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An analysis of protein synthesis patterns during early embryogenesis of the urodele – *Ambystoma mexicanum*

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SUMMARY

Changes in protein synthesis during early *Ambystoma mexicanum* (axolotl) embryogenesis were monitored using two-dimensional (2-D) polyacrylamide gel electrophoresis. No change in synthesis patterns during progesterone-induced oocyte maturation was detected. In oocytes matured *in vivo* (unfertilized eggs), however, the synthesis of several oogenetic proteins ceased, only to be resumed later in development. At fertilization, one novel non-oogenetic protein was found. A cleavage-specific protein was also detected.

Dramatic changes in protein synthesis patterns were detected at gastrulation in axolotl embryos. About 10% of the proteins synthesized at earlier stages ceased synthesis at gastrulation. Another 10% of the proteins synthesized during gastrulation were novel. A gastrulation-specific protein was also detected. After gastrulation additional novel non-oogenetic proteins were synthesized for most stages examined. A pronounced increase in the number of novel proteins synthesized was observed at the onset of neurulation and during neural fold fusion. Some of those proteins were specific to dorsal or axial structure tissue (AST) and some were specific to ventral or non-axial structure tissue (NAST).

Actin and tubulin synthesis was also monitored during axolotl development. While the cytoplasmic γ- and β-actins were synthesized at all stages, muscle-specific α-actin synthesis began at the head-process stage (stage 23/25).

INTRODUCTION

Amphibian embryos have long been used as a model system for studying the mechanisms which control embryogenesis. Until recently, very little information was available on the biochemical changes that occur during development. With the advent of a variety of new techniques, the pattern as well as the rate of protein synthesis have been studied in numerous organisms. These methods, however, have been applied to only a few species of amphibians.

The most extensively studied amphibian is the anuran, *Xenopus laevis*. Several investigators have shown that during *Xenopus* early development the patterns of protein synthesis vary substantially between the stages of oogenesis and tadpole (Brock & Reeves, 1978; Ballantine, Woodland & Sturgess, 1979; Bravo & Knowland, 1979). Some information is available concerning the synthesis of

Key words: protein synthesis, axolotl eggs, *Xenopus* eggs, developmental characteristics, 2-D gels, patterns.

several relatively ubiquitous proteins found in all species of amphibians. The embryonic synthesis of three classes of actin in *Xenopus laevis* (Sturgess *et al.* 1980; Ballantine *et al.* 1979) and *Ambystoma mexicanum* (Mohun, Mohun, Tilly & Slack, 1980) has been well characterized. In the case of *Xenopus*, the onset of α-actin synthesis was shown to begin at gastrulation (Sturgess *et al.* 1980). In contrast, in axolotl embryos, α-actin synthesis was not detected until the head process stage (Mohun *et al.* 1980).

It is evident that changes occur in the overall pattern of protein synthesis during early amphibian development. An adequate description of the general changes in protein synthesis patterns during development of an amphibian other than *Xenopus* is, however, unavailable. Statements concerning general changes in protein synthesis patterns during amphibian development may not be valid for other species of amphibians.

While *Xenopus laevis* and other species of amphibians share general features of amphibian development, they differ from each other in many important details. For example, *Xenopus* eggs are monospermic (Grey, Bastiani, Webb & Schertel, 1982). Urodele eggs, on the other hand, are polyspermic (Wakimoto, 1979). *Xenopus* embryos develop at a faster rate than axolotl embryos. While *Xenopus* embryos reach first cleavage 90 min postfertilization, axolotl embryos undergo first cleavage 5 h postfertilization. *Xenopus* eggs possess two distinct mechanisms to prevent supernumerary sperm from entering the egg. In addition to a slow block to polyspermy, *Xenopus* eggs possess a fast block mediated by a chloride-dependent change in egg membrane potential (Grey *et al.* 1982). In contrast, urodeles lack a fast (electrical) block to polyspermy. While prospective mesoderm in *Xenopus* blastula is located in the deep cell layers (Keller, 1975), presumptive mesoderm occupies the superficial layer of axolotl blastulae (Smith & Malacinski, 1983). Axolotl blastula are essentially single layered, while *Xenopus* blastula are double layered with a thin outer layer and an inner layer several cells thick (Nieuwkoop & Sutasurya, 1979). Cell movements that participate in archenteron formation differ between urodele (Pasteels, 1942) and anuran (Keller, 1976) embryos. Furthermore primordial germ cells originate from different germ layers in urodele (Sutasurya & Nieuwkoop, 1974) and anuran (Whitington & Dixon, 1975) embryos. Whether these differences in early morphogenetic pattern formation are underlain by fundamental differences in gene expression remains to be determined.

To determine whether protein synthesis patterns of other amphibians are similar to the anuran, *Xenopus laevis*, the urodele *Ambystoma mexicanum* (axolotl) was chosen as the experimental system. The axolotl egg is relatively large (>2 mm diameter) and the developmental rate slower than other common laboratory species of amphibians. The number of embryos required for each sample is, therefore, much smaller than for other amphibian species (e.g. *Xenopus*). These features also make the axolotl egg exceptionally amenable to various manipulations such as microinjection and microsurgery. Most important, however, is the large number of genetically defined mutants in the axolotl. There

are approximately three dozen mutants which affect one or another stage of early development, including cell and tissue function (Malacinski, 1975; Briggs & Briggs, 1984) as well as adult pigmentation (Frost, Briggs & Malacinski, 1984). The results from this investigation will, therefore, also be useful in experiments designed to investigate the nature of those developmental mutants.

The aim of this investigation is to develop a general understanding of the types of proteins synthesized at various developmental stages of the axolotl. ^{14}C-amino acid labelling, tissue dissection, and two-dimensional (2-D) polyacrylamide gel electrophoresis were employed to analyse and compare the overall protein synthesis patterns of axolotl embryos from oogenesis to the early stages of organogenesis. The synthesis patterns in the embryologically distinct dorsal and ventral regions of early axolotl embryos were compared and the synthesis of two known proteins, actin and tubulin, was monitored.

In general, our results indicate that axolotl embryos show changes in protein synthesis patterns during most stages of early development. Several developmental stages showed a dramatic change in the pattern of protein synthesis. Those changes are discussed in relation to similar previous analyses of *Xenopus* protein synthesis patterns.

MATERIALS AND METHODS

Source of embryos

Ambystoma mexicanum (axolotl) embryos were obtained from natural spawnings, manually dejellied, and staged according to Bordzilovskaya & Dettlaff (1979). Embryos were raised in a 10 % Steinberg's solution (pH 7·4).

Labelling procedure

To label proteins synthesized during a specific developmental stage approximately 20–25 nl (0·1 μCi) of a lyophilysed ^{14}C-amino acid mixture derived from a yeast protein hydrolysate (Amersham No. CFB.25) were microinjected into the embryo. Embryos were then incubated at room temperature for 2–6 h. The length of the incubation period coincided with the length of the developmental stage under examination.

After an appropriate incubation period, postgastrula-stage axolotl embryos were dissected into dorsal and ventral halves which were analysed separately (Fig. 1). Embryos from the stages preceding neurulation, including gastrula-stage embryos, were left intact. The embryos were placed in a small volume of sonication buffer containing 0·01 M-Tris-HCl and 5·0 mM-$MgCl_2$ (pH 7·4) and frozen until ready for protein extraction.

Sample preparation

Once the samples were thawed, 0·2 M-phenylmethylsulphonyl fluoride (PMSF: Sigma No. P-7626) was added to a final concentration of 0·2 mM. The samples were then sonicated on ice for two 30-s bursts using a Bransen JA-21 Sonicator. Sonication was used for protein extraction because it allowed for maximum recovery from small quantities of tissue. 25·0 μg ml^{-1} of DNase (Sigma No. D-4638) and 12·5 μg ml^{-1} of RNase (Sigma No. R-5125) were added and the sample was left on ice for 15 min. Solid urea was then added to a final concentration of 9·5 M followed by an equal volume of lysis buffer containing 9·5 M-urea, 2·0 % NP-40 (Calbiochem), 5·0 % B-mercaptoethanol, 2·0 % LKB ampholines (pH 3–10 and pH 5–7 in the ratio 1:4). The sample was kept frozen at −70 °C prior to electrophoresis.

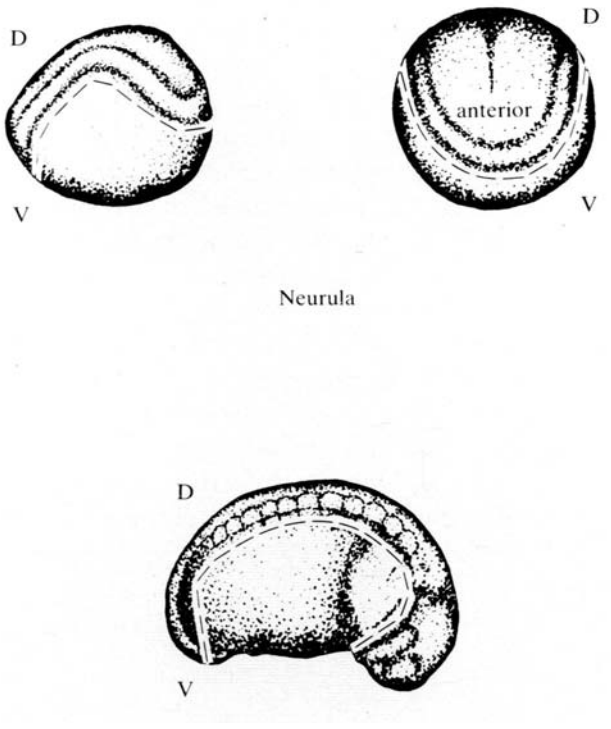


Fig. 1. Dissection of axolotl embryos into dorsal (AST) and ventral (NAST) regions. Dotted lines indicate boundaries of dissection.

First dimension separation by isoelectric focussing

Isoelectric focussing (IEF) gels were prepared according to O'Farrell (1975) using a combination of pH 3–10 and pH 5–7 LKB ampholines in the ratio of 1:4, which resulted in a pH gradient of 5·0–7·2. Samples were centrifuged at 10 000 r.p.m. in an Eppendorf 5412 centrifuge for 10 min to remove yolk, cell debris and pigment granules. The supernatant was then loaded onto the top of the IEF tube gel and run for a total of 9000 volt hours. Because the buffer in which the proteins were extracted contained substances that render standard protein assays unreliable, it was not possible to standardize the sample volume loaded onto each IEF gel according to the protein concentration. Therefore approximately 25 000–30 000 TCA-precipitable c.p.m. were routinely loaded onto each gel. This represented the labelled proteins from two to four embryos.

After electrophoresis, each IEF gel was extruded into 5·0 ml of sample buffer containing 10·0 % glycerol, 5·0 % B-mercaptoethanol, 2·3 % sodium dodecyl sulphate (SDS), 0·065 M-Tris-base and stored at −70°C prior to second-dimension electrophoresis.

Second dimension on SDS-polyacrylamide gels

The second dimension gel was a 15 cm wide, 20 cm high, and 1·8 mm thick 10·0 % polyacrylamide (BioRAd) separating gel with a 4·0 % acrylamide stacking gel. The IEF gel was fixed atop the second dimension gel using 1·0 % agarose (Sigma No. A-6013) dissolved in sample buffer. A concentration of 10·0 % acrylamide provided optimal protein separation. The gel was prerun with a high concentration SDS running buffer (0·025 M-Tris-HCl, 0·19 M-glycine, and

2·0 % SDS) in the top reservoir for 25 min at 20 mA gel^{-1}. This running buffer was replaced with a low-concentration SDS running buffer (0·025 M-Tris-HCl, 0·19 M-glycine, and 0·1 % SDS coloured with 1·0 % bromphenol blue). Electrophoresis was run at 7 mA gel^{-1} until the dye front reached the bottom of the gel. The gels were then fixed in 10·0 % acetic acid for one hour and prepared for fluorography.

Fluorography

Gels were soaked in 1·0 M-sodium salycilate for 20 min and dried under vacuum (Chamberlain, 1979). The dried gels were then packed under Kodak X-Omat X-ray film for four weeks and then developed (Kodak D-19 developer) and fixed.

Criteria used to detect changes in synthesis pattern

In most cases electrophoretic patterns, including those from identical developmental stages, were not entirely superimposable due to slight but uncontrollable variations during the separation of the proteins. Rigorous criteria were, therefore, employed for gel comparisons. Each fluorograph was first orientated with respect to several major proteins which are synthesized at most stages. Changes in gel patterns were considered significant if they were (1) detected in duplicate fluorographs and (2) present on fluorographs that contained mixed samples. In the first instance, several fluorographs prepared from embryos of different spawnings were analysed for reproducibility. Some differences were found in the 2-D gel patterns of identical stages obtained from different spawnings. These proteins (four to five spots) varied from spawning to spawning and were deleted from the analyses described below. Any change in pattern was considered significant only if it appeared in several fluorographs prepared from the same stage of development, but from different spawnings. It should also be noted that only qualitative differences were subject to analysis. Increases or decreases in spot intensities were not included in this investigation.

In the second instance, it was important to determine whether the changes in the 2-D gel pattern might be due to artifacts of the extraction procedure. Hence, embryos from different developmental stages were co-extracted and co-electrophoresed. The resulting fluorographs always represented a composite of the changes noted when stages were individually compared. This indicated that the changes were probably not due to a stage-specific difference in extraction efficiencies.

With regard to initiation and termination of the synthesis of a specific protein it is impossible with the present methodologies to determine exactly when synthesis began or ceased, or merely fell below the limit of resolution of fluorography. For brevity, therefore, the terms synthesis 'began' or 'ceased' were used in reference to the appearance or disappearance of a radioactive protein from a fluorograph.

RESULTS

PROTEIN SYNTHESIS PATTERNS DURING AXOLOTL EARLY EMBRYOGENESIS

Proteins synthesized during oocyte maturation in vitro

Axolotl oocytes were manually removed from ovaries and sorted according to size. They were staged according to a *Xenopus* oocyte staging series (Dumont, 1972) because an axolotl oocyte staging series is not available. Stage-VI oocytes (pigmented animal half, lightly pigmented vegetal half, and an unpigmented equatorial region) and stage-III oocytes (uniform pigmentation) were collected. Half of the stage-VI oocytes were then stimulated to mature *in vitro* by the addition

of 3·0 μM-progesterone to the culture medium. The stage-VI untreated oocytes, the progesterone-treated stage-VI oocytes, and the stage-III oocytes were then microinjected with ^{14}C-amino acids. After approximately 5 h incubation at room temperature the hormone-treated oocytes were observed for maturation. Maturation was scored by the appearance of a white spot at the animal pole, which indicates germinal vesicle breakdown and emission of the first polar body. Proteins were then extracted from both progesterone-matured oocytes, untreated oocytes, and stage-III oocytes, and analysed as described in Materials and Methods.

No differences were observed in the pattern of proteins synthesized in stage-III and stage-VI oocytes. The proteins synthesized in stage-VI oocytes are shown in Fig. 2 (fluorographs of proteins synthesized in stage-III oocytes are not shown). Stage-VI oocytes are postvitellogenic. Stage-III oocytes, on the other hand, are undergoing yolk accumulation (vitellogenesis) as well as undergoing changes in chromosome morphology (Dumont, 1972). Yet those physiological events were not reflected in the types of proteins synthesized during this latter stage of oogenesis.

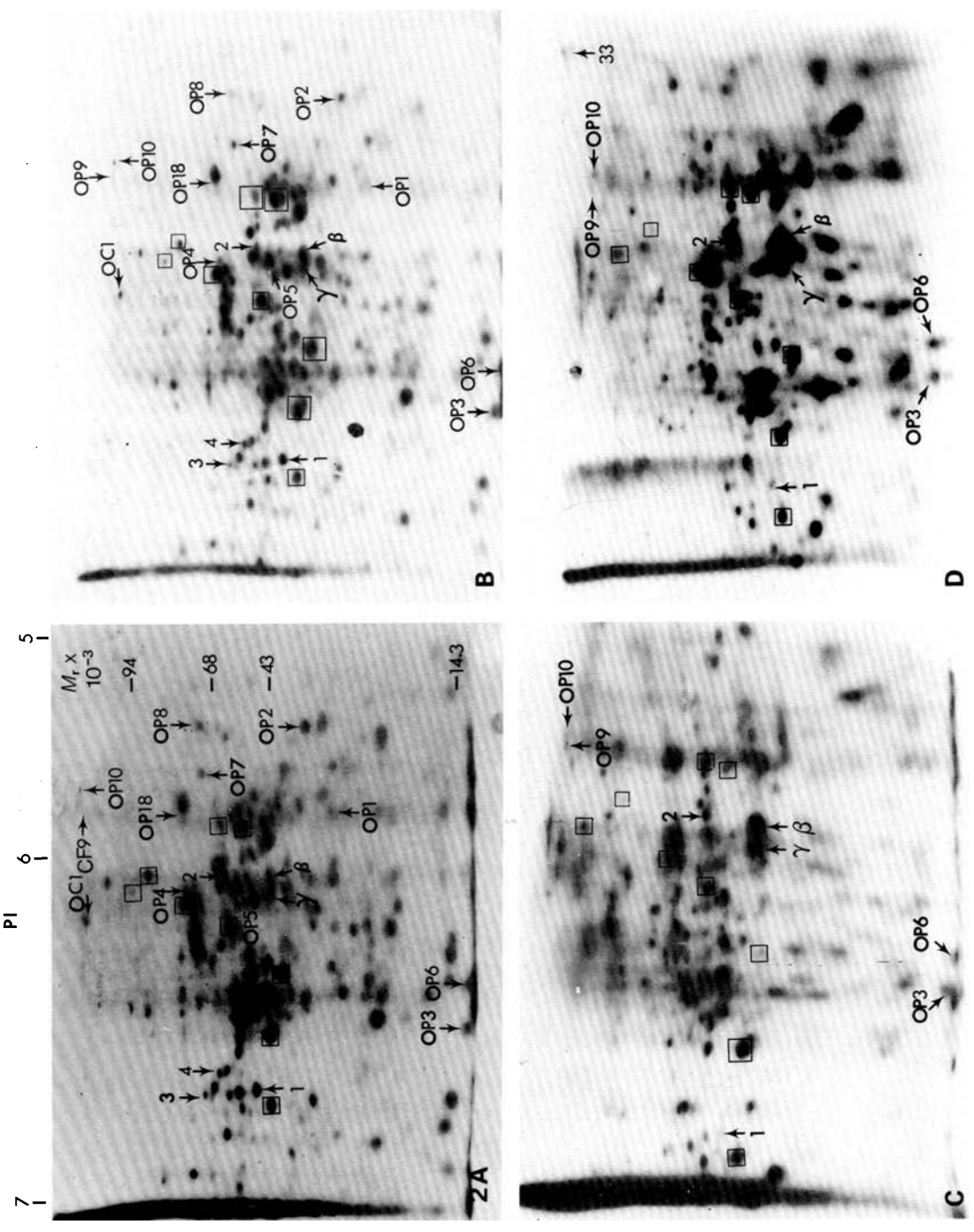
Axolotl stage-VI oocytes do not display changes in protein synthesis patterns as a result of *in vitro* maturation (Fig. 2A,B). The *in vitro* matured oocytes, untreated oocytes, and stage-III oocytes did, however, synthesize a protein found only during oogenesis (Fig. 2A,B: OC1). Since the present study examined only those stages from oogenesis through early tailbud, it is possible that stage-specific proteins, such as OC1, might actually be synthesized later in development, after stage 30/31.

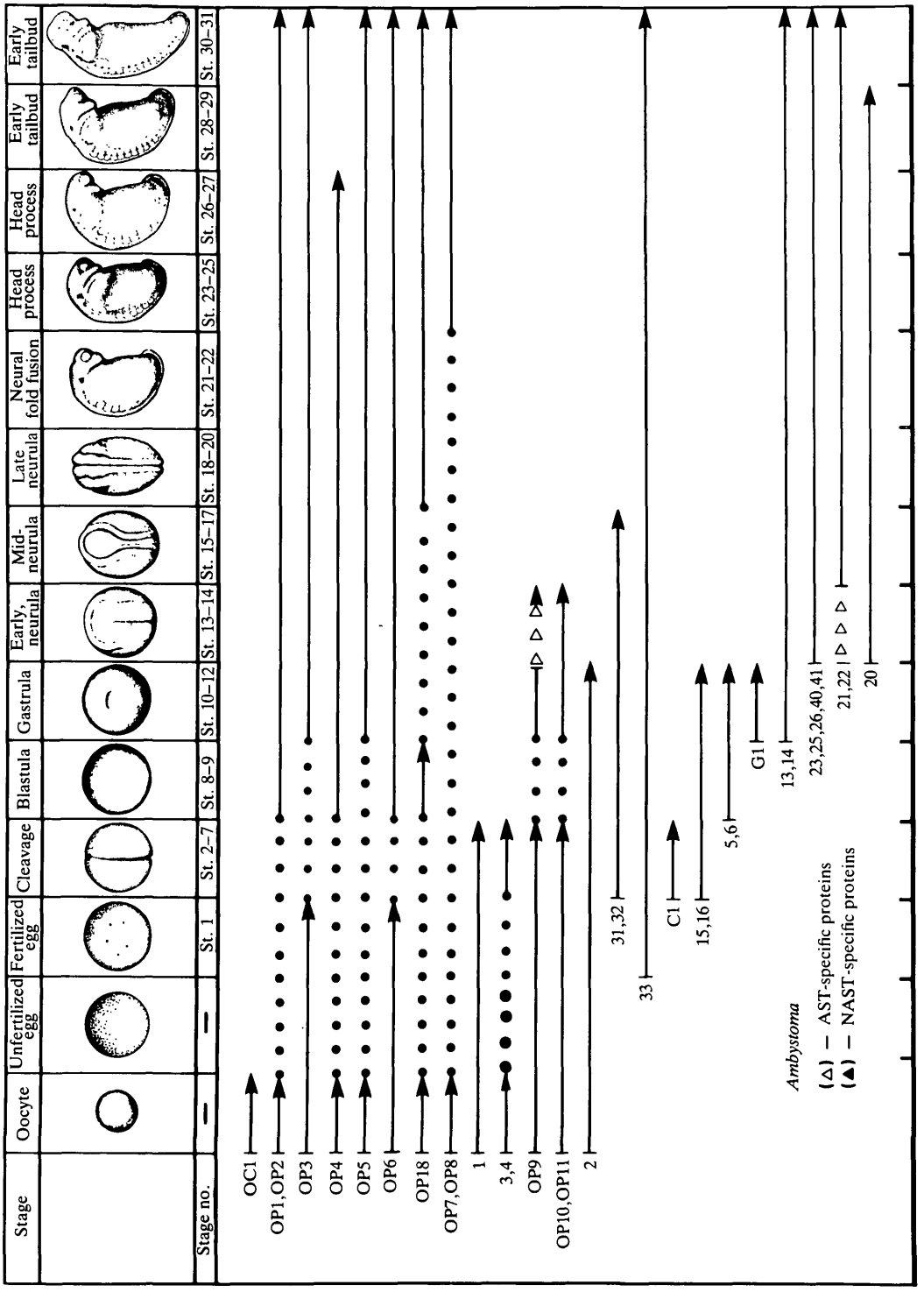
Proteins synthesized in unfertilized eggs

Unfertilized eggs were obtained by stimulating unmated axolotl females to spawn with an intramuscular injection of 40 mg of follicle-stimulating hormone. Eggs were collected as spawned, microinjected with ^{14}C-amino acids, and analysed. Unfertilized eggs were, therefore, labelled after the eggs had matured (*in vivo*) peritoneally and had passed through the oviduct. *In vitro* matured oocytes, in contrast, were labelled as the oocytes matured.

We found that several proteins synthesized in axolotl oocytes matured *in vitro* were not detected in unfertilized eggs (Fig. 2C: OP1, OP2, OP4, OP5, OP7, and OP8). This suggests that changes in protein synthesis may occur sometime between the appearance of the maturation spot and spawning since these proteins

Fig. 2. Fluorographs of 2-D gel separations of newly synthesized axolotl embryonic proteins. In all cases proteins were first separated by isoelectric focusing followed by separation in a denaturing 10 % polyacrylamide gel. (A) Stage-VI oocytes injected and incubated for 5 h. (B) Stage-VI oocytes treated with progesterone, injected, and incubated for 5 h until maturation (*in vitro* matured oocytes). (C) Unfertilized eggs injected and incubated for 5 h. (D) Fertilized eggs injected and incubated for 5 h until appearance of the first cleavage furrow. Arrows indicate major differences between these stages. Boxes indicate proteins found at all stages. OC indicates oocyte-specific proteins. OP indicates oogenetic proteins that disappear then reappear later in development.


pI
7
6
5
$M_r \times 10^{-3}$
−94
−68
−43
−14.3
OC1
CF9
OP10
OP18
OP8
OP7
OP2
OP1
OP4
OP5
OP3
OP6
1
2
3
4
γ
β
2A
B
C
D
33


Stage
Oocyte
Unfertilized egg
Fertilized egg
Cleavage
Blastula
Gastrula
Early, neurula
Mid-neurula
Late neurula
Neural fold fusion
Head process
Head process
Early tailbud
Early tailbud
Stage no.
—
—
St. 1
St. 2–7
St. 8–9
St. 10–12
St. 13–14
St. 15–17
St. 18–20
St. 21–22
St. 23–25
St. 26–27
St. 28–29
St. 30–31
OC1
OP1,OP2
OP3
OP4
OP5
OP6
OP18
OP7,OP8
1
3,4
OP9
OP10,OP11
2
31,32
33
C1
15,16
5,6
G1
13,14
23,25,26,40,41
21,22
20
Ambystoma
(△) – AST-specific proteins
(▲) – NAST-specific proteins

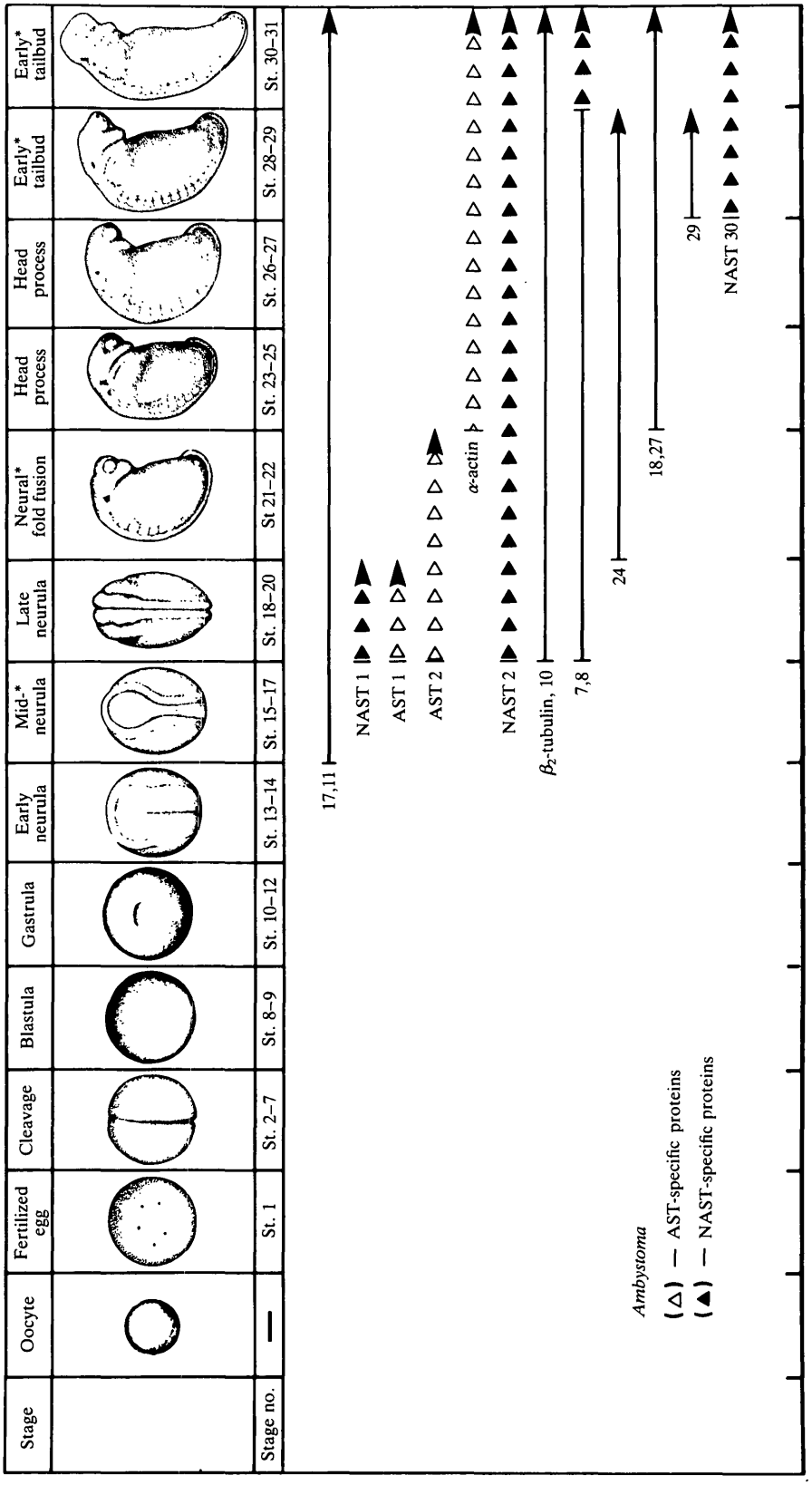


Fig. 3. Summary of the changes in axolotl proteins synthesis patterns. (A) Oogenesis to early neurula (stage 13/14). (B) Early neurula (stage 15/17) to early tailbud (stage 30/31). Solid lines indicate periods of synthesis of the protein and dotted lines indicate periods when the synthesis of the protein cannot be detected.

were detected in premature oocytes but not in eggs that had matured and passed through the oviduct.

Proteins synthesized at fertilization

Many proteins synthesized during axolotl oogenesis which were not synthesized in unfertilized eggs were also not detected immediately after fertilization. The synthesis of proteins with the same relative mobilities resumed, however, later in development. Proteins of this type are indicated by OP in Fig. 2 with a summary of these as well as the other changes in protein synthesis detected during axolotl embryogenesis listed in Fig. 3. In axolotl embryos the synthesis of proteins with the same relative mobilities as OP1, OP2, and OP4 resumed at blastulation. The synthesis of OP5 reappeared at gastrulation. The synthesis of two other proteins with the same relative mobilities as OP7 and OP8, did not resume until later in development, at the head process stage (stage 23/25). In addition, one novel non-oogenetic protein, No. 33, was detected at fertilization. This protein was subsequently detected at all later stages of development (Fig. 3).

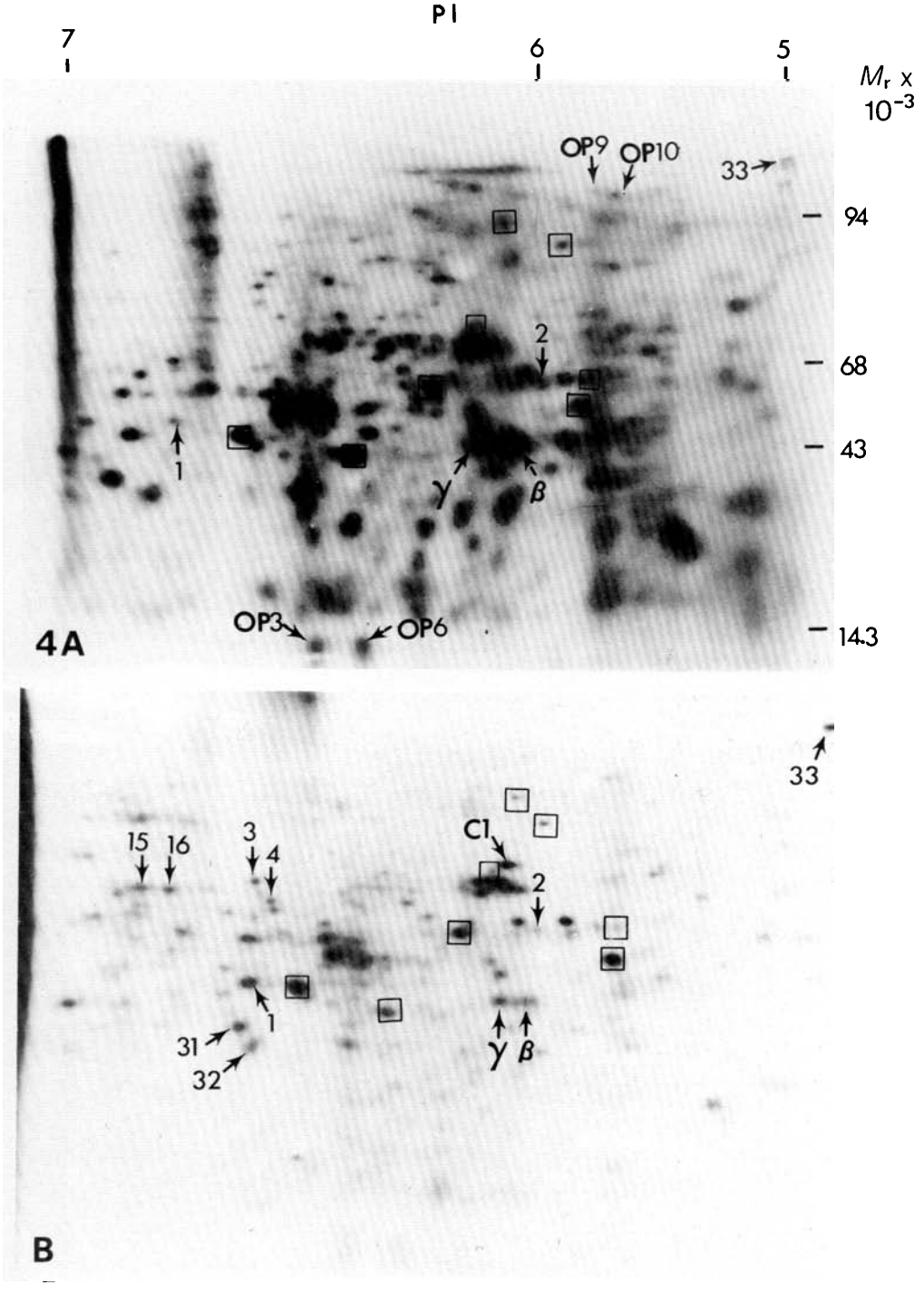
Proteins synthesized during cleavage

Several changes in gel patterns occurred during cleavage. As indicated in Fig. 4B, the synthesis of many novel non-oogenetic proteins, No. 15, No. 16, No. 31, No. 32, and C1, was detected at the onset of cleavage. The synthesis of No. 31 and No. 32 was detected at all later stages of development. The synthesis of two of those proteins, No. 15 and No. 16, ceased detectable synthesis at the beginning of neurulation (Fig. 3). The synthesis of C1 ceased after stage 8 (128 cells). C1 synthesis was apparently restricted in axolotl embryos to the period of rapid cell division. In addition, two oogenetic proteins that were synthesized in oocytes, unfertilized eggs, and fertilized eggs were not detected during cleavage (Fig. 4: OP3 and OP6). A protein with the same relative mobility as OP3 resumed synthesis at gastrulation. A protein with the same relative mobility as OP6 resumed synthesis at blastula.

Proteins synthesized during gastrulation

A pronounced change in the gel pattern was observed at gastrulation. Approximately 10 % of the proteins detected prior to gastrulation ceased synthesis at the onset of gastrulation. These proteins are shown in Fig. 5A. It was also observed that approximately 10 % of the proteins on gastrulation gels were novel, non-oogenetic proteins (Fig. 5B). Except for one protein (G1) these proteins were

Fig. 4. Fluorograph of 2-D gels of proteins synthesized in axolotl embryos after fertilization. (A) Fertilized axolotl eggs injected and incubated for 5 h until appearance of the first cleavage furrow. (B) Two-cell stage embryos injected and incubated for 6 h (to the 128-cell stage). Arrows indicate major differences between these stages. Boxes indicate proteins found at all stages. C indicates cleavage-specific proteins.


PI
7
6
5
M_r x 10^{-3}
OP9
OP10
33
94
2
68
1
43
γ
β
OP3
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4A
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15
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3
4
C1
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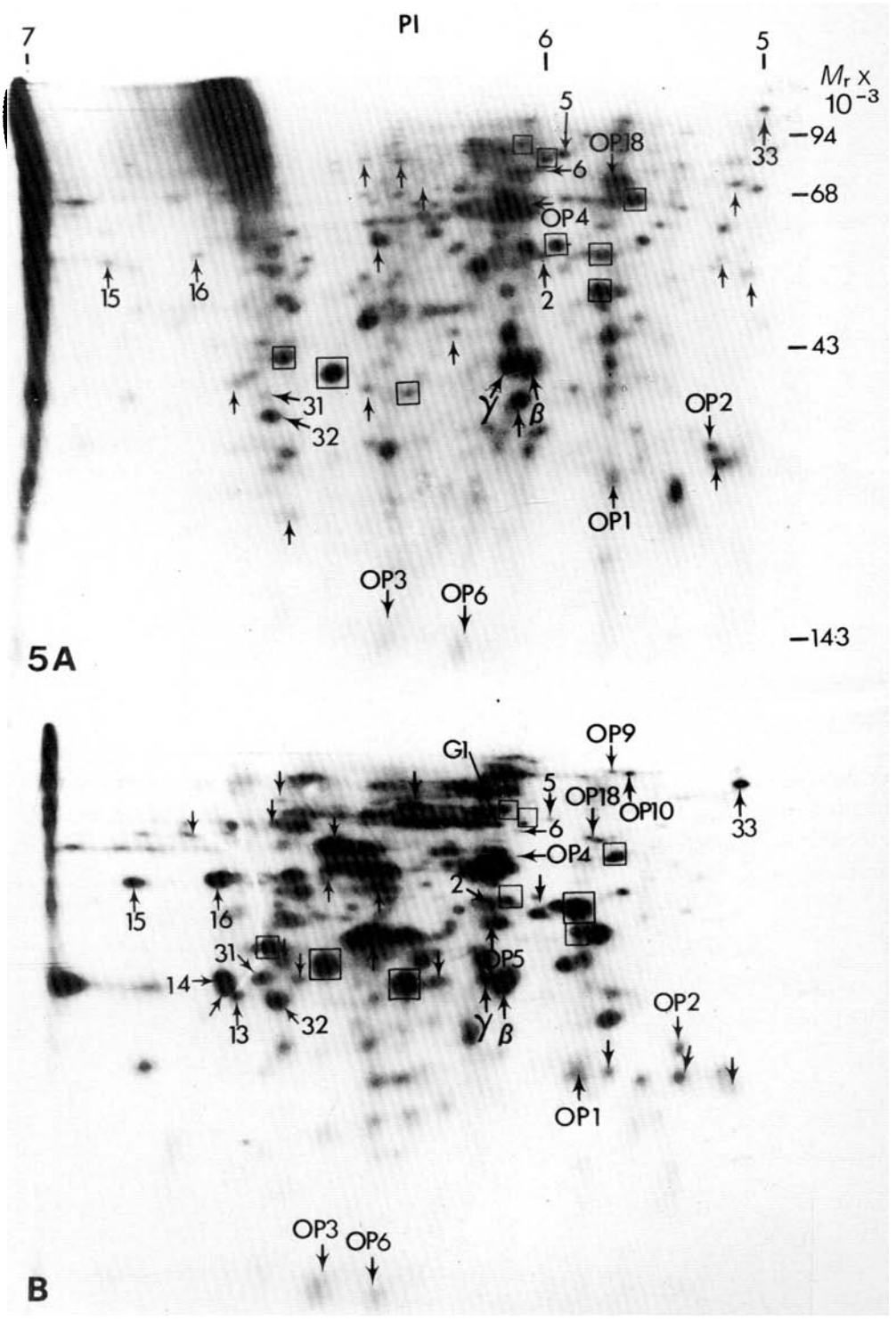
1
31
32
γ
β
B


PI
7
6
5
M_r x 10^{-3}
−94
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−43
−14·3
OP18
OP4
OP2
OP1
OP3
OP6
OP9
OP10
OP5
G1
β
γ
5A
B

subsequently detected at all later stages of development. G1 synthesis was confined to gastrulation.

Proteins synthesized in different regions

Gel patterns in dorsal and ventral halves of postgastrula-stage axolotl embryos were also analysed. The dorsal half consisted of tissue that forms neural tube, notochord, and somites. It was designated axial structure tissue (AST). The ventral half consisted of endoderm and lateral plate mesoderm that develops into gut and related organs, and was designated non-axial structure tissue (NAST).

As shown in Fig. 3, synthesis of novel non-oogenic proteins was detected at most of the developmental stages analysed. Most of these proteins were detected in both AST and NAST. A pronounced increase in the incidence of novel non-oogenetic protein synthesis was detected, however, during two stages of axolotl embryogenesis. The first pronounced increase was detected at the onset of neurulation (stage 13/14). The synthesis of proteins marked No. 20, No. 21, No. 22, No. 23, No. 25, No. 26, No. 40, and No. 41 in Fig. 6A,B was first detected at neurulation and was found in all later stages in both AST and NAST.

A second significant increase in the synthesis of novel non-oogenic proteins was detected during neural fold fusion (stage 18/20). A few of those proteins (Fig. 6C,D: No. 7, No. 8, and No. 10) were found in both AST and NAST. The synthesis of No. 7 and No. 8 was not detected after the early tailbud stage (28/29) while the synthesis of No. 10 was detected in all later stages (Fig. 3). The remainder of the proteins that first appeared at neural fold fusion (stage 18/20) were, however, found in either one or the other half of the embryo.

The synthesis of two AST-specific proteins, AST1 and AST2, was first detected during stage 18/20 and is indicated in Figs 6C and 7A. The synthesis of AST1 was detected only during stage 18/20. The synthesis of AST2, on the other hand, was detected during stages 18/20 and 21/22. During stage 21/22 the neural folds completely fuse at the midline to form the neural tube. Since the synthesis of AST1 and AST2 was found only in axial structure tissue and only during neural fold fusion, it is possible that these two proteins play some role in the process of neural fold fusion.

As shown in Fig. 3, NAST proteins were also detected during neural fold fusion (stage 18/20). The synthesis of NAST1 and NAST2, marked in Figs 6D and 3B, began at stage 18/20. NAST1 was detected only during stage 18/20, while NAST2 was detected at all later stages.

Except for the synthesis of α-actin (see below), it was not until early tailbud (stage 28/29) that another tissue-specific protein was detected (Fig. 3). The

Fig. 5. Fluorographs of 2-D gels of proteins synthesized by axolotl blastula and gastrula. (A) Blastula-stage embryos injected at stage 8 and incubated for 5 h to stage 9. (B) Gastrula-stage embryos injected at stage 10 and incubated for 6 h to stage 11. Unlabelled arrows in (A) indicate proteins found in previous stages that disappear at gastrulation. Unlabelled arrows in (B) point to proteins that appear at gastrulation. Boxes indicate proteins found at all stages. G indicates gastrula-specific proteins.

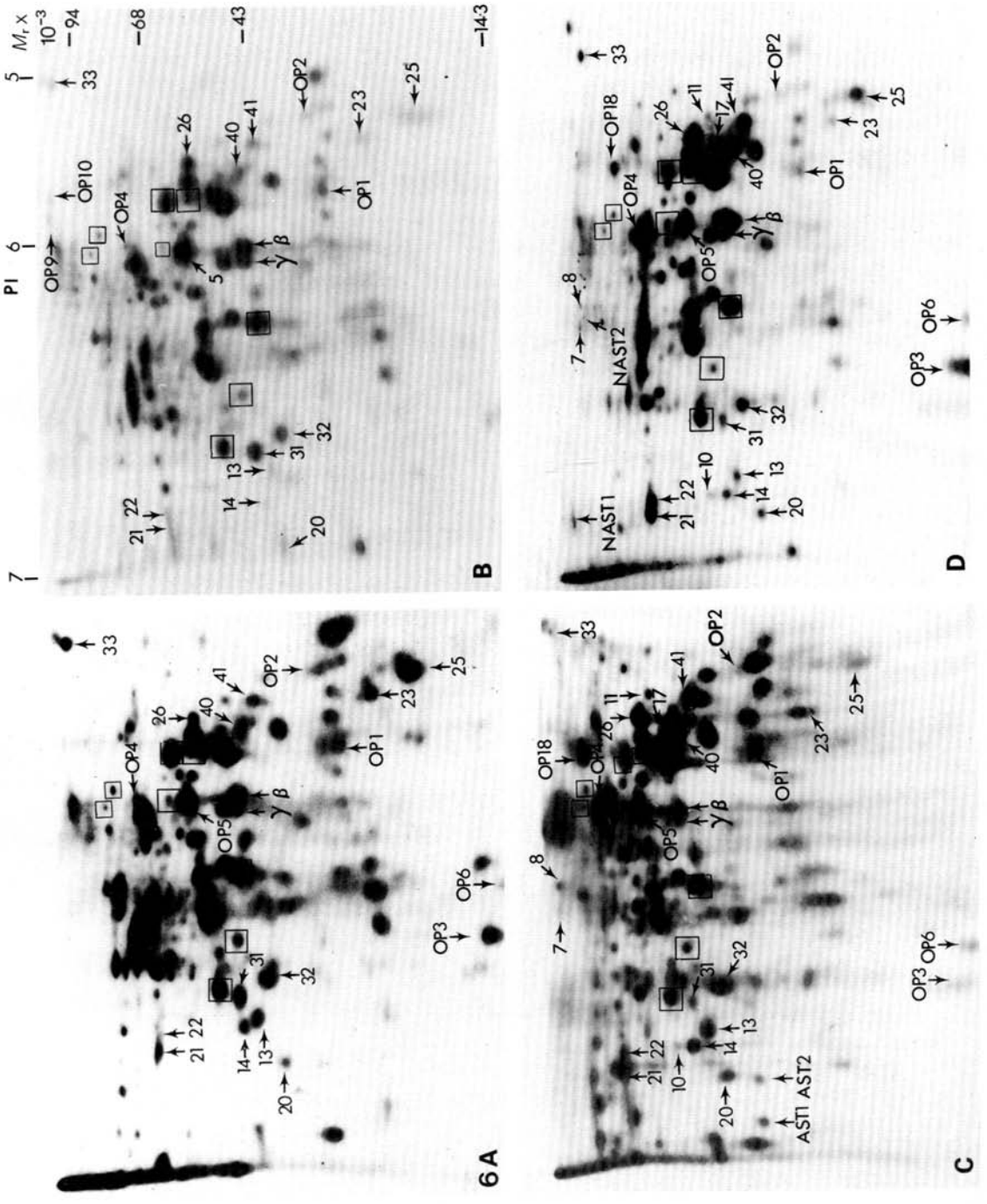

Fig. 6. For legend see p. 86.

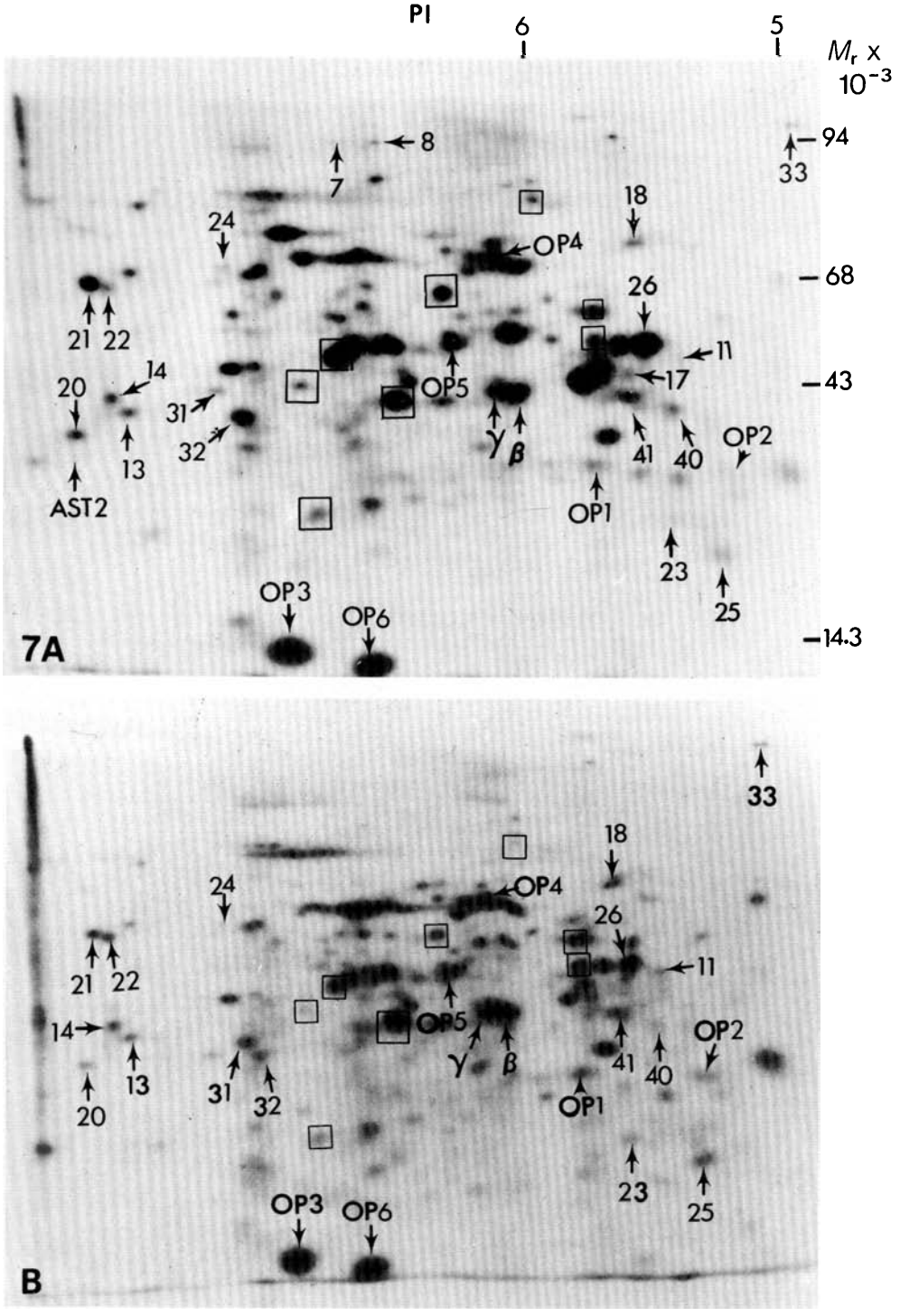

Fig. 7. For legend see p. 86.

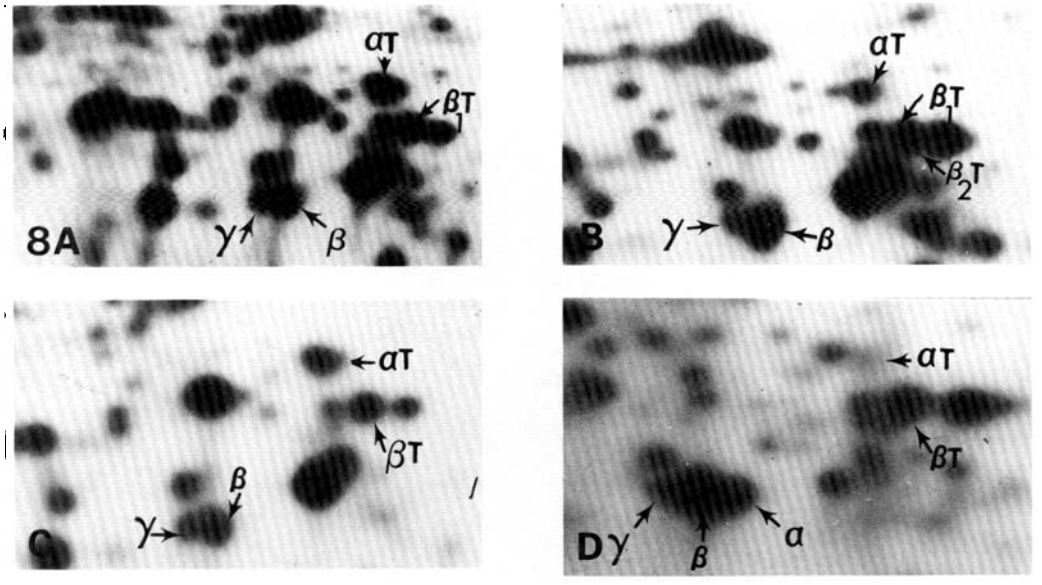

Fig. 6. Fluorographs of 2-D gels of proteins synthesized in either the dorsal (AST) or ventral (NAST) half of axolotl embryos. (A) AST and (B) NAST from embryos injected at stage12/13 and incubated for 6 h to stage 13/14. (C) AST and (D) NAST from embryos injected at stage 17/18 and incubated for 6 h to stage 18/20. Arrows indicate major differences between these stages. Boxes indicate proteins found at all stages.

Fig. 7. Fluorographs of 2-D gels of proteins synthesized in the (A) AST and (B) NAST from axolotl embryos injected at stage 20/21 and incubated for 6 h to stage 21/22. Arrows indicate major differences between these stages. Boxes indicate proteins found at all stages.

Fig. 8. Portions of fluorographs of 2-D gels of proteins synthesized in axolotl embryos indicating actin and tubulin synthesis. (A) Embryos injected at stage 14/16 and incubated for 6 h to stage 15/17. (B) Embryos injected at stage 17/19 and incubated for 6 h to stage 18/20. (C) NAST and (D) AST from embryos injected at stage 22/24 and incubated for 6 h to stage 23–25.

synthesis of NAST30 was detected during early tailbud (stages 28/29 and 30/31; data not shown).

Synthesis of specific proteins

The synthesis of actin and tubulin was monitored. Both are well characterized and therefore easy to identify on 2-D gels. Three classes of actin have been identified in amphibians: γ- and β-actins are found in the cytoplasm of all cells; α-actin is specific to muscle cells. These actin variants have the same relative molecular mass but differ slightly in their iso-electric points. The position of the actins on gels of axolotl embryonic proteins was determined by comparing peptide maps derived from a *Staphylococcus* V-8 protease digestion (Cleveland, Fischer,

Kirschner & Laemmli, 1977) of a rabbit muscle actin standard to the presumed actin spot cut from a fluorograph.

γ- and β-actin synthesis was detected in all stages of development and in all tissues. The synthesis of α-actin, however, was not detected until the head process stage (stage 23/25) and was restricted to AST (Fig. 8C,D). Furthermore, α-actin synthesis was confined to the somites (data not shown). These results agree with the report of Mohun *et al.* (1980).,

Tubulins represent another class of proteins that can be easily identified on a 2-D gel. In amphibians there are four types of tubulin subunits; α_1, α_2, β_1, and β_2. The position on axolotl fluorographs of the four tubulin subunits was tentatively identified by comparison with published reports (Mohun *et al.* 1980) and then by comparison to a purified tubulin standard (Raff, 1977). As summarized in Fig. 3, α_1, α_2, and β_1 tubulin was found during all stages of axolotl development. β_2 tubulin synthesis, however, did not commence until stage 18/20 (Fig. 8A,B).

DISCUSSION

The present study used protein labelling and 2-D gel electrophoresis to analyse changes in protein synthesis patterns during early axolotl development. Changes in protein synthesis patterns were detected for most of the stages analysed. Those changes are summarized in Fig. 3. A comparison of the changes in protein synthesis patterns between axolotl embryos and previously published reports on protein patterns during *Xenopus* embryogenesis are summarized in Table 1.

It is clear that the 2-D gel patterns do not change when axolotl oocytes were matured *in vitro*. Ballantine *et al.* (1979), using [^{35}S]methionine, also observed no change in protein synthesis patterns in *Xenopus* oocytes either before or after oocyte maturation *in vitro*.

These results were somewhat surprising for several reasons. Increases in the rate of protein synthesis during *Xenopus* oocyte maturation (Smith, 1975) as well as changes in the 1-D gel patterns during maturation in *Rana* oocytes (Ecker & Smith, 1971) have been reported. Furthermore differences in polypeptide synthesis were found during oocyte maturation in porcine oocytes (McGaughey & van Blerkom, 1977), in mouse oocytes (Schultz & Wassarman, 1977) and in rabbit oocytes (van Blerkom & McGaughey, 1978).

One possible explanation for this discrepancy concerns the separation methods. Even though the 2-D methods employed in the present study are highly sensitive for separating polypeptides it only resolves a small percentage of the embryo's total protein population. Approximately 270 newly synthesized proteins were analysed in the present study. Complexity studies performed on sea urchin embryonic RNA populations have shown that approximately 14 000 different structural genes are present on gastrula polysomes (Galau, Britten & Davidson, 1974). The complexities of the total RNA population of both sea urchin (Galau *et al.* 1976) and amphibian embryos (*Xenopus*: Davidson & Hough, 1971) have been found to be similar. The methods used in this study, therefore, probably

Table 1. *Comparison of the changes in protein synthesis patterns during axolotl and* Xenopus *embryogenesis*

Source of data	Axolotl This report*	Xenopus Bravo & Knowland†	Xenopus Ballantine *et al.*†
Oocyte maturation *in vitro*	No change; synthesis of oocyte-specific protein (OC1)	Synthesis of an oocyte-specific protein	No change
Unfertilized egg	Disappearance of many oogenetic proteins	Disappearance of many oogenetic proteins; synthesis of two unfertilized egg-specific proteins	Disappearance of many oogenetic proteins
Fertilized egg	Synthesis of one novel protein	No data	No change
Cleavage	Synthesis of a cleavage-specific protein (C1): synthesis of several novel proteins	Synthesis of many novel proteins	Synthesis of a few novel proteins
Gastrula	Disappearance of 10 % pregastrula proteins; synthesis of many new proteins including a gastrula-specific protein (G1)	No pronounced change	Synthesis of a few novel proteins, synthesis of an ectoderm-specific protein
Neurulation	Pronounced increase in new protein synthesis at stage 13/14, stage 18/20: AST-specific proteins found at stage 13/14, stage 18/20, stage 23/25; NAST specific proteins detected at stage 18/20.	No pronounced change	Synthesis of a few novel proteins including an ectoderm-specific protein at stage 17 and stage 24, an endoderm-specific at stage 17 and stage 24.
Tailbud	Synthesis of many new proteins; NAST specific proteins found at stage 28/29 and stage 30/31.	No data	No data

* Proteins labelled with ^{14}C amino acids.
† Proteins labelled with [^{35}S]methionine.

detect only a very small percentage (less than 10 %) of the total proteins which may in fact be synthesized at any one stage. Changes which occur during oocyte maturation may be below the limit of resolution of our 2-D gel methods.

Likewise, changes that went undetected during oocyte maturation could also involve proteins that have isoelectric points that are outside the pH range of the first dimension gel. The pH range (5·0–7·2) employed in the first dimension IEF gel does not resolve basic proteins (isoelectric points greater than pH 7·2). The basic proteins, including histones and most ribosomal proteins, migrate to the basic end of the first dimension gel and are not resolvable from one another in the large streak at one end of the second dimension gel. Needless to say, the proteins present in the streak were not included in the analysis. A 1-D, non-isoelectric focusing gel or a 2-D gel system with a more basic pH range in the first dimension may be helpful in identifying alterations in the protein synthesis patterns during amphibian oocyte maturation.

It was also observed that many proteins detected in axolotl oocytes were not found in unfertilized eggs. Both Ballantine *et al.* (1979) and Bravo & Knowland (1979), analysing [^{35}S]methionine-labelled proteins, reported a cessation of synthesis of many oogenetic proteins in unfertilized *Xenopus* eggs. It is possible that this change in protein synthesis patterns is a result of egg activation, since gel patterns of unfertilized eggs are similar to gel patterns from fertilized (activated) eggs. Spontaneous activation of an amphibian egg does occur after microinjection. If, however, unactivated axolotl or *Xenopus* eggs are sorted out on the basis of the absence of specific activation reactions (Signoret, Briggs & Humphrey, 1962) the same result was found (unpublished observation). This suggests that changes in protein synthesis occurs sometime between maturation and fertilization (activation), possibly as the egg moves through the oviduct.

Our results also show that axolotl embryos synthesize a novel non-oogenetic protein at fertilization. Ballantine *et al.* (1979) reported no change in protein synthesis patterns at fertilization in [^{35}S]methionine-labelled *Xenopus* embryos. During cleavage several novel proteins as well as a stage-specific protein (C1) were detected in axolotl embryos. C1 was synthesized between the first and the sixth cleavage division. Neither Ballantine *et al.* (1979) nor Bravo & Knowland (1979) reported the synthesis of a cleavage-specific protein during *Xenopus* embryogenesis. On the other hand, Bravo & Knowland (1979) detected several proteins whose synthesis began at cleavage.

After the seventh cleavage division (128-cell stage) the synchrony of cleavage divisions begins to diminish. The cleavages at animal and dorsal sides becomes slightly faster than those in other areas of both axolotl (Bordzilovskaya & Dettlaff, 1979) and *Xenopus* (Nieuwkoop & Faber, 1956) embryos. In addition, soon after, at the 12th cleavage division in *Xenopus* and the 11th cleavage divison in the axolotl, events of the mid-blastula transition occur which includes transcription initiation and blastomeres adopting variable G1 phases (Newport & Kirschner, 1982). It is possible that these cleavage-specific proteins are involved in maintaining synchronous cell divisions during the first few cleavage divisions.

The most significant developmental difference in the axolotl gel patterns was observed at gastrulation. The synthesis of approximately 10% of the proteins found in pregastrula stages ceased at the onset of gastrulation. Another 10% of the proteins synthesized during gastrulation represent new proteins found at all later stages of development. It was also observed that axolotl embryos synthesized a gastrula-specific protein (G1). Preliminary results have indicated that G1 synthesis may be restricted to vegetal mesoderm and/or endoderm. G1 was found not to be synthesized in ectodermal explants from axolotl gastrulae. These explants, however, underwent all of the other changes in protein synthesis patterns found in intact embryos.

Few changes in protein synthesis have been recognized during *Xenopus* gastrulation. Smith & Knowland (1984) have recently shown that proteins specific to dorsal regions were synthesized for the first time at gastrulation. They also identified three proteins which were synthesized earlier and became regionalized at gastrulation. Ballantine *et al.* (1979), as well as this laboratory (unpublished observation), found only a few novel non-oogenetic proteins synthesized during *Xenopus* gastrulation.

Differences in gel patterns between axolotl and *Xenopus* may be correlated with differences in gastrulation between the two species. In *Xenopus* embryos the mesoderm is found in the deep cell layers of early gastrula (Keller, 1975). In axolotls, the mesoderm is located in the superficial cells of early gastrula (Smith & Malacinski, 1983). Because of this difference in the location of presumptive mesoderm, the movements which bring the mesoderm to its final position between endoderm and ectoderm also differs (Keller, 1976). This might be reflected in the differences between these two species in the manner in which 2-D gel patterns change at gastrulation.

The synthesis of actin was also monitored during axolotl embryogensis. The onset of axolotl α-actin synthesis was first detected at the head process stage (stage 23/25). This confirms previous studies by Mohun *et al.* (1980). *Xenopus* α-actin synthesis has been shown to first appear at gastrulation (Ballantine *et al.* 1979; Sturgess *et al.* 1980). It is not easy to explain muscle-specific α-actin synthesis in the somitic mesoderm in *Xenopus* embryos at a time when the primary germ layers are first being specified. Mohun *et al.* (1980) speculated that *Xenopus* embryos precociously synthesize α-actin because they have a faster rate of development than most species of amphibians. *Xenopus* embryos were postulated to preload prospective somite cells with α-actin for later muscle cell differentiation. Axolotl embryos have a much slower rate of development and therefore perhaps do not prepare for muscle cell differentiation to the same extent as *Xenopus* embryos. Anticipatory synthesis of a protein is not without precedence in *Xenopus*. Large amounts of cytokeratins, proteins characteristic of epithelial differentiation, accumulate in *Xenopus* oocytes. These proteins were found to be continually synthesized during oogenesis and early embryogenesis (Franz *et al.* 1983). It would be interesting to determine whether other species of amphibians with rapid developmental rates similar to *Xenopus* synthesize α-actin early in embryogenesis.

In conclusion it can generally be stated that the overall pattern of protein synthesis between *Xenopus* and axolotl embryos is similar. No change in protein synthesis patterns occur during oocyte maturation *in vitro* in either *Xenopus* or axolotl oocytes. Several proteins cease detectable synthesis in both axolotl and *Xenopus* unfertilized eggs, only to reappear later in development. For both axolotl and *Xenopus* embryos, novel non-oogenetic proteins can be detected for most stages after fertilization, with the synthesis of several of those proteins restricted to specific regions of the embryo. Some changes in protein synthesis patterns during amphibian embryogenesis may, however, be species specific. Changes in protein gel patterns at gastrulation are markedly different between *Xenopus* and axolotl. A pronounced increase in the incidence of novel non-oogenetic protein synthesis was detected at the onset of neurulation and neural fold fusion in axolotl embryos but not in *Xenopus* embryos. Finally, the onset of α-actin synthesis, an indication of muscle cell differentiation, is markedly different between axolotl and *Xenopus* embryos.

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