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Retroviral insertions into a herpesvirus are clustered at the junctions of the short repeat and short unique sequences

(Marek disease virus/reticuloendotheliosis virus/long terminal repeat/insertional mutagenesis/retroviral integration)

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ABSTRACT **We previously described the integration of a nonacute retrovirus, reticuloendotheliosis virus (REV), into the genome of a herpesvirus, Marek disease virus (MDV), following both long-term and short-term coinfection in cultured fibroblasts. The long-term coinfection occurred in the course of attenuating the oncogenicity of the JM strain of MDV and was sustained for >100 passages. The short-term coinfection, which spanned only 16 passages, was designed to recreate the insertion phenomenon under controlled conditions. We found that REV integrations into MDV were common and could occur within the first passage following coinfection. Now we have mapped the integration sites. After 5–16 passages *in vitro*, 17 out of 19 REV junction sites are clustered in two 1-kilobase regions at the junctions of the short unique and short repeat region of MDV. In the long-term cocultivation experiment, 6 out of 10 insertions also mapped in this region. In both cases, integrated proviruses are unstable and undergo subsequent recombinative deletion, often leaving a solitary long terminal repeat. The long terminal repeat sequences are, however, stably maintained for many rounds of passaging *in vitro*. This clustering of insertions presumably is influenced by selection for viable and fast-growing viruses, and occurs in a region of the MDV genome which shows significant size heterogeneity in several strains.**

Marek disease virus (MDV) is an avian herpesvirus which causes malignant transformation of T cells in chickens. The genome structure of MDV is similar to that of Herpes simplex virus (HSV), with long and short unique regions (U_L and U_S) each bounded by inverted repeats (TR_L, IR_L, IR_S and TR_S). Although the gene organization is similar to that of HSV (1), novel open reading frames in both the U_S region and the two repeats have been reported (2, 3).

The disease phenotype of MDV can be altered by prolonged passage of the virus in fibroblast culture. With such long-term passage both the virulence and the oncogenicity of MDV are attenuated and changes in the genome are detected. Most studies have focused on the expansion of a repeat sequence in R_L (4–6). However, other, undefined structural changes have been noted in the R_S/U_S region as well (7, 8).

During prolonged passage of the JM strain of MDV, viruses were obtained with improved *in vitro* growth properties but no detectable replication in chickens (9). We have recently reported these high-passage JM viruses contain integrated retroviral sequences which resulted from accidental coinfection of the MDV-infected fibroblasts with reticuloendotheliosis virus (REV), a chicken retrovirus (10). Even though infectious retrovirus is no longer present in the culture, REV long terminal repeat (LTR) sequences have been stably maintained in these MDV viruses for >100 passages. These integrations are associated with large structural changes in the MDV genome in both the U_S and R_S regions (11).

By coinfection of duck embryo fibroblasts (DEFs) with both REV and MDV, we demonstrated that this process of retroviral integration can also occur within several passages of cocultivation (10). The structure of the inserted REV sequences resembles those of cellular integrated proviruses, with loss of the terminal nucleotides of the retrovirus and a 5-bp duplication of the MDV sequence at the site of insertion.

We have extended these analyses and constructed a detailed insertional map of REV sequences in the MDV genome. Although no distinct sequence is found at insertion sites, REV integrations in both experiments cluster at the junctions of U_S. We report that these junction regions are heterogeneous in several MDV strains examined.¶

MATERIALS AND METHODS

Virus Propagation. The MDV clone JM/102W (12) and the CS strain of REV (13) were used for coinfection experiments. Other MDV strains used were CU2 (14), Md5 (15), and Md11 (16). Viruses were propagated in DEFs or chicken embryo fibroblasts as described (17).

Polymerase Chain Reaction (PCR). Total genomic DNA from MDV-infected cells or MDV DNA purified by pulsed-field gel electrophoresis (18) was cut with a variety of restriction enzymes (e.g., *Eco*RI, *Sal* I, or *Ava* II) and ligated in a 100-μl volume. REV-containing fragments were amplified by inverse PCR using LTR primers (U3, 5′-CCGAGAAATGATATCAGCG-3′; U5, 5′-GGGTGGGGTAGGGATCCGG-3′ or 5′-CCGATTCGAATCTGTAATAAAAGCTTTTTCTTC-3′) which would extend into the flanking sequences (19). Ten to twenty discrete bands between 0.2 and 3 kb were seen on ethidium-stained gels with each amplification. Products were cloned by using the *Eco*RV, *Bam*HI, or *Hin*dIII sites present within the primers. Genomic regions of JM-Lo and Md11 strains were cloned by direct PCR using a U_S primer for each junction (+394, 5′-ATGGCAGTTTGAGGTTCATG-3′; +11139, 5′-GACATAACACTCATATTAAGGG-3′) or an R_S primer (−30, 5′-GCGGTATGAGATGCACG-3′) with an upstream R_S primer (−862, 5′-TAGCTCGAGCCAAAAGGGAA-3′) and directly sequenced or blunt-end cloned.

DNA Sequence Analysis. PCR products were cloned into an M13 vector and sequenced on both strands by using [α-[^{35}S]thio]dATP and T7 polymerase (Sequenase; United States Biochemical) (20). Direct DNA sequencing of PCR products was also done using the *fmol* system (Promega) with

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Abbreviations: DEF, duck embryo fibroblast; HSV, herpes simplex virus; LTR, long terminal repeat; MDV, Marek disease virus; REV, reticuloendotheliosis virus.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession no. L09061).

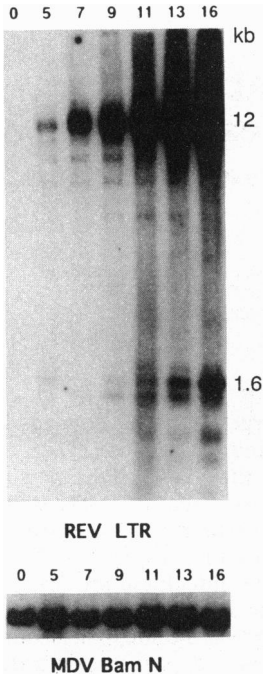

FIG. 1. Detection of LTR insertions in short-term coinfection. Total genomic DNA from REV/MDV-infected DEF monolayers was isolated, digested with *Bam*HI, and blot-hybridized to the REV LTR *Sac* I–*Bam*HI probe (10). Lane numbers (0, 5, 7, 11, 13, and 16) refer to the serial passage numbers from the time of REV/MDV coinfection. As a control for MDV copy number, the same blot was probed with the *Bam*HI N fragment from the U_L region of MDV (21). Only a small portion of the autoradiograph is shown.

[α-^{32}P]GTP-labeled primers. DNA sequence analysis was carried out with the IBI MACVECTOR program.

Pulsed-Field Gel Electrophoresis. The electrophoresis conditions and preparation of the DNA plugs were as described (18). The DNA was electrophoresed in a 1% agarose gel in 0.5× TBE (TBE is 0.09 M Tris base/0.09 M boric acid) for 20 hr at 200 V with a 50- to 90-sec switch gradient (Bio-Rad CHEF-DR II system). MDV minichromosomes from passage 16 DNA were extracted from the gel, digested with *Eco*RI (which does not cleave REV DNA), and ligated into the λ DASH vector (Stratagene) for construction of the phage libraries. Fourteen LTR-hybridizing phage clones were restriction-mapped, and three different insertion sites were identified.

RESULTS

REV Insertions into MDV in Short-Term Culture. DEFs were coinfected with MDV and REV and the cells were analyzed for evidence of integration. Fig. 1 is a Southern blot of total genomic DNA digested with *Bam*HI from various passages. The DNA samples were precalibrated so that each lane would contain similar amounts of MDV DNA, as verified by hybridization to the *Bam*HI N fragment of the cloned MDV library (21). When probed with REV LTR, faint bands appear by passage 5 following coinfection, and by passage 16 two prominent bands (12 and 1.6 kb) and many minor bands are evident. While this experiment in itself does not distinguish insertions into the host or MDV genome, we note that REV infection alone should give rise to only the 1.6-kb band, an internal viral fragment (22), and a background smear representing the randomly integrated proviruses. The presence of other bands suggests that there may be specific REV insertions in the MDV genome and that MDV genomes harboring certain REV insertions are the dominant population at late passages.

Clustered Insertions at the R_S/U_S Junctions. To effectively screen a large number of distinct insertion sites, we used inverse PCR (10). Total genomic DNA collected at each passage after coinfection was digested, ligated, and amplified by using primers specific for REV LTR. To guard against selective amplification of only certain insertion sites, several different restriction enzymes were used to generate the initial linear fragments of the MDV genome prior to ligation. Products representing REV integrants into MDV were obtained in every passage examined, including several in the first passage following coinfection.

Fig. 2 (upper arrows) shows the distribution of 19 independent REV insertions obtained after PCR amplification of DNA collected from passages 5–16. Remarkably, most of the insertions obtained are confined to two regions of the virus. The left-hand region contains 3 insertions that map in the first 50 bp of U_S; the right side contains 3 insertions that map in the last 700 bp of U_S. Eleven integrants are located in the last 400 bp of the repeat, so whether they were present on both sides could not be determined in most cases. The remaining two sites are located in the reported coding region of the gD glycoprotein in U_S (2). To confirm the results obtained from the inverse PCR, MDV DNA from passage 16 cells was purified through a pulsed-field gel and used to construct phage libraries. Three REV LTR hybridizing clones were sequenced and the insertion sites were determined. They contained solo LTRs which mapped to three sites in R_S identified above by PCR analysis (see black dots in Fig. 3 for precise locations).

To ensure that the clustered insertions were not a product of this particular experiment, we also determined the REV insertion sites in the highly passaged JM strains of MDV (JM-Hi). The JM virus stock had been in culture for 211 passages and was accidentally coinfected by REV at passage 40. We previously showed that multiple REV insertions had occurred (10). Six of 10 insertions obtained from p211 of JM-Hi virus map in this region (Fig. 2, lower arrows). The remaining JM-Hi insertions map at the R_L/U_L junction and are reported elsewhere (23). Thus, two independent experi-

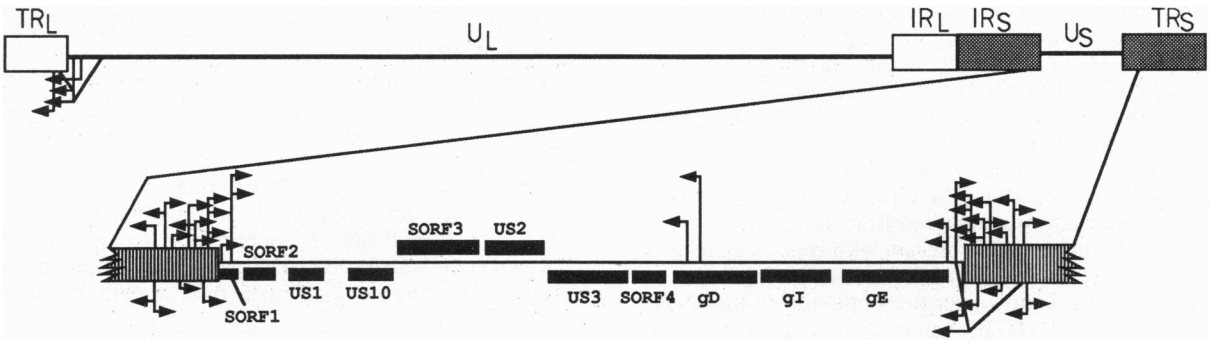

FIG. 2. Insertional map of REV LTRs in the MDV genome. U_L is the long unique region. TR_L and IR_L (open boxes) refer to the inverted terminal and internal long repeats, respectively. U_S is the short repeat. TR_S and IR_S are the repeats flanking the short region (shaded boxes). REV integrations into the MDV genome following passages 5–16 of MDV in DEFs are indicated above the map. REV integrations into JM-Hi virus (passage 211) are indicated below the map. Arrows indicate the transcriptional orientation of the LTR. Location and identity of the genes in U_S (black boxes) are based on ref. 2. The genes are named by reference to their homology to HSV-1 genes.

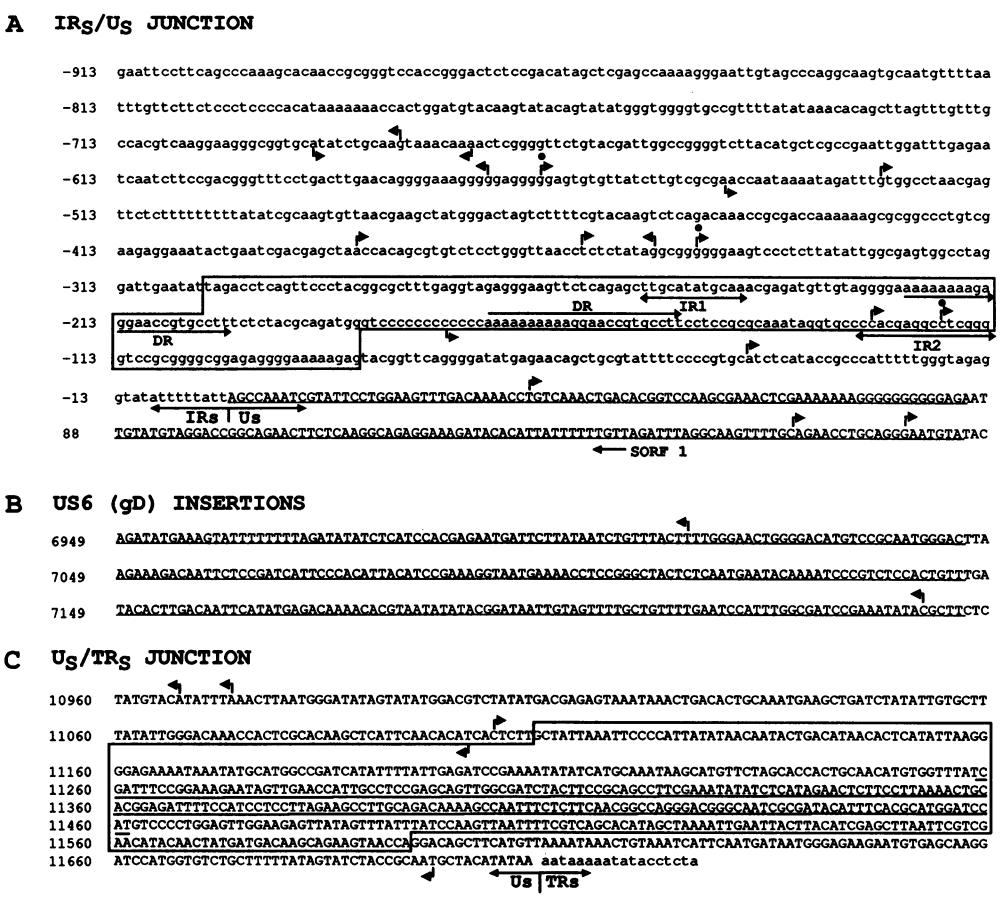

FIG. 3. Nucleotide sequences of the U_S/R_S junctions and the sites of REV integrations into MDV. Sequences from JM MDV U_S are in uppercase, and those from the R_S region are in lowercase. Nucleotide numbers correspond to the map of the GA strain reported elsewhere, with +1 assigned as the first base of the left end of U_S (2). Arrows indicate the location and the transcriptional direction of LTR inserts, obtained from inverse PCR of passages 5–16 of short-term coinfection (arrows above the sequence) or JM passage 211 (arrows below). Each arrow is centered on the third base of a 5-bp REV insertion site. Black dots represent sites also obtained from λ clones, which were isolated from a genomic library of passage 16 MDV DNA purified from a pulsed-field gel. (*A*) Boxed sequence in IR_S indicates the boundary of the direct repeat duplicated in Md11 strain. Within this sequence are two 22-bp direct repeats (DR) and two inverted repeats (IR1 and IR2). The location of the SORF1 reading frame is underlined. It terminates at the first base of U_S. (*B*) Portion of the gD glycoprotein gene sequence derived from the JM MDV U_S region, with the location of two insertion sites indicated. (*C*) Boxed sequence in U_S/TR_S junction represents region which is not present in the sequence reported from the GA strain (2). A 68-codon open reading frame in U_S is underlined.

ments under different conditions produced a similar clustered insertion pattern.

Insertion Sites and Sequences of the MDV R_S/U_S Junctions. To precisely locate the insertion sites, we determined the relevant R_S/U_S junction and gD sequences of the wild-type JM strain of MDV (Fig. 3). Unlike the U_L or U_S region, the R_S junction region of MDV shares little homology with herpesvirus of turkey or HSV. The small repeats usually found near the U_S/R_S junctions of herpesviruses (24) are absent. There is, however, a 218-bp repeat (boxed in Fig. 3*A*) which is present at variable copies in different strains of MDV (see below). Within this repeat region is a directly repeated sequence (22 of 23 bp identical; DR) which is flanked by 12 bp (IR1) and 16 bp (IR2) inverted repeats. No significant open reading frames are found in the 913 bp of R_S reported here. The REV insertion sites are marked with arrows indicating their transcriptional orientation. There is no common sequence motif at insertion sites, but most of the insertions are oriented toward the U_S region. A number of insertions are located within stretches of guanine and adenine nucleotides. Immediately after the R_S sequence is SORF1, an 83-amino acid coding region (2). Three of the insertions would disrupt its coding capacity.

At the U_S/TR_S junction (Fig. 3*C*), there are five insertions within the U_S region. We found that the sequence in JM MDV differs from the published data of the GA strain (2) by the presence of a 486-bp sequence (boxed region), within which is an open reading frame of 68 amino acids (underlined). None of the five insertions map within the JM-specific region.

Structure of REV Inserts. The most frequently found REV inserts are solitary LTRs with the last two nucleotides lost and the flanking MDV sequences duplicated. An example is shown in Fig. 4*A*. The isolation of clones containing only solo LTRs suggests that REV proviruses were rapidly deleted after several rounds of passaging *in vitro*. In most cases, deletion most likely occurred via recombination between the 5′ and 3′ LTRs to generate an intact solo LTR with a 5-bp duplication of the MDV sequence at the site of integration. Homologous recombinations between LTR and other proviral sequences were also observed (Fig. 4*B*). Recombination between adjacent MDV sequences and short stretches of proviral sequences was most likely responsible for MDV/LTR junctions like those depicted in Fig. 4*C*. Stretches of 5–7 bases of identical sequence appeared to be sufficient for recombination. The sequence GGGGAA present in both U3 of the LTR and at the 3′ LTR junction was used several times.

R_S/U_S Junctions as Sites of Heterogeneity in MDV. There are several factors that may contribute to the clustered insertion pattern (see *Discussion*). Here, we consider the possibility that the U_S/R_S junctions in MDV may be regions of unusual structural flexibility that tolerate the insertion of foreign sequences. Using Southern analysis and PCR-based

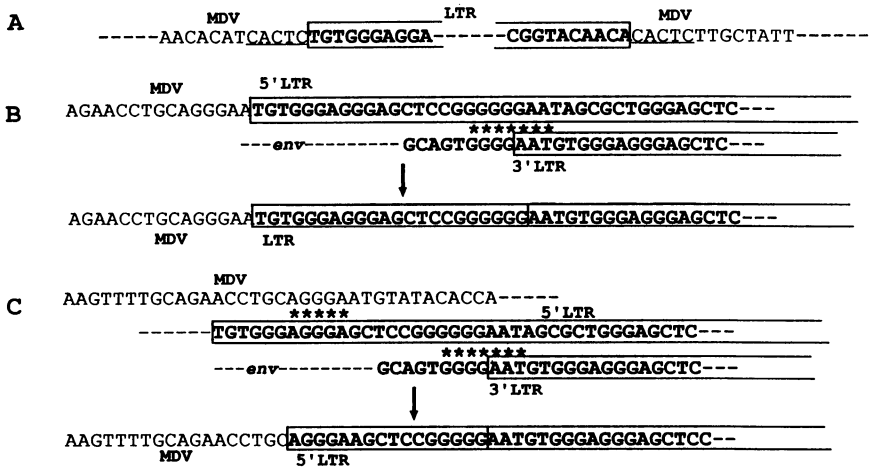

FIG. 4. Structure of REV LTR inserts. (*A*) Solo LTR inserted into MDV sequence in U_S, with 5-bp duplication underlined. (*B* and *C*) Two different inverse-PCR integration junctions with LTR sequences joined to sequences from the same region of MDV U_S (+180 of U_S). The MDV sequence at the site of these junctions is homologous to two sequences present near the U3 LTR end of REV. Stars indicate identical base pairs. LTR sequences are boxed. Arrows indicate the final inverse PCR product in each example with reiterated LTR sequences. In *B*, the 5′ LTR of an integrated provirus is shown recombining with the *env*-3′ LTR junction to generate a duplicated U3 LTR junction. In *C*, a provirus integrated at another site is shown recombining with homologous MDV sequence to generate a truncated LTR junction.

sequencing, we have analyzed the R_S/U_S junctions in several strains of MDV (JM, CU2, Md5, and Md11; maps shown in Fig. 5*A*). The structure of JM (a moderately pathogenic strain used for coinfections above) was found to be virtually identical to CU2, an MDV strain of low pathogenicity. Southern blots of *Eco*RI-digested samples (Fig. 5*B*) illustrate distinct structural alterations found in two highly pathogenic MDV isolates. Probes 1 and 2 identify the IR_S/U_S and TR_S/U_S junctions, respectively. Md5 differs from JM or CU2 by the conversion of ≈700 bp of U_S sequence from the left junction into R_S. On the other hand, Md11 displays a ladder of bands differing by ≈200 bp, due to the presence of variable number of the 218-bp repeat at the termini of R_S. This repeated region is located within the region of clustered insertion sites (boxed sequence in Fig. 3*A*). The fluidity of the R_S ends is further demonstrated by structural changes noted during the passaging of Md11 in culture (Hi/Md11 versus Lo/Md11 with probe 3 in Fig. 5*B*).

DISCUSSION

In this paper, we show that REV insertions into MDV occur rapidly and efficiently following coinfection. After a number of rounds of replication *in vitro*, MDVs containing REV LTR sequences dominate the culture. The retroviral insertions in these viruses are clustered in a small region spanning the R_S/U_S junction. We report the nucleotide sequence of both of these junctions from the JM strain of MDV. The insertional map from the short-term coinfection experiment resembles that obtained from REV insertions during the fortuitous coinfection of the JM strain during prolonged passaging. The clustering of insertions in the same region in two experiments conducted under different conditions demonstrates a selective pressure for acquisition of REV LTRs at the repeat junctions.

Retroviruses integrate into host genomes in a random fashion, although preferences for transcriptionally active chromatin (25) and certain unspecified sequences have been reported (26). We analyzed viruses that had been propagated in culture for more th ı five passages. These conditions tended to select against viruses with inserts that impair viral replication functions. Thus, we anticipated a nonrandom distribution of the viral insertion sites. However, we were surprised by the tight clustering of the insertions near the U_S/R_S junctions. There are several factors that could contribute to these clustered integrations. First, the U_S/R_S junctions are most likely dispensable for virus growth *in vitro*. Indeed, a pathogenic strain GA MDV has a 48-bp deletion at the right junction of U_S (2), and a JM high-passage viral clone with a similar deletion in this region has an

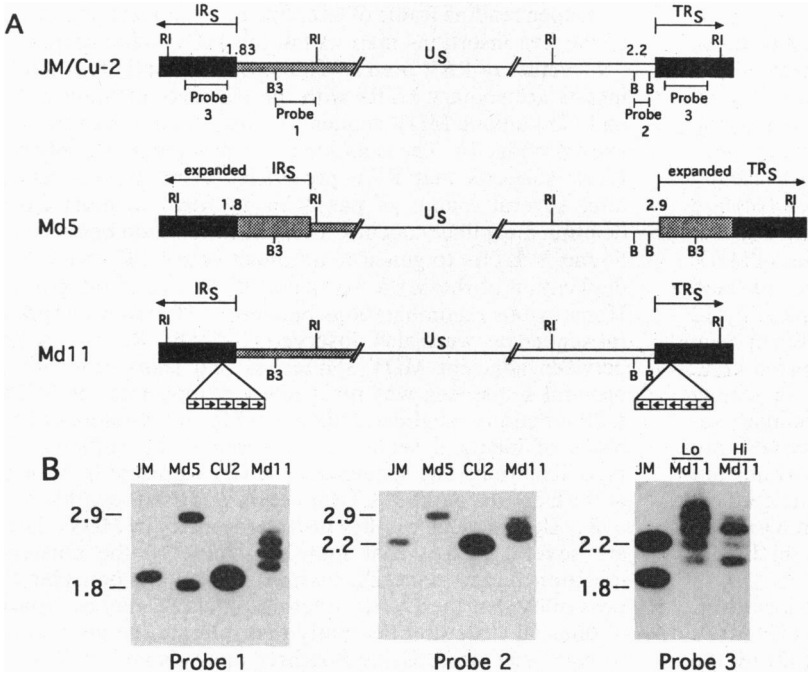

FIG. 5. Structural variation of the R_S/U_S junctions in different MDV strains. (*A*) The map of each strain was derived from the Southern blot shown in *B* and sequence data for each of the strains. Dotted regions are U_S sequences from JM, some of which are incorporated into R_S in Md5. Boxes with arrows in Md11 indicate locations of 214-bp reiterated repeats. The locations of probes used in the Southern blot are indicated on the JM/CU2 map. Numbers indicate sizes of *Eco*RI restriction fragments in kilobases. B3, *Ban*III; B, *Bam*HI; RI, *Eco*RI. (*B*) Total genomic DNA from MDV-infected DEFs was isolated, digested with *Eco*RI, and blotted with ^{32}P-labeled probes. MDV samples include low-passage JM (passage no. 14), CU2 (no. 13), Md5 (no. 10), and Md11 (no. 11) and high-passage Md11 (no. 78). High-passage Md11 was analyzed only with probe 3. Probe 1 is a *Ban*III–*Eco*RI fragment located within the 1.83-kb *Eco*RI fragment near the left junction. Probe 2 is the *Bam*HI U fragment directly adjacent to TR_S. Probe 3 is a PCR-generated subfragment of the 1.83-kb *Eco*RI fragment, which is located within the repeat region.

enhanced *in vitro* growth rate (11). Alterations of the R_S/U_S junctions (27, 28) have been noted in several strains of HSV. Insertion of foreign sequences into the repeat/unique junction of pseudorabies virus is also tolerated (29).

Second, the U_S/R_S junction appears to be a region of extraordinary flexibility tolerating insertions of foreign DNA. We have presented evidence of structural variation in this region among different strains of MDV. The junction structure of the highly pathogenic strain Md5 and Md11 is different from that of the strains of low (CU2) or moderate (JM and GA) pathogenicity. The R_S region in Md5 is expanded by incorporating parts of the left end of U_S. Similar alterations have been observed in HSV (27) and pseudorabies virus (24). In Md11, a 218-bp repeat is frequently duplicated or deleted, giving rise to viruses with heterogeneous R_S termini. The expansion or contraction of the 218-bp repeat apparently has little effect on the replicative ability of the virus; but its effect on viral oncogenicity remains to be determined.

A third factor that could affect retroviral insertion specificity is a variation in chromatin structure at the R_S junctions. We have not examined this possibility. Finally, given the potential of inserted LTRs to promote gene expression, it is possible that the U_S/R_S junction region may encode gene(s) which, upon activation by an LTR, enhance MDV growth. Recently, we isolated several MDV clones carrying LTR inserts; all of them have enhanced growth properties compared with the parental virus (11). In one MDV clone with a solo LTR insert in R_S (bp −345 in Fig. 3*A*) from the short-term coinfection experiment, high levels of a transcript initiated from the LTR promoter and expressing the SORF1, US1, and US10 coding regions were detected (unpublished data). This provides evidence of retroviral LTR promoter-insertional activation of herpesvirus genes. The above four possibilities are not mutually exclusive and it is likely that a combination of these factors are responsible for the observed insertion specificities.

The most frequently observed REV inserts in MDV are solo LTRs, presumably derived by homologous recombination between LTRs subsequent to proviral insertion. The second most frequently observed inserts are LTRs with a 23-bp iteration (TGTGGGAGGGAGCTCCGGGGGAA) at the 5′ end (Fig. 4 *B* and *C*). As diagrammed in Fig. 4, this LTR repeat could have arisen from recombination between the sequence GGGAA present in the U3 of LTR and at the junction of *env* and LTR. Deletion of integrated proviruses from cellular genomes by homologous recombination between the LTRs occurs at low frequency (30–32). The high frequency observed here could be the result of size constraints necessary for efficient herpesviral packaging. It could also result from the herpesvirus recombinative machinery. Weber *et al.* (33) demonstrated that integrated Tn*5* transposons in HSV underwent high-frequency inversion events via recombination mediated by the virus.

In summary, the present report documents efficient retroviral LTR insertion into a herpesvirus genome and the clustered insertions near the U_S/R_S junctions for viruses at late passages. The nonrandom distribution is most likely the result of selection for viable viruses *in vitro*. This study did not yield a sufficient number of insertion sites in viruses at early passages to allow us to test whether the insertion sites were random prior to extensive virus spread. It does suggest that under different selection conditions, a different insertion pattern may be found. In a manner similar to the uncovering of protooncogenes, retrovirus insertion sites may be used to identify herpesvirus genes with specific functions.

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