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Molecular Cloning and Analysis of the Endogenous Retrovirus Chemically Induced from RFM/Un Mouse Cell Cultures

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We molecularly cloned and analyzed an N-tropic ecotropic retrovirus induced with iododeoxyuridine from RFM/Un mouse cell cultures. Based on the restriction map, the RFM/Un virus appears to be indistinguishable from other induced N-tropic retroviruses. A nucleotide sequence analysis of the long terminal repeat of an infectious clone revealed structural features characteristic of murine type C retrovirus long terminal repeats. The U3 region of the RFM/Un virus long terminal repeat, however, contained no short sequence duplication or insertion found in other murine leukemia virus isolates.

Current interest in the RMF/Un mouse strain stems from the high incidence of myeloid leukemia and increased incidence of thymic lymphoma after radiation of the host mice (27). This could be of particular importance as a model because myeloid leukemia also occurs in humans exposed to high-level whole-body irradiation. Throughout the lifespan of these mice, an endogenous retrovirus is spontaneously expressed in hemopoietic cells. The involvement of this virus with any neoplasia has not been unequivocally established, although evidence indicating a retrovirus etiology for radiation-induced myeloid leukemia has been presented (14).

The RFM/Un strain has been found to yield a single biologically homogeneous host range class of endogenous virus from both normal and neoplastic cells (26). This mouse strain carries the *Fv-1*^{n} allele, and the chemically induced RFM/Un virus (RFV) is N-tropic (ecotropic); however, unlike those of AKR mice, RFM fibroblasts are quite resistant to exogenous infection with the induced virus. RFM/Un fibroblast cultures are resistant to infection by other induced N-tropic viruses (WN1802N from BALB/c cells and AKV from AKR cells) but are not resistant to the highly passaged Gross leukemia virus (GLV). We have recently reported some biological and biochemical characteristics of this virus (26), and we report here the results of restriction endonuclease mapping studies, molecular cloning, and nucleotide sequence analysis of the long terminal repeat (LTR) region of an infectious DNA clone of RFV.

The RFV used in this study was induced from RFM 3T3 cells by 5′-iodo-2-deoxyuridine treatment and passed once in SC-1 cells (26). A modification of the procedure of Hirt (12, 31) was used to prepare unintegrated RFV DNA from SC-1 cells 48 h after infection at a multiplicity of infection of 1.

A preliminary restriction endonuclease-Southern gel blot analysis (22) was made for unintegrated, linear RFV DNA (data not shown). No *Eco*RI site was observed in the viral DNA, and the restriction endonucleases *Hin*dIII, *Sal*I, *Pvu*I, *Xba*I, and *Xho*I each had a single recognition site. A comparison with published maps of other ecotropic retroviruses (4, 19, 20) and our unpublished maps of WN1802B indicated that RFV was indistinguishable from the others. This was consistent with our recent results comparing the biological and biochemical properties of RFV with those of other ecotropic retroviruses (26).

The covalently closed circular RFV DNA was purified from Hirt supernatant fluid by ethidium bromide-CsCl density gradient centrifugation and was digested with the single-cut enzyme *Hin*dIII. This DNA was cloned in the lambda phage vector Charon 9 (3), and plaques were screened by hybridization with ^{32}P cDNA of GLV (31) by the procedure of Benton and Davis (1). DNA inserts from hybridization-positive clones (8.2 and 8.8 kilobase pairs) were transferred to pBR322 to facilitate analysis. Recombi-

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TABLE 1. Host range property of parental RFV and progeny virus recovered from DNA transfection with pRFV105

Virus preparation[a]	XC titer (PFU/ml)[b] in the following cell type:			
	SC-1	NIH 3T3	C57BL	RFM 3T3
pRFV105 progeny	1.3×10^5	1.2×10^4	0.6×10^1	<1
RFV stock	5.5×10^5	1.5×10^4	1.6×10^3	1.2×10^1

[a] Virus preparations were 12-h cell-debris-free culture media harvested from DNA-transfected NIH 3T3 cells at passage 5 (pRFV105 progeny) and from SC-1 cells chronically infected with RFV (RFV stock).

[b] One to three serially diluted virus-containing media were added to 16-h cultures of different mouse fibroblast cells in 30-mm wells. At 72 h after infection, UV irradiation was applied and XC cells were added. After another 72 h, cells were stained and syncitial were plaques counted (21).

nant plasmid clones pRFV101 (8.2-kilobase-pair insert) and pRFV105 (8.8-kilobase-pair insert) were amplified (6), and DNA was extracted and analyzed by restriction endonuclease digestion. It was obvious that the inserts of these two clones have the same map of the permutated structure, with the exception of one copy of the LTR. There were no apparent deletions or gross rearrangements in either of these cloned genomes.

The plasmid clones pRFV101 and pRFV105 were tested for their biological activity by DNA transfection in NIH 3T3 cells by the calcium phosphate precipitation method (10) as previously described (13). After one or more passages, each transfected culture was assayed by the XC assay (21). The insert isolated from pRFV105 was positive, whereas the insert of pRFV101 was not. Virus isolated from the XC-positive transfected culture was characterized by titration on RFM 3T3, NIH 3T3, C57BL, and SC-1 cell cultures and was compared with our standard RFV stock (Table 1). The virus grew best in SC-1 cells, showed preferential growth in NIH 3T3 cells over C57BL cells, in agreement with the N-tropism of RFV, and was severely restricted in the original host, RFM 3T3. In addition, the morphology of XC plaques was indistinguishable from that of plaques induced by the original RFV, which are small in comparison with plaques of the other N-tropic viruses.

The overall strategy for the determination of nucleotide sequence by the method of Maxam and Gilbert (16), utilizing restriction endonuclease sites within and around the two joined LTRs of pRFV105, is shown in Fig. 1. The complete nucleotide sequence is shown in Fig. 2. There were two tandem 527-base-pair LTRs which had a 13-base-pair inverted repeat at the termini. Adjacent to the left LTR was a 21-base sequence complementary to the 3′ sequence of the $tRNA^{Pro}$ primer (01 L to −21 L) (see Fig. 2 for nomenclature). There were two overlapping GCG triplets (384 L to 386 L), one of which may be the RNA capping site. Preceding this site by 20 bases was a 5′-TATAAAA-3′ sequence which was a 7- of 8-base match to the polymerase II consensus sequence (7, 32). Another AT-rich se-

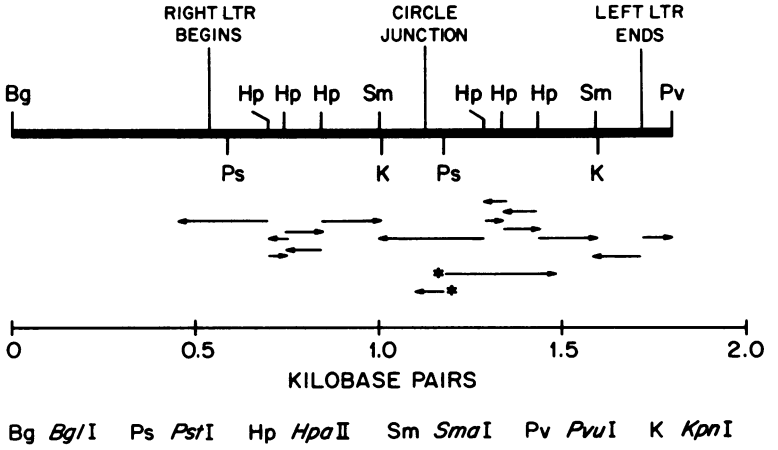

FIG. 1. Endonuclease cleavage map of RFV DNA fragment containing the LTR sequences. The arrows show the DNA fragments whose sequences were determined and the direction of sequencing. *, 3′ ^{32}P-labeled ends. All others were labeled at the 5′ ends.

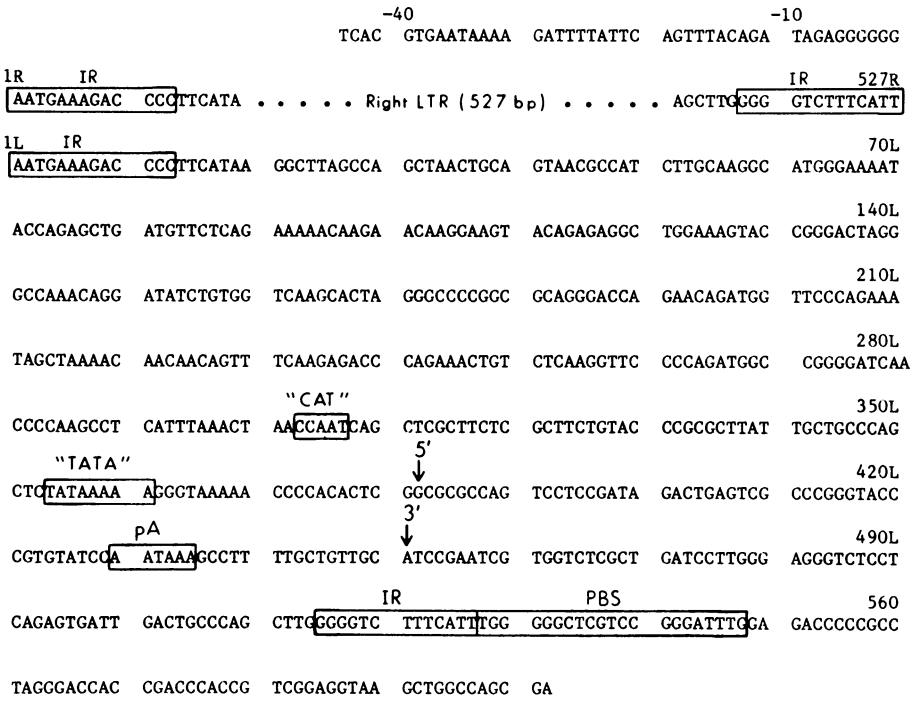

FIG. 2. Sequence of pRFV105 LTR region. Nucleotides 1R to 527R represent the LTR at the right end of the linear genome (adjacent to the *env* gene), and nucleotide 1L to 527L represent the LTR at the left end of the linear genome (adjacent to the *gag* gene). The sequences upstream of the right LTR are designated by negative numbers. The sequence shown is of the strand which has the same polarity as the genomic RNA. Sequences of structural or regulatory importance are boxed and designated as follows: IR, inverted repeat; "CAT" and "TATA", polymerase II consensus sequences; pA, polyadenylation signal; PBS, primer binding site. The 5′ (i.e., cap) and 3′ (minus polyadenylate) ends of the genomic RNA are indicated.

quence, 5′-ATTAAA-3′ was observed at positions 292–298. However, the extent of homology to the polymerase II consensus sequence and the more distal position relative to the putative RNA capping site render this sequence less likely to be the promoter for RNA synthesis. A signal for polyadenylation, 5′-AATAAA-3′ (18), was observed at positions 430–435, and a CA dinucleotide, the preferred site for polyadenylation, was observed 15 nucleotides downstream. Thus, the LTR of RFV contains all of the structural features characteristic of murine type C retroviruses (8, 24, 25, 28).

Because RFV was an endogenous virus with a minimal passage history, the LTR sequence of this virus was a good reference point with which to measure the variation in sequence which existed in other isolates. In Fig. 3 we schematically show the positions where the bases of four other mouse retroviruses differed from those of RFV. The first three, GLV, WN1802B, and AKV, were virtually identical to RFV. The exception is that both AKV and WN1802B had duplications (of different length) in the mid-U3 region, which corresponded with the duplicated "enhancer" sequence of Moloney murine sarcoma virus (15); also, GLV had an insertion in this region, part of which was a duplication of sequences downstream. There is evidence which indicates that all ecotropic mouse retroviruses have a common origin (23), and it was therefore not surprising that the LTR sequence of the endogenous viruses of RFM/Un, AKR, and BALB/c cells were similar.

The GLV (passage A) originated from an AK leukemia filtrate passaged in C3H mice (11) and was almost certainly derived from AKV. The extreme similarity of the RFV and GLV sequences was somewhat surprising given the long passage history of GLV, both in vivo and in vitro, and the fact that GLV is oncogenic. The RFV isolate has not been shown to be oncogenic (26), in agreement with studies on AKV. Although the AKV and GLV genomes are very closely related, there are oligonucleotide differences scattered throughout the genome, with the major differences in the 3′ region (5). From our study the only substantial difference between the LTRs of RFV and GLV and the 36-base-pair insertion in the U3 region of GLV, although

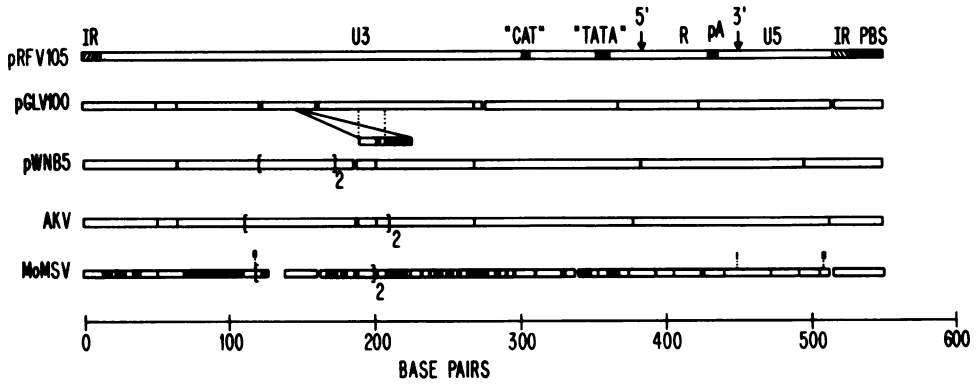

FIG. 3. Comparison of RFV LTR sequence with other murine retrovirus LTRs. The positions of structural or regulatory elements known to be characteristic of retrovirus LTRs are indicated in the pRFV105 schematic map by diagonal or crosshatched lines. Abbreviations are as described in the legend to Fig. 2. The 5′ (i.e., cap) and 3′ (minus polyadenylate) ends of the genomic RNA are indicated by arrows and separate the LTR into three regions; U3 is unique to the RNA 3′ end, R is a short terminal repeat, and U5 is unique to the RNA 5′ end. Base changes relative to the pRFV105 sequence (Fig. 2) are indicated by a solid bar through the map, and base deletions are shown as breaks in the map. Small insertions are indicated as bars above the map, and tandem duplicated regions are shown in brackets. The pGLV100 (molecular clone of GLV) and pWNB5 (molecular clone of WN1802B, isolated from BALB/cells) sequences are from our unpublished work. The AKV sequence is from reference 29, and the Moloney murine sarcoma virus (MoMSV) sequence is from reference 8. The pGLV100 sequence shows a segment which is inserted at base 145. The dotted lines indicate that part of this sequence (open symbol) is similar to a region 44 bases downstream. The origin of the remainder of the insert is unknown.

there were seven single-base changes. We do not know if these changes alone accounted for the differences in the oncogenic property of GLV relative to RFV.

Moloney murine leukemia virus, like GLV, causes T-cell leukemia (17), but the two viruses have only 40% sequence homology (2) and show many differences in restriction enzyme sites in the genome (9, 19, 30). A comparison of the RFV LTR with the Moloney murine sarcoma virus/murine leukemia virus LTR (8, 28, 29) revealed base changes scattered throughout the sequence, with a higher proportion in the U3 region (Fig. 3). It is interesting to note that there were base changes directly adjacent to the eucaryotic consensus sequences for RNA polymerase II transcription. Additional comparisons of this type will be required to determine just how great the tolerance for sequence divergence is, but it appears to be considerable. A comparison of Moloney virus and AKV LTR sequences also reveals this divergence (29).

Our analysis of the molecular clone and previous biochemical characterization indicated that RFV was a typical endogenous N-ecotropic retrovirus, although the replication was severely restricted by RFM/Un cells as compared with other *Fv-1*^{n} mouse cells (26). RFM/Un mice are therefore a unique and valuable model in which to study the role of endogenous ecotropic retrovirus in radiation-induced leukemia. A recent southern gel-hybridization analysis of the RMF/Un genome has revealed that the endogenous virus resides at a single locus, and preliminary evidence has suggested that radiogenic myeloid leukemia cells have additional integrated proviruses (unpublished data). Future work will be aimed at establishing a causal relationship between the novel proviral integrations and myeloid leukemia, understanding the mechanism of restriction of exogenous infection by the RFM/Un cells, and determining whether intragenomic transposition is an alternate pathway for normal proviral integrations.

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