Biosynthesis and In Vivo Localization of the Decapentaplegic-Vg-related Protein, DVR-6 (Bone Morphogenetic Protein-6)

Nancy A. Wall, Manfred Blessing, Christopher V. E. Wright, and Brigid L. M. Hogan

Department of Cell Biology, Vanderbilt University Medical School, Nashville, Tennessee 37232

Abstract. DVR-6 (BMP-6 or Vgr-1) is a member of the TGF-β superfamily of polypeptide signaling molecules. In situ hybridization studies have previously shown that DVR-6 RNA is expressed in a variety of cell types in the mouse embryo, but no information has been available on protein localization and biosynthesis. We have produced a polyclonal antibody to the proregion of DVR-6 and used it to localize the protein in whole mount and sectioned embryonic, newborn, and adult mouse tissues. DVR-6 protein is expressed in the mouse nervous system beginning at 9.5 days postcoitum (d.p.c.) and continues through adulthood. A variety of epithelial tissues also produce DVR-6 protein, including the suprabasal layer of the skin, bronchiolar epithelium, and the cornea. Additionally, a stably transfected cell line, BMGE+H/D6c4, is used to study the biosynthesis of DVR-6 protein and evidence is presented for translational regulation of DVR-6 expression.

T HE transforming growth factor beta (TGF-β)[1] superfamily is a large group of signaling polypeptides with multiple biological activities in vivo and in vitro (Soma and Grotendorst, 1989; Sato et al., 1990; Kehrl, 1991; Massague et al., 1991). Members of this family are secreted, disulfide-bonded dimers of $\sim$25,000–30,000 M_r, proteolytically cleaved from larger precursors. The decapentaplegic-Vg-related (DVR) subgroup of the TGF-β superfamily contains members closely related to *Drosophila* decapentaplegic (DPP), *Xenopus* Vg-1, and mammalian bone morphogenetic proteins (BMPs) (Lyons et al., 1991). Genetic analysis has shown that DPP is required at several different stages of embryogenesis, such as dorso-ventral patterning of the blastoderm, proximal-distal patterning of the imaginal discs, and midgut morphogenesis (Irish and Gelbart, 1987; Posakony et al., 1990; Panganiban et al., 1990a). Additionally, studies have been reported on the biochemical characterization of DPP protein, its localization in tissues using specific antibodies, and the identification of genes that regulate and interact with DPP during development (Panganiban et al., 1990b; Immergluck et al., 1990; Ferguson and Anderson, 1992). In contrast, much less is known about the role of *Xenopus* Vg-1 (DVR-1) and the other DVR genes in vertebrate embryonic development. In situ hybridization studies and RNase protection experiments have demonstrated that several members of this subgroup are ex-

pressed in a variety of tissues throughout development in mouse and *Xenopus*, in particular in tissues undergoing inductive interactions (Weeks and Melton 1987; Lyons et al., 1989a,b, 1990; Jones et al., 1991). However, functional studies with purified protein have been limited to either ectopic bone and cartilage induction in mammals, or mesoderm induction in *Xenopus* (Wang et al., 1990; Jones et al., 1992; Dale et al., 1992).

As stated above, specific antibodies against *Drosophila* DPP have been invaluable for studying the biosynthesis of DPP and demonstrating the in vivo localization of the protein. Clearly, this approach is an important step towards understanding the biological role of vertebrate DVR proteins. We report here the production of an affinity-purified antibody, αDVR-6, against the proregion of mouse DVR-6 that specifically recognizes this protein and not the closely related proteins, DVR-5 or -7. We have used αDVR-6 to follow biosynthesis of DVR-6 in cultured cells and to localize the precursor protein in the cytoplasm of cell lines and in a variety of embryonic, neonatal, and adult tissues. These include the suprabasal layers of the epidermis, the bronchiolar epithelium of newborn lung, adult cornea, and cells of the nervous system. In the case of neuronal tissue, protein is localized not only in the cell bodies but also at high levels in the axons, suggesting that DVR-6 is an axonally transported protein. However, we were not able to detect the protein in several other tissues and two cell lines (PYS and 49F) that express DVR-6 mRNA by in situ hybridization, Northern analysis, and RNase protection analysis. This suggests that there is posttranscriptional regulation or modification of DVR-6.

---

1. *Abbreviations used in this paper:* BMGE, bovine mammary gland epithelial; BMP, bone morphogenetic protein; d.p.c., days postcoitum; DPP, *Drosophila* decapentaplegic; DVR, decapentaplegic-Vg-related; GST, glutathione-S-transferase; TGF-β, transforming growth factor beta.

Materials and Methods

Antigen Production

A glutathione-S-transferase (GST)/DVR-6 fusion protein was made using the pGEX system described by Smith and Johnson (1988). A 256-bp XhoII/RsaI fragment (nucleotides 180 to 436) of the mouse DVR-6 cDNA designated Vgr-1 (Lyons et al., 1989a) encoding part of the proregion was inserted into the SmaI site at the 3' end of the GST-coding region in the pGEX 2T vector. This region of Vgr-1 was chosen because it encodes the part of the DVR-6 protein that has diverged most extensively from the closely related proteins DVR-5 and DVR-7 (BMP-5 and BMP-7, Celeste et al., 1990). Comparison of the predicted amino acid sequence of hDVR-6 with that of hDVR-5 and hDVR-7 in the region used for making the GST/DVR-6 fusion protein reveals only 36.8 and 33.3% identity, respectively (Celeste et al., 1990).

Preparation of antigen is described elsewhere (Wall et al., 1992).

Antibody Preparation and Specificity

Female New Zealand rabbits were injected initially subcutaneously and intramuscularly in a total of four sites with a total of 750 μg of GST/DVR-6 fusion protein emulsified in Freund's complete adjuvant. Approximately 24% of this total represents DVR-6 protein. Subsequently, 750-μg boosts were in Freund's incomplete adjuvant. Antibody preparation and specificity were determined by previously described methods (Wall et al., 1992).

In Vitro Transcription of DVR-5, -6, and -7 RNA

The full-length human DVR-5, -6, and -7 cDNAs were used for in vitro transcription of RNA. Linearized DNA template (inserted behind an SP6 promoter) was incubated with the following: 5 mM m7G(5')ppp(5')G (Pharmacia LKB Biotechnology Inc., Piscataway, NJ); 5 mM rATP, rCTP, and rUTP and 0.5 mM rGTP; RNase inhibitor; transcription buffer; and SP6 polymerase (all from Promega Corp., Madison, WI). Transcription product was then incubated with RNase-free DNase I (Promega Corp.).

In Vitro Translation

1 μl of in vitro transcription product was incubated with RNase inhibitor (Promega Corp.), translation buffer, rabbit reticulocyte lysate, and 1 mM amino acid mix minus methionine (rabbit reticulocyte lysate kit, Promega Corp.) and 2 μl all-*trans* [35S]methionine (1,000 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA). The volumes of translation product used for immunoprecipitation were adjusted to give equal amounts of TCA-precipitable radioactivity.

Immunoprecipitation

Translation product was incubated with either normal rabbit serum or αDVR-6 in 400 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, and 1% NP-40 (NET buffer). Antigen-antibody complex was bound to protein A-Sepharose (Sigma Chem. Co., St. Louis, MO) prewashed with NET buffer, and analyzed by SDS-PAGE (under reducing conditions) and autoradiography.

Transfection, Culture, and Labeling of Bovine Mammary Gland Epithelial (BMGE) Cells and PYS cells

Cells of the BMGE+H line (Schmid et al., 1983) were cotransfected with the coding region of human DVR-6 under the control of the keratin III and IV* regulatory elements (Blessing et al., 1987) and the neomycin resistance gene marker plasmid pAG60 (Colbére-Garapin et al., 1981). Transfection was performed by the calcium phosphate technique (Gorman et al., 1982). Clones were selected in DME supplemented with 20% FBS/1% penicillin/streptomycin/1% glutamine/1 μg/ml each of prolactin, insulin, and hydrocortisone/800 μg/ml geneticin. After selection, confluent monolayers in 60-mm plates were preincubated in methionine-free DME for 30 min and then labeled with 200 μg/ml [35S]methionine (ICN Biomedicals, Inc.) for 2.5 h at 37°C. The medium was collected and the cytoplasmic fraction was obtained by adding 50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, and 1% NP-40 for 3 min at room temperature. After centrifugation to remove aggregated material, 200 μl of medium or 100 μl of the cytoplasmic fraction was incubated with equal amounts of IgG from either normal rabbit serum

or αDVR-6 in 1 ml NET buffer for 1.5 h at room temperature. Antigen-antibody complex was bound to protein A-Sepharose (Sigma Chem. Co.), prewashed with NET buffer, and analyzed by SDS-PAGE and autoradiography.

PYS cells were transfected by the calcium phosphate method with a single plasmid containing hDVR-6 under the control of the β-actin promoter and a neomycin resistance gene. PYS cells were grown in DME supplemented with 5% FBS/1% penicillin/streptomycin and 1% glutamine.

RNAse Protection Assay

The 256-bp XhoII/RsaI fragment used to make the GST/DVR-6 fusion protein was also used to make the antisense RNase protection probe. Probe was labeled with [32P]UTP (800 Ci/mmol; New England Nuclear, Boston, MA). Total RNA from PYS cells was combined with 5 × 10^5 cpm of probe in 50% formamide, 40 mM Pipes, pH 7.6, 400 mM NaCl, and 1 mM EDTA (hybridization buffer). This mixture was incubated at 85°C for 5 min, and then at 50°C overnight. Subsequently, unhybridized RNA was digested in 300 μl of 10 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, 83.3 μg/ml RNase A, and 16.7 U/ml RNase T_1 for 30 min at room temperature. Digestion was stopped by adding 0.5% SDS and Proteinase K to a final concentration of 120 μg/ml and incubating at 37°C. Protected RNA was purified by phenol/chloroform extraction and ethanol precipitation, analyzed on an 8% sequencing gel, and visualized by autoradiography.

Western Blot Analysis

Dissected tissues (heads, livers, spinal cords, and limb buds) were placed directly into 10 vol of sample buffer containing 5% β-mercaptoethanol, 2.3% SDS, 62 mM Tris·HCl, pH 6.8, and 10% glycerol, and then boiled for 5 min and either used immediately or stored at −20°C. SDS-PAGE was performed using a 12% separating gel, and proteins were then electroblotted to a nylon filter (Amersham Corp., Arlington Heights, IL). The filter was blocked with 5% FBS, 1% BSA, 0.5% Tween 20, 0.1 M $MgCl_2$, 0.01 M Tris·HCl, pH 7.4. DVR-6 protein was detected by incubation with affinity-purified primary antibody diluted 1:1,000 in TBST followed by incubation with a 1:5,000 dilution of goat anti-rabbit IgG conjugated to peroxidase (Boehringer Mannheim Corp., Indianapolis, IN) and subsequently incubated with a 1:5,000 dilution of goat anti-peroxidase conjugated to peroxidase (Cappell Laboratories, Malvern, PA, 0100-1221). All antibodies were preincubated with mouse liver acetone powder before use (Harlow and Lane, 1988). The luminol solution from Amersham's ECL kit (RPN.2109) was used as a substrate for peroxidase, and the resulting chemiluminescent reaction was detected on XAR-5 film (Eastman Kodak Co., Rochester, NY).

Fixation and Immunohistochemical Staining of Embryos and Cells

Fixation of embryos is described elsewhere (Wall et al., 1992). Cells were cultured on glass coverslips, fixed in methanol/DMSO (4:1) 2 min at room temperature, treated to inhibit endogenous peroxidase activity, rinsed in PBS, and used immediately for immunohistochemistry. Immunohistochemical methods used on whole and sectioned embryos are described elsewhere (Wall et al., 1992).

Results

Specificity and Species Crossreactivity of αDVR-6

The amino acid sequence of the DVR-6 portion of the GST/DVR-6 fusion protein used for immunization (see Materials and Methods) was compared to sequences in the EMBL database (Brutlag et al., 1990) to determine if there were any other known proteins which might be recognized by the antibody. No protein sequences showed more than 22% similarity. To confirm that αDVR-6 does not cross-react with its closest family members, immunoprecipitation experiments were carried out using human DVR-5, -6, and -7 (BMP-5, -6, and -7) protein synthesized in vitro. Fig. 1 shows that αDVR-6 only recognizes DVR-6 protein.

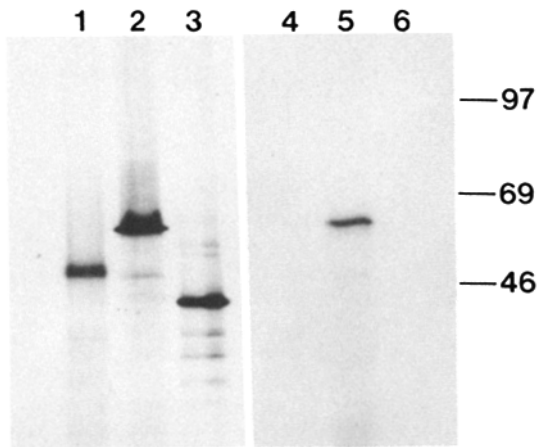Further evidence demonstrating the specificity of the DVR-6 antibody is shown in Figs. 2 and 3. BMGE+H cells

Figure 1. In vitro translation of DVR-5, -6, and -7 RNA and immunoprecipitation of translation products with αDVR-6. (Lanes 1-3) Human DVR-5, -6, and -7 protein, respectively. (Lanes 4-6) Immunoprecipitation, using αDVR-6, of human DVR-5, -6, and -7, respectively. The positions of protein markers are shown in kilodaltons on the right.
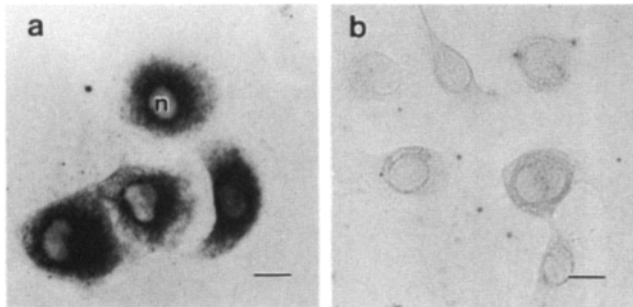

Figure 2. Immunohistochemistry of BMGE cells stably transfected with hDVR-6 using (a) αDVR-6 and (b) normal rabbit serum. n, nucleus. Bars, 20 μm.
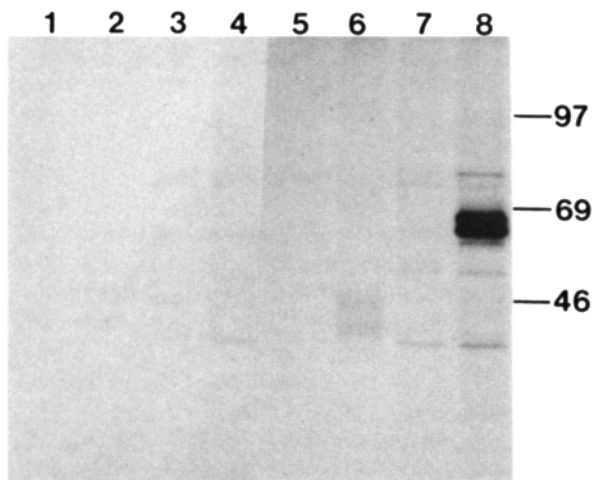

Figure 3. Immunoprecipitation of newly synthesized protein made by BMGE (lanes 1-4) and BMGE/D6c4 (lanes 5-8) cells with αDVR-6. (Lanes 1 and 5) Medium + normal rabbit serum; (lanes 2 and 6) medium + αDVR-6; (lanes 3 and 7) cytoplasmic fraction + normal rabbit serum; and (lanes 4 and 8) cytoplasmic fraction + αDVR-6. The positions of protein markers are shown in kilodaltons on the right.
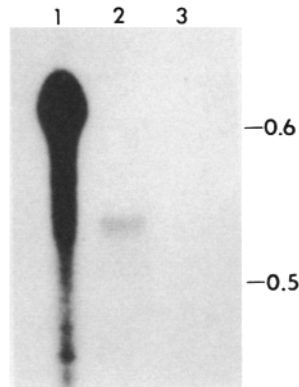

Figure 4. RNase protection of mouse DVR-6 in PYS cells. (Lane 1), 256-bp XhoII/RsaI probe; (lane 2), 512-bp protected fragment; and (lane 3), probe with water as a negative control.

were stably transfected with the entire coding region of human DVR-6 cDNA in a cytokeratin-based expression vector. Immunohistochemistry (Fig. 2) and immunoprecipitation (Fig. 3) experiments show that the antibody recognizes protein in the transfected cell line BMGE+H/D6c4 but not in untransfected control cells.

Mouse DVR-6 cDNA sequence was used to make the fusion protein antigen. However, the resulting antibody recognizes human (Figs. 1-3) and chicken (Kuratani, S., Baylor College of Medicine, personal communication) DVR-6 protein. The chicken DVR-6 amino acid sequence is not known, but the mouse and human sequence have 81.6% identity in the region used to make the antigen. Therefore, αDVR-6 is useful for studies in at least three species.

Expression in Cell Lines

Lyons et al. (1989a,b) showed, using Northern analysis, that DVR-6 (Vgr-1) RNA is expressed in three cell lines—murine PYS, human 49F, and human HT1080. However, of these three, immunoreactivity for αDVR-6 is only seen in the cytoplasm of the HT1080 cell line (data not shown). This discrepancy between mRNA and protein expression could have several explanations. For example, there may be alternative splicing of the DVR-6 transcript in PYS and 49F cells that removes the region encoding the epitope recognized by αDVR-6. However, results of RNase protection assays demonstrate that the RNA sequence encoding the epitope recognized by αDVR-6 is present in PYS cells (Fig. 4). Another explanation is that translation of the mRNA is inhibited, perhaps through 5' or 3' untranslated sequences. Preliminary experiments which support this idea show that an hDVR-6 construct, without any 5' or 3' untranslated sequence, is translated when transfected into PYS cells. Other explanations are that the translation product is complexed with another protein, obscuring the epitope recognized by αDVR-6, or that DVR-6 protein is quickly degraded upon synthesis. Experiments to distinguish between these possibilities are currently under way.

Biosynthesis of DVR-6 Protein

Biosynthesis of hDVR-6 protein was examined using the stably transfected cell line, BMGE+H/D6c4. The predicted relative molecular mass of the precursor protein is 57,207 and the in vitro–translated (therefore, unmodified) DVR-6 protein is ∼58,000 (Fig. 1). Fig. 3 shows that newly synthe-
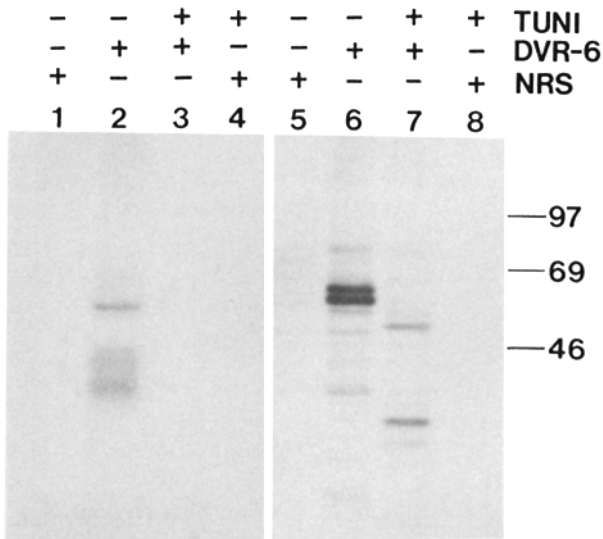
Figure 5. Immunoprecipitation of newly synthesized protein made by BMGE/D6c4 cells with and without tunicamycin treatment. (Lanes 1 and 2) Medium of untreated cells with normal rabbit serum (NRS) and αDVR-6, respectively. (Lanes 3 and 4) Medium of tunicamycin-treated cells with αDVR-6 and NRS, respectively. (Lanes 5 and 6) Cytoplasmic fraction of untreated cells with NRS and αDVR-6, respectively. (Lanes 7 and 8) Cytoplasmic fraction of tunicamycin-treated cells with αDVR-6 and NRS, respectively. The positions of protein markers are shown in kilodaltons on the right.
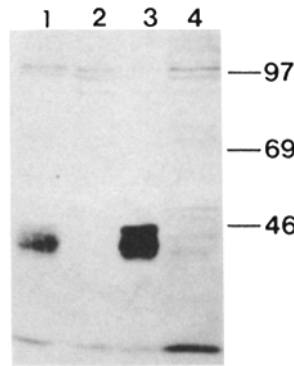

Figure 6. Western blot of 12.5-d.p.c. mouse tissue with αDVR-6. (Lane 1) Head; (lane 2) liver; (lane 3) spinal cord; and (lane 4) limb bud. The positions of protein markers are shown in kilodaltons on the right.

sized DVR-6 protein in the cytoplasm of BMGE+H/D6c4 cells exists as ∿62,000/65,000-M_r doublet. This doublet most likely represents different glycosylated forms of full-length DVR-6 protein (Wang, E., Genetics Institute, personal communication and see below). The predicted relative molecular mass for the proregion alone of DVR-6 protein is 42,370. Therefore, another doublet of ∿40,500/43,500 M_r found in the medium probably represents this part of the protein which is cleaved from the mature region around the time of secretion (lane 6, Fig. 3). The appearance of this doublet as diffuse rather than sharp bands is indicative of partially degraded, glycosylated protein.

To demonstrate that DVR-6 protein is glycosylated, immunoprecipitation experiments were repeated using cultures treated with tunicamycin (Fig. 5). The lower doublet is not seen in the medium but a protein of ∿41,500 M_r is now seen in the cytoplasm. This protein species most likely represents the cleaved proregion of DVR-6 which, perhaps due to lack of glycosylation, is not secreted into the medium. Also, the ∿62,000/65,000-M_r doublet found in the cytoplasm of control cells is no longer present, but is replaced by a single ∿55,000-M_r protein. This species most likely represents the unglycosylated form of the DVR-6 precursor.

Western blot analysis of 12.5 days postcoitum (d.p.c) embryonic tissue was performed to demonstrate that DVR-6 protein is present in vivo. Tissues that are immunoreactive for αDVR-6 by immunohistochemistry—namely, brain and spinal cord—and tissues that are not immunoreactive for the antibody—liver and limb bud—were used. Fig. 6 shows that the cleaved proregion, the ∿40,500/43,500-M_r doublet, is present in brain (head) and spinal cord, but not in liver and

limb buds. No uncleaved protein was detected by Western blot, but it is detectable by immunoprecipitation of labeled tissues (data not shown).

Localization of DVR-6 Protein In Vivo

Previous studies using in situ hybridization had revealed DVR-6 RNA in a number of embryonic tissues including the central nervous system, skin, hypertrophic cartilage, and primary oocytes. Intact and sectioned embryos were studied by immunohistochemistry to see if localization of DVR-6 protein correlates with previously published RNA expression patterns (Lyons et al., 1989a,b).

Expression of DVR-6 in the Developing Mouse Nervous System

Using whole mount immunohistochemistry with mouse embryos, DVR-6 protein is first detected at 9.5 d.p.c., in the developing trigeminal mesencephalic tracts, cranial nerves and ganglia, and spinal nerves of the anterior spinal cord (Fig. 7 a). Immunoreactivity was not seen in 9.0 d.p.c. embryos. By 10.5 d.p.c., as neural development proceeds, expression is maintained in these areas and is also seen in the spinal nerves emerging from more posterior regions of the spinal cord (Fig. 7, b and c).

Localization of protein was studied in more detail in transverse sections of embryos at 9.5, 10.5, 12.5, and 15.5 d.p.c. As the nervous system develops, the number of DVR-6-positive cells increases (Fig. 8). Immunoreactivity at 9.5 d.p.c. in the anterior regions of the spinal cord is in cells located in lateral and ventral regions of the marginal zone. DVR-6 protein is also seen in putative neural crest cells that presumably will contribute to the formation of the cranial ganglia. By 10.5 d.p.c., the cranial and dorsal root ganglia and the presumptive motor neurons are immunoreactive and express DVR-6 protein in cell bodies as well as axons. This is particularly evident in Fig. 8 e. At 12.5 d.p.c., both the cell bodies throughout the spinal cord and the axonal tracts which form the white matter are heavily stained, again showing that DVR-6 protein is present in axons as well as cell bodies and, therefore, is probably transported and secreted axonally. However, by 15.5 d.p.c., the staining of axon tracts is reduced.

In newborn mice, expression is in most regions of the brain but is more obvious in areas which contain larger cells or more concentrated neuronal populations such as the cere-
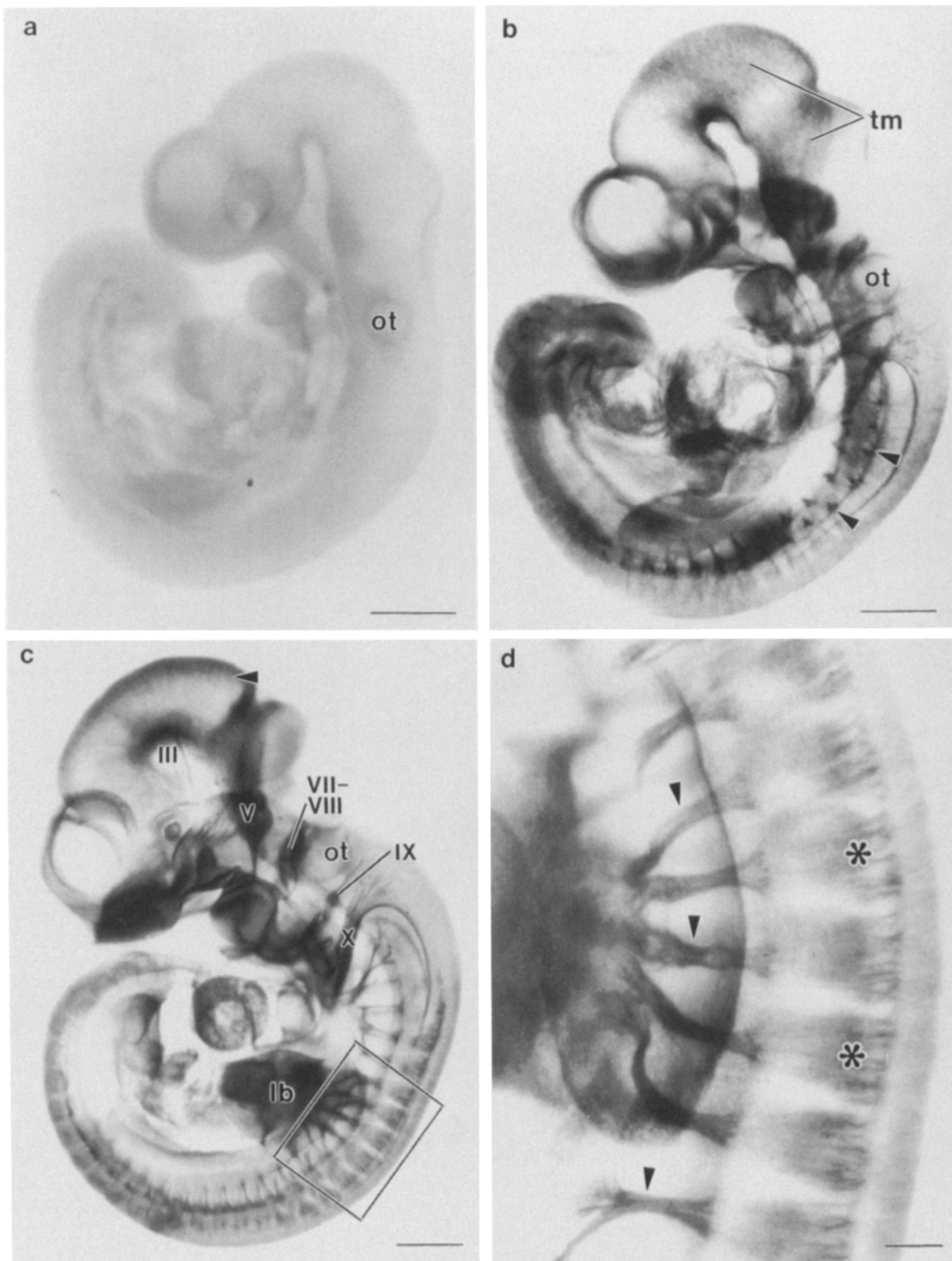
Figure 7. Immunohistochemistry of intact 9.5- and 10.5-d.p.c. mouse embryos. (*a*) 9.5-d.c.p. mouse embryo with normal rabbit serum. (*b*) 9.5-d.p.c. mouse embryo. Arrows indicate the developing spinal nerves. *tm*, trigeminal mesencephalic tract. (*c*) 10.5-d.p.c. mouse embryo. Cranial nerves are numbered. Arrow shows the trigeminal mesencephalic tract. *ot*, otic vesicle; *lb*, limb bud. (*d*) Boxed area in *c*. Arrows show the ventral motor roots of the spinal nerves. Asterisks show the dorsal roots of the spinal nerves. Bars: (*a–c*) 500 μm; (*d*) 100 μm.
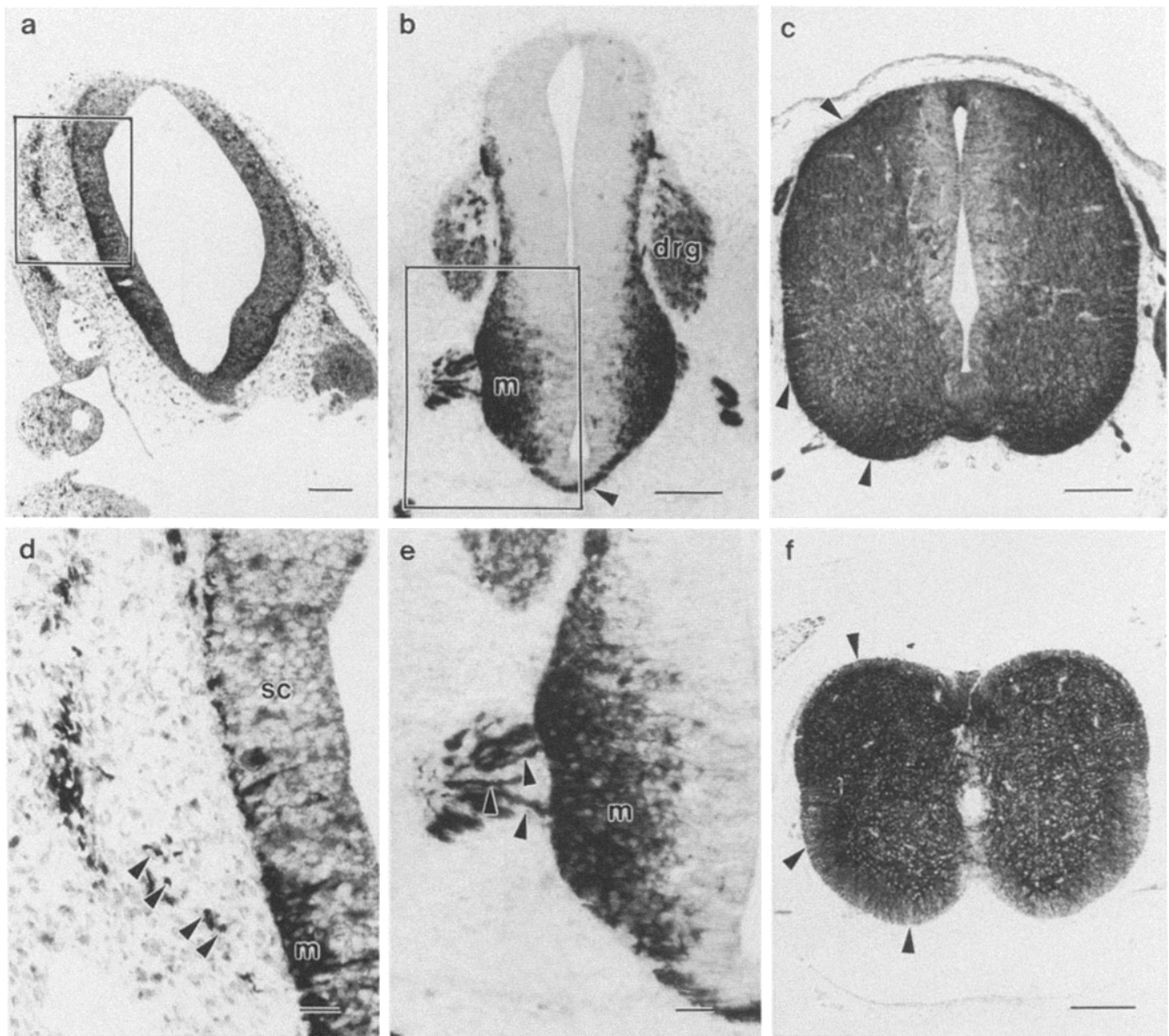
Figure 8. Immunohistochemistry of sections through the developing spinal cord and hind brain. (*a*) 9.5-d.p.c. hindbrain, branchial arch region. Note immunoreactive cells in the periphery of the neural tube, including presumptive motor neurons. (*b*) 10.5-d.p.c. spinal cord. Arrow shows the ventral commissural tract. (*c*) 12.5-d.p.c. spinal cord. Arrows show heavy staining of axonal tracts. (*d*) Magnification of boxed area from *a*. Arrows show putative neural crest cells. (*e*) Magnification of boxed area from *b*. Note strong cytoplasmic staining of motor neuron cell bodies and axons exiting from the spinal cord (*arrows*). (*f*) 15.5 d.p.c. Arrows show decreased staining of axonal tracts compared to 12.5 d.p.c. *drg*, dorsal root ganglion; *m*, motor neurons; *sc*, spinal cord. Bars: (*a* and *b*) 100 μm; (*c* and *f*) 50 μm; (*d* and *e*) 25 μm.

bellum, hippocampus, and brain nuclei (data not shown). Fig. 9 shows that this expression is maintained in the adult. DVR-6 protein immunoreactivity is seen in the cerebellum (Fig. 9, *a* and *c*) and in the hippocampus (Fig. 9, *b* and *d*). While protein can be definitely localized to neuronal cells, we cannot at present exclude the possibility that it is also expressed in some glial cells. However, in counterstained sections of the eye it was apparent that there were glial cells which did not express DVR-6 protein. Additionally, in the adult, but not in embryos or newborns, αDVR-6 immunoreactivity is seen in the choroid plexus (Fig. 9 *b*) and meninges (data not shown).

Fig. 9 also shows the developing eye (Fig. 9 *e*) and cochlea (Fig. 9 *f*) of a 15.5-d.p.c. embryo. As seen in the spinal cord, DVR-6 immunoreactive cells are limited to the most mature

region of the retina and the axon tract of the optic nerve, indicating that this protein is expressed in postmitotic neurons. In the cochlea, expression of DVR-6 protein is seen in the eighth cranial nerve and the region containing hair cells in the cochlear duct wall.

Expression in Epithelia

DVR-6 protein is first detected in the epidermis at 15.5 d.p.c., specifically in the nasal epithelium, when it is beginning to thicken and keratinize (Fig. 10, *a–d*). By 16.5 d.p.c., expression is seen in the suprabasal cells of the skin, but is absent from the basal layer and hair follicles. This correlates with the expression of DVR-6 mRNA (Lyons et al., 1989*a,b*). In newborn mice, αDVR-6 immunoreactivity is
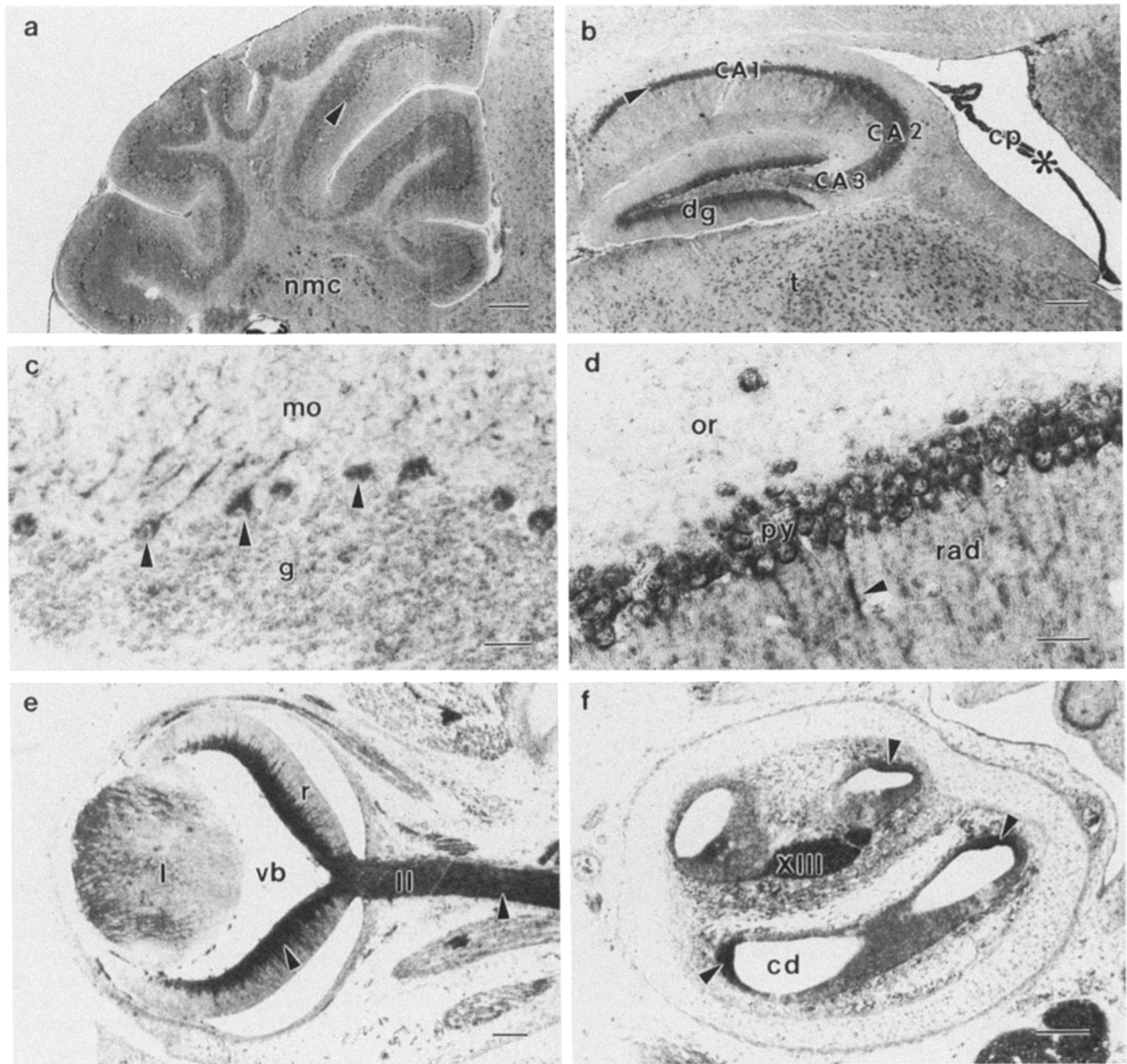
Figure 9. Immunohistochemistry of brain and sensory organs. (*a*) Adult cerebellum. Arrow shows Purkinje cell layer. (*b*) Adult hippocampus. Arrow shows pyramidal cell layer. Asterisk denotes choroid plexus which is intensely immunoreactive. (*c*) High power view of the Purkinje cell layer. Arrows show Purkinje cells. (*d*) High power view of the pyramidal cell layer. Arrow shows an axon extending from a pyramidal cell. (*e*) 15.5-d.p.c. eye and optic nerve. Arrows show immunoreactivity in the retinal ganglion cells and axons in the optic nerve (*ll*). The staining in the lens is nonspecific. (*f*) Horizontal section of a 15.5-d.p.c. cochlea. Arrows show immunoreactivity in hair cells of the sensory epithelium. Cranial nerves and hippocampal regions are numbered. *nmc*, nucleus medialis cerebellum; *dg*, dentate gyrus; *t*, thalamus; *cp*, choroid plexus; *mo*, molecular layer; *g*, granule cell layer; *or*, striatum oriens; *py*, pyramidal layer; *rad*, striatum radiens; *l*, lens; *vb*, vitrious body; *r*, retina; *cd*, cochlear duct. Bars: (*a* and *b*) 200 µm; (*c* and *d*) 25 µm; (*e*) 100 µm; (*f*) 50 µm.

still seen in the suprabasal layer of the epidermis. Additionally, all of the corneal epithelial cells of the adult are DVR-6 immunoreactive, but not the limbus (Fig. 10, *g–i*). Since the epithelial cells of the cornea migrate centripetally from the limbus, this expression pattern is analogous to that of the skin in which the basal cell layer (mitotic region) does not express DVR-6 protein, while the suprabasal layer (postmitotic) does express DVR-6 protein. Fig. 10, *e* and *f*, shows that DVR-6 protein also is expressed in the bronchiolar epithelium of newborn lung but not in alveolar epithelium.

Other Tissues

Hypertrophic cartilage, uterine stroma, and oocytes, tissues that previously had been shown by in situ hybridization to express DVR-6 mRNA, were also examined by immunohistochemistry. Hypertrophic cartilage in late-stage embryos was not immunoreactive for αDVR-6. Ovaries and uteri from each day of the estrous cycle were stained but showed no immunoreactivity in the oocytes or uterine stroma. Additionally, both fertilized and unfertilized ova taken at various
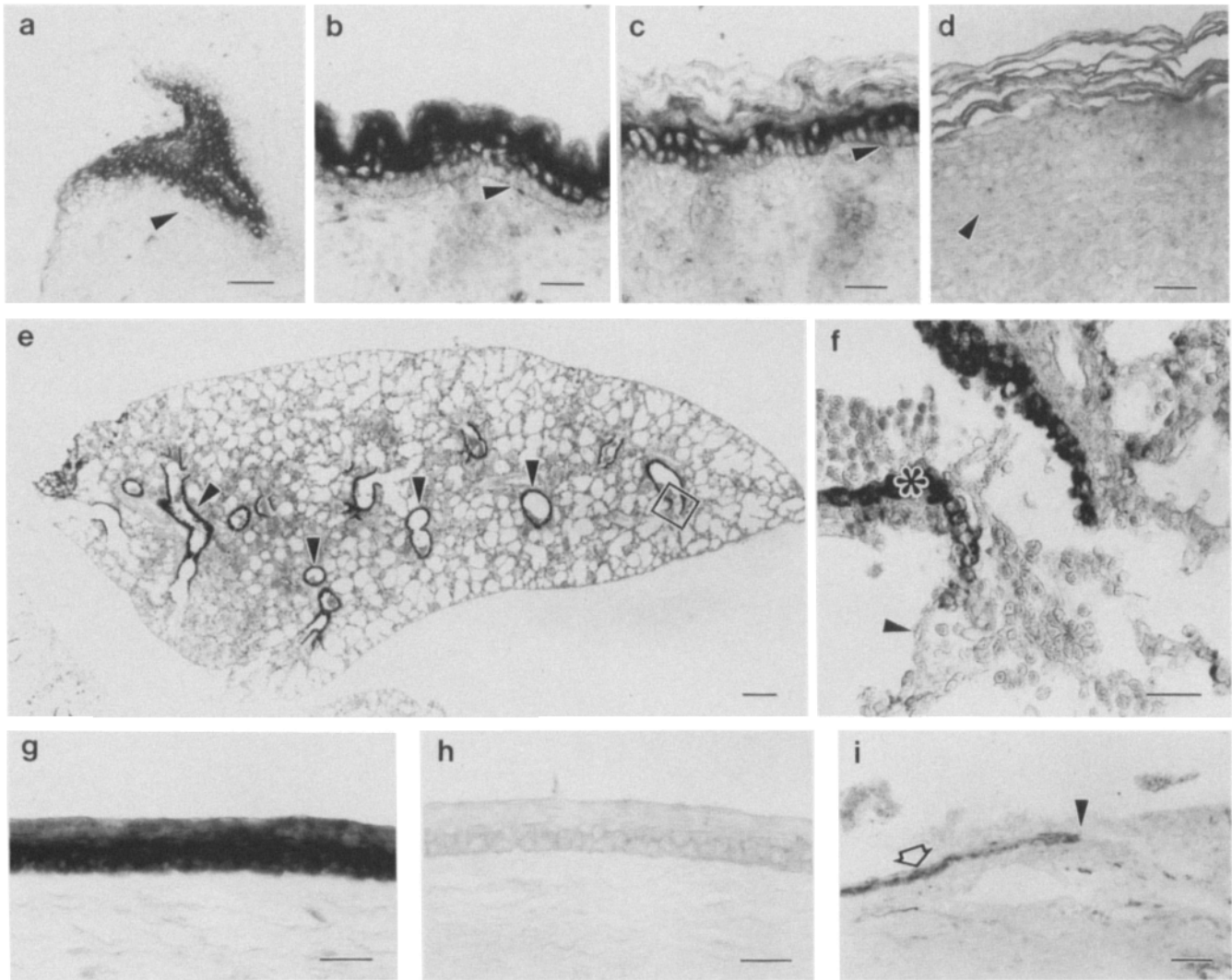
Figure 10. Immunohistochemistry of mouse epithelial tissue. (*a*) 15.5-d.p.c. suprabasal nasal epithelium. (*b*) 16.5-d.p.c. skin. (*c*) Newborn skin. (*d*) Newborn skin with normal rabbit serum. Arrows indicate basal cells which show little or no expression of DVR-6 protein. (*e*) Newborn lung. Arrows indicate areas of positive staining. (*f*) High power view of newborn lung (boxed area in *e*). Immunoreactivity is seen in the bronchiolar epithelium (*asterisk*) but not the alveolar epithelium (*arrow*). (*g*) Adult cornea. (*h*) Adult cornea with NRS. (*i*) Limbus region of adult eye. Open arrow shows trailing edge of the DVR-6 positive cornea. Arrow shows where corneal epithelium ends. Bars: (*a–d* and *f*) 25 μm; (*e*) 200 μm; (*g* and *h*) 20 μm; (*i*) 50 μm.

times after ovulation were also immunostained, but again showed no immunoreactivity with αDVR-6.

Discussion

The availability of a specific antibody for DVR-6 has provided a means for in-depth studies of its synthesis, post-translational modification, secretion, and localization in at least three species (mouse and human, this paper; chicken, Kuratani, S., unpublished observations). Although in situ hybridization studies have previously shown expression patterns for DVR-6 during development, these studies only establish where mRNA is located, not the protein. Since proteins are the agents of function, and since the presence of a transcript does not necessarily indicate translation, a protein expression pattern, as well as characterization of the protein, is important for examining the potential role of DVR-6 in the developing embryo.

Immunoprecipitations done with αDVR-6 reveal two newly synthesized protein doublets of ~62,000/65,000 and ~40,500/43,500 M_r in cells transfected with hDVR-6 and in tissues of developing mouse embryos (Fig. 3 and data not shown). Since αDVR-6 only recognizes the proregion of the protein, it is likely that the higher doublet is the full-length DVR-6 protein and the lower is the cleaved proregion. This is consistent with the model that DVR-6 protein, as a member of the TGF-β superfamily, is synthesized as a prepro secreted protein (Brunner et al., 1988; Gentry et al., 1988; Sha et al., 1989).

DVR-6 protein, which has five potential N-glycosylation sites (two in the proregion and three in the mature region), exists in two glycosylated forms and at least its proregion is secreted from cells. The significance of two glycosylated forms of this protein is presently unknown, but it appears that glycosylation may affect secretion of DVR-6 protein since tunicamycin treatment of BMGE+H/D6c4 cells shows that secretion of the cleaved proregion of DVR-6 is inhibited when it is unglycosylated (Fig. 6). Absence of glycosylation

may or may not inhibit the secretion of the presumed mature region of DVR-6 protein. An antibody to the mature region is currently being produced so that this can be determined.

In situ hybridization studies have shown that DVR-6 mRNA is expressed in epithelia and neural tissues, hypertrophic cartilage, and primary oocytes (Lyons et al., 1989a,b). Although αDVR-6 recognizes protein in neural and epithelial tissues, it does not detect DVR-6 protein in hypertrophic cartilage or oocytes. This discrepancy between mRNA and protein expression is also found in cell lines that contain DVR-6 transcripts. While HT1080 cells express both DVR-6 mRNA and protein, PYS and 49F cells, which contain DVR-6 transcripts, do not contain detectable protein. One explanation for this is that the 256-bp sequence encoding the epitope recognized by αDVR-6 is spliced out of the DVR-6 transcripts in these cell lines and tissues. However, RNase protection experiments using this 256-bp sequence as a probe in PYS cells show that the sequence is present (Fig. 4). Therefore, a more plausible explanation for this discrepancy is that DVR-6 mRNA is not translated in these tissues and cell lines. However, we cannot rule out the possibility that other mechanisms may be involved. DVR-6 protein could be degraded as it is translated or it could complex with another protein masking the epitope recognized by αDVR-6. To explore the possibility of translational control, PYS cells, a cell line in which DVR-6 transcript but not protein is detected, have been stably transfected with hDVR-6. The hDVR-6 construct excludes 5' and 3' untranslated sequences since these may be involved in translational control (Hershey, 1991). Preliminary results demonstrate that PYS cells are capable of synthesizing DVR-6 protein (data not shown). This suggests that translational control via 5' or 3' untranslated sequences has been relieved. In the embryo, it remains to be seen whether translational control plays a role in the lack of DVR-6 protein expression in hypertrophic cartilage and oocytes.

Immunohistochemical staining of epithelial and neuronal tissues shows that DVR-6 protein is expressed in vivo at different stages of development. In the epidermis, protein is first detected at 15.5 d.p.c., when the epidermal thickening is beginning to occur, in the suprabasal layers of the nasal region. By 16.5 d.p.c., expression is seen in the suprabasal layer of the skin and this pattern is maintained in newborns. Also in newborns, bronchiolar epithelium is immunoreactive for αDVR-6. Additionally, in the adult, the corneal epithelium, but not the limbus or conjunctival epithelium, expresses DVR-6. This is noteworthy because, based on keratin expression, the limbal and conjunctival epithelia more closely resemble the basal layer of the skin while the corneal epithelium resembles the suprabasal layer (Schermer et al., 1986). The role of DVR-6 in these epithelial tissues is unknown at this time. However, since the suprabasal and corneal epithelia are differentiated, postmitotic cells, secreted DVR-6 protein may act in an autocrine manner to promote withdrawal from the cell cycle and/or differentiation. Alternatively, DVR-6 may be acting in a paracrine fashion to regulate the proliferation and differentiation of basal cells.

In the nervous system, DVR-6 immunoreactive cells are first detected at 9.5 d.p.c. in the trigeminal mesencephalic tract, cranial ganglia, and spinal cord. Also at this stage, both the sensory and motor components of the spinal nerves begin expressing protein, first anteriorly, and then in more posterior sites as development proceeds. Protein is seen in both the cytoplasm and axons of neuronal cells at this time. This expression is particularly evident in the motor neurons of the 10.5-d.p.c. embryo (Figs. 7 and 8). Cross sections of spinal cord from stages 9.5–15.5 d.p.c. and sagittal sections of brain from stages 12.5–adult (data not shown) reveal that immunoreactive cells first appear in the outer layers, and then subsequently more interior layers of these tissues. This temporal and spatial expression pattern suggests that DVR-6 is expressed in newly born, postmitotic neurons. This hypothesis will be tested by correlating DVR-6 expression with bromodeoxyuridine labeling.

Flanders et al. (1991) have shown that TGF-β-2 and TGF-β-3 proteins are expressed in most regions of the embryonic nervous system, but not as extensively as DVR-6 protein. For example, while DVR-6 is present in all cranial and dorsal root ganglia, TGF-βs 1, 2, and 3 are absent from some of these ganglia. Another difference in expression of these proteins is that TGF-β is present in some glia in vivo and in vitro (Flanders et al., 1991; Saad et al., 1991; Constam et al., 1992; Lindholm et al., 1992) but, so far, no expression of DVR-6 has been detected in a number of glial cell lines studied (data not shown). Additionally, preliminary studies in which sections of the 15.5-d.p.c. optic nerve have been immunostained and counterstained, do not show staining of DVR-6 in cells presumed to be glia associated with the nerve tract (data not shown). If DVR-6 proves to be neuron specific, it can be used as an early marker distinguishing between neuronal and glial cells.

The presence of DVR-6 protein in the cell bodies and axons of postmitotic cells of the nervous system suggests a variety of potential functions, either paracrine or autocrine in nature. Since there is presumably a secreted form of the protein, one potential function is the stimulation or inhibition of cell division or the promotion of differentiation, of associated glial cells, neighboring neurons, innervating neurons, or target cells, such as muscle. Further studies to determine whether DVR-6 is secreted via dendrites or axons (or both), and, like other members of the TGF-β superfamily, it is sequestered by the extracellular matrix, will help in determining the role of this protein in neural development. It will be necessary to produce purified protein and an antibody directed against the mature protein (experiments are currently under way) before these questions can be fully addressed. Also, identification and study of a receptor for DVR-6 will aid in elucidating the function(s) of this protein.

Immunoprecipitation and Western blot analysis was done to rule out the possibility that αDVR-6 cross-reacts with a neurofilament protein. Immunoprecipitation experiments of 12.5-d.p.c. brain and spinal cord showed that the uncleaved form of DVR-6 protein was being synthesized, but at a low rate (data not shown), and the results of Western analysis show that protein of the expected relative molecular mass for the cleaved proregion of DVR-6 is present (Fig. 4). This demonstrates that the appropriate size protein is present in tissue extracts and that for steady-state protein levels, the predominant form of DVR-6 protein recognized by αDVR-6 is the cleaved species. This raises the possibility that the staining patterns seen in vivo may be those of residual, cleaved, pro-DVR-6 protein, and not full-length protein. At present, the resolution of immunostained sections by light microscopy does not allow us to distinguish whether this protein is entirely intracellular, or whether the cleaved proregion also accumulates in the extracellular environment.

Additionally, the persistence of cleaved pro-DVR-6 in steady-state protein suggests that it may have a function of its own.

One important conclusion reached using αDVR-6 is that only a subset of tissues previously shown to express DVR-6 mRNA express the protein. The PYS cell line (which expresses DVR-6 transcripts but not protein) should provide clues as to the mechanism(s) involved in this discrepancy of mRNA and protein expression. Tissues which do express both DVR-6 transcripts and protein in the mouse are epithelial and nervous tissues. While the functional role(s) of this protein is not known at this time, these expression data presented here allow us to begin proposing and testing potential functions. Additionally, αDVR-6 should be useful as an early marker for neuronal differentiation in the developing nervous system.

References

Bateman, H. E., and R. M. Mason. 1984. Applied Anatomy and Physiology of the Speech and Hearing Mechanism. Charles C. Thomas Publisher, Springfield, IL. 636.

Blessing, M., H. Zentgraf, and J. L. Jorcano. 1987. Differentially expressed bovine cytokeratin genes. Analysis of gene linkage and evolutionary conservation of 5'-upstream sequences. EMBO (Eur. Mol. Biol. Organ.) J. 6: 567-575.

Brunner, A. M., L. E. Gentry, J. A. Cooper, and A. F. Purchio. 1988. Recombinant type 1 transforming growth factor beta precursor produced in Chinese hamster ovary cells is glycosylated and phosphorylated. Mol. Cell. Biol. 8:2229-2232.

Brutlag, D. C., J. P. Dautricourt, S. Maulik, and J. Relph. 1990. Improved sensitivity of biological sequence data base searches. Comput. Appl. Biosci. 6:237-245.

Celeste, A. J., J. A. Iannazzi, R. C. Taylor, R. M. Hewick, V. Rosen, E. A. Wang, and J. M. Wozney. 1990. Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. Proc. Natl. Acad. Sci. USA. 87:9843-9847.

Colebére-Garapin, F., F. Horodniceanu, P. Kourilsky, and A. Garapin. 1981. A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. 150:1-14.

Constam, D. B., J. Philipp, U. V. Malipiero, P. ten Dijke, M. Schachner, and A. Fontana. 1992. Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia. J. Immunol. 148:1404-1410.

Dale, L., G. Howes, B. M. J. Price, and J. C. Smith. 1992. Bone Morphogenetic protein 4: a ventralizing factor in early Xenopus development. Development. 115:573-585.

Ferguson, E. L., and K. V. Anderson. 1992. Localized enhancement and repression of the activity of the TGF-β family member, decapentaplegic, is necessary for dorsal-ventral pattern formation in the Drosophila embryo. Development. 114:583-597.

Flanders, K. C., G. Ludecke, S. Engels, D. S. Cissel, A. B. Roberts, P. Kondaiah, R. Lafyatatis, M. B. Sporn, and K. Unsicker. 1991. Localization and actions of transforming growth factor-betas in the embryonic nervous system. Development. 113:183-191.

Gentry, L. E., M. N. Lioubin, A. F. Purchio, and H. Marquardt. 1988. Molecular events in processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. Mol. Cell. Biol. 8:4162-4168.

Gorman, C. M., L. F. Moffatt, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol. Cell. Biol. 2:1044-1051.

Harlow, E., and D. P. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 726.

Harrison, R. V. 1988. The Biology of Hearing and Deafness. Charles C. Thomas Publisher, Springfield, IL. 432.

Hershey, J. W. B. 1991. Translational control in mammalian cells. Annu. Rev. Biochem. 60:717-755.

Immergluck, K., P. A. Lawrence, and M. Bienz. 1990. Induction across germ layers in Drosophila mediated by a genetic cascade. Cell. 62:261-268.

Irish, V. F., and W. M. Gelbart. 1987. The decapentaplegic gene is required for dorsal-ventral patterning of the Drosophila embryo. Genes & Dev. 1:868-879.

Jones, C. M., K. M. Lyons, and B. L. M. Hogan. 1991. Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. Development. 111:531-542.

Jones, C. M., K. M. Lyons, P. M. Lapan, C. V. E. Wright, and B. L. M. Hogan. 1992. DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in Xenopus mesoderm induction. Development. 115: 639-647.

Kehrl, J. H. 1991. Transforming growth factor beta: an important mediator of immunoregulation. Int. J. Cell Cloning. 9:438-450.

Lindholm, D., E. Castren, R. Kiefer, F. Zafra, and H. Thoenen. 1992. Transforming growth factor-beta 1 in the rat brain: increase after injury and inhibition of astrocyte proliferation. J. Cell Biol. 117:395-400.

Lyons, K. M., J. L. Graycar, A. Lee, S. Hashmi, P. B. Lindquist, E. Y. Chen, B. L. M. Hogan, and R. Derynck. 1989a. Vgr-1, a mammalian gene related to Xenopus Vg-1, is a member of the transforming growth factor β gene superfamily. Proc. Natl. Acad. Sci. USA. 86:4554-4558.

Lyons, K. M., R. W. Pelton, and B. L. M. Hogan. 1989b. Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-β-like genes coordinately regulate aspects of embryonic development. Genes & Dev. 3:1657-1668.

Lyons, K. M., R. W. Pelton, and B. L. M. Hogan. 1990. Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). Development. 109:833-844.

Lyons, K. M., C. M. Jones, and B. L. M. Hogan. 1991. The DVR gene family in embryonic development. Trends Genet. 7:408-412.

Massague, J., J. Heino, and M. Laiho. 1991. Mechanisms of TGF-beta action. Ciba. Found. Symp. 159:51-65.

Panganiban, G. E., K. E. Rashka, M. D. Nietzel, and F. M. Hoffmann. 1990a. Biochemical characterization of the Drosophila dpp protein, a member of the transforming growth factor beta family of growth factors. Mol. Cell. Biol. 10:2669-2677.

Panganiban, G. E., R. Reuter, M. P. Scott, and F. M. Hoffmann. 1990b. A Drosophila growth factor homolog, decapentaplegic, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. Development. 110:1041-1050.

Paxinos, G. 1985. The Rat Nervous System. Academic Press Inc., Marrickville, Australia. 285.

Posakony, L. G., L. A. Raftery, and W. M. Gelbart. 1990. Wing formation in Drosophila melanogaster requires decapentaplegic gene function along the anterior-posterior compartment boundary. Mech. Ageing Dev. 33:69-82.

Sato, Y., R. Tsuboi, R. Lyons, H. Moses, and D. B. Rifkin. 1990. Characterization of the activation of latent TGF-beta by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. J. Cell Biol. 111:757-763.

Schermer, A., S. Galvin, and T. Sun. 1986. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J. Cell Biol. 103:49-62.

Schmid, E., D. L. Schiller, C. Grund, J. Stadler, and W. W. Franke. 1983. Tissue type-specific expression of intermediate filament proteins in a cultured epithelial cell line from bovine mammary gland. J. Cell Biol. 96: 37-50.

Sha, X., A. M. Brunner, A. F. Purchio, and L. E. Gentry. 1989. Transforming growth factor beta 1: importance of glycosylation and acidic proteases for processing and secretion. Mol. Endocrinol. 3:1090-1098.

Shambra, U. B., J. M. Lauder, and J. Silver. 1992. Atlas of the Prenatal Mouse Brain. Academic Press Inc., San Diego, CA. 327.

Sidman, R. L., J. B. Angevine, and E. T. Pierce. 1971. Atlas of the Mouse Brain and Spinal Cord. Harvard University Press, Cambridge, MA. 261.

Soma, Y., and G. R. Grotendorst. 1989. TGF-beta stimulates primary human skin fibroblast DNA synthesis via an autocrine production of PDGF-related peptides. J. Cell. Physiol. 140:246-253.

Wall, N. A., C. M. Jones, B. L. M. Hogan, and C. V. E. Wright. 1992. Expression and modification of Hox2.1 protein in mouse embryos. Mech. Dev. 37: 111-120.

Wang, E. A., V. Rosen, J. S. D'Alessandro, M. Bauduy, P. Cordes, T. Harada, D. I. Israel, R. M. Hewick, K. M. Kerns, P. LaPan, D. P. Luxenberg, D. McQuaid, I. K. Moutsatsos, J. Nove, and J. M. Wozney. 1990. Recombinant human bone morphogenetic protein induces bone formation. Proc. Natl. Acad. Sci. USA. 87:2220-2224.

Weeks, D. L., and D. A. Melton. 1987. A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-β. Cell. 51:861-867.