 33°C, selectively, the 4.0-kb RNA is processed to a spliced 3.5-kb RNA in which the *gag* and *mos* genes are rejoined in a continuous open reading frame, thus allowing synthesis of the P85^{*gag-mos*}-transforming protein. In contrast, the MuSVts110 revertant cell lines (designated 54-5A4 and 204-3) appear transformed at all growth temperatures from 33 to 39°C and express a P100^{*gag-mos*}-transforming protein from an apparently unprocessed 4.0-kb viral RNA. In the current study we established both by S1 nuclease analysis and primer extension sequencing that the revertant 54-5A4 and 204-3 4.0-kb viral RNAs suffered a 5-base deletion at the intron-exon border of the 3' splice site. The effect of this deletion is twofold. First, because of the damage to the 3' splice site, the revertant viral 4.0-kb RNAs cannot be processed to the spliced 3.5-kb RNA and, consequently, cannot be translated to P85^{*gag-mos*}. Second, the 5-base deletion excises an in-frame stop codon positioned at the intron-exon border in the parental RNA and restores the original *mos* gene reading frame. The net effect is to produce a continuous open reading frame from the *gag*, alternate *mos*, and authentic *mos* gene reading frames which are fused together in the revertant 4.0-kb RNA. This continuous open reading frame can be translated into the P100^{*gag-mos*}-transforming protein at any growth temperature.

MuSVts110 is a deletion mutant of wild-type murine sarcoma virus (MuSV-349) that uses a novel mechanism to express the product of the *v-mos* gene and thus transform target cells. At 33°C, the permissive temperature for transformation, a 431-nucleotide intron bounded by cryptic splice sites in the viral *gag* and *mos* genes is excised from the viral transcript, and the sequences are rejoined such that these genes are fused in a continuous open reading frame, yielding a spliced 3.5-kilobase (kb) RNA from which can be translated a transforming *gag-mos* serine protein kinase, P85^{*gag-mos*} (7). These splice sites are cryptic in two senses. They are present but apparently never used in the parental MuSV-349 RNA (18). Additionally, splicing between these sites is observed only at growth temperatures of 33°C or lower in MuSVts110-infected cells (5, 12). Phenotypic revertants of the 6m2 line of MuSVts110-infected cells, however, have been observed which appear transformed at both 33 and 39°C and which produce a P100^{*gag-mos*} serine kinase at both temperatures from an apparently unprocessed 4.0-kb viral RNA (5, 17). No wild-type P37^{*mos*} has been observed in these revertant cells (8). In this study we show that the revertant 4.0-kb RNA has suffered a short deletion at the 3' splice site which has at the same time eliminated the possibility of a splice and obviated its necessity by placing the *gag* and *mos* genes in frame, thus allowing the synthesis of a 100-kilodalton (kDa) *gag-mos* transformation protein.

MATERIALS AND METHODS

Cells and viruses. NRK cells infected with the transformation mutant of murine sarcoma virus MuSVts110 (6m2 cells) characteristically appear morphologically transformed at

33°C and normal at 39°C. Additionally, they readily form colonies in soft agar at 33°C but not at 39°C (1). These phenotypic changes appear to be directed by a P85^{*gag-mos*}-transforming protein that is synthesized at 33°C but absent at 39°C (6). The revertant 54-5A4 cell line was derived from a clone of parental MuSVts110 nonproducer 6m2 cells which spontaneously acquired the ability to grow well in soft agar at both 39°C and 33°C. The revertant 204-3 clone was derived by superinfection of 6m2 cells with the simian sarcoma virus-simian-associated virus complex, after which the culture medium containing MuSVts110 simian sarcoma virus-simian-associated virus complex pseudotypes was used to reinfect NRK cells. A clone which grew well in soft agar at both 33 and 39°C was selected. These cell lines were maintained in McCoy medium containing 10% fetal calf serum at 37°C. The 6m2 cell line was maintained in the same medium at 33°C.

DNA primers. DNA primers *mos*P1 and *mos*P2 for primer extension sequencing were constructed from short restriction fragments of the MuSV-124 *v-mos* gene as previously described (12). Briefly, *mos*P1 is a 52-base-pair (bp) DNA fragment located between a *Dde*I site at wild-type nucleotide 3,981 and a *Pst*I site at nucleotide 4,036 in the *v-mos* sequence. This primer has been used to sequence the *gag-mos* splice junction in the parental 6m2 3.5-kb RNA (12). Primer *mos*P2 is exactly the same as pDD39 described by Donaghue and Hunter (3), consisting of a 56-bp DNA fragment located between a *Bst*NI site at nucleotide 3,908 and a *Dde*I site at nucleotide 3,960. This primer has been used to determine the sequence of the *gag-mos* junction in the parental 6m2 4.0-kb RNA (12).

DNA probes used in S1 analysis. These probes were

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FIG. 1. Structures of viruses and DNA probes used. Structure of the wild-type MuSV-349 viral DNA is shown with several restriction sites (D, *Dde*I, X, *Xho*I, B, *Bgl*II, A, *Ava*I) and nucleotide addresses relevant to this study. Under MuSV-349 is shown the MuSV_{ts110} 4-kb RNA containing a 1,488-base central deletion relative to MuSV-349. Excision of the 431-base intron in this RNA yields the 3.5-kb viral RNA shown at the bottom of the figure. The DNA probes used in this study (pBA.36, DD.29, and pNVB 1.7) are shown at the top of the figure. The extent to which these probes are protected from S1 nuclease digestion by hybridization to the MuSV_{ts110} viral RNAs is shown both above the 4-kb transcript and below the 3.5-kb transcript.

originally described and used by Nash et al. 1984 (12) and will be briefly described here for clarity (Fig. 1).

(i) **pBA.36.** This 356-bp fragment of MuSV-124 DNA extends from a *Bgl*II site at position 3,698 across both the *gag-mos* junction point and the 3' splice site in MuSV_{ts110} to an *Ava*I site at position 4,056. The unprocessed MuSV_{ts110} viral RNA protected about 175 bases of this DNA from digestion by S1 nuclease, whereas the spliced 3.5-kb RNA protected only 122 bases from digestion in a similar experiment.

(ii) **DD.29.** DD.29 is a 290-bp fragment of the pMLV-1 *gag* gene extending from a *Dde*I site at position 1,481 across the 5' splice site used in MuSV_{ts110} to a *Dde*I site at position 1,771. These positions correspond to nucleotides 1,902 to 2,192 in the MuSV-124 sequence.

(iii) **pNVB 1.7.** This DNA is a 1,717-bp fragment of MuSV-124 DNA extending from an *Xho*I site at nucleotide 1,981 across the MuSV_{ts110} *gag-mos* boundary to the same *Bgl*II site at position 3,698 which is the 5' end of the viral DNA in pBA.36.

Purification of plasmid DNA. The various inserts and fragments described in the previous section were excised by digestion with the appropriate restriction enzyme(s), size-fractionated on an 0.8% agarose gel, and stained with ethidium bromide. DNA was purified from agarose by adsorption to glass powder (19) or by electroelution in TE buffer (10 mM Tris [pH 8], 1 mM EDTA). Electroeluted DNA fragments were further purified by chromatography on a NACS column (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) according to directions provided by the supplier.

In cases where small DNA fragments were purified from polyacrylamide gels, gel slices were crushed to a paste in 2 ml of TNE buffer (10 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA) and agitated overnight. Acrylamide granules were removed by centrifugation, and DNA was purified from the supernatants by chromatography on a NACS column.

End labeling of DNA. (i) **3' End labeling.** The 3' ends of restricted DNA fragments were labeled by filling in with the Klenow fragment of DNA polymerase. Briefly, DNA fragments to be labeled (1 to 2 μ g) were adjusted to 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.6)–7.5 mM $MgCl_2$ –10 mM 2-mercaptoethanol–50 μ g of bovine serum albumin per ml. To this mixture was added 2.5 μ l (6 to 8 U) of the Klenow fragment of DNA polymerase, 40 μ Ci of the appropriate [α - 32 P] deoxynucleoside triphosphate (dNTP) (>3,000 Ci/mmol), and 0.1 mM unlabeled dNTPs as required by the restriction site to be filled in. After incubation at 15°C for 60 min, 20 mM EDTA was added to stop the reaction. The reaction mix was desalted by passage over a G-50 column equilibrated in TNE buffer after the addition of 20 μ g of carrier DNA. Radioactivity in the void volume was pooled, ethanol precipitated, and dissolved in TE buffer.

(ii) **5' End labeling.** Restriction fragments were first dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Briefly, the DNA solution was adjusted to 50 mM Tris (pH 9), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine containing 10 U of calf intestinal phosphatase at 37°C. After 30 min, an additional 10 U of calf intestinal phosphatase was added for another 30 min. The digest was mixed with 0.1 volume of

10× TNE and 0.05 volume of 10% sodium dodecyl sulfate (SDS) for 15 min at 68°C to halt the reaction. Following phenol-chloroform (1:1) extraction, the nucleic acids were ethanol precipitated. For 5' end labeling, dephosphorylated DNA was adjusted (100 mM Tris [pH 7.6], 20 mM MgCl₂, 10 mM dithiothreitol, 0.2 mM spermidine, 0.2 mM EDTA) and 10 to 20 U of T4 kinase and 150 µCi of [γ -³²P]ATP was added for 1 h at 37°C. The end-labeled DNA fragments were then subjected to electrophoresis on a 20% sequencing gel, and the fragments of interest were isolated from the polyacrylamide as described above.

Primer extension. 5' End-labeled *mos*P1 or *mos*P2 was mixed individually with 10 µg of total cellular RNA from 6m2 or 54-5A4 cells as indicated in the text and ethanol precipitated. The pellets were thoroughly dissolved in a mixture containing 30 µl of 80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.8), 400 mM NaCl, and 1 mM EDTA, immersed in an 85°C water bath for 10 min, and transferred to a 65°C water bath which was then turned off. Hybridization was allowed to occur during cooling to room temperature overnight (3). After hybridization, the reaction mixes were ethanol precipitated and dissolved in 25 µl of 10 mM Tris (pH 7.4). Primer extension in the presence of dideoxynucleoside triphosphates (ddNTPs) was carried out essentially as described previously (3, 12). First, 5 µl of the hybrid mix was dispensed into four separate tubes, to each of which was added 1 µl of a 200 µM dNTP mixture (dATP, dCTP, dGTP, or dTTP), 1 µl of a ddNTP mixture calculated to produce the following ratios: ddATP/dATP, 1.3; ddCTP/dCTP, 0.4; ddGTP/dGTP, 0.3; ddTTP/dTTP, 0.9, 1 µl of avian myeloblastosis virus reverse transcriptase (Seikagaku; 10 U/µl), and 2 µl of a 5× buffer containing 250 mM Tris (pH 8.3), 0.5 M KCl, 50 mM MgCl₂, and 5 mM dithiothreitol. These mixtures were incubated at 40 to 42°C for 60 min to extend the primers, after which they were ethanol precipitated in the presence of 10 µg of carrier yeast RNA and 10 mM MgCl₂.

Sequence analysis. The extended primers were dissolved in 75% formamide–20 mM EDTA (pH 8.0)–0.01% bromophenol blue–xylene cyanol, heated to 100°C for 2 min, and analyzed on a 6% polyacrylamide gel containing 8 M urea in TBE buffer (50 mM Tris [pH 8.3], 50 mM boric acid, 2 mM EDTA) at 2,000 V for 105 min. After electrophoresis, the gel was either subjected to autoradiography directly with Kodak XAR-2 film and Dupont Cronex Lightning-Plus intensifying screens or fixed with 10% acetic acid–10% methanol for 20 min, dried, and then autoradiographed.

Preparation of cellular DNA. Cells growing in roller culture were rinsed with an isotonic buffer and gently lysed in 2% SDS–7 M urea–0.35 M NaCl–1 mM EDTA buffered with 10 mM Tris (pH 8). The cell lysate was extracted with an equal volume of phenol-chloroform (1:1) saturated with the above SDS-urea buffer and ethanol precipitated. Nucleic acids from each roller culture were dissolved in 3 to 5 ml H₂O, digested sequentially with 50 µg of RNase per ml at 37°C for 60 min and 100 µg of proteinase K per ml at 37°C for 120 min, extracted with phenol-chloroform, precipitated with ethanol, and finally dissolved in H₂O.

Blot hybridization of DNA. Routinely, 15 µg of cellular DNA digested with a given restriction enzyme as recommended by the supplier was analyzed on an 0.8% agarose gel in 50 mM Tris (pH 8.3)–40 mM sodium acetate–2 mM EDTA at 40 V for 16 to 18 h and transferred to nitrocellulose essentially as described by Southern (16) and modified by Wahl et al. (20) and Singh and Jones (15). The nitrocellulose

was prehybridized at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium pyrophosphate–0.2% SDS–100 µg/ml heparin. Hybridization was carried out at 42°C in the same solution containing 2 × 10⁶ cpm/ml ³²P-labeled probe (1 × 10⁸ to 3 × 10⁸ cpm/µg). After hybridization, the filters were washed four times with 3× SSC–0.1% SDS at 42°C and two times with 0.1× SSC–0.1% SDS at 55°C, dried, and autoradiographed.

Isolation of cellular RNA. Intracellular RNA was isolated from tissue culture cells by a guanidine hydrochloride extraction procedure (5, 21). Briefly, cellular pellets were dissolved in solution A (8 M guanidine hydrochloride–100 mM sodium acetate [pH 5]) and subjected to vigorous Dounce homogenization. After centrifugation of the extracts at 10,000 × *g* for 15 min, nucleic acids were precipitated from the supernatant by the addition of 0.5 volume of ethanol at –20°C for 30 min and collected by centrifugation at 10,000 × *g* for 20 min. The nucleic acid pellets were dissolved in 0.25 to 0.50 of the original volume of solution B (solution A plus 0.1 volume of 200 mM EDTA) and again precipitated with 0.5 volume of ethanol. This last step was repeated two more times, decreasing the volume of solution B by half each time. The final pellet was dissolved in 20 mM EDTA (pH 7.5) and extracted with chloroform-isobutanol (4:1). The aqueous phase was mixed with 2 volumes of 4.5 M sodium acetate (pH 6), and the RNA was precipitated at –20°C overnight. The RNA was collected by centrifugation, washed once with 3 M sodium acetate and once with 80% ethanol, and dissolved in H₂O.

S1 nuclease analysis. These analyses were done essentially as described previously (11, 12). Briefly, 5' end-labeled *Ava*I-digested pBA.36 DNA was hybridized for 3 h to 10 µg of total cellular RNA from 6m2 cells grown at 39, 33, or 28°C as indicated in the text in 80% formamide–40 mM PIPES (pH 6.8)–400 mM NaCl–1 mM EDTA at 56°C (the hybridization temperatures for pNVB 1.7 DNA and DD.29 DNA were 54 and 60°C, respectively), digested with 200 U of S1 nuclease per ml at 37°C for 30 min, phenol extracted, and ethanol precipitated. The digests were dissolved in 20 µl of 10 M urea–1 mM EDTA–0.015% bromocresol green, heated to 50°C for 5 min, and analyzed on a 4% polyacrylamide gel containing 8 M urea. After electrophoresis, the gels were fixed with 50% methanol–7% acetic acid, dried, and autoradiographed.

RESULTS

Viral RNA species in MuSVts110 revertant cell lines. We have previously shown by direct RNA blot hybridization (5, 17) that MuSVts110 revertant 54-5A4 and 204-3 cells contain a single ~4.0-kb viral RNA at all growth temperatures between 28 and 39°C. No processing of the 4.0-kb RNA to the spliced 3.5-kb RNA characteristic of the parental line of MuSVts110-infected cells (6m2 cells) growing at 33°C has been observed in the revertants. Because P85^{*gag-mos*} is translated from the 3.5-kb RNA, it appeared unlikely that these revertant cells could make P85^{*gag-mos*}. Nevertheless, 54-5A4 and 204-3 cells appear morphologically transformed at all growth temperatures and grow well in soft agar. The constitutive transformed state in these cells seems to be mediated by a P100^{*gag-mos*} fusion protein structurally distinct from but functionally similar to the P85^{*gag-mos*} produced in 6m2 cells at temperatures permissive for transformation (8, 17).

We have recently shown that the *gag* and *mos* genes in the parental 6m2 4.0-kb viral RNA are joined out of frame such that translation of this RNA is terminated 42 bases down-

FIG. 2. S1 nuclease analysis of the *mos* gene side of the *gag-mos* junction and splice acceptor sites in parental 6m2 cells and revertant 54-5A4 cells. Total cellular RNA (10 μ g) from 6m2 cells and 54-5A4 cells grown at either 37 or 33°C was hybridized to the 5' end-labeled pBA.36 probe. The hybrids were digested with S1 nuclease, and the protected fragments were analyzed on a 4% polyacrylamide gel containing 8 M urea. Lanes: 1, a 5'-end-labeled pBR322 DNA-*Hinf*I digest; 2, 54-5A4 RNA, 37°C; 3, 54-5A4 RNA, 33°C; 4, 6m2 RNA, 33°C. Fragment sizes in bases are indicated.

stream of the *gag-mos* junction (12). Thus, we originally thought that P100^{*gag-mos*} synthesis in the revertant cells might easily be explained by a point mutation at or near the *gag-mos* junction that would relieve this blockade. For this reason, we were surprised at the results of our initial S1 nuclease protection experiments on intracellular viral RNA in 54-5A4 and 204-3 cells. RNA from 54-5A4 cells grown at either 33 or 39°C protected two distinct size fragments (Fig. 2, lanes 2 and 3) of the 359-bp pBA.36 insert (see Fig. 1). The first of these, 175 bases long, is characteristic of the parental 6m2 4.0-kb RNA. The smaller protected fragment, about 115 bases in size, is very similar in size to the 122-base fragment characteristically protected by the spliced 3.5-kb 6m2 RNA that codes for P85^{*gag-mos*} (see Fig. 1). It was first thought that protection of this 115-base fragment by 54-5A4 and 204-3 cellular RNA argued for the presence of a hitherto unrecognized, spliced, but untranslated, 3.5-kb viral RNA in the revertant cells. This was an unexpected result because one cannot detect any 3.5-kb RNA or P85^{*gag-mos*} in these revertant cells at any growth temperature (5, 17).

We next attempted to confirm the presence or absence of a processed viral RNA in the revertants by additional S1 protection experiments with 3' end-labeled DNA fragments extending across the *gag-mos* junction in the 4.0-kb RNA and across the 5' splice site in the 3.5-kb RNA. The S1 probe used for analysis across the *gag-mos* boundary is designated pNVB 1.7 (see Fig. 1.). The 6m2 4.0-kb RNA will protect only 440 bases of the pNVB 1.7 probe (Fig. 3, lane 1), because of a deletion in the RNA beginning at nucleotide 2,404 (see Fig. 1) which occurred in the original generation of MuSV_{ts110} from MSV-349. Because these 440 bases lie wholly within the intron in the 4.0-kb RNA (see Fig. 1), the processed 3.5-kb RNA will not protect any of the pNVB 1.7 probe. S1 nuclease analysis of revertant RNA-pNVB 1.7 hybrids show that 54-5A4 and 204-3 cellular RNA, like the 6m2 4.0-kb RNA, will protect 440 bases of pNVB 1.7 (Fig. 3, lanes 2 and 3). We next attempted to determine if the 5'

splice site at nucleotide 2,017 is used in the revertant RNAs. Because the pNVB 1.7 probe cannot detect the processed 3.5-kb RNA, we used as a probe the 3' end-labeled DD.29 DNA which has homology across the 5' splice site as a probe (see Fig. 1). We have previously shown that the spliced 3.5-kb RNA will protect only 120 bases of the DD.29 DNA (11) (Fig. 4, lane 4), establishing that the *gag* gene in this RNA extends only up to the 5' splice site at nucleotide 2,017. In contrast, the unprocessed 6m2 4.0-kb RNA will fully protect the DD.29 DNA fragment (11) (Fig. 4, lane 6). Control experiments in which probe DD.29 alone carried through the hybridization reaction was digested with S1 nuclease yielded only a trace amount of reannealed probe (data not shown). When similar experiments were done with RNA from the revertant cells, it was found that 54-5A4 and 204-3 RNA fully protected the DD.29 fragment from S1 nuclease digestion (Fig. 4, lanes 3 and 5), suggesting that no processed 3.5-kb RNA with the 5' splice donor site at nucleotide 2,017 is present in these cell lines at 37°C.

Thus, it initially seemed that in the revertant cells there could be viral RNAs with *gag* genes extending up to nucleotide 2,404 fused to *mos* sequences at either nucleotide 3,883 (175-base-protected fragment of pBA.36) or nucleotide 3,940 (115-base-protected fragment of pBA.36). Further experimentation, however, emphasized two additional facts. First, RNA from the revertant cell lines reproducibly protects only 115 bases of the pBA.36 probe, not the 122 bases protected by the 6m2 3.5-kb RNA. Second, hybrids formed between pBA.36 and RNA from either 6m2 or revertant cells respond differently to increasing concentrations of S1 nuclease. Exposure of 6m2 RNA-pBA.36 hybrids to five times the stan-

FIG. 3. S1 nuclease analysis of the *gag* gene side of the *gag-mos* junction in parental 6m2 and revertant 54-5A4 and 204-3 cells. As described under in Materials and Methods and in Fig. 1, cellular RNA from 6m2, 54-5A4, and 204-3 cells was hybridized to the 3' end-labeled pNVB 1.7 probe, digested with S1 nuclease and was analyzed on a 4% polyacrylamide gel. Lanes: 1, 6m2 RNA; 2, 54-5A4 RNA; 3, 204-3 RNA. The unlabeled lane is a 5'-labeled pBR322-*Alu*I digest. Fragment sizes in bases are indicated.

dard amount of S1 nuclease (3,300 U/ml [Pabst Laboratories]) appeared to reduce the amount of 175- and 122-base fragments protected, but it did not alter the relative amounts of the protected fragments (Fig. 5, compare lanes 1 and 2). In contrast, exposure of a 54-5A4-pBA.36 hybrid to 5× S1 nuclease reproducibly failed to protect any 175-base fragment; only the 115-base fragment was protected (Fig. 5, lanes 3 and 4). This result is also shown quite clearly in Fig. 6, in which the pBA.36 DNA probe was hybridized to 54-5A4 RNA (lane 3), 204-3 RNA (lane 6), and RNA from 6m2 cells grown at 33°C (lane 4) or 39°C (lane 5).

Nucleotide sequence at the *gag-mos* junction in MuSVts110 revertant viral RNAs. From the above results it appeared that the viral RNA in the revertant cells was similar to that found in 6m2 cells, except that hybrids between the revertant RNA and the pBA.36 probe were more accessible to elevated concentrations of S1 nuclease at a point near the 3' splice site. To establish the nature of the possible structural alteration in the revertant cell RNAs, we sequenced these RNAs around the *gag-mos* junctions and at the 3' splice site.

Mos primer 2 (*mos*P2), a 52-base fragment of the *v-mos* gene extending from wild-type nucleotide 3,908 to 3,960 (Fig. 7) has been used to sequence the *gag-mos* junction in parental 6m2 4.0-kb RNA by the primer extension method

FIG. 4. S1 nuclease analysis of the *gag* gene side of the splice donor site in parental 6m2 and revertant 54-5A4 and 204-3 cells. Total cellular RNA from 6m2 cells grown at either 33 or 39°C and from 54-5A4 and 204-3 cells was hybridized to the 3'-end-labeled DD.29 probe, digested with S1 nuclease, and the digests were analyzed on a 4% polyacrylamide gel. Lanes: 1 and 7, a 5'-end-labeled pBR322-*Hin*I digest; 2, a size standard of DD.29 probe only; 3, 54-5A4 RNA; 4, 6m2 RNA, 33°C; 5, 204-3 RNA; 6, 6m2 RNA, 39°C. Fragment sizes in bases are indicated.

FIG. 5. Differing response of 6m2 RNA-pBA.36 and 54-5A4 RNA-pBA.36 hybrids to increasing S1 nuclease concentration. S1 digests of 6m2 RNA-pBA.36 or 54-5A4 RNA-pBA.36 hybrids were done with either 200 U (1×) or 1,000 U (5×) of the enzyme per ml. The protected fragments were analyzed on a 4% polyacrylamide gel. Lanes: 1, 6m2 RNA, 1× S1; 2, 6m2 RNA, 5× S1; 3, 54-5A4 RNA, 1× S1; 4, 54-5A4, 5× S1; 5, a 5'-end-labeled pBR322-*Hin*I digest. Fragment sizes in bases are indicated.

(12). In a similar extension experiment done with a 54-5A4 RNA-*mos*P2 hybrid, a *gag-mos* junction sequence identical to that found in the 6m2 4.0-kb RNA was found (data not shown). From these results, it was clear that there was no point mutation at or near the *gag-mos* junction in the 54-5A4 RNA that could restore the original *mos* gene reading frame. However, when the sequences surrounding the 3' splice site in the 6m2 and revertant RNAs were compared by extension of *mos* primer 1 (*mos*P1), a DNA fragment extending from nucleotide 3,981 to 4,036 in the *v-mos* gene (Fig. 7), it was found that the 54-5A4 and 6m2 4.0-kb RNAs were strikingly dissimilar. The 6m2 4.0-kb RNA contained an intact 3' splice site (Fig. 8A). However, in both revertant RNAs, a 5-base deletion (A-G-T-G-T) had occurred at the intron-exon border (Fig. 8B). Note that the sequence A-G-T-G-T at the 3' splice site in 6m2 RNA (Fig. 8A) is missing from both 54-5A4 and 204-3 RNA (Fig. 8B).

The nucleotide sequence surrounding the *gag-mos* junction and the 3' splice acceptor site in both the parental 6m2 and revertant 4.0-kb RNAs is shown diagrammatically in Fig. 9. As shown, unspliced 6m2 4.0-kb RNA uses an alternate *mos* reading frame beyond the *gag-mos* junction point. This reading frame remains open for only 42 bases until a TAG stop codon is reached. In the revertant 4.0-kb RNA, the *gag-mos* junction is identical to the 6m2 4.0-kb RNA and uses the same alternate *mos* reading frame. However, the 5-base deletion at the 3' splice site not only destroys this site but also deletes an in-frame stop codon and

FIG. 6. S1 nuclease analysis of the *mos* gene side of the *gag-mos* junction and splice acceptor sites in parental 6m2 and revertant 54-5A4 and 204-3 RNA with a $5\times$ S1 nuclease concentration. Cellular RNA-pBA.36 hybrids were treated and analyzed as described in the legend to Fig. 1, except that 1,000 units of S1 nuclease was used. Lanes: 1 and 7, a 5'-end-labeled pBR322-*HinfI* digest; 2, pBA.36 probe only; 3, 54-5A4 RNA; 4, 6m2 RNA, 33°C; 5, 6m2 RNA, 39°C; 6, 204-3 RNA. Fragment sizes in bases are indicated.

restores the original *mos* reading frame, thus bypassing another stop signal. Consequently, the revertant 4.0-kb RNA presents a continuous open reading frame across the *gag-mos* junction into the alternate *mos* reading frame, and thence across the 5-base deletion at the intron-exon border back into the original *mos* reading frame.

Proviral DNA in parental 6m2 and revertant 54-5A4 and 204-3 cells. 54-5A4 is a spontaneous revertant clone derived from 6m2 cells. The phenotypically similar 204-3 cells, on the other hand, were derived by reinfection of NRK cells with a viral harvest from 6m2 cells superinfected with the simian sarcoma virus-simian-associated virus complex. Be-

FIG. 7. DNA primers used for sequence analysis of MuSV_{ts110} viral RNA. Positions of the DNA primers *mosP1* and *mosP2* relative to the 5' splice site, the *gag-mos* gene junction, and the 3' splice site are shown.

FIG. 8. Sequence analysis of the 3' splice site in parental 6m2 and the revertant 54-5A4 and 204-3 4.0-kb viral RNAs. Total cellular RNA (10 μ g) from 6m2, 54-5A4, and 204-3 cells was hybridized to 5'-end-labeled *mosP1* primer and extended with reverse transcriptase in the presence of dideoxynucleoside triphosphates. The product was analyzed in a 6% polyacrylamide sequencing gel. (A) 6m2 RNA; (B) 54-5A4 and 204-3 RNA.

cause both revertants have identical 5-base deletions in the 3' splice site, we checked their independent origins by comparing the integration sites of the viral DNA in the parental 6m2 and revertant cell clones. As expected of a spontaneous revertant, the viral DNA in the 54-5A4 cells is at the same cellular locus as it is in 6m2 cells, as suggested by the presence of identical MuSV_{ts110}-related restriction fragments in the DNA of both cell lines (Fig. 10). As shown in Fig. 10, the numbered bands represent MuSV_{ts110} DNA fragments plus flanking sequences; the bands with no designation are *c-mos* DNA fragments. In contrast, the viral DNA in 204-3 cells is found at a different cellular locus than in 6m2 and 54-5A4 cells, as shown by the clear difference in the sizes of the 204-3 cell MuSV_{ts110}-related restriction fragments (Fig. 10C) over those found in 6m2 and 54-5A4 DNA. This is the result expected if the 204-3 cells originated from a new infection of NRK cells.

DISCUSSION

In the MuSV_{ts110}-infected 6m2 cell line, cell transformation is mediated by P85^{*gag-mos*}, a serine kinase which is temperature sensitive with regard to both its expression (6, 12) and enzymatic activity (7). Previous work has documented the basis for the thermosensitivity of P85^{*gag-mos*} expression. P85^{*gag-mos*} is translated from a 3.5-kb splice product of a larger 4.0-kb viral RNA. Splicing excises a 431-base intron that contains an out-of-frame *gag-mos* junction, replacing it with a shortened but continuous *gag-mos* open reading frame (12). For reasons that are still unclear, splicing of the MuSV_{ts110} 4.0-kb viral RNA is thermosensitive; this processing event only occurs at growth tempera-

tures between 28 and 33°C (5, 11). Because the splicing event effectively regulates the production of the P85^{gag-mos} transforming protein, 6m2 cells appeared transformed if grown at 28 to 33°C and normal if grown at 39°C. In contrast, the basis for the thermosensitivity of the P85^{gag-mos} serine kinase activity is not known.

The major question we wished to address with regard to the MuSVts110 revertants was how the transformed state was maintained in these cells at both 33 and 39°C. Like the parental 6m2 cells, both revertants contained a 4-kb viral RNA. However, unlike 6m2 cells, no 3.5-kb RNA or P85^{gag-mos} could be found in these cells (5, 17). Neither could any wild-type 5.3-kb RNA nor P37^{mos} be found (5, 17). However, previous work had also suggested that the transformed state might be mediated by a 100-kDa polypeptide, P100^{gag-mos}, expressed at both 33 and 39°C (17). Additionally, it had been reported that P100^{gag-mos} was a serine kinase that, like wild-type P37^{mos} and unlike P85^{gag-mos}, was stable at 39°C (8). It is not clear why there are differing degrees of heat stability among the *mos* gene kinases. P37^{mos} and P100^{gag-mos} are heat-stable serine kinases. P85^{gag-mos}, however, is rapidly inactivated at 39°C. Conformational changes may explain the differences in the enzymatic activity of the proteins; P85^{gag-mos} is lacking the N-terminal 20 amino acids of P37^{mos} and has acquired about 58-kDa of the *gag* precursor polypeptide. However, despite the heat stability of its kinase activity, P100^{gag-mos} more resembles P85^{gag-mos} than P37^{mos} in structure because it contains an additional 129 amino acids of the *gag* precursor and 14 amino acids from an alternate *mos* gene reading frame. These extra residues are situated between the *gag* and *mos* domains in P85^{gag-mos}.

The observations discussed above provided a superficial explanation for the temperature-independent transformed state displayed by these revertant cells, but failed to define how the revertant P100^{gag-mos} is actually expressed. In addressing this question, it was clear from the outset that the revertant 4.0-kb viral RNA which presumably codes for P100^{gag-mos} must differ from parental 6m2 4.0-kb RNA in at least two ways. First, some sort of frameshift must have

FIG. 10. Blot hybridization of 6m2, 54-5A4, and 204-3 viral DNA. Cellular DNA from 6m2, 54-5A4, and 204-3 cells was digested with various restriction enzymes, analyzed by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to a *mos* gene-specific probe. (A) 6m2 DNA; (B) 54-5A4 DNA; (C) 204-3 DNA. In each panel, the DNA was digested with Lanes: 1, *Bam*HI; 2, *Bgl*I; 3, *Hind*III; 4, *Bgl*II. Numbered bands represent MuSVts110 DNA fragments plus flanking sequences.

occurred to generate a continuous *gag-mos* reading frame. Second, either this frameshift or a second mutation that did not affect the reading frame must have occurred to destroy the capacity of the 4.0-kb RNA to be processed. The results presented in this study satisfy both conditions, since the 5-base deletion found at the revertant 3' splice site produces both of the above effects. The resulting revertant viral RNA cannot be spliced in the same way as the parental 6m2 4.0-kb RNA but does present a continuous open *gag-mos* reading frame capable of supporting the translation of a 100-kDa *gag-mos* serine kinase that can transform cells in culture.

Based on these results and on results previously published by us and others, a model for *v-mos* gene expression in MuSVts110-infected cells can be formulated (Fig. 9). In wild-type MuSV-infected cells, the viral RNA is transcribed as a 5.3-kb RNA that is not known to be spliced (18). Although the *env* gene 3' splice site is present in the 5.3-kb RNA, it is altered by a single nucleotide change and, as a consequence, is inactive (4). Translation of the wild-type MuSV 5.3-kb RNA is known to proceed from two initiation codons, one located at nucleotide 1,042 and the other at nucleotide 3,875 (18). The 63-kDa precursor to the viral core proteins, Pr63^{gag}, is translated from the reading frame preceded by the initiation codon at nucleotide 1,029, whereas the 37-kDa transforming protein, P37^{mos}, is translated from the initiation codon found at nucleotide 3,875. A third initiation codon followed by an open reading frame is found at nucleotide 2,970 in MuSV-124 RNA (15); however, it is unclear whether this reading frame is translated in infected cells. In addition, there are several initiation codons found within the *mos* gene reading frame that can be translated in vitro (2, 9, 13, 18). Whether these initiation sites are used in vivo in infected cells is not known.

In contrast to the wild-type MuSV RNA, the MuSVts110 4.0-kb genomic RNA, which was formed as a result of a

FIG. 9. Structural basis for viral *gag* and *mos* gene expression in wild-type MuSV-349, MuSVts110 precursor and spliced RNAs, and MuSVts110 revertant RNAs.

1,488-base frameshift deletion between six base direct repeats (A-A-C-G-C-C) in the viral *gag* gene and the immediate 5' end of the *mos* gene, can only be translated from the *gag* gene initiation codon into a truncated 58-kDa *gag* precursor containing 14 C-terminal amino acids derived from an alternate *mos* reading frame (12). No P37^{mos} can be formed from this RNA because the initiation codon at wild-type nucleotide 3,875 was lost as part of the original 1,488-base deletion (12). If the MuSVts110 4.0-kb RNA is translated in vitro, however, the internal *mos* gene initiation codons have been shown to be active (10). In a novel event that occurs only at growth temperatures of 33°C and lower, a 431-base intron between in-frame, previously cryptic, splice donor and acceptor sites can be excised from the MuSVts110 4.0-kb RNA. The resultant 3.5-kb RNA contains the *gag* and *mos* genes joined in frame, permitting the synthesis of the novel transforming protein P85^{gag-mos}.

A third route resulting in the transformation of cells in culture has been taken by revertants of MuSVts110-infected cells. In these cells neither the *mos* gene initiation codon nor an intact *mos* gene 3' splice acceptor site is available. However, the formation of a continuous *gag-mos* open reading frame as a consequence of the 5-base deletion at the splice acceptor site allows the synthesis of a third transforming protein, P100^{gag-mos}.

Despite varying contributions to each from the viral *gag* and *mos* genes, P37^{mos}, P85^{gag-mos}, and P100^{gag-mos} seem to have similar functions in that all are serine kinases which can transform cells in culture (7, 8). The kinase activity of P85^{gag-mos} is temperature labile, whereas the activities of P37^{mos} and P100^{gag-mos} are heat stable. Additionally, P85^{gag-mos} and P100^{gag-mos} seem to lack the toxicity associated with high-level expression of P37^{mos} in MuSV-infected cells (14).

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