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Retroviral Insertional Activation in a Herpesvirus: Transcriptional Activation of U_S Genes by an Integrated Long Terminal Repeat in a Marek's Disease Virus Clone

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Insertional activation of host proto-oncogenes has been recognized as a basic mechanism by which nonacute retroviruses induce cancer. Our previous work has demonstrated that retroviruses can efficiently integrate into DNA virus genomes. Specifically, coinfection of cultured fibroblasts with a chicken herpesvirus, Marek's disease virus (MDV), and a chicken retrovirus results in frequent stable retroviral insertions into the herpesvirus genome. Such insertions could alter the expression of herpesvirus genes, possibly resulting in novel phenotypic properties. In this article, we report the characterization of a replication-competent clone of MDV with integrated retroviral sequences. This virus was isolated from a chicken following injection of fibroblasts coinfected with MDV and the retrovirus, reticuloendotheliosis virus. Transcripts originating from the reticuloendotheliosis virus long terminal repeat promoters were found to encode the adjoining MDV genes, SORF2, US1, and US10. This virus replicates well in culture but has an unusual phenotype in chickens, characterized by an attenuated virulence which produces no nerve lesions but, rather, severe thymic atrophy. While the causal relationship between the insertion and the observed phenotypes remains to be established, our data provide the first evidence of retroviral insertional activation of herpesvirus genes.

Marek's disease virus (MDV) and reticuloendotheliosis virus (REV) are two of the most widespread and persistent viral infections in chickens. MDV, an oncogenic herpesvirus, often infects the same animal and cell types as REV, a nonacute retrovirus. MDV has a typical alphaherpesvirus genomic structure consisting of two unique regions, U_L and U_S, flanked by inverted repeats designated TR (terminal repeat) and IR (internal repeat) (Fig. 1). During MDV replication, the TR and IR frequently undergo homologous recombination, resulting in the inversion of the U_L and U_S sequences. Our previous work has demonstrated that REV can integrate into the MDV genome following both long- and short-term coinfections of chicken or duck embryo fibroblasts (DEF) (11, 15). This process is mediated by the retroviral integration machinery and can result in numerous insertions within several passages. Integrated proviruses frequently undergo deletions in which only intact solo long terminal repeats (LTRs) or partial LTR sequences are retained (15). Long-term integrations were observed in cultures of a high-passage (211 passages) JM strain of MDV fortuitously coinfected by REV (44). This gave rise to a heterogeneous population of MDV clones with variable numbers of REV insertions (17). In addition to the integration of retroviral sequences, these viruses were also found to contain deletions and rearrangements in other regions of the genome. The short-term experiment was carried out under controlled conditions. After only four passages, MDV with stably inserted LTRs was readily detected and increased in proportion to the parental viruses upon further cell passaging (11, 15). The insertions were not randomly distributed but, rather, clustered near the R_S/U_S boundaries. We have discussed several possible consequences of retroviral insertion into herpesviruses, including the transmission of retroviral information by herpesviruses, the activation or inactivation of herpesvirus genes, the alteration of herpesvirus biological properties, etc. (12, 15).

In this article, we report the isolation from short-term cultures of an MDV clone with an REV integration which retains the ability to replicate both in vitro and in chickens. This clone, RM1, was derived from passage 14 of the mixed infection. Similarly, passaged wild-type virus showed little change in either replicative or oncogenic properties in vitro or in vivo. RM1 virus contains a solo LTR integrated at the R_S/U_S boundary and has no other readily detectable genomic alterations. The in vitro growth properties of RM1 are similar to those of the wild-type virus, but RM1 has a distinctive in vivo phenotype characterized by severe thymic atrophy and attenuated oncogenicity. Most significantly, a stable transcript initiating from the inserted REV LTR promoter was found to transcribe adjacent U_S genes, providing direct evidence for insertional activation of herpesvirus genes by a retroviral LTR.

MATERIALS AND METHODS

Virus cloning and propagation. The original LTR-containing virus (RM1) was isolated from a $15I_Sx7_1$ chicken inoculated with a suspension of DEF coinfected with MDV strain JM/102W (JM) and the CSV (chicken syncytial virus) strain of REV and passaged a total of 14 times (11). Six weeks postinoculation, cell-free virus was isolated from feather follicles and plated on DEF. Single MDV plaques were then picked and expanded as clones. These MDV cultures were found to be negative for infectious REV by an immunofluorescence assay with a REV-specific monoclonal antibody (5). Clones were then screened for the presence of REV integrations. One clone, RM1, was selected for further analysis. Because of the cell-associated nature of MDV in vitro, the in vivo propagation greatly facilitates cloning of individual viruses. Different factors, alone or in combination, can account for this process eliminating coinfecting REV from the MDV cultures. These factors can include dilution of REV and an inability of REV to invade and/or replicate in feather follicles.

Two different concentrations of cloned RM1 virus (100 and 1,000 PFU/ml) were reinoculated into chickens, which were compared with JM virus-infected chickens. The first group was sacrificed at 21 days postinfection (p.i.) and examined for gross Marek's disease lesions (visceral tumors and enlarged nerves). The second group of birds was examined for gross lesions; surviving birds were

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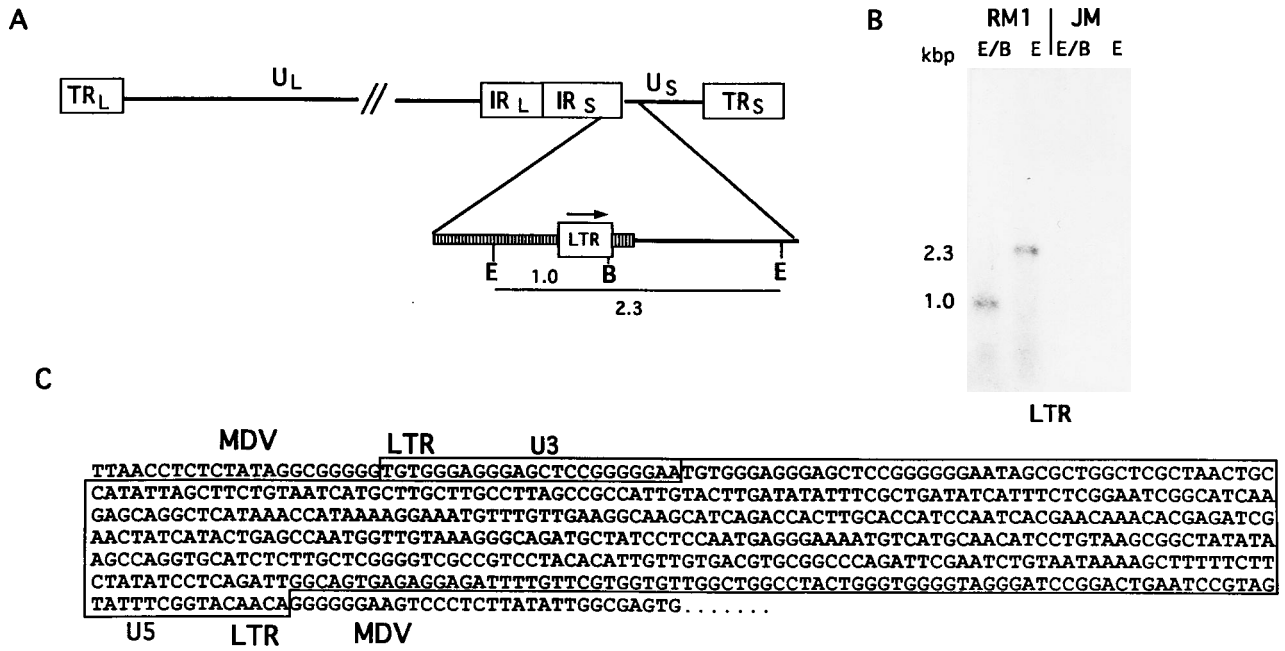

FIG. 1. Isolation of an MDV clone with a solo LTR insert. (A) The genomic map of RM1. The transcriptional direction of the LTR is indicated. The shaded box in the enlarged section denotes the inverted repeat short (IR_S). The sizes of the *Eco*RI (E) fragment and the *Eco*RI-and-*Bam*HI (B) fragment harboring the LTR are given in kilobase pairs. (B) Southern analysis of RM1 DNA. Total genomic DNA was isolated from RM1- and JM-infected cells. The RM1 stock used in this experiment was freshly isolated from a single plaque within three passages. The JM stock was the low-passage (p14), oncogenic isolate. The cultures were free of infectious REV as judged by an immunofluorescence assay. The LTR probe was derived from a 0.5-kb *Sac*I-*Bam*HI fragment. (C) Nucleotide sequence of the inserted LTR. The REV LTR sequence is boxed, with the duplicated U3 sequence indicated. Aside from the 23-bp duplication of the U3 termini, a 1-bp insertion, and 1-bp substitution, the LTR sequence is identical to a previously reported CSV LTR (38). The flanking sequences correspond to the MDV R_S region.

terminated at 48 days p.i. Histological examination for the presence of malignant T cells and reactive T and B cells was carried out on thymus, gonad, liver, and other tissues. Vagus and brachial nerves were examined for enlargement. Viremia (number of DEF plaques produced per 10^6 buffy coat cells from blood of infected chickens) was measured as previously described (45). Virus recovered from the peripheral blood of RM1-infected chickens was plaque purified from DEF. Plaque assays were used to determine in vitro growth rates and are expressed as PFU per input PFU.

DNA analysis. To identify the sites of retroviral insertion, total genomic DNA from MDV-infected cells was isolated and hybridized with REV LTR and MDV probes as described previously (11). Integration sites were initially cloned by inverse PCR using REV LTR primers as previously described (15). The integration sites were also determined by PCR amplification using primers flanking the integration cluster (upstream R_S oligonucleotide, 5′-GCCTGCAGTGCCACG TCAAGGGAAGGGC-3′; downstream R_S oligonucleotide, 5′-GCGGTATGA GATGCACG-3′). PCR products were cloned into M13 vectors and sequenced on both strands by the dideoxy chain termination method (34) using ^{35}S-ATP and T7 polymerase (Sequenase; United States Biochemical). Direct sequencing of the PCR products was carried out with the pmol sequencing system (Promega) together with the use of ^{32}P-labeled oligonucleotides. The JM-Hi virus used as a positive control for 132-bp-repeat expansion was obtained by serial passage of the JM parent virus in culture (44). The current stock is at passage 211.

RNA analysis. RNA was purified from MDV-infected DEF by guanidinium-CsCl centrifugation (4). Northern (RNA) blots were obtained following separation on 1.2% glyoxal gels (39). Blots were probed with a *Sac*I-*Bam*HI REV LTR probe (38) or MDV probes comprising the 1.8- or 2.8-kb *Eco*RI subfragment of the *Bam*HI-A clone present in the GA MDV genomic library (7). For primer extension, an REV LTR primer (5′-TTATTACAGATTCGAATC-3′) located in the R region of the LTR was end labeled with [γ-^{32}P]ATP and used to reverse transcribe 40 μg of total RNA from RM1-infected DEF (23). Products were analyzed on 6% polyacrylamide–7 M urea gels in which a sequencing ladder was employed for purposes of sizing.

cDNA isolation. cDNA cloning was done by PCR using the RACE protocol (6). One microgram of total RNA from RM1-infected DEF was reverse transcribed with murine leukemia virus reverse transcriptase using a *Not*I $(dT)_{15}$ primer and PCR amplified either with an LTR U5 sense primer (5′-GGGTGG GGGTAGGGATCCGG-3′) and the *Not*I $(dT)_{15}$ primer or with the same LTR U5 primer and a U_S antisense primer (5′-ATGGCAGTTTGAGGTTCATG-3′). The U_S antisense primer is located at approximately 0.4 kb from the R_S/U_S junction, between the SORF1 and SORF2 coding sequences. Primers were removed by Centricon 100 filtration (Amicon Corp.), and the reaction products were cloned into M13 vectors by using restriction sites present in the primers or in the amplified sequences.

RESULTS

Isolation of an MDV clone with a solo LTR insert. Having shown that REV can stably integrate into MDV following short-term coinfection, we were interested in examining the effects of such integrations on in vivo viral replication and gene expression. To that end, we isolated a biologically active, plaque-purified MDV clone, RM1, on the basis of its hybridization to an LTR probe. This clone was derived from passage 14 of the MDV-REV coinfection cultures. As shown in Fig. 1B, Southern hybridization with a REV LTR probe revealed a single band in the *Eco*RI-digested (lane E) or *Eco*RI- and *Bam*HI-digested (lane E/B) DNA of the plaque-purified RM1, indicating a single REV insertion. No signal was detected in the parental JM virus. Direct sequencing of the LTR-containing fragment of RM1 established the presence of a solo LTR in the internal repeat short (IR_S) region of MDV at a position 347 bp from the IR_S/U_S boundary (Fig. 1A and C). The LTR sequence is 99% identical to the LTR of the CSV strain of REV, except that a 23-nucleotide sequence is duplicated at the very 5′ end of the LTR. The MDV sequence GGGGG adjacent to the insertion site is duplicated, attesting to an authentic retroviral integration. No other retroviral sequences were found present in the RM1 genome. To prepare a virus stock for further in vitro and in vivo characterization, RM1 was further passaged six times on DEF. During these passages, a predominant viral fraction underwent duplication of the LTR insertion at an identical site in the terminal repeat short (TR_S; Fig. 2, upper panel) region, presumably a result of the frequent recombination between TR_S and IR_S. This is evidenced by

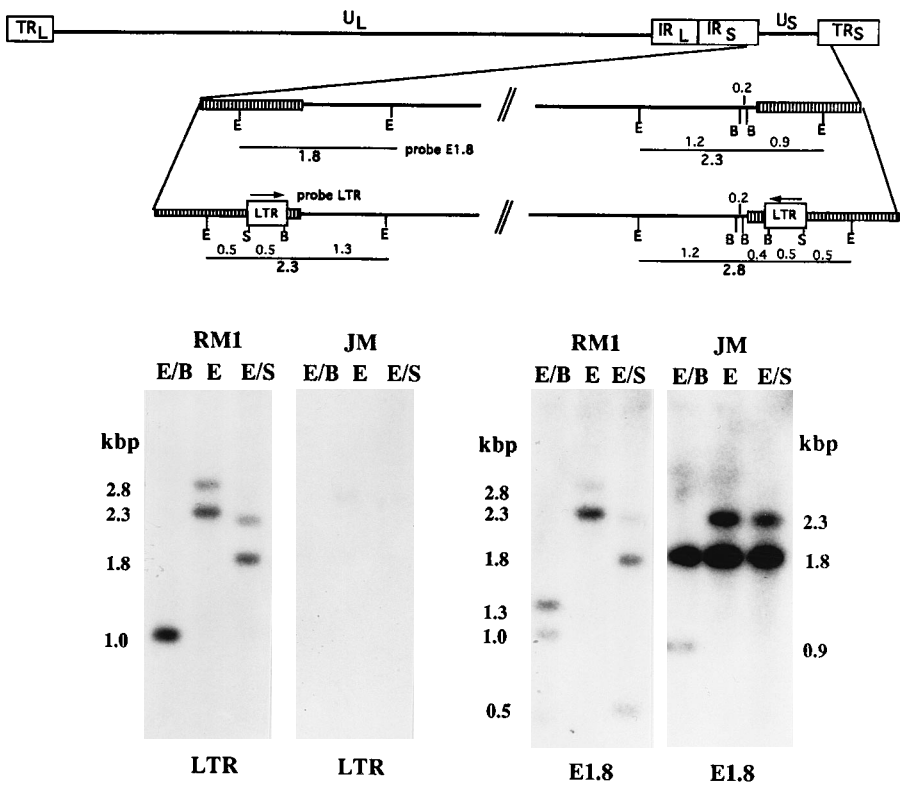

FIG. 2. Duplication of LTR in RM1 upon passage. The RM1 stock used in this experiment was derived from the original stock (Fig. 1) by further passaging six times in vitro. The genomic structure of the passaged RM1 is shown. The part carrying the duplicated LTR insert is in the enlarged section, compared with that of the JM parent virus without an LTR insert. The notations are as described in the legend to Fig. 1. The sizes of individual restriction enzyme cleavage fragments are shown. The locations of the probes used for the Southern blot analysis (lower panels) are indicated. For Southern analysis, DNAs from passaged RM1- and JM-infected cells were isolated and digested with *Eco*RI (E), *Eco*RI plus *Sac*I (E/S), or *Eco*RI plus *Bam*HI (E/B). The probes used were REV LTR and the *Eco*RI 1.8-kb fragment of MDV. The sizes of the individual bands are estimated from a lambda *Bst*EII marker run in the same gel.

Southern blot analysis (Fig. 2) using an REV LTR probe which hybridizes to two *Eco*RI fragments (2.3 and 2.8 kb) (RM1 LTR panel, lane E), instead of the one observed in the original stock (Fig. 1B, lane E). Hybridization with an MDV E1.8 probe, which can detect both repeat regions, confirmed the location of the LTR in both IR_S and TR_S (RM1 E1.8 panel, lane E). In the JM parent virus, the E1.8 probe hybridizes to a 1.8-kb fragment and a 2.3-kb fragment, which correspond to the IR_S/U_S and TR_S/U_S junctions, respectively (Fig. 2 map and JM E1.8 panel, lane E). The increased size of these two fragments in RM1 (from 1.8 to 2.3 kb and from 2.3 to 2.8 kb) is accounted for by insertions of the REV LTR (0.55 kb). Southern analysis of doubly digested DNAs (Fig. 2, lanes E/B and E/S) provided corroborative evidence. Furthermore, PCR cloning demonstrated that identical LTR junctions were present in both IR_S and TR_S (data not shown). This virus stock was used for the studies with chickens described below.

Mapping and cloning of the MDV transcript initiating from the LTR. To assess the effects of LTR insertion on MDV gene expression, Northern blots of RNA extracted from MDV-infected chicken embryo fibroblasts were carried out (Fig. 3). When probed with REV LTR, a major transcript of 3.2 kb was detected in RM1-infected cells but not in cells infected with an equally passaged JM parent virus (LTR panel). The 3.2-kb band was also detected in RM1 cells with the MDV E1.8 probe, which spans the LTR insertion site (E1.8 panel), and with the MDV E2.8 probe, located immediately downstream of the E1.8 probe (E2.8 panel). On the other hand, Northern blot analysis failed to identify transcripts initiating from the integrated LTR located in the TR_S copy of RM1 (data not shown). We have therefore focused on transcription near the IR_S/U_S junction. The size and the nature of the 3.2-kb transcript are consistent with it being initiated from the LTR promoter and transcribed across the coding sequences of SORF2, US1, and US10, terminating after the US10-proximal poly(A) signal in the U_S region. The two transcripts (2.6 and 1.7 kb) detected by the E2.8 probe in both RM1-infected cells and JM-infected cells were identical to the native MDV transcripts previously described (27, 28). These transcripts initiate from U_S region promoters and terminate near the same poly(A) site (Fig. 3, map).

The 2.6-kb transcript can also be detected by the E1.8 probe, although its intensity is much weaker than that of the LTR-directed 3.2-kb transcript (E1.8 panel). This is largely due to the greater extent of homology between the E1.8 probe and the 3.2-kb transcript.

To precisely define the initiation site in the LTR, primer extension analysis of RNA from RM1-infected cells was carried out with an LTR U5 antisense primer located downstream of the two previously identified promoter sites in the U3 region of the REV LTR (32). Two strong bands, 53 and 54 nucleotides in length, were detected, in agreement with predicted start sites near the U3-R boundary of the REV LTR (Fig. 3, primer extension). Thus, transcription from the integrated LTR is initiated at the same sites as those in the intact provirus. Several cDNA clones obtained by reverse transcription PCR (RT-PCR) confirm the linkage between the LTR sequence and MDV SORF2, US1, and US10 sequences. A 0.8-kb RT-PCR

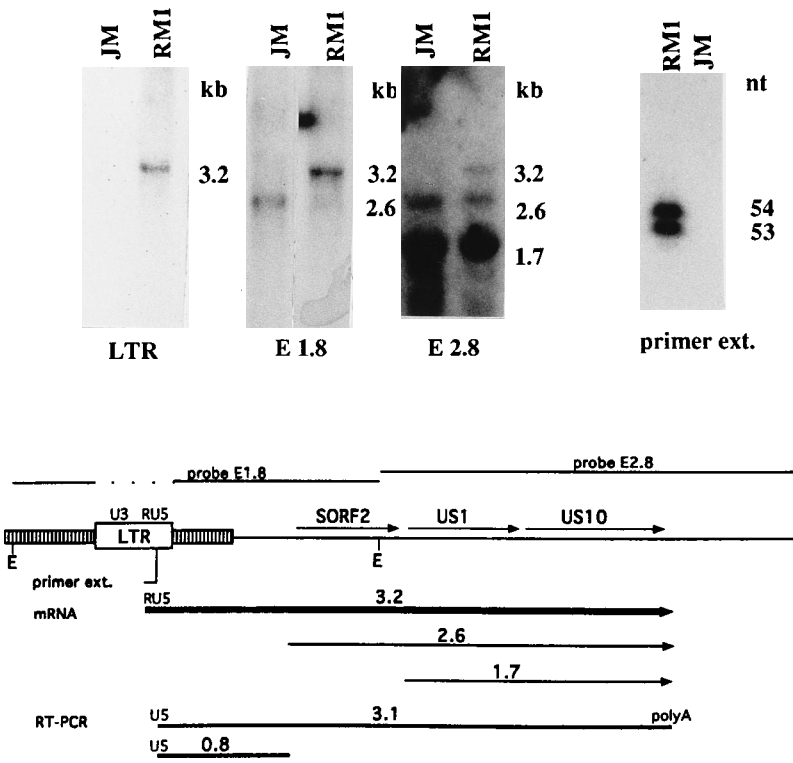

FIG. 3. Transcription from the LTR insertion in RM1. Total RNA from RM1- or JM-infected cells was prepared for Northern blot (left three panels) and primer extension (ext.) analyses. Probes for Northern blots included the *Sac*I-*Bam*HI fragment of REV LTR and the 1.8- and 2.8-kb *Eco*RI fragments (indicated in the summary diagram in the lower panel). The latter two fragments were purified from the *Eco*RI-digested *Bam*A clone of the GA MDV genomic library (8). Sizes (in kilobases) were determined with the use of poststained RNA standards. For primer extension analysis, an end-labeled antisense primer located in the R region of the LTR was used (see Materials and Methods). Two extension products were identified with RNA from RM1-infected cells (primer extension) but not with RNA from JM-infected cells. Based on Northern blot, primer extension, and RT-PCR analysis, a model for transcription in the region flanking the inserted LTR in RM1 is depicted below. The notations are the same as those for the previous figures. The locations of the REV LTR U3, R, and U5 regions are indicated as are the predicted primer extension products. The locations and identities of several U_S genes are based on previous studies (2b, 33). The directions of the transcripts are indicated by arrows, and the predicted start sites and termination sites of individual transcripts are shown. The locations and the sizes of the two RT-PCR products are indicated. The primers used for RT-PCR are described in Materials and Methods. The RT-PCR products were verified by direct sequencing of the termini and a significant portion of the products.

fragment was obtained by using a LTR U5 primer and an antisense U_S primer located near the start site of SORF2. An additional 3.1-kb fragment was obtained with the LTR U5 primer and an oligo(dT) primer. Taken together, the above data suggest that the 3.2-kb transcript is an LTR-driven polycistronic message which terminates downstream of the US1 and US10 coding sequences. Generally speaking, only the 5′-proximal open reading frame (ORF) is translated to any significant extent in eucaryotes. Extrapolating to this case, the SORF2 would be the most likely product to be expressed by this message. Although there is little information about the SORF2 product, its structure resembles several known herpesvirus transactivators (see Discussion). Without SORF2-specific antibodies we could not verify increased expression of the SORF2 polypeptide; however, our preliminary analysis showed that the US1 and US10 proteins are expressed at comparable levels in RM1- and JM-infected cells (2a).

Biological behavior of RM1. As a first step in characterizing possible biological effects resulting from the LTR insertion, we studied the in vitro and in vivo biological properties of RM1. To assess the replication properties of the RM1 virus in culture, virus growth in DEF was analyzed and the data were compared with those for the JM parent virus at passages 14 and 48. Virus production was assayed by counting plaques in triplicate cultures at 2, 5, and 8 days p.i. The results plotted in Fig. 4 demonstrate that viral replication and/or spread is at

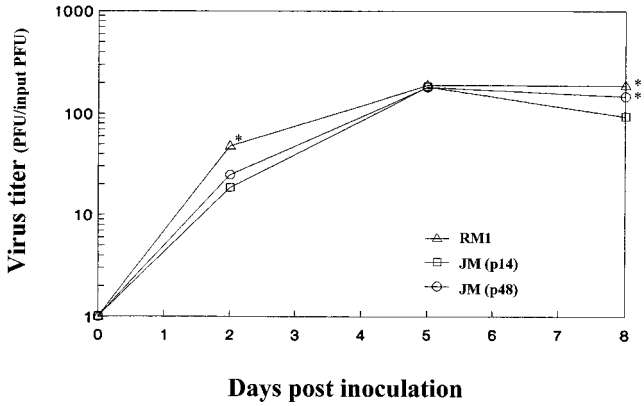

FIG. 4. In vitro growth rate of RM1. RM1- and JM-infected cells, each carrying 100 PFU eq, were inoculated onto nine plates of DEF cultures. Two different passages (p14 and p48) of JM virus stock were used in this experiment. Three plates for each group were harvested at each time point (i.e., 2, 5, and 8 days p.i.). The virus titer from each culture was determined by plaque assay. The data are expressed as total PFU per input PFU. Each datum point is the mean of three replicate cultures. The statistics were calculated by the Bonferroni *t* test. Statistically significant differences ($P < 0.05$) from the values for the p14 JM control are indicated (asterisks).

TABLE 1. In vivo behavior of RM1 compared with JM parent virus[a]

Virus PFU	No. of chickens				
	21 days p.i., gross MD	48 days p.i.			
		MD		Thymic atrophy	Viremia
		Gross	Histo		
RM1					
100	0/6	0/6	0/6	5/6	5/6
1,000	0/6	1/6	1/6	6/6	5/6
JM					
100	5/6	6/6	2/2	2/2	2/2
1,000	5/6	6/6	—	—	—
None	0/6	0/6	0/6	0/6	0/6

[a] Groups of six $15I_5x7_1$ chickens were inoculated with parental JM virus (passage 14) or the RM1 clone. At 21 days p.i., only gross Marek's disease (MD) lesions were examined in this experiment. In subsequent experiments when thymus was examined, atrophy induced by RM1 was evident at 21 days p.i. At 48 days p.i., both gross MD and other parameters shown in the table were evaluated. Viremia was determined by the production of viral plaques after incubation of DEF cultures with 1,000,000 buffy coat cells from the blood of infected chickens. Evidence for gross MD included visceral tumors and enlarged nerve lesions. Histologic (histo) examination for MD involved examination of gonad, liver, and vagus and brachial nerves for characteristic malignant and reactive lymphocytic infiltrates. —, no birds survived to the day of necropsy.

least as rapid in RM1 as in the fully virulent JM parent virus. Thus, the LTR insertion does not appear to impair the in vitro replicative ability.

To study the in vivo biological properties of RM1, chickens were inoculated with two different titers of RM1 virus or JM virus. The JM virus is highly oncogenic, and at 21 days postinfection, 10 of the 12 JM-infected chickens developed Marek's disease with visceral tumors and nerve lesions (Table 1). None of the RM1-infected chickens had demonstrable pathology. At 48 days p.i., 10 of the JM-infected chickens had already died of fatal lymphoma associated with Marek's disease; the remaining two showed aggressive Marek's disease lesions. In contrast, only one of the 12 RM1-infected chickens developed lymphomas, and none showed evidence of nerve lesions. Histological examination confirmed these observations. Interestingly, the attenuation of tumorigenicity of RM1 cannot be attributed to an inability to replicate in vivo, since 10 of the 12 RM1-infected chickens developed viremias with titers comparable to those of the JM parent virus. How efficiently RM1 replicates in the target T cells was not addressed in this study. However, RM1 induces thymic atrophy to a much greater extent than the JM parent virus. These features make RM1 somewhat unique among the JM isolates. The potential to replicate well in vivo and the potential to induce thymic atrophy usually cosegregate with oncogenic strains (28). The attenuated strains are deficient in all three characteristics. Since RM1 is intermediate between the two, it may provide a useful tool in dissecting the molecular basis for these contrasting phenotypes.

Lack of detectable genomic alterations in RM1. Alterations in MDV genome structure have been previously shown to correlate with attenuation of in vivo MDV pathogenicity. The best established correlate involves expansion of a 132-bp reiterated sequence adjacent to the origin of replication in R_L (8, 21, 36). This expansion appears to alter the transcriptional pattern characteristic of the *Bam*H gene family (for a review, see reference 20). To examine whether the attenuation of RM1 oncogenicity can be attributed to the expansion of the 132-bp repeats or other gross alterations, *Bam*HI-digested RM1 DNA

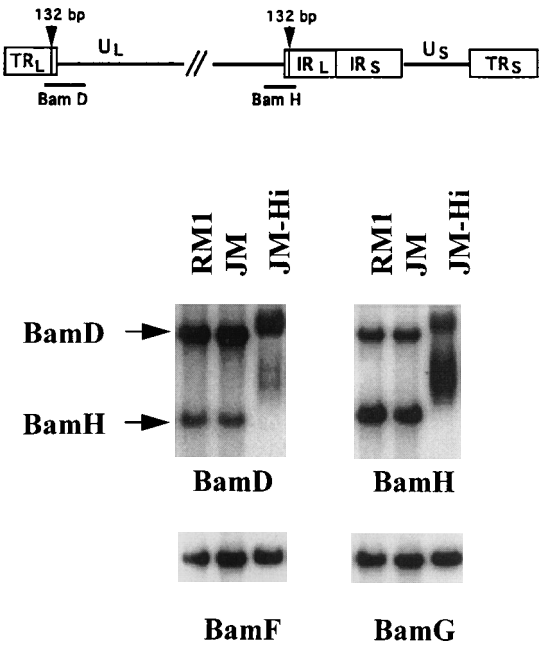

FIG. 5. Lack of 132-bp expansion in the RM1 genome. DNA isolated from RM1-, JM (parent virus, oncogenic strain)-, or JM-Hi (high-passage, attenuated strain)-infected cells was digested with *Bam*HI and Southern blotted with probes derived from the various *Bam*HI clones of the GA MDV library (8). The locations of *Bam*D and *Bam*H probes which span the TR_L/U_L and IR_L/U_L junctions are indicated. The locations of the 132-bp repeat are also shown. The *Bam*D and -H fragments are of defined sizes in RM1- and JM-infected cells but become heterogeneous in size, because of increased copy numbers of the 132-bp repeat in JM-Hi-infected cells.

was Southern blotted and hybridized with individual *Bam* fragments derived from the entire MDV library (8) (representative samples are shown in Fig. 5). Genomic DNA from the oncogenic JM parent virus (lane JM) and the high-passage, attenuated strain (lane JM-Hi) were used as controls. The 132-bp repeats are located in TR_L and IR_L regions present in the *Bam*D and -H fragments. As shown in Fig. 5, *Bam*D and *Bam*H probes detect the same two *Bam*HI fragments. Oncogenic JM DNA contains two sharp bands corresponding to the *Bam*D and -H fragments, whereas the attenuated JM-Hi DNA exhibits heterogeneity in these regions, consistent with the previously reported expansion of the 132-bp repeats (8, 11, 21, 36). RM1 DNA appears to be virtually identical to the oncogenic strain with no evidence of 132-bp expansion (lane RM1). PCR analysis confirmed a lack of expansion in this region (20a). These results suggest that attenuation of RM1 oncogenicity is likely due to a genetic mutation(s) not associated with the 132-bp repeats. *Bam*F and -G probe hybridizations are also depicted in Fig. 5. Once again, no obvious differences were detected between RM1 and JM DNAs. Multiple hybridizations failed to reveal readily detectable genomic alterations in RM1 other than those resulting from the LTR insertions described above.

DISCUSSION

Our previous studies have shown that retroviral integration into MDV is an efficient process. The integrated proviruses are initially unstable, resulting in the recombinational deletion of the internal sequences and retention of solo LTRs. Viruses with solo LTRs are genetically stable. We have identified two hot spots for REV integration at the two R_S/U_S junctions of

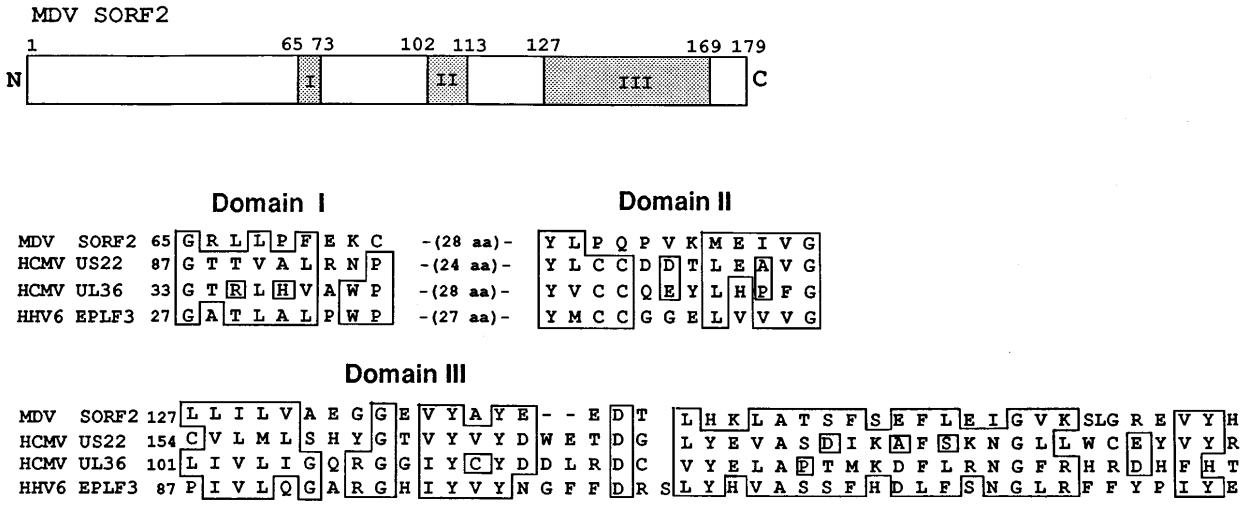

FIG. 6. Domain structure of SORF2. SORF2 contains three recognizable domains (shaded boxes) based on alignment with other proteins. The amino acid sequence similarities to human cytomegalovirus (HCMV) US22 and UL36 and human herpesvirus 6 (HHV6) EPLF3 gene products are boxed. The space between domain I and II (ranging from 24 to 28 amino acids [aa]) is relatively constant among these proteins.

MDV, where the vast majority of the stable LTR insertions are localized (15). This finding has now been corroborated by at least two other studies involving coinfection of HVT or MDV with avian leukosis virus (8a, 13). Such a clustered integration pattern contrasts with the generally nonspecific nature of retroviral insertions but is reminiscent of the region-specific integrations near proto-oncogenes in leukemias and lymphomas (for a review, see reference 19). In the latter case, tumors arise presumably as a result of selection for insertions that successfully activate proto-oncogenes. We do not know what the selective pressures for the LTR insertion clusters in MDV are but have considered several possibilities (15). One possibility is that REV LTR-mediated insertional activation of MDV genes near the R_S/U_S junctions provides a selective growth advantage in vitro or in vivo. This study provides an initial lead to this possibility. The RM1 virus described here has an LTR which is present (either duplicated or in one copy) within the R_S/U_S junctional hot spot. This positions the active promoter sequence of the LTR upstream of a number of tightly regulated MDV U_S region genes. REV LTR sequences have previously been shown to act as strong promoters in chicken fibroblasts in vitro (9) and in a variety of chicken tissues in vivo (1). Of particular relevance is our previous finding that REV LTRs can deregulate c-myc expression by promoter insertion in chicken T- and B-cell lymphomas (14, 26). A recent study based on transient transfection of the REV LTR also showed that the LTR promoter activity is generally high in chicken T cells, especially MDV-transformed lymphoblastoid cell lines (31). As such, transcription from the integrated LTR in RM1 could alter programmed gene expression following MDV infection, both quantitatively and qualitatively.

In RM1, the normal transcription pattern of a portion of U_S is altered by the LTR insertion. There is an additional 3.2-kb LTR-driven transcript which overlaps with two downstream messages (2.6 and 1.7 kb) normally present in the wild-type JM MDV. A similar group of transcripts (2.6, 1.7, and 0.9 kb) (Fig. 3) has recently been described for the GA strain of MDV by Parcells et al. (27). (In our gel, the 0.9-kb transcript was not retained and was not investigated further.) Together, these results demonstrate that the MDV homologs of the herpes simplex virus genes US1 (ICP22) and US10 as well as a putative MDV-specific gene, SORF2, are expressed as a coterminal group of transcripts. The LTR-promoted transcript in RM1 is polycistronic, encoding all three of these ORFs. While the level is not particularly high, this transcript could potentially promote the expression of these MDV products in such a way as to circumvent their tight regulation. In murine retrovirus-induced T-cell lymphoma, it is the deregulation of c-myc expression, rather than the absolute level of the LTR-driven *myc* transcript, that is critical in tumorigenesis (30).

SORF2 is the 5′-proximal ORF of the 3.2-kb transcript most likely to be translated efficiently (18). SORF2 is a novel gene, probably unique to serotype I MDV, about which virtually nothing is known. Indeed, the existence of the SORF2 gene product is only assumed and has yet to be confirmed. However, a comparison of its predicted amino acid sequence with those of other herpesvirus genes sheds some light concerning its possible function. SORF2 is a 179-amino-acid protein, consisting of three recognizable domains with homology to human cytomegalovirus US22 and human herpesvirus 6 EPLF3 (Fig. 6). These two genes are members of the human cytomegalovirus US22 extended gene family, which includes several other gene products encoded by human cytomegalovirus and human herpesvirus 6 (16, 25, 37) as well as a fowlpox virus ORF (40). A common feature of the proteins in this family that have been studied is their ability to transactivate herpesvirus genes as well as the human immunodeficiency virus LTR promoter. Experiments are in progress to raise antibody against MDV SORF2, to verify its identity, and to assess its transactivation ability.

The other two gene products that might be deregulated are US1 and US10, both known herpesvirus proteins. The herpes simplex virus US1 product, ICP22, is an immediate-early gene (22) which is dispensable for growth in cell culture but may regulate virulence or tissue tropism (24). The MDV US1 gene, however, is regulated as a late gene (2). Recent deletion mutant studies have suggested that the US1 product may be encoded by the 1.7-kb transcript described above (27). US10 is a nonessential virion gene in herpes simplex virus whose function is currently unknown. In MDV, US10 encodes a 24-kDa phosphorylated protein regulated as a late gene (2). The presence of a potential zinc finger domain in many US10 homologs has suggested a possible role in gene regulation (10). It thus

appears that all three gene products potentially affected by LTR insertion may have transactivation properties, although none of them seems to be essential for in vitro and in vivo growth, as elegantly shown by recent deletion analyses (27, 28). This finding notwithstanding, the consequence of overexpression or deregulation of these putative transactivators remains to be analyzed. RM1 may provide a convenient tool for these experiments.

During the initial in vitro passages of RM1, most of the virus population had duplicated the inserted LTR to both R_S regions. This is consistent with evidence from studies of herpes simplex virus that changes in one inverted repeat are highly unstable and recombine within one or two generations to yield identical repeats (43). In a careful study, Umene showed that herpesviruses with heterozygous repeats (A/B) quickly give rise to progeny viruses with either type of homozygous repeats (A/A or B/B) with equal frequency (42). In our work, however, most viruses in the population have duplicated the inserted LTR in IR_S to TR_S. We have yet to recover revertants (i.e., wild-type R_S). This disequilibrium is likely due to a selection pressure requiring the presence of the inserted LTR, although we do not yet know what this pressure is. The preferential retention of an insertion was also seen in pseudorabies virus when foreign sequences were inserted at the S-L junction (29). In that study, it was proposed that equalization of the inverted repeats in pseudorabies virus occurs by a copying mechanism, not by homologous recombination.

RM1 has unusual properties which differ from those of other JM isolates. It is significantly attenuated in oncogenicity, yet it replicates efficiently in vivo and induces severe thymic atrophy. Other attenuated JM isolates or nononcogenic serotypes of MDV (e.g., SB1 and HVT) replicate poorly in chickens (28, 35, 44), causing neither gross tumors, nerve lesions, nor necrosis or atrophy of the spleen, bursa, and thymus (3). In addition, previous studies showed that attenuated JM almost invariably sustains a 132-bp-repeat expansion unlike RM1. Thus, the attenuation mechanism associated with RM1, which is uncoupled from replication ability, may differ from that of other strains. Indeed, judging from the severe thymic atrophy induced by RM1, it is possible that RM1 imposes a higher level of cytolytic infection of thymocytes than the parental oncogenic strain. The fact that oncogenic strains cause a moderate level of thymic atrophy, in addition to lymphoma, indicates that there is a delicate balance between the lytic and persistent (or latent) T-cell infections by MDV. We would like to speculate that one of the deregulated transactivators such as SORF2 may tilt the balance, in favor of lytic infection. We further speculate that this is cell type specific (e.g., T cell) and would not significantly affect in vitro replicative capacity of virus in DEF. Like all other cases involving recombinant herpesviruses, a definitive causal relationship requires curing of the LTR insertion. In the absence of proper selection markers (e.g., β-galactosidase or drug resistance), such a demonstration is not at all trivial.

While this article has focused on LTR promoter activation of MDV genes, it should be noted that LTRs also function as effective enhancers that can upregulate adjacent promoters in a position- and orientation-independent manner. In the case of mammalian retroviruses, LTR enhancer activation, rather than promoter activation, constitutes the predominant mode of proto-oncogene activation in T lymphomas and other tumors (for a review, see reference 41). Since a potential promoter for the long form of the MDV ICP4 gene is located 100 to 200 bp upstream from the LTR insertion site (20b), it is conceivable that the ICP4 transactivator may also be associated with the novel phenotype of RM1. By the same token, the LTR enhancer near the U_S/TR_S junction may also activate other nearby genes. The RM1 mutant virus reported here provides a unique opportunity to identify possible genes involved in the cytolytic infection of T lymphocytes and in the establishment of latency.

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