

Activation of an oncogenic microRNA cistron by provirus integration

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Retroviruses can cause tumors when they integrate near a protooncogene or tumor suppressor gene of the host. We infected >2,500 mice with the SL3-3 murine leukemia virus; in 22 resulting tumors, we found provirus integrations nearby or within the gene that contains the mir-17-92 microRNA (miRNA) cistron. Using quantitative real-time PCR, we showed that expression of miRNA was increased in these tumors, indicating that retroviral infection can induce expression of oncogenic miRNAs. Our results demonstrate that retroviral mutagenesis can be a potent tool for miRNA discovery.

oncogene | retroviral mutagenesis

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression. They are initially transcribed by RNA polymerase II and contained within hairpins on a long primary transcript. The hairpins are then processed by two successive steps mediated by a double-stranded RNA-binding protein and RNase III (in mammals, DGCR8 and Drosha followed by TRBP and Dicer), to create the mature ≈ 21 -nt miRNA. The miRNA is loaded into the RNA-induced silencing complex and in animals, the complex is directed to mRNAs by the complementarity of six or seven bases within the miRNA. This leads to either translational repression or mRNA cleavage (1). It has been predicted that one-third of human genes may be regulated in this way (2).

Several miRNA hairpins can be encoded as a cistron on a single primary transcript. Such is the case for the human gene, *c13orf25*, and its mouse homolog. Here, a primary transcript encodes (in order, 5' to 3') mir-17-5p, mir-17-3p, mir-18a, mir-19a, mir-20a, mir-19b-1, and mir-92-1. He *et al.* (3) used a miRNA microarray to profile human B cell tumor lines and found that miRNAs encoded by *c13orf25* were overexpressed. They then showed that in mice, when B cells constitutively overexpressing c-Myc were transduced with part of the human cistron containing miRNAs 17-3p to 19b-1, lymphoma formed at an accelerated pace, suggesting that these miRNAs could be oncogenes (3). In addition, Hayashita *et al.* (4) found that the mir-17-92 cistron was overexpressed in human lung cancer. Also, O'Donnell *et al.* (5) showed that c-Myc expression leads to increased expression of miRNAs from the mir-17-92 cistron, and that mir-17-5p and mir-20 negatively regulate the cell proliferation factor E2F1, suggesting that these miRNAs could also have tumor suppressor properties.

Although microarray analysis of miRNA expression in tumors has proven quite useful in identifying candidates involved in cancer and has provided seminal insight, this method inherently cannot distinguish cause from correlation and so must be corroborated by additional data, expression of a transgene or identification of implicating deletions, translocations, and other mutations (3, 6–8). Alternatively, retroviral insertional mutagenesis might be used to identify causative cancer genes. In this method, slow-transforming retroviruses, which themselves carry no oncogene, insert provirus DNA into the host DNA. Because the provirus integrates into essentially random locations in the host genome, retroviruses can be

used as a gene discovery tool to mutagenize on a genome-wide scale (9–12). When the provirus integrates in cis near an oncogene, the insertion of the viral promoter or enhancer can induce overexpression of the oncogene and drive tumor formation (9, 13–15). Or, when the provirus integrates within a tumor suppressor gene, this can truncate and disrupt the gene and thus also lead to tumorigenesis (16). Because barriers to superinfection keep the number of integrations per cell to a minimum, and because tumors are by nature clonal, the multiple yet few retroviral integrations found in a tumor are all likely to have activated or inactivated a cancer-causing gene. Thus, identification of the locations of integration leads to the discovery of oncogenes and tumor suppressor genes (10–12, 16–20). Here, we investigate the use of retroviral mutagenesis to modulate the expression of oncogenic miRNA. We show that murine leukemia virus can induce overexpression of miRNA from the mir-17-92 cistron and cause the formation of lymphoma in mice.

Results

Retroviral Integration Sites. The murine leukemia virus strain SL3-3 causes T lymphoma almost exclusively (i.e., not B lymphoma; refs. 13 and 14) and has been used for retroviral insertional mutagenesis (16, 18, 19). We infected 2,545 newborn BALB/c mice with the retrovirus, and nearly all mice developed lymphoma. Tumors were excised from the spleen and thymus after an average of 84 ± 29 (average \pm standard deviation) days. Genomic DNA flanking the retroviral integration sites was PCR-amplified and sequenced; integration sites were then mapped to locations in the genome. In 22 tumors (average latency, 92 ± 42 days), each from a different mouse, we found that the 8.9-kb SL3-3 provirus had integrated near the mir-17-92 miRNA cistron (Table 1 and Fig. 1). Because the exact integration location differed in 20 of 22 tumors, and because we were able to sequence the junction between the provirus LTR and the genome (Table 1), we believe it less likely that the integration locations near mir-17-92 were identified because of PCR artifacts. All of the LTR proviral sequences were two bases shorter than those contained in the retroviral RNA genome. The loss of these two bases occurs when a staggered cut is made at the ends of the provirus DNA before integration, and the lack of these bases at the junction sequences helps to rule out artifacts arising from any contamination from plasmid DNA reagents.

Tumors did contain copies of the provirus at other genomic

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Abbreviation: miRNA, microRNA.

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Table 1. Location and junction sequence of proviruses near the mir-17-92 cistron in mouse T lymphomas

| No. | Chromosome 14 integration location | LTR genome junction sequence |
|-----|---------------------------------------|---|
| 1 | 113914725 | CCTTATGAAGGGGTCTTTAAGATGAGGAAATTGAGAAGT |
| 2 | 113915207 | CCTTATGAAGGGGTCTTTCAATTATTAATAAACAT |
| 3 | 113915504 | CCTTATGAAGGGGTCTTTCACCTTGCTAAAATGTAGCATT |
| 4 | 113915586 | CCTTATGAAGGGGTCTTTCAAAGGAGAGCTGATCAGGAGC |
| 5 | 113915928 | CCTTATGAAGGGGTCTTTCAAATAGTACCTAGAGTGTCAAG |
| 6 | 113921419 | CCTTATGAAGGGGTCTTTCAGAACGAGACTAGGAAATAAG |
| 7 | 113921940 | CCTTATGAAGGGGTCTTTCAGCATGTGCTTTCAGAAAGACT |
| 8 | 113921997 | CCTTATGAAGGGGTCTTTCACCTGTAGTAGATGTTATGAA |
| 9 | 113922010 | CCTTATGAAGGGGTCTTTCATTATGAATTCTCAATTCTTT |
| 10 | 113924219 | CCTTATGAAGGGGTCTTTCAGTTTTCTCCCTCCGCGCCA |
| 11 | 113924350 | CCTTATGAAGGGGTCTTTCACCCAATCAGGACCTCGTGG |
| 12 | 113924468 | CCTTATGAAGGGGTCTTTCACGTGCACGAATTAATGTGCC |
| 13 | 113924502 | CCTTATGAAGGGGTCTTTCAAAAATAAAGTTGAAAACCCA |
| 14 | 113915272 | CCTTATGAAGGGGTCTTTCATTAATAACTGGCTTTTTCT |
| 15 | 113915299 | CCTTATGAAGGGGTCTTTCAGTTATTTGTAACGCTTAGC |
| 16 | 113915504 | CCTTATGAAGGGGTCTTTCACCTTGCTAAAATGTAGCATT |
| 17 | 113917616 | CCTTATGAAGGGGTCTTTCACACTGTTCTAGTGCCTGTG |
| 18 | 113921983 | CCTTATGAAGGGGTCTTTCAGTTTGAATTGCTGCTTGTGA |
| 19 | 113922010 | CCTTATGAAGGGGTCTTTCATTAGGAATCTCAATCATT |
| 20 | 113924395 | CCTTATGAAGGGGTCTTTCAAAGCTAGAGAATACTGGCTA |
| 21 | 113924434 | CCTTATGAAGGGGTCTTTCAGATGAGTAGCAGCAAGCCT |
| 22 | 113924501 | CCTTATGAAGGGGTCTTTCAAAAATAAAGTTGAAAACCCA |

Junction sequence consists of 20 bases of the 5'LTR (reverse complement, normal type) and 20 bases of the adjacent genomic sequence (bold type). Base location is for the first base of the genomic sequence after the LTR as determined by the University of California, Santa Cruz, Mouse Genome Database (February 2006 assembly).

locations and analysis located on average of 2 ± 1 additional sites per tumor. This result is in line with the known pathology of murine leukemia virus induced lymphoma; it is generally believed multiple cancer genes need to be activated or inactivated for tumorigenesis, and the multiple integrations accomplish this. The genes near other integration sites included *Evi5*, *Notch1*, and *Jundm2*.

The mir-17-92 integration sites were clustered together at three distinct regions; sites within these regions were separated by no more than 1.2 kb (Fig. 1). The proviruses in the first and second regions were primarily inserted in a transcriptional orientation opposite that of the mir-17-92 primary transcript. The sites were upstream of the putative promoter (Fig. 1) as indicated by numerous Cap Analysis Gene Expression tags (ID 4360008.1) sequenced by the Riken Institute. The proviruses of

a third cluster were downstream of the promoter and transcribed in the same direction as the primary transcript. Unless there are retroviral biases or "hot spots" for integration, the clustering of sites suggests that there are certain positions and orientations optimal for tumorigenesis. We note that, to date, although mouse ESTs that include mir-17-92 sequences have not been deposited into the public EST database, they do exist for the human mir-17-92. For this study and in Fig. 1, the mouse EST AK053349, just downstream of the miRNA cistron, is presumed to be the 3' end of the primary miRNA transcript; the 5' boundary of the primary transcript is deduced from the location of the Cap Analysis Gene Expression tag sequences.

miRNA Primary Transcript Expression. Using quantitative real-time PCR, we measured the steady-state levels of mir-17-92 primary RNA. Using primers and probes (Fig. 1) downstream of the miRNA cistron, we measured the expression level of the mir-17-92 primary RNA [Fig. 2; supporting information (SI) Fig. 4]. Expression of the primary miRNA transcript was on average 4.2 times greater in tumors where a provirus had integrated nearby the gene than in control tumors, i.e., tumors induced by retrovirus but without the mir-17-92 integration, and also 2.9 and 3.9 times greater than adult and neonate thymus tissue, respectively. NIH 3T3 fibroblasts had the lowest amount of the primary RNA, expressing a level less than one-third of those of the control tumors. Here comparison to the control tumors is the best for evaluating the oncogenicity of mir-17-92. The other tissues are informative but less ideal points of reference. NIH 3T3 cells, although immortalized, are of a different cell lineage, and healthy thymus tissue will contain different cell types, including dendritic cells and T cells of varying stages in development.

Mature miRNA Expression. To ascertain whether the actual mature miRNA had increased because of provirus integration, we developed a method to amplify and measure miRNA by quantitative

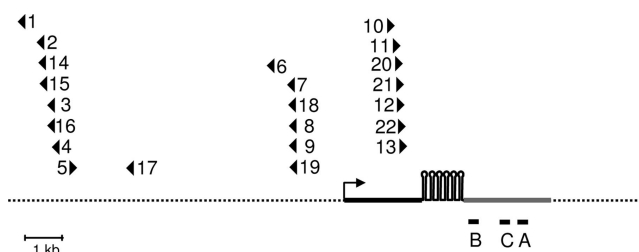


Fig. 1. Location and orientation of proviruses in relation to the gene encoding the mir-17-92 cistron in mouse T lymphomas. Integration sites (triangles) from independent tumors mapped by genomic position relative to the gene (thick solid line) that encodes the mir-17-92 cistron (five hairpin loops). Triangles pointed to the right and left represent proviruses in the 5' to 3' and 3' to 5' orientations, respectively. The transcription start site (arrow) is positioned according to Cap Analysis Gene Expression tags (ID 4360008.1). Also shown: quantitative RT-PCR primers and probes targeted to three regions, A, B, and C (horizontal bars), of the primary RNA, and the region that comprises the EST AK053349 (gray region of the gene).

System for RT-PCR, mir-17-3p cDNA was created from this RNA by using the cDNA primer, 5'-CGTCGGTGGTAGGTC-GAGCGACGTACAAGT-3', where TACAAGT is complementary to mir-17-3p. Here we used reaction conditions identical to that prescribed for random hexamer priming. A mixture containing the cDNA primer, dNTP, and RNA was first incubated at 65°C for 5 min and then placed on ice for at least 1 min. Next, a premixed solution containing reverse transcriptase buffer, MgCl₂, DTT, RNaseOUT, and SuperScriptIII reverse transcriptase was added. The mixture was then incubated at 10 min for 25°C, followed by 50 min at 50°C (elongation) and 85°C for 5 min (inactivation).

Quantitative real-time PCR was performed by using the ABI PRISM 7700 (Applied Biosystems) machine by using an annealing temperature of 50°C and an elongation temperature of 60°C. The

primers and probes for mir-17-3p were 5'-GACTGCAGTGAGG-3', 5'-CGTCGGTGGTAGG-3', 5'-[6-FAM]-CACTTGTACGTCGCTCG-[TAMRA]-3'. Synthetic RNA used for the calibration curve and doping experiments were: 17-3p, 5'-ACUGCAGU-GAGGGCACUUGUA-3', 17-3p with one nucleotide mismatch (underlined), 5'-ACUGCAGUGAGCGCACUUGUA-3', and a randomly synthesized 21-nt RNA 5'-NNNNNNNNNNNNNNNNNNNNNN-3, where N is an A, G, C, or U.

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