Analysis of Recombinant DNA Clones of the Endogenous BALB/c Murine Leukemia Virus WN1802N: Variation in Long Terminal Repeat Length

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We analyzed 15 recombinant DNA clones of the unintegrated closed circular DNA intermediate of the BALB/c endogenous ecotropic murine leukemia virus WN1802N. Thirteen of these clones had an insert which corresponded to the complete murine leukemia virus genome. Of these, six contained a single long terminal repeat (LTR) and seven contained two LTRs. The viral genomes in nine clones had an LTR of 520 base pairs (bp), one had an LTR of 570 bp, three had an LTR of 600 bp, and one had an LTR of 670 bp. Restriction endonuclease analysis demonstrated that the size variability resides in the U3 region. Seven of eight clones which yielded infectious virus by DNA transfection had the 520-bp LTR, and the other had a 600-bp LTR. More detailed examination of plasmid subclones of three isolates with different-sized LTRs revealed that the approximate position which varies in the U3 region corresponds to the 72-bp repeat region of Moloney sarcoma virus. Possible consequences of these variations are discussed.

It is now well established that the termini of unintegrated retroviral DNA and integrated provirus differ from the genomic RNA by the presence of repeated sequences termed the long terminal repeat (LTR) (10, 11, 21, 22). This structure is derived by transcription of contiguous sequences from the 5' and 3' ends of the genomic RNA by a "jumping" mechanism (3, 10, 22). Analysis of LTR sequence (6, 13, 23, 25, 28, 29) has suggested an important role for them in integration, by analogy with bacterial and eucaryotic transposons, and in the regulation of gene expression, due to the presence of 5' and 3' noncoding sequences common to eucaryotic genes.

Two size classes of murine leukemia virus (MuLV) LTR have been reported in several cases (17, 19, 27), and some isolates have recently been shown to differ in the U3 region of the LTR due to the presence or absence of sequence duplications (2, 6, 24, 28).

These findings are very intriguing because of the important multifunctional role the LTR region has in retrovirus replication. A detailed characterization of the LTR variations which exist in viral populations will be valuable in understanding structure-function relationships and constraints on sequence variations. To amplify and analyze individual genomes, we constructed recombinant DNA clones of WN1802N MuLV and report here the characteristics, with

particular emphasis on the LTR region, of 15 isolates.

BALB/c MuLV WN1802N (N-tropic), SC-1 cells, and XC cells were originally obtained from W. P. Rowe and J. W. Hartley of the National Institutes of Health, Bethesda, Md. Covalently closed circular DNA of WN1802N was isolated from SC-1 cells 48 h after infection by a modification (31) of the procedure described by Hirt (8) and digested with the single-cut restriction endonuclease *Hind*III. This DNA was ligated to purified left and right terminal *Hind*III fragments of the lambda phage vector Charon 9 (5), packaged in vitro, and plated on *Escherichia coli* DP50 Sup F. Of approximately 10,000 plaques, 50 hybridized with [³²P]cDNA (prepared from WN1802B MuLV 70S RNA [31]) by the plaque hybridization protocol described by Benton and Davis (1). Fifteen independent isolates were analyzed by *Hind*III digestion, gel electrophoresis, and DNA blot hybridization. This is shown in Fig. 1 (A and B). The insert size along with other information is given in Table 1. Seven of the clones (λWN7, λWN12, λWN26, λWN29, λWN30, λWN36, and λWN41) had an insert size which corresponded approximately to the complete MuLV genome containing two LTRs (8.8 to 9.1 kilobase pairs [Kbp]). Six of the clones (λWN1, λWN13, λWN18, λWN21, λWN22, and λWN27) had an insert which corresponded to the MuLV genome with a single LTR (8.2 to 8.3

FIG. 1. Analysis of *Hind*III insert fragment size of 15 MuLV-Charon 9 recombinant DNA clones. *Hind*III digests of cloned DNAs were subjected to electrophoresis in a submerged horizontal 0.7% agarose gel at 50 V for 16 h. The ethidium bromide-stained gel is shown in panel A and an autoradiograph of the Southern blot hybridized with WN1802B MuLV [³²P]cDNA is shown in panel B. Molecular size markers in outside lanes (M) are *Hind*III fragments of bacteriophage lambda DNA. The number above each lane designates the individual λ WN isolate.

Kbp). Clone λ WN1 contained an additional 5.8-Kbp fragment of unknown origin which did not hybridize with the viral probe. Two of the clones did not have inserts of the two categories noted above. One (λ WN33) had a 7.4-Kbp insert, presumably a genome which had a deletion, and the other (λ WN3) had a 12-Kbp insert due to a 3-Kbp duplication or insertion. λ WN3 was apparently unstable, resulting in minor subpopulations with different insert sizes.

To further characterize these clones, *Hind*III-digested DNA was religated and tested for infectivity by DNA transfection on NIH-3T3 cells, and the results are shown in Table 1. Approximately half of the isolates were infectious, including single- and double-LTR-containing clones. With the exception of λ WN33 with a 0.8-Kbp deletion, it is not known what contributed to the lack of infectivity of any clone.

It was surprising to find that λ WN3, the clone containing a 3-Kbp insertion or duplication, was infectious. This observation was made with DNA from two independent lysates of the plaque-purified isolate (as had all 15 clones) and DNA from five different plaque-purified isolates from the original plaque of λ WN3. The correct uninterrupted gene order was possible if the insertion or duplication was located between the

LTRs, and the preliminary mapping was consistent with this arrangement.

More detailed analysis by using an LTR-specific probe and restriction enzymes which cut within the LTR (*Pst*I and *Kpn*I) and ones which cut close to but outside the LTR (*Xba*I plus *Pvu*I) confirmed which clones had one or two LTRs and revealed a variability in LTR size among these clones (Table 1). The map positions where these restriction enzymes cleaved the MuLV insert are shown in Fig. 2. Nine of the 15 clones had an LTR of approximately 520 bp. Among the remaining clones, we found three different sizes: 570,600 (three isolates), and 670 bp. The LTR size of the deletion clone (λ WN33) was not determined. Restriction fragments which did not include the LTR did not exhibit this variability among clones (not shown).

Recent observations of LTR differences in endogenous provirus and exogenous isolates (17, 27) led us to believe that the more abundant small class we observed may be inherited from the endogenous provirus and the larger sizes due to insertions or duplications which occur during exogenous replication.

Seven of the nine clones of WN1802N which had a 520-bp LTR were infectious. Among the five which had a larger LTR (three distinctly

TABLE 1. Analysis of clones by restriction endonuclease digestion and DNA transfection

λWN Clone	Restriction endonuclease fragment size (Kbp) ^a						No. of LTR	Size of LTR (bp)	Infectivity ^d
	<i>Hind</i> III	<i>Pst</i> I ^b	<i>Kpn</i> I + <i>Hind</i> III ^c			<i>Xba</i> I + <i>Pvu</i> I			
			Fragment 1	Fragment 2	Fragment 3				
18	8.2	—	1.35	—	2.5	1.0	1	520	+
22	8.2	—				1.0	1	520	+
27	8.2	—				1.0	1	520	—
26	8.8	0.52				1.5	2	520	+
29	8.8	0.52				1.5	2	520	+
30	8.8	0.52				1.5	2	520	+
36	8.8	0.52	1.35	0.52	2.5	1.5	2	520	—
41	8.8	0.52	1.35	0.52	2.5	1.5	2	520	+
3	12 ^e	3 ^f				1.0, 3 ^f	2	520	+
1	8.3 ^g	—	1.38	—	2.5	1.1	1	570	—
13	8.3	—	1.43	—	2.5	1.1	1	600	+
21	8.3	—				1.1	1	600	—
12	8.9	0.60	1.43	0.60	2.5	1.6	2	600	—
7	9.1	0.67	1.48	0.67	2.5	1.7	2	670	—
33	7.4	—				— ^h	1	ND ⁱ	—

^a Southern blot (26) of *Hind*III digest was hybridized with [³²P]cDNA prepared from WN1802B MuLV 70S RNA. All others were hybridized with ³²P nick-translated LTR subclone (527-bp *Pst*I fragment) of pRFV105, a plasmid clone of the endogenous ecotropic virus from an RFM/Un mouse.

^b Only the low-molecular-weight *Pst*I fragment (diagnostic of double LTR genomes) is given. A dash (—) indicates no comparable fragment from single LTR clones.

^c The fragments which hybridized with the LTR-specific probe are listed in the left-to-right order as they occur in the map given in Fig. 2. A dash (—) indicates no comparable fragment from single LTR clones.

^d Transfection of NIH-3T3 cells was performed by a modification of the protocol of Graham and Van der Eb (7), as previously described (9). XC plaque assays (20) were conducted after each passage, and samples were scored as negative (—) if no plaques were observed by the third passage. Those exhibiting XC plaques were scored positive (+). Assays were performed on at least two independent DNA preparations of each clone.

^e This clone existed as a mixed population, presumably due to the instability of the rearrangement (insertion or duplication) which increased its size.

^f The 3-Kbp fragment suggests that the two LTRs are not joined in the typical fashion but are separated by the 3-Kbp insertion or duplication.

^g An additional 5.8-Kbp fragment of unknown origin which does not hybridize with the probe was present in this clone.

^h The deletion in this clone included the *Xba*I site, thereby excluding a comparable *Xba*I + *Pvu*I LTR-containing fragment.

ⁱ ND, Not determined, due to uncertainty of extent of deletion.

different sizes), only one (λWN13, 600-bp LTR) was infectious. Although an interesting possibility, it remains to be determined whether the LTR variation is responsible for the lack of infectivity of these clones. Molecular clones of WN1802B and Gross leukemia virus, which have LTRs of 580 and 560 bp, respectively, are infectious (unpublished results). Sequence variations have been reported which do not affect the viability of other isolates (18, 24, 30). The possibility that some sequence variations attenuate or are lethal to the virus still remains. We are currently testing the activity of the large LTRs from the noninfectious clones by restriction fragment exchange experiments. It is also possible that some variants have a selective advantage which accounts for their frequent occurrence.

Plasmid subclones were constructed to facilitate more detailed analysis of pWN7, pWN12, and pWN41, with double LTR genomes of 670,

600, and 520 bp, respectively. The *Pst*I fragment which consisted of a portion of the right and left LTR and contained the circle junction was recovered from polyacrylamide and labeled with 3'-[α-³²P]dATP at the 3' ends. This was followed by digestion of a portion with *Sma*I, which cut in the R region, and electrophoresis and autoradiography. The result indicated that the smaller fragment, which included all of U5 and the circle junction region, was the same size in all three clones, whereas the larger fragment, which included most of the U3 region, contained the size variation. This fragment, ³²P labeled at the *Pst*I site, was recovered from the gel and further analyzed by partial digestion with *Hpa*II. The resulting map is shown in Fig. 3. The same regions of pWN7 and pWN12 differed relative to pWN41. Thus the size variations of LTR found in these WN1802N MuLV clones were not due to deletions at the junction as described in some

FIG. 2. The cleavage site of restriction endonucleases used in this study indicated in a schematic map of typical double and single LTR genomes. These sites were initially determined for WN1802B, the B-tropic isolate of BALB/c (C. Y. Ou, J. O. Kiggans, W. K. Yang, unpublished results), and RFV, the N-tropic endogenous virus of RFM/Un (R. S. Liou, L. R. Boone, J. O. Kiggans, D. M. Yang, T. W. Wang, R. W. Tennant, and W. K. Yang, submitted for publication). These sites have been confirmed with molecularly cloned DNA from these strains, and the WN1802N recombinant DNA clones have been shown to share the same sites. The map is drawn to illustrate the permuted gene arrangement due to cloning at the *HindIII* site, and the LTR size is the more abundant 520-bp class (see text). Cleavage sites: □, *HindIII*; ▽, *KpnI*; ○, *XbaI*; ●, *PstI*; ▼, *PvuI*.

molecular clones of avian retrovirus (4, 13, 15), but may be analogous to the spontaneous variation detected in the U3 region of spleen necrosis virus (24). It is striking that WN1802B (unpublished results), AK virus, and Moloney leukemia and sarcoma viruses (6, 30) have tandem duplications of various lengths in this same region, and Gross leukemia virus has a 36-bp insertion, a portion of which appears to be a duplication of sequences downstream (unpublished results). It is likely that the variation in the WN1802N isolates is the same class of alteration. It is unlikely that the variations are cloning artifacts, since they are present in both LTRs in those clones with two LTRs. It is very interesting that the 72-bp tandem repeat of Moloney murine sarcoma virus LTR has recently been shown to activate a simian virus 40 genome from which a 72-bp tandem repeat had been removed (16). The simian virus 40 repeat has an important activator or enhancer role in gene expression, and it is postulated that repeats in retrovirus LTRs have a similar role. This suggests one possible selective advantage for genomes with altered or duplicated activator sequences. The location of the variability reported here for the WN1802N genome is consistent with some involvement of these activator sequences; however, the heterogeneity suggests that the structural

FIG. 3. Comparison of fine structure map of three different-sized LTRs. Schematic representation is the LTR region of RFV (from pRFV105) (Liou et al., submitted for publication) which has no large sequence duplication in the U3 region. Above the basic LTR is indicated the region which is duplicated (tandem 53-bp direct repeat) in WN1802B MuLV (pWNB5) (unpublished observations). Below the schematic is indicated the region in which a 36-bp sequence is inserted in Gross leukemia virus (pGLV100) (unpublished observations). The arrows indicate that the left portion of the insert (open box) is a duplication of sequences downstream. The large ^{32}P -labeled *PstI*-*SmaI* fragment was subjected to *HpaII* partial digestion to order the fragments. The map of pWNB5 is identical to that of pRFV105. Dotted lines indicate which restriction sites are assumed to be the same.

constraints may not be rigid. These LTR variants are currently being tested for relative activator or enhancer activity.

The mechanism which generates these sequence alterations, which are detected as restriction fragment size variations, is unknown. This may simply be a consequence of faulty reverse transcription which is not tolerated in other regions of the genome. Models of reverse transcription-recombination (3, 12, 14) could account for duplication or deletion if switches in template (RNA or DNA) occurred out of register. We are currently attempting to determine the rate at which these LTR size variations accumulate and stabilize in virus populations and the biological consequences. Continued work in this area may lead to a better understanding of the structure-function relationships in this portion of the viral genome which is vital to many aspects of the retrovirus life cycle.

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LITERATURE CITED

1. Benton, W. D., and R. W. Davis. 1977. Screening λ gt recombinant clones by hybridization to single plaques in situ. *Science* 196:180-182.
2. Berns, A. J. M., M. H. T. Lai, R. A. Bosselman, M. A.

- McKenna, L. T. Bachelier, H. Fan, E. C. R. Maandag, H. v.d. Putten, and I. M. Verma. 1980. Molecular cloning of unintegrated and a portion of integrated Moloney murine leukemia viral DNA in bacteriophage lambda. *J. Virol.* 36:254-263.
3. Coffin, J. M. 1979. Structure, replication, and recombination of retrovirus genomes: some unifying hypotheses. *J. Gen. Virol.* 42:1-26.
 4. DeLorbe, W. J., P. A. Luciw, H. M. Goodman, H. E. Varmus, and J. M. Bishop. 1980. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. *J. Virol.* 36:50-61.
 5. De Wet, J. R., D. L. Daniels, J. L. Schroeder, B. G. Williams, K. Denniston-Thompson, D. D. Moore, and F. R. Blattner. 1980. Restriction maps for twenty-one Charon vector phages. *J. Virol.* 33:401-410.
 6. Dhar, R., W. L. McClements, L. W. Enquist, and G. F. Vande Woude. 1980. Nucleotide sequences of integrated Moloney sarcoma provirus long-terminal repeats and their host and viral junctions. *Proc. Natl. Acad. Sci. U.S.A.* 77:3937-3941.
 7. Graham, F. L., and A. J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
 8. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
 9. Hsu, I.-C., and W. K. Yang. 1977. DNA transfection of ecotropic murine leukemia viruses in mouse cell cultures. *Cancer Res.* 37:1709-1714.
 10. Hsu, T. W., J. L. Sabran, G. E. Mark, R. V. Guntaka, and J. M. Taylor. 1978. Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. *J. Virol.* 28:810-818.
 11. Hughes, S. H., P. R. Shank, D. H. Spector, H.-J. Kung, J. M. Bishop, H. E. Varmus, P. K. Vogt, and M. L. Breitman. 1978. Proviruses of avian sarcoma virus are terminally redundant, co-extensive with unintegrated linear DNA and integrated at many sites. *Cell* 15:1397-1410.
 12. Hunter, E. 1978. The mechanism for genetic recombination in the avian retroviruses. *Curr. Top. Microbiol. Immunol.* 79:295-309.
 13. Ju, G., and A. M. Skalka. 1980. Nucleotide sequence analysis of the long terminal repeat (LTR) of avian retroviruses: structural similarities with transposable elements. *Cell* 22:379-386.
 14. Junghans, R. P., L. R. Boone, and A. M. Skalka. 1982. Retroviral DNA H structures: displacement-assimilation model of recombination. *Cell* 30:53-62.
 15. Katz, R. A., C. A. Omer, J. H. Weis, S. A. Mitsialis, A. J. Faras, and R. V. Guntaka. 1982. Restriction endonuclease and nucleotide sequence analyses of molecularly cloned unintegrated avian tumor virus DNA: structure of large terminal repeats in circle junctions. *J. Virol.* 42:346-351.
 16. Levinson, B., G. Khoury, G. Vande Woude, and P. Gruss. 1982. Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus. *Nature (London)* 295:568-572.
 17. Rands, E., D. R. Lowy, M. R. Lander, and S. K. Chattopadhyay. 1981. Restriction endonuclease mapping of ecotropic murine leukemia viral DNAs: size and sequence heterogeneity of the long terminal repeat. *Virology* 108:445-452.
 18. Rassart, E., L. DesGroseillers, and P. Jolicoeur. 1981. Molecular cloning of B- and N-tropic endogenous BALB/c murine leukemia virus circular DNA intermediates: isolation and characterization of infectious recombinant clones. *J. Virol.* 39:162-171.
 19. Rassart, E., and P. Jolicoeur. 1980. Restriction endonuclease mapping of unintegrated viral DNA of B- and N-tropic BALB/c murine leukemia virus. *J. Virol.* 35:812-823.
 20. Rowe, W. P., W. E. Pugh, and J. W. Hartley. 1970. Plaque assay techniques for murine leukemia viruses. *Virology* 42:1136-1139.
 21. Sabran, J. L., T. W. Hsu, C. Yeater, A. Kaji, W. S. Mason, and J. M. Taylor. 1979. Analysis of integrated avian RNA tumor virus DNA in transformed chicken, duck, and quail fibroblasts. *J. Virol.* 29:170-178.
 22. Shank, P. R., S. H. Hughes, H.-J. Kung, J. E. Majors, N. Quintrell, R. V. Guntaka, J. M. Bishop, and H. E. Varmus. 1978. Mapping unintegrated avian sarcoma virus DNA: termini of linear DNA bear 300 nucleotides present once or twice in two species of circular DNA. *Cell* 15:1383-1395.
 23. Shimotohno, K., S. Mizutani, and H. M. Temin. 1980. Sequence of retrovirus provirus resembles that of bacterial transposable elements. *Nature (London)* 285:550-554.
 24. Shimotohno, K., and H. M. Temin. 1982. Spontaneous variation and synthesis in the U3 region of the long terminal repeat of an avian retrovirus. *J. Virol.* 41:163-171.
 25. Shoemaker, C., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore. 1980. Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. *Proc. Natl. Acad. Sci. U.S.A.* 77:3932-3936.
 26. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
 27. Steffen, D. L., S. Bird, and R. A. Weinberg. 1980. Evidence for Asiatic origin of endogenous AKR-type murine leukemia proviruses. *J. Virol.* 35:824-835.
 28. Sutcliffe, J. G., T. M. Shinnick, I. M. Verma, and R. A. Lerner. 1980. Nucleotide sequence of Moloney leukemia virus: 3' end reveals details of replication, analogy to bacterial transposons, and an unexpected gene. *Proc. Natl. Acad. Sci. U.S.A.* 77:3302-3306.
 29. Van Beveren, C., J. G. Goddard, A. Berns, and I. M. Verma. 1980. Structure of Moloney murine leukemia viral DNA: nucleotide sequence of the 5' long terminal repeat and adjacent cellular sequences. *Proc. Natl. Acad. Sci. U.S.A.* 77:3307-3311.
 30. Van Beveren, C., E. Rands, S. K. Chattopadhyay, D. R. Lowy, and I. M. Verma. 1982. Long terminal repeat of murine retroviral DNAs: sequence analysis, host-proviral junctions, and preintegration site. *J. Virol.* 41:542-556.
 31. Yang, W. K., D.-M. Yang, and J. O. Kiggans, Jr. 1980. Covalently closed circular DNAs of murine type C retrovirus: depressed formation in cells treated with cycloheximide early after infection. *J. Virol.* 36:181-188.