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Rorγ (*Rorc*) Is a Common Integration Site in Type B Leukemogenic Virus-Induced T-Cell Lymphomas

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The retrovirus type B leukemogenic virus (TBLV) causes T-cell lymphomas in mice. We have identified the *Rorγ* locus as an integration site in 19% of TBLV-induced tumors. Overexpression of one or more *Rorγ* isoforms in >77% of the tumors tested may complement apoptotic effects of c-*myc* overexpression.

Type B leukemogenic virus (TBLV) is a retrovirus that is more than 98% identical to mouse mammary tumor virus (MMTV) (1, 7). Differences between MMTV and TBLV include a 440-bp deletion of U3 sequences present within the MMTV long terminal repeat (LTR). This deletion removes negative regulatory elements that inhibit viral transcription in many cell types, including lymphoid cells. LTR sequences flanking the deletion also are triplicated in the TBLV U3 region to form a T-cell-specific enhancer (24). Our previous results have shown that *cis*-acting sequences from the TBLV LTR are sufficient to convert the disease tropism of an infectious MMTV provirus from relatively long latency mammary tumors (6 to 9 months) to rapidly appearing T-cell lymphomas (2 to 3 months) (2).

Retroviruses that lack encoded oncogenes appear to induce cancer by insertional mutagenesis, leading to deregulation of nearby genes. Because retroviral integration is relatively random, identification of viral insertions within or near the same genes in different tumors suggests that there has been selection for outgrowth of cells carrying specific insertions. Such common integration sites (CISs) have been used as molecular tags to identify oncogenes, tumor suppressor genes, and oncogenic pathways (5, 12, 17, 19, 25, 31). There are at least nine MMTV CISs, which generally fall into three categories (*Wnt*, *Fgf*, and *Notch* family genes [4, 16, 20, 21, 33]), whereas only two CISs, *Tblvi1* and c-*myc*, have been described for TBLV. The *Tblvi1* CIS was identified in 20% of 55 TBLV-induced T-cell lymphomas examined (26) and mapped to the mouse X chromosome, but the target gene(s) remains unknown. We have detected integrations within or near the c-*myc* locus in 23% of TBLV-induced tumors (references 3 and 28 and data not shown). However, unlike many other murine retroviral studies, our previous analysis of 35 TBLV-induced tumors revealed only two tumors with detectable c-*myc* arrangement by Southern analysis, while PCR analysis confirmed that those two tumors plus nine others had TBLV integrations near or within this locus (3). Surprisingly, one tumor (T623B) had at least seven TBLV insertions at four sites within or near the c-*myc* locus. These studies suggested that TBLV-induced lymphomas are polyclonal and that many of the integrations could not be detected by Southern blotting due to the presence of multiple tumor cell clones.

***Rorγ* is a common TBLV integration site.** Using PCR analysis, we identified proviral insertions within the *Rorγ* (*Rorc*) locus, which encodes at least two protein isoforms. Rorγ and its thymus-specific isoform, Rorγt, are members of the nuclear hormone receptor superfamily that includes ligand-regulated transcription factors and receptors for which a specific ligand has not been identified (29). Rorγ also is known as RORC, RZR, thymus orphan receptor, and nuclear receptor 1F3 (22, 23, 27). Rorγ and Rorγt are highly related proteins that use distinct promoters and differ only at their amino termini (11, 13, 34). *Rorγ*-knockout mice, which lose expression of both RNA isoforms, lack lymph nodes and Peyer's patches, demonstrating a requirement for Rorγ/γt in lymphoid organogenesis (10, 14, 30). These mice also show a 75% reduction of total T cells and greatly reduced expression of the antiapoptotic gene Bcl-XL (14, 30). Exogenous expression of either isoform in T-cell hybridomas inhibited interleukin-2 and Fas ligand expression and blocked T-cell receptor-induced cell proliferation and apoptosis (11, 18). *Rorγ*-null mice also have an increased rate of apoptosis and proliferation, resulting in rapid T-cell lymphoma formation (32). A recent report of *Rorγt*-deficient mice has ascribed most of the gross anomalies to the thymus-specific form (9).

In this study we screened a panel of 47 TBLV-induced tumors by PCR analysis using 26 primer pairs consisting of a forward or reverse TBLV-LTR primer and a *Rorγ* genomic primer (combinations of those given in Table 1) as previously described (3). We detected TBLV integrations within or near the *Rorγ* locus for 9 of the 47 tumors tested (19%) (Fig. 1). Several of these tumors (T7, T9, T12, T623B, and T670) contained multiple TBLV integrations in the same tumor but in different locations. Insertions occurred near the beginning or end of the gene, consistent with an enhancer insertion mechanism. No integrations interrupting the coding regions were detected since the two integrations in exon 11, T670 and T7, are located in the 3′ untranslated region (UTR). The most common clustering of integrations occurred within intron 2 of *Rorγ*, with six TBLV insertions detected in five different tumors. TBLV integrations also were clustered near the 3′ end of the *Rorγ* locus, which may alter promoter activity (36) or transcript stability (insertions in the 3′ UTR). None of the inte-

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TABLE 1. Primers used to identify TBLV integrations within the *Ror*γ/γt locus

Primer name	Sequence (5′–3′)	Location-orientation[a]
TBLV-LTR408(+)	CCAATAAGACCAATCCAATAGGTAGAC	TBLV-LTR U3, forward
TBLV-LTR408(+)long	TTTACCAATAAGACCAATCCAATAGGTAGAC	TBLV-LTR U3, forward
TBLV-LTR786(−)	CACTCAGAGCTCAGATCAGAAC	TBLV-LTR U3, reverse
TBLV-LTR786(−)long	AAAATAGAACACTCAGAGCTCAGATCAGAAC	TBLV-LTR U3, reverse
RORCex1(−)	GTGCCGTCCTTGCTGCCC	Exon 1, reverse
RORCex3(+)	GATTCGTGGGGACAAGTCATC	Exon 3, forward
RORCt170(−)	CTCATGACTGAGAACTTGGCTC	Exon 1(t), reverse
RORC281(+)	CAGTTCAGGAGGCATGAGTGAA	Exon 2, forward
RORC(−)5	TCCTTCCTCCAGATCACTTTGACAGCCC	Exon 11, reverse
RORCintron10(−)	TAGGAGGGAATGAGTACTTCG	Intron 10, reverse
RORC(−)	GAGGTGTGGGTCTTCTTTGCAGC	Exon 2, reverse
RORC488(+)	CAGCAGCAAGTGATGGAG	Exon 11, forward
RORCex/in8(−)	TCACCCAAGGCTCGAAACAGC	Exon 8, reverse
T670-646(−)	GCCTAGGATACATGCTTGCC	3′ of exon 11, forward
T670-616(−)	GTGTCAGATTCGTTAGCAGTC	Exon 11, forward
RORCt(+)	ACCTCCACTGCCAGCTGTGTGCTGTC	Intron 2 [exon 1(t)], forward
RORCex4(−)	CACATTACACTGCTGGCTGC	Exon 4, reverse

[a] Primer location in either the TBLV-LTR or *Ror*γ/γt locus; orientation relative to provirus or gene coding sequence.

grations localized to the *Ror*γ locus by PCR could be detected by Southern analysis with either a 4.3-kb genomic probe spanning *Ror*γ exons 1 and 2, intron 1, and part of intron 2 through *Ror*γt exon 1(t) or a 3.9-kb probe including the 3′ UTR of *Ror*γ/γt exon 11 and downstream region, suggesting that only a portion of the tumor population contained the TBLV integration. The majority of the TBLV-induced tumors appeared to be at least semiclonal as judged by Southern blotting with T-cell receptor β and γ probes (data not shown).

The average size of the PCR products obtained (ca. 5 kb) may limit detection of all integrations within the *Ror*γ locus. We attempted to overcome such limitations by using sufficient primer sets to completely scan the locus (Table 1). Nevertheless, 5 kb represents the approximate region screened on either end of the gene, whereas retroviral insertions have been shown to affect gene expression at distances over 200 kb (15). Furthermore, *Ror*γ locus intronic sequences include many single nucleotide runs and *Alu* repeats, which may have further diminished PCR product detection.

The *Ror*γ locus was recently identified as a Moloney murine leukemia virus (MuLV) CIS in studies identifying $p27^{Kip1}$ collaborating oncogenes (12). Complementarity between these two genes was suggested by Winoto and Littman (35) and indicates the utility of using numerous genetic and viral models to examine oncogenic pathways since other large-scale retroviral tagging studies using MuLV failed to detect the *Ror*γ locus as a CIS (19, 25). Furthermore, 8.5% of the TBLV-induced tumors showed integrations in both c-*myc* and *Ror*γ (Table 2).

*Ror*γ/γt expression in developing thymocytes is tightly controlled and is necessary for T-cell maturation (10, 13). Two recent studies using retroviral tagging identified the locus *Sox4*, encoding a transcription factor involved in B- and T-cell development, as a CIS for Moloney MuLV (25, 31). Although *Sox4* was the most frequently targeted CIS in the study by Suzuki et al. (31) (55 of 194 tumors), we were unable to detect any TBLV integrations near *Sox4*. As previously suggested (8), these studies indicate that the unique TBLV enhancer is likely to identify additional cellular genes that are not identified by MuLV insertional mutagenesis.

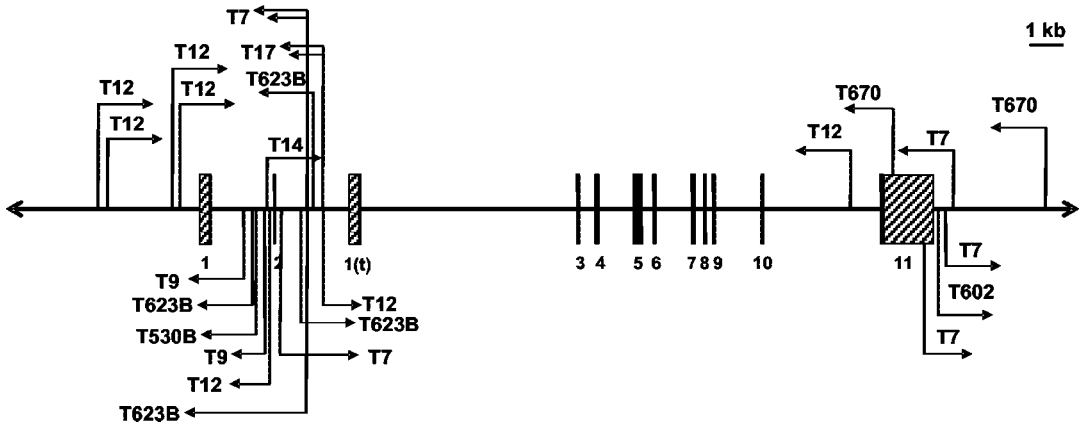

FIG. 1. Location of TBLV insertions within the *Ror*γ locus in T-cell lymphomas as detected by PCR. Black arrows represent the location and orientation of TBLV proviruses. The tumors (designated T) containing the integrations are indicated closest to the arrow. Black bars represent *Ror*γ exons; 5′ and 3′ UTRs are indicated by hatched boxes.

TABLE 2. TBLV tumors with integrations at multiple loci

Tumor	No. of integrations at locus:	
	c-*myc*	*Ror*γ/γt
T9	1	2
T17	1	2
T602	1	1
T623B	7	4

TABLE 3. Primers used for real-time RT-PCR analysis

Primer name	Sequence (5′ to 3′)
RORC(−)	GAGGTGTGGGTCTTCTTTGCAGC
RORC(+)6	GGAGGGCAGCAAGGACGGCAC
RORCt170(−)	CTCATGACTGAGAACTTGGCTC
RORCt(+)	ACCTCCACTGCCAGCTGTGTGCTGTC
c-myc568(+)	TTCTGACAGAACTGATGCGCT
c-myc695(+)	TATGGCTGAAGCTTACAGTCC
gapdh197(+)	CACGGCAAATTCAACGGCA
gapdh247(−)	GATGACAAGCTTCCCATTCTCG

***Ror*γ and *Ror*γt are overexpressed in TBLV-induced tumors.** *Ror*γ, *Ror*γt, c-*myc* and *Gapdh* mRNA levels in the TBLV-induced lymphomas were analyzed and compared to those from normal thymus (Fig. 2). Quantitative real-time reverse transcription-PCR (RT-PCR) was performed using an ABI PRISM 7700 sequence detection system and SYBR Green Universal Master Mix according to the instructions of the manufacturer (Applied Biosystems). (Primers are shown in Table 3.) *Ror*γ levels varied from 0.2- to 38-fold that observed in normal thymus, and 7 of the 18 tumors tested (~39%) showed at least twofold overexpression (and significant differences at the 95% confidence level by Student *t* tests). In contrast to previous reports (11), we routinely detected little or no *Ror*γ expression in the thymus, yet expression was abundant in the liver (33-fold higher than that of normal thymus). TBLV integrations were identified in two of the tumors showing *Ror*γ overexpression (T12 and T14). Seven different integration sites were detected in T12, four upstream of *Ror*γ exon 1 and one in intron 2 in the same transcriptional orientation, and one each within introns 1 and 10, both in the reverse orientation. *Ror*γt expression levels in the tumors tested ranged from 0.2- to 52-fold that detected in normal thymus. Nine of the 18 lymphomas tested (~50%) showed at least twofold *Ror*γt overexpression, which was significantly different from levels in normal thymus; together, more than 77% of the TBLV-induced tumors overexpressed one or more *Ror*γ isoforms. We have not yet detected TBLV integrations near the *Ror*γ locus in any of the tumors showing *Ror*γt overexpression. However, proviral insertions may occur at more distal locations than those that were examined in this study, or *Ror*γt may be indirectly activated.

Consistent with our previous analysis (28), c-*myc* overexpression was observed in 89% (16 of 18) of the TBLV-induced tumors tested. TBLV integrations have been detected in four of the primary tumors (T9, T10, T623B, and T700) with c-*myc* overexpression (Fig. 2 and data not shown). Elevated c-*myc* RNA levels also were detected in T16 and T17 cells passaged in syngeneic mice. Unfortunately, primary tumor RNA was available from only two of the four tumors that contain TBLV insertions at both c-*myc* and *Ror*γ loci. Of these two tumors, T9

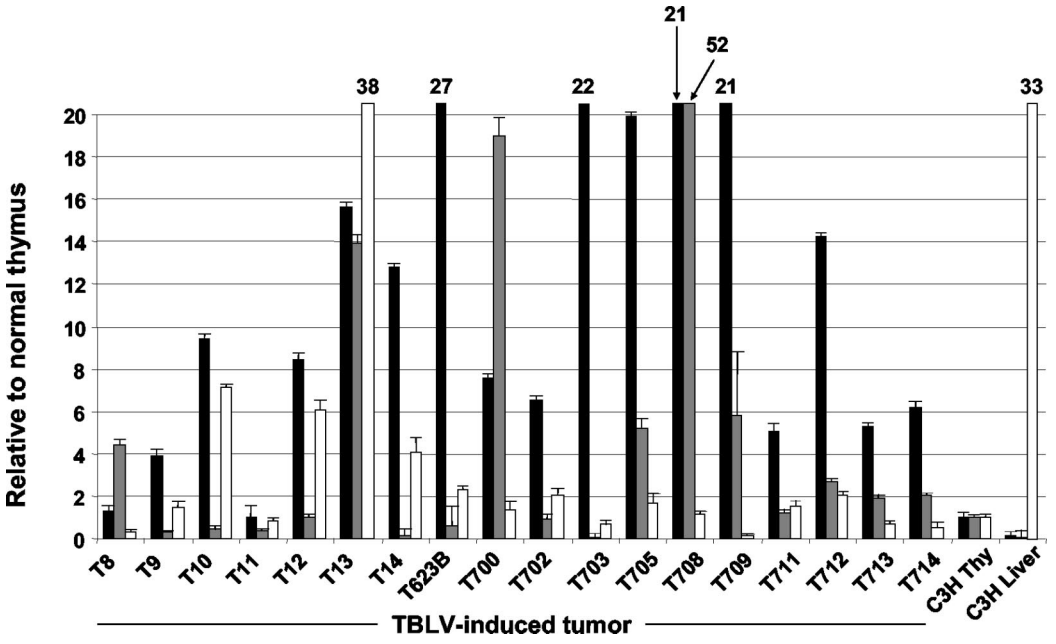

FIG. 2. Both c-*myc* and *Ror*γ/γt are overexpressed in the majority of TBLV-induced tumors. c-*myc* (black bars), *Ror*γt (gray bars), and *Ror*γ (white bars) expression levels from real-time RT-PCR analysis are shown relative to that from normal thymus. The standard errors for gene expression levels greater than 20-fold that of normal thymus are as follows: T13, *Ror*γ, 38 ± 0.4; T623B, c-*myc*, 27 ± 0.1; T703, c-*myc*, 22 ± 0.1; T708, c-*myc*, 21 ± 0.4; T708, *Ror*γt, 52 ± 0.2; T709, c-*myc*, 21 ± 0.3; C3H liver, *Ror*γ, 33 ± 0.02. Gene expression experiments were performed in triplicate three to five times depending on the availability of tumor RNA. Real-time RT-PCR primers were used at a final concentration of 0.1 to 0.2 μM and had been previously determined to have similar amplification efficiencies (slopes of <0.1).

showed ca. fourfold c-*myc* overexpression and did not show *Ror*γ or *Ror*γt overexpression; T623B showed high levels of c-*myc* overexpression (27-fold) and seven detected integrations and modest (ca. twofold) *Ror*γ overexpression (with four detected integrations). We have previously demonstrated that the proviral location and orientation relative to c-*myc* and the composition of the enhancer within the TBLV LTR all affect target gene expression (3).

The observations that many tumors overexpressed both c-*myc* and *Ror*γ/γt and that ~9% of tumors had detectable insertions in both genes suggest that these transcription factors are important individually in the progression toward disease and may collaborate during T-cell lymphomagenesis. The idea that the antiapoptotic factors Rorγ and Rorγt (14, 30, 32) may antagonize the known proapoptotic effects of c-Myc (6) is being explored.

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REFERENCES

1. **Ball, J. K., L. O. Arthur, and G. A. Dekaban.** 1985. The involvement of a type-B retrovirus in the induction of thymic lymphomas. Virology **140:**159–172.
2. **Ball, J. K., and G. A. Dekaban.** 1987. Characterization of early molecular biological events associated with thymic lymphoma induction following infection with a thymotropic type-B retrovirus. Virology **161:**357–365.
3. **Broussard, D. R., J. A. Mertz, M. Lozano, and J. P. Dudley.** 2002. Selection for c-*myc* integration sites in polyclonal T-cell lymphomas. J. Virol. **76:**2087–2099.
4. **Callahan, R., and G. H. Smith.** 2000. MMTV-induced mammary tumorigenesis: gene discovery, progression to malignancy and cellular pathways. Oncogene **19:**992–1001.
5. **Chatterjee, G., A. Rosner, Y. Han, E. T. Zelazny, B. Li, R. D. Cardiff, and A. S. Perkins.** 2002. Acceleration of mouse mammary tumor virus-induced murine mammary tumorigenesis by a p53 172H transgene: influence of FVB background on tumor latency and identification of novel sites of proviral insertion. Am. J. Pathol. **161:**2241–2253.
6. **Dang, C. V.** 1999. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol. Cell. Biol. **19:**1–11.
7. **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.
8. **Dudley, J. P.** 2003. Tag, you're hit: retroviral insertions identify genes involved in cancer. Trends Mol. Med. **9:**43–45.
9. **Eberl, G., and D. R. Littman.** 2003. The role of the nuclear hormone receptor RORγt in the development of lymph nodes and Peyer's patches. Immunol. Rev. **195:**81–90.
10. **He, Y. W., C. Beers, M. L. Deftos, E. W. Ojala, K. A. Forbush, and M. J. Bevan.** 2000. Down-regulation of the orphan nuclear receptor RORγt is essential for T lymphocyte maturation. J. Immunol. **164:**5668–5674.
11. **He, Y. W., M. L. Deftos, E. W. Ojala, and M. J. Bevan.** 1998. RORγt, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. Immunity **9:**797–806.
12. **Hwang, H. C., C. P. Martins, Y. Bronkhorst, E. Randel, A. Berns, M. Fero, and B. E. Clurman.** 2002. Identification of oncogenes collaborating with p27Kip1 loss by insertional mutagenesis and high-throughput insertion site analysis. Proc. Natl. Acad. Sci. USA **99:**11293–11298.
13. **Jetten, A. M., S. Kurebayashi, and E. Ueda.** 2001. The ROR nuclear orphan receptor subfamily: critical regulators of multiple biological processes. Prog. Nucleic Acid Res. Mol. Biol. **69:**205–247.
14. **Kurebayashi, S., E. Ueda, M. Sakaue, D. D. Patel, A. Medvedev, F. Zhang, and A. M. Jetten.** 2000. Retinoid-related orphan receptor gamma (RORγ) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. Proc. Natl. Acad. Sci. USA **97:**10132–10137.
15. **Lazo, P. A., J. S. Lee, and P. N. Tsichlis.** 1990. Long-distance activation of the Myc protooncogene by provirus insertion in *Mlvi-1* or *Mlvi-4* in rat T-cell lymphomas. Proc. Natl. Acad. Sci. USA **87:**170–173.
16. **Lee, F. S., T. F. Lane, A. Kuo, G. M. Shackleford, and P. Leder.** 1995. Insertional mutagenesis identifies a member of the *Wnt* gene family as a candidate oncogene in the mammary epithelium of *int-2*/*Fgf-3* transgenic mice. Proc. Natl. Acad. Sci. USA **92:**2268–2272.
17. **Li, J., H. Shen, K. L. Himmel, A. J. Dupuy, D. A. Largaespada, T. Nakamura, J. D. Shaughnessy, Jr., N. A. Jenkins, and N. G. Copeland.** 1999. Leukaemia disease genes: large-scale cloning and pathway predictions. Nat. Genet. **23:**348–353.
18. **Littman, D. R., Z. Sun, D. Unutmaz, M. J. Sunshine, H. T. Petrie, and Y. R. Zou.** 1999. Role of the nuclear hormone receptor RORγ in transcriptional regulation, thymocyte survival, and lymphoid organogenesis. Cold Spring Harbor Symp. Quant. Biol. **64:**373–381.
19. **Lund, A. H., G. Turner, A. Trubetskoy, E. Verhoeven, E. Wientjens, D. Hulsman, R. Russell, R. A. DePinho, J. Lenz, and M. van Lohuizen.** 2002. Genome-wide retroviral insertional tagging of genes involved in cancer in *Cdkn2a*-deficient mice. Nat. Genet. **32:**160–165.
20. **MacArthur, C. A., D. B. Shankar, and G. M. Shackleford.** 1995. *Fgf-8*, activated by proviral insertion, cooperates with the *Wnt-1* transgene in murine mammary tumorigenesis. J. Virol. **69:**2501–2507.
21. **Marchetti, A., F. Buttitta, S. Miyazaki, D. Gallahan, G. H. Smith, and R. Callahan.** 1995. *Int-6*, a highly conserved, widely expressed gene, is mutated by mouse mammary tumor virus in mammary preneoplasia. J. Virol. **69:**1932–1938.
22. **Medvedev, A., A. Chistokhina, T. Hirose, and A. M. Jetten.** 1997. Genomic structure and chromosomal mapping of the nuclear orphan receptor *ROR*γ (*RORC*) gene. Genomics **46:**93–102.
23. **Medvedev, A., Z. H. Yan, T. Hirose, V. Giguere, and A. M. Jetten.** 1996. Cloning of a cDNA encoding the murine orphan receptor RZR/RORγ and characterization of its response element. Gene **181:**199–206.
24. **Mertz, J. A., F. Mustafa, S. Meyers, and J. P. Dudley.** 2001. Type B leukemogenic virus has a T-cell-specific enhancer that binds AML-1. J. Virol. **75:**2174–2184.
25. **Mikkers, H., J. Allen, and A. Berns.** 2002. Proviral activation of the tumor suppressor E2a contributes to T cell lymphomagenesis in Eμ*Myc* transgenic mice. Oncogene **21:**6559–6566.
26. **Mueller, R. E., L. Baggio, C. A. Kozak, and J. K. Ball.** 1992. A common integration locus in type B retrovirus-induced thymic lymphomas. Virology **191:**628–637.
27. **Ortiz, M. A., F. J. Piedrafita, M. Pfahl, and R. Maki.** 1995. TOR: a new orphan receptor expressed in the thymus that can modulate retinoid and thyroid hormone signals. Mol. Endocrinol. **9:**1679–1691.
28. **Rajan, L., D. Broussard, M. Lozano, C. G. Lee, C. A. Kozak, and J. P. Dudley.** 2000. The c-*myc* locus is a common integration site in type B retrovirus-induced T-cell lymphomas. J. Virol. **74:**2466–2471.
29. **Stehlin-Gaon, C., D. Willmann, D. Zeyer, S. Sanglier, A. Van Dorsselaer, J. P. Renaud, D. Moras, and R. Schule.** 2003. All-trans retinoic acid is a ligand for the orphan nuclear receptor RORβ. Nat. Struct. Biol. **10:**820–825.
30. **Sun, Z., D. Unutmaz, Y. R. Zou, M. J. Sunshine, A. Pierani, S. Brenner-Morton, R. E. Mebius, and D. R. Littman.** 2000. Requirement for RORγ in thymocyte survival and lymphoid organ development. Science **288:**2369–2373.
31. **Suzuki, T., H. Shen, K. Akagi, H. C. Morse, J. D. Malley, D. Q. Naiman, N. A. Jenkins, and N. G. Copeland.** 2002. New genes involved in cancer identified by retroviral tagging. Nat. Genet. **32:**166–174.
32. **Ueda, E., S. Kurebayashi, M. Sakaue, M. Backlund, B. Koller, and A. M. Jetten.** 2002. High incidence of T-cell lymphomas in mice deficient in the retinoid-related orphan receptor RORγ. Cancer Res. **62:**901–909.
33. **van Leeuwen, F., and R. Nusse.** 1995. Oncogene activation and oncogene cooperation in MMTV-induced mouse mammary cancer. Semin. Cancer Biol. **6:**127–133.
34. **Villey, I., R. de Chasseval, and J. P. de Villartay.** 1999. RORγt, a thymus-specific isoform of the orphan nuclear receptor RORγ/TOR, is up-regulated by signaling through the pre-T cell receptor and binds to the TEA promoter. Eur. J. Immunol. **29:**4072–4080.
35. **Winoto, A., and D. R. Littman.** 2002. Nuclear hormone receptors in T lymphocytes. Cell **109**(Suppl.)**:**S57–S66.
36. **Zeidler, R., S. Joos, H. J. Delecluse, G. Klobeck, M. Vuillaume, G. M. Lenoir, G. W. Bornkamm, and M. Lipp.** 1994. Breakpoints of Burkitt's lymphoma t(8;22) translocations map within a distance of 300 kb downstream of *MYC*. Genes Chromosomes Cancer **9:**282–287.