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Molecular Cloning and Characterization of Mouse Mammary Tumor Proviruses from a T-Cell Lymphoma

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Five mouse mammary tumor virus proviruses and their flanking cellular DNA sequences have been cloned from a transplanted C57BL/6 (B6) T-cell lymphoma containing additional copies of mouse mammary tumor virus DNA. Characterization of these proviruses and their flanking DNA indicates that B6 lymphomas contain many newly integrated mouse mammary tumor virus copies synthesized by a mechanism(s) which generates polymorphism or deletions or both.

The number of mouse mammary tumor virus (MMTV) proviruses is increased in the DNA of many T-cell lymphomas compared with that found in the germ line (10, 11, 18). To further investigate the mechanism of this phenomenon, we have molecularly cloned several MMTV proviruses and their adjacent cellular sequences from a C57BL/6 (B6) lymphoma carrying additional MMTV proviruses. Analysis of these clones indicates that MMTV DNA acquisition does not involve localized replication (1, 9, 25) of a single chromosome, but rather replication and reintegration of MMTV proviruses into different chromosomes by a mechanism(s) which promotes polymorphism among new proviruses.

Isolation and restriction mapping of molecularly cloned MMTV proviruses. To examine the structure of MMTV proviruses and their flanking cellular sequences, we prepared a library of B6 lymphoma DNA. Five recombinant clones were isolated by screening in vitro packaged phage by hybridization to cloned MMTV probes.

Restriction mapping of three clones, AA Cl 1, 2, and 6, is shown in Fig. 1 and compared with maps of two endogenous proviruses of B6 mice, Mtv-8 (unit II) (15, 23) and Mtv-9 (unit III) (7, 23). Initial observations show that the overall order of viral genes is similar to that seen for other MMTV proviruses. Conserved sites between Mtv-8 and clones 1, 2, and 6 appear to be the internal *Kpn*I and *Bgl*II sites within the *gag-pol* region as well as the single *Eco*RI and *Hin*dIII sites. However, examination of sites 3′ to the *Hin*dIII site reveals a number of restriction site polymorphisms among clones using the enzymes *Pst*I, *Bam*HI, and *Bgl*II. This degree of polymorphism in our molecular clones has not been observed in published maps of other cloned MMTV proviruses (14, 20, 22). As expected from previous data (11), the AA Cl 1 and 2 proviruses lack the 1.0-kilobase (kb) *Pst*I fragment typical of milk-borne MMTV; therefore, it is unlikely that these proviruses derive from exogenous MMTV which is found in B6 mice only as a consequence of laboratory infection (8, 11).

Subcloning of cellular flanking regions from clones 1 and 2. Because we previously observed similar sizes of newly acquired MMTV-cell junction fragments in distinguishable tumors of the same mouse strain (11), we used Southern blotting (26) to examine the integration sites of the clone 1 and 2 proviruses in B6 and other tumors. Chromosomal DNAs from normal B6 liver and from B6 T-cell lymphomas were digested with *Eco*RI, subjected to electrophoresis on agarose gels, and then transferred to nitrocellulose. The DNA then was hybridized to a 5′ flanking probe from AA Cl 1 (probe B, Fig. 2A). Liver DNA samples contain a single *Eco*RI band of 9.4 kb (Fig. 2A, lane 4), whereas two B6 lymphomas with amplified MMTV DNA contain a single additional fragment of 13.4 kb (Fig. 2A, lanes 5 and 6). The latter corresponds approximately to the size predicted from the AA Cl 1 provirus (Fig. 1). Examination of additional lymphomas from A/J and BALB/c mice (ASL1, RL♂1, and S49) and a GR mouse mammary tumor line revealed a germ line 11.4-kb fragment in all cases (Fig. 2A, lanes 2, 3, 8, and 9). These results indicate that the site detected by probe B is occupied by a single new MMTV provirus and that similar new proviruses are present in other B6 lymphomas (B6Mo3 and EL4) containing additional MMTV DNA (Fig. 2A, lanes 5 and 6). Moreover, the relative intensity of the tumor-specific band does not appear to be greater than that of the germ line band, suggesting that there has been no amplification of cellular DNA flanking the clone 1 provirus.

To characterize the integration site of a second provirus, regions of unique DNA were located in cellular DNA flanking AA Cl 2. Probe D (Fig. 1) was hybridized to Southern blots containing normal or lymphoma-derived chromosomal DNA which had been digested with *Hin*dIII (Fig. 2B). Liver DNA from B6 mice showed a single 5.4-kb fragment (Fig. 2B, lane 1), wherease B6 tumors with amplified MMTV proviruses did not (Fig. 2B, lanes 2 and 3). Instead, the tumors RBL-5 and EL4 contained a fragment of 7.0 kb. In addition, the tumor EL4 contained two faint bands of 14.5 and 5.4 kb (Fig. 2B, lane 2). Similar faint bands have been observed in additional experiments with probe C (Fig. 1) (data not shown). These data suggest the loss of all or part of the chromosome containing the unoccupied proviral integration site.

DNA samples from BALB/c mice or a GR mammary tumor line revealed a single 5.4-kb fragment with probe D (Fig. 2B, lanes 4 and 5; data not shown). The latter suggests that several B6 lymphomas but not other tumors contain the clone 2 provirus. Amplification of proviral flanking DNA was not detected with either probe C or D (Fig. 2B and 2C; data not shown). Moreover, hybridization experiments with flanking probes from Mtv-8, -9, and -17 (the probable pro-

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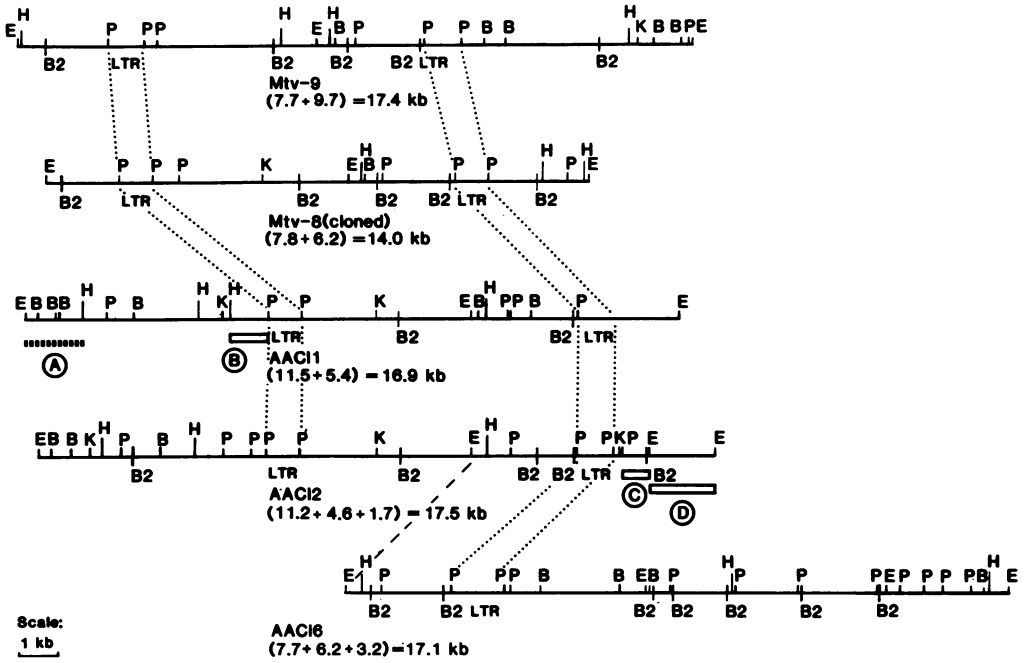

FIG. 1. Restriction maps of MMTV proviruses. Data for the Mtv-9 map were compiled from Cohen et al. (7) and Peterson et al. (23). The Mtv-8 map was derived from data of Hynes et al. (15) and Peterson et al. (23). The lymphoma-derived MMTV clones were constructed by ligation of purified Charon 4A arms (4) to partial *Eco*RI digests of size-selected (10 to 20 kb) DNA from the B6 lymphoma line P3C. Subsequently the DNA was packaged in vitro (3), titers were determined, and the DNA was plated without amplification. Nitrocellulose replicas (2) were screened by hybridization to MMTV probes. Clones were plaque purified before isolation of DNA and restriction enzyme analysis. The restriction enzymes used to generate maps of lymphoma-derived proviruses (AA Cl 1, 2, and 6) were *Bam*HI (B), *Kpn*I (K), *Eco*RI (E), *Pst*I (P), *Hin*dIII (H), and *Bgl*II (B2). Subcloned probes in plasmid vector pUC9 (28) are designated by the circled letters A through D. The open boxes represent unique sequences, and the closed boxes are repetitive DNA.

genitors of the clone 1 and 2 proviruses) revealed no amplification or rearrangement of the three endogenous B6 proviruses (data not shown).

Mapping of clone 1 and 2 proviruses by somatic cell hybridization. Hybridization analyses with cloned flanking probes from either amplified or endogenous proviruses did not support localized gene amplification as the mechanism of MMTV DNA acquisition in lymphomas. To investigate

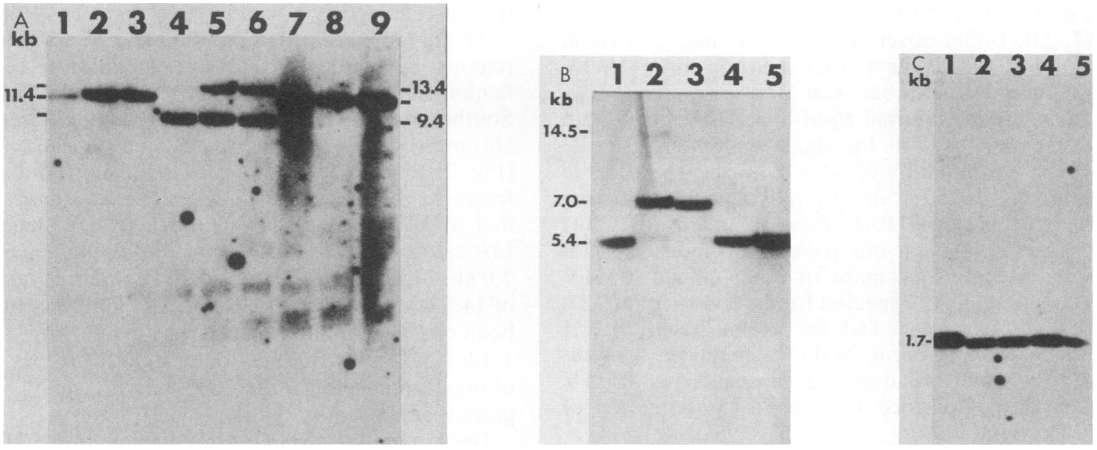

FIG. 2. Southern blots of normal or tumor-derived DNA samples. (A) Hybridization of *Eco*RI-digested DNA to probe B. Lanes: 1, BALB/c liver; 2, S49G3 (BALB/c lymphoma); 3, RLδ1 (BALB/c lymphoma); 4, B6 liver; 5, B6Mo3 (B6 lymphoma); 6, EL4 (B6 lymphoma); 7, A/J liver; 8, ASL1 (A/J lymphoma); 9, GR mouse mammary tumor cells. (B) DNA samples (10 μg) were digested with *Hin*dIII, blotted onto nitrocellulose, and hybridized to probe D. Lanes: 1, B6 liver; 2, EL4 (B6 lymphoma); 3, RBL-5B (B6 lymphoma); 4, BALB/c liver; 5, RLδ1 (BALB/c lymphoma). (C) Samples were digested with *Eco*RI and hybridized to probe D. Lanes: 1, B6 thymus; 2, C6XL (B6 lymphoma); 3, ERLD (B6 lymphoma); 4, P4C (B6 lymphoma); 5, RBL-5B (B6 lymphoma). All lymphomas except ERLD and C6XL had increased MMTV DNA copies. Southern blots and nick translations were performed as described previously (11). The origin and passage of these tumors and cell lines have been reported (11). The C6XL line was obtained from J. Allison (University of Texas, Science Park).

TABLE 1. Mapping of AA Cl 1 proviruses

Mouse chromosome	No. of hybrid clones[a] (AA Cl 1/chromosome retention[b])				% Discordant
	+/+	−/−	+/−	−/+	
1	0	9	0	11[c]	55
2	0	11	0	9	45
3	0	10	0	6	38
4	0	12	0	9	43
5	0	17	0	4	19
6	0	10	0	9	47
7	0	6	0	15	71
8	0	15	0	5	25
9	0	13	0	7	35
10	0	17	0	3	15
11	0	20	0	0	0
12	0	8	0	8	50
13	0	9	0	7	44
14	0	16	0	4	20
15	0	2	0	14	88
16	0	11	0	6	35
17	0	7	0	10	59
18	0	9	0	8	47
19	0	10	0	9	47
X	0	9	0	9	50

[a] Hamster-mouse somatic cell hybrids were prepared by fusion of HGPRT-minus Chinese hamster and mouse (BALB/c, A, or NFS) spleen cells using β-propriolactone-inactivated Sendai virus (6, 16). Hybrids were isolated in medium containing hypoxanthine, aminopterin, and thymidine or back-selected in medium containing 8-azaguanine. Mouse chromosomes retained in each hybrid line were identified by trypsin-Giemsa banding and by isoenzyme analysis.

[b] Of the 21 hybrids analyzed for AA Cl 1 cellular flanking DNA, 16 were karyotyped by Giemsa-trypsin banding, and 5 were typed for expression of various mouse isozyme markers.

[c] Nine hybrids lacked AA Cl 1 and chromosome; 11 hybrids retained chromosome but did not have AA Cl 1.

whether novel proviruses were localized to a single chromosome, we used the technique of somatic cell hybridization. Southern blots of DNA derived from mouse-hamster hybrids containing known mouse chromosome complements were hybridized to probes for unique cellular flanking regions from either clone 1 or 2 (Tables 1 and 2). These data indicate that the clone 1 provirus resides on chromosome 11, whereas the clone 2 provirus is on chromosome 3. We conclude that the additional MMTV proviruses in T-cell lymphomas are not localized to a single mouse chromosome.

Transcription of MMTV DNA in lymphomas. Although previous data indicated that no mature MMTV proteins are made in B6 lymphomas containing increased MMTV DNA (11), it was unclear whether this block was at the transcriptional or translational level. Northern blots (27) of lymphoma samples and GR mouse mammary tumor cells producing milk-borne virus revealed that the total level of RNA is similar in these cell types (Fig. 3A). The level in lymphomas was not affected by the presence of the hormone dexamethasone (compare lanes 3 and 4 of Fig. 3A). As previously reported (12), there are two species of mRNA in GR tumor cells of 8.4 and 3.3 kb (Fig. 3A, lanes 1 and 2). Comparison with RNA species found in lymphomas with additional MMTV proviruses reveals two species of 8 and 2.9 kb in total RNA (Fig. 3A, lanes 3 through 7). Poly(A)-containing RNAs are similar to those seen with total RNA (Fig. 3B), except that longer film exposures revealed an additional band of 1.6 kb. Polyadenylated RNAs from lymphomas containing a germ line complement of MMTV proviruses show no detectable RNA on this or longer exposures (Fig. 3B).

We previously have shown that many transplanted T-cell lymphomas of murine origin contain increased copies of MMTV DNA (11). In this report we have cloned five independent MMTV proviruses from a B6 lymphoma carrying additional MMTV DNA and have characterized three of them with respect to their viral and flanking cellular DNA. This analysis indicated that additional MMTV proviruses did not arise by gene amplification (1, 9, 25). Southern blotting experiments with cloned cellular flanking DNA confirm that clones 1 and 2 are novel proviral insertion events present only in B6 lymphomas, whereas clones 6 and 7 appear to be the 3′ ends of the endogenous proviruses Mtv-17 and Mtv-9 (L. Hsu, A. Glasgow, and J. Dudley, unpublished data).

Our present results reveal several novel findings. First, restriction mapping of cloned MMTV proviruses indicated an unexpected degree of polymorphism in viral DNA. This degree of polymorphism could arise through error-prone reverse transcription, although such extensive variation has not been observed previously in cells infected by exogenous MMTV (14, 20, 22). Second, a part or all of the chromosome harboring the unoccupied clone 2 provirus integration site has been lost from several B6 lymphomas containing additional MMTV DNA. Although the loss of homologous alleles is not unprecedented in nonvirally induced tumors (13, 21, 24), the findings in B6 lymphomas may indicate selection for proviral integration events whose effects are expressed as recessive traits. A third observation of this study reveals high levels of truncated RNAs in B6 and BALB/c lymphomas. Such transcripts are consistent with deletions reported in the U3 region of the long terminal repeat of other lymphoma-derived MMTV proviruses (17, 19). In summary, our findings suggest a novel mechanism for replication and dispersal (possibly retrotransposition [5]) of endogenous MMTV proviruses whose unique cellular locations may lead to aberrant gene activation in T-cell lymphomas.

TABLE 2. Mapping of AA Cl 2 proviruses

Mouse chromosome	No. of hybrid clones (AA Cl 2/chromosome retention[a])				% Discordant
	+/+	−/−	+/−	−/+	
1	10	8	4	5	33
2	7	9	7	4	41
3	9	10	0	0	0
4	6	9	10	4	48
5	2	11	14	2	55
6	10	9	5	4	32
7	10	4	5	9	50
8	4	10	10	3	48
9	5	13	8	1	33
10	2	13	13	0	46
11	0	11	12	0	52
12	4	5	5	4	50
13	4	5	6	3	50
14	5	13	10	1	38
15	8	3	1	6	39
16	4	9	6	2	38
17	10	5	3	5	35
18	6	8	3	2	26
19	6	7	6	3	41
X	11	11	5	3	27

[a] Eighteen of the 30 clones were karyotyped by Giemsa-trypsin banding, and 12 were typed for expression of various mouse isozyme markers. Ten hybrids had chromosome 1 and AA Cl 2 flanking DNA (+/+); 8 hybrids had neither AA Cl 2 flanking DNA nor chromosome 1 (−/−); 4 hybrids had AA Cl 2 flanking DNA but lacked chromosome 1 (+/−); 5 hybrids lacked AA Cl 2 flanking DNA but retained chromosome 1 (−/+).

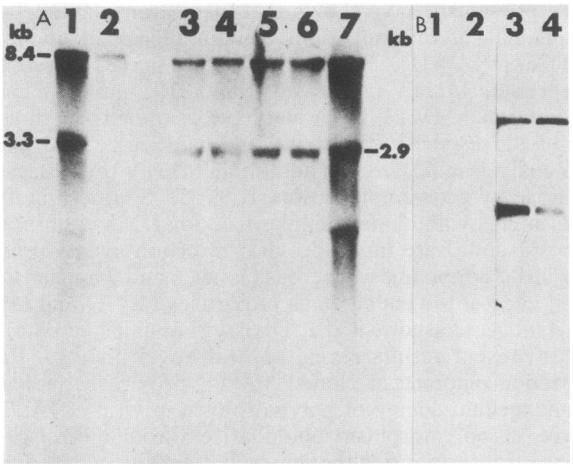

FIG. 3. Northern blot analysis of RNA from lymphomas. (A) Total RNA was extracted from cells, and 30 μg was run on 1.2% formaldehyde gels as described by Thomas (27). After blotting onto nitrocellulose, the samples were hybridized to nick-translated, cloned MMTV LTR. Lanes: 1, GR mouse mammary tumor cells grown in 10^{-5} M dexamethasone for 24 h; 2, GR cells without dexamethasone; 3, RBL-5B B6 lymphoma cells grown with dexamethasone; 4, RBL-5B cells without dexamethasone; 5, B6Mo3 B6 lymphoma cells grown with dexamethasone; 6, B6Mo3 cells without dexamethasone; 7, S49 BALB/c lymphoma cells without dexamethasone. Dexamethasone was toxic for S49 cells. (B) Samples of 2 μg of poly(A)-containing RNA were run on denaturing gels, blotted onto nitrocellulose, and hybridized to labeled MMTV long terminal repeat probe. Lanes: 1, ERLD (B6 lymphoma); 2, C6XL (B6 lymphoma); 3, RBL-5B (B6 lymphoma); 4, P4C (B6 lymphoma). ERLD and C6XL did not have additional MMTV DNA.

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LITERATURE CITED

1. **Alt, F. W., R. E. Kellems, J. R. Bertino, and R. T. Schimke.** 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J. Biol. Chem. **253**:1357–1370.
2. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. Science **196**:180–182.
3. **Blattner, F. R., A. E. Blechl, K. Denniston-Thompson, H. E. Faber, J. E. Richards, J. L. Slightom, P. W. Tucker, and O. Smithies.** 1978. Cloning human fetal γ-globin and mouse α-type globin DNA: preparation and screening of shotgun collections. Science **202**:1279–1284.
4. **Blattner, F. R., B. G. Williams, A. E. Blechl, K. Denniston-Thompson, H. E. Faber, L.-A. Furlong, D. J. Grunwald, D. O. Kiefer, D. D. Moore, E. L. Sheldon, and O. Smithies.** 1977. Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. Science **196**:161–169.
5. **Boeke, J. D., D. J. Garfinkel, C. A. Styles, and G. R. Fink.** 1985. Ty elements transpose through an RNA intermediate. Cell **40**:491–500.
6. **Callahan, R., D. Gallahan, and C. Kozak.** 1984. Two genetically transmitted BALB/c mouse mammary tumor virus genomes located on chromosomes 12 and 16. J. Virol. **49**:1005–1008.
7. **Cohen, J. C., J. E. Majors, and H. E. Varmus.** 1979. Organization of mouse mammary tumor virus-specific DNA endogenous to BALB/c mice. J. Virol. **32**:483–496.
8. **Cohen, J. C., P. R. Shank, V. L. Morris, R. Cardiff, and H. E. Varmus.** 1979. Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissue of the mouse. Cell **16**:333–345.
9. **Dalla Favera, R., F. Wong-Staal, and R. C. Gallo.** 1982. *onc* gene amplification in promyelocytic leukaemic cell line HL-60 and primary leukaemic cells of the same patient. Nature (London) **299**:61–63.
10. **Dekaban, G. A., and J. K. Ball.** 1984. Integration of type B retroviral DNA in virus-induced primary murine thymic lymphomas. J. Virol. **52**:784–792.
11. **Dudley, J., and R. Risser.** 1984. Amplification and novel locations of endogenous mouse mammary tumor virus genomes in mouse T-cell lymphomas. J. Virol. **49**:92–101.
12. **Dudley, J. P., and H. E. Varmus.** 1981. Purification and translation of murine mammary tumor virus mRNAs. J. Virol. **39**:207–218.
13. **Fearon, E. R., B. Vogelstein, and A. P. Feinberg.** 1984. Somatic deletion and duplication of genes on chromosome 11 in Wilm's tumours. Nature (London) **309**:176–178.
14. **Groner, B., E. Buetti, H. Diggelmann, and N. E. Hynes.** 1980. Characterization of endogenous and exogenous mouse mammary tumor virus proviral DNA with site-specific molecular clones. J. Virol. **36**:734–745.
15. **Hynes, N., N. Kennedy, U. Rahmsdorf, and B. Groner.** 1981. Hormone responsive expression of an endogenous proviral gene of mouse mammary tumor virus after molecular cloning and gene transfer into cultured cells. Proc. Natl. Acad. Sci. USA **78**:2038–2042.
16. **Kozak, C. A., and W. P. Rowe.** 1980. Genetic mapping of the ecotropic virus inducing locus (Akv-2) of the AKR mouse. J. Exp. Med. **150**:1419–1423.
17. **Kwon, B. S., and S. M. Weissman.** 1984. Mouse mammary tumor virus-related sequences in mouse lymphocytes are inducible by 12-*O*-tetradecanoyl phorbol-13-acetate. J. Virol. **52**:1000–1004.
18. **Michalides, R., E. Wagenaar, J. Hilkens, J. Hilgers, B. Groner, and N. E. Hynes.** 1982. Acquisition of proviral DNA of mouse mammary tumor virus in thymic leukemia cells from GR mice. J. Virol. **43**:819–829.
19. **Michalides, R., E. Wagenaar, and P. Weijers.** 1985. Rearrangements in the long terminal repeat of extra mouse mammary tumor proviruses in T-cell leukemias of mouse strain GR result in a novel enhancer-like structure. Mol. Cell. Biol. **5**:823–830.
20. **Nusse, R., and H. E. Varmus.** 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell **31**:99–109.
21. **Orkin, S. H., D. S. Goldman, and S. E. Sallan.** 1984. Development of homozygosity for chromosome 11p markers in Wilms' tumour. Nature (London) **309**:172–174.
22. **Peters, G., S. Brookes, R. Smith, and C. Dickson.** 1983. Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. Cell **33**:369–377.
23. **Peterson, D. O., K. G. Kriz, J. E. March, and M. G. Toohey.** 1985. Sequence organization and molecular cloning of mouse mammary tumor virus DNA endogenous to C57BL/6 mice. J. Virol. **54**:525–531.
24. **Reeve, A. E., P. J. Housiaux, R. J. M. Gardner, W. E. Chewings, R. M. Grindley, and L. J. Millow.** 1984. Loss of a Harvey *ras* allele in sporadic Wilms' tumour. Nature (London) **309**:174–176.
25. **Roberts, J. M., L. B. Buck, and R. Axel.** 1983. A structure for amplified DNA. Cell **33**:53–63.
26. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
27. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
28. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.