Galectin-4 and Galectin-6 Are Two Closely Related Lectins Expressed in Mouse Gastrointestinal Tract*

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Galectins are a family of carbohydrate-binding proteins that share a conserved sequence and affinity for β-galactosides. Some, such as galectin-1, are isolated as dimers and have a single carbohydrate recognition domain (CRD) in each monomer, whereas others, such as galectin-4, are isolated as monomers and have two CRDs in a single polypeptide chain. In the course of studying mouse colon mRNA for galectin-4, we detected a related mRNA that encodes a new galectin that also has two CRDs in a single peptide chain. The new galectin, galectin-6, lacks a 24-amino acid stretch in the link region between the two CRDs that is present in galectin-4. Otherwise, these two galectins have 83% amino acid identity. Expression of both galectin-4 and galectin-6 is confined to the epithelial cells of the embryonic and adult gastrointestinal tract. Galectin-4 is expressed at about equal levels in colon and small intestine but much less in stomach, whereas galectin-6 is expressed at about equal levels throughout the gastrointestinal tract.

Although the functions of galectins are not established, there is evidence that the best studied, galectin-1 and galectin-3, play roles in cell adhesion and signaling by cross-linking of glycoconjugate ligands (2, 11). Galectin-4 has been found in the epithelium of the rat (12–14), porcine (15), and human (16) alimentary tract, and may sometimes be associated with adherens junctions (17).

In the course of cloning of galectin-4 cDNA from mouse colon, we isolated an additional closely related cDNA. Since the nomenclature of galectins was being established at that time (1), we reserved the name galectin-6 for the protein it encodes. Here, we provide the sequence and demonstrate carbohydrate binding activity of this new galectin. In addition, we have begun to address the biological functions of galectin-4 and galectin-6 by determining their distribution in embryonic and adult mouse tissues.

MATERIALS AND METHODS

General Information—All materials, equipment, and experimental conditions were identical to those described by Gitt et al. (3, 18) unless stated otherwise.

Polymerase Chain Reaction (PCR)—We used the following conditions for all amplifications. We used Ampli-Taq (Perkin-Elmer Cetus), 250 μM deoxynucleotides, 25 pmol of each primer, and buffer provided by the enzyme manufacturer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin). We amplified for 45 cycles, with each cycle consisting of a 40-s denaturation at 95 °C, a 1-min annealing at 60 °C, and a 1.5-min extension at 72 °C. Amplified fragments were visualized on ethidium bromide-stained 1% agarose gels.

Isolation and Characterization of Galectin-4 and Galectin-6 cDNAs—Total RNA was purified from mouse colon using RNAzol (Tel-Test, Friendswood, TX), following the manufacturer's protocol. Concentration was determined by absorbance at 260 nm. Ten micrograms of the RNA was reverse-transcribed for 2 h at 42 °C using 200 units of Moloney murine leukemia virus reverse transcriptase (U. S. Biochemical Corp.) in a 20-μl solution containing 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 mM MgCl₂, 100 mg/ml nuclelease-free bovine serum albumin, 1.0 mM deoxynucleotide triphosphates, 20 units RNasin (Promega, Madison, WI), and using 50 pmol of a poly(dT)-containing primer (GACCGAAGTCGATCGATTGTTTTTTTTTGGTGGT). After reverse transcription, the solution was heated for 5 min at 95 °C, immediately cooled on ice, and diluted to 1 ml with 10 mM Tris-HCl, pH 7.6, 1 μM EDTA. Ten μl of this solution was used as a template in PCR with different gene-specific primer pairs as described in Figs. 1 and 2. The amplified products were ligated directly to plasmid pCR1000 or pCR3 (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Expression of Galectin-6 Domain 2—A construct encoding the amino acid 161–301 fragment of galectin-6 was generated in the pET11d vector (Novagen, Madison, WI) using the same primers as used for expression of domain 2 of rat galectin-4 (12), and expressed in the BL21 host. In brief, the cDNA clone pmG6–2 (Fig. 1), constructed to provide a galectin-6-specific template encompassing all of domain 2, was used as template in a PCR amplification with primers rG4E and rG4K (Fig. 2) and the product cloned into pCR3 (Invitrogen). Primer rG4E has an engineered NcoI site encompassing the ATG encoding Met-161, and rG4K has additional sequence generating a BamHI site downstream of the stop codon. The plasmid DNA prepared from this clone was digested with NcoI and BamHI, and the insert was ligated into NcoIBamHI-
digested pET11d (Novagen, Madison WI). A clone containing the correct galectin-6 fragment was grown and induced by isoprrol-1-thio-β-D-galactopyranoside according to the manufacturer’s protocol. A pET11d clone with no insert was also grown and induced as a negative control. Lyons were prepared by sonication in MEBPS (58 mM Na₂HPO₄, 18 mM KH₂PO₄, 75 mM NaCl, 2 mM mercaptoethanol) supplemented with 2.5% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride, and β-galactoside-binding proteins purified by affinity chromatography on lactose-Sepharose as described by Gitt et al. (18). Western blot conditions are described below.

**Northern Blots**—Total RNA was isolated from various mouse tissues using RNAzol (Tel-Test Inc.). The amount of RNA in each sample was estimated by absorbance at 260 nm. Samples were electrophoresed along with size markers (Life Technologies, Inc.) on a 1.2% agarose gel containing 6% formaldehyde and 20 mM MOPS and the gel transferred to MagnaGraph nylon filters (Micron Separations, Inc., Westborough, MA) in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate). Ultraviolet cross-linking, hybridization buffer, and wash solutions were the same as described (19). For probes, we used PCR-amplified cDNA containing all the coding sequence but no untranslated sequence of rat galectin-4 (12), or, as a control, human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA amplified from a plasmid kindly provided by Dr. Alex Bulfone, Department of Psychiatry, University of California, San Francisco). Both probes were labeled with ³²P by random primer polymerization (20). Hybridization and washing was done at 45 °C. After hybridization with the galectin-4 probe and autoradiography, the galectin-4 probe was removed by washing for 1 h at 65 °C in 50% formamide, 2 × SSC, followed by a 0.1 × SSC rinse at room temperature. The blot was then incubated with the GAPDH probe under the same conditions. Quantitation of bound radioactivity was done using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**RESULTS AND DISCUSSION**

**Identification of Galectin-4 and Galectin-6**—To isolate mouse galectin-4 cDNA, we amplified a portion of cDNA prepared from mouse colon RNA by PCR using different pairs of primers designed on the basis of the sequence of rat galectin-4. To our surprise, we obtained two sets of clones, pmG6–1, and pmG6–2, respectively (Fig. 1), that included a fragment of human GAPDH cDNA. The genomic clones (pmG6–1, pmG6–2, respectively) and the galectin-6 cDNA clones (pmG6–1, pmG6–2) were linearized with HindIII and EcoRI and then incubated with 200 µg/ml proteinase K for 30 min at 37 °C to remove RNases, then phenol-extracted and ethanol-precipitated. For sense transcription templates, pmG6–1 and pmG6–2 inserts were released by HindIII/EcoRI digestion and recloned into similarly digested pBluescript SK+ (Stratagene, La Jolla, CA). RNA of the resultant clones and a plasmid containing a fragment of human GAPDH cDNA were linearized with HindIII, and treated as above with proteinase K and phenol. A pBluescript SK+–derived plasmid containing a fragment of human GAPDH cDNA was linearized with EcoRI. Galectin-4 and galectin-6 antisense transcripts were generated with T7 RNA polymerase, and sense galectin-4 and galectin-6 transcripts and antisense GAPDH transcripts were prepared from mouse colon RNA by PCR using different pairs of primers designed on the basis of the sequence of rat galectin-4. The genomic clones (pmG6–1, pmG6–2, respectively) and the galectin-6 cDNA clones (pmG6–1, pmG6–2) were linearized with HindIII, and treated as above with proteinase K and phenol. A pBluescript SK+–derived plasmid containing a fragment of human GAPDH cDNA was linearized with EcoRI. Galectin-4 and galectin-6 antisense transcripts were generated with T7 RNA polymerase, and sense galectin-4 and galectin-6 transcripts and antisense GAPDH transcripts were generated with T3 RNA polymerase, using the Maxiscript system (Ambion, Austin, TX) with 10× each of ATP, CTP, TTP, UTP, (5 µM CTP, and 75 µCi of [α³²P]CTP per transcription. The transcripts were purified on 5% polyacrylamide gels and eluted by diffusion into 0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS. The RNase protection assay was done using an Ambion RNase protection assay kit II. In brief, the antisense transcripts were hybridized overnight at 42 °C with different amounts of total tissue RNA plus yeast RNA carrier. In control experiments, tissue RNA was replaced by in vitro synthesized sense transcripts or omitted entirely. After hybridization, the mixtures were digested with 1 unit/ml RNase A + 200 units/ml RNase T1. The products were concentrated by precipitation and then electrophoresed on an 8% urea 5% polyacrylamide gel. The gel was autoradiographed with intensifying screens after drying. Densitometric quantitation was done with the PhosphorImager system. Data were corrected for background and different sizes of protected bands, and then normalized relative to GAPDH mRNA in each sample, as detected with GAPDH-specific primers. The cDNA fragments were then sequenced by direct cycle sequencing using the Sequenase 2.0 kit (US Biological, Swampscott, MA) with M13F and M13R primers.

**Antisense ³⁵S-cRNA probes** were prepared by T7 polymerase transcription of clones pmG6–1 and pmG6–2 (Fig. 1) as described below. In situ hybridization was performed as described by Wilkinson and Green (21), using mouse embryos starting at first day of gestation (0.5 day post coitum (dpc)).

**In Situ Hybridization of Mouse Embryos—**Antisense ³²P-RNA probes were prepared by T7 polymerase transcription of clones pmG4–1 and pmG6–1 (Fig. 1) as described below. In situ hybridization was performed as described by Wilkinson and Green (21), using mouse embryos starting at first day of gestation (0.5 day post coitum (dpc)).

**RNase Protection Assay (RPA)—**For generation of antisense galectin-4 and galectin-6 transcripts, DNA from clones pmG4–1 and pmG6–1 (Fig. 1) was linearized with EcoRI and then incubated with 200 µg/ml proteinase K for 30 min at 37 °C to remove RNases, then phenol-extracted and ethanol-precipitated. For sense transcription templates, pmG4–1 and pmG6–1 inserts were released by HindIII/EcoRI digestion and recloned into similarly digested pBluescript SK+ (Stratagene, La Jolla, CA). DNA of the resultant clones and a plasmid containing a fragment of human GAPDH cDNA were linearized with HindIII, and treated as above with proteinase K and phenol. A pBluescript SK+–derived plasmid containing a fragment of human GAPDH cDNA was linearized with EcoRI. Galectin-4 and galectin-6 antisense transcripts were generated with T7 RNA polymerase, and sense galectin-4 and galectin-6 transcripts and antisense GAPDH transcripts were generated with T3 RNA polymerase, using the Maxiscript system (Ambion, Austin, TX) with 10× each of ATP, CTP, TTP, UTP, (5 µM CTP, and 75 µCi of [α³²P]CTP per transcription. The transcripts were purified on 5% polyacrylamide gels and eluted by diffusion into 0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS. The RNase protection assay was done using an Ambion RNase protection assay kit II. In brief, the antisense transcripts were hybridized overnight at 42 °C with different amounts of total tissue RNA plus yeast RNA carrier. In control experiments, tissue RNA was replaced by in vitro synthesized sense transcripts or omitted entirely. After hybridization, the mixtures were digested with 1 unit/ml RNase A + 200 units/ml RNase T1. The products were concentrated by precipitation and then electrophoresed on an 8% urea 5% polyacrylamide gel. The gel was autoradiographed with intensifying screens after drying. Densitometric quantitation was done with the PhosphorImager system. Data were corrected for background and the different sizes of protected bands, and then normalized relative to GAPDH mRNA in each sample, as detected with GAPDH-specific probes in either a Northern blot (Fig. 5) or in an RNase protection assay (data not shown). Since dilutions of tissue RNAs resulted in corresponding decreases in amount of protected probe for both galectin-4 and galectin-6, the assay is RNA-limiting under the conditions employed. Moreover, the weight markers were prepared by incubation of nuclear DNA (Life Technologies, Inc.) with 0.08 pmol/µl [α³²P]dCTP, a mixture of 80 µM each dATP, dGTP, dTTP, and Kmew fragment of DNA polymerase I. In addition, a sequencing ladder was run in an adjacent lane.

**Western Blot Analysis of Tissue Extracts—**Total soluble proteins were extracted from mouse stomach, small intestine, and colon using
from genomic DNA of a highly inbred mouse strain (129/SV) that is homozygous at all loci proves that they must indeed be encoded by separate genes, rather than being encoded by alleles of the same genetic locus. Genomic Southern blots offer further support for this conclusion (22). Hence, they are clearly different proteins. The protein sequence that most closely resembles rat galectin-4 in mouse is designated galectin-4, and the other protein is designated galectin-6.

Structures of Galectin-4 and Galectin-6—In the structures determined for galectin-1 (23), and galectin-2 (24) by x-ray crystallography, the CRD consists of a tightly folded "β-sandwich" of about 130 amino acids. The homologous sequences that are predicted to form these domains in galectin-4 and galectin-6 are indicated by a black bar in Fig. 3. Hence, both galectin-4 and galectin-6 have two CRDs in tandem, each containing all the conserved amino acid residues involved in β-galactoside binding in other galectins (*asterisked* residues in Fig. 3).

In addition, each galectin has three stretches of sequence that are not part of the tightly folded CRDs (indicated by open bars in Fig. 3). One is found as the link region joining the two CRDs, another is found at the N terminus, and a third consists of a few amino acid residues at the C terminus. The major difference between galectin-4 and galectin-6 is that the link region connecting the two CRDs is shorter in galectin-6 (by 22 amino acids compared with rat galectin-4, and by 24 amino acids compared with mouse galectin 4). Excluding this part of the link region, mouse galectins-4 and -6 are 93% and 83% identical.

![Fig. 2] Composite sequences of mouse galectin-4 and galectin-6 cDNAs (mG4 and mG6) compared with rat galectin-4 cDNA (rG4). The mouse sequences were derived from a composite of cDNA and genomic DNA clones (Fig. 1), and the rat sequence is from Oda et al. (12). In the mouse sequences, residues identical to the corresponding rat galectin-4 residue are shown as dots. Dashes represent gaps introduced for alignment. Numbers give the last residue on the line, with the first translated nucleotide as 1 and assuming that the corresponding translational initiation site is conserved in mG4. Locations, orientations, and names of oligonucleotides used as primers are indicated by arrows underneath the relevant sequence. The oligonucleotides labeled with an asterisk had additional "anchor sequence" or other modifications and are described in full in Table II of Ref. 12, where primer 10rG4D, 11rG4E, 4rG4H, and 12rG4L.
Mouse, rat, and pig galectin-4 differ to the extent expected for cal on the nucleic acid and amino acid levels, respectively. Dashes indicate gaps introduced for alignment. Residues below the sequences denote the position of other regions. Asterisks below the sequences denote the position of other regions. The only sequence. However, in heterologous hybridizations, no fragments were protected with RNA from yeast (lanes c and g). When galectin-6 probe was mixed with small intestinal RNA and digested with RNAses, the size of the largest protected fragment was 335 nt (Fig. 4 lane b), which is equal to the length of the galectin-6-specific sequence in this probe (Fig. 4B). Similarly, with the galectin-4 probe, a 465-nt band was protected (Fig. 4A, lane f), equal to the length of the mouse galectin-4-specific sequence of this probe. In this case, the 42-nt sequence from the 3' primer (rG4G) used to generate mG4–1 was probably not protected because it had been derived from the rat galectin-4 amino acid sequence (12), and has multiple sequence differences compared with the corresponding mouse galectin-4 nucleotide sequence (Fig. 4B).

To further validate the specificity of the RPA, we also tested each probe with galectin-4 sense RNA and galectin-6 sense RNA generated by in vitro transcription of pmG4–1 and pmG6–1, respectively. When galectin-4 sense RNA was probed with the galectin-4 antisense probe, one fragment of about 570 nt was protected (data not shown) corresponding to all the mouse galectin-4-specific sequence (465 nt) in the probe plus 42 nt of overlapping vector sequence (gray in Fig. 4B). Similarly, when galectin-6 sense RNA was probed with the galectin-6 antisense probe, one fragment of 395 nt was protected (data not shown), which is 335 nt of galectin-6-specific RNA + 60 nt of overlapping vector sequence. However, in heterologous hybridizations, i.e. when galectin-4 sense RNA was probed with galectin-6 antisense RNA or galectin-6 sense RNA was probed with galectin-4 antisense RNA, the 570- and 395-nt fragments were not detected (Fig. 4A, lanes d and h). Hence, the RPA allows specific detection of galectin-4 and galectin-6.

For each of these heterologous hybridizations, however, smaller fragments, mainly of about 210 nt and 280 nt, were seen instead. The undigested probes had the expected length (425 and 600 nt, respectively; Fig. 4A, lanes a and c), and no fragments were protected with RNA from yeast (lanes c and g). When galectin-4 probe was mixed with small intestinal RNA and digested with RNAses, the size of the largest protected fragment was 335 nt (Fig. 4A, lane b), which is equal to the length of the galectin-6-specific sequence in this probe (Fig. 4B). Similarly, with the galectin-4 probe, a 465-nt band was protected (Fig. 4A, lane f), equal to the length of the mouse galectin-4-specific sequence of this probe. In this case, the 42-nt sequence from the 3' primer (rG4G) used to generate mG4–1 was probably not protected because it had been derived from the rat galectin-4 amino acid sequence (12), and has multiple sequence differences compared with the corresponding mouse galectin-4 nucleotide sequence (Fig. 4B).

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FIG. 4. Selective detection of galectin-4 and galectin-6 mRNA by RNase protection assay. A, results with the galectin-6 probe (antisense of pmG6–1) are shown in lanes a–d and i–n. Results with the galectin-4 probe are shown in lanes e–h and o–t. The undigested probes are shown in lanes a and e, respectively. To test the specificity of the probes, they were first mixed with small intestinal RNA (lanes b and f), yeast RNA (lanes c and g), or sense in vitro transcripts of pmG4–1 (lane d) or pmG6–1 (lane h). Then the probes were tested with RNA from colon (lanes i and o), ileum (lanes j and p), jejunum (lanes k and q), stomach (lanes l, r, and s), liver (lanes m and t), and yeast (lane n). The exposure of lanes s and t (galectin-4 probe with stomach and liver mRNA, respectively) was 5 times longer. The sizes of the indicated bands were calculated based on the mobility of sequencing ladders and restriction digests of model DNA used as size markers (data not shown). B, schematic of fragments of galectin-4 and galectin-6 protected in the RPA. Galectin CRD sequence is filled, link sequence is open, and pertinent (see text) vector sequences are gray. The sequence due to the 3′-primer used (rG4G) to generate pmG4–1 is indicated (see text). The large type numbers indicate sizes of predicted protected fragments (nt) and the small type numbers indicate other pertinent distances (nt) mentioned in the text.

The undigested probes are shown in lanes a and e, respectively. To test the specificity of the probes, they were first mixed with small intestinal RNA (lanes b and f), yeast RNA (lanes c and g), or sense in vitro transcripts of pmG4–1 (lane d) or pmG6–1 (lane h). Then the probes were tested with RNA from colon (lanes i and o), ileum (lanes j and p), jejunum (lanes k and q), stomach (lanes l, r, and s), liver (lanes m and t), and yeast (lane n). The exposure of lanes s and t (galectin-4 probe with stomach and liver mRNA, respectively) was 5 times longer. The sizes of the indicated bands were calculated based on the mobility of sequencing ladders and restriction digests of model DNA used as size markers (data not shown). B, schematic of fragments of galectin-4 and galectin-6 protected in the RPA. Galectin CRD sequence is filled, link sequence is open, and pertinent (see text) vector sequences are gray. The sequence due to the 3′-primer used (rG4G) to generate pmG4–1 is indicated (see text). The large type numbers indicate sizes of predicted protected fragments (nt) and the small type numbers indicate other pertinent distances (nt) mentioned in the text.

When RNA from different parts of the gastrointestinal tract were probed with the galectin-6 probe, about equal amounts of the 335-nt fragment were found in all cases (Fig. 4A, lanes i–l). The RNA from liver and yeast did not protect any of the fragments mentioned above (lanes e, g, m, and n), not even if visualized after 5-fold prolonged exposure (exemplified by lane t; not shown for other samples); nor did RNA from brain, tongue, lung, heart, skeletal muscle, or kidney (data not shown). The many smaller fragments observed with the galectin-4 probe used in lanes o–s that cannot be explained by cross-hybridization with galectin-6 may be due to the presence of incomplete antisense transcripts in the probe solution protected by galectin-4 RNA. These bands, like the “intact” 465-nt band, are much less abundant with stomach RNA (lane r), and RNA from tissues outside the gastrointestinal tract (e.g., lane t).

To confirm the tissue-specific expression of galectins-4 and -6 and to obtain information on the size of the mature mRNAs, we also used a Northern blot (Fig. 5). Although the great similarity between galectin-4 and galectin-6 prevents their separate detection on a Northern blot, this assay allowed us to confirm their lack of detectable expression in non-gastrointestinal tissues.

Bands hybridizing with rat galectin-4 cDNA were found only in gastrointestinal tissue extracts, including stomach, small intestine, and colon. The strongest hybridization to gastrointestinal RNA was found as a broad band corresponding to about 1.0 kb. This is similar in size to the major hybridizing RNA for galectin-4 cDNA in rat intestine (12) and is consistent with the size expected for the mature mRNA of both galectin-4 and galectin-6 in mouse (22). Hence, the broad band at 1.0 kb probably contains the mature mRNA for both galectin-4 and galectin-6, which are not well resolved.
A weaker band at 2.8 kb was also detected only in gastrointestinal tissues, after the long exposure of the blot (Fig. 5). Quantitation by PhosphorImager showed that this band accounts for less than 5% of the hybridizing radioactivity. This band probably does not represent mature mRNA for either galectin-4 or galectin-6 because it does not have the expected size, and it is not present in the relative amount of either galectin as detected by RPA (about 50/50 in intestine, Fig. 4A, i–k). Moreover, a probe made from just the link region present in galectin-4 but absent in galectin-6 hybridized strongly to the 1.0-kb band and weakly (again about 5%) to the 2.8-kb band (data not shown), suggesting that the latter band contains incompletely processed galectin-4 RNA.

Despite the fact that the blot in Fig. 5 was exposed for a long time, no signal was detected in the lanes (a–e, h, and l) with RNA from the non-gastrointestinal tissues tested (brain, tongue, kidney, skeletal muscle, heart, liver, and lung). The PhosphorImager quantitation would be able to detect a band corresponding to about 1% of the radioactivity in the 1-kDa band. To check for the possibility that the lack of detection of galectins-4 and -6 in non-gastrointestinal tissues was due to loss of RNA, the blot was reprobed for GAPDH mRNA, known to have a relatively even distribution in most tissues (including the gastrointestinal tract) and commonly used as a marker of RNA integrity (e.g. Ref. 25). Except for lane b (tongue), the amount of RNA in the lanes from the non-gastrointestinal tissues was within the same order of magnitude as for lanes f–j, as estimated by the amount of GAPDH mRNA. The signal for GAPDH mRNA from the tongue sample was significantly lower (about 5% compared with intestine, lanes g and h) than for the other tissues but still clearly detectable. Hence, galectin-4 and galectin-6 expression is high in gastrointestinal tissue, but undetectable in non-gastrointestinal tissues. The result of the RPA assay was consistent with this conclusion because, as mentioned above, no signal for galectins-4 or -6 was detected with mRNA from non-gastrointestinal tissues.

Expression of Galectin-4 or Galectin-6 during Embryogenesis—To examine the tissue origin of galectins-4 and -6 further, we examined their expression in mouse embryos of different age by in situ hybridization (Fig. 6) with antisense RNA probes transcribed from clones pmG4–1 and pmG6–1 (Fig. 1). As expected, the two probes gave identical signals, each probably reacting with both galectin-4 and galectin-6 mRNAs. Hence, the observed signal represents galectin-4 or galectin-6 or a mixture of the two.

As in the adult, expression of galectin-4 or galectin-6 was found only in the gastrointestinal tract in the embryo. The expression is detected in the gastrointestinal epithelial cells starting from day 13.5 of embryogenesis (Fig. 6, A–F). At this stage, we observed reactive RNA all along the digestive tract, except the glandular region of the stomach (data not shown). As shown in Fig. 6 (A and B), all other tissues of the 13.5-day embryo are devoid of signal from galectin-4 or galectin-6 transcripts, notably skin, brain, spinal cord, tongue, notochord, cartilage, heart, lung, bladder, liver, thymus, and blood cells. Expression was not detectable at any site, including intestine, between day 0.5 and 12.5 (Fig. 6, C and D, and data not shown).

Expression of galectin-4 and galectin-6 persists in the adult intestine, in the endoderm (epithelium) but not mesoderm (mesenchyme) (Fig. 6, G and H).

It is thus clear that the expression of galectin-4 and galectin-6 is much more limited compared with galectin-1 and galectin-3, which are both expressed in many different tissues (26, 27). Galectin-1 and galectin-3 are also present in intestine, but their patterns of expression differ. Galectin-1 is present in the mesoderm but not the endoderm, the inverse of the galectin-4/6 pattern (26). Galectins-3, -4, and -6 are co-expressed in intestinal Galectin-4 and Galectin-6

**Fig. 5. Detection of galectin-4 or galectin-6 mRNA by Northern blot of total RNA from adult mouse tissues.** The upper panel shows hybridization with a GAPDH-specific probe. The rest of the figure shows the blot probed with rat galectin-4 cDNA under medium stringency. Calculated sizes of detected RNAs are indicated. Roughly 20 μg (determined by A260) of total RNA from each of the following tissues was applied: brain (a), tongue (b), kidney (c), skeletal muscle (d), heart (e), stomach (f), proximal small intestine (g), medial small intestine (h), ileum (i), colon (j), liver (k), and lung (l).

**Fig. 6. Detection of galectin-4 or galectin-6 in sections of embryonic and adult mouse tissues by in situ hybridization.** The sections were probed with antisense galectin-4 cDNA (35S-labeled) transcribed from plasmid pmG4–1 (Fig. 1). Brightfield (A) and darkfield (B) photographs of a midsagittal section of a 13.5-day post coitum (dpc) embryo. Note the signal restricted to intestinal loops. Brightfield (C, E, and G) and darkfield (D, F, and H) views of intestine from 12.5-dpc embryo (C and D), from 13.5-dpc embryo (E and F), and from an adult mouse (G and H). Panels E and F are details taken from A and B. The signal is first detected on the 13th day of embryogenesis and is then detected through adulthood. Scale bars, 700 μm in A and B and 200 μm in C–H.
the epithelium of the adult intestine, but galectin-3 is not expressed there before birth (27). Galectin-9 is also expressed in the adult intestine but in many other tissues as well (9).

Expression of Galectin-4 and Galectin-6 Proteins—To examine the expression of galectin-4 and galectin-6 at the protein level, we probed Western blots of tissue extracts with an antisera raised against the N-terminal CRDs of rat galectin-4. This antisera detected a band migrating as about 36 kDa in small intestine and colon but not stomach (Fig. 7). The mobility of this band is the same as for recombinant rat galectin-4, and hence likely to be mouse galectin-4. Its detection in small intestine and colon, but not stomach, is consistent with the results of the RPA.

Galectin-6 is expected to migrate slightly faster than galectin-4, by an amount corresponding to 2–3 kDa. A very weak band at 33 kDa was detected in small intestines and colon as an adherens junction-associated protein (17), it is possible that this band is the same as for recombinant rat galectin-4, and hence likely to be mouse galectin-4. Its detection in small intestine and colon, but not stomach, is consistent with the results of the RPA.

Functional Correlates—The organization of galectins-4 and -6 and other bi-CRD galactins such as galectins-8 and -9 suggests that they function by cross-linking ligands for each of the CRDs. In a simple model, this functional activity would then depend on the specificity of each of the two carbohydrate binding sites, as determined by the structure of the CRDs, and the distance and relative orientation of the two CRDs, as determined by the structure of the link region between the CRDs. The latter property is the most likely functional difference between galectin-4 and galectin-6 since they differ mainly in the length of their link regions. Interestingly, galectin-9 also occurs in two forms differing only in their link regions. The form specifically expressed in intestine has a longer link region, while the form expressed in other tissues has a shorter link region (Ref. 9; Fig. 3). This provides further evidence that expression of different link regions may be functionally significant whether by their effects on CRD spacing, as suggested above, or by other effects. Nevertheless, it is also possible that galectins-4 and -6 are functionally redundant.

Whether or not galectins-4 and -6 are functionally distinct, their prominent expression in gastrointestinal epithelial cells suggests that they function in these cells. Prior to day E12.5, when neither galectin-4 nor galectin-6 are expressed, the intestine consists of a multilayered endodermal epithelium surrounded by mesenchyme. Around day E13.5, when galectin-4 or galectin-6 expression starts, there is a wave of morphological change in the intestine, starting in the ileum and extending both proximally and distally as the epithelium differentiates to a single-layered epithelium surrounded by the developing muscles and connective tissue of the gut. The formation of villi and various cell-cell junctions also occurs at this time (28). Therefore, major changes of cell-cell interactions are taking place in the intestinal epithelium between days 12.5 and 13.5 of development. Since porcine galectin-4 (15) was originally isolated as an adherens junction-associated protein (17), it is possible that galectin-4 or galectin-6 is involved in these processes.

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