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NOTES

The Oncogenic Potential of an Activated Hox-2.4 Homeobox Gene in Mouse Fibroblasts

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The homeobox gene Hox-2.4 is transcriptionally activated in cells of the mouse myeloid leukemia WEHI-3B. The constitutive Hox-2.4 expression in WEHI-3B cells is due to insertion of a transposable element belonging to the family of intracisternal A particles. In this study, we demonstrated the oncogenic potential of this activated homeobox gene. NIH 3T3 fibroblast clones bearing the activated Hox-2.4 gene produced fibrosarcomas in nude mice.

Genes involved in carcinogenesis have in most cases been isolated from oncogenic viruses (3) or by a transfection assay with DNA from tumors (16). In addition, a candidate gene approach can be used to search for alterations in the structure and expression of genes that are known to play a key role in normal development and that directly control the transcriptional program in the nucleus. Given the knowledge of their normal function, homeobox genes seem to be good candidates for such a role. They were first shown to play a major role in the control of *Drosophila* development by genetic studies (18, 22, 24). Homeobox genes have been highly conserved in evolution, being found in the mouse and human genomes (13, 17, 19, 20), and their protein products are known to function as sequence-specific regulators of transcription (7, 10, 11, 15). In our initial search for genomic changes of homeobox genes in tumor cells, we found a rearrangement of the Hox-2.4 gene in WEHI-3B mouse myeloid leukemia cells as a result of insertion of an intracisternal A particle (IAP) upstream of Hox-2.4 (4, 5). This resulted in constitutive transcription of this homeobox gene (referred to as IAP-Hox-2.4) and the production of an abnormal transcript (4, 14). To directly show the biological effect of this Hox-2.4 activation and its possible role in tumorigenesis, we have used DNA-mediated gene transfer into cells that do not express the gene.

NIH 3T3 fibroblasts, which have been extensively used to detect transforming genes by DNA transfection (16), were used as recipient cells. We used the natural construct of the rearranged Hox-2.4 gene as found in WEHI-3B leukemic cells. This construct included the genomic DNA fragment encoding Hox-2.4 and the integrated viral element to drive transcription of the gene (λWR20; Fig. 1). Phage DNA was digested with *Eco*RI to release the 13-kb insert, and the DNA mixture (40 μg) was used directly for transfection by the calcium phosphate precipitation technique (9). In parallel experiments, NIH 3T3 cells were transfected with phage DNA of λWR9 (Fig. 1), which contains the *Eco*RI insert of the normal gene obtained from the same WEHI-3B genomic library. Since no foci were observed in the Hox-2.4-transfected cells in this experiment, we used the strategy of selection for transfected and drug-resistant cells and testing for tumorigenicity in nude mice as described previously for other genes (8). Plasmid DNA of pSV2neo (10 μg) containing the selectable G418 resistance marker (27) was cotransfected with λWR20 or λWR9 DNA. Transfection with pSV2neo DNA alone served as the negative control, whereas transfection with pEJ6.6 DNA (activated human Ha-*ras* gene) (25) served as the positive control for tumor formation. Three weeks after transfection, the G418-resistant colonies were isolated and subcultured. Cells of 30 drug-resistant colonies were screened for Hox-2.4 expression by a slot blot analysis of total cytoplasmic RNA preparations. Ten of these clones were positive for expression of Hox-2.4 RNA expressing similar amounts of RNA. Three clones, numbers 17, 25, and 28, which were the first to grow rapidly, were used for further analysis of integration and expression of the exogenous Hox-2.4 gene. High-molecular-weight DNA was prepared for Southern blot analysis (26) to determine integration of the rearranged IAP-Hox-2.4 gene by hybridization to probe a. This probe includes the 5′ region of the gene (Fig. 1) and detects the 2.2-kb *Bam*HI fragment specific to the rearranged Hox-2.4 gene and different from the endogenous 6-kb *Bam*HI fragment (Fig. 2).

Cytoplasmic RNA was isolated from cells lysed with 0.5% Nonidet P-40, enriched for poly(A)$^+$ RNA (1), and electrophoresed in 1% agarose gels containing 3% formaldehyde. Northern (RNA) blot analyses of RNA hybridized to probe a are shown in Fig. 3. All clones containing the IAP-Hox-2.4 gene expressed a high level of Hox-2.4 RNA, comparable to that in the WEHI-3B leukemic cells. In addition, the size of the RNA transcript was about 1.9 kb as found in WEHI-3B cells (Fig. 3), whereas the size of the normal Hox-2.4 transcript in the spinal cord was about 2.3 kb. The recipient NIH 3T3 cells did not express the endogenous Hox-2.4 gene (Fig. 3), nor did the clones transfected with the normal Hox-2.4 gene (e.g., clone 41; Fig. 3). This finding indicates that sequences of the IAP elements in *cis* to Hox-2.4 are responsible for activation of transcription of the gene in transfected cells. Three transfected clones, numbers 17, 25, and 28, that contained the transfected IAP-Hox-2.4 gene (Fig. 2) were used for further study. The IAP-Hox-2.4- and the *ras*-transfected clones grew to a higher cell density than did the parental NIH 3T3 cells (Fig. 4). In addition, the

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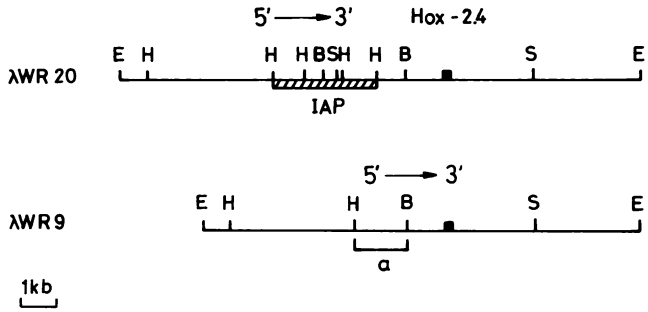

FIG. 1. Restriction map of inserts of Hox-2.4 phage clones isolated from a WEHI-3B genomic library. Symbols: ■, position of the homeobox; ▨, inserted IAP. Restriction endonucleases: *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), and *Sal*I (S). The *Hin*dIII-*Bam*HI fragment indicated as probe a was isolated from an overlapping region of the phage clone wh38 as described previously (4).

IAP-Hox-2.4-transfected cells had a disordered multilayer growth of cells in culture (clone 28; Fig. 5), and the morphology of the cells differed from the morphology of the cells transfected with Ha-*ras* (Fig. 5).

The capability of the cells of clones 17, 25, and 28 for anchorage-independent growth was tested by plating the cells in soft agar as described previously (23) except that 20% fetal calf serum was used. None of 10^4 Hox-2.4-transfected cells formed colonies in soft agar by 3 weeks, whereas the plating efficiency of clone 11 of a myeloid leukemia was 30% in the same experiment.

To determine the tumorigenic potential of the IAP-Hox-2.4 gene, *nu/nu* mice were injected subcutaneously with the transfected cells. Tumors developed in all of the mice

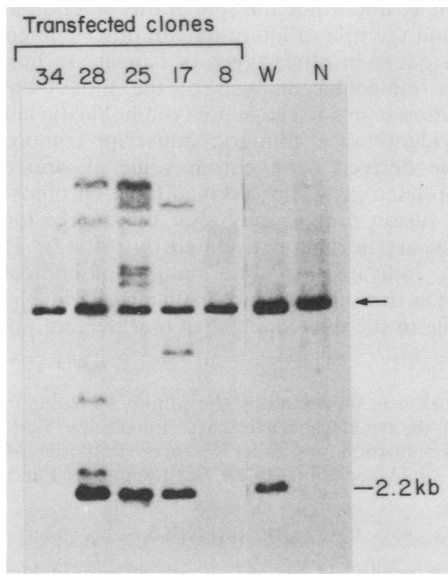

FIG. 2. Detection of integrated sequences of Hox-2.4 in transfected cells. Southern blots (26) were prepared from *Bam*HI-digested DNA and hybridized with ^{32}P-labeled probe a (see Fig. 1). The arrow indicates endogenous mouse Hox-2.4 sequences. The presence of the 2.2-kb hybridizing band indicates integration of the IAP-Hox-2.4 in clones 17, 25, and 28 but not in clone 8. N, NIH 3T3 cells (obtained from Varda Rotter of this institute); W, WEHI-3B cells. Numbers above the lanes indicate clones. Clone 34 was transfected with pSV2neo.

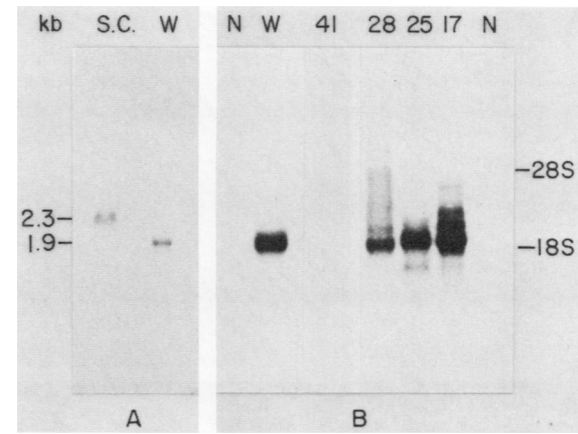

FIG. 3. Northern blot analysis of transfected clones. RNA blots were hybridized with ^{32}P-labeled probe a (see Fig. 1). S.C., Spinal cord total RNA; W, WEHI-3B cells; N, NIH 3T3 cells. Numbers above the lane indicate transfected clones (clone 41 was transfected with normal Hox-2.4; other clones were transfected with IAP-Hox-2.4). Panels A and B were electrophoresed on different gels.

injected with IAP-Hox-2.4-transfected clones 17, 25, and 28 but not with three clones that were transfected only with pSV2neo (Table 1). All tumors from clones 17, 25, and 28 grew progressively. We also injected a lower number of cells of clone 28. Injection of 10^5 cells induced tumors in 4 weeks, and injection of 10^4 cells induced tumors in 5 to 6 weeks. We cultured one of the tumors from clone 28. The cultured tumor cells were morphologically the same as the parental clone 28 and expressed large amounts of the abnormal Hox-2.4 mRNA of the expected size. This finding shows that the tumor was derived from the cells that were injected.

The activated Hox-2.4-induced subcutaneous tumors de-

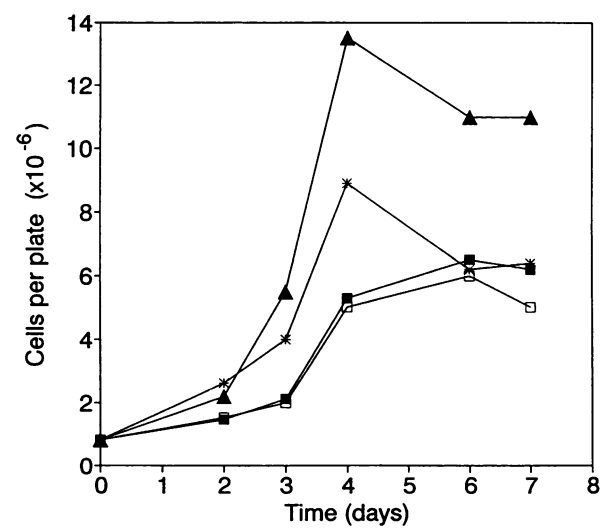

FIG. 4. Growth curves of NIH 3T3 cells (□) and NIH 3T3 cells transfected with the normal Hox-2.4 gene (■; clone 82, which does not express Hox-2.4), the pEJ6.6 Ha-*ras* gene (*; clone 8), and the IAP-Hox-2.4 gene (▲; clone 28). A total of 0.8×10^6 cells were seeded in 60-mm petri dishes with 5 ml of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, and the number of cells was counted for 7 days.

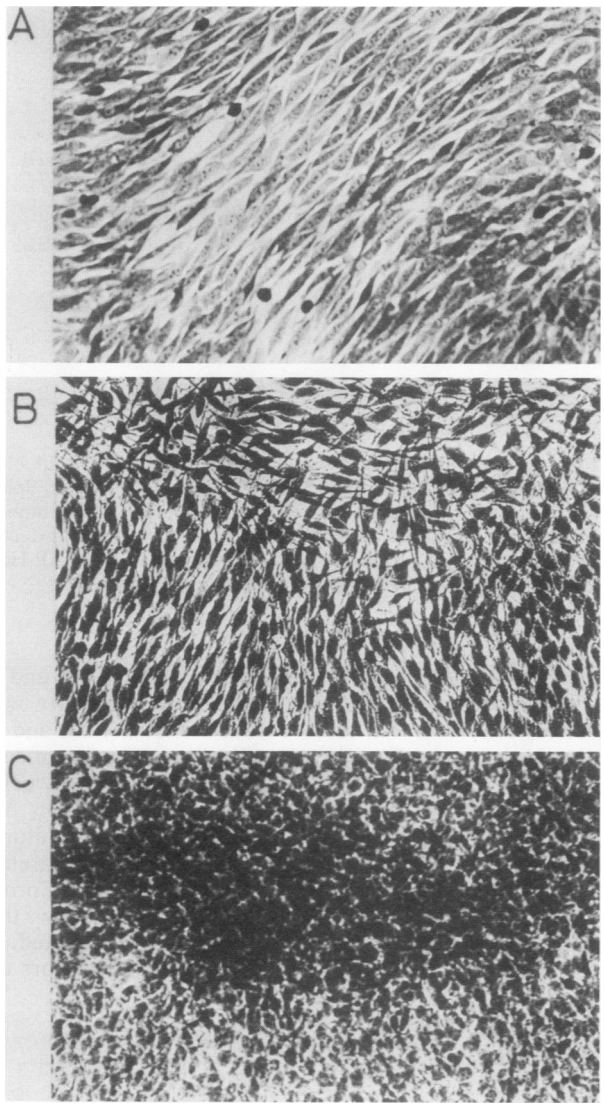

FIG. 5. Photographs of NIH 3T3 cells (A), cells transfected with the pEJ6.6 Ha-*ras* gene (clone 8) (B), and cells transfected with the IAP-Hox-2.4 gene (clone 28) (C). The cells were stained with May-Grünwald-Giemsa stain.

veloped more slowly than the *ras*-induced tumors. A noticeable difference between the two types of tumors was observed in sections of tumor tissues. The *ras*-induced tumors seemed to be rich in blood vessels, whereas the Hox-2.4-induced tumors had a pale appearance, indicating a lower degree of neovascularization. The genetic program of gene expression in the activated Hox-2.4-transformed fibroblasts may not include angiogenic factors, which may explain the slower growth of the subcutaneous tumors. We also injected nude mice with cells of clone 28 directly into the liver. We observed aggressive growth of tumors in the liver and intraperitoneally, causing death of the animals by 5 to 6 weeks after injection, whereas the life span of mice injected subcutaneously ranged from 4 to 6 months. The behavior of the IAP-Hox-2.4 gene as a weak or a strong oncogene thus depends on the tissue in which the tumor develops.

It will be important to determine whether the expression of Hox-2.4 protein is continuously required for the transformed phenotype, which can be tested by regulating its expression with an inducible regulatory element. The availability of specific antibodies in the future will facilitate the biochemical studies that are required to determine whether a mutant Hox-2.4 protein is produced in the transfected cells. At present, the possibility that an altered function of the Hox-2.4 protein is responsible for cell transformation cannot be excluded.

TABLE 1. Tumorgenicity of transfected NIH 3T3 cells in nude mice[a]

Clone no.	Transfected with:			Hox-2.4 gene expression	Tumors/ mice injected	Time of tumor development (wk)
	pSV2neo	IAP-Hox-2.4	pEJ6.6			
28	+	+	−	+	6/6	3
25	+	+	−	+	3/3	4
17	+	+	−	+	3/3	6–8
94	+	−	−	−	0/3	
49	+	−	−	−	0/3	
34	+	−	−	−	0/3	
8	+	−	+	−	4/4	2

[a] CD1 *nu/nu* mice were injected subcutaneously with 2×10^6 cells. The number of tumors was monitored weekly for 12 weeks after injection. All tumors grew progressively.

Our results show that an activated Hox gene can have tumorigenic potential when expressed in NIH 3T3 fibroblasts. Another genetic change in homeobox genes in cancer cells is a deletion of one copy of the Hox-4.1 gene (6) in mouse myeloid leukemia cells which show a deletion in one chromosome 2 (2). This deletion is the most common chromosome aberration in mouse myeloid leukemia cells, and it is not present in WEHI-3B leukemic cells (2, 6). It will be interesting to determine the role of this deletion in leukemogenesis and the role of alterations in other homeobox genes that may occur in other types of cancer. In human leukemias, the molecular analysis of the t(1;19) chromosome translocation in pre-B-cell acute lymphoblastic leukemia has recently identified a chimeric transcript composed of the E2A gene derived from chromosome 19 and a new homeobox-related gene, *prl*, derived from chromosome 1 (12, 21). The fusion protein may alter the expression of target genes that are normally regulated by prl (12). The results, therefore, indicate that abnormalities of homeobox genes which act as transcription factors in normal development can play a role in the development of murine and human leukemias.

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