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Epidermal development in *Xenopus laevis*: the definition of a monoclonal antibody to an epidermal marker

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SUMMARY

A monoclonal antibody 2F7.C7 which identifies an epidermal antigen common to both anuran and urodele amphibia is described. This antigen has a high molecular weight and is expressed at stage $12\frac{1}{2}$ first inside and then on the surface of epidermal cells. It is not expressed on presumptive nervous tissue. It is expressed on all cells of the larval epidermis including ciliated and non-ciliated cells and sucker. The relationship of the epidermal antigen to 'epimucin' is investigated by blocking experiments using Peanut agglutinin. These studies suggest that either the molecule recognized by 2F7.C7 is not epimucin, or that its epitope is far away from the lectin-binding site. Studies using this antibody in grafting and sandwich experiments are discussed and an overall scheme of epidermal development in *Xenopus* is presented.

INTRODUCTION

Although the amphibian embryo has been at the centre of attention of developmental biologists for about 100 years, little is known about the basic mechanisms of developmental determination of the three embryonic germ layers, the ectoderm, the mesoderm and the endoderm. Explant isolation experiments clearly suggest that the ectoderm and the endoderm are already specified at the 8-cell stage (Nakamura, Takasaki, Okumoto & Iida 1971). The isolated vegetal half of the embryo when cultivated in a neutral environment forms gut, notochord, muscle and blood cells, whereas the isolated animal half of the embryo is highly pigmented and develops into atypical epidermis which contains ciliated cells, epidermal cells, adhesive gland cells and sometimes neuroepithelium. Mesoderm specification is thought to occur later, at the 64-cell stage, and arises from an inductive interaction between the cells of the ectoderm and endoderm (Holtfreter & Hamburger, 1955; Nieuwkoop, 1969, 1973; Nakamura, Takasaki & Mizohata 1970). Subsequent development of the early embryo depends on sequential interactions between these three embryonic germ layers. The ectoderm and endoderm are therefore the fundamental cell types on which the whole process of early development is based.

Key words: monoclonal antibody, epidermis, epimucin, specification, *Xenopus laevis*

Ectodermal differentiation has been studied in a variety of ways. The normal fate map of the early amphibian gastrula, established through vital stain experiments, shows that the ectodermal cells become either epidermis or neural plate prior to the differentiation of the sensory organs and other epidermal structures (Vogt, 1929; Keller, 1975, 1976). The general importance of the morphogenetic movements which take place during gastrulation, in the future organization of the embryo, have been illustrated in embryos that have been prevented from gastrulating by placing them in hypertonic medium (Holtfreter & Hamburger, 1955). The ectoderm of the resulting exogastrulae develops into a ciliated bag of atypical epidermis in an analogous fashion to isolated animal pole explants from amphibian blastulae. This demonstrates the role of the tissues underlying the ectoderm in the normal embryo in stimulating the further differentiation of the ectoderm into nervous system, sense organs and other epidermal structures. In the neutral environment of an ectoderm explant or exogastrula, however, the ectoderm develops into a mass of relatively undifferentiated epidermal cells.

In recent years there has been increasing efforts to find biochemical markers which are temporally or regionally restricted in early amphibian development (Ballantine, Woodland & Sturgess 1979; Bravo & Knowland, 1979; Slack, 1984a). Slack (1984a, 1984b) has demonstrated that epidermis of normal embryos and ectodermal explants synthesize a number of epidermis-specific proteins including the cytokeratins and a major glycoprotein, epimucin, with a native M_r of 470×10^3 and a subunit M_r of 170×10^3. This glycoprotein has been shown to be the main epidermal receptor for Peanut agglutinin in *Ambystoma* (Slack, 1985). It has been hoped that the definition of such markers will help to identify the intermediate steps in the specification and determination of embryonic tissues which, before their identification, could only be recognized by their terminally differentiated products.

This paper reviews the description of a monoclonal antibody, 2F7.C7, which identifies an antigen common to both urodele and anuran amphibia, which is first expressed within the cells of the prospective epidermis, but not the neural plate, in stage-$12\frac{1}{2}$ embryos. This antigen is subsequently expressed on the epidermal surface of the developing larva, but not elsewhere. This antibody has been used to study the specification and determination of the epidermis (Jones & Woodland, 1985) and the role of cell interaction and cell division in epidermal differentiation (Woodland & Jones, 1985). The possible relationship between the antigen identified by immunoprecipitation with 2F7.C7 and epimucin is investigated and discussed.

METHODS

Fixation, embedding and antibody staining

Xenopus laevis and *Xenopus borealis* embryos were obtained from either natural matings or artificial fertilizations. *Ambystoma mexicanum* embryos were a gift from Dr. Jonathan Slack, and were obtained from a natural mating. All embryos were fixed after staging (Nieuwkoop & Faber, 1967; Bordzilovskaya & Detlaff, 1979) in

2% TCA for > 1 h at 4 °C. Embryos were then transferred into embedding acrylamide following the method described by Dreyer, Singer & Hausen (1981). Equilibrated embryos were finally set into acrylamide blocks and frozen by plunging into isopentane cooled to − 140 °C over liquid nitrogen. Blocks were stored at −20 °C.

Sections were cut at 10 μm thickness on a Jung Frigocut and collected on 0·1 % gelatin-subbed slides. The slides were then air dried and postfixed in acetone for 10 minutes at room temperature. Sections were then drawn around with a marker pen and either used immediately or stored at −20 °C.

Sections of embryos were incubated in 1 % BSA/PBS for 30 minutes at room temperature in a damp box. This was then removed by suction and replaced by either 50 μl of monoclonal antibody supernatant or PBS and the slides incubated for 30 minutes. Slides were then washed by immersing in PBS for 15 minutes with three changes. 50 μl of FITC-conjugated rabbit anti-mouse IgG (FITC-RAMIgG, Miles) at 1/50 dilution was added to each section, and the slides incubated for 30 minutes at room temperature. Slides were then washed in PBS for 15 minutes with three changes and mounted in 50:50 PBS: glycerol. Slides were scored using a Standard Zeiss microscope fitted with epifluorescence and recorded on Tri-X-pan film.

Peanut agglutinin (PNA) blocking studies were carried out by incubating sections with 20 μg PNA (Vector Laboratories) prior to labelling with either 2F7.C7 monoclonal supernatant and rhodamine RAM IgG as described previously, or 25 μl of FITC-conjugated PNA (Vector Laboratories) at 100 $\mu g ml^{-1}$. Fluorescence was scored and recorded as before.

Iodogen labelling and immunoprecipitation

20 ectodermal explants were made from dejellied stage-9 *Xenopus laevis* embryos. The explants were incubated until control embryos reached stage 19. ^{125}I-labelling of explants was carried out essentially as described by (Tuszynski, Knight, Kornecki & Srivastava (1983)). 1 mg of IODO-GEN (Pierce Chemical Co.) was dissolved in 1 ml chloroform in a glass tube and the chloroform evaporated under pressure. The iodogen-coated tube was filled with 0·5 ml Barths saline (88 mM-NaCl, 1 mM-KCl, 24 mM-$NaHCO_3$, 15 mM-Tris, 0·33 mM-$Ca(NO_3)_2$, 0·41 mM-$CaCl_2.2H_2O$ and 0·82 mM-$MgSO_4.7H_2O$), and the ectodermal explants + 30 $\mu Ci^{125}I$ added. The mixture was incubated for 30 minutes at room temperature with occasional shaking. The explants were then washed five times in 10 ml volumes of Barths saline and homogenized in 300 μl detergent buffer (Valle, Besley & Colman, 1981). The homogenate was spun for 10 minutes in an Eppendorf centrifuge and the supernatant removed and stored at −20 °C. 50 μl aliquots of this extract were then immunoprecipitated following the method described in Valle *et al.* (1981) either with 50 μl 2F7.C7 five times concentrated monoclonal supernatant or with five times concentrated NS1 supernatant from the parent myeloma. A duplicate 2F7.C7 immunoprecipitated tube was then digested with 1 μg V8 protease (Cleveland, Fischer, Kirschner & Laemmli, 1974) prior to running on at 12 % polyacrylamide gel (Laemlli, 1970). Gel electrophoresis was carried out as described in Jones & Rughani (1984).

RESULTS

An antibody 2F7.C7 has been identified from a monoclonal antibody fusion between NS1 and Balb/c spleen cells immunized with TX114 soluble membrane proteins (Bordier, 1981) from *Xenopus laevis* embryos. This antibody reacts with all three amphibians tested: *Xenopus laevis*, *X. borealis* and *Ambystoma mexicanum*. The antigen recognized by this antibody is not expressed prior to stage $12\frac{1}{2}$. As development proceeds the antigen is expressed very strongly on the epidermis of the developing embryo including non-ciliated epidermis, ciliated epidermis and sucker (Fig. 1). It is expressed on a small minority of epidermal cells and in the rectum of the adult.

The antigen can first be detected within the presumptive epidermis of a stage-$12\frac{1}{2}$ embryo (Fig. 2A,B). Soon after this the majority of the antigen is found on the outermost wall of the epidermis and little, if any, expression in the inner sensorial

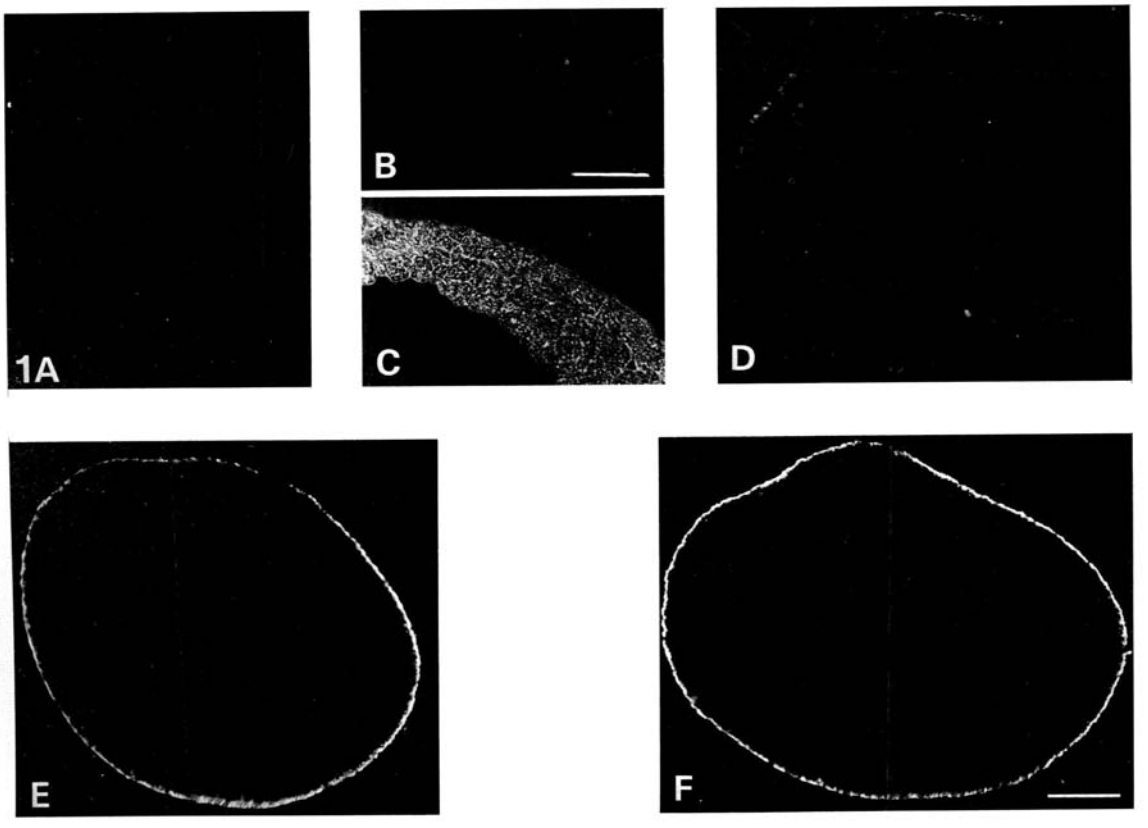

Fig. 1 Developmental expression of 2F7.C7 on *X. borealis* embryos Cryostat sections of different stages of development stained with 2F7.C7 and FITC conjugated Rabbit anti mouse IgG. (A) Stage-11 gastrula (B/C) High-power stage-11 gastrula (D) Stage $12\frac{1}{2}$ (E) Stage 15 neurula (F) Stage 19 neurula. Bar = 180 μm (A, D, E, F); bar = 75 μm (B, C)

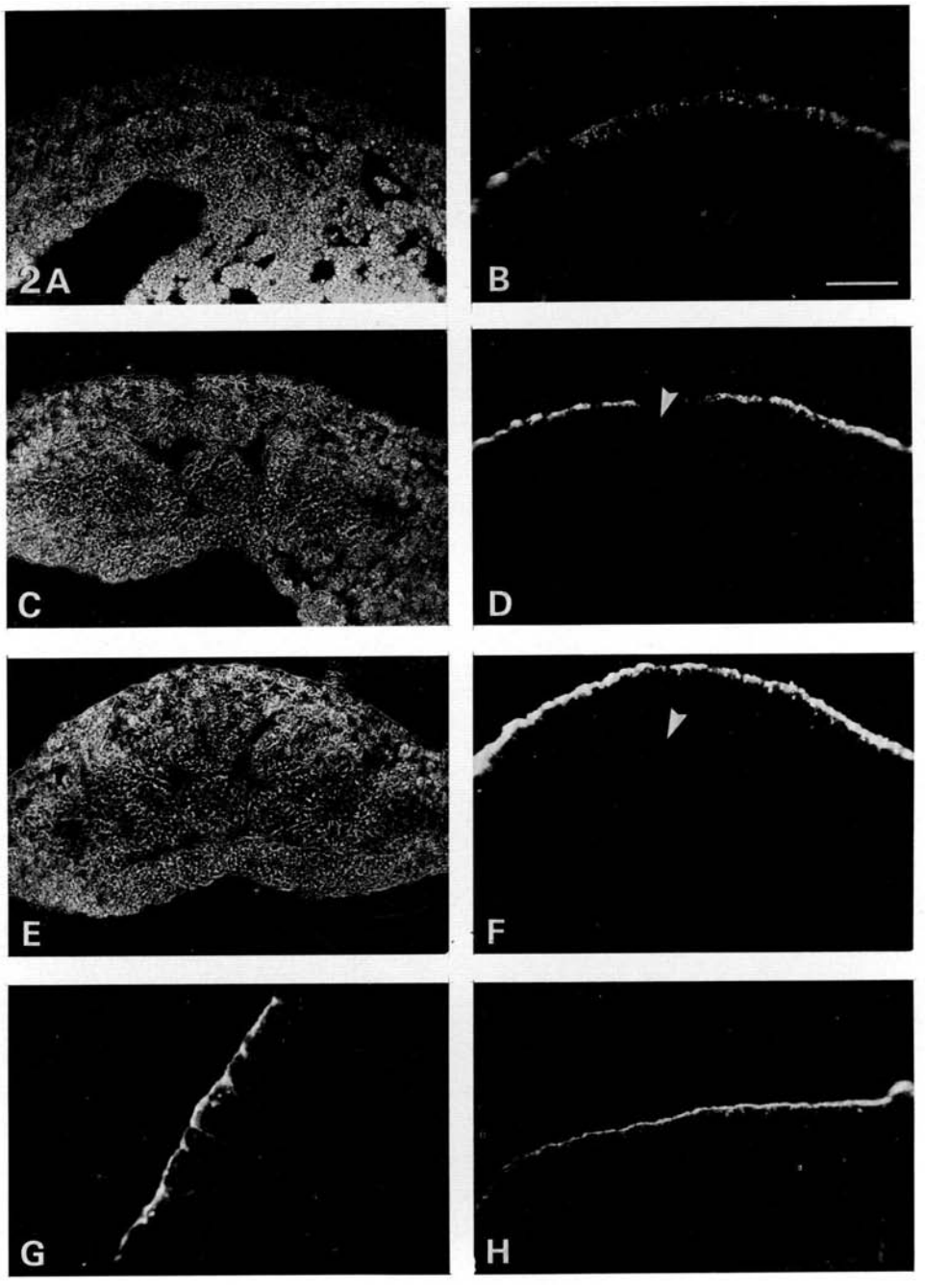

Fig. 2 Regional distribution of 2F7.C7 on *X. borealis, X. laevis* and *A. mexicanum* High-power phase and fluorescence of cryostat sections from neurulae stained with 2F7.C7 and FITC-conjugated rabbit antimouse IgG. (A, B) Stage-12½ *X. borealis* epidermis (C, D) Stage-15 *X. borealis* with developing neural tube (arrow) (E, F) Stage-19 *X. borealis* with complete neural tube (arrow) (G) Stage-19 *A. mexicanum* epidermis (H) Stage-19 *X. laevis* epidermis. Bar = 75 μm

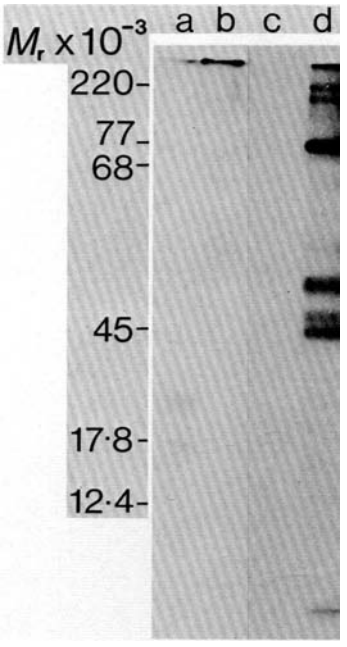

Fig. 3 Molecular identification of the epidermal marker defined by 2F7.C7 Immunoprecipitation of iodogen-labelled ectodermal extract with 2F7.C7 run on 15 % SDS PAGE and autoradiographed. (a) V8 protease treated 2F7.C7 immunoprecipitate (b) 2F7.C7 immunoprecipitate (c) NSI immunoprecipitate (d) Total labelled ectodermal extract

layer (Fig. 2C,D). The presumptive nervous system does not express the antigen (Fig. 2C,D,E,F), An identical distribution of fluorescence is seen in *X. laevis* and *X. borealis* (Fig. 2 compare F & H). In *Ambystoma mexicanum* the antigen is distributed less on the external surface of the epidermis and tends to stain the whole outer membrane of the cells (Fig. 2G).

Iodogen labelling of the surface of ectodermal explants labels seven major protein bands. Immunoprecipitation with 2F7.C7 yields 1 major band of very high molecular weight, greater than 220×10^3, which hardly enters the gel (Fig. 3, track b). A control track immunoprecipitated with $5 \times$ NSI supernatant does not show such a band (Fig. 3, track c). Digestion of a duplicate immunoprecipitate with 1 μg of V8 protease yields peptides with M_r of 17×10^3 and 24×10^3, indicating that the antigen identified by 2F7.C7 has a protein component, but because of its high molecular weight is probably a glycoprotein with a high carbohydrate moeity (Fig. 3, track a). Experiments are now in progress to determine whether this is the case and to establish which part of the molecule bears the antigenic site recognized by 2F7.C7.

The distribution of 2F7.C7 was compared with that of FITC-conjugated Peanut

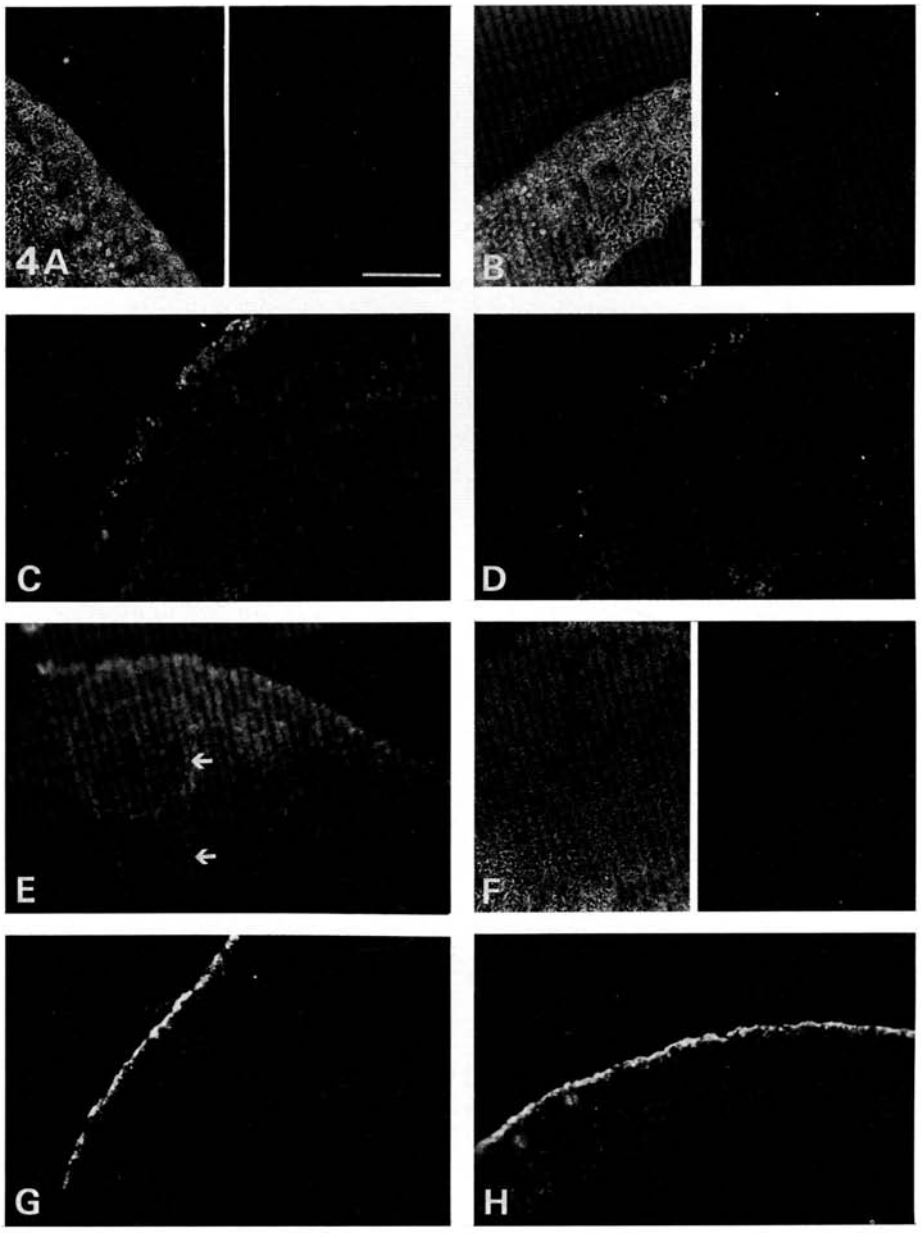

Fig. 4 Blocking of 2F7.C7 with Peanut Agglutinin Cryostat sections of Stage-12½ (A, B, C, D) or stage-19 (E, F, G, H) *X. borealis* embryos. (A, E) Stained with FITC-conjugated PNA (B, F) Blocked with 20 μg PNA and stained with FITC-conjugated PNA (C, G) Stained with 2F7.C7 (D, H) Blocked with 20 μg PNA and stained with 2F7.C7. Bar = 75 μm

agglutinin in *X. borealis* (Fig. 4). As described by Slack (1985) no PNA binding was detected until after the onset of neurulation. At stage 14 the receptor appeared in the epidermis, but gave a much weaker labelling than 2F7.C7. In stage-19 and later

neurulae more binding was seen surrounding the neural tube and the notocord and in the epidermis and sucker. Epidermal binding was still considerably less bright than with 2F7.C7. Attempts were made to block the binding of the antibody 2F7.C7 to sections presaturated with unlabelled PNA, to determine whether 2F7.C7 identified the major Peanut agglutinin receptor on the epidermis. Sections preincubated with 20 μg of unlabelled PNA prior to antibody labelling failed to decrease the amount of 2F7.C7 binding subsequently (Fig. 4D,H), but did completely block binding of FITC-conjugated PNA (Fig. 4B,F).

DISCUSSION

The distribution of an epidermal antigen defined by the monoclonal antibody 2F7.C7 is described. The antigen is first expressed at stage $12\frac{1}{2}$ in *Xenopus laevis* and *X. borealis*, the time at which the nervous system is normally first evident (Nieuwkoop & Faber, 1967). This antigen is conserved in both urodele and anuran amphibia despite the difference in epidermal organization of these two classes. In anuran amphibia the epidermis is composed of two distinct cell sheets which can be identified by morphological criteria (Keller, 1980; Nieuwkoop & Florschutz, 1950). The epidermis in urodeles is a much more homogeneous single layer of cells (Løvtrup, 1975; Vogt, 1929).

The antigen defined by 2F7.C7 has a very high molecular weight on SDS polyacrylamide gel electrophoresis which on V8 proteolysis (Cleveland *et al.*, 1974) identifies two major peptides. It seems likely that the antigen is a highly glycosylated molecule. Slack (1984a) has identified a high molecular weight glycoprotein specific for the epidermis in the axolotl. This marker, epimucin, has a native M_r of 470×10^3 and a subunit M_r of 170×10^3. He has shown that this molecule serves as the major epidermal receptor of Peanut agglutinin. In *Xenopus* the definition of the receptor for Peanut agglutinin is much less clear, PNA-agarose affinity columns binding a polydisperse smear of high molecular weight proteins (Slack, 1985). Could 2F7.C7 be identifying the major *Xenopus* equivalent of epimucin?

The epidermal antigen starts to be expressed at approximately the same time as epimucin and with the same regional restrictions, first appearing within presumptive epidermal cells at stage $12\frac{1}{2}$. Neither molecule is present on neural plate or in the differentiated nervous system. The distribution of epimucin, defined by FITC-conjugated PNA binding, to epidermis in *Ambystoma* showed that the receptor was less concentrated on the outer surface of the cells and tended to surround the cells (Slack, 1985). Its distribution in *Xenopus* was much more concentrated on the outer surface of the epidermis. This distribution is identical to that seen for 2F7.C7.

However, blocking studies with PNA failed to prevent binding of 2F7.C7 to sections although all epidermal receptor sites for PNA are blocked to the further binding of FITC-conjugated PNA. This experiment does not categorically show that 2F7.C7 recognizes a different molecule to epimucin, but suggests that if 2F7.C7

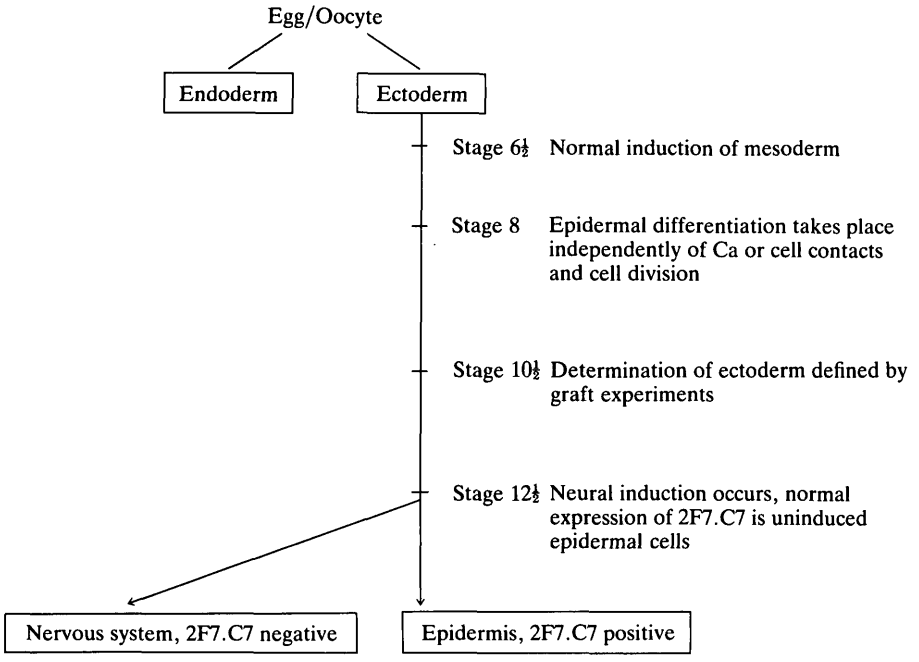

Fig. 5 Scheme or epidermal differentiation in *Xenopus*

does recognize *Xenopus* epimucin, that the antigenic site of the molecule is too far from the PNA receptor site for blocking studies to be effective. Further biochemical studies will be necessary to distinguish these possibilities.

The antibody 2F7.C7 has been used in a series of experiments to try to establish the timing of epidermal specification and determination (Jones & Woodland, 1985) and the role of cell disaggregation and cell division in epidermal differentiation (Woodland & Jones, 1985). Isolated explants from the animal pole of embryos from stage 3 upward express the normal epidermal antigen at approximately the same time as in the whole embryo. Vegetal explants do not. The two layers of the *Xenopus* epidermis can be separated and cultured independently (Asashima & Grunz, 1983). The inner, sensorial layer and the outer layer differ in their expression of 2F7.C7. The majority of the outer layer is positive whereas only isolated positive cells are found in the inner, sensorial explants. Slack (1984b) has suggested that the inner region of a whole ectodermal explant is not epidermis on the basis of its glycolipid biosynthesis. He suggested that it might be (a) residual totipotent undifferentiated ectoderm, (b) neuroepithelium, or (c) epidermis which is unable to express its markers because of its inside position. We have demonstrated that culture of the inside layer alone does not increase the number of cells expressing the epidermal marker, supporting Slack's view that (c) is unlikely to be the case (Jones

& Woodland, 1985). Since the inner sensorial layer does not normally express the antigen in the whole embryo, the few cells positive for 2F7.C7 seen in inner explants could be destined either for an outside position, moving possibly in response to the axial stretching of the embryo, or they could not express the antigen at all in the normal embryo due to some effect of the underlying layers of mesoderm and endoderm. These questions can now be resolved using cell marking experiments (Heasman, Wylie, Hansen & Smith, 1984; Jones & Woodland, 1985) in conjunction with antibody staining. These experiments support the view of Nakamura *et al.* (1971) that specification of the epidermis occurs from the 8-cell stage in animal pole cells.

Epidermal determination, as defined by the expression of the 2F7.C7 defined antigen, has been tested by graft experiments (Jones & Woodland, 1985). Ectodermal sandwiches (Holtfreter, 1933) made from stage-9 *Xenopus* embryos with inner grafts from stages 6 to 9 indicate that epidermal differentiation occurs regardless of an inside position in highly pigmented cells normally destined to become the outer layer of the epidermis. These experiments also show that little movement of implanted cells takes place to the surface of the sandwich suggesting, perhaps, that the normal organization of epidermis is imposed by the underlying cells of the mesoderm. These grafts however are in an ectodermal environment. Ectodermal cells from stage-6 to -10 *Xenopus* embryos have been transplanted into the endoderm of stage-8 or-9 acceptor embryos and allowed to develop to stage 23 either as normal embryos or as exogastrulae. Under conditions where exogastrulation occurs the graft usually remains at the surface of the endoderm. The grafts express the epidermal antigen if derived from stage 8 or later, but not from stages before this. If the grafted embryos are allowed to develop normally the graft occupies an entirely internal position within the endoderm. Only grafts from stage-10½ embryos are capable of differentiating to form the epidermal antigen. This suggests that from stage 10½ the ectoderm is determined to form a marker of normal epidermis. However, it is not terminally differentiated since it is still competent to respond to induction by underlying dorsal mesoderm, to form nervous system, until the end of gastrulation. It is at the end of this period of competence that the epidermal antigen identified by 2F7.C7 can first be recognized.

Further steps in the epidermal differentiation pathway have been defined by experiments involving cell disaggregation and inhibition of normal cell division with Cytochalasin B (Woodland & Jones, 1985). *Xenopus* embryos which have been disaggregated in Ca^{2+}-free medium prior to stage 8 and then allowed to develop until controls reach stage 19 do not express the epidermal antigen, whereas disaggregated embryos from subsequent stages do. Furthermore, embryos which have been disaggregated before stage 8 can be reaggregated by addition of Ca^{2+} up to at least stage 9 and expression of the antigen restored. This experiment defines a requirement for either calcium ions or cell contact prior to stage 8 for normal epidermal development to occur.

Experiments using the epidermal marker 2F7.C7 can thus provide information

on the essential steps in early epidermal differentiation (Fig. 5), the initial determinant(s) specifying ectoderm and endoderm presumably being laid down during oogenesis.

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