Isolation of a chick cytokeratin cDNA clone indicative of regional specialization in early embryonic ectoderm

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Summary

During early vertebrate development, a series of inductive tissue interactions appear to be involved in establishing regional specializations that are eventually elaborated in the basic body plan of the embryo. These early inductive interactions are particularly difficult to study because they often occur in the absence of any associated morphological changes. In the chick embryo, the regional subdivision of the early ectoderm is evidenced by a marked lens-forming bias in the head ectoderm, which is absent from the presumptive dorsal epidermis of the trunk region. This striking divergence in developmental state is present long before any differentiation into lens or epidermal phenotypes can be detected. As a strategy for isolating genes whose differential expression might be a reflection of this regional subdivision, a cDNA library was prepared from early embryos and screened for differential hybridization to radiolabelled probes prepared from head ectoderm and trunk ectoderm. Two related cDNA clones were isolated that hybridize to transcripts present at much higher levels in trunk ectoderm than in head ectoderm. Sequence analysis of one of these clones revealed a high degree of similarity to members of the type II subfamily of intermediate filament cytokeratins. This clone (pCKsel) was used to examine cytokeratin gene expression in ectodermal tissues. A large increase in the level of CKsel transcripts was found to take place in trunk ectoderm, approximately coordinate with neurulation, contrasting sharply with the much lower levels detected in head ectoderm and neural ectoderm at all stages tested. These results indicate that differential cytokeratin gene expression can occur within a contiguous layer of simple embryonic epithelia, and that this expression pattern coincides closely to the subdivision of the early ectoderm into regions with distinct developmental potencies. This type of regulation has not been described previously for members of the cytokeratin gene family.

Key words: chick embryo, regional specialization, cytokeratin, amino acid sequence, RNA.

Introduction

Embryonic development in vertebrates appears to rely extensively on cell and tissue interactions to specify the progressive and orderly establishment of a diverse array of cellular phenotypes (see Jacobson, 1966; Nieuwkoop et al. 1985; Gurdon, 1987; Jacobson and Sater, 1988, for reviews). While the importance of inductive phenomena has been recognized for many years (see Spemann, 1938 for review of early literature), both the nature of the information relayed during such interactions and the underlying mechanisms involved remain poorly understood. This is perhaps due to the enormous complexity of the vertebrate embryo and its relative inaccessability to genetic analysis. Instead, methods of experimental embryology have been employed to document the interactive nature of embryonic tissues in vertebrates. The objective interpretation of such experiments is greatly facilitated when the precise origins of cells in various experimental combinations can be ascertained (for examples, see Gimlich and Cooke, 1983; Jacobson, 1984; Grainger et al. 1988). Likewise, it is useful to have objective criteria for evaluating the phenotypic outcome of a given manipulation. In recent years, highly sensitive molecular assays for cell type-specific differentiation have been successfully exploited for this purpose (e.g. Gurdon et al. 1985; Dale et al. 1985). However, in a number of systems, it appears that induction is a sequential process (reviewed by Wessells, 1977; Jacobson and Sater, 1988). Thus, it is likely that some important developmental decisions occur long before the appearance of terminal differentiation prod-
ucts. This suggests the importance of identifying markers that are associated with events as early as possible in an inductive process.

The induction of the vertebrate lens is a classical system in which extensive evidence implicates the involvement of a sequence of events leading to the elicitation of the lens phenotype (see Henry and Grainger, 1987; Grainger et al. 1988; Jacobson, 1966; Jacobson and Sater, 1988, for reviews). The lens forms by invagination of the head ectoderm overlying the optic vesicle (presumptive retina), an outgrowth of the forebrain that develops in close association with the lens anlage. The optic vesicle appears to play a role in the growth, differentiation, and maintenance of the normal lens phenotype. However, substantial evidence indicates that earlier inductive events play a major role in lens induction, before the optic vesicle has even begun to form (reviewed by Grainger et al. 1988). These early events lead to the establishment of what has been referred to as a ‘lens-forming bias’, a term that reflects the increased ability or propensity of certain regions of ectoderm to participate in lens formation (Karkinen-Jaaskelainen, 1978a; Henry and Grainger, 1987). In the early ectoderm, this property extends beyond the presumptive lens region to encompass a large area of head ectoderm (see Barabanov and Fedtsova, 1982; Henry and Grainger, 1987). The action of the optic vesicle appears only to be effective on ectoderm in which a ‘lens-forming bias’ is already present (Grainger et al. 1988); the optic vesicle may serve to help localize the response to the presumptive lens ectoderm, while the lens-forming bias is eventually lost from other regions of head ectoderm.

The lens constitutes a distinct morphological structure, and a number of marker proteins (crystallins; see Piatigorsky, 1981 for review) are produced at very high levels. However, these phenotypic characteristics of the differentiated lens appear much later in development than the lens-forming bias (e.g. compare Shinohara and Piatigorsky, 1976, to Barabanov and Fedtsova, 1982), which appears to be established in the absence of any overt differentiation. In order to characterize further the important early changes that are occurring in the early ectoderm, we felt it would be desirable to obtain markers capable of recognizing events that precede the onset of lens differentiation. In the present report, we describe our strategy for identifying such a marker and report the cloning of a chick cytotkeratin cDNA that permits the recognition of an early molecular distinction between head ectoderm (possessing the lens-forming bias) and body ectoderm which apparently has not been exposed to early aspects of lens induction.

Materials and methods

Embryos and tissue fragments
Fertile White Leghorn chicken eggs (Truslow Farms, Chester-town, MD) were incubated at 38°C, 60% relative humidity, to reach the desired stages (all stages are according to Hamburger and Hamilton, 1951). Embryos were placed in phosphate-buffered saline (PBS), pH 7.4, for staging and removal from the peripheral tissues of the zona pellucida. For the isolation of ectodermal fragments, embryos were first incubated on ice in 2.25 % trypsin (Gibco), 0.75 % pancreatic (Difco) in PBS. Incubation times were 40–60 min for anterior head ectoderm and 15–30 min for trunk ectoderm. Embryos were then transferred to 20 % chick serum (Gibco; heat inactivated at 56°C) in PBS and ectodermal fragments were dissected within 15 min before healing could occur. At stage 10 (10–12 somites), the anterior head ectoderm (used for screening and for Northern blot analysis) consisted of virtually all the ectoderm overlying the optic vesicle and prosencephalon, except for a small region along the dorsal midline that was more difficult to remove. Trunk ectoderm was defined at stages 8–16 as the dorsal ectoderm overlying the somites and lateral plate mesoderm, on either side of the neural tube (or plate) ectoderm. Presumptive trunk ectoderm was collected from stage 4 (definitive streak stage) embryos based on previously published fate mapping data (see Rudnick, 1948; Rosenquist, 1966). This tissue was isolated from the lateral portion of the zona pellucida, at the mid-streak level. Tissue fragments (or whole stage 10 embryos, dissected in PBS without trypsinization for cDNA library construction) were pooled in PBS until at least 50 or more were collected, rinsed in PBS, quickly frozen in liquid nitrogen and stored at -70°C for up to 12 months before use in RNA isolations.

cDNA library construction
RNA was prepared by thawing whole stage 10 (10–12 somite) embryos in homogenization buffer (10 mM-Tris–HCl, pH 7.5, 100 mM-NaCl, 0.25 M sucrose, 5 mM-MgCl2, 0.5 % NP-40, 10 mM-panadyl ribonucleoside complex [BRL]), followed by homogenization in 20 volumes of this buffer at 4°C using a dounce homogenizer with a loose-fitting pestle. The homogenate was immediately centrifuged at 10000 g for 5 min at 4°C. The supernatant was then adjusted to 25 mM-EDTA, 1 % SDS and extracted repeatedly with phenol/chloroform/isoamyl alcohol (25:24:1), and the RNA was precipitated with ethanol. Poly(A)+ RNA was selected by two binding and elution cycles using oligo d(T)-cellulose (type III, Collaborative Research), essentially as described by Maniatis et al. (1982). cDNA was prepared using AMV reverse transcriptase as described by Schwarzbauer et al. (1983), and rendered double-stranded by treatment with RNase H and E. coli DNA disintegrase I (Gubler and Hoffman, 1983). EcoRI sites were double-stranded by treatment with RNase H and E. coli DNA disintegrase I (Gubler and Hoffman, 1983). EcoRI sites were methylated using EcoRI methylase, and the double-stranded cDNA was then made blunt using the Klenow fragment of DNA polymerase and subsequently ligated to EcoRI linkers. The sample was digested with EcoRI and cDNA was separated from linkers by chromatography on Biogel A50m (BioRad) as described (Huynh et al. 1985). Size-selected cDNA (>250bp) was ligated to λgt 10 arms (Huynh et al. 1985), and packaged using a commercial packaging extract (Stratagene). The complexity of the library prior to amplification was estimated to be 3X10⁶ independent recombinants; inserts visualized by agarose gel electrophoresis and ethidium bromide staining ranged from approximately 0.5–4 kilobases. Hybridization to cloned probes indicated that approximately 5 % of the clones correspond to either 28S or 18S ribosomal RNA (rRNA).

RNA isolation from ectodermal tissues
Approximately 100–200 ectodermal fragments were thawed in 200 μl of 4.2 mM-guanidine thiocyanate, 0.5 % sarkosyl, 25 mM-Tris–HCl, pH 8.0, 100 mM-2-mercaptopethanol, and homogenized by vortexing and pipetting up and down several times. The homogenate was added to 200 μl of 0.1 M-Tris–HCl, pH 8.0, 10 mM-EDTA, 1 % SDS, and the mixture
was extracted several times with phenol/chloroform/isoamyl alcohol (25:24:1) followed by a final extraction with chloroform/isoamyl alcohol (24:1). After addition of 10 μg glycogen (molecular biology grade, Boehringer–Mannheim), nucleic acids were precipitated with an equal volume of isopropanol at −20°C. For differential screening (see below), DNA was removed by digestion with 100 units ml−1 DNase (RQI DNase, Promega). Typically, 2.5–5.0 μg of total RNA were obtained per 100 ectodermal fragments.

**Differential screening**

Because of the small amounts of RNA obtainable from stage 10 head ectoderm and trunk ectoderm, [32P]cDNA was prepared by reverse transcription of total RNA templates using oligo (dT) as primer. The reaction mixture contained 4 μg of total RNA template, 50 mM-Tris-HCl, pH8.3, 140 mM-MgCl₂, 5 mM-dithiothreitol, 25 μM dT oligo d(T)₁₂₋₁₈, 1 mM-d(GAT)₆TP, 5 μM-dCTP, 1.67 μM-[32P]dCTP (10 μl of 10 mCi/ml−1 at 3000 Ci/mmol−1; New England Nuclear), and 500–1000 units ml−1 of AMV reverse transcriptase (Life Sciences), in a volume of 21 μl. Approximately 7–25×10⁶ cts min⁻¹ of [32P] cDNA per microgram of input total RNA was obtained using this method; it is likely that a significant fraction of such probes was derived from reverse transcription of rRNA templates. Duplicate filters of the stage 10 cDNA library were hybridized to equivalent concentrations of head ectoderm and trunk ectoderm cDNA probes. Radiolabelled cDNA (2×10⁶ cts min⁻¹ per 100 mm filter) was hybridized in 2 ml per filter of 50% formamide, 1 M-NaCl, 1% SDS, 10% dextran sulfate, and 250 μg ml⁻¹ sheared, denatured salmon sperm DNA at 42°C for approximately 16 h. Filters were washed to 67°C in 2×SSC, 1% SDS, followed by 0.1× SSC at room temperature. Exposures of 2–3 days with X-Omat AR film (Kodak) at −70°C with intensifying screens were generally sufficient to visualize any plaques that hybridized to an extent significantly above background. After plaque purification, the differentially expressed cDNA clones (see text) containing a 2.3 kb insert was subcloned into 

**RNA sequencing**

DNA was not removed from the samples prior to densitometric quantitation or electrophoresis. We have found that the concentration of nucleic acid obtained from the absorbance (A₂₆₀) values was divided in half. This method proved to be a reliable indicator of the relative amounts of total RNA in dissected tissue samples. In all cases, gels were stained with ethidium bromide and the intensity of rRNA staining was compared to known amounts of more carefully quantitated rRNA. Stained gels were photographed prior to transfer, and both gel and filter were checked after blotting to ascertain that all rRNA had been transferred. Because it is important that lanes to be compared have similar amounts of total RNA, negatives of ethidium bromide-stained gels were also scanned using a Bioimge Visage 2000 computerized image analysis system, to confirm the relative amounts of rRNA in the various lanes. In all cases but one (Fig. 5, lane 6) for the purposes of such comparisons, the scanning data agreed within a factor of two with the value predicted by absorbance. In Fig. 5, lane 6 the absorbance estimate was 2.5-fold greater than that determined by scanning. Small differences among lanes were therefore not significant relative to the dramatic differences seen in CKsel gene expression, for example between the very high level of CKsel RNA in stage 8–15 trunk ectoderm and the much lower levels seen in head ectoderm and neural ectoderm (see Results).

[32P]RNA (antisense) probes were synthesized using XbaI-linearized pCKsel as template, and T7 RNA polymerase (Promega) to produce probes that span the EcoRI insert of the cDNA clone. RNA blots were hybridized for 12–16 h at 62°C in 50% formamide, 5×SSC, 0.1% SDS, 0.05% ficoll, 0.05% polyvinylpyrrolidone, 100 μg ml⁻¹ salmon sperm DNA, and 1–5×10⁶ cts min⁻¹ ml⁻¹ of radioactive probe. Two 5 min washes at room temperature were followed by six washes (30 min each) in 0.1× SSC at 67°C. All exposures were carried out using X-Omat AR film at −70°C with intensifying screens. Selected autoradiograms were scanned on a Gildford multimedia densitometer and signals quantified with a Perkin–Elmer LCI-100 integrator.

**In situ hybridization**

In situ hybridization was carried out based on the method of Cox et al. (1984). Embryos were fixed in 4% paraformaldehyde (in PBS) at 4°C for 12–24 h, dehydrated in ethanol and embedded in paraaffin. 8 μm sections were dried on slides, paraffin was removed and sections were treated with proteinase K and acetic anhydride prior to hybridization. [35S]RNA (antisense) probes spanning the pCKsel insert were prepared as for Northern blots (see above) except that [α-thio][35S]UTP was substituted for [α32P]dCTP, and probes were subjected to limited alkaline hydrolysis. Hybridization was carried out at 65°C in 50% formamide, 0.3 m-NaCl, 20 mM-Tris–HCl (pH 8.0), 5 mM-EDTA, 0.02% bovine serum albumin, 0.02% ficoll, 0.02% polyvinylpyrrolidone, 10% dextran sulfate, 10 mM-dithiothreitol, and 1.3×10⁷ cts min⁻¹ ml⁻¹ of radioactive probe for 16 h. Slides were washed in 4× SSC, 10 mM-dithiothreitol at room temperature and treated with RNase A (20 μg ml⁻¹) at 37°C for 30 min. Slides were then washed in 50% formamide, 2× SSC at 50°C for 45 min followed by 0.1× SSC at 50°C for 45 min. Dried slides were dipped in Kodak NTB2 emulsion and exposed for approximately 3 days. Control sections were hybridized under identical conditions to a sense probe prepared from the 3’ PurI fragment of a chick δ-crystallin cDNA clone (pOCr-2; Bhat and Piatigorsky, 1979). No specific pattern of hybridization to this control probe was observed; only a very low and uniform level of signal could be seen throughout these sections (Charlebois, 1988).

**Results**

**Screening strategy and cDNA clone isolation**

To identify markers indicative of early regional specialization within the ectoderm, we screened for differen-
The regional establishment of the lens-forming bias can be detected (+) and the region of trunk ectoderm in which the lens-forming bias is absent (−). Data concerning the lens-forming bias reflect the results of Karkinen-Jaaskelainen (1978a); Barabanov and Fedtsova (1982) and unpublished observations from our laboratory. Structures abbreviated at right in the illustration: ov, optic vesicle; pie, presumptive lens ectoderm (overlying the optic vesicle); nlhe, nonlens head ectoderm; som, somite; te, trunk ectoderm (overlying the somite and flanking lateral plate regions).

In contrast, body ectoderm from the dorsal surface overlying the somites always fails to yield a lens-forming response in isolated explants (Karkinen-Jaaskelainen, 1978a; Barabanov and Fedtsova, 1982; R.M. Grainger et al. in preparation). In these studies, it has been shown that in the stage 10 embryo (10–12 somites; see Fig. 1) presumptive lens ectoderm, which overlies the optic vesicles, has the capacity to form morphologically distinguishable lentoids and synthesize crystallin proteins (the major lens proteins) in vitro. In contrast, body ectoderm from the dorsal surface overlying the somites always fails to yield a lens-forming response in isolated explants (Karkinen-Jaaskelainen, 1978a; Barabanov and Fedtsova, 1982; R.M. Grainger et al. in preparation). The ability to differentiate into lens under these conditions is not limited to the true lens precursor cells, however. A large region of head ectoderm, outside the presumptive lens region, can also exhibit the lens phenotype when cultured in isolation (Barabanov and Fedtsova, 1982). This is consistent with the data in amphibians, where the initial establishment of the lens-forming bias appears to extend to a large region of nonlens head ectoderm (Henry and Grainger, 1987; Grainger and Mannion, in preparation).

We reasoned that the functional distinction apparent in the stage 10 ectoderm might be reflected in the profiles of gene expression in the head and trunk ectoderm, and therefore initiated a search for genes which are preferentially expressed in either region. A cDNA library was constructed from stage 10 whole embryos, and differential screening of the library was performed using probes prepared from dissected fragments of head ectoderm and trunk ectoderm. Approximately 30000 clones were screened, and two clones were identified based on clear differential representation in the stage 10 ectoderm. These clones appear to be homologous based on hybridization tests; one of these clones, containing an EcoRI insert of approximately 2.3 kb, was selected for further characterization.

Examination of the RNA gel blot analysis shown in Fig. 2 reveals that this cDNA clone hybridizes strongly to a 2.9 kb RNA in trunk ectoderm, which is present at much lower levels in head ectoderm. Quantitation of densitometric scans indicates a difference of at least 50-fold in the levels of this RNA between these regions of ectoderm. Additional lower molecular weight bands were sometimes observed in dissected tissue fragments as well (see also Fig. 5); these bands were only detected in samples also exhibiting high level expression of the 2.9 kb transcript. We do not yet know if these transcripts are degradation products, alternate products of the same gene, or the products of closely related genes. The isolation of ectodermal tissues required treatment in a crude trypsin/pancreatin preparation, and (because of their very small size) fragments from many embryos were pooled before being frozen, a period of at least several hours during which some degradation could have occurred. In RNA preparations of whole stage 10 embryos, which were collected much more quickly and were not subjected to trypsin/pancreatin treatment, only the 2.9 kb band was detected under identical hybridization and wash conditions (Fig. 2). This result indicates that in stage 10 embryos, the 2.9 kb RNA is by far the most predominant species detected by our cloned probe (see Discussion). In any case, it is clear that the cDNA probe isolated by our differential screening procedure demonstrates that an overt molecular distinction exists between the head ectoderm and trunk ectoderm of the stage 10 embryo.

Fig. 3 shows the nucleotide sequence and deduced amino acid sequence of the cloned cDNA isolated as described above. The EcoRI fragment contained in this clone is 2280 nucleotides in length, as compared with an approximate length of 2.9 kb observed for the major RNA transcript on agarose/formaldehyde gels. Analysis of this sequence reveals a major open reading frame which extends for 390 amino acids (nucleotides 1–1170) before reaching a translation termination signal. The remaining sequence of approximately 1.1 kb appears to
4.5 X 10^6 cts min^{-1} ml^{-1} of radioactive probe, and exposed 22 h (see Materials and methods); whole embryo lane was shown). Abbreviations: head ect, head ectoderm (1.5 μg); trunk ect, trunk ectoderm overlying the somite region (1.5 μg); whole emb, whole embryo (0.6 μg). Two separate experiments are shown: head ectoderm and trunk ectoderm lanes were hybridized to 2.5 X 10^6 cts min^{-1} ml^{-1} of radioactive probe, and the autoradiogram was exposed for 22 h (see Materials and methods); whole embryo lane was hybridized under identical conditions with 4.5 X 10^6 cts min^{-1} ml^{-1} of radioactive probe, and exposed for 65 h.

**Fig. 2.** Detection of differential CKse1 RNA levels in stage 10 ectoderm. Total RNA was electrophoresed on a 1.2% agarose/6% formaldehyde gel, blotted to nylon membrane, and hybridized to antisense [32P]RNA spanning the insert of plasmid subclone pCKse1. Horizontal bars at left indicate the migration of 28S (upper band) and 18S (lower band) rRNAs. The major hybridizing band migrates at approximately 2.9 kb as determined by comparison to a ladder of RNA size standards in a separate experiment (not shown). Abbreviations: head ect, head ectoderm (1.5 μg); trunk ect, trunk ectoderm overlying the somite region (1.5 μg); whole emb, whole embryo (0.6 μg). Two separate experiments are shown: head ectoderm and trunk ectoderm lanes were hybridized to 2.5 X 10^6 cts min^{-1} ml^{-1} of radioactive probe, and the autoradiogram was exposed for 22 h (see Materials and methods); whole embryo lane was hybridized under identical conditions with 4.5 X 10^6 cts min^{-1} ml^{-1} of radioactive probe, and exposed for 65 h.

Considerable sequence similarity (about 30%) is present among different classes of IF subunits; this similarity is found primarily in the rod domains of the various IFs (Steinert and Parry, 1985). The cytokeratins can be subdivided into two subtypes on the basis of charge and sequence similarity; individual members of the type I (more acidic) and type II (more basic) subfamilies exhibit a much higher degree of sequence similarity (greater than 50% identity) with each other than with those of the other (type I or type II) subfamily, or with other types of IF (reviewed by Fuchs et al. 1987; Quinlan et al. 1985). Within the conserved, central rod domain, the chick cytokeratin is approximately 75% identical at the level of amino acid sequence to a number of type II cytokeratins. These include the simple epithelial cytokeratins corresponding to cytokeratins no. 7 (HK7; Glass et al. 1985; Glass and Fuchs, 1988) and 8 (HK8; Leube et al. 1988) of the human catalog (Moll et al. 1982), as well as bovine (Magin et al. 1986), mouse (referred to as endo A; Morita et al. 1988; Semat et al. 1988) and Xenopus (Franz and Franke, 1986) counterparts of HK8. In addition, human 56k type II cytokeratins (HK6a, Hanukoglu and Fuchs, 1983; HK6b, Tyner et al. 1985) present in epidermis under conditions of hyperproliferation (Weiss et al. 1984) also share approximately 75% identity in this highly conserved region.

In Fig. 4, the deduced amino acid sequence of the chick cytokeratin is shown in alignment with several of the cytokeratins that are most closely related. It is evident that our cDNA clone contains all of the conserved, rod domain of the protein, but is apparently lacking the amino terminus as well as any homologous 5' untranslated regions. The degree of sequence identity is high in the rod domains of each of these type II cytokeratins, making it difficult to ascertain the precise relationship of the chick cytokeratin to these cytokeratins based on sequence similarity in this region. While the nonhelical 'tail' region at the C-terminus is generally more divergent (Fuchs et al. 1987), it has been observed that within this region, several segments exist where significant cross-species homology can be detected (Hoffman et al. 1985; Morita et al. 1988). In particular, the region immediately following the transition from the central (α-helical) rod domain to the nonhelical 'tail' (designated T1 by Morita et al. 1988) was seen to be highly conserved among mammalian equivalents of human cytokeratin no. 8 (HK8), and partially conserved with the equivalent cytokeratin in Xenopus (Morita et al. 1988). As a group the HK8 equivalents of these various species were observed to be much more similar to one another in this region than to epidermal cytokeratins within their respective species (Morita et al. 1988).

Examination of the C-terminal segments aligned in Fig. 4 indicates that the chick cytokeratin is more similar to HK7 of human simple epithelia in this region than to any of the other related type II cytokeratins. In the T1 region of the tail (see Fig. 4) the chick sequence is identical at 19 of 25 positions (76%) to HK7. In contrast, the chick cytokeratin exhibits striking diver-
Fig. 3. Nucleotide sequence and deduced amino acid sequence of chick embryo cDNA clone pCKsel. The nucleotide sequence is numbered in the lefthand margin. The polyadenylation signal sequence is underlined, and the translation of HK8 (see Fig. 4). Similar results are seen in comparisons with the corresponding human, bovine, and frog equivalents in this region. For example, only 8 of 25 amino acids (32%) are identical between the chick and 52 % identity with HK6 (shown in Fig. 4; Tyner et al. 1989). This segment is not seen in the chick embryo cDNA we have cloned, and is also absent from the human cytokeratins HK5 (Lersch and Fuchs, 1988), HK6 (Hanukoglu and Fuchs, 1983; Tyner et al. 1989). The above data suggest that among the type II cytokeratins expressed in embryonic and simple epithelia (for reviews, see Quinlan et al. 1985, and O’Guin et al. 1986; mouse, Semat et al. 1988 and Morita et al. 1986; bovine, Magin et al. 1985), the second conserved segment (Morita et al. 1988; Giordano et al. 1989; underlined in Fig. 4) is present just before the C-terminus of cytokeratin 8 in all cases so far examined (human, Leube et al. 1986; bovine, Magin et al. 1986; mouse, Semat et al. 1988 and Morita et al. 1988; frog, Franz and Franke, 1986; goldfish, Giordano et al. 1985). In this regard, it should also be noted that a second conserved segment (Morita et al. 1988; Giordano et al. 1989; underlined in Fig. 4) is present just before the C-terminus of cytokeratin 8 in all cases so far examined (human, Leube et al. 1986; bovine, Magin et al. 1986; mouse, Semat et al. 1988 and Morita et al. 1988; frog, Franz and Franke, 1986; goldfish, Giordano et al. 1985). This segment is not seen in the chick embryo cDNA we have cloned, and is also absent from the human cytokeratins HK5 (Lersch and Fuchs, 1988), HK6 (Hanukoglu and Fuchs, 1983; Tyner et al. 1985), and HK7 (Glass et al. 1985). The above data suggest that among the type II cytokeratins expressed in embryonic and simple epithelia (for reviews, see Quinlan et al. 1985, and O’Guin
Cytokeratin gene expression in chick embryo

Fig. 4. Comparison of amino acid sequence deduced from clone pCKsel with several closely related cytokeratins: HK7, human cytokeratin 7 (Glass et al. 1985); endo A, mouse endo A (murine equivalent of human cytokeratin 8; Morita et al. 1988; Semat et al. 1988); HK6b, human cytokeratin 6 (Tyner et al. 1985). Gaps (denoted by \[\cdot\]) have been introduced to obtain maximum identity among the sequences. A dash (—) at a given position indicates amino acid identity with the chick cytokeratin CKsel. Solid overlines indicate regions of \(\alpha\)-helical rod predicted by Morita et al. (1988) for endo A to be used as a reference indicating the portion of these cytokeratins which overlap with clone pCKsel. The open box indicates the 'T1' segment (Morita et al. 1988) of the C-terminal tail, where CKsel is most closely related to HK7 (see text). The underlined segment at the C-terminus of the endo A sequence is highly conserved among all K8 cytokeratins, but is absent from HK6, HK7, and CKsel.

et al. 1987), the chick cytokeratin represented by our cDNA clone is most closely related to cytokeratin no. 7. However, in view of our current lack of knowledge concerning the cytokeratin gene family in the chick (see Discussion), we have chosen to designate the putative cytokeratin encoded by our cDNA clone as chick CKsel. This designation reflects the observation that CKsel transcripts represent a major component of type II cytokeratin gene activity in simple embryonic epithelia in the chick.

Cytokeratin gene expression during development of the ectoderm

To evaluate the temporal establishment of differential expression in the ectoderm, we performed Northern blots on dissected fragments of ectoderm from a series of stages of developing embryos (Fig. 5). At the primitive streak stage (stage 4), we isolated fragments of epiblast lateral to the streak which, based upon previous fate mapping studies (Rudnick, 1948; Rosenquist, 1966), includes the presumptive trunk ectoderm. As shown in Fig. 5, the levels of CKsel expression detected at this stage (lane 1) are much lower than those seen in the stage 10 trunk ectoderm (lane 3). We believe that even this reduced level is likely to be an overestimate of actual CKsel expression in the presumptive trunk ectoderm, due to probable contamination with presumptive extraembryonic (cytokeratin positive) cells at the outer margin of the area pellucida (see Discussion).

Soon after the definitive streak stage (stage 4), the process of neurulation occurs in ectoderm, leading to the formation of a morphologically distinguishable neural plate. By stage 8, the early trunk ectoderm can be identified as overlying the developing somites, and is readily separable from the thickened neural folds. As seen in Fig. 5 (lane 2), the CKsel RNA level is substantially increased in stage 8 trunk ectoderm relative to that prior to neurulation. CKsel gene expression increases further during the subsequent development of trunk ectoderm (Fig. 5, lanes 3–5). By day 6 of embryonic development, the earliest indications of feather differentiation are detectable in the dorsal epidermis (Wessells, 1965; Haake et al. 1984). When we looked for cytokeratin transcripts homologous to our CKsel probe after this point (stage 31–32, 7 days of development), it was observed that the levels were greatly reduced (Fig. 5, lane 6).

Relative to the trunk ectoderm, the ectoderm of the lens anlage appears to express greatly reduced levels of CKsel RNA. To test CKsel expression in very early presumptive lens ectoderm, we isolated tissue frag-
Fig. 5. Detection of cytokeratin RNA in ectoderm of epidermal (lanes 1–6) and nonepidermal (lanes 7–9) ectoderm at various stages of chick development. Total RNA (amounts given below in parentheses) were electrophoresed on 1.2% agarose/6% formaldehyde gels, transferred to nylon membrane and hybridized to antisense [32P]pRNA spanning the insert of clone pCKsel. Lane 1, stage 4 presumptive trunk ectoderm (2 μg); lane 2, stage 8 trunk ectoderm (1 μg); lane 3, stage 10 trunk ectoderm (0.5 μg); lane 4, stage 13 trunk ectoderm (1 μg); lane 5, stage 15–16 trunk ectoderm (1 μg); lane 6, stage 31.5 dorsal epidermis (1 μg); lane 7, stage 4 presumptive lens and adjacent head ectoderm (0.5 μg); lane 8, stage 15–16 lens (0.5 μg); lane 9, stage 10 neural tube (1 μg). Two separate experiments using the same hybridization and wash conditions (see Materials and methods) are represented: (1) lanes 1–4, 1 x 10^6 cts min^{-1} ml^{-1} of probe was applied, and autoradiographic exposure was for 48h, and (2) lanes 5–9, 1.3 x 10^6 cts min^{-1} ml^{-1} of probe was applied, and exposure was for 45h. Arrow indicates the major hybridizing band which migrates at 2.9 kb. Additional lower molecular weight RNA species seen in lane 3 migrated at approximately 1.7 kb.

To assess the regional embryonic distribution of CKse1 transcripts in more detail, in situ hybridization of antisense CKse1 transcripts to tissue sections of stage 10 embryos was performed (Fig. 6). In close agreement with the Northern blot analysis shown above, the sagittal section shown in Fig. 6 demonstrates the striking differential expression in the ectoderm. Very strong hybridization signal is derived from the trunk ectoderm along the length of the body axis. This signal abruptly diminishes at a point anterior to the somites. The presumptive lens ectoderm, as well as a large region of head ectoderm posterior to the optic vesicles, shows greatly reduced hybridization. The signal in this head ectoderm is indistinguishable from the background level obtained in control sections which were hybridized to a sense probe prepared from a chick α-crystallin cDNA clone (see Materials and methods).

It is also evident from Fig. 6 that transcripts detected by our CKse1 probe are not restricted to the trunk ectoderm but are extensively represented in endoderm as well as in regions of mesoderm. In addition to the strongly hybridizing heart tissue seen in the sections shown in Fig. 6, strong hybridization could be observed in the mesoderm in more lateral sections. Additional characterization of CKse1 expression patterns in the various germ layers will be presented elsewhere (Charlebois et al. 1989; Henry et al. in preparation). With respect to the spatial and temporal expression of
Cytokeratin gene expression in chick embryo

Fig. 6. *In situ* hybridization of antisense CKsel RNA to a sagittal section of a stage 10 embryo. Anterior of the embryo is at left, and the dorsal surface is at the top of the figure. Phase contrast (A) and darkfield illumination (B) of the same section, which was cut lateral to the midline. Strong hybridization is seen to ectoderm in the trunk region, but not to head ectoderm extending anterior to the somites. High levels of cytokeratin RNA are also seen in the endoderm and in heart mesoderm in the plane of this section. Abbreviations: ov, optic vesicle; he, head ectoderm; te, trunk ectoderm; som, somite; ht, heart; en, endoderm. The bar represents 200 μm.

CKsel in the ectoderm, these studies have corroborated the conclusions reached in the present work by Northern blot analysis.

**Discussion**

The divergent developmental states of early head ectoderm and trunk ectoderm were originally detected in a biological assay designed to evaluate the lens-forming capabilities of these regions. These studies supported a model suggesting that a large region of head ectoderm gains a lens-forming bias by virtue of inductive interactions during early development. The success of the cloning strategy described here provides the first evidence that this early biological difference is reflected by distinct patterns of genetic activity in these regions of ectoderm. In addition, the finding that the cDNA clone isolated on the basis of this differential expression corresponds to a cytokeratin directs attention to a striking type of regulation that has not been described previously for members of this gene family. In the discussion below, we first address the identity and expression characteristics of this cytokeratin in relation to the growing body of knowledge concerning the cytokeratin gene family, and then consider the implications of our findings in the context of the regionalization events that take place in the early ectoderm.

**Cytokeratin gene expression in early ectoderm**

Among the various types of intermediate (8-10 nm) filaments, the cytokeratins, which are expressed in epithelial cells and their derivatives, are by far the largest and most diverse class (reviews by Fuchs *et al.* 1987; Quinlan *et al.* 1985). Two distinct subfamilies of cytokeratins can be distinguished on the basis of their amino acid sequences: the generally smaller and relatively acidic type I cytokeratins, and the larger and more basic type II class. At least one member of each
subfamily must be expressed in a given epithelial cell, in order to permit the formation of the heterotypic tetrameric subunit of the IF (Fuchs et al. 1987; Quinlan et al. 1985). Expression studies have indicated that specific type I and type II cytokeratins are frequently co-expressed in pairs in a highly regulated manner from one cell type to another (reviewed by O'Guin et al. 1987). It is not understood why the cytokeratin pattern varies dramatically in different epithelial cell types, but examination of the specific cytokeratin profile can provide useful information concerning the type and degree of differentiation in a particular epithelium (O'Guin et al. 1987).

Members of the cytokeratin gene family have been found to be among the earliest genes to be differentially expressed during development (see Jamrich et al. 1987; Duprey et al. 1985; Page, 1989). The pattern of cytokeratin gene expression appears initially to be very simple, due to the less diverse and less differentiated state of epithelia in the early embryo (reviewed by O'Guin et al. 1987). The predominant cytokeratins of human simple (one-layered) epithelia are the type II cytokeratins 7 and 8, and their expression partners, type I cytokeratins 18 and 19 (Moll et al. 1982; Quinlan et al. 1985). The simple epithelial cytokeratins, in particular the cytokeratin pair 8/18, are prominent in the simple epithelia of early embryos (Brulet et al. 1980; Brulet and Jacob, 1982; Jackson et al. 1980, 1981; Kemler et al. 1981; Oshima et al. 1983; Singer et al. 1985). The cytokeratin pattern becomes more complex with the formation of complex, stratified and pseudo-stratified epithelia (Fouquet et al. 1988). The cDNA clone (CKsel) described in the present study is the first cytokeratin clone that has been isolated from the chick embryo, and the evidence indicates that it represents a major component of cytokeratin gene expression in simple embryonic epithelia.

We have used the CKsel probe to examine cytokeratin gene expression in early embryonic tissues. As shown in Fig. 2, the 2.9 kb RNA band represents by far the most prominent band detected by this probe in early embryos. This band has been seen in abundance on Northern blots in which microdissected tissue from each germ layer (i.e. ectoderm, mesoderm and endoderm) was tested separately for hybridization to the CKsel probe (Charlebois, 1988). However, it is not yet known how many cytokeratin genes there are in the chick or if closely related type II cytokeratins are expressed in early development. The probe used in the expression studies described here contained sequences encoding the conserved, a-helical portion of the protein. Thus, although high stringency hybridization and wash conditions were employed, we cannot entirely rule out the possibility that the CKsel probe detected transcripts derived from several genes. This might explain the occasional detection of lower molecular weight species in some tissue samples (e.g. see Fig. 5). For example, if other cytokeratin RNAs are present in only a small proportion of the cells of the embryo (i.e. specific regions of ectoderm which would be highly enriched in dissected samples relative to whole embryos), such weakly cross-reactive species might escape detection in whole embryo RNA. If this explanation were correct, these genes would be of interest because these minor bands are also differentially represented in the early embryo, apparently always expressed in cells also exhibiting high levels of the 2.9 kb band. As there is precedent for several closely related genes encoding highly similar cytokeratins (see for example Tyner et al. 1985; Miyatani et al. 1986), further studies will be required to characterize the cytokeratin gene family in the chick and the transcripts derived from its individual members. The chick cytokeratin gene isolated in the present study can now be used as a starting point to consider the extent to which different cytokeratin genes are expressed during development.

The CKsel sequence deduced from our chick cDNA clone shows a high degree of sequence similarity to previously identified mammalian and amphibian cytokeratins. Since the clone isolated in the current study was identified because it is expressed in a simple embryonic epithelium (ectoderm), it was not surprising to find that it was most closely related to a simple epithelial cytokeratin, i.e. HK7. However, since no information is yet available concerning the cytokeratin gene family in the chick, it seems premature to classify our cDNA clone as encoding the chick equivalent of HK7. Specifically, we do not yet know whether there are distinct cytokeratins corresponding to HK7 and HK8 in the chick. In several previous studies of avian development, the profile of cytokeratin immunoreactivity was explored using antibodies that specifically recognize cytokeratins of human simple epithelia (Erickson et al. 1987; Page, 1989). Monoclonal antibodies that recognize the human cytokeratin pair 8/18 (Erickson et al. 1987), cytokeratin 8 alone or cytokera-
tins 7 and 8 (both used by Page, 1989), have been used to detect cytokeratins in various epithelia during early quail and chick development. Whether the putative protein product of the CKsel gene corresponds to the cytokeratin(s) identified by these antibody reagents has not yet been determined.

Certain aspects of the expression data presented here are consistent with previous reports of K8 immunoreactiv-
ity (Erickson et al. 1987; Page, 1989), although clear differences have also been observed which could be due to the cDNA (CKsel) and antibody probes recognizing the products of different cytokeratin genes. In the epiblast at the primitive streak stage, the region containing the presumptive trunk ectoderm was found to exhibit a much lower level of CKsel RNA than does the trunk ectoderm at later stages. The immunohistochemical data of Page (1989) indicate that most or all K8 gene expression in the epiblast is restricted to the area opaca and outer margins of the area pellucida, which are destined to form extraembryonic tissue (Rosenquist, 1966; Wolk and Eyal-Giladi, 1977). We have detected a similar pattern of CKsel hybridization in situ in early embryos (in preparation). The dissected fragment of stage 4 epiblast used for the presumptive trunk ecto-

derm RNA shown in Fig. 5, lane 1 is likely to have included a small proportion of this presumptive extra-
embryonic ectoderm, and thus could account for most or all of the hybridization signal in this lane. Our Northern blot data indicate a substantial increase in CKs1 RNA levels in presumptive trunk ectoderm during the period between stage 4 and stage 8. Page (1989) found that significant K8 immunoreactivity in the body ectoderm is first observed at stages 5–7, i.e. during neurulation. In contrast to the high level expression observed in trunk ectoderm, neither CKs1 RNA (this study) nor CK8 immunoreactivity (Page, 1989; also Erickson et al., 1987, in quail embryos) can be detected in neural ectoderm. Apparently, coordinate with the formation of neural ectoderm, there is a large increase in cytokeratin gene expression in trunk ectoderm, which is destined to form epidermis. Interestingly, others have reported the appearance of ‘epidermal’ markers in the nonneural ectoderm at the late gastrula/early neurula stage in amphibian embryos (Akers et al., 1986; Slack, 1984; Jones and Woodland, 1986), some of which are cytokeratin polypeptides (Slack, 1984).

Previous studies of cytokeratin gene expression in avian embryos (Erickson et al., 1987; Page, 1989) have not, however, reported differential expression, as we have shown here, between head and trunk ectoderm. It is not clear whether the head ectoderm was included in these previous analyses, or if so whether the antibodies used by these investigators did in fact detect cytokeratin in the region of head ectoderm where we observed drastically reduced CKs1 hybridization. If this were the case, it may be that the antibodies used in these studies recognize a different cytokeratin, which is not differentially expressed in the ectoderm, than that encoded by CKs1. Alternately, it is at least theoretically possible that there is not a perfect correspondence between RNA and protein levels for the same cytokera-
tin. For example, protein levels and immunoreactivity could remain significant for a period of time after CK8 transcription was repressed (i.e. in head ectoderm), or after the half-life of newly synthesized CKs1 transcripts was greatly reduced.

It is noteworthy that head ectoderm and trunk ectoderm, which are morphologically indistinguishable regions of a contiguous sheet of ectoderm, exhibit differential patterns of cytokeratin gene expression. It has been pointed out elsewhere (see Erickson et al., 1987; Page, 1989) that it is difficult to relate cytokeratin patterns to various aspects of early embryonic organization. For example, the differential distribution of cytokeratins does not appear to be characteristic of either the germ line derivation or cellular organization (e.g. epithelium or mesenchyme) of a particular tissue. Thus, the finding that CKs1 expression is regulated within the ectoderm suggests that rather than relating to overt cellular organization, cytokeratin gene expression in some cases reflects more subtle events associated with the establishment of divergent developmental pathways. In the discussion below, we focus on how these data bear on our current understanding of the establishment of early regional specializations in the ectoderm.

Early regional specialization in embryonic ectoderm

CKs1 RNA serves as a marker that allows for the direct assessment of a regional specialization event in early ectoderm, prior to any morphological evidence that this event has occurred. By monitoring CKs1 gene expression, it is therefore possible to gain some insight into the spatial and temporal characteristics associated with the establishment of diversity in early ectoderm. This information provides a critical framework for devising experiments (in which CKs1 should be an excellent marker) to evaluate the cellular and molecular mechanisms involved in directing tissues onto particular developmental courses.

The regional distribution of cytokeratin RNA within the stage 10 ectoderm has important implications both for the interpretation of previous work on lens induction and in the design of future experiments. In the chick, the ‘lens-forming bias’ is manifest in the ability of isolated explants of head ectoderm to form lens-like structures and synthesize large amounts of crystallin protein in vitro, a property that sharply distinguishes it from trunk ectoderm, which appears to have no lens-forming bias when cultured in isolation (Karkinen-Jaaskelainen, 1978a; Barabanov and Fedtsova, 1982; our unpublished observations). This difference could be due to the exposure of head ectoderm to inductive influences that do not extend far enough posteriorly to affect the trunk ectoderm. This issue has been explored in experiments testing the ability of the optic vesicle to elicit a lens-forming response in post-neurula (stage 8–11) trunk ectoderm (Karkinen-Jaaskelainen, 1978a,b; Grainger et al. in preparation). While a positive response was reported in some optic vesicle/trunk ectoderm combinations (Karkinen-Jaaskelainen, 1978a,b), the results of many such experiments in our laboratory have convinced us that the capacity of trunkectoderm to exhibit aspects of lens differentiation under these conditions is quite low (Grainger et al. in preparation). These data argue that the region-specific lens-forming properties of chick ectoderm are similar to those in Xenopus, where we have observed: (1) a lens-forming bias exists in a large region of head ectoderm (Grainger and Mannion, in preparation); (2) the ability of trunk ectoderm to participate in lens formation becomes restricted during neurulation (Henry and Grainger, 1987). We have shown in the present study that, with respect to cytokeratin gene expression, the stage 10 trunk ectoderm has clearly become differentiated from the head ectoderm. Additionally, the fact that cytokeratin RNA levels increase dramatically in trunk ectoderm at the time of neurulation raises the possibility that activation of cytokeratin gene expression may signify a restriction in the lens-forming potential of the ectoderm. In order to test the action of putative lens inductors on trunk ectoderm which has not begun to overtly diverge from head ectoderm, it therefore would appear necessary to select ectoderm from a relatively early stage, prior to the large increase in CKs1 gene expression that occurs during neurula-

It is likely that inductive interactions specify the
development of other placodal structures, such as nose and ear, in the head ectoderm at approximately the same developmental stage that the lens-forming bias is acquired in this region (Jacobson, 1966; Jacobson and Sater, 1988). This leads us to consider more generally the implications of our results in terms of the hierarchy of decisions that occur in the ectoderm. The earliest overt morphological distinction that takes place in the development of other placodal structures, such as nose and ear, in the head ectoderm at approximately the same developmental stage that the lens-forming bias is acquired in this region (Jacobson, 1966; Jacobson and Sater, 1988). This leads us to consider more generally the implications of our results in terms of the hierarchy of decisions that occur in the ectoderm. The earliest overt morphological distinction that takes place in the ectoderm appears to be its subdivision into neural and nonneural components. It would appear that an early regionalization event within the nonneural ectoderm delineates a placodal (head) region possessing developmental potencies (see Jacobson and Sater, 1988) and a molecular phenotype (this study) that are distinct from the rest of the body ectoderm.

It has been noted previously that an early step in both neural (see London et al. 1988) and mesodermal (Symes et al. 1988) induction seems to involve the suppression of epidermal differentiation, and we now suggest that a similar event occurs during the establishment of the placodal ectoderm. Such a phenomenon could reflect an early event in a multistep induction process, and might be required to establish or maintain competence of the placodal ectoderm to respond to subsequent inductive stimuli which are involved in specifying the appropriate structures such as lens, nose and ear. A similar mechanism could also be important in very early neural and mesodermal inductions, both of which appear to be processes involving several steps (London et al. 1988; Symes et al. 1988).

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