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Protein synthetic patterns of tissues in the early chick embryo

ROBIN H. LOVELL-BADGE

MRC Mammalian Development Unit, Wolfson House (University College London), 4 Stephenson Way, London NW1 2HE, U.K.

MARTIN J. EVANS

Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, U.K.

AND RUTH BELLAIRS

Department of Anatomy and Embryology, University College London, Gower Street, London WC1 6BT, U.K.

SUMMARY

Tissues dissected from early chick embryos were labelled *in vitro* with [^{35}S]methionine, and their patterns of polypeptide synthesis investigated using the technique of two-dimensional (2-D) polyacrylamide gel electrophoresis. Apart from providing a preliminary description of the molecular changes associated with the processes of gastrulation and segmentation in the chick embryo, this study has revealed a number of polypeptides that may be useful as markers of cell type or function.

The protein synthetic patterns of hypoblast from early and late gastrulae (stages 2 and 4, respectively: Hamburger & Hamilton, 1951) and of definitive endoblast and junctional endoblast from late gastrulae all resemble one another closely, but differ markedly from that of the epiblast at either stage. The lower layer tissues are characterized by the presence of eleven polypeptides that are largely absent from the epiblast. These findings are discussed with reference to current theories on the origins of the lower layer tissues.

Comparisons between the 2-D patterns for tissues dissected from gastrulae and from embryos undergoing segmentation (stage 12) have revealed ten polypeptides showing stage-specific rather than tissue-specific expression. Apart from these ten polypeptides, the 2-D patterns for epiblast and ectoderm were practically identical, and distinguishable from those of other tissues by a lack of any unique polypeptides. On the other hand, stage-4 endoblast and stage-12 endoderm differed in the expression of many polypeptides.

One polypeptide was found that may be considered as a marker of mesodermal cell type, as it was present in lateral plate, segmental plate and somitic mesoderm, but not in tissues of the other germ layers. Lateral plate could be distinguished from the other mesodermal tissues in the expression of a number of polypeptides, but the similarity in the 2-D patterns for segmental plate and somites suggest that the separation of somites from the anterior end of the segmental plate is not accompanied by the synthesis of new polypeptides.

Key words: Chick embryo, 2-D electrophoresis, polypeptide synthesis, endoderm, somites, ectoderm.

INTRODUCTION

Two-dimensional (2-D) polyacrylamide gel electrophoresis is a useful technique both for revealing polypeptides specific to particular cell types, and for giving an overall impression of the extent of changes in gene expression that occur in developing systems. The technique has been used in analyses of development in a number of species, such as in the sea urchin (Bedard & Brandhorst, 1983), frog (Ballantine, Woodland & Sturgess, 1979) and extensively in the mouse, covering stages from oogenesis to organogenesis (Van Blerkom & McGaughey, 1978; Handyside & Johnson, 1978; Evans, Lovell-Badge, Stern & Stinnakre, 1979; Johnson & Rossant, 1981; Van Blerkom, Janzen & Runner, 1982). In the present investigation we have used 2-D electrophoresis to study some of the molecular events occurring in chick embryogenesis.

Our main aim has been to seek specific polypeptides which might arise as tissues differentiate in the early embryo. We have confined this analysis, therefore, to tissues taken from early gastrulae, late gastrulae and somite stages. In the first place we have looked at the derivation of the embryonic endoderm. By the time that the hen's egg is laid the *area pellucida* already consists of two layers, though the lower one is not yet complete. The upper one is the epiblast, the lower is the hypoblast. Subsequently the hypoblast layer is invaded by other tissues, the definitive endoblast and the junctional endoblast. It is generally accepted that the fate of the hypoblast is to become the extraembryonic endoderm which lines the yolk sac stalk, whilst that of the definitive endoblast and junctional endoblast is to form the embryonic endoderm (see discussion by Bellairs, 1982). There is, however, less agreement as to the precise origin of these tissues and of their relationship to one another (Stern & Ireland, 1981; Bellairs, 1982). We have therefore compared the protein synthetic patterns in the hypoblast, definitive endoblast, junctional endoblast and the epiblast. Apart from the possibility of revealing some polypeptides that may be useful as markers of cell type or function it was hoped that the results would clarify any such relationship between the tissues. Secondly, the polypeptide patterns obtained from endoderm and ectoderm of somite stage embryos have been related to those of the endoblast and epiblast of the gastrula stages, specifically to look for lineage markers. Finally, we have studied the various types of mesoderm at the time of somite formation, since it is possible to separate newly formed somites, segmental plate (which is destined to give rise to somites) and lateral plate (which does not give rise to somites). 2-D polypeptide patterns from these tissues have been compared, and also related to those from tissues of the other germ layers.

MATERIALS AND METHODS

Embryonic tissues

Eggs (White Leghorn) were supplied by Winter Egg Farms, Fowlmere, Cambridge.

Embryos at stages 2, 4 and 12, as defined by Hamburger & Hamilton (1951), were explanted into cold HEPES buffered Tyrodes solution and dissected with tungsten needles. Some mild trypsin treatment was necessary to obtain a clean dissection of the stage-12 embryos, but not of the two younger stages. Eight stage-2 embryos were dissected into hypoblast and epiblast (see Fig. 1A); eight stage-4 embryos were dissected into hypoblast, junctional endoderm, definitive endoblast and (mesoderm-free) epiblast, attempts being made to avoid regions where the tissues overlapped one another (see Fig. 1B). The posterior halves of twelve stage-12 embryos were dissected into endoderm, segmental plate, somites, lateral plate and ectoderm (see Fig. 1C).

Cell labelling and sample preparation

The dissected tissues derived from four to six embryos were pooled and placed in wells of Terasaki multiwell dishes (Falcon plastics, type 3034), and the Tyrodes solution was replaced with 2·5 μl of methionine-free Dulbeccos modified Eagles medium (DMEM) supplemented with insulin (1 μg/ml) and transferrin (5 μg/ml). They were incubated in this medium for 1 h at 37°C (5 % CO_2, 95 % humidity) to deplete endogenous methionine pools. This pre-incubation was found to increase TCA precipitable counts approximately 40-fold (results not shown). The medium was pipetted off and replaced with 2·5 μl of labelling medium made up as follows: three parts 1·33-strength methionine and potassium-free DMEM (with insulin and transferrin) plus one part neat [^{35}S]methionine as supplied by Amersham (SJ 204; 800 Ci/mmol). This gave final concentrations of about 1·6 μM-methionine (1·25 mCi/ml) and 5mM-potassium. They were then incubated for a further 3 h at 37°C.

The medium was withdrawn and the cells taken up in a total of 10 μl SDS lysis buffer (9·5 M-urea, 2 % Ampholines, 0·5 % SDS, 5 % β-mercaptoethanol). 0·5 μl aliquots were taken for determining TCA-precipitable counts, and the remainder stored frozen at −70°C.

Total counts ranged from $1{\cdot}1\times10^5$ to 2×10^6 (mean $7{\cdot}6\times10^5$). Aliquots containing approximately 5×10^5 c.p.m., or the whole sample if less, were diluted to 15 μl with SDS lysis buffer, and then 20 μl triton lysis buffer added (recipe as for SDS lysis buffer but with 4 % triton X-100 instead of SDS) before loading onto isoelectric focussing gels.

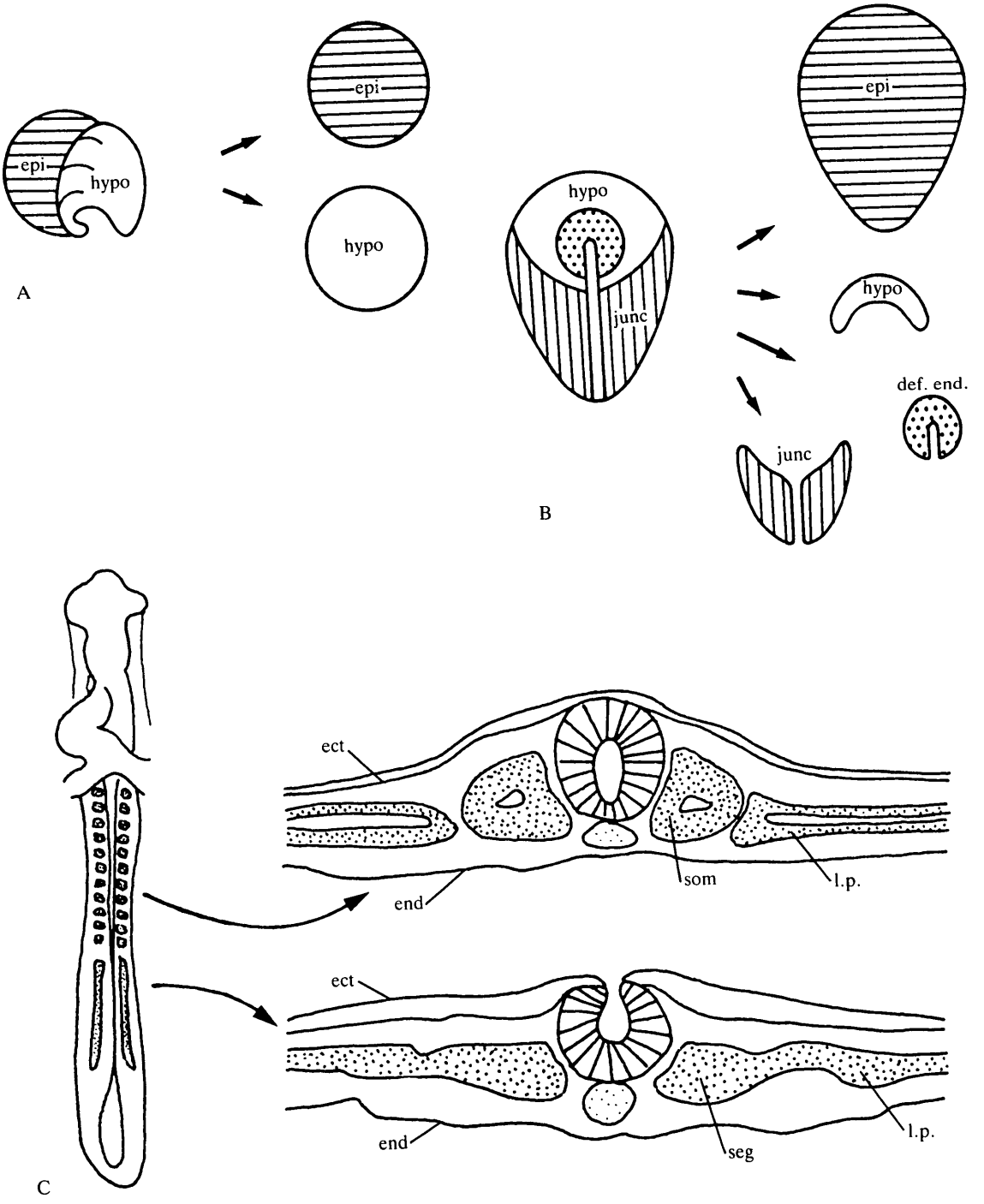
2-D electrophoresis

The proteins were separated into two dimensions by isoelectric focussing followed by SDS polyacrylamide gel electrophoresis essentially as described in Lovell-Badge & Evans (1980), except that the focussing gels were run at a constant power of 1·4 W for 16 h followed by 1·7 W for 2·5 h, and then equilibrated with two changes of 10 ml equilibration buffer for 30 min before being stored frozen at −70°C. This was found to improve separation in the second dimension. The fixed, washed gels were impregnated with PPO for autofluorography, dried under vacuum and exposed to preflashed Fuji RX medical X-ray film at −70°C (Laskey & Mills, 1975), by vacuum packing them together in black polythene bags (Lovell-Badge, 1978).

Comparisons are based on at least two separate samples of each cell type, and where possible two gels of the same sample.

RESULTS

High-resolution 2-D electrophoresis was used to compare the polypeptides synthesized during 3 h labelling periods of the tissues dissected from early chick embryos. A total of about 800 polypeptide spots were compared for each of the gels and differences in the expression of some 27 of these between the various tissues have been identified. It is possible that a few quantitative and qualitative differences have been missed due to a limited problem of irreproducibility. This


epi
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end
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C

Fig. 1.

consisted of variation in intensity or absolute position, of a small number of polypeptide spots in a characteristic but not tissue-specific manner; a problem that has been encountered by other workers (e.g. see Bedard & Brandhorst, 1983). In our case, this was perhaps due to differing amounts of yolk proteins in the samples. Unless otherwise stated, the polypeptides discussed below demonstrated consistent and reproducible differences between tissues. These polypeptides are indicated by arrows in Figs 2–4; a summary of the results and our interpretation of whether differences are qualitative or quantitative is shown in Table 1.

Fig. 2 shows representative gels from stage-2 hypoblast and epiblast. A number of major differences are seen, in particular the polypeptide spots labelled 1–6, 10 and 11 which are very abundant in hypoblast. Spots 7, 8 and 9 are also present in higher amounts in hypoblast. It is interesting to note that there are no polypeptides specific to the epiblast.

The 2-D patterns shown by stage-4 hypoblast and epiblast are essentially the same as that from the stage-2 tissues (gels not shown). Gels from endoblast and junctional endoderm are shown in Fig. 3. They are very similar to each other, and to the hypoblast, possessing all the abundant polypeptides and other differences that distinguished it from epiblast.

Representative gels from the various tissues dissected from stage-12 embryos are shown in Fig. 4 (A–E). There are few novel polypeptides synthesized at this stage. Spot 14 is fairly abundant in endoderm, but is also present in lateral plate (and weakly in stage-2 hypoblast). Spots 15 and 16 were found in only one of the two samples of segmental plate, so, without further experimentation, there is doubt as to their validity as markers, although they were absent from all other tissue samples. There is, however, one polypeptide, spot 27, that may be characteristic of mesoderm as it is present in all three mesodermal tissues, but absent from all the others studied. The endoderm has lost the abundant polypeptides that were characteristic of the lower layer, including endoblast, in the earlier stage embryos (spots 1–7 and 10), although it retains some of the others (8, 9 and 11), as does the lateral plate mesoderm (8 and 9). Two other polypeptides, 12 and 13, present in all tissues, are much more abundant in both endoderm and

Fig. 1. (A) Diagram of the area pellucida of stage-2 embryo to show dissection into epiblast (epi) and hypoblast (hypo). The area opaca was discarded. (B) Diagram of the area pellucida of stage-4 embryo to show dissection into epiblast (epi), hypoblast (hypo), definitive endoblast (def. end.) and junctional endoblast (junc.). The area opaca was discarded. (C) Diagram of a stage-12 embryo, together with diagrams of transverse sections at two levels. The following tissues were dissected: endoderm (end.), segmental plate (seg.), posteriorly situated somites (som.), lateral plate mesoderm (l.p.) and ectoderm (ect.).

Fig. 2. Two-dimensional protein patterns from (A) epiblast and (B) hypoblast both from stage-2 embryos.

Fig. 3. Two-dimensional protein patterns from (A) definitive endoblast and (B) junctional endoblast both from stage 4 embryos.

Fig. 4. Two-dimensional protein patterns from (A) ectoderm, (B) endoderm, (C) segmental plate, (D) somites and (E) lateral plate, all from stage-12 embryos.

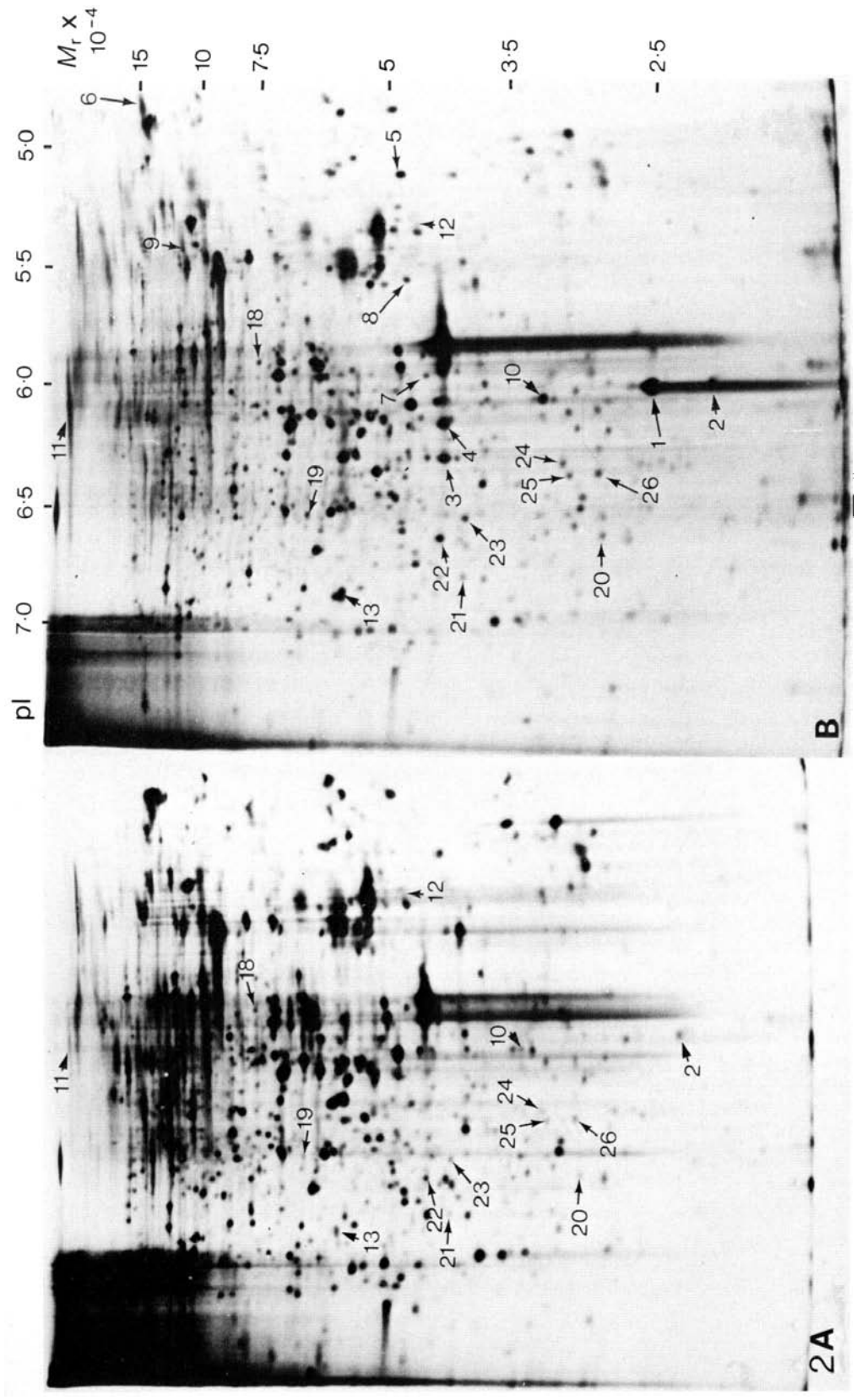

Fig. 2. For legend see p. 69.

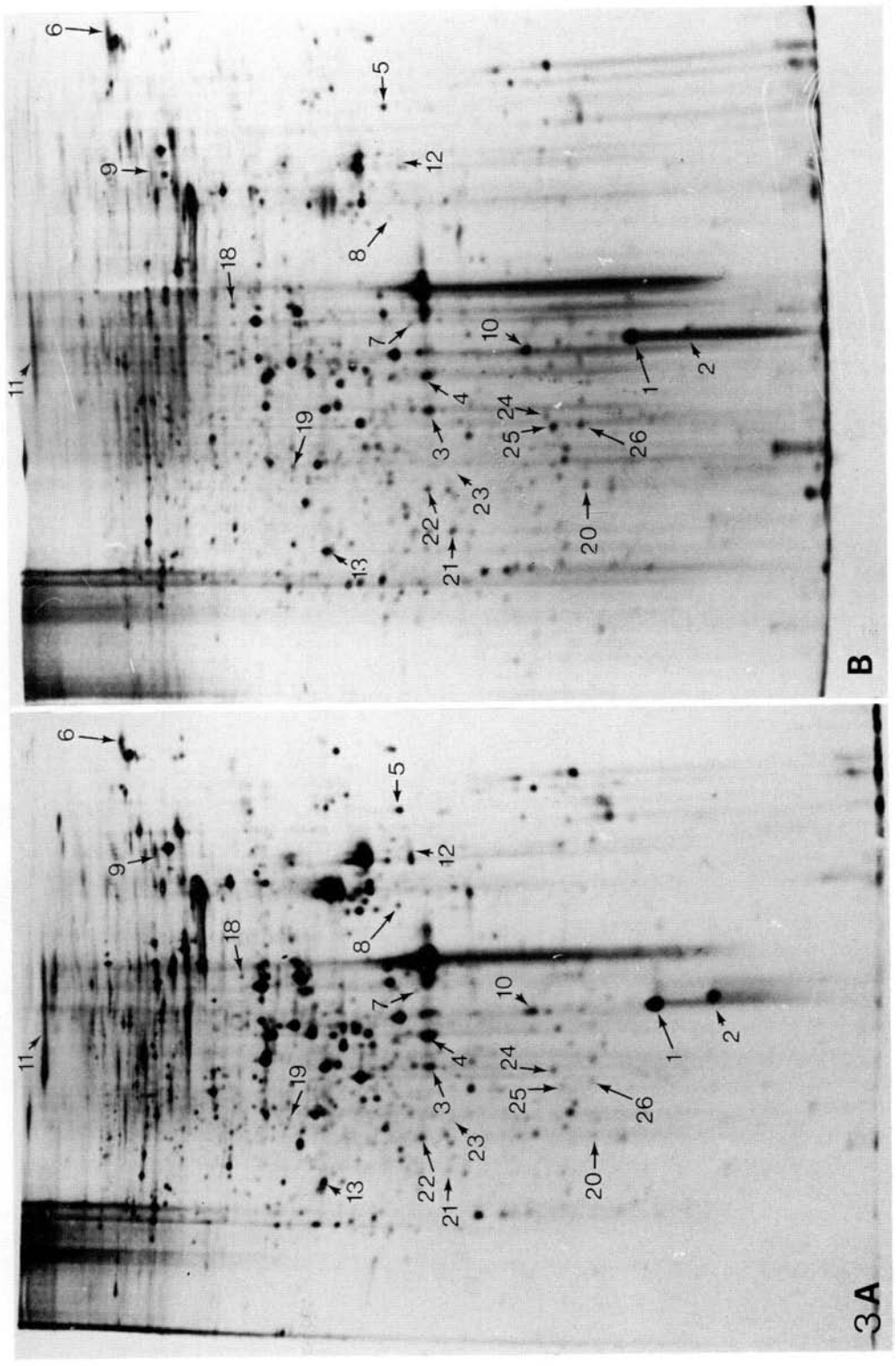

Fig. 3. For legend see p. 69.

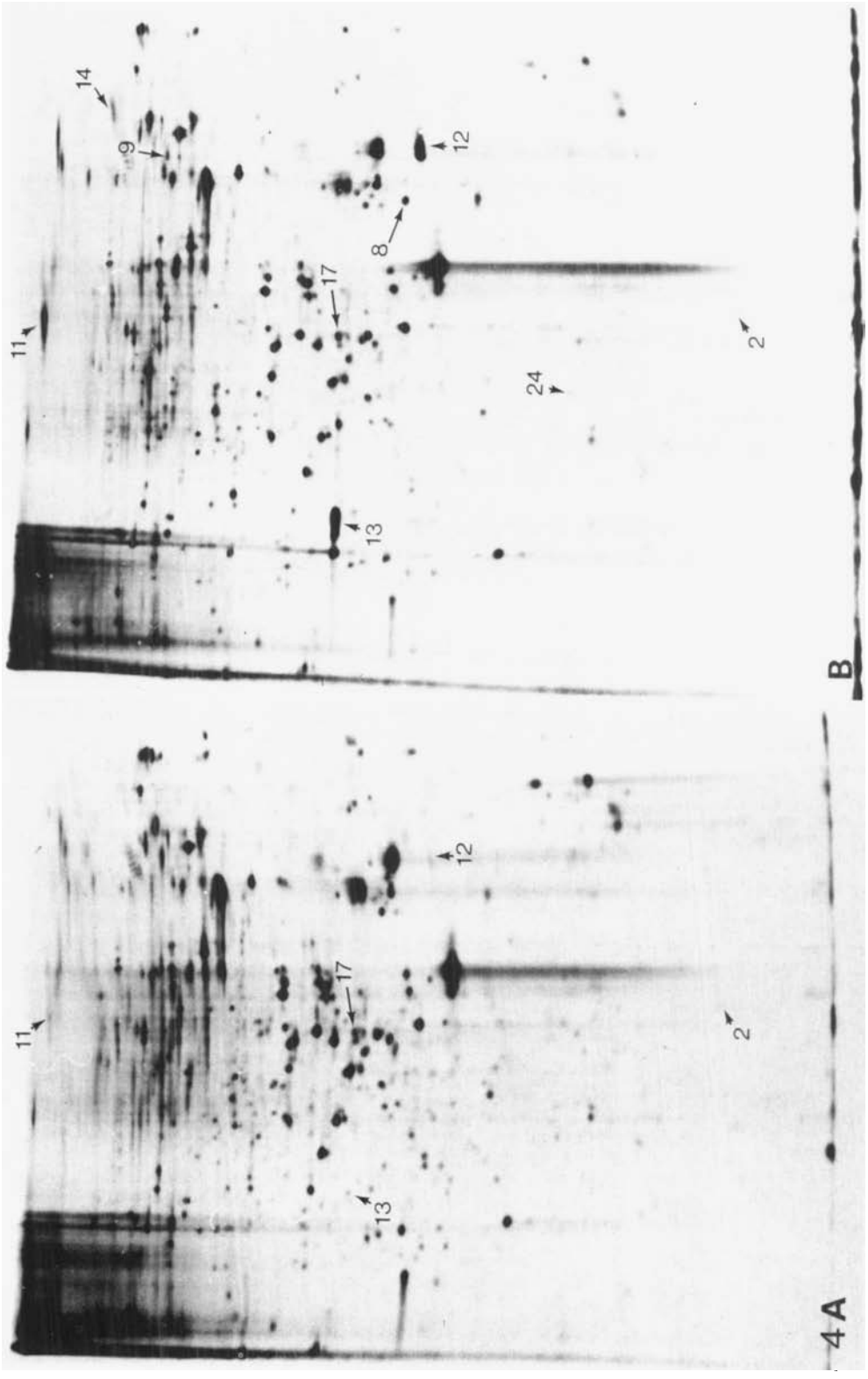

Fig. 4. For legend see p. 69.

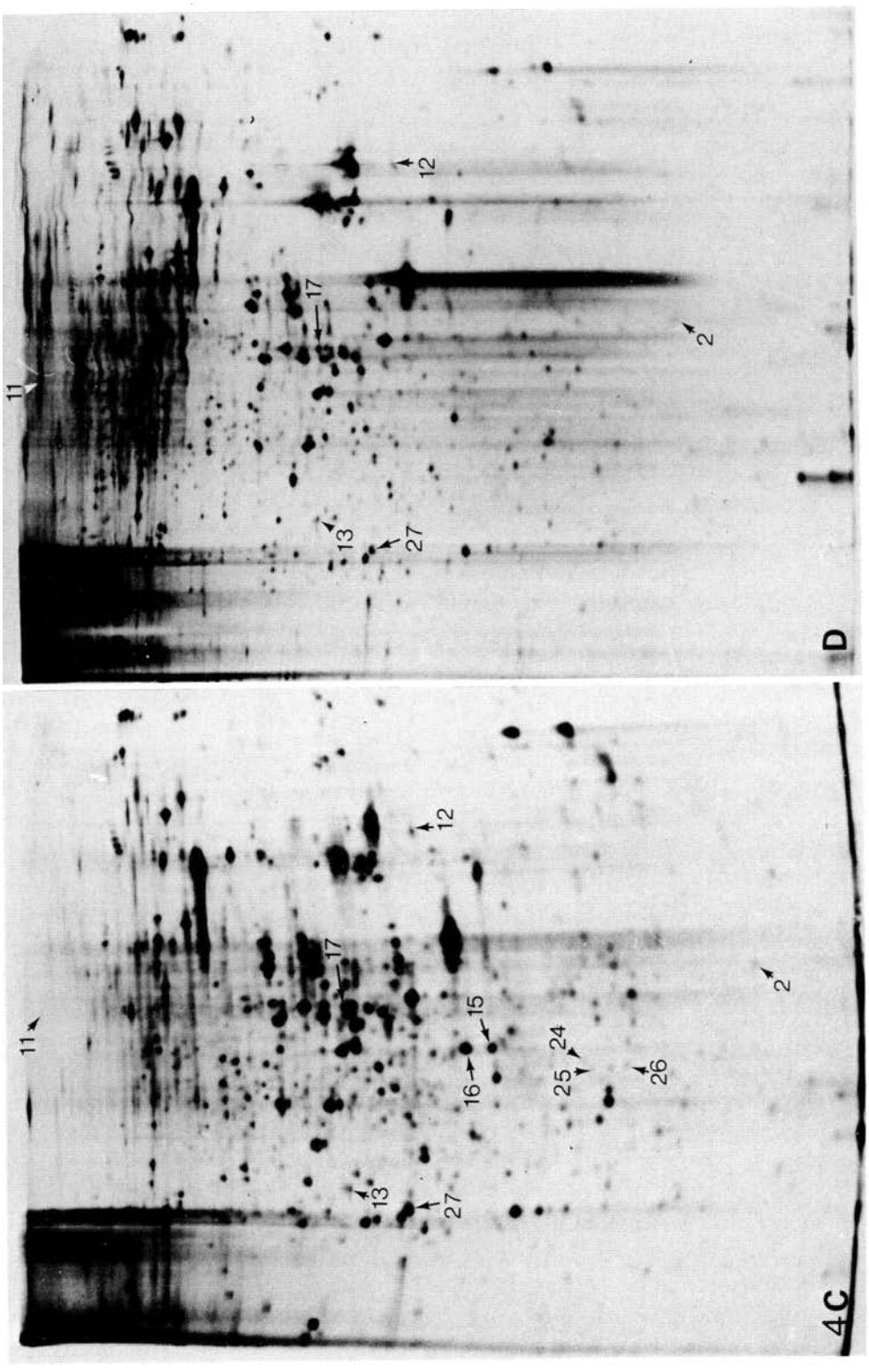

Fig. 4. For legend see p. 69.

lateral plate mesoderm and there is one polypeptide – numbered 17, present in all the stage-12 tissues, that was completely absent from the earlier stages.

All the other differences between stage 12 and the earlier tissues involve the loss or reduction in intensity of spots that were previously ubiquitous. Somites and ectoderm lack all of these (spots 18–26), endoderm lacks all but trace amounts of 24, and segmental and lateral plate mesoderm lack 18–23, and have only trace amounts of 24–26.

The differences in polypeptide synthesis between all the stages and tissues studied are summarized in Table 1. From this it is readily seen that epiblast and ectoderm are characterized by a lack of any 'specific' polypeptides. This is also exhibited to some extent by the mesodermal tissues, in particular the somites. The lateral plate, however, can be distinguished from the other mesodermal tissues by the presence of polypeptides 8, 12 and 13, and the segmental plate with 15 and 16 (given the reservations expressed above), and the mesodermal tissues in general may be distinguished from others by the expression of polypeptide 27.

DISCUSSION

Using the technique of two-dimensional electrophoresis we were able to resolve at least 800 newly synthesized polypeptides in each of the tissues dissected from early chick embryos. A comparison of these patterns of synthesis has revealed qualitative or large quantitative changes in the expression of a total of 27 polypeptides amongst the range of tissues studied. This number is seemingly small compared to the extent of differences between the cell types in terms of their morphology, behaviour and commitment. It must be remembered, however, that the gels only allow detection of a subset of polypeptides, either because they fall outside the pI and relative molecular mass ranges resolved, or because they are in the low abundance class of proteins (see Lovell-Badge & Evans, 1980 and Bedard & Brandhorst, 1983 for discussion). The differences between the excluded polypeptides may be significant. It is also possible that minor quantitative changes in polypeptide synthesis are important in determining tissue characteristics, but the application of accurate quantitative methods of comparison to gels run under highly reproducible conditions is essential if this is to be investigated (Garrels, 1979). Many workers have commented on the similarities in patterns of protein synthesis amongst embryonic tissues (see, for example, Van Blerkom *et al.* 1982 and Evans *et al.* 1983). However, any difference found may be assumed to be relevant and potentially useful to the study of differentiation.

One of the main findings in this investigation is that the protein synthetic patterns of the lower layer samples resemble one another closely, irrespective of whether they were of hypoblast, definitive endoblast or junctional endoblast, and of whether they were dissected from the early or the late gastrula. Moreover, these patterns all differ markedly from those of the upper layer, the epiblast. The lower layer tissues are characterized by the presence of 11 polypeptides that are largely

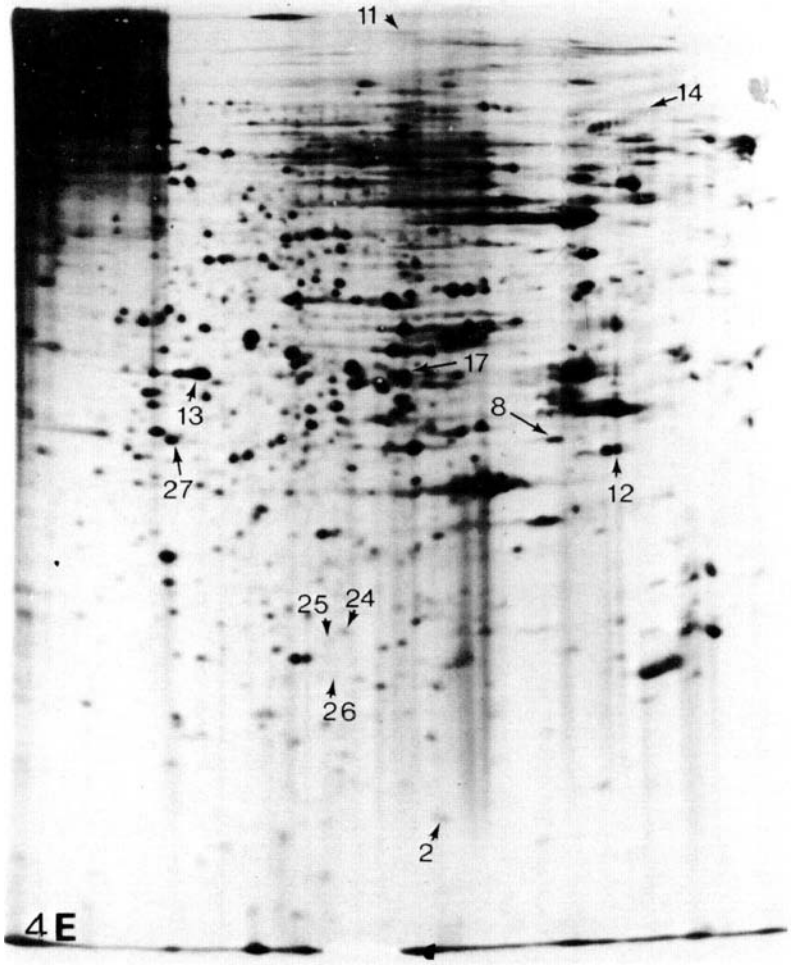

Table 1. *The tissue distribution and estimated relative abundance of polypeptides 1–27 in the early chick embryo*

	Stage 2		Stage 4				Stage 12				
	hypo	epi	hypo	def junc	end	epi	end	l.p.	seg	som	ect
1	+++		+++	+++	+++						
2	+++	++	+++	+++	+++	+	+	+	+	+	+
3	+++		+++	+++	+++						
4	+++		+++	+++	+++						
5	++		++	++	++						
6	++		++	++	++			±		±	
7	+		+	+	+			±		±	
8	+		+	+	+		+	+			
9	+		+	+	+		+				
10	++	±	++	++	++	±					
11	+++	+	+++	+++	+++	+	+++	+	+	++	+
12	+	+	+	+	+	+	+++	+++	+	+	+
13	+	+	+	+	+	+	+++	+++	+	+	+
14	±						++	+			
15									(++)		
16									(++)		
17							++	++	++	++	++
18	+	+	+	+	+	+					
19	+	+	+	+	+	+					
20	+	+	+	+	+	+					
21	+	+	+	+	+	+					
22	+	+	+	+	+	+					
23	+	+	+	±	±	+					
24	+	+	+	+	+	+	±	+	±		
25	+	+	+	+	+	+		±	±		
26	+	+	+	+	+	+		±	±		
27								++	++	++	

±, just detectable, +++, very abundant.

Fig. 4. For legend see p. 69.

absent from the epiblast. On the other hand no polypeptides unique to the latter were found, and in this context it is interesting to note that Wolk & Eyal-Giladi (1977) found that the hypoblast possessed some specific antigens whilst the epiblast did not.

There are several possible explanations for the close similarity we have found between the hypoblast, definitive endoblast and junctional endoblast. The first is that these are not discrete tissues, but that there is some intermingling of their cells *in situ*. Indeed, Stern & Ireland (1981) have suggested as a result of chick/quail grafting experiments that there is some intermingling of definitive endoblast and junctional endoblast in the centre of the *area pellucida*. Although this might result in an overlap of the 2-D patterns obtained for these two tissues, it would, however, not account for the fact that they each resemble the pattern for the hypoblast.

A second possibility is that, contrary to generally held views, all the lower layer cells have a common origin. It is usually considered that the hypoblast cells form by delamination or polyinvagination from the overlying epiblast, though these ideas are based predominantly on morphological evidence and have never been tested satisfactorily by experiment (discussed by Bellairs, 1982). Some investigators have, however, suggested that many, if not all, of the cells of the hypoblast arise by migrating forward from the marginal region at the posterior end of the *area pellucida* (Spratt & Haas, 1961; Vakaet, 1970). Subsequently, it is from this region that the junctional endoblast is also derived (Stern & Ireland, 1981) and it is even possible that it is the source of the definitive endoblast. The latter is formed from cells which migrate from the primitive streak (Vakaet, 1962; Nicolet, 1970; Fontaine & Le Douarin, 1977; Vakaet, 1970), but there is no clear evidence as to the origin of these cells. It is often implied that they have entered the primitive streak by ingression from the epiblast, but it is also possible that they have never been in the epiblast but have been carried forward in the middle layer of the primitive streak as it formed. The primitive streak itself is derived from the marginal zone (Spratt & Haas, 1961; Azar & Eyal-Giladi, 1979).

The third possible explanation is that cells which have come to lie in the lower layer of the *area pellucida* acquire certain characteristics irrespective of their origin. These could be polypeptides associated with yolk metabolism or proximity to the subgerminal fluid, or could be related to some aspect of behaviour common to cells of this lower layer. Many of these polypeptides are very abundant (e.g. spots 1–6) and would therefore reflect quite significant changes in gene activity over quite a short time period (about 12 h), between the early and late gastrula, if cells of the junctional and definitive endoblast do indeed originate from the epiblast. Whether such changes in gene activity are a result of regulation of transcription, translation or of post-translational modification could be investigated by carrying out *in vitro* translation of mRNA isolated from these tissues (see Van Blerkom, 1981 and Evans *et al.* 1983).

The polypeptides characteristic of the lower layer may prove to be useful as biochemical markers of the tissues involved in gastrulation, and, given means of

detecting them *in situ* (for example, antibodies) it may be possible to distinguish between the above three alternative explanations for their presence in the endoblast tissues.

The definitive endoderm of stage-12 embryos might be expected to share characteristic polypeptides in common with stage-4 endoblast. However, they differ in 21 out of the 27 spots identified as showing changes in expression during this, 28 h, period (see Table 1). Of the three polypeptides they do share (8, 9 and 11) only spot 9 can be considered specific to the endodermal lineage as the other two are also found in mesoderm. On the other hand, at least seven of the differences between endoderm and endoblast are due to polypeptides whose expression may be considered as stage specific. Thus spot 17 is found in all stage-12 tissues examined, but in none of the stage-2 or -4 tissues, and 18–23 show the inverse distribution. Polypeptides 24–26 also show stage specificity to some extent as they are absent or only just detectable in the later tissues. There are no polypeptides unique to the definitive endoderm, but it may be distinguished from all other tissues by the set of markers expressed.

Ectoderm is characterized from other stage-12 tissues, as is the epiblast in the early embryos, by a lack of 'markers'. Indeed, if the 'stage-specific' polypeptides (17–26), as discussed above, are ignored, the 2-D patterns for ectoderm and epiblast are almost identical. This may reflect a lack of specialization in the ectoderm even though the embryo at this stage is undergoing extensive morphogenesis at the start of organogenesis. In the mouse, epiblast and early ectoderm also share similar 2-D electrophoretic patterns, again characterized by a lack of marker polypeptides when compared to other cell types (Evans *et al.* 1979).

Unlike the ectoderm and endoderm of stage-12 embryos, the three mesodermal tissues studied show expression of a common novel polypeptide – spot No. 27. This is a fairly abundant polypeptide and deserves further investigation as a marker of mesodermal type.

The differences between the three types of mesoderm – somitic, lateral plate and segmental plate are of particular interest, not only because these tissues exhibit clear behavioural differences when explanted *in vitro* (Bellairs, Sanders & Portch, 1980), but also because of their bearing on the process of somite segmentation. The lateral plate does not contribute to the somites, so it is not surprising that its polypeptide pattern is different; in fact it shares spots in common with stage-12 endoderm (8, 12, 13 and 14). By contrast, the segmental plate does give rise to somites and it might be expected that segmentation would be accompanied by the synthesis of new proteins. However, apart from an apparent increase in the expression of one high relative molecular mass polypeptide(s), spot 11, no new spots were visible in the gels derived from somites. This may reflect the fact that the changes, cell shape, etc., which accompany somite segmentation (Bellairs, 1979) have already started to take place within the segmental plate (Meier, 1979; Bellairs, 1984). The separation of individual somites from the anterior end of the segmental plate is, therefore, probably not accompanied by the

synthesis of new polypeptides, at least none that are resolvable by the techniques used here.

Little is known about specific protein synthesis in the early chick embryo. Cell surface and extracellular matrix proteins, such as fibronectin, which may have a role in guiding cell locomotion, are known to be made during gastrulation, but it is not clear by which cell type(s) (Thiery, Duband & Delouvée, 1982; Sanders, 1982; Lash, 1984). Zalik and coworkers (Zalik, Milos & Ledsham, 1983) have recently identified two β-D-galactoside-binding lectins. One of these is particle associated and appears to be confined to the *area opaca*, with the highest activity found in the extraembryonic endoderm; the other, soluble lectin, is distributed throughout the embryo. The histone variants, H2B-2 and H3-3 have been found to be made for the first time at about the 1-somite stage (Urban & Zweidler, 1983); but possibly mark the appearance of slower dividing cells rather than any particular cell type. Studies with antisera (e.g. Wolk & Eyal-Giladi, 1977) have revealed a number of cell-type-specific antigens, but it is not known whether these are polypeptide or, as is frequently found in early mouse embryos, carbohydrate determinants (see Muramatsu, Gachelin, Moscona & Ikawa, 1982).

In this investigation, by using two-dimensional gel electrophoresis, we have found 27 polypeptides that show changes in expression in the early chick embryo. Although further work is necessary to characterize their function and level of control, they show promise as markers of differentiation.

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