Determinants of Thymotropism in Kaplan Radiation Leukemia Virus and Nucleotide Sequence of Its Envelope Region

LAURENT POLIQUIN, DOMINIQUE BERGERON, JEAN-LUC FORTIER, YVES PAQUETTE,* RICHARD BERGERON, AND ERIC RASSART*

Département des Sciences Biologiques, Université du Québec à Montréal, C.P. 8888, Succursale A, Montréal, Québec, Canada H3C 3P8

Received 24 January 1992/Accepted 4 May 1992

RadLVs are a group of murine leukemia viruses which are induced by radiation and cause T-cell leukemia. Viral clones isolated from the BL/VL3 lymphoid cell line derived from a thymoma show variable tropism and leukemogenic potential. We have constructed chimeric viruses by in vitro recombination between two viruses, a RadLV that is thymotropic and an endogenous ecotropic virus that is nonthymotropic. We show here that, in contrast to thymotropism determinants identified previously, which lie in the long terminal repeat (LTR), it is the envelope region that is responsible for the thymotropism of BL/VL3 RadLV. The nonthymotropic virus which we have rendered thymotropic by transfer of the env region of RadLV in the present study has been shown previously to become thymotropic when the LTR of another thymotropic virus is inserted in its genome. Thus, the LTR and envelope gene may be involved in complementary action to lead to thymotropism.

The radiation leukemia virus (RadLV) was first isolated by Kaplan after induction of T-cell lymphoma in C57BL/6 mice, following fractionated irradiation (17, 18). Although the initial isolate was only weakly leukemogenic (17), propagation in C57BL/6 mice gave rise to a virus mixture which was highly leukemogenic (18) and selectively thymotropic, both in vivo and in vitro (3, 4, 22), and which grew poorly on fibroblasts and lost its leukemogenicity when propagated on mouse fibroblasts (24).

From thymomas induced by highly leukemogenic, in vivo-passaged RadLV, lymphoid cell lines were established (23). One of them, BL/VL3, was shown to produce the same type of virus mixture as the parental RadLV (3, 23). Individual ecotropic viral genomes were cloned from BL/VL3 (33) and were shown to be highly leukemogenic, thymotropic, and unable to replicate on mouse fibroblasts in vitro, like the parental passaged RadLVs (34). The inability to grow on fibroblasts in vitro was shown to be related to the long terminal repeats (LTR) of BL/VL3 RadLVs (32). Differences between proviral genomes of primary, nonthymotropic (Ti-8 and Ti-9) and passaged, thymotropic (BL/VL3) clones were shown to exist in the envelope region, as well as in LTR, by use of specific probes (34). In order to better identify the determinants of thymotropism in the BL/VL3 RadLVs, we have constructed recombinant viral DNA molecules between one BL/VL3 thymotropic clone [BL/VL3(V13)] and a nonthymotropic endogenous BALB/c virus (clone p75-2) (7). In contrast to what has been found for other thymotropic murine leukemia viruses (MuLVs), in which the LTR has been shown to carry the sequences responsible for thymotropism (7, 14), we show here that the determinants for BL/VL3(V13) RadLV thymotropism reside in the envelope region of its genome. Since this p75-2 clone has also been shown previously to be rendered thymotropic by the LTR of another thymotropic virus (BC111) (7), the LTR and envelope gene may be involved in complementary action to lead to thymotropism.

Constructions of viral chimeras. The parental viral genomes used to construct chimeric recombinants were those of BL/VL3(V13) RadLV and a recombinant clone of BALB/c endogenous ecotropic virus, clone p75-2, both described previously (7, 34). BL/VL3(V13) is a molecular clone derived from the BL/VL3 lymphoid cell line. It is B tropic, highly leukemogenic, and selectively thymotropic (T') (34). The p75-2 recombinant MuLV was constructed from endogeneous, ecotropic, B- and N-tropic viruses (5, 7). It is nonthymotropic (T-) and B tropic, thus allowing both the parental and the recombinant viruses to be tested on Fv-1/B6 cells and mice.

Recombinant DNA molecules were constructed by ligating appropriate DNA restriction fragments purified from the cloned parental genomes, and chimeric infectious viruses were recovered after electroporation on Ti-6 cells (30, 34). Three such chimeric viruses obtained in the present study are depicted in Fig. 1E. The first construct, p75-2/VL3-XoK, inserts the LTR, the env gene, and part of the pol gene from BL/VL3(V13) into the p75-2 genome. A second recombinant, labeled p75-2/VL3-XoK, inserts the 3' part of the pol gene and most of the env gene from BL/VL3(V13) into the p75-2 genomic background. The reciprocal recombinant, VL3/p75-2-XoK, in which the pol and env genes from p75-2 are inserted into the V13 genome, was also constructed. In Fig. 1, DNAs from cells chronically infected with the various viruses (parental or recombinant) were digested with KpnI, PstI, and SacI. These sites, as illustrated in Fig. 1E, identify the origin of each genomic region of interest, allowing the confirmation that the desired chimeras were effectively obtained. Thus, the origin of the 3' half of the proviral genomes could be determined after KpnI digestion and hybridization with the gp70 ecotropic MuLV-specific probe (1, 11), in which detection of a 3.4-kbp fragment in BL/VL3(V13) and in the VL3/p75-2-XoK recombinant indicates...
the presence of a second KpnI site of V13 origin just 5’ of the XhoI site used in the construction but detection of a 3.9-kbp fragment in p75-2 and in the p75-2/VL3-XoX and p75-2/VL3-XoK recombinants indicates the p75-2 origin (Fig. 1A). The presence of a 3.9-kbp band in lanes b and d of Fig. 1A is due to the endogenous ecotropic provirus in Ti-6 cells (also detected in Fig. 1B as a 12-kbp band). In Fig. 1B, detection of a 3.7-kbp SacI fragment in the p75-2/VL3-XoX and p75-2/VL3-XoK recombinants (lanes c and e) confirms the origin of the 5’ and 3’ portions of their genomes from p75-2 and V13, respectively. The hybridization of a BL/VL3 U3 LTR-specific probe (34) to a 1.35-kbp KpnI restriction fragment allowed the identification of the recombinants containing the LTR of BL/VL3 in their structure (VL3/p75-2-XoX and p75-2/VL3-XoK) (Fig. 1C, lanes c and d). The same probe hybridized to an 8.2-kbp band in the PstI digests (Fig. 1D), confirming the V13 origin of the 5’ LTR on these recombinants. The presence of additional bands in Fig. 1C at positions of 5.35 and 3.9 kbp and in Fig. 1D at positions of 7.5 and 4.6 kbp is due to the detection of endogenous LTR-containing sequences (31). Thus, all of the chimeric viruses had the expected structure. Three more recombinants described previously (32) were tested in the present study. Briefly, they consist of the p75-2 genome in which the LTR was replaced with VL3 XbaI-KpnI or PstI-KpnI fragments (p75-2/VL3-XK and p75-2/VL3-PK, respectively) or the reciprocal recombinant in which the LTR PstI-PvuI fragment from p75-2 was inserted into the VL3 genome (VL3-pBR7) (see Table 1).

**Thymotropism of parental and chimeric MuLVs.** To identify the viral determinants responsible for the thymotropism of BL/VL3(V13), the parental and recombinant viruses were injected intraperitoneally into newborn C57BL/6 (Fv-Jbb) mice to measure their replication potential in the thymus. Twenty days after injection, the mice were sacrificed and thymocytes were plated on indicator cells. Appearance of reverse transcriptase activity in the culture supernatant was monitored (16). The results of these experiments are given in Table 1. As described earlier (7, 34), the parental viruses, BL/VL3(V13) and p75-2, are thymotropic (T+), and nonthymotropic (T−), respectively. Because previous studies have demonstrated that numerous retroviruses owe their ability to grow on thymocytes to the properties of their LTR sequences (7, 14) and that these sequences are also often related to the pathogenesis of the viruses (8, 12, 19), we first directed our analysis toward chimeric recombinants involving exchanges of the LTR sequences between thymotropic (T+) and nonthymotropic (T−) parental viruses. Clearly, in the case of BL/VL3(V13) RadLV, the LTR does not solely account for its thymotropism. Indeed, the first three recombinants studied placed the LTR (p75-2/VL3-PK) or the LTR plus the COOH-terminal portion of p15E (p75-2/VL3-XK) of T+ V13 into the background of T− p75-2, or reversely the LTR of T− p75-2 in the background of T+ V13 (VL3-pBR7). In each case, the chimera produced maintained the thymotropic phenotype corresponding to the parent from which the env portion of the genome was derived, the exchange of LTRs not affecting it in any way (Table 1). The sequences from the LTRs of both viruses, T− p75-2 and T+ BL/VL3(V13), have been obtained previously (7, 32). They show important differences in the U3 region, namely within the 99 bp that is directly repeated in tandem in Akv (20). p75-2 has a single copy of it, and BL/VL3 has three 43-bp direct repeats separated by two intervening 11-bp sequences which are also perfect direct repeats. These differences in the organization of the LTRs may play a role in the leukemogenicity of the viruses (34) but obviously do not explain the thymotropic differences.

However, when we turned to chimeras which exchanged parts of the central genome, it became clear that the envelope region is a determinant of BL/VL3(V13) thymotropism; p75-2/VL3-XoK, which has both the envelope region and the LTR from V13, is thymotropic, as well as p75-2/VL3-XoX, which contains only the envelope region from V13, in the p75-2 background. In contrast, VL3/p75-2-XoX, which is all V13 except for the envelope region, is nonthymotropic (Table 1). The BL/VL3(V13) genomic region encompassed by the XhoI and XbaI restriction sites (positions 4098 and 7345 of the genome, respectively) is therefore necessary and sufficient to confer thymotropism upon p75-2. In addition to this primary determination by the env region, one can note that the thymotropic effect is further strengthened when the LTR (in p75-2/VL3-XoK) or the gag-pol region (in VL3/pBR7) from V13 is transferred along with env (100% of positive mice, shorter delay; Table 1).

**Sequence of the envelope region of BL/VL3(V13).** The nucleotide sequence of the BL/VL3(V13) fragment identified above as being responsible for its thymotropism was deter-
TABLE 1. Thymotropism assays for the parental and recombinant viruses

<table>
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<tr>
<th>Recombinant</th>
<th>Ratio of positive mice to mice injected*</th>
<th>Delay in appearance of RT (days)*</th>
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<tr>
<td>P K</td>
<td>Xo X P K</td>
<td>BL/VL3(V13)</td>
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<td></td>
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<td>7/9</td>
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<td>VL4/p75-2-XoX</td>
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* The thymotropism tests were conducted as described in Material and Methods.

* All mice were sacrificed at 3 weeks. The delay represents the time after the thymocytes were put in culture, until the reverse transcriptase (RT) assay was positive (>2 times the background).

This region covers not only the env gene but also the COOH-terminal portion of the polymerase protein and the integrase protein (21, 37). Therefore, we cannot state definitively that the envelope protein itself is responsible for the thymotropism, although several reasons tend to point to this interpretation rather than the polymerase. The polymerase gene is better conserved, and the envelope has also been shown to play a role, if not in tropism, at least in pathogenesis, in certain systems (12, 13, 28, 35). When we sequenced the region involved in thymotropism determination in our assays, we indeed found several changes in the pol gene. We found that the envelope amino acid sequence is well conserved between BL/VL3(V13) and pMolS02 viruses (Fig. 3). This does not come as a surprise, since they are both T* and they both originate from the BL/VL3 cell line (15, 34). Still, O’Neill (29) has found that they do not bind to BL/VL3 cells with the same efficiency, indicating that their envelopes do differ. In any case, finer recombinants and site-directed mutants will have to be tested before we can demonstrate conclusively that the envelope is directly responsible.

The nonthymotropic property of p75-2 cannot be explained by an inability to enter these cells due to its envelope protein, since a recombinant of p75-2 containing only the LTR from the BALB/c endogenous, T* BC111 virus can replicate in the thymus (7). The envelope protein of p75-2 therefore cannot be the only factor responsible for its inability to replicate in the thymus.

Since the LTR of BL/VL3(V13) does not by itself confer thymotropism to a nonthymotropic virus, although it certainly functions in thymocytes because V13 is thymotropic, and since p75-2 can be rendered thymotropic by replacement of either its LTR (with that of BC111) or its envelope [with that of BL/VL3(V13)], one has to think of a mechanism in which the right combination of envelope, LTR, and target cell must coexist for replication in the thymus. Since there are many different T-cell subsets in the thymus at various stages of maturation, either expressing or shutting off expression of several cell surface proteins (for a review, see reference 9), it is possible that the envelopes of p75-2 and VL3 recognize and bind to receptors on different types of T cells. If, for example, the p75-2 envelope binds to a T cell in which its LTR promoter is inactive, replacing the LTR with one that is active in that cell (e.g., BC111) would render that recombinant virus thymotropic. Alternatively, exchanging the envelope protein of p75-2 with that of V13 could allow it to enter a cell type in which its LTR is now active, again making it thymotropic. There is also the possibility that

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FIG. 2. Nucleotide sequence of the VL3 DNA from the XhoI site in the polymerase gene to the beginning of the LTR. The translation into polymerase and envelope proteins is indicated above the nucleotide sequence, as identified at the left. The XhoI site at the very beginning and the XbaI site used in the construction of chimeric viruses are indicated.

some intracellular step requires interaction between the envelope and the LTR or between some envelope-induced intracellular modification and the LTR, but that seems less likely, since no such effect has been observed in any murine retroviruses studied to date.

It will also be interesting to examine the relationship between tropism and leukemogenicity in our system. Although there is a definite link between tropism and leukemogenicity, it has been shown that they can be distinguished in some systems (2, 12, 13, 26). Involvement of more than one gene, including the envelope, in the leukemogenic potential has also been seen in several other systems (6, 12, 27, 38). Our preliminary tests also point toward that direction. In this case, the interaction(s) alluded to above could represent sequential events in multistep carcinogenesis.

With the RadLVs, we have a full complement of variants from the fibrotropic (F'), T' nonleukemogenic (L') virus to F'T' L', to F'T' L'++, and finally to F'T' L'++ (34).
permutating envelopes and LTRs between these various viruses and testing for both tropism and leukemogenicity, we hope to be able to shed some light on the various mechanisms involved.

**Nucleotide sequence accession number.** The nucleotide sequence presented in Fig. 2 is available through GenBank, accession number M93052.

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**REFERENCES**


