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DNA rearrangement of a homeobox gene in myeloid leukaemic cells

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A homeobox gene rearrangement has been detected in WEHI-3B mouse myeloid leukaemic cells. The rearranged gene was identified as Hox-2.4 which is a member of the Hox-2 gene cluster on mouse chromosome 11. Both the normal and the rearranged genes were cloned and analysed, and the rearranged genomic Hox-2.4 gene was sequenced. The results indicate that the rearrangement is due to insertion of an intracisternal A particle 5′ upstream to Hox-2.4 and that this resulted in constitutive expression of the homeobox gene. It is suggested that constitutive expression of the homeobox gene may interrupt the normal development program in these leukaemic cells.

Key words: DNA rearrangement/constitutive gene expression/homeobox gene/intracisternal A particle/myeloid leukaemia

Introduction

The homeobox containing genes are thought to have an important function in the control of pattern formation in embryonal development of *Drosophila*. This is based on genetic analysis of *Drosophila* mutants which exhibit disordered morphogenesis such as *bithorax*, *antennapaedia*, *engrailed*, *evenskipped*, *fushi tarazu* and others (reviewed in Gehring, 1987; Scott and Carroll, 1987). The regulation of body morphology of *Drosophila* is probably achieved by selection between alternative developmental programs determining the polarity, number and identity of the body segments. Homeobox sequences have been highly conserved through evolution and are found also in mice and humans (McGinnis *et al.*, 1984; Levine *et al.*, 1984).

In fruit fly embryogenesis the homeobox genes are expressed in a stage-specific and site-specific manner (Akam, 1983; Beachy *et al.*, 1985). Similar observations of temporal- and differentiation-specific expression were reported also for mouse and human embryos (Colberg-Poley *et al.*, 1985; Hauser *et al.*, 1985; Gaunt *et al.*, 1986; Mavilio *et al.*, 1986; Krumlauf *et al.*, 1987) and in specific tissues of adults (Jackson *et al.*, 1985; Augulewitsch *et al.*, 1986; Rubin *et al.*, 1986). These experiments have raised the possibility that mammalian homeobox genes may have analogous morphogenetic functions to those that control *Drosophila* embryonic development. The high degree of conservation of the homeobox genes sequence and structure might have resulted from selective pressure to retain these functions (McGinnis *et al.*, 1984). Sequence analysis of homeoboxes has shown homology to a domain of DNA binding proteins in bacteria and yeast (Laughon and Scott, 1984; Shepherd *et al.*, 1984) and DNA binding activity was detected for three gene products (Desplan *et al.*, 1985; Fainsod *et al.*, 1986; Hoey and Levine, 1988). These results and their nuclear localization suggest that homeobox gene products may function by regulating *in trans* sets of genes involved in development. Systematic genetic analysis of mutants, as was done for development of *Drosophila*, has not been possible yet for mammalian development. Here we report the characterization of a homeobox gene rearrangement in a mouse myeloid leukaemia cell line WEHI-3B (Blatt *et al.*, 1987) which is the first detection of a possible abnormal function of a mammalian homeobox gene.

Results

Detection of the rearranged homeobox gene in WEHI-3B DNA

Initially Southern blots of several leukaemic cell lines were hybridized with homeobox probes previously described (Lonai *et al.*, 1987). In this analysis an additional hybridizing fragment was detected upon digestion with *Hin*dIII and *Eco*RI in WEHI-3B DNA when compared to normal BALB/c spleen DNA, the mouse strain in which this leukaemia originated (Burgess and Metcalf, 1980). Further analysis has shown that the new fragment in the *Hin*dIII

Fig. 1. Southern blot hybridization to Hox-2.3 unique probe (probe a in Figure 3). s, BALB/c spleen DNA; W, WEHI-3B DNA.

digest of WEHI-3B is a fragment that contains one homologue of Hox-2.3 and Hox-2.4 genes (see Figures 1 and 2). Figure 2 shows the results of hybridization of DNA from several different normal tissues of a BALB/c mouse and two sub-lines of WEHI-3B with a probe that flanks the Hox-2.3 box sequences (probe a, Figure 3). *Hin*dIII digest of WEHI-3B DNA contains two copies of the gene, one identical in size to the normal gene of BALB/c mice (9.5 kb) and the second smaller in size (8.5 kb). This indicates that only one homologue of chromosome 11 is rearranged. An identical pattern is obtained with DNA from the two sub-lines of WEHI-3B. All the normal cells, which included normal macrophages, did not show this rearrangement (Figure 2). Since WEHI-3B cells are leukaemic precursors of macrophages, this result rules out the possibility that this rearrangement in WEHI-3B could represent somatic rearrangement in normal macrophage development. This rearrangement was also not found in three clones of myeloid leukaemic cells (clones 1, 10 and 7-M12) derived from three different leukaemias induced by X-irradiation (Lotem and Sachs, 1977).

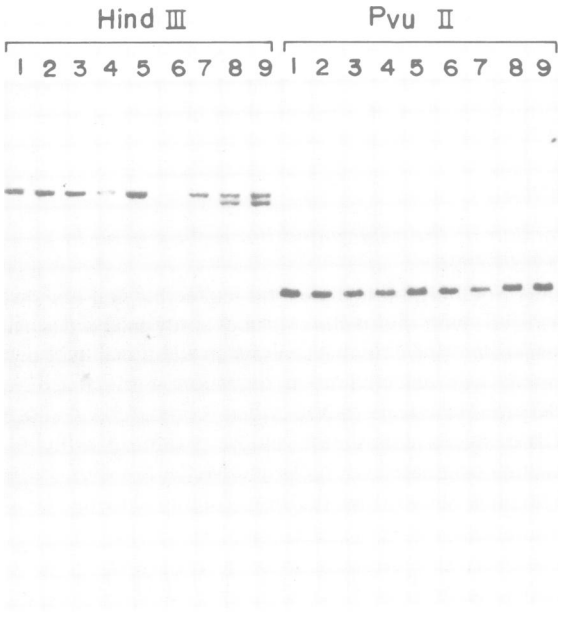

Fig. 2. Southern blot hybridization of DNA from different BALB/c tissues to probe a. 1 and 5, spleen; 2, liver; 3, brain; 4, kidney; 6, non-adherent peritoneal cells; 7, macrophages (adherent peritoneal cells); 8 and 9, W_1 and W_2 WEHI-3B sub-lines. In lane 6, the DNA was not digested with *Hin*dIII.

Isolation of the normal and the rearranged Hox-2 genomic clones from WEHI-3B cells

To understand the nature of the rearrangement we isolated genomic clones of Hox-2 genes from WEHI-3B DNA. The genomic library was constructed in λ EMBL-3 (Frischauf *et al.*, 1983) by using a *Sau*3A partial digest of WEHI-3B DNA. The library was screened with a subclone of Hox-2.3, probe a, containing unique sequences flanking the Hox-2.3 homeobox (Figures 2 and 3). Three recombinant phages (wh5, wh28 and wh38) were isolated and analysed. Although phage wh28 includes a region of ~11 kb downstream of Hox-2.3 it does not include Hox-2.2. The recombinant phages wh5 and wh38 contain the same DNA insert except for reversed orientation with respect to the λ EMBL-3 arms. Initially the two clones wh5 and wh38 were recognized as the normal counterparts of Hox-2.3 and Hox-2.4 genes in WEHI-3B DNA, based on the *Hin*dIII fragment containing the box sequences (9.5 kb). *Hin*dIII digestion of clone wh28, however, gave rise to the 8.5 kb fragment of the rearranged gene (Figure 4). In contrast, a *Bam*HI site, which resides 6 kb 5′ to Hox-2.3 box sequences was not affected by the rearrangement. We conclude therefore that the border of the rearranged area is located between the *Bam*HI and the *Hin*dIII sites (included in fragment b, Figure 3) and also that the rearrangement involves the Hox-2.4 and not Hox-2.3 gene.

The rearrangement is due to an IAP integration

The DNAs of the isolated clones, wh5, wh28 and wh38 were screened for hybridization with retroviral probes, such as MoLTR (Bacheler and Fan, 1981), an *env* probe for ecotropic MuLV (Chan *et al.*, 1980) and an intracisternal A particle (IAP) probe, pMIA10-3 (Lueders and Kuff, 1980) to reveal a possible insertion element in DNA of clone wh28. This analysis resulted in the detection of IAP-related sequences only in the rearranged clone wh28 (Figure 5). The IAP probes did not hybridize to wh38 or wh5 phage DNAs although they include a 1.5 kb region overlapping with the rearranged area. *Kpn*I digest of wh38 yields a 4.5 kb *Kpn*I fragment and a 3.5 kb *Kpn*I−*Sal*I fragment which are

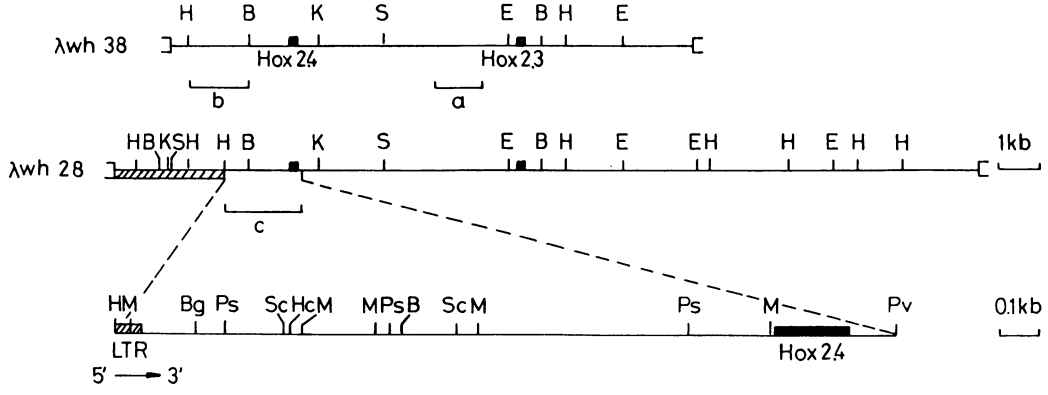

Fig. 3. Restriction map of clones isolated from WEHI-3B genomic library. λwh38 representing the normal gene and λwh28 representing the rearranged gene. The solid boxes mark the location of the homeoboxes of Hox-2.3 and Hox-2.4. The cross-hatched box marks the IAP insertion site. The restriction endonucleases used for mapping were: *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K) and *Sal*I (S). The following restriction sites in fragment c were derived by the nucleotide sequence analysis: *Bgl*II (Bg), *Hin*cII (Hc), *Msp*I (M), *Pst*I (Ps), *Pvu*II (Pv) and *Sac*I (Sc).

recognized by probe b (*SalI* site exists in the multiple cloning site of EMBL-3) (Figure 3). These fragments did not hybridize to the IAP probe. However, digest of wh28 DNA yields two fragments of 3.6 kb and 2.7 kb by *Kpn*I and two fragments of 3.5 and 1.2 kb by *Kpn*I−*Sal*I double digest which hybridized to probe b and to the pMIA10-3 probe (Figure 5).

Nucleotide sequence of Hox-2.4

Our sequence analysis was performed on a 2 kb *Hin*dIII−*Pvu*II fragment, isolated from the rearranged wh28

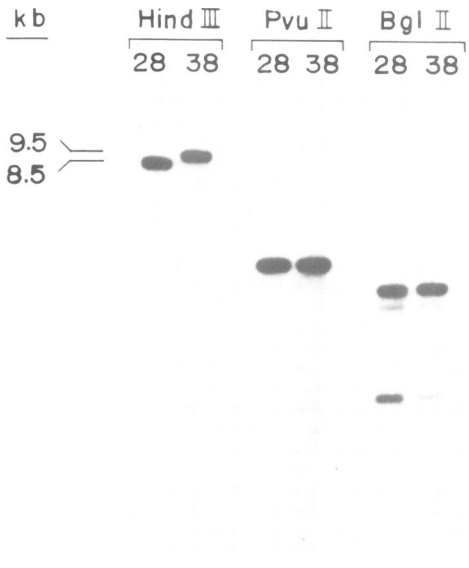

Fig. 4. Analysis of clones wh28 and wh38 by hybridization to probe a.

Fig. 5. Hybridization of an IAP probe (pMIA10-3) to DNA of clones wh28 and wh38. A, digestion with *Kpn*I; B, *Kpn*I and *Sal*I.

phage DNA and subcloned in Bluescript M13-plasmid (probe c, Figure 3). Our strategy involved sequencing of overlapping subclones, which contain unidirectional deletions generated by *Exo*III and sequencing by the dideoxy chain termination method (Sanger *et al.*, 1977).

The nucleotide and deduced amino acid sequence of Hox-2.4 are shown in Figure 6. The deduced amino acid sequence indicates that it includes the entire coding region of the gene; that it includes the highly conserved octapeptide of the amino terminus of most sequenced murine homeobox genes (Breier *et al.*, 1988) (including the ATG initiation codon) as shown in Figure 7A and also boxed in Figure 6, and that it includes the carboxy terminal homeobox region described previously (Hart *et al.*, 1987). The sequence also shows another possible initiation codon at position −283, which is followed immediately by a termination codon (−274) and three other termination codons downstream in another reading frame. Another common feature, which the Hox-2.4 gene shares with other homeobox genes, is the presence of the highly conserved hexapeptide encoded by the first exon (Krumlauf *et al.*, 1987; Breier *et al.*, 1988, Le Mouellic *et al.*, 1988) which appears boxed in Figure 6 and is also shown in Figure 7B. This hexapeptide is usually located several amino acids upstream to the homeobox domain in the mature product. Indeed a 5′ intron splice site sequence AAG/GTGAG is present at position 418 of our sequence (two amino acids downstream of the hexapeptide) and a 3′ intron splice acceptor site sequence TTCCCAG/CA is present at position 1186 (predicted as three amino acids upstream of the box sequence). Both are in good agreement with the splice consensus sequences (Shapiro and Senapathy, 1987).

The box sequence of Hox-2.4 (Hart *et al.*, 1987) is closely related to that of the Hox-3.1 gene (60 of 61 identical amino acids) and both are relatively divergent from other homeoboxes (Augulewitsch *et al.*, 1986; Breier *et al.*, 1986). We therefore compared (in Figure 8) the deduced amino acid sequence of the open reading frame of Hox-2.4 to that of the published Hox-3.1 cDNA (Breier *et al.*, 1988; Le Mouellic *et al.*, 1988). This alignment requires an insertion of six amino acids in Hox-3.1 at position 58 in order to get the best fit, and predicts 240 amino acids for Hox-2.4 compared to 242 amino acids for Hox-3.1. It also shows that in addition to the box itself, regions of homology are scattered throughout the two genes. Two such blocks are, 23 of 28 amino acids at the N′-terminus (amino acids 1−28) and 8 of 10 amino acids at the extended conserved hexapeptide (amino acids 133−142). In contrast to the almost complete homology of the box sequences of Hox-2.4 and Hox-3.1 the overall homology of the deduced amino acid sequence of the two genes is 53%. A notable difference between the two sequences resides at the carboxy terminus. The Hox-2.4 deduced sequence is not rich in glutamic acid residues as is Hox-3.1 (8 versus 15 residues in Hox-3.1 in a region of 21 amino acids) (Breier *et al.*, 1988).

Sequence of the 5′ upstream region

Our sequence analysis includes 413 nucleotides upstream of the coding region. Analysis of this sequence reveals two overlapping stretches of mirror-repeat sequences called H-palindromes (Mirkin *et al.*, 1987) as shown in Table I. Such sequences are present at the promoter region of several genes and can undergo a structural change as has been shown for the binding site of the large T antigen at the origin of

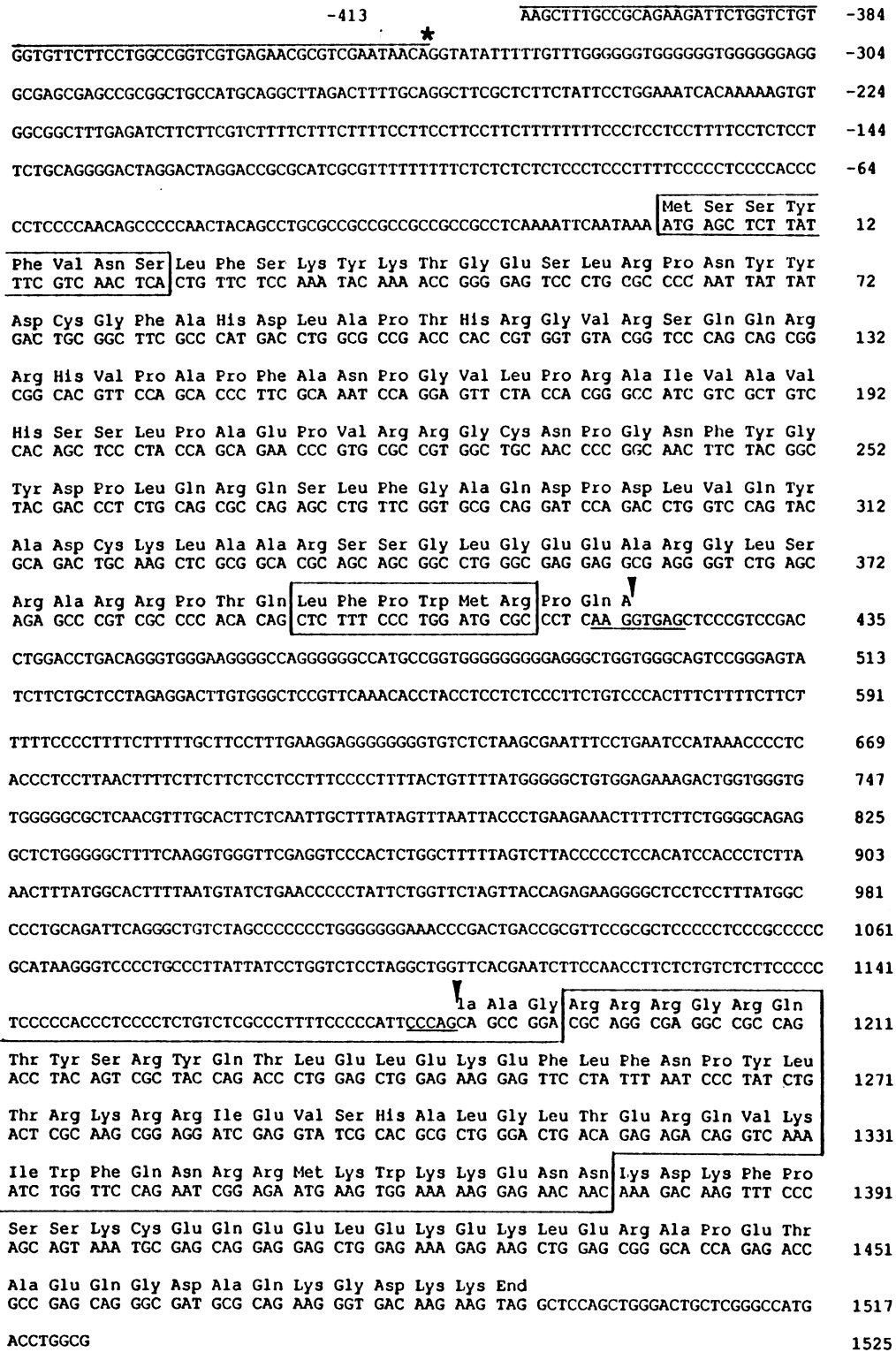

Fig. 6. Nucleotide and deduced amino acid sequence of the genomic Hox-2.4 gene. The splice junctions are, therefore, given on the basis of sequence similarities to other genes (Shapiro and Senapathy, 1987). Potential splice sites are underlined and splice junctions are indicated by vertical arrows. The conserved amino terminal sequence, the consensus sequence of the hexapeptide and the homeo domain are boxed. Nucleotides upstream of the translation initiation site are given in negative numbers. The 3′ LTR sequence is overlined and the LTR/cellular junction is marked by an asterisk.

replication of SV40 (Riley *et al.*, 1986). This Hox-2.4 region also contains a stretch of marked purine/pyrimidine asymmetry. Over 98% of the nucleotides between positions −210 to −141 and −113 to −57 are pyrimidines.

Sequences related to IAP

The DNA fragment which was sequenced includes 70 nucleotides of the viral element. It was inserted 342 bp upstream of the coding region of Hox-2.4 (Figure 6,

A

Hox 2.4	MET	SER	SER	-	TYR	PHE	VAL	ASN	SER
Hox 3.1	MET	SER	SER	-	TYR	PHE	VAL	ASN	PRO
Hox 2.1	MET	SER	SER	-	TYR	PHE	VAL	ASN	SER
Hox 1.3	MET	SER	SER	-	TYR	PHE	VAL	ASN	SER
Hox 2.3	MET	SER	SER	LEU	TYR	TYR	ALA	ASN	ALA
Antp	MET	THR	SER	-	TYR	PHE	THR	ASN	SER

CONSENSUS :

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                            PHE              SER
   MET SER SER  -   TYR          VAL ASN
                            TYR              ALA
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B

Hox 2.4	LEU	PHE	PRO	TRP	MET	ARG
Hox 3.1	MET	PHE	PRO	TRP	MET	ARG
Hox 2.1	ILE	PHE	PRO	TRP	MET	ARG
Hox 1.3	ILE	TYR	PRO	TRP	MET	ARG
Hox 2.3	ILE	TYR	PRO	TRP	MET	ARG
Hox 1.1	ILE	TYR	PRO	TRP	MET	ARG
Antp	LEU	TYR	PRO	TRP	MET	ARG

CONSENSUS :

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ILE TYR                 ARG   4-18 aa
        PRO TRP MET           -------> HOMEO DOMAIN
LEU PHE                 LYS
                              splice
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Fig. 7. (**A**) Conserved amino-terminal sequences of murine homeo proteins and the deduced consensus sequence. (**B**) Conserved hexapeptide region in homeobox genes of different species. A consensus sequence is indicated below. The distance of the hexapeptide upstream of the homeobox in several genes is indicated above the arrow.

sequences overlined). Comparison of this sequence to that of other IAP elements reveals complete homology to the sequence of the 3′ long terminal repeat (LTR) of the IAP inserted upstream to the interleukin-3 (IL-3) gene in the same WEHI-3B cells (Ymer *et al.*, 1986). It is also identical in this region to the 3′ LTR of the IAP inserted into the immunoglobulin $\varkappa$ chain gene in a hybridoma line (Hawley *et al.*, 1984). The sequence of the LTR is in the same 5′−3′ orientation as the Hox-2.4 gene. It possesses the 4-base terminal inverted repeat (AACA), typical to the ends of the LTRs of the IAP genomes (Kuff *et al.*, 1983) which marks the viral/cellular junction at position −342 (marked with an asterisk in Figure 6). In addition it includes a short sequence,

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  1 MSSYFVNSLFSKYKTGESLRPNYYDCGFAHDLAPTHRGVRSQQRRHVPAP  50
  1 MSSYFVNPLFSKYKGGESLEPAYYDCRFPQSVGRSHALVYGPGGSAPGFQ  50

 51 FANPGVL......PRAIVAVHSSLPAEPVRRGCNPGNFYGYDPLQRQSLF  94
 51 HASHHVQDFFHHGTSGISNSGYQQNPCSLSCHGDASKFYGYEALPRQSLY 100

 95 GA.QDPDLVQYADCKLAARSSGLGEEARGLSRARRPTQLFPWMRPQAAGR 143
101 GAQQEASVVQYPDCKSSANTNSSEGQGH.LNQNSSPSLMFPWMRPHAPGR 149

144 RRGRQTYSRYQTLELEKEFLFNPYLTRKRRIEVSHALGLTERQVKIWFQN 193
150 RSGRQTYSRYQTLELEKEFLFNPYLTRKRRIEVSHALGLTERQVKIWFQN 199

194 RRMKWKKENNKDKFPSSKCEQEELEKEKLERAPETAEQGDAQKGDKK*   240
200 RRMKWKKENNKDKLPGARDEEKVEEEGNEEEEKEEEEKEENKD*       242
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Fig. 8. Amino acid sequence similarities between the deduced Hox-2.4 and Hox-3.1 proteins. The sequences are in one-letter code and aligned by the Bestfit program of the UWGCG package. Solid vertical lines correspond to identical amino acids. Sequences showing 100% positional identity are boxed. This alignment requires the consideration of insertion of six amino acids in Hox-3.1 as compared to Hox-2.4 in the first exon (position 58) while the C′ terminal end of Hox-2.4 contains an additional four amino acids.

TGTTCT, which serves as a possible glucocorticoid responsive element, present also in the LTR of MMTV (Scheidereit *et al.*, 1983).

Expression of Hox-2.4 in WEHI-3B cells

Northern blot analysis of cytoplasmic RNA of WEHI-3B cells resulted in the detection of Hox-2.4 RNA with both probes b and c. Probe b does not include the homeobox and was therefore chosen for further study in order to avoid cross hybridization with products of related homeobox genes. Figure 9 shows the expression of Hox-2.4 in WEHI-3B and in spinal cord. Hox-2.4 is not expressed in clones derived from three other myeloid leukaemias induced by X-irradiation nor in normal bone marrow RNA (data not shown). Comparison to normal Hox-2.4 RNA expressed in adult spinal cord shows that the WEHI-3B Hox-2.4 RNA is smaller in size, that is 1.9 kb versus 2.3 kb transcript of the spinal cord. The signal obtained with the total RNA of the spinal cord without enrichment for poly$(A)^+$ RNA indicates a relatively high level of expression of Hox-2.4 in this tissue.

We also compared the amount of Hox-2.4 RNA expression in two WEHI-3B sub-lines, both containing the rearranged gene. Identical Northern blots were prepared with equal amounts of RNA of each WEHI-3B clone, as seen from hybridization to c-*myc* and β actin probes (Figure 9). However, the W_1 clone expressed about twice as much Hox-2.4 RNA as the W_2 clone, judging from the intensity of the signals. W_1 clone is a subtetraploid clone (~73 chromosomes) while W_2 is a near diploid clone. The increased expression of the rearranged Hox-2.4 in clone W_1 is presumably due to additional copies of this gene.

In order to check from which strand the rearranged Hox-2.4 is expressed, we probed a Northern blot with single-stranded probes for Hox-2.4. This was prepared by subcloning the *Kpn*I−*Bam*HI fragment, containing Hox-2.4, in Bluescript M13-vector and using a primer extension reaction to synthesize the plus strand as a probe. Both the normal (spinal cord) and the rearranged Hox-2.4 RNAs hybridized to this probe. In contrast, the complementary strand synthesized by primer extension of probe c failed to detect

Table I. H palindrome sequences

Source	Position	Sequence
Hox-2.4 gene	− 78	. . . TCCCCTCCCCACCCCCT . . .
Hox-2.4 gene	− 89	. . . TCCCTCCCTTTTCCCCCTCCCT . . .
Human c-*myc* gene	−300	. . . CCCTCCCCATAAGCGCCCCTCCC . . .
Chicken α 2(1) collagen promoter	−200	. . . TCCCTCCCCTTCCTCCCTCCCT . . .

H palindrome sequences found 5′ upstream of the coding sequence of the Hox-2.4 gene were aligned with similar sequences for the human c-*myc* gene (Boles and Hogan, 1987) and the chicken α2(1) collagen promoter (McKoen *et al.*, 1984). The symmetry is underlined.

Hox-2.4 RNA (data not shown). We therefore conclude that the rearranged Hox-2.4 is transcribed from the same strand as the normal gene.

Discussion

The present study has characterized the homeobox gene rearrangement which is due to a viral integration of IAP upstream of the coding region of the Hox-2.4 gene in a myeloid leukaemia cell line (Blatt *et al.*, 1987). Our nucleotide sequence of the Hox-2.4 gene and the deduced amino acid sequence show homology to conserved regions of other homeobox genes. These include the highly conserved regions of the N′-terminal octapeptide (Breier *et al.*, 1988), the hexapeptide of the first exon (Krumlauf *et al.*, 1987; Breier *et al.*, 1988) and the homeobox itself (Hart *et al.*, 1987) (Figures 6, 7 and 8). The analysis of the genomic clone was required to determine the precise site of the viral integration and further confirmation of the protein sequence can be obtained from the sequence of cDNA clones or the protein itself. Our data indicate that Hox-2.4 shares the basic structural features of other homeobox genes (Krumlauf *et al.*, 1987; Odenwald *et al.*, 1987; Hart *et al.*, 1987; Meijlink *et al.*, 1987; Fibi *et al.*, 1988; Breier *et al.*, 1988) and that Hox-2.4 is organized in two exons and encodes a protein of 240 amino acids. The conservation at the N-terminal end in addition to the peptide sequences just upstream to the homeo domain, which is shared by different homeobox genes, suggest a similar functional role for these regions.

The insertion of the IAP element is found 342 bp upstream of the initiation of the Hox-2.4 gene in the same 5′−3′ orientation. This insertion presumably removed the *cis*-acting control elements, usually present 5′ to the coding region of genes, which are necessary for the normal function of the gene. In turn, the LTR sequences of the viral element most likely provide a transcriptional promoter which causes the constitutive expression of Hox-2.4 in WEHI-3B cells. Expression of this homeobox gene could not be detected in three other myeloid leukaemias, nor in normal bone marrow RNA. Since expression of the Hox-2.4 gene is, therefore, not typical of myeloid cells, this suggests that in WEHI-3B it is due to the viral integration. WEHI-3B is a myeloid leukaemia which has been used as a source for the constitutively produced growth factor IL-3 (Lee *et al.*, 1982). It has been shown that the constitutive expression of IL-3 in these cells is due to insertion of an IAP upstream of the IL-3 gene (Ymer *et al.*, 1985, 1986). The Hox-2.4 gene rearrangement described here is the second IAP integration detected in these cells and furthermore, both occurred in one homologue of chromosome 11. However,

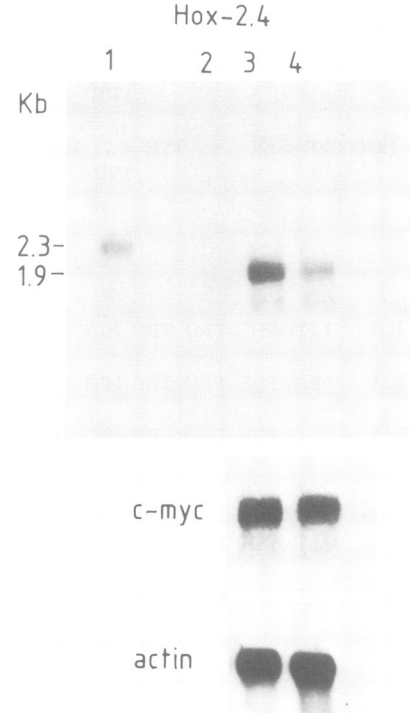

Fig. 9. Expression of Hox-2.4 RNA in normal spinal cord and in two sublines of WEHI-3B cells. This RNA was hybridized to probe b (Figure 3). **Lane 1**, total RNA from adult mouse spinal cord; **lane 2**, poly(A)$^-$ RNA from the W_1 clone of WEHI-3B; **lane 3**, poly(A)$^+$ RNA from the W_1 clone of WEHI-3B; **lane 4**, poly(A)$^+$ RNA from the W_2 clone of WEHI-3B. The RNA from clones W_1 and W_2 was also hybridized to c-*myc* and β actin probes to quantitate the amount of RNA loaded on the gels. rRNAs from *Escherichia coli* and mouse cells served as mol. wt markers.

it has not yet been determined if both occurred on the same chromosome. Apparently the physical distance between the Hox-2.4 and IL-3 genes is large since no significant linkage between them could be detected by genetic analysis of RFLPs in a set of AXB recombinant inbred strains (Lonai *et al.*, 1987). The sequence analysis performed by us on the inserted viral sequences indicates homology between the inserted Hox-2.4 and IL-3-IAP and perhaps both IAP transpositions may have occurred at the same time.

Several examples exist which demonstrate changes in gene expression as a result of novel IAP insertions. Gene activation was shown for IL-3 (Ymer *et al.*, 1985) c-*mos* (Canaani *et al.*, 1983) in cell lines and for *ren-2* in a germline integration (Burt *et al.*, 1984). Gene inactivation was reported for two κ immunoglobulin light chains (Hawley *et al.*, 1984). The result of such integrations probably

depends on whether or not the structure of the cellular gene is interrupted and also on the functional properties of the transcription control elements carried by the viral genome. In WEHI-3B cells it seems that the Hox-2.4 gene remained intact and only the determination of its transcription was affected. Normal development requires that genes express themselves at the right time, in the right cells and in the proper amounts. Exceptions to these rules can lead to abnormal development and to malignancy (Sachs, 1980, 1987). The constitutive expression of Hox-2.4 in WEHI-3B cells may interrupt the normal developmental program. Moreover, in WEHI-3B cells it is accompanied by the constitutive expression of the growth factor IL-3. It was shown that expression of IL-3, as a result of retroviral mediated gene transfer, can cause continued growth of hematopoietic cells, but tumorigenicity depends on the acquisition of additional genetic changes (Wong *et al.*, 1987). We suggest that the constitutive expression of both IL-3 and Hox-2.4 may give the combination of these two gene rearrangements an oncogenic potential, and that an altered function of this or other homeobox genes may also play a role in the development of other tumours.

Materials and methods

Cell lines

WEHI-3B (Burgess and Metcalf, 1980) is a myelomonocytic leukaemia induced by mineral oil injection in a BALB/c mouse. Two sub-lines of WEHI-3B were used, W_1, obtained from Dr T.M.Dexter (Paterson Labs., Manchester, UK), and the other, W_2, from Dr M.A.S.Moore (Memorial Sloan-Kettering Cancer Center, New York). The line obtained from M.A.S.Moore is a near diploid line that was used for the construction of the genomic library. The clone W_1 (from T.M.Dexter) has a higher chromosome number. The other cell lines used were clones 1, 10 and 7-M12 each derived from a different X-irradiation induced myeloid leukaemia in SJL/J mice (Lotem and Sachs, 1977). All cells were cultured in Dulbecco's modified Eagle's medium and 10% horse serum.

DNA probes

The probes for Hox-2.2, Hox-2.3 and Hox-2.7 were kindly given by P.Lonai and described before (Lonai *et al.*, 1987).

Probe a: an *Xba*I–*Pvu*II fragment of unique flanking sequences 3′ of the box of Hox-2,3, isolated from λmh22 (Lonai *et al.*, 1987). In Figure 3 its position is shown with respect to the overlapping region of λwh38.

Probe b: a *Hin*dIII–*Bam*HI fragment of Hox-2.4 isolated from λwh38 and subcloned in pUC-19 (Figure 3).

Probe c: a *Hin*dIII–*Pvu*II fragment including the box of Hox-2.4, isolated from λwh28 and subcloned in Bluescript M13 (Stratagene, San Diego, USA).

Mo-LTR-1: Moloney MuLV LTR probe kindly supplied by Drs M.Vogt, Salk Institute, La Jolla, CA and M.Haas, University of California, San Diego, La Jolla, CA (Bacheler and Fan, 1981).

p-400 *env*: a probe for the envelope gene of ecotropic MuLV, kindly supplied by Dr M.Martin, Laboratory of Molecular Microbiology, NIH, Bethesda (Chan *et al.*, 1980).

pMIA10-3: a plasmid that contains 6 kb of an IAP genome, kindly supplied by Dr K.Lueders, Laboratory of Biochemistry, NCI, NIH, Bethesda (Lueders and Kuff, 1980).

c-myc was kindly supplied by S.Kim (Shen-Ong *et al.*, 1982); β actin was kindly supplied by H.Czosnek (Nudel *et al.*, 1982).

Preparation of RNA

Tissue culture cells were lysed in 0.3% NP40 in buffer containing 20 mM Tris–HCl pH 8.0, 0.2 M NaCl and 20 mM $MgCl_2$ on ice. The nuclei were spun down at 800 *g* and the supernatant containing the RNA was collected. SDS was added to a concentration of 1%, EDTA to 20 mM and proteinase K (Merck, Darmstadt, FRG) to 0.2 mg/ml. After 10 min the RNA was extracted with phenol/chloroform and precipitated with ethanol. Poly(A)$^+$ RNA was purified as described (Aviv and Leder, 1972). RNA from spinal cord was isolated after homogenization in lithium-urea as described (Auffray and Rougeon, 1980).

Preparation of cellular DNA

Cells were lysed in 0.3% NP40 in buffer containing 0.3 M sucrose, 10 mM Tris–HCl pH 7.5 and 3 mM $CaCl_2$. The nuclei were spun down at 800 *g* and then resuspended in buffer containing 10 mM Tris–HCl pH 7.5, 0.1 M NaCl and 10 mM EDTA. One volume of 2% SDS was added followed by proteinase K (Merck) to a final concentration of 0.1 mg/ml. Digestion was allowed to continue overnight with a second aliquot of proteinase K. The DNA was then purified with the standard phenol/chloroform extractions and precipitated with ethanol.

Preparation of λ phage DNA

Phage DNA was prepared essentially as described (Maniatis *et al.*, 1982). Phages were precipitated from lysed cultures by 10% polyethylene glycol (PEG 6000) and residual amounts of PEG were removed by chloroform extraction. The phages were purified by glycerol step gradient and dissolved in 0.5% SDS, 20 mM EDTA and 50 μg/ml proteinase K at 65°C. The DNA was purified with standard phenol/chloroform extractions and ethanol precipitation.

Restriction endonuclease digestion

Digestion of high mol. wt mouse DNA or Phage DNA was carried out in a 4- to 6-fold enzyme excess. The enzymes were purchased from New England Biolabs, Beverly, USA or Pharmacia, Uppsala, Sweden and reactions were carried out under conditions recommended by the manufacturers.

Southern blots and hybridization

Digested DNA was electrophoresed through 0.8% agarose gel (7 μg of mouse DNA or 0.2 μg of phage DNA per lane) and transferred to nylon membrane (Gene Screen Plus, New England Nuclear Corp., Boston, USA) as described (Southern, 1975). The probes were labelled by nick-translation (Rigby *et al.*, 1977). Hybridization was carried out in 5.5 × SSC (20 × SSC being 3 M NaCl and 0.3 M Na citrate), 50% formamide, 0.5% SDS, 50 mM Tris–HCl pH 7.5 and 0.1 mg/ml denatured Salmon sperm DNA at 37°C, and 10% dextran sulphate was added for mouse genomic blots. The hybridized blots were generally washed in 2 × SSC at 65°C followed by 0.2 × SSC at 65°C.

Construction of WEHI-3B genomic library

Most steps involved in the construction and the screening of the library were carried out according to standard procedures as described (Maniatis *et al.*, 1982) and according to the special steps of cloning in λEMBL-3 (Frischauf *et al.*, 1983) and instructions by Promega Biotech (Madison, USA). Briefly, WEHI-3B DNA was partially digested with *Sau*3A (0.03 U/μg) treated with calf intestinal phosphatase and cloned into the *Bam*HI site of EMBL-3. The vector DNA was also pre-digested with *Eco*RI to prevent ligation of the stuffer region of EMBL-3. The library (2×10^6 p.f.u.) was screened with probe a (see Figure 3).

Construction of nested deletions

The DNA fragment to be sequenced (probe c) was subcloned in Bluescript M13-vector. The plasmid DNA was digested with *Kpn*I and *Sal*I (present in the polylinker site) and subjected to unidirectional deletions by *Exo*III at 34°C which were terminated at time intervals of 30 s. After S1 nuclease reaction and ligation deleted subclones were obtained which differ in an average size of 150–200 bp.

DNA sequencing

The nucleotide sequencing was determined by the dideoxy chain termination method (Sanger *et al.*, 1977), by using the Sequenase™ sequencing kit (United States Biochemical Corporation, Cleveland, USA).

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Note added in proof

We have also found that mouse myeloid leukemias with a deletion in one chromosome 2 [Azumi and Sachs (1977), *Proc. Natl. Acad. Sci. USA*, **74**, 253–257] have a deletion of one copy of the Hox-4.1 gene [Blatt and Sachs, (1988), *Biochem. Biophys. Res. Commun.*, **156**, 1265–1270]. This shows a second change in homeobox genes in tumour cells.