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# Expression of Hox-2.4 homeobox gene directed by proviral insertion in a myeloid leukemia

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## ABSTRACT

The presence of an altered Hox-2.4 gene in the WEHI3B murine myeloid leukemia suggests that homeobox genes may contribute to neoplasia. A survey of 31 leukemia cell lines of the myeloid, lymphoid and erythroid lineages revealed that Hox-2.4 was expressed only in WEHI3B and the pre-B lymphoid line 70Z/3, in which no DNA rearrangement was observed. To clarify the WEHI3B alteration and normal Hox-2.4 structure, we have sequenced near full length cDNA clones from WEHI3B and 70Z/3, and the 5′ portion of the normal Hox-2.4 gene. A WEHI3B cDNA clone demonstrates that an intracisternal A-particle (IAP) provirus has inserted within the first exon of the gene and generated a Hox-2.4 mRNA with a 5′ sequence derived from the IAP long terminal repeat. A remarkable degree of similarity found between the amino acid sequences of Hox-2.4 and Hox-3.1, which reside on different chromosomes, supports the notion that an ancient homeobox gene cluster has been duplicated and dispersed early in vertebrate evolution.

## INTRODUCTION

The homeobox is a highly conserved domain of 60 residues first identified in many morphology-determining genes of Drosophila but subsequently found throughout the higher animal kingdom (1). It is generally thought that homeobox proteins regulate transcription of other genes by binding to cis-acting DNA sequences. Although many vertebrate homeobox genes have now been cloned, and a number of the mouse genes assigned to specific clusters (Hox-1 to Hox-7), their functions remain to be established (2-4). While a role in early development is supported by their restricted patterns of expression during embryogenesis (5), and by the defects induced in Xenopus embryos by altered homeobox gene expression (6,7), increasing evidence also favors some roles in adult differentiation. Most of the genes are expressed in specific adult tissues [reviewed in (8)], and several tissue or cell type-specific transcription factors of adult animals have recently been found to contain homeo domains (9-13).

We have been pursuing the hypothesis that homeobox genes participate in

 

the control of hemopoiesis, the complex process that generates the diverse blood cell types throughout life. In support of this hypothesis, we isolated a number of homeobox cDNA clones from hemopoietic sources and showed that these genes are expressed in a complex pattern among hemopoietic cell lines (14). During those studies, we observed that the Hox-2.4 gene was expressed by the promyelocytic leukemia cell line WEHI3B (15) in association with a DNA rearrangement, consistent with a preliminary report by Blatt et al (16) of a proviral insertion in that vicinity.

As the Hox-2.4 rearrangement may have contributed to neoplastic transformation in WEHI3B, we have characterized the normal Hox-2.4 gene and this alteration. Genomic clones spanning Hox-2.4 have been isolated previously (17,18), and its homeobox sequence has been reported (19). We describe here nearly full length Hox-2.4 cDNA clones from WEHI3B as well as the pre-B lymphoid leukemia line 70Z/3, and a survey of Hox-2.4 expression in other hemopoietic cell lines. The results show that an endogenous retrovirus-like element, namely an intracisternal A-particle (IAP) genome, has inserted within the first exon of Hox-2.4 in WEHI3B and directs its expression. Moreover, a remarkable degree of conservation is demonstrated between Hox-2.4, which resides on mouse chromosome 11 (20) and Hox-3.1, on chromosome 15 (21). This finding strongly supports the hypothesis (eg. 19,22) that an ancestral homeobox gene cluster has been duplicated and dispersed early in the evolution of vertebrates.

Since submission of this manuscript, Blatt et al (23) have described the rearranged Hox-2.4 gene in WEHI-3B. Most of our conclusions are in accordance with theirs, but there are substantial differences in the reported Hox-2.4 sequences (see Discussion).

## MATERIALS AND METHODS

### Isolation of cDNA clones and nucleotide sequencing

The WEHI3B cDNA library was synthesized using poly $A^+$ mRNA and an oligo (dT)-XbaI primer, by a unidirectional cloning procedure (Promega). EcoRI-methylated double-stranded cDNAs were ligated to EcoRI linkers, digested with EcoRI and XbaI, and size-selected cDNAs (>1kb) cloned into EcoRI/XbaI-digested lambda GEM4 (Promega). Recombinant phage were screened with a Hox-2.4 genomic fragment (probe a in Fig. 2A), which derived from the lambda L2 clone (17), kindly provided by Dr. F. Meijlink, and with an oligonucleotide from the sequence 3′ to the Hox-2.4 homeobox (19) (underlined in Fig.4). Clones hybridizing with both probes were plaque purified.

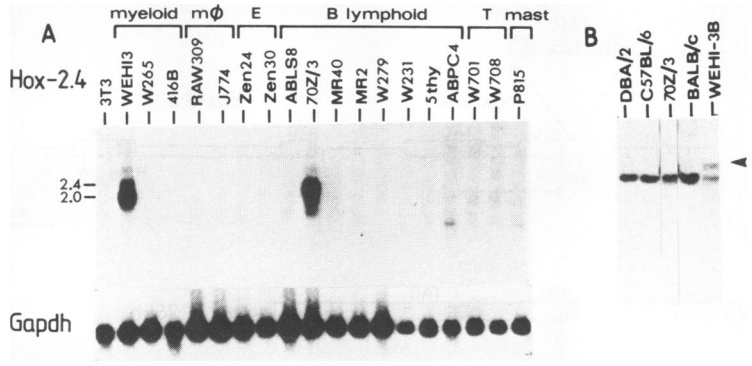


Fig. 1. Northern and Southern blot analysis. **(A)** Expression of Hox-2.4 gene in representative myeloid, macrophage (MΦ), erythroid (E), B and T lymphoid, and mast cell lines and in BALB/c 3T3 fibroblasts. Each lane has 4 µg of poly(A)$^+$ RNA. Hybridization was at high stringency (14), and the probe was the 800-bp SacI cDNA fragment (probe b, Fig. 2B); similar results were obtained with genomic probe a in Fig. 2A. Hybridization with the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) probe confirmed that all the mRNAs loaded were intact and of comparable abundance. Sizes are in kb. **(B)** EcoRI digests of genomic DNA from WEHI3B and 70Z/3. DNA from BALB/c, C57BL/6 and DBA/2 liver was used as controls, since the WEHI3B leukemia arose in a BALB/c mouse and 70Z/3 in a C57BL/6 X DBA/2 mouse. Each lane contained 5 µg DNA, and the probe was the 800-bp SacI fragment from the 70Z cDNA, b in Fig. 2B.

The 70Z/3 cDNA library (24), kindly provided by Drs. David Baltimore and Lynn Corcoran, was screened initially at low stringency with a mixture of $^{32}$P end-labelled oligonucleotide probes from different classes of homeoboxes (14), and a Hox-2.4 clone was subsequently identified.

For sequencing genomic and cDNA clones, restriction fragments were subcloned into M13 vectors and plasmid pGEM3Zf (Promega). Single and double stranded sequencing were carried out with ($\alpha$ $^{35}$S) dATP by dideoxynucleotide chain termination, and all sequences were read from both strands.

Cell lines surveyed for Hox-2.4 expression

Cell lines examined included BALB/c 3T3 fibroblasts; myeloid or macrophage lines WEHI3B, WEHI265, WEHI274, WR19, 416B, J774, P388D1, RAW264 and RAW309; B lymphoid lines 70Z/3, MR2, MR40, LyH7, ABLS8, 5thy, WEHI231, WEHI279, P3, MPC11 and ABPC4; T lymphoid lines W701, W703, W708, Tikaut, and ST4; erythroid lines FL-F4N, Zen21, Zen24, Zen29, Zen30; and mast cell line P815. The origins of these lines have been described (14), except for the immature B lymphoid lines (MR lines), which derive from transgenic mice expressing myc

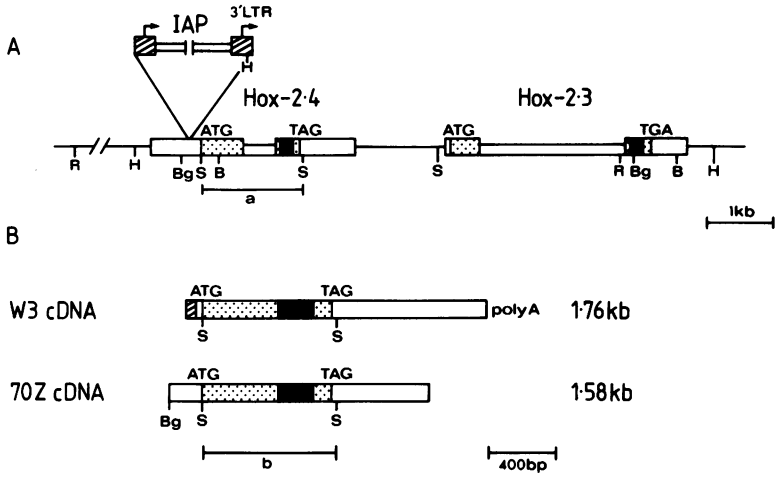


Fig. 2. Hox-2.4 gene and its alteration in WEHI3B. **(A)** Part of the Hox-2 locus, spanning the Hox-2.4 and Hox-2.3 genes. The IAP integration within the Hox-2.4 5′ exon is shown with the proviral LTRs cross-hatched. Filled boxes denote the two homeoboxes, stippled boxes represent coding regions, and unfilled boxes the 5′ and 3′untranslated sequences. Restriction sites, derived from refs. 17 and 18 are HindIII (H), BglII (Bg), BamHI (B), EcoRI (R) and SacI (S). **(B)** Structure of cDNA clones from WEHI3B and 70Z/3 cell lines. The hatched region at the 5′ terminus of the W3 cDNA represents part of the IAP 3′ LTR.

plus ras, and the Zen lines, which were induced by a myc retrovirus. Poly(A)$^+$ RNA was prepared and Northern blots were run as described (14).

## RESULTS

### Hox-2.4 is expressed in only two of 31 murine hemopoietic cell lines

To determine the pattern of Hox-2.4 expression among hemopoietic cell types, we have examined poly A$^+$ RNA from 31 leukemia cell lines, including nine of myeloid or macrophage origin, eleven B and five T lymphoid lines, five

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IAP        AGAAGATTCTGGTCTGTGGTGTTCTTCCTGGCCGGTCGTGAGAACGCGTCGAATAACA
           ************************************************************
    W3     AGAAGATTCTGGTCTGTGGTGTTCTTCCTGGCCGGTCGTGAGAACGCGTCGAATAACAGCCCCCCAACTACAGCCTGCG
Hox-2.4          ** *          * * * ***        * *    ** *       **********************
 germline GTTTTTTTTTCTCTCTCTCTCCCTCCCTTTTCCCCCTCCCCACCCCCTCCCCAACAGCCCCCAACTACAGCCTGCG
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Fig. 3. A hybrid viral-Hox-2.4 transcript in WEHI3B. The aligned sequences are from the 3′ LTR of the IAP genome upstream from the IL-3 gene in WEHI3B (25,26), the 5′ end of the W3 cDNA and the normal Hox-2.4 5′ exon, obtained by sequencing the 250-bp BglII/SacI genomic fragment in Fig. 2A.

erythroid lines and a mast cell line (see above). As illustrated by the northern blot in Fig. 1A, we detected Hox-2.4 transcripts only in WEHI3B and the 70Z/3 pre-B lymphoid line. WEHI3B exhibits a major transcript of 2.0 kb and a minor one of 2.4 kb, whereas a 2.2 kb mRNA predominates in 70Z/3.

The expression in WEHI3B is associated with a DNA rearrangement in the vicinity of the Hox-2.4 gene, detectable by digestion with EcoRI (Fig 1B), HindIII (14), or KpnI. For 70Z/3, however, rearrangement was not observed in digests made with EcoRI (Fig 1B), nor with BamHI, HindIII or KpnI (data not shown). Collectively, these experiments examine a region from 5 kb upstream of the gene to 5 kb downstream (see Fig 2A and maps in refs 17,18).

**Hox-2.4 expression in WEHI3B results from an IAP insertion in the 5′ untranslated region**

To clarify the rearrangement in WEHI3B and the normal Hox-2.4 structure, we first isolated long cDNA clones from WEHI3B and 70Z/3 (Fig 2B). Using a genomic clone isolated by Meijlink et al (17), we could then map the 5′ exon of the Hox-2.4 gene to a position only 0.4 kb upstream from the previously identified (19) homeobox-bearing exon (Fig 2A). When the sequence in the 5′ portion of the Hox-2.4 gene (BglII-SacI fragment) was compared with that of the 70Z/3 cDNA, no differences were found. However, the sequence of the longest WEHI3B cDNA clone (denoted W3) differed from that of the 70Z/3 cDNA and relevant genomic segment over its 5′ terminal 54 nucleotides. This sequence is identical to the 3′-terminal sequence from the long terminal repeat (LTR) of an intracisternal-A particle provirus previously found upstream of the interleukin-3 growth factor gene in WEHI3B (25,26). This conclusion is illustrated in Fig. 3, where the relevant sequences of the IAP 3′ LTR, W3 cDNA and Hox-2.4 genomic region are aligned. Thus an IAP provirus has perturbed Hox-2.4 as well as the IL-3 gene of WEHI3B, as suggested by Blatt et al (16,23).

Since the WEHI-3B cDNA has the IAP sequence only 53 bp upstream of the Hox-2.4 initiator methionine codon (Figs 2 and 3), the gene must be subjugated to viral control. The detection of the hybrid IAP-Hox-2.4 cDNA suggests that transcription initiates at the strong promoter in the 3′ LTR and continues into the Hox-2.4 gene (see Discussion). The major WEHI3B 2.0 kb mRNA has a size consistent with a transcript initiating at that promoter, since the LTR 5′ untranslated sequence will add 120 bases (26) to the 1.7 kb of Hox-2.4 sequences found in the WEHI3B cDNA clone, and the poly(A) will add another 0.1-0.2 kb. The minor 2.4 kb transcript is most likely the nuclear precursor, since the Hox-2.4 intron is approximately 400 bp long.

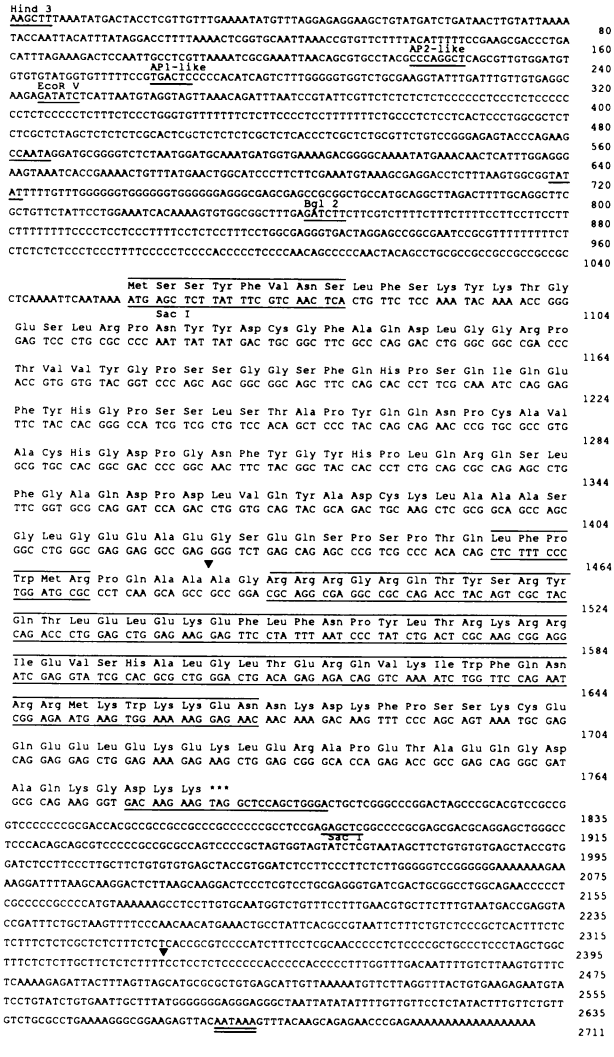


Fig. 4. Composite of Hox-2.4 genomic and cDNA sequences and the predicted amino acid sequence of the protein. Nucleotides 1-1060 were determined from the genomic HindIII/SacI fragment in Fig 2A, and nucleotides 1001-2711 from the W3 clone. Confirmatory data from nucleotides 825-2418 came from the 70Z/3 cDNA. The homeobox and the conserved hexapeptide and amino-terminal regions are boxed. The splice site is indicated by the arrowhead following nucleotide 1485, and another arrowhead indicates the 3′ end of the 70Z cDNA. The polyadenylation signal is underlined twice. Relevant restriction sites are indicated and an oligonucleotide probe used in the initial screening is underlined. In the 5′ untranslated region, potential binding sites for transcription factors AP-1 and AP-2 (31), the TATA-like box and the CCAAT box are underlined.

**Structural features of the Hox-2.4 mRNA and protein**

A 2711-bp composite Hox-2.4 sequence derived from the 5′ portion of the Hox-2.4 gene and the cDNA clones is shown in Fig. 4. The major open reading frame extends from the ATG potential initiator codon at nucleotide 1057 to the TAG at 1786. The sequence of the 7OZ/3 cDNA clone indicates that the 5′ untranslated region is at least 215 bp long, but a preliminary S1 nuclease analysis using a genomic probe (HindIII-BglII fragment in Fig 2A) on 7OZ/3 polyA$^+$ mRNA suggests that the non-coding region may be nearly 400 bp long, which is consistent with the size of the 7OZ/3 mRNA. Whether the TATA-like element and CCAAT box underlined in Fig. 4 are part of the Hox-2.4 promoter is not yet clear. The 3′ untranslated region of the Hox-2.4 mRNA is 905 bp long, with an AATAAA polyadenylation signal 22 bp before the 3′ terminal poly(A) tract.

The predicted Hox-2.4 amino acid sequence is 243 residues long. The sequence in the homeobox region, which agrees with that reported previously (19), places Hox-2.4 in the Antennapedia class, since 51 out of 60 amino acid residues match those in Antennapedia itself. Hox-2.4 also contains the conserved hexapeptide sequence (consensus leu/ile/val-tyr/phe-pro-trp-met-arg/lys) that features in a number of homeobox proteins (eg. 27,28); this is located only seven codons upstream from the homeobox in Hox-2.4 but is encoded on a separate exon, as is commonly observed. The N-terminal eight amino acid residues of the Hox-2.4 protein are also very similar to those in a number of other Antennapedia-like murine homeobox proteins (eg. 28,29). As the next five residues are also shared with Hox-2.3 (17) and Hox-1.1 (30) as well as Hox-3.1 (29) (see below), these genes appear to represent a sub-family. The conservation of the N-terminal and hexapeptide regions among many homeobox proteins suggests that these domains have some important functions, such as mediating protein-protein interaction or transport to the nucleus. Although a number of vertebrate homeobox proteins possess a C-terminal glutamic acid stretch, which might activate transcription of target genes, no long highly acidic regions are present in Hox-2.4.

**Marked similarity of Hox-2.4 and Hox-3.1 proteins**

Figure 5 shows that the Hox-2.4 protein, encoded on mouse chromosome 11, is strikingly similar to Hox-3.1 (29), which resides on chromosome 15. The two homeoboxes have 59 out of 60 amino acids in common. There is also extensive similarity near the amino terminus, where 24 of the first 30 amino acids are the same, and indeed considerable conservation exists throughout the entire region preceding the homeobox, including the hexapeptide motif. Homology is

```
                10         20         30         40         50         60         70
Hox 2.4  MSSYFVNSLF SKYKTGESLR PNYYDCGFAQ DLGGRPT.VV YGPSSGGS.. .FQHPSQ.IQ EFYH.GPS..
         ******* ** **** ****  * **** * *  |* *   |* ***  ***    *** *  |* |*|* * *
Hox 3.1  MSSYFVNPLF SKYKGGESLE PAYYDCRFPQ SVG.RSHALV YGP..GGSAP GFQHASHHVQ DFFHHGTSGI

                80         90        100        110        120        130        140
Hox 2.4  SLSTAPYQQN PCAVACHGDP GNFYGYHPLQ RQSLFGAQ.D PDLVQYADCK LAAA..SGLG EEAEGSE.Q.
         * *   **** ** | ****    ****  *  ****|*** |   |*** ***   *   * *     *
Hox 3.1  SNSG..YQQN PCSLSCHGDA SKFYGYEALP RQSLYGAQQE ASVVQYPDCK SSANTNSSEG Q...GHLNQN

               150        160        170        180        190        200        210
Hox 2.4  SPSPTQL.FP WMRPQAAAGR RRGRQTYSRY QTLELEKEFL FNPYLTRKRR IEVSHALGLT ERQVKIWFQN
         * **| * ** **** *  ** * ******** ********** ********** ********** **********
Hox 3.1  S.SPS.LMFP WMRPHAP.GR RSGRQTYSRY QTLELEKEFL FNPYLTRKRR IEVSHALGLT ERQVKIWFQN

               220        230        240        250        260        270
HOX 2.4  RRMKWKKENN KDKFPSS... .KCEQE..EL EKEKLERAPE TAEQGDAQKG DKKstop
         ********** *** *       * * *  *  * ** *   *   *     *  *
Hox 3.1  RRMKWKKENN KDKLPGARDE EKVEEEGNEE E.EKEEEEKE ..EN....K. Dstop
```

Fig. 5. Similarity of the predicted Hox-2.4 and Hox-3.1 (29) amino acid sequences. Identical amino acid residues are indicated by an asterix and similar ones by a vertical line. Dots indicate gaps introduced to maintain alignment. The homeoboxes are bracketed.

lower in the region C-terminal to the homeobox. Overall, with the alignment presented in Fig.5, Hox-2.4 is similar to Hox-3.1 at 175 out of 242 positions (72%), if gaps are allowed and very conservative changes (Thr/Ser, Asn/Gln, Asp/Glu, Val/Ile/Leu, Phe/Tyr) are counted. This high degree of homology strongly suggests that Hox-2.4 and Hox-3.1 have arisen from a common ancestral gene, which may have been duplicated as part of a cluster of homeobox genes before dispersion (see below). However, the 5′ and 3′ untranslated regions of Hox-2.4 and Hox-3.1 have not been conserved.

## DISCUSSION

Our identification of an IAP-Hox-2.4 hybrid transcript in WEHI3B (Fig. 3) shows that the anomalous expression of the Hox-2.4 gene in this myeloid leukemia results from the insertion of an IAP genome within the 5′ untranslated region of the gene (Fig. 2A). Since the Hox-2.4 promoter and upstream sequences are displaced by the provirus, which has its 3′ LTR juxtaposed with the body of the gene, it is very likely that the relatively abundant Hox-2.4 expression in WEHI3B (Fig. 1) is driven by the powerful promoter in that LTR. The IAP genome may be equivalent to that inserted upstream from the IL-3 gene in WEHI3B (25,26), since the 3′ terminal 58 nucleotides of their 3′ LTRs are identical (Fig. 3).

Blatt et al (23) have very recently reported a sequence for the rearranged Hox-2.4 gene from WEHI-3B. Although our results support their conclusion that the rearrangement reflects an IAP insertion, there are major discrepancies (including frameshifts) between the reported sequences. These affect 62 amino acid residues within the first Hox-2.4 exon. A review of sequencing data from both the WEHI-3B and 70Z/3 cDNAs and an unrearranged genomic clone supports the assignments made here (Fig.4); hence the homology with Hox-3.1 is much higher than previously reported (23). A comparison of our WEHI-3B cDNA with their WEHI-3B genomic sequence (23) reveals an unsuspected feature of the insertion. Their genomic sequence places the IAP insertion 342 bp upstream of the coding region (23). To account for the fusion of IAP and Hox-2.4 sequences at -51 bp in our WEHI-3B cDNA (Fig 3), we suggest that the sequence from the insertion point to position -51 is excised from the IAP-Hox-2.4 precursor RNA by splicing. The relevant sequences of the postulated splice points, AG/GUAUA and CCCCCUCCCCAACAG/C, are similar to consensus donor and acceptor splice sites.

Since the Hox-2.4 alteration is present in two independent WEHI3B cell lines (14,23), it probably was present in the original tumor and may well have contributed to its development. There are numerous examples of cellular oncogenes activated by retroviral insertion, including c-mos activation by IAP insertion (32). Presumptive transcription factors with oncogenic potential include the c-fos, c-jun and evi-1 genes. The c-jun gene appears to encode the AP-1 transcriptional factor, which can complex with the c-fos product (33). Evi-1, which encodes a zinc-finger protein, is activated by retroviral insertion in a number of myeloid leukemias (34).

The concomitant alteration of Hox-2.4 and IL-3 by IAP genomes in WEHI3B may indicate that the two gene products played complementary roles in its transformation. Due to one insertion, WEHI3B secretes abundant IL-3, which stimulates the proliferation of myeloid cells but is normally made by another cell type (25). Hence the enforced Hox-2.4 expression may provide an oncogenic function distinct from the growth factor pathway; for example, it may promote immortalization by interfering with terminal differentiation. A similar role has been suggested for evi-1 (34). Activation of Hox-2.4 itself cannot be common in murine leukemias, because the gene is expressed in only two of 31 cell lines examined, but certain other homeobox genes might perform similar functions. We cannot as yet account for the Hox-2.4 expression in 70Z/3, but it may be relevant that this line also expresses Hox-2.1 and that expression of Hox-2.2 is elevated; hence the activating event in 70Z/3 may

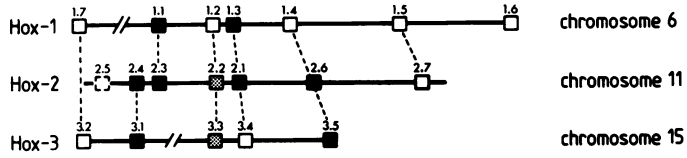


Fig. 6. Alignment of similar homeobox genes from Hox clusters on different mouse chromosomes. Similar genes are joined by dotted lines. Filled boxes denote genes with pronounced similarity to at least one other gene throughout the coding region; stippled boxes, strong homology between the homeoboxes but lower elsewhere; unfilled boxes, genes with closely related homeoboxes, but other sequences not available. Spacing within the Hox-1 locus is from ref.41 and of the Hox-2 locus from ref.19 and 22. The spacing of Hox-3.3 to Hox-3.5 is from the human data (39), assuming that c8 is Hox-3.3, cp11 is Hox-3.4 and cp19 is Hox-3.5. The murine gene previously designated Hox-6.1 (43) presumably should be denoted Hox-3.3, as it seems to correspond to the human c8 gene (14,39). Hox-1.7 has not been linked at the DNA level to the other Hox-1 genes (42) and its position above simply reflects its homology with Hox-3.2 (29) and reportedly Hox-2.5 (19). Similarly, Hox-3.1 and Hox-3.3 have not been physically linked.

affect more genes.

We found that Hox-2.4 is remarkably similar to Hox-3.1 throughout the coding region (Fig.5). Their clear homology is analogous to that previously observed between certain members of the Hox-2 and Hox-1 homeobox gene clusters, most notably Hox-2.1 and Hox-1.3 (35-37) and Hox-2.3 and Hox-1.1 (17,29). The pairwise comparisons that we have made between all published murine and human amino acid sequences (including available sequences outside the homeobox) indicate that the genes joined by dotted lines in Fig. 6 are closely related. It is evident that there is extensive conservation and a colinear gene order between a substantial portion if not all of the Hox-1, Hox-2 and Hox-3 clusters. Moreover, the Hox-5.1 gene on mouse chromosome 2 (38) is closely related to the human Hox-3.5 (cp19) gene (39) and the murine Hox-2.6 gene (22), while the available data on Hox-4.1 suggests that it is related to Hox-1.5 and Hox-2.6 (40). Thus, the emerging data strongly supports the hypothesis (see 19,22,36,37) that an ancestral homeobox gene cluster has been replicated and dispersed to different chromosomes early in the evolution of vertebrates. Presumably the expanded homeobox repertoire facilitates the more complex control required for vertebrate ontogeny. Why the clustered organization has been maintained remains obscure, but it may well allow some form of coupled transcription within the cluster, such as the shared promoter found in the human Hox-3 locus (39), or alternative splicing between different homeobox genes to generate even more homeobox polypeptides.

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