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Construction and Analysis of Deletion Mutations in the U5 Region of Moloney Murine Leukemia Virus: Effects on RNA Packaging and Reverse Transcription

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A collection of deletion mutations was generated in the U5 region of cloned DNA copies of Moloney murine leukemia virus or a related retrovirus. Cell lines expressing the mutant DNAs were generated by cotransformation, and the virions released were characterized biochemically. Deletions in the 5′ part of U5 profoundly reduced packaging of the viral RNA into virions; one deletion in the 3′ part of U5 did not block packaging but affected reverse transcription. One mutant with a deletion in the central part of U5 was fully viable and served to separate the two functional parts of U5.

The linear proviral DNA of retroviruses contains repeated sequence blocks termed long terminal repeats (LTRs) arranged in direct orientation at the two termini. The LTRs are each divided into three regions termed U3, R, and U5 on the basis of their appearance in the viral RNA: the R region is repeated at both ends of the RNA, U3 is unique and present only near the 3′ end of the RNA, and U5 is unique and present only near the 5′ end of the RNA (52). In most retroviruses these sequences do not code for proteins but are the *cis*-acting sites of recognition by proteins responsible for mediating several phases of the life cycle. The U3 region, for example, contains the potent transcriptional promoter responsible for the formation of viral RNAs from the integrated provirus (24); the R region is used by reverse transcriptase to mediate the translocation or jump of a DNA intermediate during viral DNA synthesis (13, 26). Our understanding of the functions mapping to the U5 region is particularly incomplete.

In addition to the *cis*-acting regions within the LTRs, there are other noncoding regions also needed in *cis* for replication that lie within the viral genome between the two LTRs. A large sequence block near the 5′ end of the Moloney murine leukemia virus (M-MuLV) RNA, termed the ψ region, has been defined by a deletion mutation (28). This region is necessary for selective encapsidation of viral RNA into assembling virion particles; a mutant proviral genome lacking this region can direct the synthesis of all of the proteins needed for virion formation but fails to package its mutant RNA and thus releases empty particles. A spontaneous mutant in the avian virus system with a related but more complicated phenotype (25) also has a deletion in this region (43). Such mutants have found wide application as helper viruses for the transfer of retroviral vector constructs to recipient cells without concomitant transfer of the helper genome (10, 28, 31, 45, 51). The ψ region could be reintroduced at downstream locations into a retroviral construct lacking the natural ψ site and could still, at least partially, direct the selective encapsidation of the viral RNA (27). But evidence has accumulated that other parts of the viral genome harbor sequences necessary or stimulatory for packaging. The presence of the M-MuLV ψ region alone in a heterologous nonviral RNA is poor at targeting the RNA to virions (3), and portions of the *gag* gene have been shown to enhance packaging of viral vector RNAs dramatically (2, 3); addition of the *gag* region can even make the ψ region sufficient for targeting (1). Similarly, analysis of avian virus mutants has revealed at least three sites that are involved in packaging (20, 21, 23, 35, 44). In the spleen necrosis virus system, it has been observed that the *env* mRNA, which lacks the major packaging region (50), is still packaged at much higher efficiency than nonviral RNAs, indicating that weakly functional signals are present on the *env* mRNA (12). Some of these auxiliary sequences might lie in the R or U5 sequences present at the 5′ end of all viral RNAs.

The U5 region of M-MuLV is 77 base pairs (bp) long; its sequence is highly conserved among all murine and even feline and simian retroviruses (52). A portion of U5 has even been noted as similar to a sequence in the corresponding region of the distantly related hepatitis B virus (32). The bases at the 3′ edge of U5 and forming the 3′ end of the whole proviral DNA are the bases joined to cell DNA during proviral integration. The 13 bp at this edge constitute a short perfect inverted repeat sequence with the corresponding bases at the 5′ edge of U3 and of the provirus. Previous studies have shown that at least some of this sequence is essential for efficient integration of the provirus; deletion of 2 or 8 bp at the 3′ edge of U5 blocked replication and formation of integrated proviruses (6). The full extent of the recognition site for the integration reaction in M-MuLV has not been determined. In most other retroviruses, including the avian viruses, the length of the inverted repeat is much shorter and there sequences of U5 upstream of the inverted repeat bases have been shown to be essential for integration (4, 11, 34). The function of the rest of U5, especially the 5′ part of the region, is unknown.

To determine the roles of U5 in replication of mammalian retroviruses, we generated a series of overlapping deletions which spanned the entire U5 region. Analysis of the viruses produced by these mutant DNAs showed a total of three distinct phenotypes. Some of these new mutants are defective in encapsidation of viral RNA, while one is defective in reverse transcription. These mutants can be added to previously described mutants defective in proviral integration (6,

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9), extending the known functions mapping to this small part of the genome.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3, Rat-2 (48), and XC cells were maintained in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum (GIBCO Laboratories) and 100 μg of gentamicin sulfate (Sigma Chemical Co.) per ml. Wild-type M-MuLV was harvested from clone 4 producer cells (38) or NIH 3T3 or Rat-2 cells after transformation with viral DNA. Recombinant virus *dl*587rev was as previously described (7). Infections were performed in the presence of 8 μg of Polybrene per ml at 37°C for 2 h.

Transformation of mammalian cells with cloned DNA. Transient transfections of NIH 3T3 and Rat-2 cells with viral DNAs to test for replication-competent virus were performed by the DEAE-dextran method (30); viable virus was measured by the XC plaque assay (37) and by determination of the levels of reverse transcriptase activity released into the medium (14). Stable cotransformation of cells by a mixture of pSV2neo and viral DNAs was by the calcium phosphate method (15, 53). Recipient cells were selected in medium containing 400 μg of G418 (GIBCO) per ml.

Cloned DNAs. Plasmid pNCA consists of a full-length nonpermuted copy of the M-MuLV proviral DNA in a pBR322-based vector (9). pNBRN was constructed by replacing the 2.2-kilobase *Cla*I-*Xho*I fragment of pNCA with the corresponding fragment of p*dl*587rev, a fully infectious, permuted copy of the recombinant viral genome *dl*587rev cloned in pBR322 (8). This virus is a recombinant between M-MuLV and closely related mouse endogenous retroviral sequences (7). Plasmid pSV2neo (46) encodes G418 resistance, and pTZ18R (Pharmacia) is a vector containing a polylinker cloning site.

Enzymatic manipulations. Two sets of nested deletions were constructed, one in each of two parental DNAs. One set was generated with plasmid pNBRN as the parent to take advantage of an *Ava*I site located at base 578, close to the 3′ edge of U5, in the recombinant virus *dl*587rev. To make the deletions, a 1.5-kilobase *Kpn*I-*Xho*I fragment was subcloned from pNBRN into vector pTZ18R. DNA of the resulting plasmid, pKX1, was linearized by partial digestion with *Ava*I (New England BioLabs, Inc.), and the full-length linear DNA was treated with BAL 31 nuclease (International Biotechnologies, Inc.; mixed species) for 30, 60, or 90 s at 30°C. The DNA was circularized with T4 DNA ligase and used to transform *Escherichia coli* HB101. DNAs from clones (18) were screened for loss of the appropriate *Ava*I site. A *Kpn*I-*Mst*II fragment containing each deletion was excised from selected clones and used to replace the corresponding fragment in a subclone of the 5′ end of M-MuLV; a 2.2-kilobase *Eco*RI-*Xho*I fragment from these subclones was then used to replace the corresponding fragment of pNCA. The resulting plasmids contain the *Ava*I site deletions in the U5 region of the 5′ LTR of *dl*587rev. During viral replication, this copy of U5 is transcribed into RNA and transmitted to progeny.

The second set of deletions was generated by using the *Sty*I site at position 541 relative to the 5′ end of the proviral DNA in the U5 region of wild-type M-MuLV. A 1-kilobase *Aat*II fragment containing U5 was excised from pNCA and subcloned into the *Aat*II site of pBR322. The resulting plasmid, pNCA-T, was linearized by partial digestion with *Sty*I, purified, treated with BAL 31 for 20, 40, or 90 s at 30°C, circularized, and used to transform *E. coli* HB101. Fragments from selected clones were then used to replace the corresponding fragment of pNCA.

The sizes and positions of the deletions were determined by DNA sequence analysis (29).

Analysis of unintegrated viral DNA. Rat-2 cells were infected with undiluted virus preparations, and after 28 h the low-molecular-weight DNA was harvested (17), separated by agarose gel electrophoresis, blotted to nitrocellulose, and analyzed by hybridization (49) with nick-translated viral DNA.

Analysis of virion RNA. Virus supernatants were collected from subconfluent producer cell lines at 2-h intervals. Pooled collections (40 ml) were filtered (Nalgene; 0.45-μm pore size), and the virions were pelleted by centrifugation (23,000 rpm; 3 h; SW27 rotor) and suspended in RNA lysis buffer (0.2 ml; 10 mM Tris hydrochloride [pH 7.4], 100 mM NaCl, 5 mM EDTA, 0.5% sodium dodecyl sulfate, 100 μg of carrier RNA per ml). The RNA was treated with proteinase K (50 μg/ml; Boehringer Mannheim Biochemicals) for 20 min at 37°C, extracted sequentially with phenol, phenol-chloroform (1:1), and chloroform, and collected by ethanol precipitation. For slot blot analysis, the RNA from 2.5 ml of medium was denatured by heat treatment at 68°C for 10 min in buffer (50% formamide, 6% formaldehyde, 1× SSC [0.15 M NaCl plus 0.015 M sodium citrate]), diluted 10- to 100-fold into 20× SSC, and loaded onto nitrocellulose with a slot blotting manifold (Schleicher & Schuell, Inc.). The filter was baked (80°C, 2 h), hybridized (49) with labeled viral DNA, and exposed to X-ray film. To score for RNA dimer formation, the native RNA from 10 ml of medium was separated by electrophoresis (30 V, 1 h) on a 1% agarose gel in buffer (20 mM MOPS [morpholinepropanesulfonic acid; pH 7.0], 5 mM sodium acetate, 1 mM EDTA) and transferred to Zeta-probe paper (Bio-Rad Laboratories) by electroblotting in 10 mM NaOH at 200 mA for 16 h at room temperature (22). The viral RNAs were visualized by hybridization with a nick-translated probe (pNCA) followed by autoradiography.

Analysis of endogenous reverse transcription reactions. Virus was collected from subconfluent producer cell lines at 12- to 15-h intervals and clarified and pelleted as for RNA analysis. The pelleted virus was suspended in 0.005 volume of TNE buffer (100 mM Tris hydrochloride [pH 8.3], 100 mM NaCl, 1 mM EDTA). Reaction mixtures for minus-strand strong-stop DNA were as previously described (16). The reaction products were treated with RNase (10 μg/ml, 30 min, 37°C), precipitated, suspended in 80% formamide, heated (95°C, 2 min), separated by electrophoresis on 10% polyacrylamide in TBE, and visualized by autoradiography. To determine whether a primer tRNA was present and could serve as a primer for added deoxynucleotides, a tRNA tagging assay was used (47). Virus collected as described above (25 μl) was incubated (37°C for 5 min) in a cocktail (50 mM Tris hydrochloride [pH 8.3], 50 mM NaCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.01% Nonidet P-40) containing final concentrations of 2.5 μM each [α-^{32}P]dATP, [α-^{32}P]dGTP, and [α-^{32}P]dTTP (400 Ci/mmol) and 100 μM ddCTP. The reaction was stopped by addition of EDTA to a final concentration of 10 mM and 10 μg of carrier RNA. The products were diluted to 200 μl with TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), extracted with phenol-chloroform (1:1), precipitated with ethanol, suspended in 80% formamide, heated (95°C for 2 min), and analyzed by electrophoresis on a 10% polyacrylamide gel.

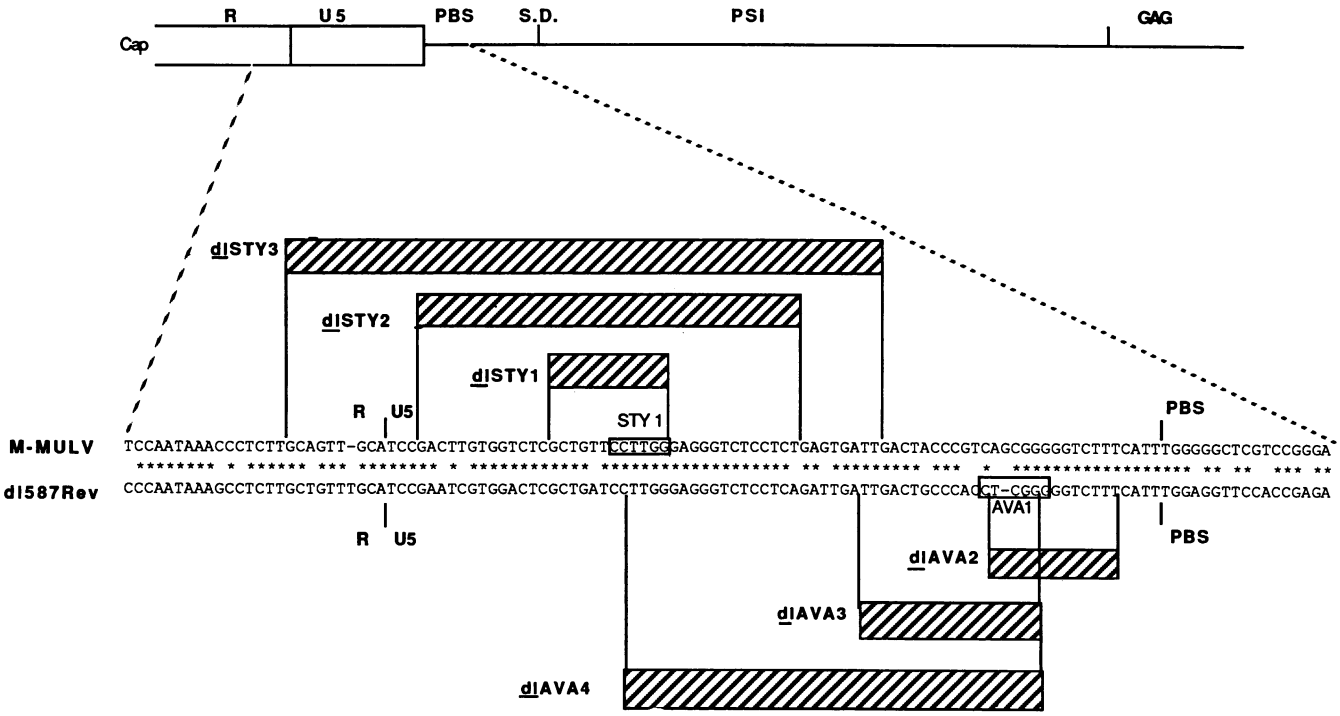

FIG. 1. Position and size of deletion mutations. At the top is a schematic drawing of the 5′ region of the M-MuLV genome. At the bottom is a blow-up of a portion of the R, U5, and primer-binding site (PBS) regions. The DNA sequences of M-MuLV and *dl*587 *rev* in this region are shown, aligned to maximize sequence matches; asterisks indicate sequence identity. The positions of the deletions in this study are indicated by hatched boxes. Those above the sequences were made in the M-MuLV parent, and those below the sequence were made in the *dl*587 *rev* parent. PSI, ψ region; S.D., splice donor.

RESULTS

Construction of deletion mutations in the U5 region. Two series of deletions were introduced into the U5 region of retroviral DNAs. In one series, small deletions were made in the 5′ part of U5 of wild-type M-MuLV by partial cleavage of a plasmid with *Sty*I, treatment of the linear DNA with BAL 31, and reclosure with ligase (see Materials and Methods). Cloned DNAs arising after transformation with the resulting DNA were screened for deletions of the appropriate size; the exact positions and sizes of the deletions were determined by DNA sequence analysis. Fragments from selected clones were then transferred into the U5 region of a complete DNA copy of the M-MuLV genome.

The bulk of the U5 region of wild-type M-MuLV contains few recognition sites for restriction enzymes. Accordingly, a second series of deletions was generated in the U5 region of a highly homologous recombinant retrovirus, *dl*587rev, which contains an *Ava*I site in the 3′ part of U5. A plasmid containing this region was cleaved with *Ava*I, treated with BAL 31 as before, and reclosed with ligase. Clones were screened, analyzed, and used to reconstruct complete viral genomes. The sizes and positions of the selected deletions are summarized in Fig. 1.

Test for viability of mutant proviral genomes: transient transformation assays. To test the deletion mutants for their ability to give rise to fully replication-competent virus, we introduced the mutant DNAs into NIH 3T3 cells by the DEAE-dextran procedure. The appearance of virus was monitored by the XC syncytial plaque assay and by measurement of reverse transcriptase activity in the supernatant culture medium. DNAs applied to cells in this way are not stably retained but are only transiently expressed, and thus the recovery of progeny virus requires efficient spread of replication-competent virus throughout the cultures.

Of the mutants tested, only *dl*Ava3 yielded progeny virus with normal kinetics; here the rate of appearance of reverse transcriptase and the number of XC syncytial plaques were indistinguishable from those of the wild-type DNA. All of the other mutant DNAs were defective and failed to yield virus at the usual time. We conclude that each of these deletions was profoundly deleterious to viral replication and by this assay potentially affected any step in the life cycle.

Prolonged maintenance of the recipient cell populations in some cases resulted in eventual recovery of virus. Mutants *dl*Sty1 and *dl*Sty2 yielded virus after a delay of 5 to 10 days, while mutants *dl*Ava2 and *dl*Ava4 gave virus after longer delays of 10 to 20 days. Mutant *dl*Sty3, containing the largest deletion, never gave rise to virus, as judged by either the reverse transcriptase or the XC plaque assay. There were two possible explanations for the delayed appearance of virus. One explanation was that these mutants were only partially defective and proceeded slowly through the replication cycle. The second explanation was that the mutants were essentially fully defective but gave rise to replication-competent virus, after a delay, by reversion or recombination with endogenous sequences in the host cell. Similar reversion events in other mutant viruses under other conditions have been well documented (7, 36, 40). To distinguish between these possibilities, the virus recovered from the recipient cells was harvested and applied to fresh NIH 3T3 cells. If the original mutants were inherently slow, the recovered virus should also be slow; if reversion events had occurred, the recovered virus should be normal. Analysis of the kinetics of replication showed that the recovered virus

was indistinguishable from the wild type, suggesting that reversion events had occurred. Analysis of the structure of one recovered virus (see below) supported this notion. We conclude that the original deletions are indeed profoundly defective and that the delayed appearance of the virus recovered can be attributed to reversion.

Transient transformation of Rat-2 cells with mutant DNAs. The tendency of retroviral mutants to revert by recombination with endogenous sequences can be precluded by utilizing as hosts not mouse but rat cell lines. To test the viability of the mutant DNAs without the complication of reversion, each DNA was introduced into Rat-2 cells by DEAE-dextran as before, and the appearance of progeny was monitored by reverse transcriptase assays. Mutant *dl*Ava3 was indistinguishable from the wild type in rat cells, as it was in mouse cells. Mutants *dl*Ava2, *dl*Ava4, *dl*Sty1, *dl*Sty2, and *dl*Sty3 showed no evidence of virus production even after 4 weeks of passage; in the absence of recombinational repair, these mutants are inviable.

To prove that the viable virus recovered from NIH 3T3 cells could have been detected if it had arisen in rat cells, we exposed fresh Rat-2 cells to the four active viral supernatants harvested from NIH 3T3 cells. These infected cultures yielded progeny virus quickly and at high levels, with kinetics indistinguishable from those of M-MuLV. Thus, the revertants from NIH 3T3 cells were not host range mutants; the failure to recover such revertants in Rat-2 cells must reflect the limited repertoire of homologous sequences in the cell or limited recombinational machinery.

Isolation of stable producer cell lines. To characterize in more detail the defects associated with the deletion mutations, cell lines stably expressing each mutant genome were generated. Each mutant DNA was mixed with pSV2neo DNA and applied to Rat-2 cells, and recipient clones were selected in G418. Analysis of the supernatants from these cultures revealed the presence of substantial virion-associated reverse transcriptase activity. Cloned cell lines carrying each mutant were prepared for further analysis, with screening of individual clones for continued release of virions. The level of reverse transcriptase activity released by these clones ranged from 2- to 10-fold less than that released by wild-type-infected cells. Subsequent analysis of intracellular virion proteins and cellular RNA confirmed the enzyme assays by showing good expression. This level of production is typical for retroviral genomes introduced into cells by transfection as opposed to infection (19, 39).

Supernatants collected from the producer cells were then applied without dilution to fresh Rat-2 cells, and production of progeny virus was tested by reverse transcriptase assays of the supernatants. The wild-type virus induced progeny release within 24 h. Mutant *dl*Ava3 induced formation of progeny with identical kinetics. All of the other mutants were completely defective and did not induce progeny virus, even when the recipient cells were maintained for 3 weeks. These results indicate that although all of the mutant DNAs were able to produce high levels of viral particles, the particles assembled by most of the mutants were uninfectious.

Reverse transcription after infection by mutant virus: analysis of viral DNA synthesis. To determine whether the defective mutants were able to perform reverse transcription, viral supernatants were collected from selected cloned producer cell lines and applied without dilution to fresh Rat-2 cells. After 24 h, the low-molecular-weight DNA was harvested by the Hirt procedure (17) and the viral DNAs were analyzed by electrophoresis followed by blot hybridization.

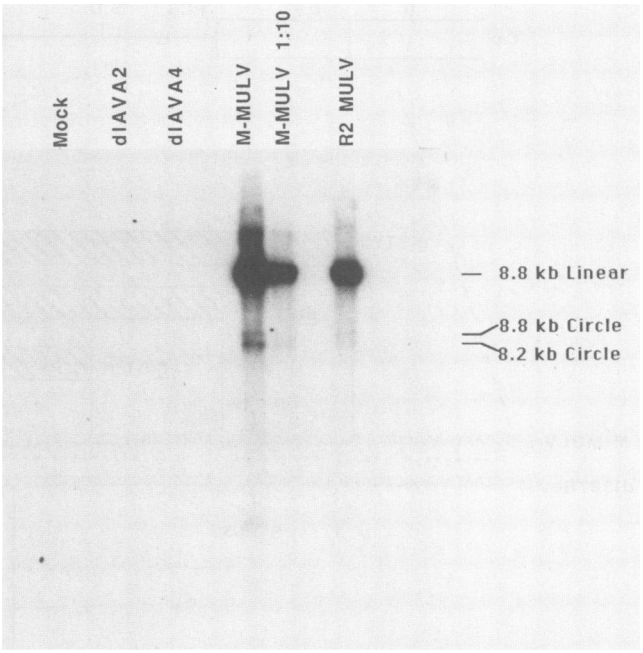

FIG. 2. Blot analysis of unintegrated viral DNAs synthesized soon after infection of Rat-2 cells. Virus supernatants from the indicated producer cells were collected and used for infection without dilution or at a 1:10 dilution. The DNA was harvested after 28 h, subjected to gel electrophoresis, and blotted, and viral sequences were detected by hybridization with labeled probes. Lanes: Mock, infection with harvests from nonproducer Rat-2 cells; *dl*AVA2 and *dl*AVA4, infection with harvests from producer Rat-2 cells; M-MULV and M-MULV 1:10, infection with harvests from wild-type clone 4 producer 3T3 cells; R2 MULV, infection with harvests from wild-type M-MuLV-infected Rat-2 cells. kb, Kilobase.

Mutants *dl*Ava2, *dl*Ava4, and *dl*Sty1 were unable to induce the synthesis of any detectable viral DNAs. Although these mutants produced only 2- to 10-fold fewer viral particles than did the wild-type virus, we estimate that they made at most 1,000-fold less viral DNA (Fig. 2). Thus, the deletions in U5 apparently blocked some stage before completion of reverse transcription. Mutant *dl*Ava3, as expected, directed the synthesis of normal levels of all of the viral DNAs (data not shown).

Packaging of genomic RNA into mutant particles: analysis of virion RNA. One possible explanation for the failure of the mutant virions to direct the synthesis of DNA was a failure of the particles to contain normal levels of virion RNA. To determine whether the deletions did affect RNA packaging, we directly measured the level of viral RNA in particles. Virions were collected from each mutant producer and purified, the RNA was extracted and loaded onto nitrocellulose, and viral sequences were measured by hybridization (slot blot assay). In all cases, the amount of RNA loaded was normalized to the virion yield as measured by reverse transcriptase assays. All of the *dl* Sty mutants, as well as *dl*Ava4, showed profound defects in packaging viral RNA (Fig. 3; Table 1). These mutants packaged 50- to 100-fold less viral RNA per virion particle, than did the wild-type virus. Mutant *dl*Sty1 showed the least pronounced defect in packaging but clearly assembled less RNA than did the wild type. To compare this behavior with that of the classical ψ^- mutants, we harvested virus from a line producing *dl*DEC6.

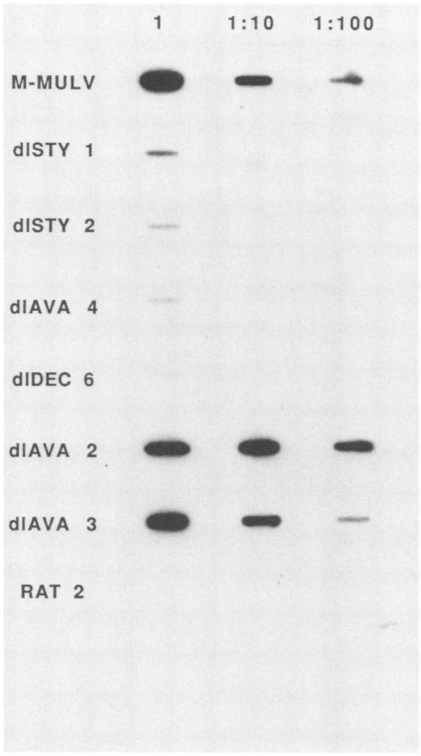

FIG. 3. Slot blot analysis of viral RNA packaged into virion particles. Virions were collected from Rat-2 cells expressing the indicated mutants, and the virion RNA was purified, loaded onto filters, and detected by hybridization. RNA extracted from an equal number of virion particles, measured by reverse transcriptase assays, was loaded in lane 1. A 1:10 or a 1:100 dilution of each sample was loaded in the indicated lane.

This mutant contains a 135-bp deletion extending from a *Bal*I site (map position 663) to an *Xma*III site (position 798) and is widely used to generate helper-free stocks of retroviral vectors (33; map positions are relative to the 5′ edge of the 5′ LTR of full-length linear proviral DNA). These mutant particles, normalized to reverse transcriptase activity, contained about 200-fold less viral RNA than the wild type. We conclude that many of the small deletions in U5 were nearly as disruptive of normal packaging of virion RNA as deletions in the major packaging region.

Mutant *dl*Ava3, the replication-competent mutant, generated virions with normal levels of genomic RNA, in accord with its viability (Table 1). The deletion in this mutant serves to define the 3′ edge of the sequences in U5 that are required for packaging and localizes the important region to the 5′ half of U5. Mutant *dl*Ava2, lacking only more 3′ sequences, was not significantly defective in RNA packaging. When normalized to the virion yield, this mutant displayed at most a two- to fivefold reduction in packaged RNA. This result suggests that the U5 sequences involved in packaging are restricted to the 5′ half of U5.

The viral RNAs in wild-type virions are contained as dimers condensed into a tightly folded structure sedimenting at 70S. To characterize further the structure of the RNAs in the mutant virions that did package RNA, we collected virus at short intervals and extracted the native RNAs (see Materials and Methods). The RNAs were then separated on a nondenaturing agarose gel, transferred to Zeta-probe paper, and analyzed by hybridization with a viral DNA probe. Packaging-defective mutant *dl*Sty2 did not contain sufficient RNA for detection in this assay. Mutant *dl*Ava2 RNA was readily detected and migrated as a high-molecular-weight species with the same mobility as the wild-type 70S viral RNA (Fig. 4). We conclude that this mutant was able to dimerize and efficiently package its genomic RNA and that the failure of this mutant to form viral DNAs must reflect defects in DNA synthesis itself.

Reverse transcription in isolated virions: analysis of minus-strand strong-stop DNA. To determine whether any of the mutants could properly initiate the process of viral DNA synthesis, endogenous reverse transcription reactions were performed with purified virions, and the labeled DNA products were analyzed by polyacrylamide gel electrophoresis (see Materials and Methods). This reaction measures the ability of the viral reverse transcriptase to elongate the tRNA primer and copy to the 5′ end of the viral genome, yielding the minus-strand strong-stop DNA species, normally 145 nucleotides long.

All of the virus harvested from Rat-2 producer cells revealed the synthesis of low levels of a DNA larger than the wild-type M-MuLV strong-stop DNA. The identity of this DNA is uncertain, but its correlation with the rat host cell and its constant presence whether the MuLV RNA was packaged or not suggest that it may derive from reverse transcription of packaged rat VL30 RNAs. These RNAs, derived from endogenous retroviruslike elements, are abundantly expressed in rat cells (41, 42) and efficiently packaged into murine leukemia virus virions. Hybridization of virion RNA with a rat VL30 probe confirmed the presence of a high level of VL30 RNA in the mutant particles (C. Meric,

TABLE 1. Phenotypes of deletion mutants

Mutant	Map position of deletion	Size (bp)	Region	Viability	Viral DNA[a]	Virion RNA[b]
Wild type				+	1.0	1.0
*dl*Sty1	534–546	12	5′ half	−	N.D.	0.04
*dl*Sty2	523–561	38	5′ half	−	N.D.	0.02
*dl*Sty3	509–567	58	5′ half	−	N.D.	0.01
*dl*Ava2	578–590	12	3′ half	−	<0.01	0.2–0.5
*dl*Ava3	566–583	17	3′ half	+	1.0	1.0
*dl*Ava4	542–582	40	3′ half	−	<0.01	0.02
*dl*DEC6	663–798	135	S.D.-*gag*[c]	−	N.D.	<0.01

[a] Viral DNA synthesized after infection, corrected for the level of applied reverse transcriptase activity. Entries are relative to the wild-type level, defined as 1.00. N.D., Not determined.

[b] Viral RNA released into virion particles, corrected for the virus yield as measured by reverse transcriptase assay. Entries are relative to the wild-type level, defined as 1.00.

[c] S.D.-*gag*, Splice donor to *gag* gene.

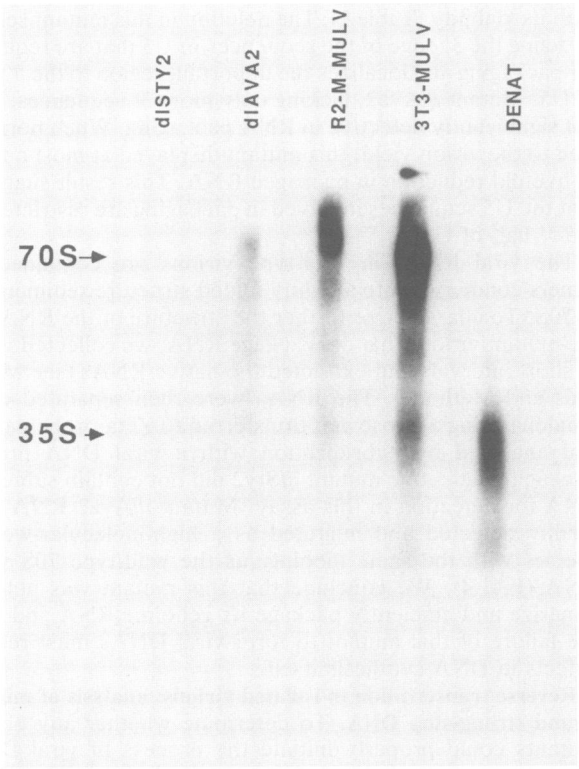

FIG. 4. Nondenaturing Northern (RNA) gel analysis of virion RNA. Virion particles were collected from Rat-2 cells releasing *dl*Sty2 and *dl*Ava2 mutant viruses, Rat-2 cells releasing wild-type M-MuLV, or NIH 3T3 cells releasing M-MuLV. RNAs were extracted and analyzed without denaturation by agarose gel electrophoresis, blotting, and hybridization. The RNA in the lane labeled DENAT was boiled for 1 min before electrophoresis.

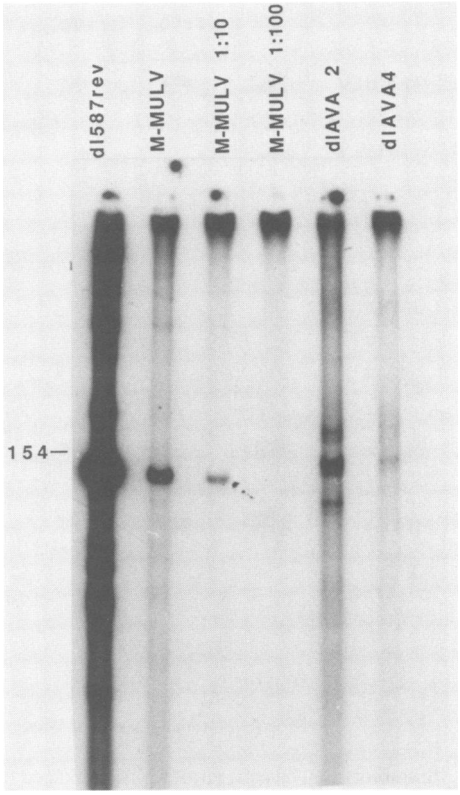

FIG. 5. Minus-strand strong-stop DNAs synthesized by mutant virions. Virions of the indicated mutants were harvested and used in endogenous reverse transcription reactions, and the DNA products were analyzed by acrylamide gel electrophoresis. The position of migration of a 154-nucleotide marker DNA is indicated. Whereas the wild-type and *dl*587rev virions synthesized high levels of a normal minus-strand strong-stop DNA (145 nucleotides), *dl*Ava2 made a mixture of its own strong-stop DNA and the putative VL30 strong-stop DNA, and *dl*Ava4 made only the larger rat VL30 DNA.

unpublished data). The presence of this species could be used as an internal control for the recovery of virions and reaction products.

The results of the strong-stop DNA analysis of the mutants supported the earlier findings. The packaging-defective mutants *dl*Ava4 (Fig. 5), *dl*Sty1, and *dl*Sty2 showed no detectable synthesis of M-MuLV strong-stop DNA, consistent with the absence of RNA. Viable mutant *dl*Ava3 produced normal levels of a strong-stop DNA which migrated more rapidly than the wild-type species, indicating that the deletion had been maintained without recombinational repair. Analysis of the revertant virus harvested after transfection of mutant *dl*Sty1 revealed the formation of normal levels of full-length strong-stop DNA, confirming that recombinational repair of the deletion had occurred (data not shown).

Analysis of mutant *dl*Ava2, which packaged RNA but failed to make DNA, gave quantitatively variable results. Only very low levels of a strong-stop DNA could be detected; the mobility of the species was slightly faster than that of the wild type, consistent with the presence of the 12-bp deletion (Fig. 5). The level of this DNA detected was variable from preparation to preparation, even when comparable levels of virions, as judged from reverse transcriptase activity, were assayed. To confirm that the tRNA was present in these virions and capable of initiating reverse transcription, we performed a tRNA tagging assay (47) in which endogenous DNA synthesis reactions were performed in the presence of ddCTP in place of dCTP. In this procedure, synthesis results in the addition of only 11 nucleotides to the primer tRNA in *dl*Ava2 before termination by incorporation of the dideoxynucleotide occurs. This analysis showed that *dl*Ava2 virions contained the tRNA and were able to prime synthesis at levels consistent with the level of packaged genomic RNA (data not shown). Although the level of priming observed was thus less per virion than for the wild type, it was not as low as the level of completed viral DNAs made in vivo as measured by Southern analysis of the low-molecular-weight DNA. We surmise that for this mutant later steps in reverse transcription are also affected and that the total reduction in DNA yield represents the accumulated defects in all steps of synthesis.

DISCUSSION

The mutants with deletions in the U5 region that we have described here can be divided into three classes based on phenotypes. Mutants with lesions in the 5′ part of U5 are defective in packaging of the viral RNA into virions, a single mutant in the central part of U5 is fully viable, and a mutant altered in the 3′ part of U5 is defective in reverse transcription both in vivo and in vitro. To these we should add the mutants described previously (6, 9), with deletions at the very 3′ edge of U5, which are defective in formation of the integrated provirus. These diverse phenotypes show that the

functions of the U5 region are many and complex. It is likely that all of these mutations are *cis* acting; in all of the mutants tested, the formation of viral proteins and the assembly of virion particles are not significantly affected. Although specific nucleotide sequences may be involved in some of these processes, it may also be that the overall three-dimensional structure of the RNA encoded by the region is most important for RNA packaging and reverse transcription. We imagine that the folded RNA could be recognized at many steps of the life cycle, including RNA dimer formation, RNA packaging, and reverse transcription.

Deletions in U5 affect RNA packaging. The major defect associated with deletions in the 5′ half of U5 is in the packaging of the viral RNA into virion particles. These mutations define a new, previously unrecognized part of the murine leukemia virus genome that seems to be important in this process. While the precise boundaries of the region are not known, analysis of the mutants described here and other mutants allows some definition of its extent. Deletions in the 5′ half of the R region, including a 30-nucleotide deletion from the 5′ cap to the *Sma*I site in R, did not abolish packaging (26). Thus, the 5′ boundary of this packaging region is no further upstream than the *Sma*I site in R. Similarly, mutants *dl*Ava2 and *dl*Ava3 show no profound defect in packaging and suggest that the 3′ boundary is no further downstream than the middle of U5, at map position 566. The deletions in this 5′ part of the U5 region have a substantial effect on packaging, reducing the level of viral RNA per virions about 100- to 200-fold below that of the wild type.

The 5′ half of U5 joins several other regions of the M-MuLV genome that are known to be involved in RNA packaging. The most important region, the ψ region, lies between the primer-binding site and the start of the *gag* gene (28). The boundaries and extent of this region are also not fully defined, but some earlier mutants (38) and new ones (S. T. Kalnik and S. P. Goff, unpublished data) suggest that only the 5′ half of the region is essential. Deletions in this section have the most profound effects on packaging of any single mutation, reducing viral RNA content 300- to 1,000-fold below that of the wild type. The ψ region is essential but not completely sufficient on its own for packaging (1); incorporation of this region into a fully heterologous mRNA did not efficiently direct the selective encapsidation of that hybrid RNA into virions (1, 3). Another region known to be important lies in the 5′ part of the *gag* gene (2, 3). This region is not absolutely necessary, since many retroviral vectors lacking this region still encode RNAs which are selectively packaged, and it is clearly not sufficient on its own, since the ψ region is always required. But the retention of the *gag* region in vectors has been shown to improve the efficiency of retroviral transmission of such vectors significantly (2, 3). Recent results show that the ψ and *gag* regions acting together in some vector constructs are sufficient; and in this context the U5 sequences are dispensible (1). In murine leukemia viruses, the U5 region and the *gag* packaging regions are thus quite similar functionally: not absolutely required in all contexts but necessary in some contexts. It might be proposed that they act as auxiliary sequences supporting the primary packaging function of the ψ region. The ψ region of the related Moloney murine sarcoma virus, curiously, is apparently sufficient on its own to direct specific packaging of heterologous RNAs (1).

The large number and size of the regions involved in packaging present a major puzzle. How can such a large number of sequences be recognized by the proteins responsible for selective encapsidation of the RNA? It seems unlikely that the capsid proteins could directly recognize the primary sequence of three long separate domains. The diversity of primary sequences that exist in different virus strains (e.g., Akv, Friend murine leukemia virus, and feline leukemia virus), all of which can be packaged into murine leukemia virus virion coats, also suggests that structure rather than primary sequence is important. We suppose that the RNA might fold into structures, especially in forming dimers, such that various separate regions are base paired. One speculative model for the dimer linkage has in fact been proposed in which U5 is paired with a region 3′ to the LTR (52). In such dimers, secondary and tertiary structures may constitute the recognition sites. A surprising aspect of the structure, whatever it may be, is the flexibility in the position of at least one region, the ψ domain; it can be moved far toward the 3′ end of the RNA and still, at least partially, help direct packaging (27). Such flexibility suggests that the structures that are recognized must be tolerant of considerable loops of RNA between them.

The complexity of the packaging region also suggests that the effect of mutations on that structure must be viewed with caution. Thus, a given deletion may disrupt overall secondary and tertiary structures involving large and distant parts of the genome; that deletion may not define a region that is normally intimately involved in packaging. The U5 region, the *gag* region, and even much of the ψ region may individually not be directly involved in the wild-type virus in forming a structure recognized during packaging. Rather, we can say only that the deletions in these regions are capable of disrupting correct structures. The genuine need for the retention of these domains remains to be evaluated. Indeed, it seems that neither U5 nor *gag*, for example, is indispensible in various vector constructs (1–3). It is clear that the mutations, however, will be helpful in defining the indispensible regions by virtue of their disruptive effects on the structure.

Deletions in U5 affect reverse transcription. The recovery of one mutant, *dl*Ava2, with a defect in reverse transcription shows that the 3′ part of U5 may be important in the process. This region is the first part of the RNA to be copied, and the reverse transcriptase is normally poised at the 3′ hydroxyl group of the primer tRNA to copy into U5. It may even be that all of U5 is important here; mutations which affect packaging preclude a determination of whether reverse transcription could have occurred normally on that template. The 3′ U5 deletions could affect several aspects of DNA synthesis. Initiation of synthesis and minus-strand strong-stop DNA formation were only marginally affected, and the decrease may be fully attributable to the slightly reduced level of packaged viral RNA. The most likely subsequent events that could account for the reduced level of full-length duplex DNA include strong-stop DNA translocation or jumping or removal of the tRNA primer by RNase H. An effect on this last step is consistent with the phenotype associated with a 2-bp insertion mutation at the 3′ edge of U5, namely, a tendency to retain the tRNA and aberrantly copy it into retained cDNA (8).

The results with mutant *dl*Ava2 are somewhat similar to recent results in the avian retrovirus system (5). Mutations altering the 3′ part of U5 were found to have little effect on RNA packaging but to reduce DNA synthesis on the viral RNA; here the problem was found to lie specifically in the initiation of DNA synthesis on the primer tRNA. Furthermore, the defect was strongly linked by reversion analysis to alterations in a secondary structure formed between the 3′

part of U5 and sequences downstream of the primer-binding site. Mutant *dl*Ava2 is altered in a similar position; it seems to differ somewhat in being more defective in later steps of DNA synthesis.

The defective mutants with lesions in U5 all exhibited an extraordinarily rapid tendency to revert after introduction into mouse cells. We have observed reversion of many mutations by recombination with endogenous elements, but normally the detection of these events requires high-multiplicity infection of cells by a mutant virus stock and a very long latency period (7, 36, 40). In our experience, most defective mutants have not reverted after simple transient transformation of mouse cells. The rapid reversion seen here is probably a consequence of the special position of U5 and the timing of its replication. If an endogenous viral RNA were copackaged with the mutant, the deletions could be efficiently repaired by a *trans* jump of the strong-stop DNA (and its U5 region) from the endogenous RNA to the 3′ end of the mutant RNA.

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