Expression of Hex mRNA in early murine postimplantation embryo development

Vincent W. Keng[a], Kazuhiro E. Fujimori[b], Zaw Myint[a], Nobuaki Tamamaki[b], Yoshiaki Nojyo[b], Tamio Noguchi[a,*]

[a]*Department of Biochemistry, Fukui Medical University, Fukui 910-1193, Japan*
[b]*Department of Anatomy, Fukui Medical University, Fukui 910-1193, Japan*

Received 29 January 1998; revised version received 13 March 1998

Abstract The onset of Hex expression and its role in early murine development was analyzed using in situ hybridization. Hex mRNA was first detected in the chorion of the ectoplacental cavity and weakly at the visceral endoderm of the future yolk sac at embryonic age (E) 7.5. Expression in embryonic tissues was detected exclusively in the hepatic anlage and thyroid primordium at E 9.5. At E 12.5 and E 15.5, Hex expression persisted in the fetal liver and thyroid, and was also detected in the fetal lung. These results suggest that Hex has its role in differentiation and/or organogenesis of several embryonic tissues.

Key words: Hex; Liver; Thyroid; Lung; In situ hybridization; Murine embryo

1. Introduction

Homeobox genes are a large family of transcription factors that regulate cell differentiation during development [1,2]. They are distinguished by their highly conserved approximately 60 amino acid motif called the homeodomain that mediates DNA binding. These genes were first discovered in *Drosophila* as regulators of body segment formation during early development by acting as DNA-binding transcription factors [3]. Most human homeobox genes are found in four distinct genomic clusters of 9 to 11 homeobox genes called HOXA, HOXB, HOXC and HOXD, which are located on chromosomes 7, 17, 11 and 2, respectively [4]. These genomic clusters are expressed along the anterior to posterior body axis according to their position in the HOX cluster. Homeobox genes outside of these clusters are called orphans.

Hex is one of such orphans. This homeobox is also called Prh, since its N-terminal region is rich in proline residues, which may function as a transcriptional regulatory domain. Hex cDNAs have been isolated from the mouse, human and chicken [5–7]. Its human gene has been mapped to chromosome 10 [6]. Northern blot analysis has indicated that this gene is expressed in B-cell and monomyeloid lineages, and also in pluripotent progenitor lines. However, Hex is not expressed in mature erythroid and T-cell lineages. Based upon these results, it has been postulated that Hex plays a role in hematopoietic differentiation [7].

We have recently isolated rat Hex cDNA during screening of transcription factors interacting with the transcriptional regulatory regions of the liver-specific L-type pyruvate kinase gene (unpublished data). Further analysis revealed that Hex was not a transcription factor of this gene. However, we are interested in this gene since high Hex mRNA levels were observed in adult rat hepatocytes and embryonic age (E) 15 fetal rat livers by Northern blot analysis (unpublished data), raising the possibility that Hex may play a role in hepatocyte differentiation. Thus, we decided to examine the developmental expression of the mouse Hex gene using in situ hybridization analysis.

Here we report that Hex gene expression is first observed during early murine development at E 7.5, primarily in the chorion of the ectoplacental cavity and weakly at the visceral endoderm in the region of the future yolk sac. Expression in embryonic tissue is first detected in the hepatic anlage and thyroid primordium at E 9.5. From E 12.5, Hex was predominantly expressed in the fetal liver and developing thyroid, and weakly in the developing fetal lung. These results suggest that Hex plays a role in the differentiation and/or organogenesis of several embryonic tissues.

2. Materials and methods

2.1. Animals

Mice of strain C57BL/6NCrj were obtained from Charles River Japan Inc., with noon on the day of finding a vaginal plug designated E 0.5. Mice were killed by cervical dislocation, fetuses removed and cleaned in 0.9% NaCl. E 12.5 and E 15.5 fetuses were removed from the uterus sac before fixation. E 5.5, E 7.5 and E 9.5 fetuses were left in the uterus sac but cut into individual segments to allow for fixation.

2.2. Fixation

Fetuses were fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (PB) at 4°C before being transferred to 10% sucrose/PB solution for overnight incubation at 4°C. Fetuses were then embedded in OCT compound (Miles Inc., USA) and stored at −80°C in sealed bags until cryostat sectioning.

2.3. Probe preparation

A sequence of 417 bp was obtained from the 3′ non-coding region of the mouse Hex genomic sequence (unpublished data) by restriction enzyme digestion with *Acc*I and *Eco*RI (Takara Shuzo, Japan), and subcloned into pBluescript SK (Stratagene, USA). The plasmid was linearized with appropriate restriction enzymes and used as templates for the synthesis of ^{35}S-labelled RNA probes. T7 and T3 polymerases were used to synthesize the sense and antisense probes, respectively. Sections were hybridized with either labelled sense strand RNA, which showed no signal above background (all sense data not shown), or labelled antisense RNA.

2.4. In situ hybridization

We essentially followed a protocol previously described [8], except for a few modifications. Briefly, serial sections (20 μm thick) were cut

*Corresponding author. Fax: (81) (776) 61-8102.
E-mail: tnoguchi@fmsrsa.fukui-med.ac.jp

Abbreviations: E, embryonic age; PB, phosphate buffer; HNF, hepatocyte nuclear factor; TTF1, thyroid transcription factor 1

PII S0014-5793(98)00342-1

by cryostat, mounted onto poly-L-lysine (Sigma, USA) coated Superfrost slides (Matsunami, Japan) and dried in a vacuum chamber. Slides were fixed with 4% paraformaldehyde in PB, treated with proteinase K (10 μg/ml), acetylated with acetic anhydride and dehydrated through a graded series of ethanols.

RNA probes (final concentration 5×10^6 cpm/ml) were added to hybridization buffer containing 50% deionized formamide, 0.3 M NaCl, 1× Denhardt's solution, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 10 mM dithiothreitol, 500 μg/ml yeast tRNA, 10% dextran sulfate. Sections were covered with the hybridization buffer and incubated at 56°C for 16–20 h in a humidified chamber. The sections were washed under conditions of high stringency, treated with RNase A (20 μg/ml) in 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), 0.01 mM EDTA (pH 8.0) and dehydrated with a graded series of ethanols before exposure to phosphorimaging plate for Bioimaging analyzer BAS1500 (Fujifilm Co., Japan) for 12 h and X-ray film (Hyperfilm β Max Amersham, USA) for 3–4 days. Hybridized sections were then coated with autoradiography emulsion (Kodak NBT-3, USA) and exposed for 2–4 weeks. After developing, sections were lightly counter-stained with crystal violet.

2.5. Northern blot analysis

Total RNA was prepared from various adult mouse tissues as previously described [9]. Electrophoresis of total RNA (10 μg), subsequent transfer to a nylon filter and hybridization of the filter with labelled probes were essentially carried out as described before [10]. The probe for Hex was described above and that for β-actin was described previously [11]. They were labelled with [α-^{32}P]dCTP by random priming (Megaprime Kit, Amersham, USA).

3. Results

3.1. Northern blot analysis

Northern blot analysis using the 417-bp probe for Hex detected specific hybridization to about 1.9 kb transcript strongly in the adult mouse liver and weakly in the spleen (Fig. 1).

3.2. In situ hybridization

At E 5.5, there was no detectable level of Hex mRNA in either the ectoderm or the primary endoderm of the developing embryo. Screening the serial sections using darkfield microscopy could not detect any signal above background (data not shown).

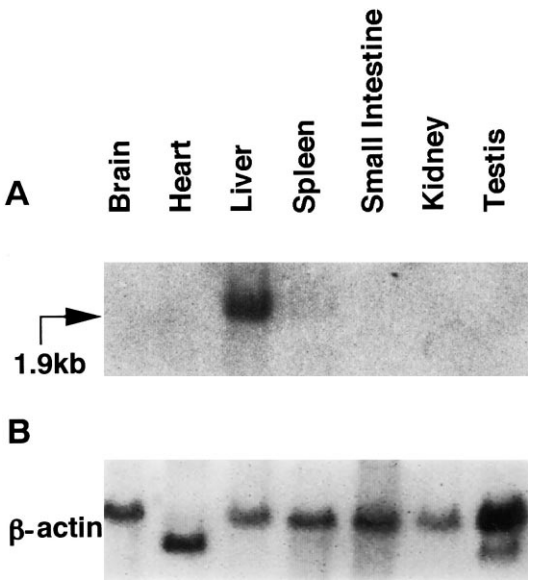

Fig. 1. Northern blot analysis of Hex expression in various adult mouse tissues. The filter was first hybridized with the probe for Hex (A), then it was stripped and rehybridized with β-actin probe to check for RNA integrity and to compensate for loading differences (B).

Parasagittal sections through the early primitive-streak stage embryo (E 7.5) demonstrated strong Hex expression restricted to the chorion of the ectoplacental cavity and weakly at the visceral endoderm in the region of the future yolk sac (Fig. 2A,B). Hex was not detected in any embryonic tissues up to this stage of development.

Two areas of strong Hex expression were observed in sections through the early 'turned' embryo (E 9.5). At this stage of development, the hepatic anlage interacting with the mesenchyme of the septum transversum strongly expressed Hex mRNA (Fig. 2C,D). High levels of Hex mRNA were also detected in the thyroid primordium (Fig. 2E,F).

Expression of Hex mRNA was observed in the fetal liver, developing thyroid and fetal lung of the E 12.5. Hex was strongly expressed in the fetal liver (Fig. 2G,H) and the developing thyroid (Fig. 2I,J), while the developing fetal lung weakly expressed Hex mRNA (Fig. 2G,H).

At E 15.5, Hex continues to be expressed in the fetal liver, developing thyroid and fetal lung (data not shown as morphology was similar to E 12.5).

4. Discussion

The present studies suggest that Hex has a role in early murine embryonic development, especially in the fetal liver and thyroid.

Several liver-enriched transcription factors have been shown to be expressed in the embryo at early stages of development. Duncan et al. [12] has demonstrated the onset of hepatocyte nuclear factor (HNF) 4α at E 4.5 in the primary endoderm. At E 5.5 and E 7.5, HNF4α was observed in the visceral endoderm surrounding the embryo where it is likely to induce secreted proteins to nourish the developing embryo. The onset of Hex expression was only observed from E 7.5, in the chorion of the ectoplacental cavity and weakly in the visceral endoderm of the future yolk sac, the former of which later in development becomes the placenta and takes over the function of the visceral endoderm. Up to this stage of development, expression of both Hex and HNF4α mRNA remained extraembryonic. HNF1β (variant HNF1) is also detected at E 5 in the visceral endoderm [13] and HNF3β is expressed in the primitive streak [14]. The different extraembryonic sites of expression of Hex and other HNFs may be due to different roles in extraembryonic tissue, contributing to the growth of the embryo.

During preparation of the manuscript, Thomas et al. [15] reported Hex expression in the mouse embryo at early stages of development by in situ hybridization. They detected Hex transcript in the primitive endoderm at E 4.5 and in the visceral endoderm at E 5.5. They also found transient Hex expression in endothelial precursors by E 7.5. The discrepancy between these findings and our findings may be due to differences in probe selection. While Thomas et al. utilized the coding region of the Hex gene as a hybridization probe, we used the 3′ non-coding region since the coding region may contain sequences highly similar to those of other homeobox genes. Northern blot analysis using this probe revealed that the size of mRNA hybridized and its expression pattern in adult tissues corresponded to Hex mRNA reported previously

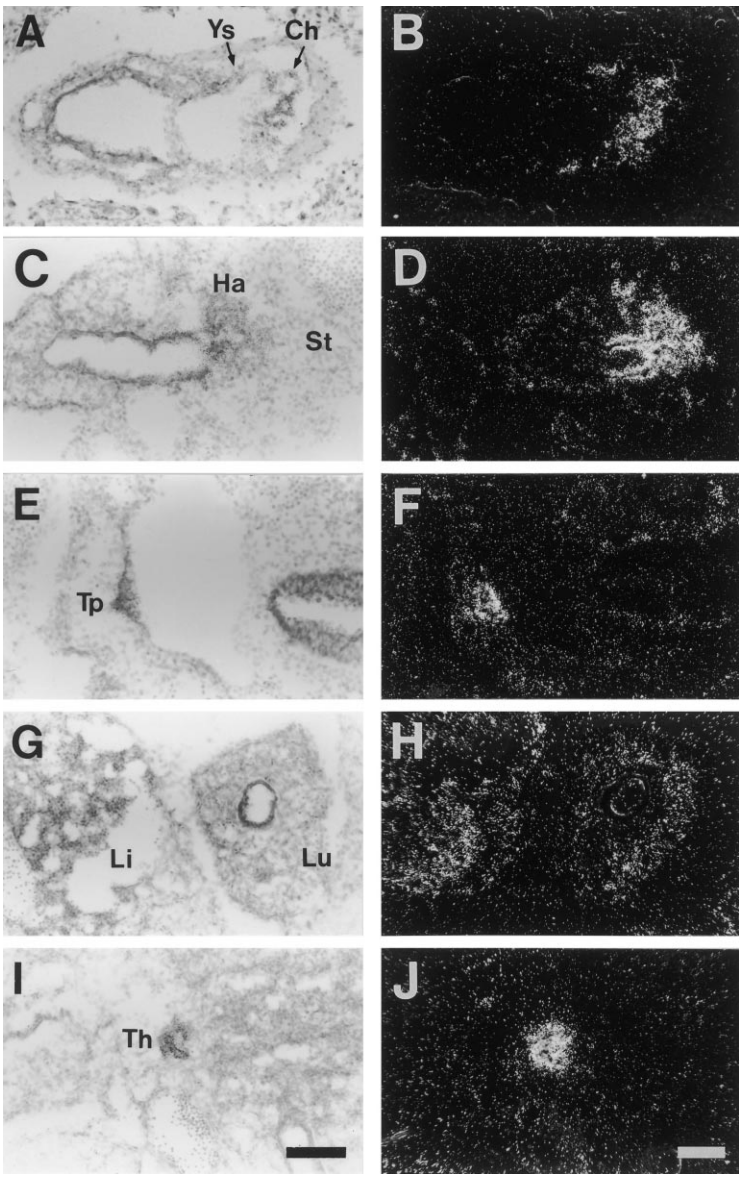

Fig. 2. Brightfield and corresponding darkfield photographs of Hex expression at various stages of murine development. A,B: Parasagittal section through the early primitive-streak stage embryo (E 7.5) hybridized with Hex. Expression was restricted to the chorion (Ch) of the ectoplacental cavity and weakly at the visceral endoderm in the region of the future yolk sac (Ys). C–F: Expression of Hex in sections through the early 'turned' embryo (E 9.5). C,D: The hepatic anlage (Ha), interacting with the mesenchyme of the septum transversum (St) strongly expressed Hex. E,F: Expression was also strongly observed in the thyroid primordium (Tp). G–J: Hex expression at E 12.5. G,H: Composite photograph demonstrated strong expression in the fetal liver (Li) and weak expression in the developing fetal lung (Lu). I,J: Specific expression was also observed in the developing thyroid (Th). Similar scale for C, E, G and I; A, B, D, F, H and J. Bars, 100 μm.

[5–7]. However, we cannot exclude the possibility that we failed to detect very low levels of Hex transcript. Further analysis is required to determine the exact onset time of Hex expression in embryo.

In the early 'turned' embryo (E 9.5), there was a shift in expression of Hex mRNA from the extraembryonic tissue to embryonic tissue. Hex mRNA was strongly detected in the hepatic anlage and thyroid primordium. Liver development is thought to occur in three stages: interaction of cardiac mesoderm with foregut endoderm, interaction with the mesenchyme of the septum transversum and finally, further differentiation [16–18]. Our observation that Hex is expressed in the hepatic anlage of the early 'turned' embryo interacting with the septum transversum strongly suggests the role of Hex in liver development. Hepatic differentiation is known to begin at this early stage of liver development, well before the structure of the organ is defined. Other transcription factors which may be involved in liver development include members of HNF1, HNF3 and HNF4 [19,20]. HNF3 proteins define the regionalization within the definitive endoderm and are expressed early in the definitive endoderm lineage [21]. Transcription factors induced at the onset of liver differentiation include HNF4α and HNF1β; HNF1α only appears at the time of liver organogenesis. Hex gene activation appears to occur after the HNF3 gene family, around the same time as HNF1β/HNF4α genes and before the HNF1α gene. Thus, we postulate that Hex is also involved in the complex transcriptional regulatory network controlling liver development.

However, further studies are required to clarify the relation between Hex and these factors.

Hex has been previously detected in the developing thyroid of *Xenopus* [22]. From E 9.5, the developing fetal thyroid continues to express Hex strongly. This expression pattern is similar to thyroid transcription factor 1 (TTF1), a homeodomain-containing transcription factor expressed at the onset of thyroid morphogenesis [23]. Therefore, there may be a relation between Hex and TTF1 in early thyroid development.

In addition, Hex may play a role in lung development since we observed Hex expression in the fetal lung at E 12.5 and E 15.5.

Acknowledgements: The authors thank Professor Akio Wanaka, Department of Cell Science, Institute of Biomedical Sciences, Fukushima Medical College, for his valuable advice.

References

[1] Wright, C.V., Cho, K.W., Oliver, G. and De Robertis, E.M. (1989) Trends Biochem. Sci. 14, 52–56.
[2] Gehring, W.J., Affolter, M. and Burglin, T. (1994) Annu. Rev. Biochem. 63, 487–526.
[3] Gehring, W.J. (1987) Science 236, 1245–1252.
[4] Acampora, D., D'Esposito, M., Faiella, A., Pannese, M., Migliaccio, E., Morelli, F., Stornaiuolo, A., Nigro, V., Simeone, A. and Boncinelli, E. (1989) Nucleic Acids Res. 24, 10385–10402.
[5] Crompton, M.R., Bartlett, T.J., MacGregor, A.D., Manfioletti, G., Buratti, E., Giancotti, V. and Goodwin, G.H. (1992) Nucleic Acids Res. 20, 5661–5667.
[6] Hromas, R., Radich, J. and Collins, S. (1993) Biochem. Biophys. Res. Commun. 195, 976–983.
[7] Bedford, F.K., Ashworth, A., Enver, T. and Wiedemann, L.M. (1993) Nucleic Acids Res. 21, 1245–1249.
[8] Fujimori, K.E., Takauji, R., Yoshihara, Y., Tamada, A., Mori, K. and Tamamaki, N. (1997) J. Histochem. Cytochem. 45, 455–459.
[9] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[10] Matsuda, T., Noguchi, T., Takenaka, M., Yamada, K. and Tanaka, T. (1990) J. Biochem. (Tokyo) 107, 655–660.
[11] Hotta, K., Nakajima, H., Yamasaki, T., Hamaguchi, T., Kuwajima, M., Noguchi, T., Tanaka, T., Kono, N. and Tarui, S. (1991) Eur. J. Biochem. 202, 293–298.
[12] Duncan, S.A., Manova, K., Chen, W.S., Hoodless, P., Weinstein, D.C., Bachvarova, R.F. and Darnell Jr., J.E. (1994) Proc. Natl. Acad. Sci. USA 91, 7598–7602.
[13] Cereghini, S., Ott, M.O., Power, S. and Maury, M. (1992) Development 116, 783–797.
[14] Monaghan, A.P., Kaestner, K.H., Grau, E. and Schütz, G. (1993) Development 119, 567–578.
[15] Thomas, P.Q., Brown, A. and Beddington, R.S.P. (1998) Development 125, 85–94.
[16] Douarin, N.M. (1975) Med. Biol. 53, 427–455.
[17] Houssaint, E. (1980) Cell Differ. 9, 269–279.
[18] Cascio, S. and Zaret, K.S. (1991) Development 113, 217–225.
[19] Cereghini, S. (1996) FASEB J. 10, 267–282.
[20] Zaret, K.S. (1996) Annu. Rev. Physiol. 58, 231–251.
[21] Ang, S., Wierda, A., Wong, D., Stevens, K.A., Cascio, S., Rossant, J. and Zaret, K.S. (1993) Development 119, 1301–1315.
[22] Newman, C.S., Chia, F. and Krieg, P.A. (1997) Mech. Dev. 66, 83–93.
[23] Lazzaro, D., Price, M., De Felice, M. and Di Lauro, R. (1991) Development 113, 1093–1104.