Molecular Cloning of Viral DNA from Leukemogenic Gross Passage A Murine Leukemia Virus and Nucleotide Sequence of Its Long Terminal Repeat

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The viral DNA genome of the leukemogenic Gross passage A virus was cloned in phage Charon 21A as an infectious molecule. The virus recovered by transfection with this infectious DNA was ecotropic, N-tropic, fibrotropic, and XC+. It was leukemogenic when reinjected into newborn SIM mice, indicating that ecotropic murine leukemia virus (MuLV) from an AKR mouse thymoma can harbor leukemogenic sequences. Its restriction map was similar to that of nonleukemogenic AKR MuLV, its putative parent, but differed at the 3' end and in the long terminal repeat (LTR). The nucleotide sequence of the Gross A virus LTR was identical to the AKR MuLV LTR sequence (Van Beveren et al., J. Virol. 41:542–556, 1982) in U5, R, and part of U3. All differences between both LTRs were found in U3. Only one copy of the U3 tandem direct repeat was conserved in the Gross A virus LTR, and it was rearranged by the insertion of a 36-base-pair sequence and by five point mutations. Only one additional point mutation common to several oncogenic MuLVs was present in U3. These structural changes in the U3 LTR and at the 3' end of the genome may be related to the leukemogenicity of this virus.

AKR mice spontaneously develop thymic lymphoma at a very high frequency at 6 to 9 months of age (12, 17). Recombinant leukemogenic murine leukemia viruses (MuLVs), the mink cell focus-inducing (MCF) viruses, have been isolated from these preleukemic and leukemic AKR mouse thymomas (20) and are thought to be involved in the disease process (8, 10, 29). These are XC- and have a xenotropic or dual-tropic host range. However, the appearance of this disease seems to depend on the expression of endogenous ecotropic MuLV (23). Indeed, an ecotropic, N-tropic, fibrotropic, XC+ MuLV (AKR virus) is expressed in several tissues of these animals throughout their life spans (38), but it is nonleukemogenic (8, 28). Gross (14, 15, 17) first demonstrated the presence of a leukemogenic virus in cell-free extracts of AKR thymoma by inoculating them into C3H newborn mice. Serial passages of these C3H thymoma cell-free extracts in C3H mice enhanced the leukemogenic potential of this virus, which was designated Gross passage A virus (16). This preparation appears to contain a mixture of ecotropic, xenotropic, and recombinant dual-tropic MuLVs (10). One viral isolate of Gross passage A MuLV that was propagated and cloned in vitro on NIH cells retained its leukemogenic potential (6). This leukemogenic MuLV appears to be ecotropic, N-tropic, fibrotropic, and XC+. Fingerprint analysis of 70S genomic RNA from Gross A MuLV and AKR virus revealed major differences within their 3'-termina 1,000 nucleotides (5). Other leukemogenic ecotropic, N-tropic, fibrotropic, and XC- MuLVs have been isolated from lymphoid cell lines established from spontaneous AKR thymoma (28). Fingerprint analysis of their RNAs revealed that they were very similar to Gross A MuLV RNA at the 3' end (31, 32).

In view of the complexity of the different leukemogenic MuLVs isolated from AKR thymoma, and because some leukemogenic virus preparations might contain more than one type of MuLV, we decided to molecularly clone the DNA genome of the virus harboring the leukemogenic sequences of Gross A MuLV preparations. This allowed us to study its general organization. By restriction analysis, we found that its long terminal repeat (LTR) and the region adjacent to its LTR at the 3' end had different restriction sites than did the genome of AKR MuLV, its putative parent. Sequencing of the LTR revealed that all differences were located in the U3 region.

MATERIALS AND METHODS

Cells and viruses. The origin of NIH/3T3 cells has been given elsewhere (35). The Gross passage A MuLV was obtained from W. A. Haseltine (Sidney
Farber Cancer Institute) and was characterized previously (5, 6). It was propagated on NIH/3T3 cells. Subconfluent NIH/3T3 cells were infected in roller bottles at a multiplicity of infection of 1 to 2 in the presence of 8 μg of polybrene per ml as described earlier (35).

Preparation of viral DNA. Unintegrated viral DNA was prepared from the Hirt supernatant of newly infected NIH/3T3 cells 20 h after infection as described earlier (35). Supercoiled and linear viral DNAs were separated on propidium iodide-cesium chloride gradients essentially as described earlier (34, 35).

Agarose gel electrophoresis and transfer of DNA. Agarose gel electrophoresis and DNA transfer onto a nitrocellulose membrane were done by the technique of Southern (42) as described previously (36). Virus-specific DNA fragments or recombinant DNA transferred to nitrocellulose membranes were detected by hybridization with 32P-labeled B-tropic MuLV DNA cloned into pBR322 (34) as previously described (35).

Molecular cloning and screening of recombinants. Unintegrated supercoiled Gross A viral DNA extracted from the Hirt supernatant of 2 × 108 newly infected NIH/3T3 cells was used for cloning. It was linearized with HindIII, enriched on sucrose density gradients, and cloned in Charon 21A essentially as described earlier (4, 34).

Transfection assay. The cloned viral DNA fragments (5 μg) were excised from λ Charon 21A or pBR322 recombinants by cleavage with HindIII and then circularized by ligation with T4 DNA ligase (New England Biolabs) as previously described (34). Transfections were performed on NIH/3T3 cells with 0.2 to 0.5 μg of ligated viral DNA by a modification (47) of the original calcium phosphate precipitation method (13), as previously described (34). After transfection, cells were passaged every 3 days. Viral production in the supernatant was measured by the reverse transcriptase and XC assays (39) as previously described (34).

DNA sequencing. Appropriate restriction fragments were treated with alkaline phosphatase (Boehringer Mannheim Corp.) and labeled at the 5′ end with 600 to 900 μCi of (γ-32P)ATP (3,000 Ci/mmol, New England Nuclear Corp.) and 20 U of polynucleotide kinase (Bethesda Research Laboratories) as previously described (25). The same fragments were also labeled by filling out the 3′ ends with 50 to 100 μCi of one [α-32P]dNTP (3,000 Ci/mmol; New England Nuclear Corp.) and the Klenow fragment of PolI (11). This labeling reaction was performed in 20 μl of 50 mM potassium phosphate (pH 7.4)-5 mM MgCl2-10 mM dithiothreitol-50 to 100 μCi of one [α-32P]dNTP-0.5 U of Klenow fragment for 20 min at 37°C. End-labeled DNA fragments were cleaved with appropriate restriction endonucleases or strand separated and then isolated by polyacrylamide gel electrophoresis as previously described (25). The nucleotide sequence was determined by the procedure of Maxam and Gilbert (25).

RESULTS

Molecular cloning of Gross A MuLV DNA. To analyze the structure of Gross A viral DNA and choose the appropriate enzyme for molecular cloning, we first mapped several restriction sites on the unintegrated viral DNA genome. HindIII and SaI were found to cleave the genome only once (Fig. 1), and EcoRI and XbaI did not cleave it at all (Fig. 1). PstI generated an 8.1-kilobasepair (kbp) fragment and two shorter ones of 450 and 140 base pairs (bp) (Fig. 1), indicating the presence of additional PstI sites compared with the ecotropic AKR MuLV (33).

For molecular cloning, the two forms of unintegrated supercoiled viral DNA were cleaved with HindIII, enriched on sucrose density gradients, and ligated to HindIII-cleaved λ Charon 21A DNA (4 μg). Packaging of this ligated DNA generated 1.6 × 103 plaques. Recombinant phages containing a permuted viral insert were identified by hybridization with 32P-labeled B-tropic MuLV DNA. About 40 MuLV recombinants were obtained, and 5 were selected and

FIG. 1. Digestion of Gross A viral DNA with restriction endonucleases. In vivo circular (a–f) or linear (g–h) unintegrated viral DNA was digested with (lane) SaI (b), XbaI (c), PstI (d and h), EcoRI (e), HindIII (f), or PstI and PvuII (g). After digestion, samples were run on 1 (a–f) or 2.0% (g–h) agarose gels, transferred to nitrocellulose filters, and hybridized to 32P-labeled B-tropic MuLV DNA (a–f) or a 32P-labeled LTR-specific probe (g and h). In lane a, A and B represent undigested open circular and supercoiled viral DNAs, respectively. The minor SaI fragments (4.1 and 4.4 kbp) in lane b and the minor HindIII fragments (5.8 and 2.9 kbp) in lane f were generated by cleavage of the contaminating linear viral DNA (8.8 kbp).
further analyzed with HinIII to detect nondefective viral genomes. Two cloned DNA fragments of 8.2 kbp (with one LTR copy) representing the full-length genome were mapped with several restriction endonucleases. An example of some of these digestions for one DNA clone (GD-17) is shown in Fig. 2. For comparison, digestion of nonpermutated AKR clone 623 DNA (24), the genome of the putative nonleukemogenic AKR MuLV parent, is also shown, along with digestions of the permuted λN-20 DNA, the cloned genome of endogenous N-C1-35 BALB/c MuLV (34). The λN-20 and AKR MuLV genomes share the same restriction sites.

FIG. 2. Restriction endonuclease mapping of GD-17 Gross A viral DNA clone. (Top) Lanes: GD-17 Gross A λ recombinant DNA digested with HinIII (a and b); UV fluorescent photograph of ethidium bromide-stained agarose gel (a) and blot analysis of the gel (b); GD-17 Gross A λ recombinant DNA digested with HinIII-XbaI (c) or HinIII (d); SmaI digestion of GD-17 viral DNA (e) and of nonpermutated AKR clone 623 DNA (24). (f); permuted GD-17 viral DNA (g, i, k, m, o, and q) and BALB/c N-tropic (N-C1-35) MuLV cloned DNA (clone λN-20) (34) (h, j, i, n, p, and r) were first digested with HinIII and then cleaved with SmaI (g and h), PvuI (i and j), XhoI (k and l), KpnI (m and n), PstI (o and p), or BamHI (q and r). DNA fragments were subjected to 1.4% agarose gel electrophoresis, blotted onto nitrocellulose membranes, and hybridized with 32P-labeled B-tropic MuLV DNA as described in the legend to Fig. 1. GD-17 viral DNA was cleaved with PstI (t) and subjected to 5% polyacrylamide gel electrophoresis (25) with HincI-digested φX174 DNA as a marker (s). (Bottom) Restriction maps of the BALB/c N-tropic MuLV DNA clone (34), the putative parental AKR virus DNA genome (33), and the Gross A virus GD-17 DNA clone are shown. The open blocks represent the LTR. AKR clone 623 DNA has lost its HinIII site, although it was present on the uncloned viral DNA genome of the endogenous AKR MuLV (33). The arrow shows the 135-bp PstI fragment. B, BamHI; H, HinIII; K, KpnI; P, PstI; Pvu, PvuI; Pv, PvuII; S, SmaI; Sc, Saci; Sl, SalI; X, XbaI; Xh, XhoI.
for the 14 restriction endonucleases we tested (33, 35). As shown, the restriction map of the leukemogenic Gross A MuLV genome was similar but not identical to the map of ecotropic nonleukemogenic AKR virus or BALB/c N-tropic MuLV. Gross A virus DNA had an additional PsiI site in the U3 LTR but lost the XbaI site, and its LTR was shorter than that of the AKR virus. Its BamHI-HindIII fragment was also shorter (by about 50 bp) than the corresponding fragment of the N-tropic BALB/c genome (Fig. 2).

**Infectivity of clone GD-17 Gross A viral DNA.** The infectivity of the 8.2-kbp recombinant GD-17 viral DNA was tested by the calcium phosphate transfection procedure (13). The HindIII-digested and religated GD-17 cloned DNA was transfected onto NIH/3T3 cells and tested for its ability to generate infectious viral particles as detected by the XC assay (39) or the reverse transcriptase assay. It was positive in these assays after 6 to 10 days. The virus recovered after this transfection had the same biological characteristics as the parental MuLV: it was XC+, N-tropic, ecotropic, and fibrotropic. Its titer reached $5 \times 10^6$ PFU/ml.

**GD-17 Gross A viral DNA clone harbors leukemogenic sequences.** Gross A MuLV has been shown to be leukemogenic when inoculated into newborn mice of the low-incidence C3H strain (6, 16, 18). We first tested the leukemogenic potential of the uncloned Gross A MuLV in SIM mice, another low-incidence strain. Like the NIH/Swiss mice, SIM mice do not harbor endogenous ecotropic MuLV genome. Of 12 SIM mice inoculated intraperitoneally with $10^5$ PFU of this uncloned MuLV, 7 were leukemic after a 3- to 5-month latent period.

To determine whether the GD-17 Gross viral DNA clone harbored sequences responsible for leukemogenicity, we determined titers for the virus recovered after transfection with GD-17 viral DNA by the XC assay (39) on NIH/3T3 cells, and $10^3$ PFU were injected intraperitoneally and intrathymically into 15 newborn SIM mice. Despite this low virus titer, 5 of 15 mice were leukemic after a latent period of 7 to 8 months and had markedly enlarged thymuses and spleens. This result indicates that the ecotropic MuLV genome that we cloned harbors the sequences responsible for the leukemogenic potential of Gross A MuLV.

**DNA sequence of Gross A virus LTR.** Because the major differences between the nonleukemogenic AKR virus genome and leukemogenic Gross A virus genome lay in the LTR, and because of the critical role the LTR plays in the promoter insertion mechanism of oncogenesis by nonacute avian leukosis virus (21, 27, 30), we determined the nucleotide sequence of this re-
FIG. 4. Sequence of Gross passage A virus LTR. The upper line shows the sequence of the Gross A virus LTR and its flanking region. For comparison, the sequence of the AKR viral LTR clone 614 reported previously (46) is shown on the lower line. The major structural features of this lower line are indicated.

region by the Maxam and Gilbert technique (25). The GD-17 viral DNA insert was first subcloned into pBR322 at the HindIII site. This cloned viral DNA (pGD-17) retained its infectivity. Appropriate fragments were then prepared from this DNA clone by digestion with restriction endonucleases. Figure 3 shows the strategy used to sequence the various restriction fragments, and Fig. 4 shows the nucleotide sequence obtained. For comparison, the AKR virus LTR nucleotide sequence published previously (46) is also shown. The Gross A virus LTR was 563 bp long. Nucleotides 309 to 626, representing U3, R, and part of the U3 region, were identical to the corresponding AKR virus sequences. These include the 13-bp-long inverted repeat at the termini of the LTR, TATA-like sequences, CCAAT box, and polyadenylate addition signals (AATAAA). The major differences between these LTRs lay in the 99-bp tandem repeat. The Gross A virus LTR had only one copy of it, and it seemed to be rearranged. A new 36-bp sequence (containing the new PstI site) had been inserted. Part of this insertion formed an almost perfect direct repeat with another sequence found downstream (position 183). The inserted sequence CAGACACTGAGCA showed good homology with the U3 LTR sequence CAGACACTGAGCA from spleen necrosis virus (40), an avian retrovirus that has been shown to have homology with mammalian retroviruses (1, 2). Other parts of U3 were identical to the AKR virus LTR except for six point mutations at positions 65, 122, 123, 162, 198, and 202 which differentiated both genomes. The 74-bp-long sequence at the right of the LTR comprised the tRNAPro primer-binding site and the putative consensus 5' donor splice sequence (AGGTAAG) (41). It differed from the Moloney MuLV DNA sequence (41) by only five bases at positions 646, 656, 679, 680, and 701. Among the possible frames of translation in the 5'-3' direction for a putative protein encoded by the Gross A virus LTR, one is for a protein of 32 amino acids with a carboxyl terminus coded by the new 36-bp sequence, and the other is for two proteins of 45 and 33 amino acids, respectively. The amino terminus of this 33-amino acid peptide would also be encoded by the new 36-bp sequence.

DISCUSSION

From a stock of leukemogenic Gross A MuLV, we cloned an infectious viral DNA molecule which generated an ecotropic, N-tropic, fibrotropic, and XC+ MuLV after transfection. Like the parental MuLV, the virus recovered was leukemogenic, indicating unambiguously that the ecotropic MuLV component of Gross A MuLV stock harbors sequences responsible for leukemogenicity. Lately, experimental evidence was presented that MCF MuLV plays a role in the development of AKR leukemia (10, 20, 29), and XC+, dualtropic viruses related to the MCF MuLV were isolated from Gross passage A virus extracts (10). Our results do not favor an etiological role for any putative MCF MuLV that
may have contaminated our original Gross A MuLV stocks. The leukemogenicity of these stocks was most likely provided by the presence of the leukemogenic ecotropic, N-tropic MuLV, the genome of which we have cloned. This ecotropic, N-tropic MuLV, which harbors leukemogenic sequences, could trigger the transformation process by itself, for example by more efficient replication in target cells or by a promoter insertion mechanism (21, 27, 30). Alternatively, its specific sequences might promote easier recombination with endogenous sequences to generate recombinant viruses, possibly MCF MuLV. These could be more directly responsible for the development of the disease. It has recently been suggested, indeed, that the induction of leukemia by Gross passage A ecotropic MuLV involved the generation of a dual-tropic (MCF-type) recombinant MuLV (10).

The restriction map of leukemogenic Gross A viral DNA was strikingly similar to that of the nonleukemogenic AKR MuLV genome (33) but was not identical, most notably in the region corresponding to the 3' end of the RNA genome, confirming earlier results obtained by T1 oligonucleotide mapping (5). Although several changes could be observed at the 3' end and in the U3 LTR of the Gross A MuLV genome as compared with its putative nonleukemogenic AKR MuLV parent, we have not yet established that these modifications are related to the leukemogenic potential of this virus. Modifications which might be present on other regions of the Gross A MuLV genome might have escaped detection with restriction endonucleases, and these regions of the genome might also contribute to its leukemogenic potential. Indeed, changes have previously been detected by T1 oligonucleotide fingerprinting in the gag-pol and the env regions (5). We also detected a shorter BamHI-HindIII fragment in the Gross A gag region (Fig. 2). Robinson et al. (37) recently reported that at least two regions of the viral genome contribute to the oncogenic potential of avian leukemia viruses. Until further work is performed in vitro-constructed recombinant MuLV to localize unambiguously the "leuk" region, the contribution of these modifications to the disease process will remain unclear. However, the association of specific 3'-end T1 oligonucleotides with the leukemogenic potential of several AKR ecotropic MuLV isolates has been well documented (31, 32), and studies with recombinant avian retroviruses suggest that the U3 region of the LTR is involved in the oncogenic potential of these viruses (37).

In this regard, our nucleotide sequence for the Gross A virus LTR revealed a structure having all the characteristics of other retrovirus LTRs (44). This sequence was identical to the AKR virus LTR sequence (46) in U5, R, and most of U3, indicating the noninvolvement of these sequences in the leukemogenic process. Six point mutations were detected in the Gross A virus U3 LTR as compared with that of the AKR virus. Moreover, mutation at position 65, which has been recognized before in T1 oligonucleotide 36B (AAAAATACCAAG) (5), was found to correlate with leukemogenicity in several other ecotropic AKR MuLVs (31, 32). The sequence (AAAAATAC) is also present in the LTR of oncogenic Friend spleen focus-forming virus (7), leukemogenic Moloney MuLV (41, 46), and one leukemogenic BALB/c MuLV (Des Grosellers, Rassart, and Jolicoeur, manuscript in preparation). Such close correlation of this point mutation with the oncogenic potential of several MuLVs might be of biological importance. Our sequencing data provided its exact location in U3.

Our data also showed that sequences in the 99-bp tandem repeat of AKR MuLV, the putative parent, have been significantly rearranged in the Gross A virus LTR. Similar tandem repeats of various length have been observed in other murine (9, 41, 46) and avian (40) retroviral LTRs and in simian virus 40 (19). Their function seems to be primarily to enhance transcription (22, 26). However, some murine LTRs were found to contain only one copy of the sequences forming the repeat (43, 45, 46), and this loss of one copy of the repeat did not seem to affect their infectivity (3). In simian virus 40 the function of the tandem repeat was also not affected by loss of one of its copies (19, 26). Only one copy of this repeat has been conserved in the Gross A virus LTR, and it has been rearranged by the insertion of a 36-bp sequence and by five point mutations. The origin of this inserted sequence is unknown, but it probably arose by recombination of the putative AKR MuLV with endogenous virus-related sequences. The other point mutations could have been present on the endogenous parental provirus or could have arisen independently. This insertion includes the T1 oligonucleotide 215 (ACCACTCAGT) previously described as being unique to Gross A MuLV (5). We do not know yet whether these sequences are involved in promoting the development of leukemia, but this rearranged segment may have a more potent enhancing effect on the transcription of viral genes or adjacent cellular oncogenes, as was shown for nonacute avian leukemia viruses (21, 27, 30).

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