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Original Article

The LIM homeodomain protein Lim-1 is widely expressed in neural, neural crest and mesoderm derivatives in vertebrate development

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ABSTRACT **Polyclonal antibodies to Xlim-1 homeodomain protein of *Xenopus laevis* were used to study the developmental expression pattern of this protein in *Xenopus*, rat and mouse. Western blotting of embryo extracts injected with different *Xlim-1* constructs confirmed the specificity of the antibody. Beginning at the gastrula stage, Xlim-1 protein was detected in three cell lineages: (i) notochord, (ii) pronephros and (iii) certain regions of the central nervous system, in agreement with earlier studies of the expression of *Xlim-1* RNA (Taira *et al., Development 120:* 1525-1536, 1994a). In addition, several new locations of Xlim-1 expression were found, including the olfactory organ, retina, otic vesicle, dorsal root ganglia and adrenal gland. Similar expression patterns were seen for the Lim-1 protein in frog and rodent tissues. These observations implicate the *Xlim-1* gene in the specification of multiple cell lineages, particularly within the nervous system, and emphasize the conserved nature of the role of this gene in different vertebrate animals.**

KEY WORDS: *LIM domain, homeodomain, Xenopus, Lim-1 antibody*

Introduction

The homeobox genes *lin-11* (Freyd *et al.*, 1990), *isl-1* (Karlsson *et al.*, 1990) and *mec-3* (Way and Chalfie, 1988) were founding members of a new family of genes called LIM genes. Proteins encoded by this family are characterized by the presence of the cysteine-rich LIM domain (C-xx-C-x_{17-19}-H-xx-C-xx-C-xx-C-x_{16-20}-C-x_{2-3}-D/C/H-x) which may be associated with different functional domains such as kinase, GAP and protein interacting domains as well as homeodomains. Recent evidence suggests that LIM domains are involved in protein-protein interactions regulating the function of associated proteins or of distinct domains in the same protein (reviewed in Sánchez-García and Rabbitts, 1994; Dawid *et al.*, 1995; Taira *et al.*, 1995). In the case of LIM homeodomain proteins it appears that LIM domains carry out a negative regulatory function (German *et al.*, 1992; Sánchez-García and Rabbitts, 1994; Taira *et al.*, 1994b).

The class of LIM-homeodomain proteins is represented at present by 15 members, all of which are expressed in various regions of the central nervous system (CNS) and in many neuroendocrine tissues (Sánchez-García and Rabbitts, 1994; Dawid *et al.*, 1995). These expression patterns, and especially the tight correlation of LIM homeobox gene expression with neuronal fates (Tsuchida *et al.*, 1994; Appel *et al.*, 1995), implicate this class of genes in lineage determination in the vertebrate CNS, a role assigned to members of this family in invertebrates (Ferguson *et al.*, 1987; Way and Chalfie, 1988, 1989; Freyd *et al.*, 1990; Bourgouin *et al.*, 1992; Cohen *et al.*, 1992; Lundgren *et al.*, 1995).

We have studied several members of the LIM-homeodomain family, in particular *Xenopus Xlim-1* and *Xlim-5*, and their homologs in other animals; these two genes encode a pair of closely similar proteins, yet are expressed in distinct patterns during development. *In situ* hybridization techniques have shown that the *Lim-1* gene in *Xenopus* (Taira *et al.*, 1992, 1994a), the mouse (Barnes *et al.*, 1994; Fujii *et al.*, 1994; Shawlot and Behringer, 1995), and the zebrafish (Toyama *et al.*, 1995b) is expressed in three major locations, one of which arises very early in embryogenesis. At the gastrula stage, *Lim-1* RNA is found in the Spemann organizer region of the *Xenopus* embryo and in its equivalent, the shield, in the zebrafish and in similar dorsal mesodermal regions of the mouse. During the late gastrula stage in *Xenopus*, this gene continues to be expressed in the notochord and head mesoderm. This expression pattern is transient, and *Xlim-1* RNA is rapidly lost from the axial mesoderm except from the tip of the tail, where notochord formation continues (Taira *et al.*, 1994a). *Lim-1* expression in the kidney and in many regions of the CNS arises at the late gastrula stage and continues throughout life (Barnes *et al.*, 1994; Fujii *et al.*, 1994; Taira *et al.*, 1994a).

Abbreviations used in this paper: GST, glutathione-S-transferase.

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These observations led to the proposal (Taira *et al.*, 1994a) that *Xlim-1* gene plays a dual role in vertebrate development: a global role in the establishment of the body plan at early stages, and a secondary role in the specification of particular cell types during subsequent development.

A remarkable aspect of LIM gene expression and function is the fact that the *Xlim-1* and *Xlim-5* genes, whose protein products are 73% identical overall, with 98% in the homeodomain and 95% in the LIM domains, are expressed in quite distinct patterns (Taira *et al.*, 1992, 1994a; Toyama *et al.*, 1995a). *Xlim-5* RNA arises at the same time in the late blastula as *Xlim-1* RNA, but its location is entirely ectodermal in contrast to the dorsal mesodermal location of *Xlim-1*. Later, *Xlim-5* is expressed only in the CNS in a distinct though partially overlapping pattern with *Xlim-1*, as will be further discussed below. Quite clearly, the *Lim-1* and *Lim-5* genes are not functionally redundant, since a disruption of the mouse *Lim-1* gene (also named *Lhx-1*) leads to embryo lethality due to failure to develop the fore- and midbrain (Shawlot and Behringer, 1995). This observation, together with data suggesting a role for *Xlim-1* in neural induction (Taira *et al.*, 1994b) indicate that the *Lim-1* gene has a major function in the development of the anterior CNS.

While the expression pattern visualized by *in situ* hybridization is informative in terms of the range of gene expression, functional inferences require knowledge about the localization of the protein. To this end we produced a polyclonal antibody to a fusion protein between glutathione-S-transferase (GST) and the C-terminal part of the Xlim-1 protein. In the present paper we first provide evidence that this antibody is specific for the Xlim-1 protein and does not cross react with the closely similar Xlim-5. At the same time, the antibody does cross react with

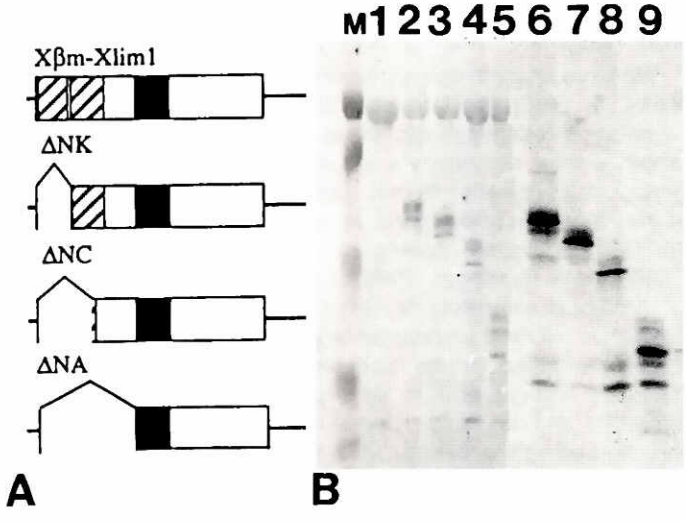

Fig. 1. Synthesis of full-length and truncated Xlim-1 proteins *in vivo* and *in vitro*. **(A)** *Constructs used for injection (see Taira et al., 1994b). The full-length construct contains ß-globin UTRs; three truncated constructs of decreasing size were prepared from Xßm-Xlim1.* **(B)** *Electrophoretic separation of proteins translated from the constructs shown in* **(A)**, *detected by Western blotting with anti-Xlim-1 antibody of embryo extracts (lanes 1-5), and by radioautography of ^{35}S-labeled in vitro translation products of the same constructs (lanes 6-9). m, markers; lane 1, uninjected embryos; lanes 2 and 6, Xßm-Xlim1; lanes 3 and 7, ΔNK; lanes 4 and 8, ΔNC; lanes 5 and 9, ΔNA.*

Lim-1 proteins of the mouse and rat, which is consistent with the high degree of conservation of this protein in different vertebrate species (Taira *et al.*, 1992; Barnes *et al.*, 1994; Fujii *et al.*, 1994; Furuyama *et al.*, 1994; Toyama *et al.*, 1995b). We show that the expression pattern of Xlim-1 protein during the development of *X. laevis* generally corresponds to that of *Xlim-1* mRNA, and the observations in mouse and rat embryos emphasize the conservation of the expression patterns of the *Lim-1* gene in different animals. However, the technical advantages of antibody staining allowed us to identify several locations of Lim-1 expression in the retina, olfactory organ, otic vesicle and adrenal gland that had not been recognized in earlier studies.

Results

The specificity of anti-Xlim-1 antibodies

The first test for recognition of Xlim-1 by our antibody preparation involved the staining of full-length and truncated proteins translated in whole embryos from synthetic mRNAs. Several constructs of *Xlim-1* were prepared (Fig. 1A), RNAs transcribed from these constructs were injected into *Xenopus* embryos, and protein extracts were subjected to gel electrophoresis (Fig. 1B). After transfer to a membrane and staining with anti-Xlim-1 antibody, three tightly spaced bands were visualized in all samples with mobilities consistent with the predicted size of the full-length or truncated Xlim-1 proteins. The fact that a triplet of bands was detected in each case suggests that a portion of the protein was subject to post-translational modification in the embryo. This suggestion is compatible with the fact that the fastest band of the triplet of full-length protein comigrates with the product obtained from the same RNA in a cell-free translation system when run on the same gel (Fig. 1B). The nature of the putative modification has not been determined. These results indicate that the antibody recognizes the Xlim-1 protein.

The high degree of similarity between Xlim-1 and Xlim-5 (Toyama *et al.*, 1995a; see Introduction) made it important to test the specificity of our antibody preparation. To this end, the patterns of *Xlim-1* and *Xlim-5* expression in the embryonic brain were compared by *in situ* hybridization and by antibody staining of parallel sections (Fig. 2). While the expression domains overlap in part, the *Xlim-5* gene is prominently expressed in the anterior region of the telencephalon (arrow, Fig. 2B) where *Xlim-1* RNA is not present (Fig. 2D). The antibody staining of a very similar section (Fig. 2E) shows that the anterior telencephalon is negative (arrow), while staining closely approximates the distribution of *Xlim-1* RNA (Fig. 2D). Thus we conclude that the anti-Xlim-1 antibody does not recognize the closely related Xlim-5 protein. While it is possible that the antibody cross reacts with the product of another closely related gene that has not yet been discovered this possibility is made less likely by the fact that 9.5-day embryos in which the *Lim-1* has been disrupted (Shawlot and Behringer, 1995) did not stain with anti-Xlim-1 antibody (not shown). These observations are compatible with the fact that the antibody was generated against the C-terminal domain of Xlim-1 which is quite similar to that of Xlim-5 but not as highly conserved as the LIM and homeodomains (see Introduction).

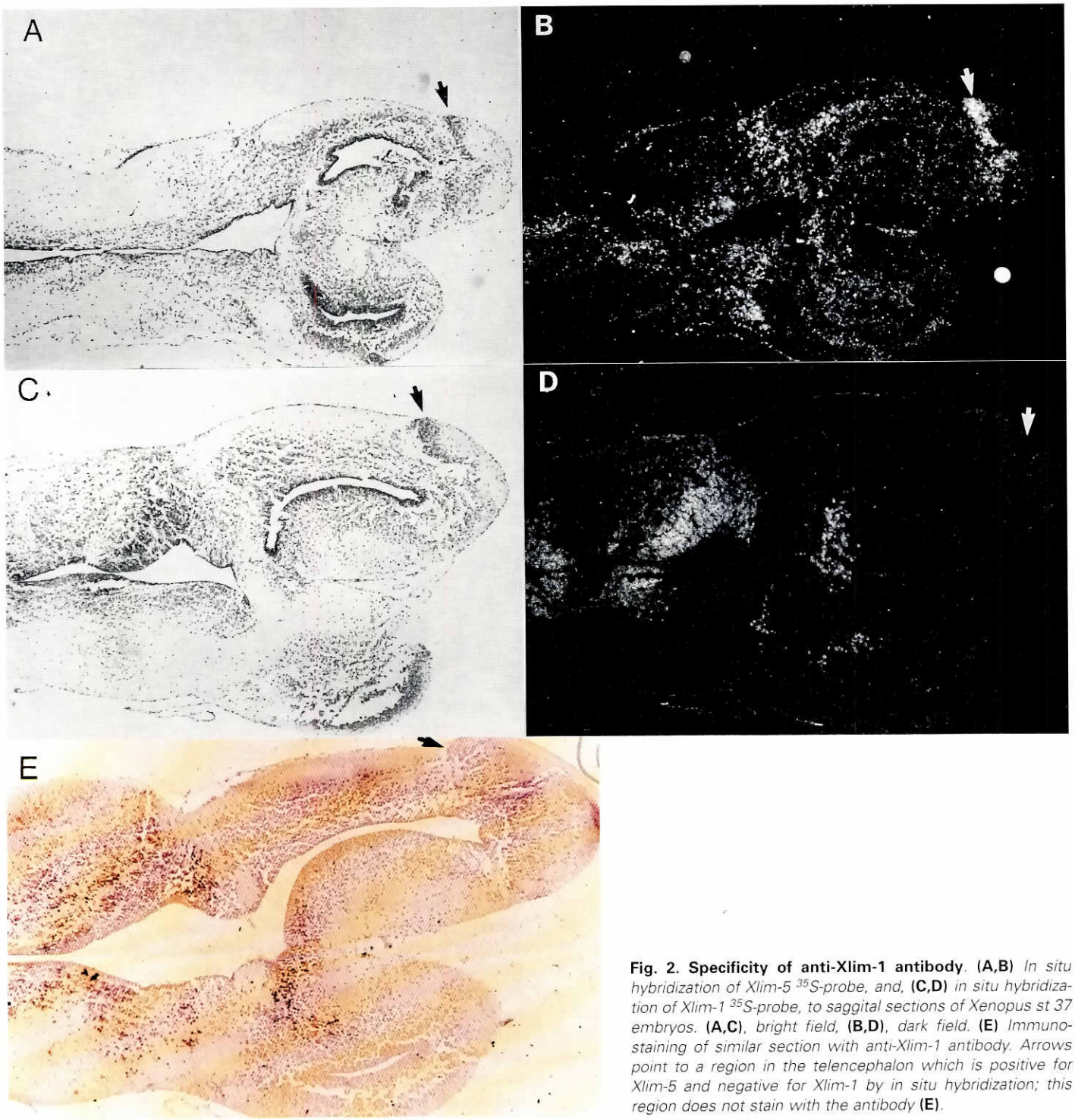

Fig. 2. Specificity of anti-Xlim-1 antibody. **(A,B)** *In situ hybridization of Xlim-5 ^{35}S-probe, and,* **(C,D)** *in situ hybridization of Xlim-1 ^{35}S-probe, to saggital sections of Xenopus st 37 embryos.* **(A,C)**, *bright field,* **(B,D)**, *dark field.* **(E)** *Immunostaining of similar section with anti-Xlim-1 antibody. Arrows point to a region in the telencephalon which is positive for Xlim-5 and negative for Xlim-1 by in situ hybridization; this region does not stain with the antibody* **(E)**.

The localization of Xlim-1 protein during early development

Xlim-1 protein was first visualized by antibody staining during mid-gastrula (stage 11/12) (Fig. 3A,B), slightly later than the first appearance of mRNA (Taira *et al.*, 1992). In good agreement with RNA localization, antibody staining is seen in the nuclei of the involuting dorsal mesoderm; the nuclear restriction of the antigen is consistent with the putative function of this homeodomain protein as a transcription factor. Most if not all nuclei of the invaginating mesoderm stain with the antibody.

At early tailbud stages (st 25), Xlim-1 is seen in nuclei of the notochord, the lateral region of the spinal cord, and the pronephros (Fig. 3C,D). *In situ* hybridization demonstrated that *Xlim-1* RNA disappears from the notochord during neurula stages, and at stage 25 can be found only in the tail tip (Taira *et al.*, 1994a). In contrast, the protein can be visualized in the entire

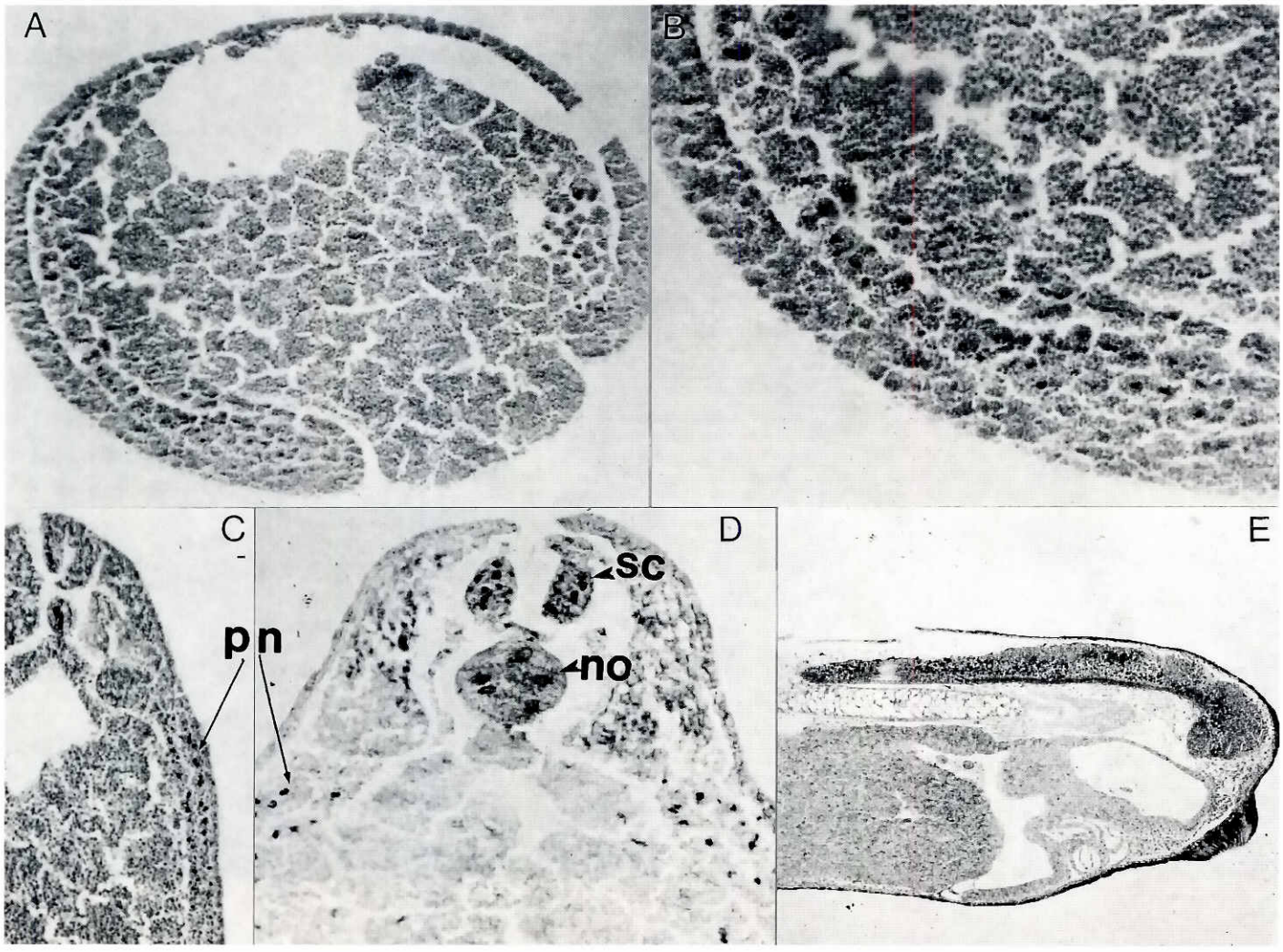

Fig. 3. Expression of Xlim-1 in *Xenopus* gastrula and tailbud embryos. (A,B) *Midgastrula embryos (st 11.5) show expression in the nuclei of dorsal mesoderm, and in a few nuclei of the underlying endoderm.* **(C,D)** *In cross sections at st 25* **(C)** *and st 28* **(D)**, *expression is seen in spinal cord (sc), notochord (no) and pronephros (pn).* **(E)** *Intense expression of Xlim-1 in many areas of the brain is seen in this parasaggital section at st 32.*

notochord at this stage, indicating persistence of the protein well after the degradation of the RNA. After stage 28, Xlim-1 protein disappears from the body of the notochord as well. Thus, as expected, the protein product follows the appearance and loss of the RNA in the notochord.

Like *Xlim-1* mRNA, the corresponding protein is found in the kidney (pronephros) and in many parts of the CNS of tailbud embryos and persists in these tissues to the adult. The pattern of expression in the brain becomes more widespread and complex as development proceeds, so that by stage 32 many regions stain for Xlim-1 (Fig. 3E). These include multiple areas of the diencephalon, midbrain, hindbrain and spinal cord, whereas the telencephalon is largely negative (see also Fig. 2C-E). A more detailed study of *Xlim-1* gene expression in the *Xenopus* brain will be presented elsewhere.

Xlim-1 expression in sensory organs

The use of the antibody permitted us to identify the presence of Xlim-1 protein in several locations in which the cognate RNA had not been observed by *in situ* hybridization (Taira *et al.*, 1994a). As shown in Figure 4A-C, expression of Xlim-1 is highly characteristic of subsets of cells in sensory organs, including the olfactory organ (4A), the eye (4B), and the otic vesicle (4C). In each case only one layer of cells is stained, and all staining is nuclear. In the retina, the positive cells can be identified by their morphology as cells belonging to inner nuclear layer (Fig. 4B). The morphology and position of Xlim-1-positive cells in the olfactory organ and otic vesicle suggest that they represent subpopulations of olfactory receptor cells and hairy receptor cells, respectively, but this tentative assignment remains to be established using secondary markers. Interestingly, optic tectum (Fig. 4D,E), and a small subpopulation of cells in the olfactory bulb (Fig. 4F,G) also express Xlim-1, suggesting the possibility that this protein may play a role in specifying the fate of neural cells of sensory systems.

The fact that *Xlim-1* expression had not been observed in sensory organs in earlier work using *in situ* hybridization does not necessarily point to a discrepancy between RNA and protein

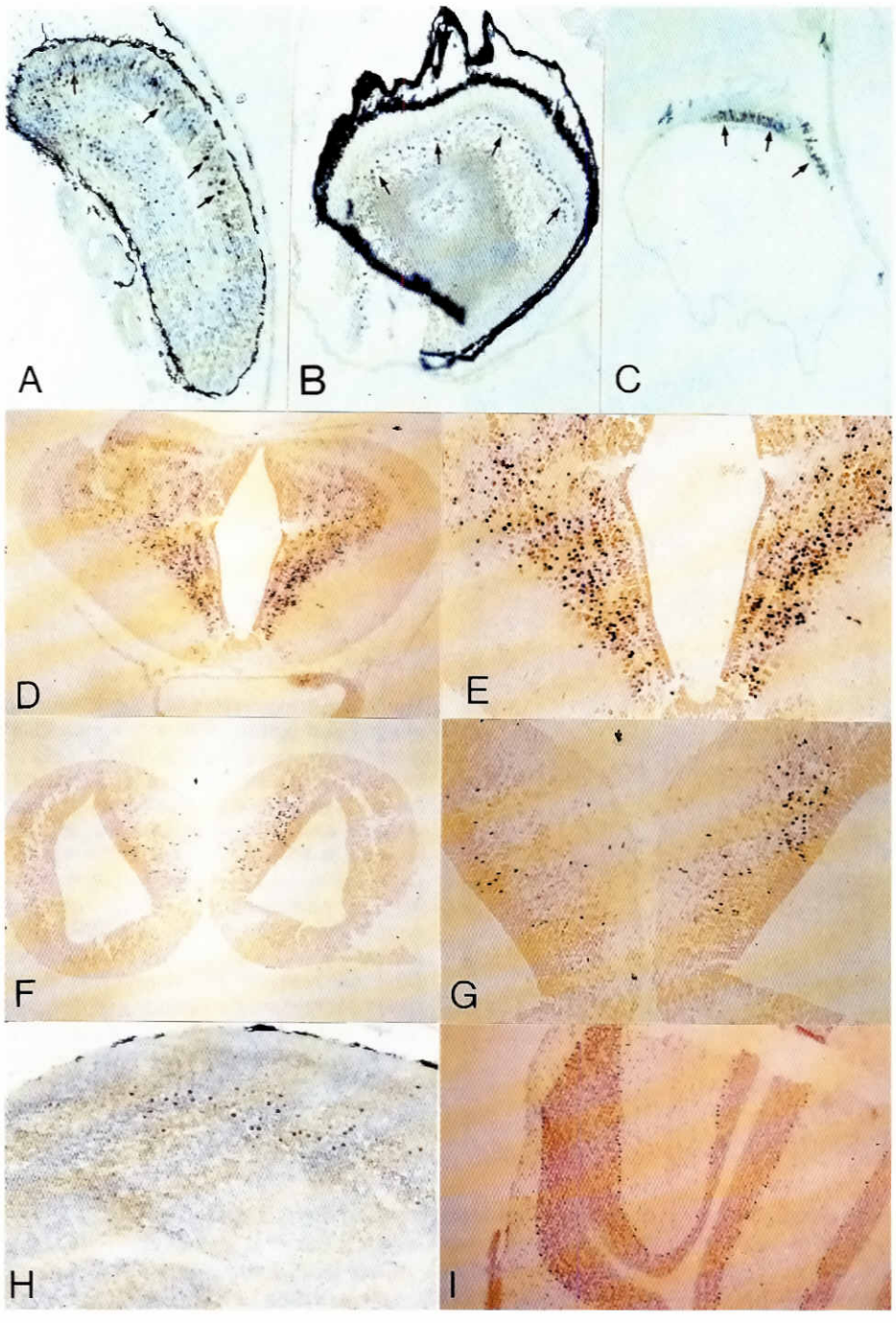

Fig. 4. Lim-1 protein expression in sensory organs and certain regions of the brain. (A-H) *Xenopus tadpoles at st 52.* **(A)** *Olfactory organ, stained nuclei arranged on entire circumference at variable density, as indicated by arrows.* **(B)** *Retina, stained nuclei arranged in single layer (arrows). Note dense pigment layer at the periphery of olfactory organ and retina.* **(C)** *Otic vesicle, asymmetric arrangement of Xlim-1-positive cells.* **(D,E)** *Optic tectum, high level of Xlim-1 expression in many but not all cell nuclei.* **(F,G)** *Olfactory bulb, Xlim-1 found in sparse population of cell nuclei.* **(H)** *Xenopus cerebellum, and,* **(I)** *rat cerebellum; stained nuclei represent Purkinje cells. Alkaline phosphatase was used as the secondary reagent in all panels.*

localization but more likely is technical in nature. Expression of RNA in a single layer of cells (e.g. Fig. 4A,B) is difficult to detect by whole-mount *in situ* hybridization, but nuclear staining is distinctive even in very few cells.

Cell types expressing Xlim-1 gene are highly conserved among different vertebrate species

The amino acid sequences of Lim-1 proteins are highly conserved among vertebrates (Taira *et al.*, 1992; Barnes *et al.*, 1994; Fujii *et al.*, 1994; Furuyama *et al.*, 1994; Toyama *et al.*, 1995b), suggesting that our antibody preparation may recognize the Lim-1 protein of vertebrates other than *Xenopus*. A comparison of staining of several organs of mouse, rat and *Xenopus* showed that the antibody reacts with highly conserved regions and cell types in these three species. Figure 4H,I illustrates the presence of Lim-1 antigen in the Purkinje cells of *Xenopus* and rat cerebellum; *in situ* hybridization studies have confirmed the presence of *Lim-1* RNA in these cells (W.P. Hayes *et al.*, unpublished). Anti-Lim-1 antibody staining was also observed in the adrenal medulla of the rat (Fig. 5A,B), and *Xenopus* (not shown).

The kidney is known as a major site of *Lim-1* gene expression (Fujii *et al.*, 1994; Taira *et al.*, 1994a). Antibody staining was used

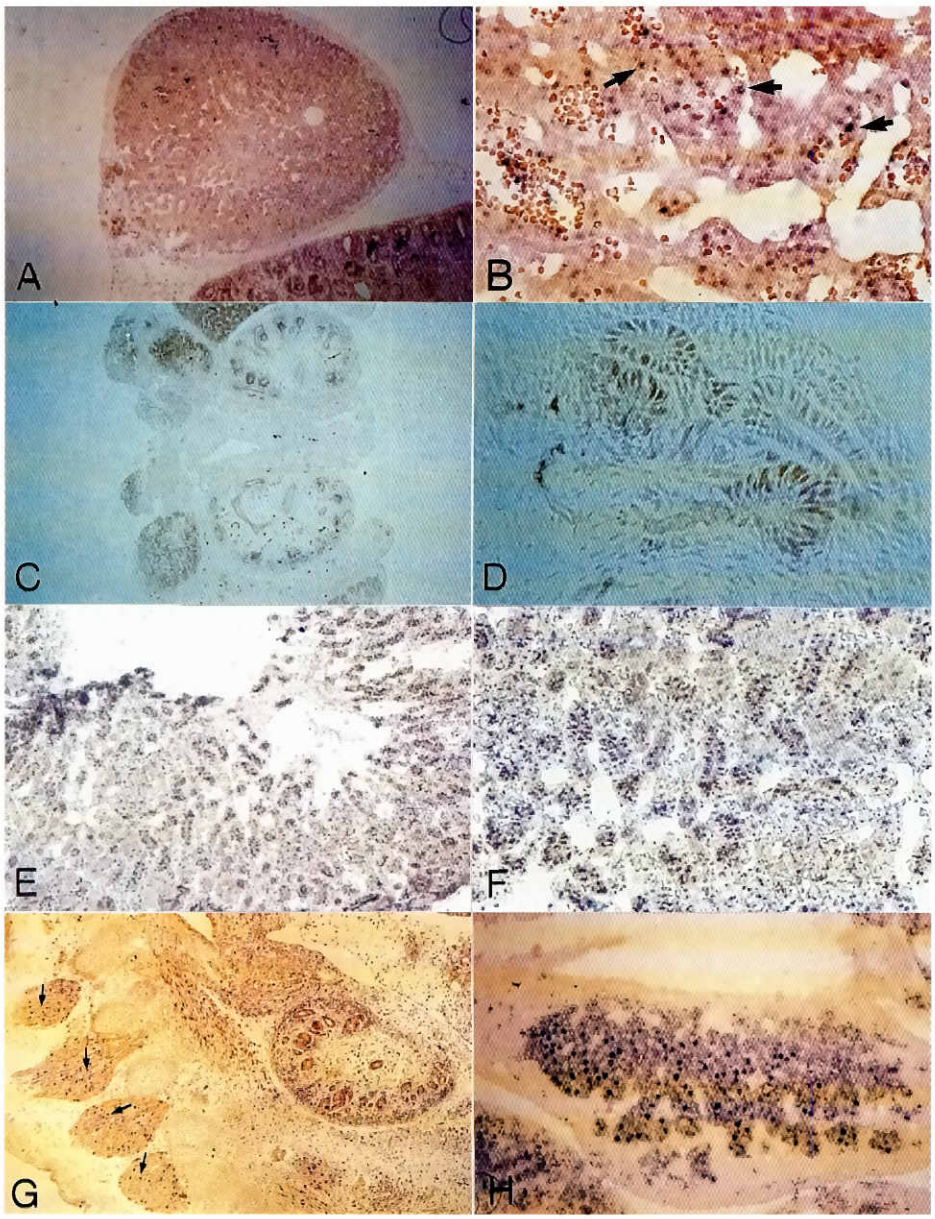

Fig. 5. Lim-1 expression in adrenal, kidney, ganglia and testes. (A,B) *In the medulla of adult rat adrenal gland, the Lim-1 antibody stains multiple nuclei; the blue nuclei are indicated by arrows in* **(B)**, *while the tan dots represent erythrocytes.* **(C,D)** *Rat embryonic kidney at E14.5 shows Lim-1 expression in the nuclei of coma-shaped bodies.* **(E,F)** *Adult Xenopus mesonephric kidney, showing nuclear staining in collecting ducts.* **(G)** *A section from a mouse embryo at E16.5 illustrates Lim-1 expression in the metanephric kidney where it is seen in coma-shaped and S-shaped bodies, and in dorsal root ganglia (indicated by arrows).* **(H)** *Rat testes at E14.5 showing Lim-1 staining in most nuclei. Panels A, B show alkaline phosphatase stained sections, all other sections in this figure were stained with horse radish peroxidase as secondary reagent.*

to further characterize the expression pattern in this organ. In the mesonephric frog kidney and metanephric kidneys of mouse and rat embryos, similar epithelial structures stained with anti-Lim-1 antibody. These include the ureteric bud and its derivatives, as well as epithelial structures differentiating from the metanephric mesenchyme including coma-shaped and S-shaped bodies (Fig. 5C-G). In fully differentiated podocytes Lim-1 protein was not detected.

Figure 5G also illustrates the nuclear expression of Lim-1 protein in dorsal root ganglia in the mouse. This observation expands the expression range of this gene to the peripheral nervous system where it has not been reported previously. The presence of Lim-1 in dorsal root ganglia and in the adrenal medulla indicates that this gene is expressed in different lineages derived from the neural crest.

Earlier work showed the presence of *Xlim-1* transcripts in adult *Xenopus* testes, but the size of the mRNA which is smaller than in other tissues, raised the question whether Lim-1 protein is produced in this organ (Taira *et al.*, 1992). Antibody staining of rat testes shows clearly positive nuclei (Fig. 5H), indicating the presence of the Lim-1 protein in these cells. However, this staining cannot establish whether the testis protein is full-length or whether it could be a truncated product.

Discussion

The *Lim-1* gene, also named *Lhx-1* in the mouse, is a member of the LIM-homeobox gene family which presently contains 15 known members (reviewed in Sánchez-García and Rabbitts, 1994; Dawid *et al.*, 1995; Taira *et al.*, 1995). The *mec-3* and *lin-11* genes of *C. elegans* and the *apterous* gene of *Drosophila* have been shown to be involved in cell lineage decisions in the differentiation of different cell types (Ferguson *et al.*, 1987; Way

and Chalfie, 1988, 1989; Freyd *et al.*, 1990; Bourgouin *et al.*, 1992; Cohen *et al.*, 1992; Lundgren *et al.*, 1995). In vertebrates, more extensive functional information is available on the *Lim-1* gene than on other genes in this class. Both ectopic expression and inactivation analyses demonstrated the critical role of *Lim-1* in organizer function during early embryogenesis. In *Xenopus*, where the *Xlim-1* gene was first shown to be expressed in the Spemann organizer (Taira *et al.*, 1992; see also Fig. 3A,B), ectopic expression of an activated form of the Xlim-1 protein elicited neural and muscle induction in responsive cells, thus emulating the major known functions of the organizer (Taira *et al.*, 1994b). In the mouse, targeted disruption of the *Lim-1* gene resulted in embryonic lethality at day E9.5 with total failure to form anterior structures, specifically the forebrain and midbrain (Shawlot and Behringer, 1995). This result demonstrates the non-redundant essential function of Lim-1 for head formation in the mouse, most likely because of its requirement in the so called "head organizer" (see also De Robertis, 1995).

One limitation of both ectopic expression in the frog embryo and disruption in the mouse embryo is the fact that only the earliest essential function of a gene is revealed. Nevertheless, the work of Shawlot and Behringer (1995) provided additional information: in a few cases where mutant mice survived to be still born, the urogenital system was completely missing. This observation is in excellent agreement with the extensive expression of the *Lim-1* gene in the kidney of different vertebrates (Taira *et al.*, 1994a; Fujii *et al.*, 1994; and this paper), and shows that this gene has an essential function in the formation of this organ system in addition to its role in head formation.

Additional insight into the specific role of Lim-1 in different tissues and cell types requires detailed information on the expression pattern of this protein throughout development. We have used a polyclonal antibody prepared against the C-terminal domain of Xlim-1 to approach this question. As expected from the high conservation of sequences, the antibody cross reacts effectively with the Lim-1 proteins of the mouse and rat, enabling us to study expression in a comparative manner. Our observations confirmed the major sites of *Lim-1* gene expression deduced by *in situ* hybridization, and discovered additional tissues and cell types that express this protein. Further, the antibody verified the general prediction that Lim-1, as homeodomain proteins in general, should have a predominantly nuclear localization.

The ability to visualize Lim-1 protein expanded our understanding of its potential roles in the notochord. *Xlim-1* RNA disappears rapidly from the notochord during neurula stages (Taira *et al.*, 1994a), but the protein persists much longer (Fig. 2). This observations implies an extended function of Lim-1 in the notochord beyond the very early functions in which this protein has been implicated so far (Taira *et al.*, 1994b; Shawlot and Behringer, 1995). During the patterning of the neural plate, signals from the notochord have been implicated in the induction of ventral neural fates, in particular in the specification of the floor plate (van Straaten *et al.*, 1988; Smith and Schoenwolf, 1989; Placzek *et al.*, 1990; Yamada *et al.*, 1993); these signals appear to be mediated, at least in part, by members of the hedgehog family (Echelard *et al.*, 1993; Roelink *et al.*, 1995). In addition, the notochord also provides signals required for somite differentiation, again mediated in part by hedgehog (Fan *et al.*, 1995). These activities may depend on several nuclear factors that are expressed in the notochord during this period, among which the forkhead class protein HNF-3ß is known to be essential (Ang and Rossant, 1994; Weinstein *et al.*, 1994). Our observations suggest that Lim-1, although not indispensable for the formation of the notochord, spinal cord and somites (Shawlot and Behringer, 1995), may nevertheless play a role in the control of notochord signalling during the neurula stage of embryogenesis.

Staining with anti-Lim-1 antibody revealed an even wider expression for this protein in late stages of development of *Xenopus*, rat and mouse than had been recognized by *in situ* hybridization. With respect to the wide range of positive cell types, though not with respect to the actual cell types involved, this expression pattern resembles that of the LIM homeodomain protein Isl-1; a monoclonal antibody against Isl-1, which probably also recognizes Isl-2, a related gene not yet discovered at the time (Tsuchida *et al.*, 1994), stained a very wide range of neurons and endocrine cells in the rat (Thor *et al.*, 1991). Widespread expression was also reported for the LIM homeobox genes *LH-2 (Lhx-2)* (Xu *et al.*, 1993). In contrast, the *Lim-3 (Lhx-3)* (Taira *et al.*, 1993; Seidah *et al.*, 1994; Zhadanov *et al.*, 1995) and *Lim-5* genes (Toyama *et al.*, 1995a) are more restricted to small sets of neural and neuroendocrine cells. However, even the *Lim-1* and *Isl-1* genes are widely but not ubiquitously expressed and clearly are highly specific in their restriction to certain cell types.

The restriction of Lim-1 protein by cell type is especially apparent in sensory organs, as shown in Figure 4A-C, which illustrates staining of single layers of nuclei in the olfactory organ, retina and otic vesicle. In the retina, the *Xlim-3* gene is also restricted to a single layer of cells, the inner nuclear layer (Taira *et al.*, 1993). While the relationship between the cells expressing different LIM homeobox genes in the retina requires further analysis by double label techniques, one might suggest on the basis of present information that different cell types in this tissue may be specified by their expression of such genes, as suggested for different types of motoneurons in the chicken and zebrafish spinal cord (Tsuchida *et al.*, 1994; Appel *et al.*, 1995). Thus it is possible that combinatorial expression of LIM homeobox genes is an important factor in assigning identity to certain cell types in parts of the CNS including the spinal cord and sensory organs. In addition, we observe that Lim-1 is expressed is some cases in the sensory organs and regions of their central target. For example, limited numbers of cell nuclei in the olfactory bulb and many cell nuclei in the optic tectum of *Xenopus* stain with the anti-Lim-1 antibody (Fig. 4D-G). Whether this commonality of expression entails a functional consequence remains to be elucidated.

Materials and Methods

Plasmid construction expression of protein

A fragment of *Xlim-1* cDNA which encodes the C-terminal region of the protein just downstream of the homeodomain (amino acids 265-403; Taira *et al.*, 1992) was generated by polymerase chain reaction and inserted into the BamHI and EcoRI sites of pGEX2T plasmid (Pharmacia). The presence of an in-frame Glutathione S transferase-XLIM-1 (GST-XLIM-1) fusion was verified by sequencing.

The fusion protein was expressed in *Escherichia coli* strain BL21(DE)Lys (Novagene) as follows. One colony of transformed cells was inoculated into 100 ml of LB-medium containing 100 µg/ml of ampi-

cillin and incubated at 37°C with shaking overnight. Twenty-five ml of this culture were used to inoculate 500 ml culture in LB. After this culture reached an OD_{600} of 0.7 it was induced by 1 mM IPTG for 4 h at 30°C. Cells were collected by centrifugation, freeze-thawed and sonicated in a volume of 20 ml of a solution containing 1% Triton-X100, 1% Tween 20, 1% CTAB, 10 mM DTT in MTPBS (Smith and Johnson, 1988) buffer, four times for 30 sec. The solution was twice clarified by centrifugation for 10 min at 13,000 rpm, and the supernatant was subjected to column affinity purification on gluthatione-agarose beads (Sigma) as described (Smith and Johnson, 1988). All steps of induction and purification were checked by SDS PAGE, with uninduced cultures as control. Purified soluble protein was injected into rabbits to produce antibodies.

Immunostaining of paraffin sections

IgG fractions were obtained from antisera with the aid of the Sure-Pure IgG purification kit (Pierce), used according to the manufacturer's protocol.

Xenopus laevis embryos and adult tissues were fixed in MEMFA (100 mM MOPS, 1 mM $MgSO_4$, 2 mM EGTA, 3.8% formaldehyde) for 1 h, washed twice in methanol for 15 min, followed by xylene, and embedded in paraplast; 6 µm sections were collected on glass slides. Sections were deparaffinized, rehydrated, and incubated in 2% Boeringer-Mannheim blocking solution for 1 h. Staining was done by incubation with anti-Xlim-1 IgG, 1/500 diluted, in 2% Boeringer-Mannheim blocking reagent overnight at 4°C, followed by three 10-min washes in PBS, and by anti-rabbit Ig conjugated to peroxidase or to alkaline phosphatase (Boehringer-Mannheim) for 1 h at room temperature. Enzymatic reaction with peroxidase-conjugated secondary antibodies was performed using Pierce reagent, and staining was enhanced with osmium tetroxide (0.4%). Alkaline phosphatase reaction was done according to the protocol of manufacturer (Boeringer-Manheim) for 35 min. The reaction was stopped in PBS, sections were dehydrated, and mounted in Permount (Fisher). The secondary reaction used in each photo is indicated in the figure legends.

To decrease background staining we used anti-Xlim IgG depleted prior to staining by incubation at 1/100 dilution with hyperfixed *X. laevis* embryos (st 25) for 1.5 h. Hyperfixation was done in 4% formaldehyde for 48 h at room temperature, followed by methanol wash and storage at -20°C. Before depletion, these embryos were rehydrated for 2x15 min in PBS, and blocked for 1 h in 2% Boehringer-Mannheim blocking reagent. We found that this procedure greatly enhanced signal-to-noise ratio.

Mouse and rat embryos and tissues were fixed in 4% PFA (Wilkinson and Green, 1990). Procedures for paraffin embedding and sectioning were the same as for *X. laevis*. Signal was enhanced by the following step: after rehydration, sections were boiled in 6 M urea for 5-6 min in a microwave at 90% power output (Cattoretti *et al.*, 1992).

In situ hybridization of paraffin sections

Xlim-1 and *Xlim-5 in situ* hybridization probes were prepared by subcloning of 3'-untranslated region of corresponding cDNAs into Bluescript SK^+ vector (Stratagene). [S^{35}]-UTP-labeled (Amersham) RNA probes transcribed from these constructs were used for *in situ* hybridization essentially as described (Wilkinson and Green, 1990).

In vitro translation and Western blot analysis

In vitro translation of different *Xlim-1* cDNA constructs was performed using [S^{35}]-methionine (Amersham) and the coupled transcription-translation system of Promega as described by the manufacturer. Following *in vitro* translation, 5 µl of the reaction per lane was used for electrophoresis in 8% SDS-PAGE (Laemmli, 1970).

Four RNA-injected or uninjected embryos were dissolved in 40 µl of 2 X sample buffer, and 10 µl per lane was used for electrophoresis in 8% SDS-PAGE (Laemmli, 1970). Following electrophoresis, protein from the gel was transferred to 0.22 µm nitrocellulose membrane using semi-dry Western blot protocol and apparatus (Owl Scientific Plastics, Inc). The membrane was blocked with 6% BSA in 10 mM Tris-HCl, pH 7.5, 0.15M NaCl, 0.05% Tween 20, 0.05% Triton X100, for 1 h, and treated with anti-Xlim-1 IgG at 1/6000 dilution overnight at 4°C. Following three 10-min washes in the same buffer, secondary anti-rabbit Ig-conjugated to peroxidase (Amersham) at 1/700 dilution was added for 1 h, washed three times in the same buffer, and stained with DABA and H_2O_2 (Sigma) as described by the manufacturer.

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