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Characterization of the 3′ untranslated region of the mouse homeobox gene *HoxB5*

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Abstract. The mouse pre-B cell line, 70Z/3, expresses multiple transcripts of the homeobox gene, *HoxB5*. We show here that this heterogeneity is due, at least in part, to the usage of alternative poly-A addition sites in the 3′ untranslated region (UT) of the primary *HoxB5* transcript. Furthermore, upon analysis of the subcellular distribution of the different *HoxB5* RNA species, we found that the transcripts are present mainly in the nucleus, with two-to-five-fold less RNA present in the cytoplasm. These studies suggest that multiple post-transcriptional regulatory mechanisms are involved in the expression of *HoxB5* RNA.
Key words. 70Z/3 pre-B cells; poly-A addition site; post-transcriptional regulation.

The 'homeobox' encodes a highly conserved, DNA-binding homeo-domain, which consists of 61 amino acid residues in a helix-turn-helix motif[1]. The regions flanking the homeo-domain, in general, determine the specificity of the DNA binding factor encoded by the homeobox gene[2,3]. The homeobox sequence was first identified in *Drosophila* homeotic mutants[4–6]. Since that time, homeobox gene products have fulfilled the original prediction that they would have a pivotal function in development, not only in insects but also in mammals. For example, homeotic mutations such as *Antennapedia* and *Bithorax* resulted in major disruptions of embryonic development and the organization of the *Drosophila* body plan[7–11]. Disruption of the normal pattern of expression of mouse *Hox* homeobox genes led to gross phenotypic changes. Mice transgenic for *HoxA7* (previously *Hox1.1*; see Scott[12] for nomenclature) had craniofacial abnormalities as well as duplicated vertebrae[13]. Overexpression of *HoxA4* (previously *Hox1.4*) led to abnormal gut development[14]. Replacement of *HoxA3* (previously *Hox1.5*) or *HoxA1* (previously *Hox1.6*) by null mutations led to major organ defects resulting in death early in development, usually before birth[15,16].

Evidence is accumulating that homeobox genes play a role in hematopoiesis[17–27]. Early evidence came from observations of abnormal expression of *Hox* genes in myeloid and lymphoid leukemias[22,27–30]. The group of Magli, for example, examined the expression of *Hox* genes in human leukemic and other transformed cells of hematopoietic origin. They found that specific *Hox* genes were expressed in individual lineages. Within a lineage the *Hox* genes were expressed in an ordered sequence, with the earliest cells of that lineage expressing the proximal genes in a cluster, and the more mature cells expressing the distal genes[22]. This intriguing regulatory pattern is reminiscent of the regulation of β-globin expression[31]. The correlation between the order of expression and the spatial/temporal expression pattern of *Hox* genes has also been demonstrated in transgenic *Drosophila* models, suggesting that the functional hierarchy of homeotic genes[32] is generally conserved. More direct evidence of *Hox* function in hematopoiesis has come from altered expression of *Hox* genes in mice. Mice transgenic for HB24 (a human homeobox gene) had abnormal thymic development[25] and a loss-of-function mutation in mouse *Hox11* led to absence of the spleen[33]. These and other studies make it clear that *Hox* genes are expressed in many hematopoietic lineages. Significantly, targets of some homeoproteins have been determined. For example, the gene product of human *HoxB2* (previously *Hox2H*) has been found to bind to the γ-globin enhancer[34]. In most cases, however, the function of homeobox gene products remains to be elucidated.

As part of our ongoing program to identify genes that direct B cell development, we found that RNA from the mouse pre-B cell line, 70Z/3, hybridized to probes derived from *HoxA7*, *HoxB5*, *HoxB7*, *HoxC8*, and *HoxC6* (previously *Hox1.1*, *2.1*, *2.3*, *3.1*, and *6.1*, respectively)[35]. Moreover, *HoxA7*, *HoxB5*, and *HoxC6* were found to be differentially expressed in two alternative phenotypes of 70Z/3[35].

HoxB5 is expressed in 70Z/3 sublines with a pre-B cell phenotype but not in sublines with a macrophage phenotype[35]. We show here that *HoxB5* transcripts of multiple sizes are present in 70Z/3 pre-B cells as well as in a variety of mouse tissues. As these transcripts are difficult to detect by Northern analysis, they may be classified as rare messages. We show that the multiple transcripts result, at least in part, from the usage of

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distinct poly-A addition sites in the 3′ UT of the gene. In 70Z/3 pre-B cells, *HoxB5* transcripts are present mainly in the nucleus, with two-to-five-fold less RNA present in the cytoplasm. We suggest that expression of *HoxB5* is regulated post-transcriptionally by the use of multiple poly-A addition sites and differential localization within the cell of the mature messages so produced.

Materials and methods

Tissues and cell cultures. Tissues were obtained from 5–6 week old (BALB/c × C57BL/6) F1 mice maintained in the Ontario Cancer Institute animal facility. 70Z/3 pre-B cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 10 mM HEPES, 5×10^{-5} M β-mercaptoethanol, and antibiotics, at 37 °C, 5% CO_2 as described[35].

RNA isolation and cDNA library construction. RNA for cDNA library construction was isolated from 70Z/3 pre-B cells as described[35,36]. Poly-A$^+$ RNA was isolated with an oligo dT column (Pharmacia) according to the manufacturer's instructions. The cDNA library was constructed as described[37]. Total RNA for the PCR assays was isolated by a single-step method involving acid guanidinium thiocyanate-phenol-chloroform extraction as described[38], typically from $1-2 \times 10^6$ cells or 1–2 g of homogenized tissue. Following extraction, RNA was treated with 150 U/ml RNAse-free DNAse (Pharmacia) in a buffer containing 50 mM Tris-Cl (pH 7.8), 1 mM EDTA (pH 8.0), 10 mM $MgCl_2$, and 0.1 mM dithiothreitol (DTT)[36]. RNAguard (Pharmacia) was added at 400 U/ml to prevent degradation of RNA. Cytoplasmic and nuclear RNA fractions were isolated as described[36] with modifications. Typically, $1-2 \times 10^6$ cells were pelleted by centrifugation at 12,000 rpm for 10 seconds in a microfuge (Eppendorf Centrifuge 5415C) and resuspended in 200 μl of lysis buffer containing 10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 1 mM DTT, and 400 U/ml RNAguard. After incubation on ice for 5 min, the supernatant (containing cytoplasmic RNA) was separated from the unlysed nuclei (as monitored by light microscopy) by centrifugation at 12,000 rpm for 45 s in a microfuge and added to an equal volume of a solution containing 10 mM Tris-Cl (pH 7.8), 5 mM EDTA (pH 8.0), 0.5% SDS, 100 mg/ml proteinase K. After incubation at 37 °C for 30 min, the solution was purified with phenol/chloroform extraction and isopropanol precipitation. The pelleted nuclei were washed three times with 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, and 3 mM $MgCl_2$, resuspended in 200 μl lysis buffer, and treated with proteinase K as described above. Both RNA fractions were also treated with DNAse.

RT-PCR assay. First-strand cDNA was synthesized with M-MLV reverse transcriptase (Gibco BRL) as specified by the manufacturer and described elsewhere[39,40]. 0.5 μg total RNA was used in a 30-μl reaction with 1 μM R1-dT primer, 5′-ATGTCGTCCAGGCCGCTCTGGACAAAATATGAATTCT(24)-3′. *HoxB5* cDNA was amplified by PCR with the primer pair *Hox*-P3/R1. *Hox*-P3, 5′-TCCGTGCTCCCCACTTCTCTTGG-3′, begins at nt 1146 (*HoxB5* cDNA is numbered as in Krumlauf et al.[41]). The R1 primer is the 5′ 36 nucleotides of the R1-dT primer described above. For amplification of the β-actin transcript, a 5′ primer, 5′-GTGGGCCGCTCTAGGCACCAA-3′, and a 3′ primer, 5′-CTCTTTGATGTCACGCACGATTTC-3′, were used. Amplification with this primer pair gave rise to a 540 bp product[42]. In a typical PCR, 0.5 μl of the cDNA mix was amplified in a 50 μl-reaction for 30 cycles of 1 min at 94 °C, 1.5 min at 65 °C, and 2 min at 72 °C in the DNA Thermal Cycler (Perkin Elmer Cetus), using AmpliTaq polymerase (Perkin Elmer Cetus) as specified by the manufacturer.

Southern blot analysis. One-fifth of the PCR mix was electrophoresed in 1.5% agarose. DNA was blotted onto a nylon membrane (Zetaprobe, BioRad) and hybridized to various probes. To detect *HoxB5* PCR products, we used a 1.2 kb EcoRI-SalI fragment (gift of B. Hogan) of the published *HoxB5* cDNA sequence[41] from the EcoRI site within the homeobox (nt 736) to the 3′ end of the cDNA (nt 1895). To detect the β-actin PCR product, a 500 bp PstI fragment from pAL41 was used[42]. XAR film (Kodak), with or without intensifying screens, was used for autoradiography.

Quantification. Two methods were used for quantification of Southern blots: 1) exposure to XAR film without intensifying screens and analysis with a Molecular Dynamics 300A computing densitometer running ImageQuant v. 2; 2) exposure to a storage phosphor screen and analysis with a Molecular Dynamics PhosphorImager running ImageQuant v. 3.2.

Cloning and sequencing PCR products. PCR mixes were purified from the overlaying mineral oil by phenol/chloroform and chloroform extractions. The samples were treated by standard methods[36]. The modified PCR products were then ligated to the vector pBS(-) (Stratagene), which had been linearized with HincII restriction enzyme and dephosphorylated with calf intestine alkaline phosphatase (BRL). XL1-blue bacteria (Stratagene) were made competent and transformed with ligated DNA by electroporation using a GenePulser (BioRad). Bacterial colonies carrying recombinant plasmids were screened by X-gal (Sigma) induced color selection. DNA sequences were analyzed by the Sanger dideoxy-mediated chain termination method[43] with a T7 Sequencing Kit (Pharmacia).

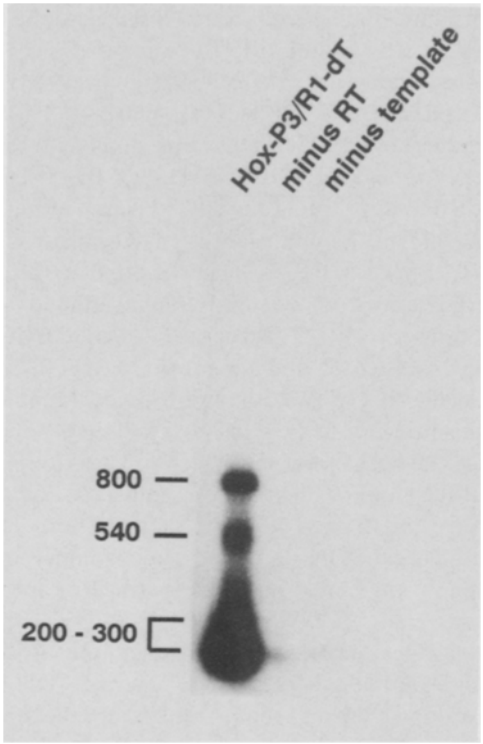

Figure 1. *HoxB5* RT-PCR products generated from 70Z/3 pre-B RNA with the *Hox*-P3/R1-dT primer pair and probed on a Southern blot with the published *HoxB5* cDNA. In lane 'minus RT', the cDNA synthesis reaction was carried out with no reverse transcriptase. In lane 'minus template', no cDNA synthesis mix was added to the PCR. Sizes are given in base pairs.

Results

3′ UT RT-PCR assay for *HoxB5* transcripts. Initial detection of *HoxB5* transcripts in lymphoid cell lines was variable and depended upon the method used to isolate the RNA[35]. Although slower, more exacting methods yielded purer RNA preparations, they rarely led to interpretable Northern blots for analyzing *HoxB5* RNA. Consistent detection was best achieved by fast RNA extraction procedures and long exposures of the blots. In all cases, transcripts of varying sizes were detected[35]. The simplest interpretation of this finding is that primary *HoxB5* transcripts are processed in multiple ways. We examined the 3′ untranslated region (UT) in some detail, as this region is often involved in RNA processing. For this purpose, we developed a 3′ UT reverse transcription-polymerase chain reaction (RT-PCR) assay.

The results of a typical 3′ UT RT-PCR assay for *HoxB5* transcripts are shown in figure 1. The 3′ primer, R1-dT, was anchored with poly-T to ensure that only poly-A$^+$ transcripts would be detected, and the 5′ primer, *Hox-P3*, was derived from the published sequence[41] in the region just 3′ of the coding sequence (fig. 2). With the *Hox-P3*/R1-dT primer pair, there were three major products of ca. 800, 540, and 200 bp. The relative abundance of the three products varies (figs 1, 2 and 4). The reason for this variation is unclear but could be an intrinsic property of the RT-PCR assay. In addition, a group of products ranging from 200 to 300 bp could be detected in small amounts on Southern blots upon long exposure (fig. 1). Thus, the 70Z/3 pre-B cell seems to contain at least three *HoxB5* transcripts that differ in the 3′ UT.

Identification and sequencing of *HoxB5* cDNA clones. To characterize the heterogeneity, we cloned the *HoxB5* transcripts from 70Z/3 pre-B cells by two methods. The first one relied on traditional RNA isolation and cDNA cloning in a λgt11 phage library constructed from poly-A$^+$ RNA of 70Z/3 pre-B cells. The second approach used direct cloning from PCR products generated with the *Hox*-P3/R1-dT primer pair. In all, we isolated four types of cDNA clones, designated *HoxB5*-200A, *HoxB5*-200B, *HoxB5*-540, and *HoxB5*-800 (fig. 2).

HoxB5-200A, a clone of about 500 bp, was isolated by the first method. This clone contains an embedded EcoRI site (nt 736) within the homeobox, and it ends in a poly-A tract at nt 1276 (fig. 2). (Numbering is based on the published *HoxB5* cDNA[41].) The second approach yielded clones of lengths expected from the three major PCR products shown in figure 1. Representative clones of each size were sequenced and are shown aligned in figure 2. All isolated clones begin at the *Hox*-P3 primer site (nt 1146–1168). *HoxB5*-200B ends in a poly-A tract at nt 1237. The actual size of this clone is 152 bp, including the 3′ primer; this is considerably less than the estimated size of 200 bp based on gel electrophoresis. The 540 bp product, *HoxB5*-540, ended in a poly-A tract at nt 1646. The 800 bp product, *HoxB5*-800, ended in a poly-A tract at nt 1895. Thus, *HoxB5*-800 corresponded to the full length 3′ UT of the published embryo *HoxB5* cDNA clone[41].

Multiple poly-A addition sites in the 3′ UT of *HoxB5* gene. The poly-A tract is usually added to RNA at a specific site[44,45]. The sequence AAUAAA is usually found 10–30 nucleotides 5′ of the poly-A tract. However, other sequences, including AAUAUA, AUUAAA, AAUAAU, AAGAAA, and AACAAA, can function in this position as polyadenylation signals[44,46]. There is also a conserved sequence that begins about 5–10 nucleotides 3′ of the polyadenylation signal. This sequence, called the G/T cluster, has the consensus YGTGTTYY (Y denotes a pyrimidine)[47].

In the published sequence of a 1.9 kb *HoxB5* cDNA[41], there are three putative polyadenylation signals at nt 1241, 1632, and 1875 (fig. 2a). In *HoxB5*-200A cDNA, there are three repeats of a putative polyadenylation signal, AAGAAA, at nt 1241 to 1257, upstream of the poly-A addition site. There is also a G/T cluster downstream at nt 1291. In *HoxB5*-540, there is a consensus polyadenylation signal, AATAAT, starting 15 bp upstream (nt 1632) of the poly-A addition site and a G/T cluster starting 10 bp downstream (nt 1656). In *HoxB5*-

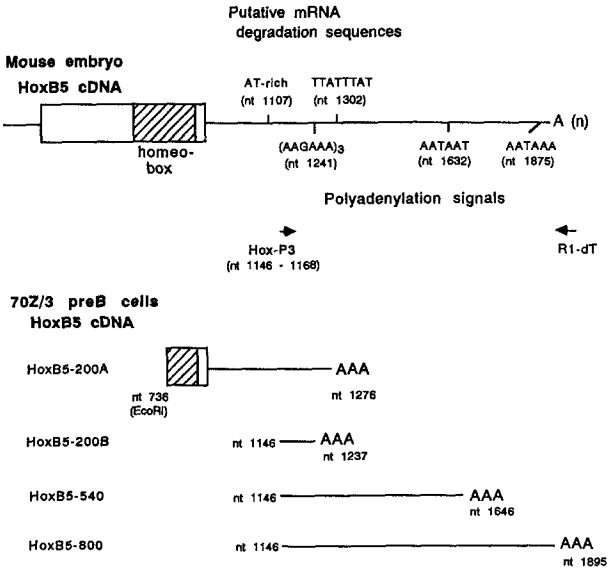

Figure 2a. Schematic diagram of the *HoxB5* cDNA from mouse embryo and 70Z/3 pre-B cells. The numbering of nucleotides (nt) is based on Krumlauf et al.[41]. Putative mRNA degradation sequences and polyadenylation signals are indicated. The primers *Hox*-P3 and R1-dT are indicated by arrows.

800, there is a consensus polyadenylation signal, AATAAA, starting 21 bp upstream (nt 1875) of the poly-A addition site; no further genomic 3′ sequences were available in the literature for analysis. These data are compatible with the notion that at least some of the multiple transcripts of *HoxB5* resulted from usage of alternative poly-A addition sites.

In *HoxB5*-200B, there is no polyadenylation signal within 30 bp of the poly-A addition site nor is there a 3′ G/T cluster at the appropriate position. It is possible that the *HoxB5*-200B sequence utilizes novel sequences for polyadenylation; it is also possible that it was cloned because the R1-dT primer annealed to the AAGAAA repeats and primed the amplification.

We have not excluded the possibility of the presence of other transcripts with even shorter 3′ UTs, as the RT-PCR assay set up here would not have detected those generated at the poly-A addition sites upstream of the primer, *Hox*-P3, used. For example, two overlapping sequences of AATAAT, starting at nt 1118, may be a polyadenylation signal for shorter transcripts.

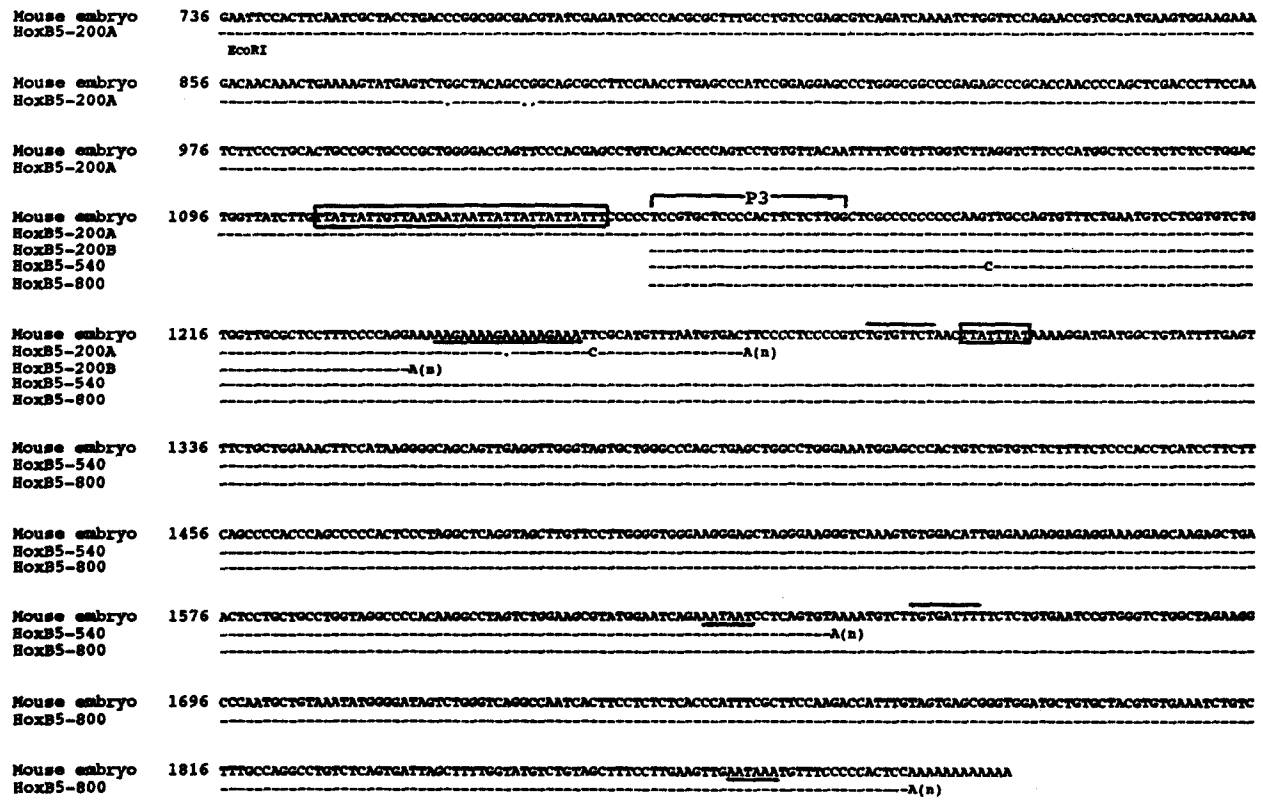

Figure 2b. Comparison of the DNA sequences of the *HoxB5* cDNA from whole embryo and 70Z/3 pre-B cells. The sequence from the EcoRI site (GAATTC) within the homeobox to the poly-A addition site of the published embryo cDNA is shown. The four clones from 70Z/3 pre-B cells are aligned to the embryo sequence; with matched nucleotides indicated by '-' (hyphen), mismatched nucleotides by the substituted ones, and missing nucleotides by '.' (period). The *Hox*-P3 primer site is marked P3. The site of termination of each cDNA product is indicated by the poly-A tract, A(n). An AT-rich region and a putative mRNA degradation signal, TTATTTAT, are marked by large and small boxes, respectively. Consensus polyadenylation signals are underlined; and G/T clusters (nt 1291 and nt 1656) are marked by lines above the sequences.

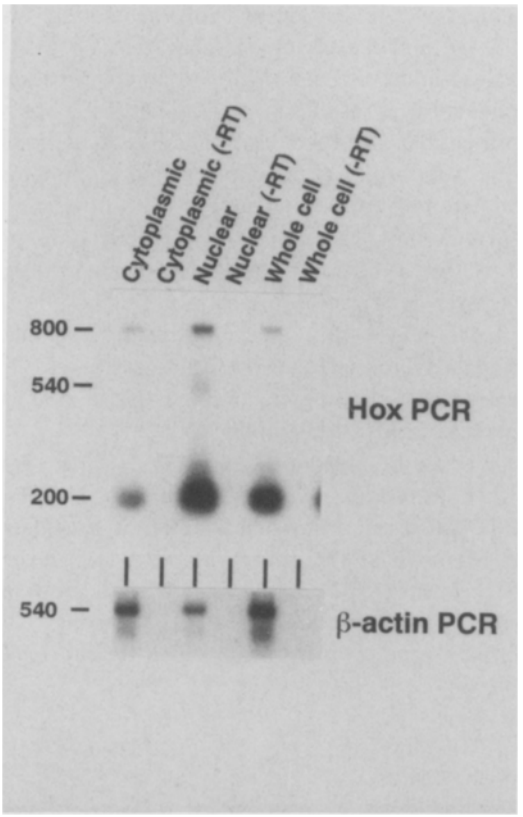

Figure 3. *HoxB5* RT-PCR products from RNA isolated from the cytoplasmic fraction, the nuclear fraction, and whole cells of 70Z/3 pre-B. PCR was carried out with the *Hox*-P3/R1-dT primer pair, and the β-actin primer pair. Southern blots were probed with *HoxB5* (upper panel) and β-actin (lower panel) (see 'Materials and methods'). Controls for cDNA-specific PCR are shown in lanes labeled (-RT). RT: reverse transcriptase. Sizes in base pairs are shown on the left.

Subcellular distribution of *HoxB5* transcripts in 70Z/3. Cytoplasmic and nuclear fractions of total RNA were isolated and examined by *HoxB5*-specific amplification. In Southern analysis (fig. 3), all three *HoxB5* RT-PCR products were more abundant in the nucleus than in the cytoplasm. Furthermore, there was no differential distribution among the three transcripts. By densitometry quantification in duplicate experiments, the ratio of the nuclear to cytoplasmic RNA of the 200, 540, and 800 bp products was 2.3:1, 4.7:1, and 2.5:1, respectively. This is in contrast to the distribution pattern of β-actin mRNA, which was less abundant in the nucleus than in the cytoplasm (1:3) (fig. 3).

Tissue distribution of *HoxB5* transcripts. We determined the prevalence and distribution of *HoxB5* transcripts in several tissues. Amplifications lacking reverse transcriptase (-RT) were included to control for the presence of residual DNA in the RNA preparations. The RNA quality of the various tissues was verified by the detection of the β-actin transcript by Northern blot analysis (data not shown). Only the samples with significantly more intense signals for the amplified products in the

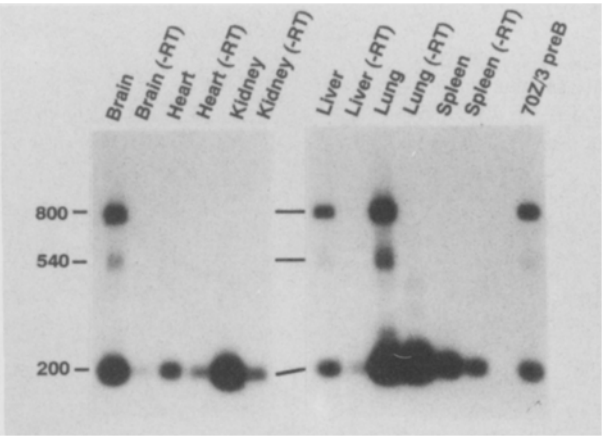

Figure 4. *HoxB5* RT-PCR products from mouse tissues. Southern blot was probed with *HoxB5* (see 'Materials and methods'). Lanes labeled (-RT) serve as controls for cDNA-specific PCR of the corresponding tissues. Sizes in base pairs are shown on the left.

RT-PCR lane than in the '-RT' lane can be interpreted as positive for the presence of the corresponding transcripts. In lung and spleen, there was a significant 200 bp band in the '-RT' lanes due to amplification of the residual DNA in the RNA preparations. Thus the 200 bp signals in these tissues are uninterpretable. However, as seen in figure 4, the distribution and amounts of the three PCR products varied with the source of the RNA. All three transcripts were present in the RNA from brain and liver. In kidney and heart, only the 200 bp transcript was detected. In lung, two major products of 540 and 800 bp and an additional product of 300 bp were observed. This 300 bp fragment could be detected in very low amounts in other tissue and cell samples. The identity of this 300 bp product is not known; our inability to clone the product is probably due to its extremely low abundance. In spleen, there are almost no RT-PCR products detectable. This observation is particularly interesting in view of our finding of all three transcripts in 70Z/3, a pre-B cell line.

Discussion

We have shown here that multiple *HoxB5* transcripts with different lengths of 3′ UT exist. Data from other systems[48–50] have revealed an important role for 3′ UT of mRNA in regulating gene expression by diverse mechanisms. These include suppression of transcription of the β-actin gene by its own 3′ UT during myogenesis[49], control of the mRNA stability mediated by AU-rich sequences on the 3′ UT of various oncogenes and growth factor genes[50], and control of gene expression of myogenin by the *trans*-acting 3′ UT of actin, troponin I, and tropomyosin mRNA during differentiation of muscle cells[48]. The presence of *HoxB5* transcripts with different lengths of 3′ UT would allow regulation of gene expression by one or more of the mechanisms

described above. The end effect would be a tight control of the steady-state levels of *HoxB5* RNA. Considering the widespread expression of *HoxB5* (fig. 4), it might be important for the cells to have this tight control if the *HoxB5* gene is to play its role in cell differentiation or growth.

We have also demonstrated that the *HoxB5* transcripts are relatively abundant in the nucleus. Several possibilities exist to account for this. First, upon leaving the nucleus, *HoxB5* RNA might be rapidly degraded by cytoplasmic RNAses. Sequence-specific RNA-binding proteins in the cytoplasm have been identified in other systems[51,52]. These proteins recognize AU-rich sequences and may mediate degradation of the RNA of genes whose products are involved in the regulation of cell growth and differentiation[50,53–55]. Similar sequences are found on the 3′ UT of *HoxB5* RNA: AT-rich sequences starting at nt 1107 and a putative mRNA degradation signal (TTATTTAT) at nt 1302 (fig. 2). It would be important to examine the role of these sequences for regulating the stability of *HoxB5* transcripts. Second, *HoxB5* RNA might be retained in the nucleus by default due to the lack of a positive signal for export to the cytoplasm. Notable features of polymerase II transcribed RNA, such as the monomethylated cap at the 5′ terminus of U1 snRNA[56–58] and sequences at the 3′ region of histone RNA[59,60], have been shown to effect or accelerate the export of homologous and heterologous RNA from nucleus to cytoplasm. However, export signal sequences have not been well defined. It remains to be seen whether a lack or deficiency of an export signal may account for the relative abundance of *HoxB5* transcripts in the nucleus. Third, the *HoxB5* transcripts could be actively retained in the nucleus. A negative signal retaining pre-mRNA in the nucleus is supplied by introns[56,61]. Normally, these pre-mRNAs would be spliced and exported to the cytoplasm very shortly after transcription has taken place. However, inefficient splicing can lead to preferential localization of these RNAs in the nucleus, probably due to prolonged association of the pre-mRNA with the splicing complex[61]. For *Hox* genes, alternative splicing involving coding regions of the neighboring genes of the loci has been detected[62]. It is conceivable that this complicated splicing process may also result in prolonged association of the pre-mRNA with the splicing machinery, and, subsequently, the differential subcellular distribution of the *HoxB5* transcripts. This possible mechanism could also account for the nuclear localization of *Hox5.1* (ref. 63). Expression studies of transfected *Hox* genes could test this hypothesis. It would be of great interest to find out whether nuclear localization is common for *Hox* RNA. If so, transcript sequestration might add another level of regulation to expression of *Hox* genes.

Another interesting observation in this study is the differential distribution of the *HoxB5* transcripts in the various tissues examined. We believe that the observed distribution of these transcripts is due to differential regulation of their expression, for several reasons. First, preferential amplification of the different products due to size does not contribute significantly to their relative abundance, because there is little correlation between the sizes of the amplified products and the intensities of the bands. This conclusion is evident from the observation that the band corresponding to the 540 bp fragment is always the weakest. Second, there is variation in the generation of the PCR products from the 70Z/3 pre-B RNA, especially the 200 bp fragment (figs 1, 2 and 4), further supporting the notion that size alone does not result in preferential amplification in this assay. Third, the absence of the longer PCR products from the heart, kidney, and spleen RNA samples is also unlikely to be due to non-specific degradation of RNA, as the RNA quality of these tissues was verified by Northern blot analysis. Therefore, the longer transcripts are either not present due to differential polyadenylation, or selectively depleted due to degradation mechanisms mediated by the putative RNA degradation signals that are present on the longer transcripts. Therefore, the results suggest that expression of the *HoxB5* transcripts may be differentially regulated in the various tissues examined.

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