Developmental Expression of 2ar (Osteopontin) and SPARC (Osteonectin) RNA as Revealed by In Situ Hybridization

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Abstract. 2ar has been identified as a gene inducible by tumor promoters and growth factors in a variety of cultured mouse cell lines (Smith, J. H., and D. T. Denhardt. 1987. *J. Cell. Biochem.* 34:13–22). Sequence analysis shows that it codes for mouse osteopontin, an RGDS-containing, phosphorylated, sialic acid-rich Ca^{++}-binding protein originally isolated from bone (Oldberg, A., A. Franzen, and D. Heinegard. 1986. *Proc. Natl. Acad. Sci. USA.* 83:8819–8823; Prince, C. W., T. Oosawa, W. T. Butler, M. Tomana, A. S. Brown, and R. E. Schrohenloer. 1987. *J. Biol. Chem.* 262:2900–3907.). In this paper we use Northern blot analysis and in situ hybridization to localize

expression of 2ar during mouse embryogenesis. 2ar RNA is first detected in developing limb bones and calvaria at 14.5 d p.c., in a population of cells distinct from those expressing SPARC (osteonectin). High levels of 2ar expression are also seen in the bone marrow–derived granulated metrial gland cells of the deciduum and placenta, and in a number of epithelial tissues, including embryonic and postnatal kidney tubules, uterine epithelium and sensory epithelium of the embryonic ear. The temporal and spatial pattern of 2ar expression seen in vivo suggests that the protein plays a wider role than previously realized, in processes which are not confined to bone development.

IN recent years, a number of cell cycle or cell proliferation–regulated genes (including cellular proto-oncogenes) have been shown to have specific temporal and tissue-specific patterns of expression during mouse embryogenesis. These findings have given us valuable information about the possible in vivo function of these genes, as well as the nature of molecular mechanisms underlying mammalian development (Müller, 1983; Jakobovits et al., 1985; Mason et al., 1985; Maness et al., 1986; Müller et al., 1986; Emilia et al., 1986; Wilkinson et al., 1987; for review see Adamson, 1987). Genes regulated by growth factors are of particular interest in this regard, since their pattern of expression may give insights into the in vivo role of growth factors during embryogenesis.

Here we extend such an analysis to 2ar, a gene originally isolated on the basis of its increased expression in the mouse JB6 epidermal cell line after treatment with the tumour promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Smith and Denhardt, 1987, and manuscript in preparation). Further studies have shown that 2ar expression is also induced in quiescent Swiss 3T3 and C3H 10T1/2 cells by serum and by growth factors such as epidermal growth factor, basic fibroblast growth factor (bFGF)[1], and embryonal carcinoma–derived growth factor (ECDGF) (our unpublished observa-

tions). This increase is due, at least in part, to an increase in gene transcription (Smith and Denhardt, 1987a, and manuscript in preparation). The predicted protein sequence of 2ar has recently been shown to be >92% identical to that of a cDNA isolated by screening an expression library of the rat ROS 17/2.8 osteosarcoma cell line with antibody against osteopontin (Oldberg et al., 1987; Denhardt et al., 1988). Osteopontin (also known as bone sialoprotein and 44 kD bone phosphoprotein) is a sialic acid-rich phosphorylated glycoprotein previously thought to be unique to bone (Herring, 1976; Franzen and Heinegard, 1985; Prince et al., 1987). Since there appears to be only a single gene for 2ar in the mouse (Smith and Denhardt, 1987a), it is probable that the protein represents the murine homologue of rat osteopontin. The main features of osteopontin are as follows. It contains the Arg-Gly-Asp-Ser (RGDS) amino acid motif found in fibronectin and a number of other extracellular proteins recognised by a family of adhesion-promoting cell surface receptors (Pytela et al., 1986; Tamkum et al., 1986). It has a high content of O-linked oligosaccharide chains rich in sialic acid which may be associated with a repeated Ser-X-Glu sequence found in the protein, contains phosphothreonine and phosphoserine, and binds tightly to hydroxylapatite, possibly via the sequence of nine consecutive Asp residues (Oldberg et al., 1986; Prince et al., 1987). These features have led to the suggestion that osteopontin is an extracellular matrix associated molecule involved in some aspect of cell adhesion, for example the binding of cells to the mineralized

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; ECDGF, embryonal carcinoma–derived growth factor; GMG; granulated metrial gland; PAS, periodic-acid Schiff; p.c., postcoitum.

type I collagen matrix of bone. In support of this hypothesis the purified protein promotes the RGD-sensitive attachment of rat osteosarcoma cells and fibroblasts to tissue culture plastic (Oldberg et al., 1986; Somerman et al., 1987). Recent immunohistochemical studies have also localized the protein to the newly formed matrix of neonatal rat bone and to the Golgi complex of fibroblast-like presumed osteoprogenitor cells, as well as osteoblasts (Mark et al., 1987).

The experiments reported here were designed to learn more about the possible role of 2ar/osteopontin in early mouse development. It was found that 2ar transcripts are localized to specific sites during normal embryogenesis such as the metrial gland cells of the deciduum, and the kidney, as well as osteogenic tissues. In the latter, the distribution of transcripts is consistent with expression in presumed progenitor cells. These findings strongly suggest that the protein plays an important role in processes other than, or in addition to, osteoblast adhesion. Such alternative functions for the protein may explain why it is expressed in a variety of cell lines in culture, and is elevated in response to tumor promoters and growth factors.

Materials and Methods

Embryos

All embryos were derived from either the CBA inbred strain (in situ hybridization) or from matings of CBA × C57BL/6 F1 mice (Northern analysis). Noon of the day of vaginal plug is 0.5 d postcoitum (p.c.). To induce the development of deciduomata, CBA females were mated with vasectomized males, and on the fourth day of pseudopregnancy ~10 μl of sterile paraffin oil was injected intraluminally into one uterine horn near the uterotubular junction using a 30-g needle (Hetherington, 1968). 5 d later both the injected horn and the control, uninjected horn were fixed and processed for in situ hybridization.

Cell Lines

Swiss 3T3 and 10T1/2 cells were grown to confluence in 175 cm² flasks containing DME/Ham's F12 (50:50 (vol/vol) DME/F12) supplemented with 10% (vol/vol) FCS (Gibco, Grand Island, NY; selected batches), in an atmosphere of 95% air, and 5% CO_2 (vol/vol). The cells were then transferred into serum-free defined medium (Heath, 1987) (basal medium supplemented with transferrin, plasma lipoproteins, and insulin). Additions of growth factors or hormones to the cell cultures were made 24 h after the transfer into serum-free medium. Undifferentiated F9 teratocarcinoma-derived stem cells were maintained on gelatin-coated tissue culture dishes and differentiation into parietal endoderm-like cells was initiated by addition of 5 × 10⁻⁸ M retinoic acid, 10⁻⁴ M dibutyryl cyclic AMP and 10⁻⁴ M isobutylmethyl xanthine, as described (Mason et al., 1986b).

Growth Factors and DNA Probes

bFGF was purified from bovine brain (Slack et al., 1987) and ECDGF was purified according to the protocol of Heath (1987).

The following mouse cDNA probes were used: 2ar (Smith and Denhardt, 1987), SPARC (Mason et al., 1986a) and murine glyceraldehyde 3-phosphate dehydrogenase obtained from Dr. P. Curtis (Wistar Institute, Philadelphia, PA). The 2ar cDNA probe was 1226-bp long, covering the entire 3′-noncoding region and ~90% of the protein coding region, as judged by alignment to the sequence of rat osteopontin (Oldberg et al., 1986; Denhardt et al., 1988).

Preparation of RNA

Embryos were dissected in medium (50:50/[vol/vol], DME/F12) containing 20 mM HEPES, pH 7.0, and 0.5% BSA, and tissues placed immediately in guanidinium isothiocyanate and homogenized using an Ultra-turrax. Total RNA was prepared from the homogenates by the guanidinium isothio-

cyanate method (Maniatis et al., 1982). Cytoplasmic RNA was isolated from cells as described by Edwards et al. (1985).

Northern Blots

Electrophoresis of RNA was performed according to the formaldehyde/agarose system described by Maniatis et al. (1982). The RNA was transferred to Hybond-N (Amersham International, U.K.) and fixed to the filters by baking at 80°C for 2 h. Hybridization was performed as described by Edwards et al. (1985) using 5 × 10⁶ cpm of nick-translated probe (sp act: 10⁸ cpm/μg) prepared from plasmids carrying cDNA copies of the appropriate gene. Autoradiography was carried out at −70°C using preflashed Kodak XAR-5 film and a single intensifying screen.

In Situ Hybridization

Preparation of tissue sections was essentially as described by Wilkinson et al. (1987) with the following modifications. Embryo and adult organs were fixed in 4% paraformaldehyde in PBS, pH 7.4, at 4°C for 16 h, dehydrated, and embedded in paraffin wax (Fibrowax pastillated, Raymond Lamb formulation). Sections of 6 μm were transferred to polylysine-coated slides and heated at 40°C for 2 h. Slides were stored dessicated at 4°C for up to 6 w. For in situ hybridization, slides were treated essentially as described by Hafen et al. (1983) with modifications suggested by Ingham et al. (1985). A 984-bp Hind III fragment of 2ar was subcloned into pGEM1 and pGEM2 (Promega Biotec, Madison, WI). [³⁵S] UTP-labeled single-stranded sense and antisense RNA probes (sp act 2.2 × 10⁹ dpm/μg RNA) were prepared as described by Melton et al. (1984), reduced to an average size of 100 bases by limited alkaline hydrolysis (Cox et al., 1984), and used in hybridizations at a final concentration of 2 × 10⁴ cpm/μl. Hybridization conditions were essentially as described (Ingham et al., 1985; Hogan et al., 1986; Holland et al., 1987). After hybridization, washing conditions were also as described (Holland et al., 1987) except that the RNase A treatment was with 5 μg/ml RNase A at 37°C for 90 min. After washing, slides were dehydrated and dipped in emulsion (K5; Ilford, Knutsford, Cheshire, England) diluted 1:1 with 2% glycerol in distilled water, placed on a cool metal plate for 10 min, dried at room temperature and humidity for 1 h, then overnight at room temperature in the presence of dessicant (Rogers, 1979). Slides were exposed at 4°C for 2–4 d, developed, and counter stained as described (Holland et al., 1987), except that the developing time was reduced to 1 min. For identifying metrial gland cells, slides were stained with periodic-acid Schiff (PAS) after digestion of glycogen with diastase (Drury and Wallington, 1980).

Results

Northern Analysis of 2ar Expression in Embryonic RNA

Initial experiments used Northern blot analysis to follow 2ar expression in a range of embryonic and extraembryonic tissues. For comparison, the filters included equivalent amounts of cytoplasmic RNA from both quiescent and ECDGF stimulated 10T1/2 fibroblasts. As reported previously, 2ar transcripts are present at low levels in quiescent fibroblasts and increase markedly in response to growth factor stimulation (Fig. 1 A, lanes A and B, and Smith and Denhardt, 1987). In addition, significant levels are detectable in 18.5 d p.c. calvaria, backbone, kidney, and placenta, and to a lesser extent in 14.5-d p.c. calvaria. The steady-state levels of RNA in these tissues, which are composed of a mixture of cell types, are approximately equivalent to those found in pure populations of growth factor stimulated fibroblasts. However, the majority of tissues examined (including major organs such as brain, liver, gut, and yolk sac) only express low, or undetectable, amounts of 2ar.

The temporal pattern of 2ar expression was then examined more closely in calvaria, kidney, placenta, and deciduum. (The latter represents the maternally derived portion of the implantation site before the formation of the definitive placenta) (Fig. 1 B). Transcripts can be detected in 14.5-d

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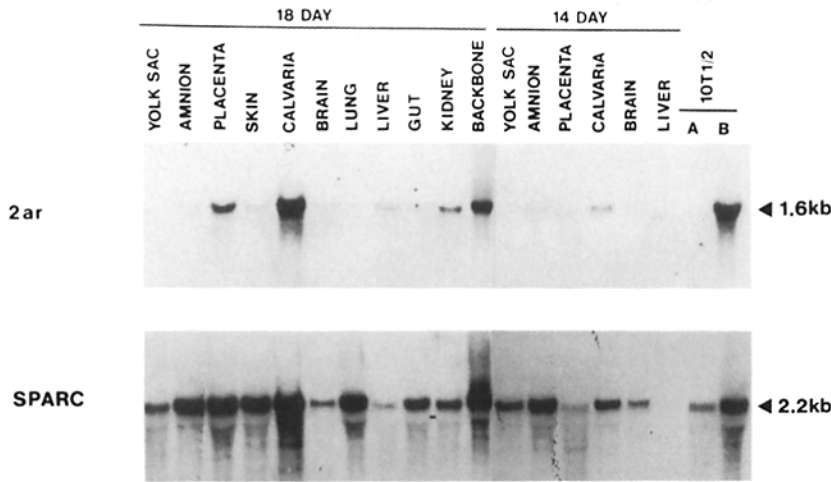
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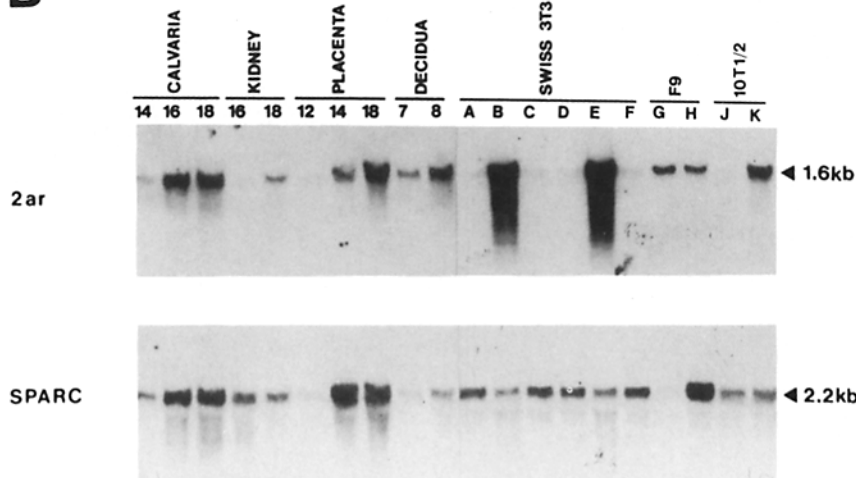
Figure 1. Distribution of 2ar/osteopontin and SPARC/osteonectin mRNA transcripts in embryonic mouse tissues, and the effects of growth factors and hormones on the steady-state RNA levels in cultured cells. The dating of the embryo tissues is to the nearest whole day (i.e., 18 d in the figure corresponds to 18.5 d in the text). (*A*) Confluent, quiescent 10T1/2 cells in serum-free defined medium, untreated (lane *A*) or exposed to ECDGF (10 ng/ml, lane *B*) for 8 h. (*B*) The dating of the embryo tissues is again to the nearest whole day. Confluent, quiescent Swiss 3T3 cells in serum-free medium, untreated (lane *A*) or exposed to: bFGF (10 ng/ml, lane *B*); progesterone (3.15 g/ml, lane *C* and 0.315 g/ml, lane *D*); bFGF + progesterone (10 ng/ml and 3.15 g/ml, respectively, lane *E*) and dexamethasone (3.7 g/ml, lane *F*) for 8 h. Undifferentiated F9 teratocarcinoma-derived stem cells (lane *G*) differentiated into parietal endoderm-like cells and harvested after 5 d (lane *H*). (Lanes *J* and *K*) 10T'/2 cells, as in *A*.

p.c. calvaria and increase progressively through 16.5 to 18.5 d p.c. A similar pattern of increasing levels of transcripts in late gestation is also observed in the kidney, and the placenta. 2ar RNA is also found in decidual tissue derived from 7.5 and 8.5 d p.c. conceptuses. As before, maximal 2ar expression in the embryonic tissues is roughly equivalent to that observed in 3T3 or 10T1/2 fibroblasts exposed to bFGF or ECDGF (Fig. 1 *B*, lanes *B*, *E*, and *K*). 2ar expression did not increase in fibroblasts exposed to nonmitogenic hormones such as progesterone and dexamethasone (Fig. 1 *B*, lanes *C*, *D*, and *F*), and there was no difference in levels between differentiated and undifferentiated F9 embryonal carcinoma cells (Fig. 1 *B*, lanes *G* and *H*).

The filters were then rehybridized with a probe for SPARC, an acidic phosphorylated Ca^{++}-binding glycoprotein identical to osteonectin (Mason et al., 1986*a*, *b*; Engel et al., 1987; Holland et al., 1987). In contrast to the pattern of 2ar expression, SPARC transcripts are detected in virtually all of the tissues examined, the relative levels corresponding to those already reported (Mason et al., 1986*b*; Holland et al., 1987). In particular, they can be seen clearly in quiescent fibroblasts, and exhibit only modest, if any, increases after growth factor stimulation (Fig. 1 *B*, lanes *J* and *K*) while the largest change in expression occurs upon retinoic acid/cyclic AMP induced differentiation of F9 EC cells (Fig. 1 *B*, lanes *G* and *H*, and Mason et al., 1986*b*). The filters were also rehybridized with a control murine glyceraldehyde 3-phosphate dehydrogenase probe to confirm that equivalent amounts of RNA were loaded in each lane (results not shown).

These results show that, in addition to being induced by growth factors in fibroblast cell lines, 2ar is expressed in a temporal and tissue specific manner during normal embryogenesis. Since the positive tissues are made up of many
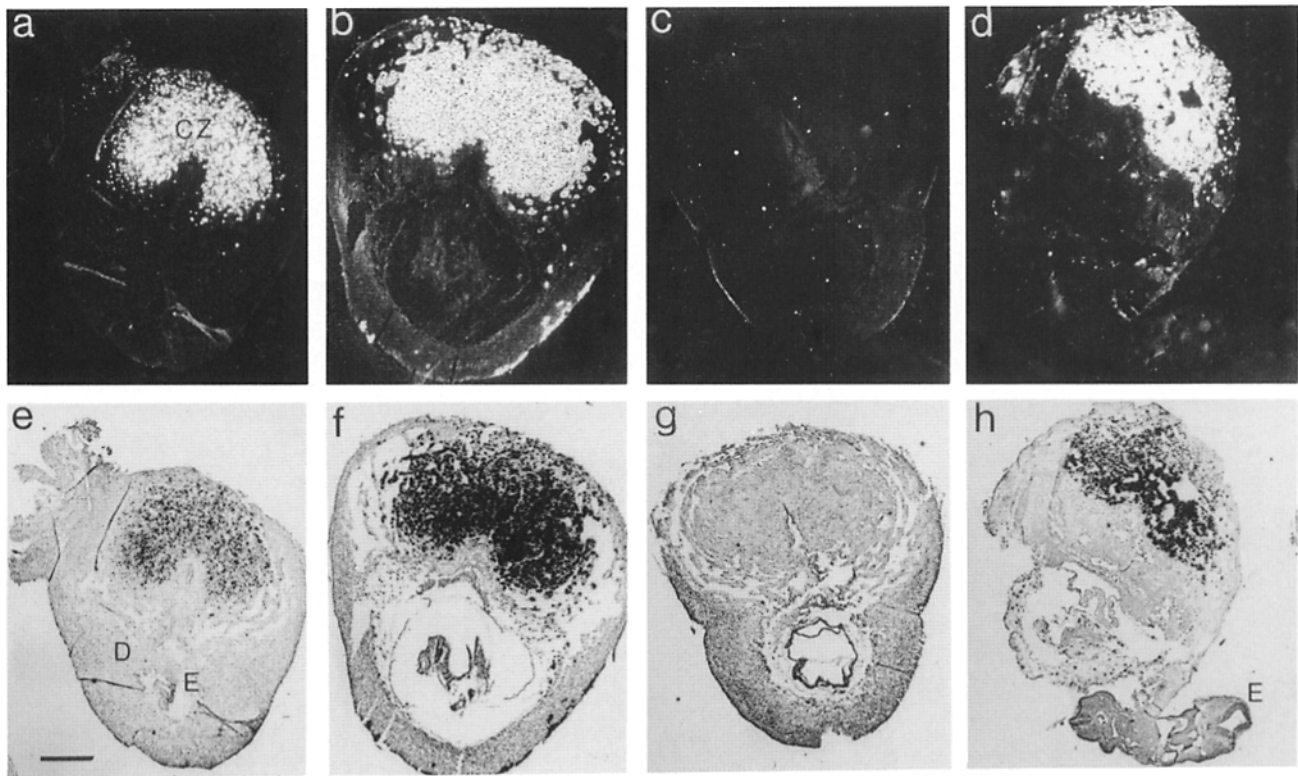
Figure 2. Distribution of 2ar (osteopontin) transcripts in the mouse deciduum. (*a* and *e*) Section through deciduum (*D*) and embryo (*E*) at 7.5 d p.c., hybridized with −ve (antisense) strand 2ar probe. Autoradiographic grains are associated with metrial gland cells in the central zone (*CZ*); (*b* and *f*) as before, but at 9.5 d p.c. The apparent hybridization at the edge of the antimesometrial deciduum is an edge-effect artifact. (*c* and *g*) section of deciduum and embryo at 8.5 d p.c., hybridized with control +ve (sense) strand probe, similar negative results were obtained at 7.5 d through to 10.5 d p.c.; (*d* and *h*) section through 10.5 d p.c. deciduum and embryo (*E*), hybridized with −ve (sense) strand probe. The embryo, which does not hybridize, has popped out of the yolk sac membranes. (*a–d*) Dark ground illumination; (*e–h*) bright field illumination. Sections were exposed to emulsion for 3 d. Bar, 0.5 mm.

Figure 3. Distribution of 2ar/osteopontin transcripts in granulated metrial gland cells in the placenta, and in embryonic bone. Deciduum (*a–e*) (*a*) section through a 14.5 d p.c. placenta hybridized with −ve (antisense) strand 2ar probe and counter stained with toluidine blue. Autoradiographic grains are black. *DB*, decidua basalis. (*b*) Section through the same placenta stained with PAS after digestion of glycogen with diastase. (*c*) High power magnification of a region of the decidua basalis in (*b*) showing granulated metrial gland cells with PAS-positive granules, decidual cells and a maternal blood vessel (*BV*); (*d*) dark ground illumination of an area in the decidua basalis of the 14.5 d p.c. placenta after both in situ hybridization and staining with PAS. Hybridization grains are specifically associated with cells containing PAS-positive granules; (*e*) high power detail under bright field illumination of a metrial gland cell from (*d*) showing PAS-positive granules, and hybridization grains associated with the extensions of the cytoplasm. The plane of focus does not allow all the hybridization grains associated with the cytoplasm to be shown, but has been chosen to illustrate the PAS-positive granules most clearly. Sections were exposed to emulsion for 3 d. (*f–o*) Embryonic bone (*f* and *g*) transverse section through the central region of the humerus of a 14.5 d p.c. embryo hybridized with −ve (antisense) strand 2ar probe. Hybridization grains are present over small, fibroblast-like cells internal to the periosteum and its associated matrix (stained pink with toluidine blue) (*arrows*); (*h* and *i*), adjacent section hybridized with −ve (antisense) strand SPARC probe showing that transcripts are confined to cells in the outer periosteal layer (*arrows*). The yellow/orange fluorescence is an artifact due to the toluidine blue staining; (*f* and *h*) bright field; (*g* and *i*) dark ground illumination; (*j*) longitudinal section of 15.5 d p.c. forelimb hybridized with 2ar −ve (antisense) strand probe. Hybridization grains (*black*) are associated with cells in the newly excavated marrow region (*arrow*); (*k*) nearby section of the same serially sectioned limb hybridized with SPARC −ve (antisense) strand probe. Transcripts are detected in cells in the outer periosteal layer, hypertrophic cartilage, and tendons (*arrows*); (*l*) high power detail of *j* showing absence of 2ar transcripts in the hypertrophic cartilage (*) and positive cells in the central marrow region where endochondrial ossification is being initiated; (*m*) high power detail of *k* showing SPARC transcripts in hypertrophic cartilage (*) and cells in the outer periosteal layer (*arrows*); (*n*) high power detail of alveolar bone in the jaw of a 17.5 d p.c. embryo showing hybridization grains over individual cells closely associated with bone matrix; (*o*) detail of nearby section hybridized with SPARC probe showing hybridization grains over more flattened, elongated cells than in (*n*). Sections were exposed to emulsion for 4 d. Bars, (*a* and *b*) 0.5 mm; (*c* and *d*) 25 µm; (*e*) 12.5 µm; (*f–i, n* and *o*) 20 µm; (*j* and *k*) 0.5 mm; (*l* and *m*) 40 µm.
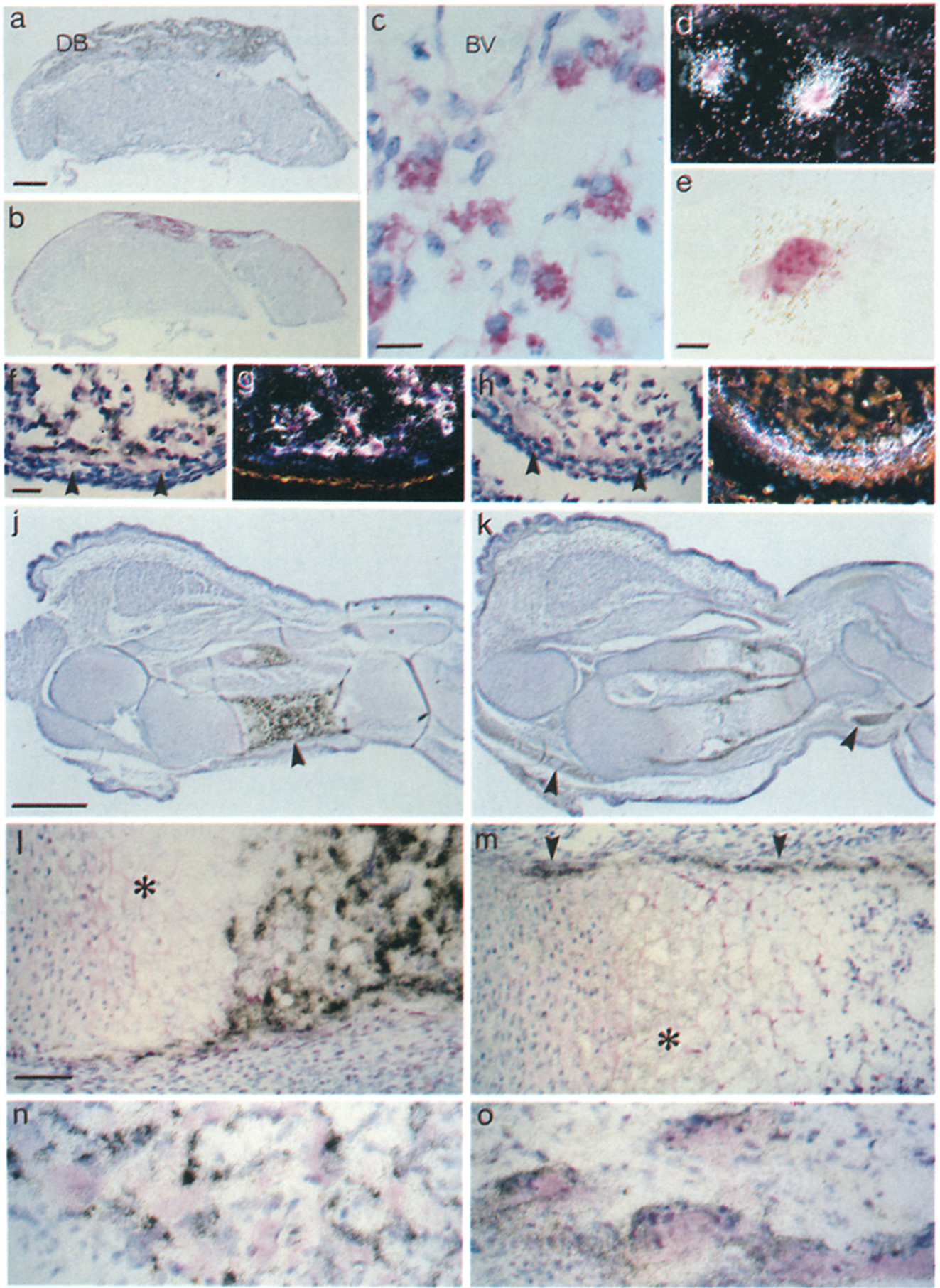
different cell types we undertook a more detailed analysis of this expression using in situ hybridization with ^{35}S-labeled single-stranded RNA probes.

Analysis of 2ar Expression by In Situ Hybridization: Expression in Granulated Metrial Gland Cells in the Deciduum and Placenta

Sections through decidual swellings at different times after implantation were hybridized with 2ar antisense RNA. Fig. 2 shows that transcripts are confined to a distinct population of individual cells, corresponding in size and distribution to granulated metrial gland (GMG) cells of maternal bone marrow origin (Bell, 1983; Bulmer et al., 1987). From serial sections of the 6.5 d p.c. deciduum the number of positive cells was estimated to be ~10[4], localized in the so-called central zone and metrial triangle (Bell, 1983) (data not shown). Between 8.5 and 10.5 d p.c. the number of cells in this location increases, and some can also be seen in more antimesometrial locations, for example adjacent to primary trophoblast giant cells (Fig. 2, b and d). By 14.5 d, the expansion of the embryo has compressed the decidual tissue to a thin outer layer, and the metrial gland is now limited to a "cap" or "decidua basalis" over the placenta (Fig. 3, a and b). Metrial gland cells are characterized by cytoplasmic granules which stain strongly with PAS even after diastase digestion (Bulmer et al., 1987). Fig. 3, a–c shows that the distribution of hybridization grains in the 14.5 d p.c. placenta corresponds to a region containing many cells with intense diastase-resistant PAS staining. Although histology is not well preserved after in situ hybridization, at high magnification it can be seen that autoradiographic grains are associated specifically with cells containing PAS positive granules. This is particularly clear in individual GMG cells lying in maternal blood vessels where their extensive cytoplasmic extensions are not obscured by surrounding decidual cells (Fig. 3, d and e). Control experiments with sense strand RNA show only background levels of hybridization (Fig. 2, c and g). By contrast, SPARC RNA levels are high throughout the deciduum, and in the parietal endoderm (our unpublished observations, and Holland et al., 1987).

There is good evidence that metrial gland cells colonize the uterus from the bone marrow, and can be found in the endometrium in the absence of an embryo (Peel et al., 1983). Fig. 4, a–d shows that 2ar positive cells are present in both the deciduoma elicited in a pseudopregnant female by the intraluminal injection of oil and in the endometrium of the control, uninjected, uterine horn. Interestingly, the uterine epithelium adjacent to the deciduoma is also strongly positive for 2ar expression (Fig. 4, a, b, e, and f), while the epithelium in the contralateral horn is negative (Fig. 4, a and b). 2ar positive metrial gland cells were absent from the endometrium of uteri fixed in either oestrus or dioestrus (data not shown).

2ar Expression in Developing Bone

Fig. 1 A and B shows that the level of 2ar RNA increases significantly in calvaria between 14.5 and 16.5 d p.c., and is also high in backbone at 18.5 d p.c. We therefore used in situ hybridization to investigate 2ar expression during bone development, and compared this with SPARC (osteonectin), a gene which is also expressed at high levels in embryonic bone (Holland et al., 1987).

With respect to the forelimb (humerus, radius, and ulna), 2ar RNA is first detected in more advanced embryos of litters fixed at 14.5 d p.c. The RNA is confined to individual cells in the center of the developing bone, in the region excavated after the vascular bud breaks through the ossification collar. By contrast, SPARC RNA can be detected earlier in the development of the forelimb, at 13.5 d p.c., and is initially localized in a densely packed layer of cells around the outer surface of the shaft of the cartilage model, in a region corresponding to the periosteum of the so-called membranous ossification "collar". This difference in distribution is seen clearly in transverse sections through the central region of 14.5 d p.c. developing bone (Fig. 3, f–i). The 2ar probe hybridizes strongly to fibroblastic-like cells with small, round, nuclei which are internal to the matrix layer beneath the periosteum and not always in contact with it (Fig. 3, f–i). By contrast, at this stage SPARC hybridization is confined to cells in the outer periosteal layer (Fig. 3, h and i). A distinct difference in the distribution of SPARC and 2ar transcripts is also apparent at 15.5 d, in longitudinal sections through the developing limb. High levels of SPARC hybridization grains are seen over the hypertrophic cartilage, as well as in the periosteum of the ossification collar (Fig. 3, k and m). In nearby sections of the same limb hybridized with 2ar probe, only background levels of grains are present over hypertrophic cartilage, but high levels of RNA are detected in numerous cells in the center of the bone where the dead cartilage is being replaced by bone matrix (Fig. 3, j and e).

At later stages of development (e.g., 17.5 d p.c.) SPARC transcripts can be detected in both the periosteum around the bone and in cells on the surface of the internal bone trabeculae (endosteum). The distribution of SPARC hybridization grains suggests that the positive cells are rather flattened and extended (Fig. 3 o). By contrast, the distribution of 2ar hybridization grains suggests that the positive cells have a different morphology, and are more individual and rounded (Fig. 3 n).

2ar in the Embryonic and Newborn Kidney

In the embryonic kidney, 2ar transcripts were detected by Northern analysis at 16.5 d p.c., and at 18.5 d p.c. had increased (Fig. 1 B). By in situ hybridization 2 ar expression could not be detected at 14.5 d p.c., but in the 16.5 d p.c. kidney a small proportion of tubules were strongly positive. It was not possible to identify this subpopulation unambiguously, but S-shaped structures and glomeruli were negative. By 18.5 d p.c. the number of hybridizing tubules had increased (Fig. 5), and by 3 d after birth transcripts can be detected in both proximal and distal tubules and loops of Henle. Glomeruli and collecting ducts are still negative (Fig. 5). In contrast, SPARC expression in the kidney is very low in the tubules, but is high in the glomeruli, which are sites of active basement membrane synthesis (Hogan et al., 1987).

2ar in the Embryonic Ear

Hybridization of sections of both 16.5 and 17.5 d p.c. embryo heads revealed high levels of 2ar expression in localized regions of the developing ear (data not shown). In the cochlea, the distribution of grains is consistent with 2ar expression in cells in the ganglion of the auditory nerve. By contrast, in the vestibular portions of the inner ear, the hybridization grains were localized over the sensory epithe-
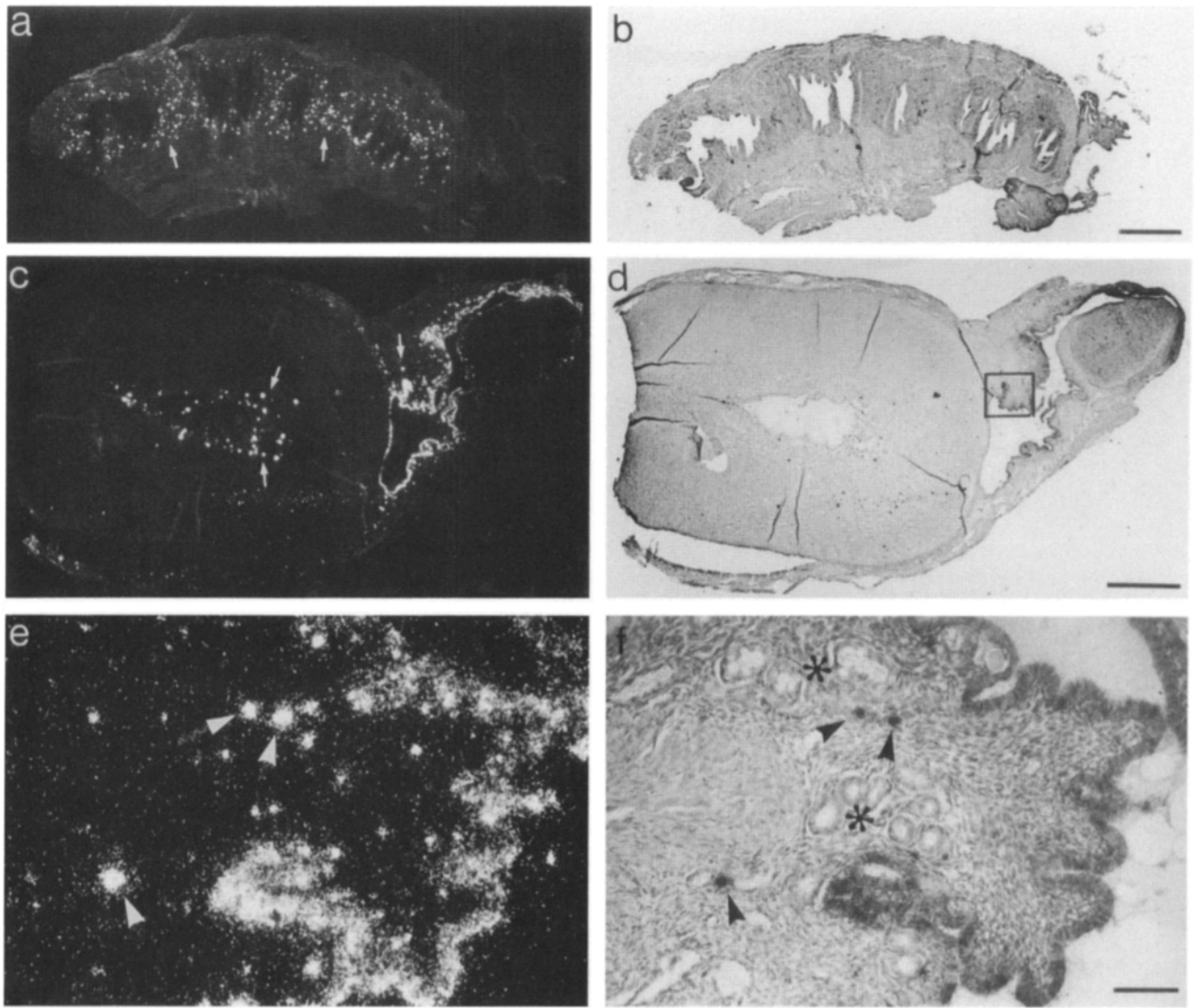
Figure 4. Distribution of 2ar/osteopontin transcripts in the oil-induced deciduoma. Oil was introduced into one horn of the uterus on day four of pseudopregnancy. 5 d later both horns were fixed, sectioned, and processed for in situ hybridization using −ve strand 2ar probe. (*a* and *b*), section through control, uninjected horn showing numerous individual 2ar-positive metrial gland cells in the endometrium (*arrows*) but no hybridization to uterine epithelium or glands; (*c* and *d*), section through oil-induced deciduoma and adjacent uterus. 2ar-positive metrial gland cells are present (*arrows*) in both the deciduoma and the adjacent endometrium; (*e* and *f*) high power magnification of region boxed in *d* showing expression in individual metrial gland cells, the most prominent of which are marked with arrows, and in the uterine epithelium (which has been shed in the deciduoma), but not in the uterine glands (*). (*a, c,* and *e*) Dark ground and *b, d,* and *f* with bright field illumination. 4 d exposure to emulsion. Bars, (*a* and *b*) 0.5 mm; (*c* and *d*) 1 mm; (*e* and *f*) 10 μm.

lium of the uticle and saccule. These epithelia produce an overlying extracellular matrix (the otoconial membrane), embedded in which are calcium-containing crystals.

2ar in Adult Tissues

Sections of a number of adult tissues in addition to the uterus were also hybridized with 2ar probe. Most were negative (e.g., lung, ovary, liver), but small clusters of 2ar positive cells were detected in the adrenal gland of a pregnant female, near the junction of the zona fasciculata and reticularis (data not shown).

Discussion

The experiments described in this paper were designed to ex-

plore the possibility that 2ar, a gene initially cloned on the basis of its inducibility in cultured mouse cells by tumor promoters and growth factors, plays a role in embryonic development. Our observations have revealed that 2ar expression during embryogenesis is confined to a small number of unrelated tissues, for example the metrial gland cells of the deciduum and placenta, kidney tubules, and uterine epithelium, as well as osteogenic tissues such as calvaria, backbone, and long bones. Given the homology between mouse 2ar and rat osteopontin, the expression in developing bone was not unexpected. However, the observation that 2ar transcripts occur in nonmineralizing cell types suggests that the 2ar/osteopontin protein has a wider role than previously realized, and that the original hypothesis of a specific function in attachment of cells to mineralizing bone matrix may have to be reassessed. Similar conclusions have recently been
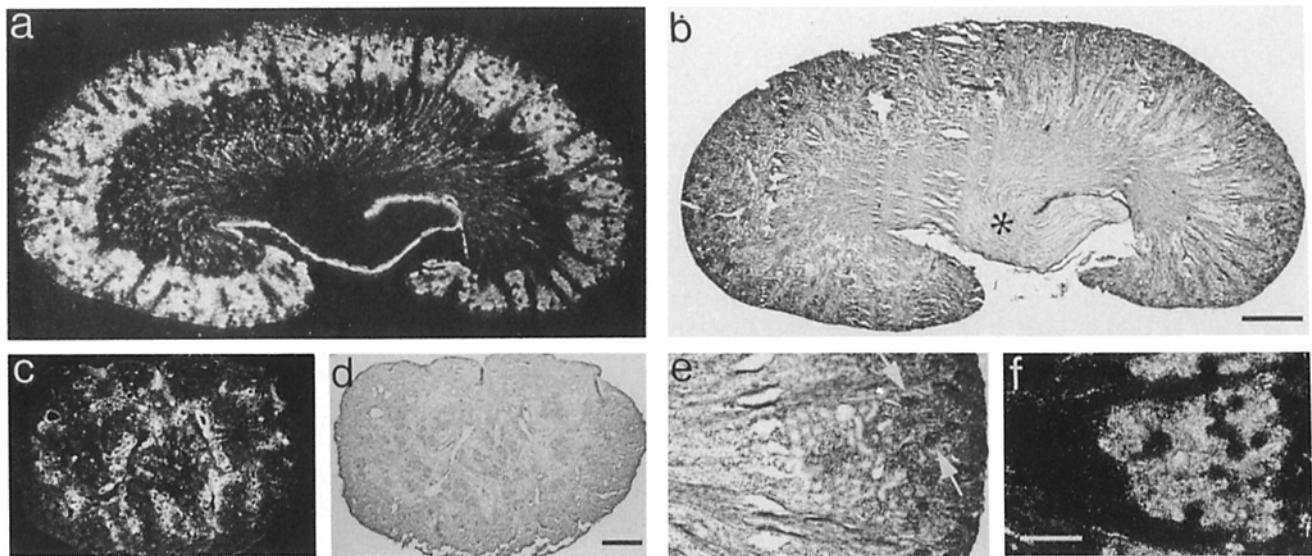
Figure 5. Distribution of 2ar/osteopontin transcripts in newborn and embryonic kidney. (*a* and *b*) Section through a 3-d postnatal kidney hybridized with 2ar −ve (antisense) strand probe. Hybridization is seen over tubules in the cortex which extend into the medulla (loops of Henle). Collecting ducts (*) are negative but show an edge-effect artifact due to the toughness of the outer fibrous sheath not seen in other tissues. (*c* and *d*) Section of an 18.5-d p.c. embryonic kidney hybridized with −ve (antisense) 2ar probe; (*e* and *f*) high magnification detail of cortex region in *a* and *b* to show hybridization in the proximal and distal tubules, while the glomeruli (*arrows*) are negative. *a*, *c*, and *e* Dark ground illumination, and *b*, *d*, and *f* with bright field illumination. Sections exposed to emulsion for 4 d. Bars, (*a* and *b*) 0.5 mm; (*c* and *d*) 0.25 mm; (*e* and *f*) 60-μm.

drawn for the Ca^{++}-binding protein SPARC (osteonectin) which is also present at high levels in nonmineralized tissues as well as developing bone (Holland et al., 1987).

2ar and SPARC Expression during Embryonic Bone Development

During mammalian limb development the cartilage model is replaced by bone in a complex sequence of events which involves both membranous and endochondral ossification (Jee, 1983). These processes are still incompletely understood at both the cellular and molecular level. About the same time as the cartilage in the central region hypertrophies and dies, cells in the densely packed outer sheath, or periosteum, begin to lay down type I collagen in an intramembranous ossification collar. Soon after, cells from the periosteum and associated blood vessels invade the region of dead cartilage, bringing in endothelial cells, precursors to bone marrow stromal cells, and osteogenic and haematopoietic stem cells (Weiss, 1981). Among the latter are precursors to the osteoclasts which decalcify and degrade the cartilage and bone matrix, excavating the central space within which endochondral ossification begins. From our in situ hybridization studies it appears that 2ar is first expressed by cells within the bone after invasion of the vascular bud and, unlike SPARC, is not switched on in cells of the outer periosteal layer and ossification collar. At least some of the 2ar positive cells may be osteoblasts, since these cells have been shown by immunohistochemistry to contain intracellular osteopontin in vivo (Mark et al., 1987). However, our studies on 14.5–15.5 d p.c. embryonic bone (Fig. 3) are also consistent with expression in earlier precursor cells, as suggested by Mark et al. (1987). These may be osteoprogenitor cells and/or progenitors to marrow stromal cells or haematopoietic cells, in-

cluding osteoclasts (Thesingh, 1986). This later idea is supported by the observation that metrial gland cells, which are derived from the haematopoietic lineage, also express high levels of 2ar (see later). A definitive answer to the question of which cells express 2ar must await the availability of further immunological and molecular markers for different bone cell types, and more information about the cell lineage relationships between mesenchymal cells in the periosteal layer, endothelial cells of the vascular bud, osteoblasts and stromal and haematopoietic cells of the bone marrow.

Concerning the expression of SPARC in bone, in situ hybridization clearly shows that expression of this gene is initiated earlier than that of 2ar, and is first detected at 13.5 d p.c., in cells in the periosteum associated with the ossification collar. Again, it is not possible, on the data presented here, to identify these cells unambiguously. They have been assumed to be osteoblasts or osteoblast precursors (Termine et al., 1985; Holland et al., 1987). However, SPARC is known to be expressed at high levels by aortic endothelial cells (Mason et al., 1986a), and the possibility that the positive cells in the periosteum, and later also the endosteum, are members of the endothelial lineage cannot be excluded.

Fig. 3, *k* and *m* also shows high levels of SPARC RNA in hypertrophic cartilage cells, but only background levels in hyaline cartilage, as previously reported (Holland et al., 1987). No significant expression of 2ar RNA is seen in either cartilage phenotype, confirming the results with antibody staining (Mark et al., 1987).

2ar Expression in the Deciduum and Placenta

Implantation of the mouse embryo elicits a dramatic cascade of proliferative and phenotypic changes in the hormone primed uterus (see Finn, 1983 for comparative review). This

results in the formation of a decidual swelling, which is invaded by the embryonic trophoblast cells (Welsh and Enders, 1987). A decidual reaction, or deciduoma, is also elicited in pseudopregnant females by inert substances such as air or oil (Hetherington, 1968). In both cases, the decidual swelling is populated by granulated metrial gland (GMG) cells. Experiments with irradiated mice have shown that these cells are derived from the bone marrow (Peel et al., 1983) but they may undergo local proliferation in the deciduum. In rodents, GMG cells are clustered into a so-called "gland" which gradually becomes compressed into a cap or decidua basalis over the placenta. In humans, GMG cells are more dispersed throughout the endometrium (Finn, 1983). The in situ hybridization results presented here show that GMG cells in the mouse express very high levels of 2ar. Identification of the 2ar positive cells in the deciduum as GMG cells is based on their number and characteristic location, and the presence of diastase-resistant, PAS-positive, cytoplasmic granules. At present there is very little evidence for the function of GMG cells (for review see Bulmer et al., 1987; Bell, 1983). The cytoplasmic granules are thought to contain various hydrolytic enzymes, including acid phosphatase, b-galactosidase, b-glucuronidase, leucine aminopeptidase, and aryl sulphatase (Bulmer, 1968). This suggests that GMG cells play a role in breaking down and remodeling the stroma of the uterus and maternal blood vessels, to allow expansion of the embryo and development of the extensive maternal blood sinuses. An immunological role for GMG cells in the rat has been proposed on the basis of the detection of cytoplasmic IgG, but this has not been seen in mouse cells (Stewart, 1985). The experiments described here open up the possibility of using a wide variety of in situ hybridization probes to throw new light on this previously rather obscure population of cells. In addition, it appears that the number of 2ar positive GMG cells is higher in the deciduum around an embryo than in the oil-induced deciduoma or in control, pseudopregnant endometrium. This suggests that growth factors produced by the embryo may stimulate the migration and/or proliferation of GMG cells.

Speculations Concerning the Function of 2ar/Osteopontin

In this paper we have shown that 2ar is expressed by a number of nonosteogenic cell types. 2ar is encoded by a single gene and in all tissues the probe hybridizes to a single 1.6-kb RNA species (Fig. 1). This suggests that they are producing the same protein, although small differences due to alternative splicing or posttranslational processing, including glycosylation, cannot be excluded. As outlined previously, these results suggest a wider role for 2ar/osteopontin than promotion of osteoblast adhesion during bone development. However, until more is known about the precise in vivo location of cytoplasmic and secreted 2ar protein in cells and tissues such as the metrial gland, uterine epithelium, kidney tubules, and developing ear, as well as the properties of the protein produced by different cell types, ideas about a functional role must be speculative. For example, it is possible that osteopontin binds Ca^{++} (Oldberg et al., 1986). If so, the protein may sequester or compartmentalize Ca^{++} or Ca^{++}-liganded molecules in the matrix or extracellular secretions, or present them in a biologically active configuration to RGD-sensitive receptors on the surface of cells. A

somewhat analogous role has been proposed with respect to haematopoietic growth factors for the glycosaminoglycans of the bone marrow stroma (Gordon et al., 1987).

Earlier studies showed that 2ar mRNA is expressed by a variety of mouse cell lines in vitro and can be induced by exposure of the cells to tumor promotors or growth factors. However, we have not observed expression of 2ar in vivo in fibroblasts, epidermal or mesenchymal cells, a finding supported by the immunocytochemical studies of Mark et al. (1987). This suggests that 2ar expression by these cell types in vivo is in some way constrained, either by the absence of appropriate inducing signals or by additional, unidentified, inhibitory mechanisms which are released upon taking cells into culture. These considerations suggest caution in the extrapolation of 2ar expression by specific cell types in vitro to their counterparts in vivo, and raises the possibility that the expression of 2ar by some cell lines is a response to cell culture, rather than a reflection of their tissue origin.

The relevance of the in vitro regulation of 2ar expression by growth factors to its pattern of expression in vivo is also unclear. The strikingly localized pattern of expression we have observed would imply that, if expression in vivo is entirely controlled by specific growth factors, their availability in vivo must be similarly restricted. It is of interest, therefore, that the non-GMG cells of the rat decidua have been recently reported to express transcripts for the epidermal growth factor-like growth factor, transforming growth factor alpha (Han et al., 1987). An alternative possibility is that 2ar gene expression is also controlled by cell-type specific genetic regulatory elements whose action overrides that of growth factor-inducible regulatory elements. On this model, the former elements would predominate in the control of 2ar expression in vivo and may be inoperative when cells are cultured in vitro.

To resolve these issues, future experiments must be directed towards dissecting the 5' flanking regions of the 2ar gene to determine the location of elements which confer tissue-specific and growth factor inducible expression both in vivo and in vitro.

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