Isx Participates in the Maintenance of Vitamin A Metabolism by Regulation of β-Carotene 15,15’-Monooxygenase (Bcmo1) Expression

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Isx (intestine specific homebox) is an intestine-specific transcription factor. To elucidate its physiological function, we generated Isx-deficient mice by knocking in the β-galactosidase gene (LacZ) in the Isx locus (IsxLacZ/LacZ mice). LacZ staining of heterozygous (IsxLacZ +/-) mice revealed that Isx was expressed abundantly in intestinal epithelial cells from duodenum to proximal colon. Quantitative mRNA expression profiling of duodenum and jejunum showed that β-carotene 15,15’-monooxygenase (EC1.14.99.36 Bcmo1) and the class B type I scavenger receptor, respectively, were drastically increased in IsxLacZ/LacZ mice. Although mild vitamin A deficiency decreased Isx expression in duodenum of wild-type (Isx +/-) mice, severe vitamin A deficiency decreased Isx mRNA expression in both duodenum and jejunum of Isx +/- mice. On the other hand, vitamin A deficiency increased Bcmo1 expression in both duodenum and jejunum of Isx +/- mice. However, Bcmo1 expression was not increased in duodenum of IsxLacZ/LacZ mice by mild vitamin A deficiency. These data suggest that Isx participates in the maintenance of vitamin A metabolism by regulating Bcmo1 expression in the intestine.

The epithelium of the small intestine comprises four types of cells: absorptive epithelial cells that absorb nutrients in the intestine, goblet cells that secrete mucus, Paneth cells that secrete antibacterial lysozyme, and gut endocrine cells that secrete various intestinal hormones (1). All of these cells are known to differentiate from common stem cells located at the lower half of crypts. Regulation of various gene expression by transcription factors is essential in the process of differentiation of progenitor cells into functionally matured cells (2–4). Of the various transcription factors, homeodomain (HD)2 transcription factors play a critical role in differentiation, proliferation, and organogenesis (5). For example, HD transcription factors such as Hex (6), Pdx-1 (7), and Cdx (Cdx1 (8) and Cdx2 (9, 10)) are involved in the development of endodermal-derived tissues such as liver, pancreas, and intestine, respectively.

We previously identified an HD transcription factor Dmbx1, which is expressed specifically in the brain during development (11) and involved in the maintenance of energy homeostasis (12). To identify an HD transcription factor resembling Dmbx-1, we screened the entire murine genomic data base and identified a genomic sequence encoding a novel HD transcription factor. This transcription factor was found to be specifically expressed in midgut-derived intestine and so was initially named midgut homeobox transcriptional factor 1. However, midgut homeobox transcriptional factor 1 was later shown to be identical to Isx (intestine-specific homeobox) by Choi et al. (13). They reported that transgenic mice expressing Cdx2 in the stomach exhibited ectopic Isx expression in stomach and that homozygous Isx knock-out mice showed increased expression of SR-BI in duodenum and ileum.

To clarify the physiological role of Isx, we generated Isx-deficient mice by knocking in LacZ in the Isx locus (IsxLacZ/LacZ mice) and found that expression of Bcmo1, a carotinoid cleavage enzyme that converts β-carotene to retinal (precursor of retinol and retinoic acid) (14, 15), was drastically induced in duodenum and jejunum of IsxLacZ/LacZ mice. Thus, Isx participates in the maintenance of vitamin A metabolism by regulating Bcmo1 expression in the intestine.
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EXPERIMENTAL PROCEDURES

Generation of IsxLacZ/LacZ Mice and Transgenic Mice Overexpressing Isx—Isx knock-out (IsxLacZ/LacZ) mice ( accession number CD80475K, Center for Developmental Biology, RIKEN, Kobe, Japan) were generated by replacing the amino acid coding sequences in exon 1 of Isx with LacZ as described (supplemental Fig. S1). Embryonic stem cells used to generate germline chimera were TT2 (16), and the mutant mice were backcrossed to mouse strain C57BL/6. All of the animal procedures were approved by the Animal Care Committees of Kobe University and the Center for Developmental Biology. The details of IsxLacZ/LacZ mice production will be provided upon request. Transgenic mice overexpressing Isx were generated by crossbreeding CAG-Cre mice (ubiquitously expressing Cre recombinase) (17) and CAG-stop-Isx mice, in which Isx expression is induced after genomic recombination by Cre recombinase (supplemental Fig. S2).

In Situ Hybridization—Two nonoverlapping antisense oligonucleotide probes (45-mer in length) were designed for each mRNA of mouse Isx, Bono1, and SR-BI. The antisense probes used were complementary to the following sequences: 631–675 and 1031–1075 of mouse Isx (GenBankTM accession number AB219123); 427–471 and 1520–1564 of mouse Bono1 (GenBankTM accession number NM_021486); and 329–373 and 953–997 of mouse SR-BI (GenBankTM accession number NM_016741). Hybridization was performed at 42 °C for 10 h with a hybridization buffer containing 10,000 cpm/μl 32P-labeled oligonucleotide probe. Sections were exposed by dipping in autoradiographic emulsion (NTB-2; Kodak) at 4 °C for 8–12 weeks. The specificity of the hybridization was confirmed by identical leveling of two nonoverlapping probes and by the disappearance of the signals upon the addition of an excess of unlabeled antisense probe.

Southern Blot Analysis and Northern Blot Analysis—Southern blot analysis of mouse tail genomic DNA and Northern blot analysis of the tissues were performed by standardized procedures. The probe used for Southern blotting was a genomic DNA fragment as shown in supplemental Fig. S1. The probes for Northern blotting were the cDNA fragments of mouse Isx (corresponding to nucleotides +1 to +369 of GenBankTM accession number AB219123), mouse Cdx1 (corresponding to nucleotides +950 to +1283 of GenBankTM accession number NM_009880), and mouse Cdx2 (corresponding to nucleotides +487 to +891 of GenBankTM accession number NM_007673).

Real Time Reverse Transcriptase (RT)-PCR Analysis—Real time quantitative RT-PCR was performed by TaqMan probes (PerkinElmer Life Sciences) using a PE ABI PRIZM 7000 apparatus (PerkinElmer Life Sciences) as previously described (12). The amount of mRNA of the gene of interest was normalized by amplification of a fragment of mouse hypoxanthine-guanine phosphoribosyltransferase (Hprt) was carried out in parallel in each sample. RT-PCR experiment was performed under standardized conditions. PCR primers for mouse Hprt and Isx were; Isx, 5′-CACCTCAACCATTACCTGAC-3′ and 5′-CTAT-GTTGAAATTGCACAGAT-3′, and Hprt, 5′-TCTTTTCGTGACCTGCGTGGATT-3′ and 5′-GCTTTTGTATTTGCT-TTTCC-3′. Denaturation was at 94 °C for 30 s, annealing was at 56 °C for 30 s, and extension was at 72 °C for 1 min for 35 cycles with hot start at 94 °C for 5 min.

LacZ Staining—For X-gal histochemistry, tissues were fixed in 2.5% glutaraldehyde and stained with buffer containing X-gal solution (phosphate-buffered saline, 5 mm potassium ferrocyanide, 5 mm potassium ferricyanide, 2 mm MgCl2) for 3–6 h.

Vitamin A-deficient Conditions—The vitamin A-sufficient (VAS) diet (containing 27 IU in reduced value of retinol/g) and the vitamin A-deficient (VAD) diet (less than 1 IU in reduced value of retinol/g) were from CLEA, Japan, Inc. The mice had free access to food and water. Isx+/+ (n = 4–9) and IsxLacZ/LacZ (n = 4–8) adult mice were fed a VAS diet for more than 4 weeks and then placed on a diet with marginal or sufficient vitamin A content for 18 days. Female mice on the VAD diet were placed in a mating cage. After getting pregnant, these female mice were housed individually and maintained on the VAD diet. After 49 days including the lactation period, the mice were euthanized, and the duodenum and jejunum were sampled.

Statistical Analysis—The data are expressed as the means ± S.E. Comparisons were made using Student’s t test. A probability level of p < 0.05 was considered statistically significant.

RESULTS

Isolation of Isx from Different Species—Mouse Isx cDNA encodes 240 amino acids, with one HD in the middle and glutamine at the 50th position in the HD. Isx is structurally a paired-like HD transcription factor. We also cloned the human, rat, and bovine homologs of this transcription factor (supplemental Fig. S3).

Spatial and Temporal Expression Patterns of Isx—Northern blot analysis showed expression of Isx in the small intestine, but none at all in the esophagus and stomach (Fig. 1A). To further examine expression sites in the intestine, 0.5-cm-thick samples collected each 2 cm from the duodenum to the rectum were subjected to Northern blot analysis (Fig. 1B). Isx was expressed at very high levels from the ileum to the proximal colon and at low levels in the proximal duodenum. Expression patterns of Isx closely resembled those of Cdx2, except that Cdx2 was expressed in the distal colon, whereas Isx was not. Furthermore, to investigate expression of Isx in the crypt-villus axis of the intestine, the adult intestinal tract (duodenum, jejunum, and ileum) was subjected to in situ hybridization. mRNA expression of Isx in the small intestine was detected from the upper crypt to the villus along the axis, but in each villus, expression weakened toward the tip (Fig. 1C). Analysis by RT-PCR of the temporal profile of Isx expression during embryogenesis indicated that Isx was clearly expressed after E9.5 (Fig. 1D). To investigate the expression and physiological role of Isx, we generated mice in which LacZ was knocked in the Isx locus (supplemental Fig. S1). First, adult IsxLacZ/LacZ (heterozygous) mice were analyzed by
In adults, the major part of the intestine was intensely labeled, but the proximal end of duodenum was weakly stained, and the distal half of the large intestine was mostly unstained, a finding consistent with the results of Northern blot analysis for Isx. In addition, expression of Isx during the prenatal period (E15.5) and 2 days after birth (P2) was examined in IsxLacZ/+ fetuses and neonates (Fig. 1F). At E15.5, Isx was expressed throughout the intestine, including the lower half of the large intestine, but at P2, the expression in the lower half of large intestine was markedly reduced, indicating that the Isx expression pattern in neonates resembles that in adults. This suggests that Isx expression in the intestine becomes restricted to the midgut region with fetal development.

**Increased Expression of SR-BI in IsxLacZ/LacZ Mice**—To comprehensively analyze the effects of Isx on gene expression, we performed microarray analysis of duodenal samples from both Isx<sup>+/+</sup> and Isx<sup>LacZ/LacZ</sup> mice. We focused on genes expressed at relatively high levels in the duodenum of Isx<sup>+/+</sup> mice. We found that expression of the SR-BI transcript (18, 19) was elevated in Isx<sup>LacZ/LacZ</sup> mice. mRNA expression of SR-BI was then analyzed by real time RT-PCR. Compared with Isx<sup>+/+</sup> mice, mRNA expression of SR-BI for Isx<sup>LacZ/LacZ</sup> mice was significantly increased in duodenum, jejunum, and terminal ileum (Fig. 2A). These data were confirmed morphologically by in situ hybridization (Fig. 2B). Previous studies showing that expression of SR-BI is strong in duodenum but weak in jejunum and ileum (20, 21) also accord with the present results (Fig. 2B).

Compared with Isx<sup>+/+</sup> mice, no significant increase in SR-BI was detected in Isx<sup>LacZ/LacZ</sup> mice (data not shown).

**Increased Expression of Bcmo1 in IsxLacZ/LacZ Mice**—Because it has been reported that SR-BI is involved in absorption of various substrates such as β-carotene, lutein, and vitamin E (22–26) in addition to cholesterol (18, 19, 27), we reanalyzed the microarray data to include the genes associated with transport of these substrates. Interestingly, we found that expression of Bcmo1, an enzyme that catalyzes the conversion from β-carotene to retinal (14, 15), was higher in Isx<sup>LacZ/LacZ</sup> mice than in Isx<sup>+/+</sup> mice. To ascertain changes in Bcmo1 in Isx<sup>LacZ/LacZ</sup> mice, real time RT-PCR was performed (Fig. 3A). In Isx<sup>LacZ/LacZ</sup> mice, expression of Bcmo1 was increased markedly in duodenum and jejunum and somewhat in terminal ileum. Expression of Bcmo1 in Isx<sup>LacZ/LacZ</sup> mice was significantly higher in duodenum and jejunum compared with Isx<sup>+/+</sup> mice. In situ hybridization confirmed that Bcmo1 expression was drastically induced in the epithelium of duodenum and jejunum of Isx<sup>LacZ/LacZ</sup> mice (Fig. 3B).

**Changes in Expression of SR-BI and Bcmo1 in Isx<sup>LacZ/+</sup> and Isx<sup>LacZ/LacZ</sup> Fetuses**—Expression of Bcmo1 and SR-BI was examined in the intestines of E17.5 Isx<sup>LacZ/+</sup>, Isx<sup>LacZ/LacZ</sup>, and Isx<sup>LacZ/LacZ</sup> fetuses (Tables 1 and 2). As in adult, expression of Bcmo1 in Isx<sup>LacZ/LacZ</sup> fetuses was markedly increased in duodenum and jejunum (Tables 1 and 2). Expression of SR-BI in Isx<sup>LacZ/LacZ</sup> fetuses also was significantly higher in duodenum and in jejunum compared with Isx<sup>+/+</sup> fetuses (Tables 1 and 2). Expression of SR-BI in Isx<sup>LacZ/LacZ</sup> fetuses was moderately but significantly higher in duodenum, jejunum, and ileum compared with Isx<sup>+/+</sup> fetuses (Tables 1 and 2). The degree of increase in SR-BI in fetuses was less than in adults.

**Effects of Overexpression of Isx on Transcriptional Regulation of Bcmo1 and SR-BI**—The effect of overexpression of Isx was further studied in mouse intestine using a transgenic approach. We generated CAG-stop-Isx mice, in which Isx expression is induced only after genomic recombination by Cre recombinase (supplemental Fig. S2A). Expression of Isx was then induced by crossing the mice with CAG-Cre mice (17). The double mutant (CAG-stop-Isx::CAG-Cre) mice exhibited a high mortality rate at birth (the relative number of the double mutants at postnatal day 1 (P1) was approximately one-tenth that expected from Mendelian law), but the surviving individuals had no apparent gross abnormality in adults. The expression of Isx in intestine was confirmed by Northern blot analysis (supplemental Fig. S2B), and mRNA expression of Bcmo1 and SR-BI in duodenum,
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Jejunum, ileum, and liver was examined by real time RT-PCR. Interestingly, the transgenic expression of Isx significantly decreased Bcmo1 expression in the ileum (the relative expression of Bcmo1 (Bcmo1/β-actin) in CAG-stop-Isx::CAG-Cre mice was 0.46-fold lower compared with control (CAG-Cre) mice) (Table 3). Bcmo1 expression in the duodenum and jejunum of CAG-stop-Isx::CAG-Cre mice seemed to show a similar tendency, but there was no statistical significance because of a large variation among the samples. By contrast, the expression of SR-BI did not change in duodenum, jejunum, or ileum.

Changes in Expression of Isx, Bcmo1, and SR-BI by Vitamin A Deficiency—The effects of vitamin A deficiency in the intestine on the expression of Isx were first examined in Isx+/+ mice. Expression of Bcmo1 in Isx+/+ mice fed a VAD diet for 18 days was significantly elevated in duodenum, jejunum, and ileum compared with Isx+/+ mice fed a VAS diet (Fig. 4A). Although no differences in expression of SR-BI in duodenum and ileum between the two groups were found, its expression in jejunum of mice fed a VAD diet was higher than in mice fed a VAS diet (Fig. 4A). Isx expression in duodenum of mice fed a VAD diet was significantly decreased compared with that in mice fed a VAS diet (Fig. 4A).

Compared with Isx+/+ female mice fed the VAS diet, the expression of Isx in Isx+/+ female mice fed the VAD diet for 49 days including the pregnancy and lactation period was markedly decreased in both duodenum and jejunum (Fig. 4B).

Changes in expression of Bcmo1 and SR-BI in duodenum and jejunum also were examined in IsxLacZ/LacZ mice fed the VAD diet for 18 days (Fig. 4C). Compared with IsxLacZ/LacZ mice fed the VAS diet, the expression of Bcmo1 and SR-BI in jejunum and IsxLacZ/LacZ mice fed the VAD diet was significantly higher (Fig. 4C). However, unlike Isx+/+ mice, the expression of Bcmo1 in duodenum of IsxLacZ/LacZ mice was not increased by the VAD diet for 18 days (Fig. 4C).

### TABLE 1

|                | +/+     | LacZ/+   | LacZ/LacZ |
|----------------|---------|----------|-----------|
| Duodenum       | 1.0 ± 0.1 | 13.4 ± 3.9<sup>a</sup> | 2.3 ± 0.2<sup>b</sup> |
| Jejunum        | 1.0 ± 0.1 | 24.6 ± 3.2<sup>b</sup> | 140.8 ± 21.3<sup>c</sup> |
| Ileum          | 1.0 ± 0.2 | ND       | 3.3 ± 0.7<sup>b</sup> |

<sup>a</sup>p < 0.05 vs. +/+. <sup>b</sup>p < 0.001 vs. +/+. <sup>c</sup>p < 0.0001 vs. +/+. ND, not determined

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**FIGURE 2.** Changes in SR-BI expression in IsxLacZ/LacZ mice. A, real time RT-PCR of SR-BI in duodenum, jejunum, and ileum of adult mice. mRNA expression of SR-BI (normalized by β-actin) in IsxLacZ/LacZ mice (n = 5–10) is expressed relative to that in Isx<sup>+/+</sup> mice (n = 5–9). B, in situ hybridization of SR-BI in adult Isx<sup>+/+</sup> and IsxLacZ/LacZ mice. Samples from duodenum, jejunum, and ileum of both genotypes were used. +/+, Isx<sup>+/+</sup>; LacZ/LacZ, Isx<sup>+/+</sup>.
DISCUSSION

HD transcription factors play critical roles in cell differentiation, cell proliferation, and organogenesis. Because Isx, an HD transcription factor, is expressed in crypts, the Isx gene likely is involved in intestinal differentiation and function, but no abnormalities in length or morphology of villi of small intestine and upper colon were apparent in Isx<sup>LacZ/LacZ</sup> mice. In addition, bromodeoxyuridine labeling showed no differences in proliferation of intestinal epithelium between Isx<sup>+/+</sup> and Isx<sup>LacZ/LacZ</sup> mice (data not shown), indicating that Isx is not essential for transcription of intestinal epithelium.

**TABLE 3**
Effect of Isx overexpression on expression of Bcmo1 and SR-BI in mice

| mRNA expression of Bcmo1 and SR-BI (normalized by β -actin) in duodenum, jejunum, ileum, and liver of transgenic mice overexpressing Isx (CAG-stop-Isx:CAG-Cre) mice (n = 4–6) is expressed relative to those of control (CAG-Cre) mice (n = 4–6). |
|---|---|---|---|
| CAG-Cre | CAG-Isx | CAG-Cre | CAG-Isx |
| **Bcmo1** | | | |
| Duodenum | 1.00 ± 0.22 | 0.84 ± 0.11 | 1.00 ± 0.06 | 0.87 ± 0.15 |
| Jejunum | 1.00 ± 0.51 | 0.44 ± 0.13 | 1.00 ± 0.08 | 0.80 ± 0.02 |
| Ileum | 1.00 ± 0.13 | 0.46 ± 0.03<sup>a</sup> | 1.00 ± 0.18 | 0.81 ± 0.10 |
| Liver | 1.00 ± 0.36 | 1.18 ± 0.20 | 1.00 ± 0.18 | 0.91 ± 0.13 |
| **SR-BI** | | | |
| Duodenum | 1.00 ± 0.14 | 7.39 ± 1.83<sup>a</sup> | 1.00 ± 0.12 | 1.24 ± 0.16 |
| Jejunum | 1.00 ± 0.32 | 53.7 ± 11.7<sup>b</sup> | 1.00 ± 0.12 | 21.79 ± 6.53<sup>a</sup> |
| Ileum | 1.00 ± 0.10 | 2.79 ± 0.63<sup>a</sup> | 1.00 ± 0.24 | 1.04 ± 0.16 |

<sup>a</sup>p < 0.01 vs. CAG-Cre.

Microarray analysis and subsequent real time RT-PCR for quantification revealed that SR-BI mRNA expression was elevated in Isx<sup>LacZ/LacZ</sup> mouse intestine. SR-BI is a molecule originally identified as a member of the CD36 protein family (28, 29). Although SR-BI is involved in cholesterol absorption in intestine (18, 19, 27), no differences were found in fasting levels of cholesterol and triglyceride and in expression levels of genes involved in cholesterol absorption between Isx<sup>+/+</sup> and Isx<sup>LacZ/LacZ</sup> mice (data not shown).

Because SR-BI is known to have low specificity for substrates and to transport various molecules in addition to cholesterol (22–26), Isx may regulate SR-BI transcription to control the absorption of molecules other than cholesterol. Accordingly, we reanalyzed the microarray data to determine whether any genes involved in transport or metabolism of substrates of SR-BI were expressed differently in Isx<sup>LacZ/LacZ</sup> mice. The expression of Bcmo1 in duodenum was found to be markedly elevated. It has been shown that the ninaB and ninaD mutations of the Bcmo1 and SR-BI gene, respectively, are responsible for visual abnormalities in Drosophila (30). Because Bcmo1 controls the synthesis of vitamin A from β-carotene, and SR-BI participates in β-carotene absorption (22, 23, 30), Isx may well be involved in β-carotene absorption and/or vitamin A production and metabolism.

The drastically increased mRNA expression of Bcmo1 in Isx<sup>LacZ/LacZ</sup> mice might be secondary to insufficient β-carotene absorption or impaired retinol production in Isx<sup>LacZ/LacZ</sup> mice or to removal of the suppressive effect of Isx on Bcmo1 by Isx deficiency. In fetuses, vitamin A is supplied through blood flow via the placenta (31). Thus, in adult Isx<sup>LacZ/LacZ</sup> mice, whereas insufficient intestinal β-carotene absorption or impaired intestinal vitamin A synthesis might induce compensatory increases in Bcmo1 expression, the expression of intestinal Bcmo1 in E17.5 Isx<sup>LacZ/LacZ</sup> fetuses should not be increased. However, the expression of Bcmo1 in duodenum and jejunum in E17.5 Isx<sup>LacZ/LacZ</sup> fetuses was found to be markedly increased, as in adults. This suggests that Isx suppresses the expression of Bcmo1 directly. The finding that expression of Bcmo1 in duodenum and jejunum of Isx<sup>LacZ/LacZ</sup> fetuses is significantly higher than that of Isx<sup>+/+</sup> fetuses also suggests Isx suppression of transcription of Bcmo1.

To further investigate Isx regulation of expression of Bcmo1 and SR-BI, we investigated changes in mRNA expression using the intesti-
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A Mild vitamin A deficiency

B Severe vitamin A deficiency

![Diagram of vitamin A synthesis in intestine in vitamin A-deficient states.](Image)

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