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# Deletions in a Recombinant Retrovirus Genome Associated with Its Expression in Embryonal Carcinoma Cells

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**We analyzed embryonal carcinoma cell lines infected with a recombinant Moloney murine leukemia virus. Lines that carried but did not express the *neo* gene retained a provirus of LTR-*gag-pol-neo*-LTR, where LTR is a long terminal repeat, whereas all G418-resistant lines deleted regions that included the primer binding site and the splicing donor site. This suggested the presence of multiple inhibitory elements.**

Murine embryonal carcinoma (EC) cells are refractory to infection by viruses such as retroviruses and papovaviruses (7, 8, 11). Earlier, we isolated clonal EC cell lines that express an integrated *neo* gene introduced by a recombinant transducing Moloney murine leukemia virus (Mo-MuLV) (9). The *neo* gene in these EC cells was expressed by *cis*-acting mechanisms. When proviruses and their flanking cellular sequences were cloned and transfected into EC cells, the *neo* gene was expressed at significantly higher frequencies than the control long terminal repeat (LTR)-*neo* sequence alone, which indicated that the genetic signal(s) that allowed *neo* gene expression in EC cells resided in the cloned DNA segments. One such clone carried (upstream of the proviral genome) a cellular enhancer element that is active in EC cells (10), underscoring the thesis that the LTR enhancer does not function in EC cells (4).

To study the proviral structure, we first analyzed the genomic DNA of various cell lines, namely, six G418-resistant EC cell lines, four "silent transductant" lines (EC cell lines that carried but did not express the proviral *neo* gene), and four virus producer cell lines, by Southern hybridization. If the proviral genome retained the original structure of the plasmid construct placed into NIH 3T3 cells (9), a 4.7-kilobase (kb) *Sac*I band was expected to hybridize with a *neo* gene probe (see Fig. 2B). Instead, the virus producer cell lines Fvc 11 and Fvc 12 and two silent transductant EC cell lines showed bands of 5.2 kb (Fig. 1, lanes 10, 11, 8, and 9, respectively). Two other virus producer cell lines showed the 4.7-kb bands expected from the original structure (lanes 12 and 13). On the other hand, all G418-resistant EC cell lines, except clone H, showed single bands of various sizes (lanes 2 through 6). In clone H, two bands hybridized with the *neo* probe; one was at 5.2 kb and the other was at 2.3 kb (lane 7). These results raised several points. First, the *neo*-containing 5.2-kb bands of the virus producer cell lines and silent transductants were larger than expected from the original plasmid structure even though the thymidine kinase gene had been lost (9). Second, the *neo*-containing *Sac*I bands of various sizes in the G418-resistant EC cell lines strongly suggested rearrangements or point mutations in these proviral genomes or both. To investigate the first point, we determined the structure of the proviral genome isolated from the virus producer Fvc 12 cells and a silent transductant cell line by cloning, restriction endonuclease mapping, and DNA sequencing (data not shown). A homologous recombination that took place in the NIH 3T3 cell between the original *neo*-containing virus and the helper Mo-MuLV genomes generated a recombinant virus with the new genome structure of LTR-*gag-pol-neo*-LTR (Fig. 2).

To determine the proviral structure in the G418-resistant EC cell lines, the cloned DNA segments (10) were analyzed by Southern hybridization. Results with *neo* and LTR probes revealed that the 5′ LTR was lost in three of six clones and suggested that upstream cellular sequences recombined with the *neo* region of the proviral genomes (data not shown). We determined the deletion-recombination points between the cellular and proviral sequences by DNA sequencing. The break point in clone A was far upstream of the *neo* gene, beyond the *Hin*dIII site (Fig. 3). On the other hand, deletions in clones B and C took place immediately upstream of the *neo* gene (17 and 36 bases, respectively, from the *Eco*RI site). As predicted from the Southern analysis of genomic DNA (Fig. 1), there were internal deletions even in clones D, F, and H that retained both 5′ and 3′ LTR sequences (Fig. 3). In these three clones, the downstream ends of the deletions were in the same region of about 100 bases as in clones B and C, although none of them were at the same base. The upstream end of the deletion in clone D was inside the R region of the 5′ LTR (eight bases downstream from the cap site), whereas clones F and H had exactly the same deletion point (two bases upstream from

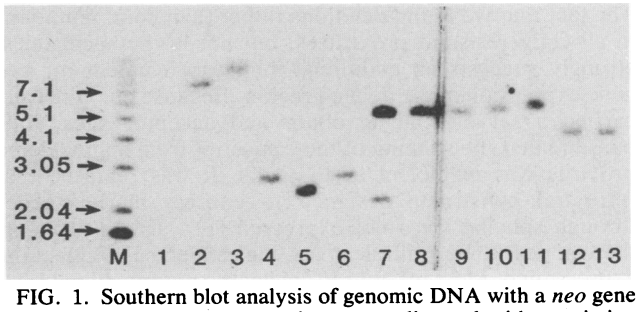


FIG. 1. Southern blot analysis of genomic DNA with a *neo* gene probe. Genomic DNA preparations were digested with restriction endonuclease *Sac*I and blotted after agarose gel electrophoresis. The filter was hybridized with a *neo* gene probe. Lane M, Size markers (in kilobase pairs); lane 1, parental EC cell line F9*tk*$^-$; lanes 2 through 7, G418-resistant EC cell lines A, B, C, D, E, and H, respectively; lanes 8 and 9, silent transductant EC cell lines SP-1 and SP-2, respectively (two other silent transductant EC cell lines, SF-1 and SF-2, showed the same band; data not shown); lanes 10 and 11, virus producer NIH 3T3 cell lines Fvc 11 and Fvc 12, respectively; lanes 12 and 13, virus producer NIH 3T3 cell lines Fvc 3 and Fvc 7, respectively (10).

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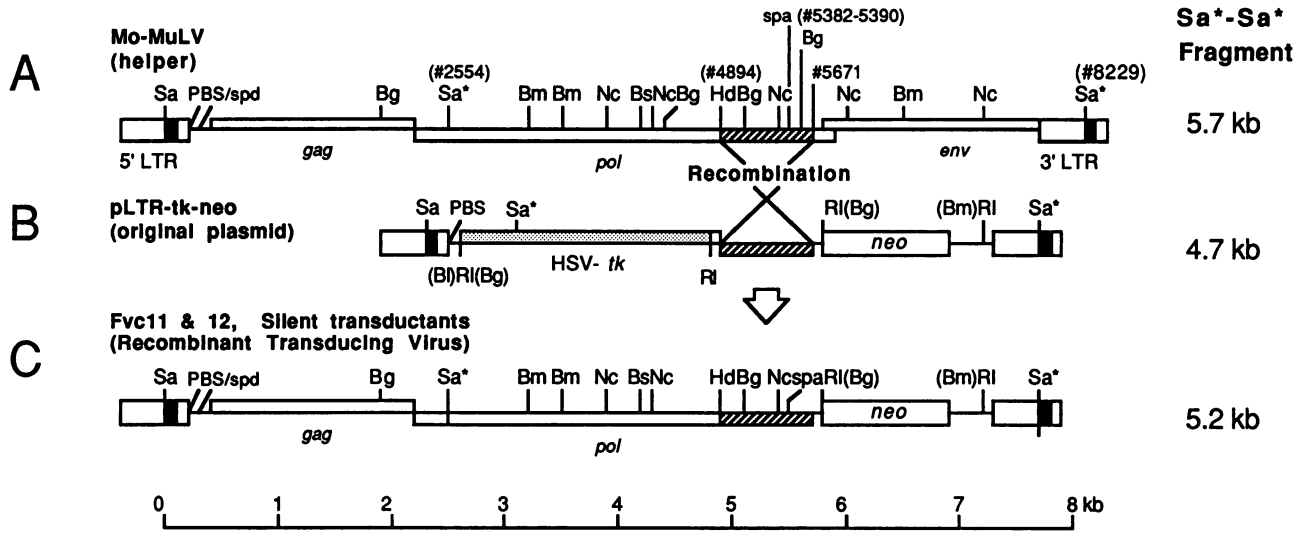


FIG. 2. Schematic diagram of generation of the recombinant transducing retrovirus strains Fvc 11 and Fvc 12. (A) Restriction map of helper Mo-MuLV. (B) Restriction map of the original plasmid construct, pLTR-*tk-neo*, that had been placed into NIH 3T3 cells to generate G418-resistant transfectants (10). (C) Proviral structure in the recombinant transducing virus producer NIH 3T3 cell lines Fvc 11 and Fvc 12. Sizes of restriction fragments generated between *Sac*I sites with asterisks (Sa*) and expected to hybridize with the *neo* gene probe are shown on the right. The hatched region represents the 780-base-pair sequence common to the helper Mo-MuLV and the original plasmid, pLTR-*tk-neo*, in which homologous recombination took place to generate the Fvc 11 and Fvc 12 viruses. PBS, tRNA primer-binding site; spd, splicing donor site for *env* mRNA; spa, splicing acceptor site for *env* mRNA. Abbreviations for restriction endonuclease sites: Sa, *Sac*I; Bg, *Bgl*II; Bm, *Bam*HI; Nc, *Nco*I; Bs, *Bst*EII; Hd, *Hin*dIII; RI, *Eco*RI.

the 3′ end of the 5′ LTR). In all of these clones, deleted regions included the tRNA primer binding site and the splicing donor site. In addition, all clones except clone A lost the *env* mRNA splicing acceptor site originally placed into the construct.

Using another retroviral construct, Barklis et al. (1) isolated 12 transductant F9 EC cell lines that expressed a proviral *neo* gene. One such clone contained a single base mutation in the primer binding site (1) which is responsible for expression of the proviral *neo* gene (13). Loh et al. demonstrated that a region around the primer binding site caused an inhibitory effect on the transient expression of an LTR-driven chloramphenicol transferase gene in F9 cells (5). This inhibitory effect is caused at the level of transcription and is independent of the orientation of the sequence (2, 6). The fact that we found deletions rather than point mutations in all G418-resistant proviruses, but not in the silent ones, strongly suggests an additional inhibitory element for *neo* gene expression in the deleted region. Because our construct carried a set of splicing donor and acceptor sites, it is possible that the splicing of the transcript from an undeleted provirus was inefficient in EC cells. It was reported that retroviral constructs designed to express marker genes through splicing were not expressed (12). Taken together, there appear to be multiple *cis*-acting elements that affect the expression of Mo-MuLV in EC cells: the nonfunctional LTR enhancer, the region inhibitory to proviral transcription around the primer binding site, and probably an inefficient splicing of the transcript. High-frequency deletions of the 5′ LTR in Fvc 12 provirus in EC cells offer a "promoter trap" to isolate promoters active in EC cells by expression selection (3). These promoters will be useful in the construction of expression vectors for EC and embryonic stem cells, as well as murine embryos.

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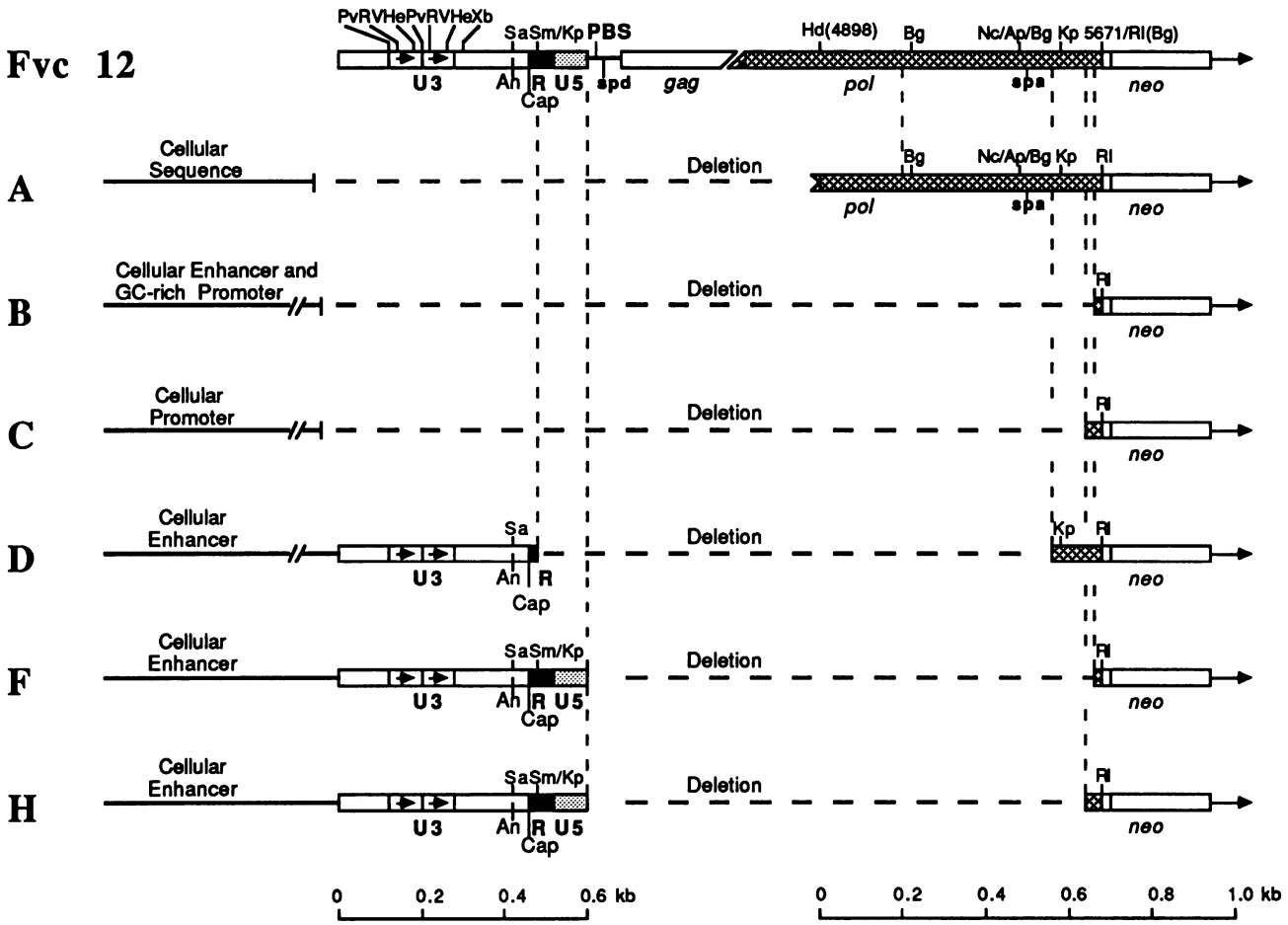


FIG. 3. Deletions in the 5′ LTR-*neo* region of the proviral genomes in the G418-resistant transductant EC cell lines. Diagrams are based on the results of restriction endonuclease and DNA-sequencing analyses. Large portions of the *gag-pol* genes in the recombinant virus Fvc 12 are not shown. Cap, mRNA capping site; PBS, tRNA primer binding site; spd, splicing donor site; spa, splicing acceptor site; An, polyadenylation signal. Restriction sites are abbreviated as follows: Sa, *Sac*I; Bm, *Bam*HI; Nc, *Nco*I; Bs, *Bst*EII; Hd, *Hin*dIII; Pv, *Pvu*II; RV, *Eco*RV; He, *Hae*III; Xb, *Xba*I; Sm, *Sma*I; Kp, *Kpn*I; Bg, *Bgl*II.

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