Cell-type-specific expression of epidermal cytokeratin genes during gastrulation of *Xenopus laevis*

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Analysis of the spatial pattern of expression of embryo-specific epidermal cytokeratin genes in *Xenopus laevis* shows earliest activity in the animal pole cells of stage-9 blastulae. These genes are transcribed predominantly in the epithelial or outer ectoderm, to a lesser extent in the sensorial or inner ectoderm, and at low levels if at all in other regions of the embryo. In the early gastrula the entire ectoderm, including preneural and preepidermal regions, expresses cytokeratin mRNAs; accumulation of these mRNAs in preneural cells is terminated after contact is made with involuting chordamesoderm. On the basis of this and earlier work (Sargent et al. 1986) we suggest that the pattern of expression of cytokeratin genes in frog embryogenesis is based on prelocalized components modulated by the inductive influence of involuting chordamesoderm. The cytokeratin proteins are deposited in the form of filamentous networks in both layers of the epidermis. In the epithelial layer, a much denser mesh of filaments is facing the outside of the embryo. This polarity is established at the onset of the polymerization of these filaments. Thus, intraembryonic and intracellular localization of keratin gene expression and protein deposition is established at the onset of activation of these genes.

[Key Words: Keratin; cytokeratin; gastrulation; *Xenopus laevis*; ectoderm; neural induction]

Received December 29, 1986; revised version received and accepted February 3, 1987.

From the transcriptional point of view, amphibian development can be divided into three distinct periods. The first period is devoted to the transcription of maternal RNA and takes place during the many months of oogenesis (Anderson et al. 1982; Smith and Richter 1985; for review, also see Davidson 1986). Fertilization starts the second period, which is characterized by an absence of measurable RNA synthesis. After 7 hr, transcriptional activity is initiated at what is called the midblastula transition (Bachvarova and Davidson 1966; Newport and Kirschner 1982). The onset of transcription results in the apparent reactivation of some of the genes already transcribed during oogenesis as well as in the activation of a set of genes that had not been expressed before (Sargent and Dawid 1983). Shortly after the onset of zygotic transcription, the different cell lineages start to differentiate into morphologically distinguishable tissues. Whereas maternal RNAs are transcribed within one cell, i.e., the oocyte, a switch from uniform to cell-type-specific gene expression must take place to allow development of the multicellular embryo. This cell-type-specific expression could start as early as the midblastula transition, when the zygotic genome is activated, or could coincide with or follow the appearance of morphologically distinct tissues.

In general terms, the appearance of cell-type-specific gene expression can be the result of two distinct though

nonexclusive phenomena: prelocalization of determinants of maternal origin, or induction through cell-cell contacts established during development. Both processes are known to play an important role in amphibian development (see Gerhart 1980; Dawid and Sargent 1986). Two fundamental cell types (ectoderm and endoderm) which give rise to all other cell types, are already specified at the eight-cell stage and their establishment does not require inductive interactions (Nakamura et al. 1971). Mesoderm specification begins in early cleavage and requires inductive interactions between cells of ectoderm and endoderm (Holtfreter and Hamburger 1955; Nieuwkoop 1969, 1973; Nakamura et al. 1970; Gurdon et al. 1985; Jamrich et al. 1985). Differentiation of all other cell types depends on elaboration of and interactions between these three cell types. Ectoderm differentiates into epidermis or neural tissue, depending on whether it comes in contact with chordamesoderm (Vogt 1929; Holtfreter and Hamburger 1955; Keller 1975, 1976). To evaluate the relative role of prelocalization of determinants versus induction, there has been an effort to find tissue- and stage-specific biochemical markers (Ballantine et al. 1979; Bravo and Knowland 1979; Slack 1984, 1985). It has been shown that certain antigens are expressed only in epidermal cells by stage 12 1/2 (Jones 1985; Jones and Woodland 1985; Akers et al. 1986). However, these markers are first activated after gastru-

lation is well underway, and therefore do not provide information about the role of prelocalization versus induction in their initial expression.

In this work, we have used cytokeratin genes as markers for ectodermal differentiation during blastula, gastrula, and neurula stages of *Xenopus laevis*. Our laboratory has isolated and characterized a set of embryo- and tadpole-specific cytokeratin genes from *Xenopus* and has shown that these genes are activated in the late blastula stage (Jonas et al. 1985; Winkles et al. 1985; E. Jonas et al., unpubl.). In this paper, we use in situ hybridization and immunocytochemistry to detect keratins and their mRNAs in the embryo. Keratin gene expression is the earliest cell-type-specific marker available in amphibian development, allowing the study of the mechanism of tissue specification during early ectoderm differentiation.

Results

Expression of cytokeratin RNA in Xenopus tadpoles

Three cDNA clones expressed specifically in embryonic and tadpole stages have been identified by sequencing as encoding epidermal cytokeratins. The structures of these three clones, DG70, DG76, and DG81, have been described previously (Jonas et al. 1985; Winkles et al. 1985; E. Jonas et al., unpubl.). The three genes have an identical spatial expression pattern during development and therefore they were used interchangeably. Figure 1

Figure 1. In situ hybridization of DG81 to sections of stage-40 tadpole. Hybridization is almost exclusively to the epidermis. The ectoderm-derived neural tube (N) does not show any hybridization. (A) Dark-field illumination; (B) bright-field illumination.

shows in situ hybridization of cRNA prepared from clone DG81 to a methacrylate-embedded section of a stage-40 tadpole. This and all subsequent in situ hybridization experiments used albino embryos to avoid confusion of silver grains with pigment granules. Silver grains are located almost exclusively in the ectoderm-derived epidermis, whereas the ectoderm-derived neural tube is negative. Having established the final pattern of expression of these genes, we proceeded to analyze their expression from the time of activation.

Transcription of cytokeratin RNA in Xenopus blastulae

The earliest time at which we detected cytokeratin mRNA was stage 9 (late blastula). The absence of detectable signal at earlier stages indicates that the hybridization seen in older embryos is not due to cytokeratin mRNA of maternal origin. There is at least one keratin mRNA in the egg (Franz and Franke 1986), but its sequence is quite different from DG70, DG76, and DG81, and there is no cross-hybridization. Figure 2A shows a Northern blot analysis of RNA isolated from the animal and vegetal third of 7- to 9-hr-old (stages 9–10) embryos. Hybridization is found in the animal-third RNA; this region gives rise to the ectoderm. Figure 2B shows a Northern blot analysis of RNA isolated from epithelial and sensorial ectoderm of stage-10 embryos; hybridization is predominantly to the epithelial (outer) layer of the ectoderm. This is also clearly visible in Figure 3 which shows in situ hybridization of DG76 probe to a section of stage-10 1/2 ectoderm: The signal is almost exclusively in the epithelial region. At this or any other stage studied there is little or no detectable hybridization to endoderm (not shown).

Expression of cytokeratin mRNA in preneural ectoderm

At the end of blastula development, the ectoderm is radially symmetric; its differentiation into epidermal and neural pathways is initiated during gastrulation. It is generally thought that neural differentiation is induced in otherwise undetermined ectoderm by the involuting chordamesoderm (see Jacobson and Rutishauser 1986). Although the position of the future neural plate is predictable once a dorsal lip forms at the beginning of gastrulation (and from cytoplasmic movements earlier; see Sharf and Gerhart 1983; Vincent et al. 1986), there is no known physical attribute associated with preneural ectodermal cells in the early gastrula. Cytokeratin gene expression was studied during gastrulation to ask at which stage the neural and epidermal lineages diverge with respect to activity of these genes. The sections shown in Figure 4 cut an early gastrula (stage 10 1/2) along its dorsal/ventral midline, i.e., through the center of the dorsal lip. This section is one of a set of serial sections, assuring correct orientation. In situ hybridization shows that the animal pole region, much of which will develop into epidermis, contains keratin mRNA (Fig. 4B). The critical region is shown in Figure 4E: This ectodermal subregion will become neural plate in a few

Figure 2. Localization of cytokeratin mRNA in dissected regions of *Xenopus* embryos. (A) Northern blot analysis of RNA from the animal and vegetal third of 7-, 8-, and 9-hr-old embryos using nick-translated DG70 DNA as a probe. The cytokeratin RNA is present in the animal pole cells from 7 hr (stage 9) on. (B) Northern blot analysis of the RNA extracted from epithelial and sensorial ectoderm of stage-10 embryos using the same probe as in A. The DG70 gene is predominantly expressed in the epithelial ectoderm.

hours' development. Yet, keratin mRNA is present in much of this region (upper or animal pole-proximal half of the segment in Fig. 4E). Transcription of keratin genes has terminated in the progressively enlarging neuroectoderm (lower or vegetal pole-proximal half of the Fig. 4E segment). At any stage of gastrulation, ectoderm in contact with the leading edge of chordamesoderm still contains epidermal cytokeratin mRNA, as it does in Figure 4E. If the preneural ectoderm were already determined

Figure 3. In situ hybridization of DG76 to a section of the blastocoel roof of stage-10 1/2 embryo. The hybridization is to the epithelial layer of ectoderm. Outside of embryo at top and blastocoel cavity at bottom of figure. (A) Dark-field illumination; (B) bright-field illumination.

in terms of transcription of these genes (i.e., did not transcribe them), a region of hybridization-free ectoderm would be seen leading the edge of the chordamesoderm; this is not the case.

During midgastrula (stage 12) epiboly of animal region cells leads to an expanded area of ectoderm around much of the embryo. As seen in Figure 5B, cytokeratin mRNAs are expressed over this entire area. The plane of this section does not cut through the neuroectoderm; in a section that does, the absence of hybridization in the preneural region is observed (Fig. 5E). The difference in cytokeratin expression between the neural and epidermal pathways becomes progressively more obvious during the process of neurulation. This is apparent in Figure 6, which shows a cross section through a stage-14 embryo: The epidermis shows strong hybridization while the neural plate no longer expresses cytokeratin genes. The signal is not present in the neural tissues of any of the subsequent stages.

Localization of cytokeratins in *Xenopus* embryos

To investigate the distribution of translation products of cytokeratin mRNAs we prepared antibodies against a conserved carboxy-terminal peptide of type I cytokeratins. These antibodies recognize most, if not all, type I embryonic cytokeratins and visualize their overall distribution (see Materials and methods). No localized immunoreaction was detected in blastulae before the beginning of transcription of embryonic cytokeratin genes; therefore, no cross-reacting maternal cytokeratins are

Figure 4. In situ hybridization of DG76 to sections of stage-10 1/2 embryos. Boxed areas in A and D indicate the regions enlarged in B, C and E, F, respectively. Dark-field (B) and phase-contrast (C) show hybridization of DG76 to the animal region of a stage-10 1/2 embryo. The entire epithelial ectoderm shows hybridization. Dark-field (E) and phase-contrast (F) show the hybridization to the preneural (dorsal) region. Arrow shows where ectoderm is in contact with the leading edge of chordamesoderm.

present in the early embryo. Figure 7 shows the immunoperoxidase staining of a frozen section of a stage-20 embryo, demonstrating the epidermal localization of the cytokeratins.

Immunofluorescence staining of stage-18 embryos reveals that both layers of the epidermis react with these antibodies, with the outer layer showing much stronger fluorescence (Fig. 8A). This concentration in the outside layer is in agreement with the in situ hybridization results, and suggests that the differences in the abundance of cytokeratins are regulated at the transcriptional level. To investigate the intracellular distribution of keratins further, we dissociated the cells of this layer and stained the individual cells with cytokeratin antibody. As seen in Figure 8C, the cytokeratins are organized in the cell in the form of filaments, forming meshworks of uneven density. The dense meshwork of filaments faces the outside surface of the embryo, and a much reduced density is visible in regions of the cells that have contact with other cells. This polarity is established from the very beginning of filament polymerization (Fig. 8D). Figure 8E presents a view of cytokeratin networks in the epidermis of a stage-12 gastrula as seen from outside of the embryo. The localization of the filaments is reminiscent of the localization of maternal cytokeratin in the egg cortex (Wylie et al. 1985), suggesting the presence of localized determinants that promote cytokeratin polymerization. It is possible that these determinants remain in the same location during early development, much the same way the pigment does, and play a role in the assembly of the embryo-specific cytokeratin fibers.

Discussion

We have followed the accumulation of developmentally regulated epidermal cytokeratin mRNA in *Xenopus* embryos. At the earliest stage at which they can be detected (stage 9) cytokeratin mRNAs are expressed in a cell-type-specific manner. This observation indicates that already in the blastula, before the beginning of gastrulation, different cell types operate according to different transcriptional programs. This is the earliest cell-type-specific transcription reported in amphibian development to date. The cytokeratins are expressed intensely in the entire epithelial ectoderm, much less in the sensorial ectoderm (inner layer of ectoderm or epidermis), and cannot be reliably detected in mesoderm and endoderm. These results may be interpreted to suggest that localized maternal components are responsible for the regulation of epidermal cytokeratin genes. Previous experiments have shown that the activation of these genes is autonomous, i.e., does not require any cell-cell contact (Sargent et al. 1986), a result consistent with the idea of prelocalized components. During gastrulation, the pattern of cytokeratin gene expression is changing. Whereas the entire epithelial ectoderm of the early gastrula is expressing these genes, their transcription in future neural cells is terminated by midgastrula, presumably through inductive interaction with cells of the chordamesoderm. It is worth noting that this inductive effect is a negative one. This result is consistent with a pattern of keratin gene expression based on uneven distribution of maternal components subsequently modified by inductive interactions.

Figure 5. In situ hybridization of DG76 to sections of stage-12 *Xenopus* embryos. Planes of sections of *B*, *C* and *E*, *F* are indicated in *A* and *D*, respectively. Arrows in *A* and *D* indicate the direction of chordamesodermal involution. Dark-field (*B*) and phase-contrast (*C*) show hybridization of DG76 to the extending epidermis; the plane of this section does not cross the neuroectoderm. *E* and *F* show a cross section; note the lack of hybridization in the neuroectoderm. The apparent signal in the chordamesoderm (arrows) is due to trapped air bubbles and not to silver grains.

The results reported here are the first demonstration that the preneural and preepidermal ectoderm initially express some identical genes, which are subsequently inactivated in preneural ectoderm. The potential of epidermal and neural cells to express the same genes has been recognized previously (Akers et al. 1986; Jones and Woodland 1986), using markers that can first be detected by late gastrula. The progressive divergence in gene expression between epidermal and neural ectoderm helps explain the decreasing competence of ectodermal cells to be neutralized or mesodermalized (Chuang 1955; Leikola 1963; Grunz 1968). One could argue that the expression of a certain set of cell-type-specific genes reflects a commitment of these cells to the corresponding

developmental direction. For example, as cells express certain epidermal genes, it might become increasingly more difficult to change their fate. In the case of cytokeratins, this fixation could be due in part to the assembly of intermediate filament structures in the cells.

The mechanism of neural induction is unknown, although a number of substances have been identified that mimic the action of *in vivo* induction (Tiedemann and Tiedemann 1956; Yamada 1958; Faulhaber 1970; Kawakami et al. 1977). Our experiments do not provide further insight into this process except to emphasize the observation that induction is not restricted to the cell layer in contact with chordamesoderm. This follows from the fact that the layer of cells that is expressing the

Figure 6. In situ hybridization of DG76 to a section of stage-14 neurula. Neural plate is at top. The absence of cytokeratin RNA in the neural plate region is obvious. (A) Dark-field microscopy; (B) phase-contrast.

bulk of cytokeratin mRNA, i.e., the outer or epithelial ectoderm, does not come into direct contact with the inducing tissue. The major inductive effect on cytokeratin expression must, therefore, "pass through" the inner ectodermal layer.

During accumulation of cytokeratins and their mRNAs, the protein distribution tends to reflect the distribution of mRNA. Intermediate filaments form and are assembled into intricate meshworks. The polymerization of these filaments starts in the epithelial layer of the epidermis at stage 9 1/2, only slightly later (1–2 hr) than the first appearance of cytokeratin transcripts, indi-

cating that there is no appreciable delay between transcription and translation of cytokeratins. Polymerization of filaments starts in the region of the cell that is facing the outside of the embryo, and this polarity is maintained through development. The filaments in the sensorial layer of the epidermis never reach the density of the outside layer and they are distributed uniformly throughout the cell.

This is the first time that in situ hybridization has been successfully used for the analysis of gene expression in early amphibian embryos. In situ hybridization to later stages of *Xenopus* embryos was performed using frozen sections (Dworkin-Rastl et al. 1986), but due to fragility of sections from early stages, this technology has a limited value for analysis of such material. In situ hybridization to paraffin and methacrylate sections (Jamrich et al. 1984), together with feasibility of analysis of transcription in *Xenopus* oocytes by in situ hybridization to nascent transcripts on lampbrush chromosomes (Jamrich et al. 1983), provides cytogenetic means to study gene expression in this amphibian from oogenesis through all of embryonic development.

Materials and methods

Animals

Adult *Xenopus laevis* were purchased from Nasco (Wisconsin). Albino animals were obtained from Dr. R. Tompkins (New Orleans). Embryos and tadpoles were reared from in vitro-fertilized eggs. Developmental stages were classified according to Nieuwkoop and Faber (1967).

Antibody production

A 14-residue synthetic polypeptide corresponding to the common carboxyl terminus of the proteins encoded by DG81 and DG70 (Winkles et al. 1985) was used to immunize rabbits by the method described by Benoit et al. (1982). The antiserum was shown to react specifically with several type I keratins found in *Xenopus* embryos, including species identified as corresponding to DG70 and DG81 (J.A. Winkles et al., unpubl.).

Figure 7. Immunoperoxidase staining of a frozen section of a stage-25 embryo using cytokeratin antibodies. Staining is limited to the epidermis of the embryo. (A) Immunoperoxidase staining; (B) phase-contrast.

Figure 8. Indirect immunofluorescence of frozen section and dissociated cells with cytokeratin antibodies. Two epidermal cell layers are decorated by these antibodies. The epithelial layer shows a concentration of antibodies in the region facing the outside of the embryo. (A) Immunofluorescence. (B) Phase-contrast. (C) Indirect immunofluorescence of a single dissociated epidermal cell from stage-18 epithelial ectoderm; the epidermal cytokeratins are assembled into a network of filaments with well-defined intracellular polarity. (D) Indirect immunofluorescence of a dissociated cell from stage-11 embryo; the polarity of the filament network is already apparent. (E) Indirect immunofluorescence of partially dissociated epidermis of stage-12 embryos as viewed from outside the embryo.

Cell dissociation

Microdissected ectoderm was placed in Barth media without Ca^{++} and Mg^{++} (Gurdon 1977) and containing 5% of Newport and Kirschner dissociation buffer (Newport and Kirschner 1982). Under these conditions, the epithelial layer of cells will separate from the sensorial cells. The epithelial layer can be picked up as a sheet of cells; the sensorial layer dissociates rather quickly into single cells. We transferred the cells in a drop of media onto a polylysine-coated slide and added a drop or two of 95% ethanol. This generally causes further disruption of the ectodermal layer and the attachment of some cells to the slide. At this point, the slide is immersed in ice-cold 95% ethanol for 10 min. Slides were washed in phosphate-buffered saline (PBS) for 10–20 min prior to immunocytochemistry.

In situ hybridization

Methacrylate embedding is as described in Jamrich et al. (1984) and was used in Figure 1. All other experiments used paraffin embedding as modified from various published procedures (Angerer and Angerer 1981; Akam 1982; Ingham 1985). Albino embryos were used in most experiments, including all figures shown in this paper. Embryos were dejellied in 2% cysteine (pH 7.8) and fixed in 4% paraformaldehyde in PBS for 15–60 min. Embryos were dehydrated through an alcohol series, cleared in xylene, and embedded in paraffin. Five-micrometer sections were cut on an ultramicrotome, and placed on a drop of water on a polylysine-coated slide. When dried, the slides were placed in xylene for 15 min to remove paraffin and rehydrated in an alcohol series prior to hybridization. The probes used were

single-stranded ^3H -labeled cRNAs prepared from M13 clones (Diaz et al. 1981).

Indirect immunofluorescence

Sections and dissociated cells were reacted with anti-cytokeratin antibodies diluted in PBS containing 16% fetal calf serum, 1% bovine serum albumin (BSA), 0.05% Tween 20 for 30 min, washed for 30 min in PBS, reacted with secondary antibodies for 30 min, and washed again with PBS.

Frozen sections

Embryos were frozen in OCT embedding medium by placing the specimen on the metal specimen holder on dry ice. Eight-micrometer sections were cut on a cryostat. Sections were dried and used without further fixation.

RNA extraction and analysis

RNA purification and gel blot hybridization techniques were carried out as described previously (Sargent et al. 1986). In each experiment, RNA from equal numbers of cells (usually 1000) was compared. Cell number was determined by hybridization of alkali-hydrolyzed nucleic acid to a subclone of the 1723 element, a highly repeated sequence present in the genome (Kay and Dawid 1983). If the RNA : DNA ratio of two samples differed by more than a factor of 2, RNA from unfertilized eggs, which does not hybridize with the keratin probes (Sargent and Dawid 1983), was added to equalize the amount of material loaded onto the nylon filter.

Acknowledgments

We wish to thank Erzsebet Jonas and Jeff Winkles for providing us with materials for these experiments, Robert Tompkins for albino frogs, and Kathi Mahon for a critical reading of this manuscript.

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Genes Dev. 1987, 1:

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