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# Molecular differences between the rostral and caudal halves of the sclerotome in the chick embryo

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## Summary

**It is known that both neural crest cell migration and motor axon outgrowth in most vertebrate embryos are segmented because of restrictions imposed upon their distribution by the neighbouring sclerotomes, each of which is divided into a rostral and a caudal half. The caudal half does not allow crest migration or axon outgrowth, while the rostral half does. In this paper, we investigate the expression of proteins and glycoproteins in the two halves of the sclerotome of the chick embryo at stages between 20 and 32 pairs of somites by two-dimensional SDS–polyacrylamide gel electrophoresis. We find that the patterns of expression are complex, and that polypeptides and glycoproteins vary both spatially and temporally: of those that are expressed differentially by the sclerotome, some differ quantitatively and others qualitatively. Some macromolecules change their spatial distribution with developmental age, and some appear or disappear as the embryos become older.**

Key words: segmentation, 2-D gel electrophoresis, glycoproteins, chick embryo, somite, sclerotome, peripheral nervous system.

## Introduction

A striking feature of higher vertebrate embryos is their segmented body plan, which is first obvious during development in the arrangement of the somites, lying adjacent to the neural tube. Segmentation is also reflected in the pattern of the peripheral nerves as they emerge from the spinal cord, and indeed there is much evidence that it is the segmentation of the somitic mesoderm that determines the segmented pattern of the peripheral nervous system (reviewed by Keynes & Stern, 1988). Lewis *et al.* (1981) showed that in the absence of somite tissue, motor nerves grow out of the neural tube in an unsegmented fashion. Both the migration of neural crest cells and the outgrowth of motor axons occurs only through the rostral half of each sclerotome (Keynes & Stern, 1984; Rickmann *et al.* 1985); this pattern is determined by differences between rostral and caudal half-sclerotome cells, since rostrocaudal rotation of somitic tissue by 180° at an appropriate stage of development results in a reversed pattern of motor nerves (Keynes & Stern, 1984) and neural crest migration (Stern & Bronner-Fraser, in preparation). The rostral–caudal subdivision of the sclerotome also appears to play a role in modulating the interactions between the cells of the two halves of the sclerotome (Stern & Keynes, 1987) and results in the formation of an intrasclerotomal boundary, now known as von Ebner's fissure (von Ebner, 1888; Keynes & Stern, 1984).

The molecular nature of the differences between rostral and caudal half-sclerotome cells is not understood. However, the cells of the rostral half appear to express cytotactin (Tan *et al.* 1987), tenascin (Mackie *et al.* 1988) and butyrylcholinesterase activity (Layer *et al.* 1988), while the cells of the caudal half bind peanut lectin (Stern *et al.* 1986) and antibodies directed against a cytotactin-binding proteoglycan ('CTB-proteoglycan'; Tan *et al.* 1987). However, no differences in the distribution of these molecules can be detected until some time *after* the onset of neural crest migration into the rostral half-sclerotome. Nevertheless, it is possible that one or more of these molecules might influence motor nerve outgrowth. Other molecules known to influence neural crest migration *in vitro*, such as laminin, fibronectin, integrin and N-CAM show no difference in their distribution within the sclerotome (Rickmann *et al.* 1985; Krotoski *et al.* 1986; Tosney *et al.* 1986). In this study, using two-dimensional polyacrylamide gel electrophoresis and Western blotting, we report the existence of novel differences in macromolecules present in the two halves of the sclerotome of embryos at much earlier stages than those used in previous investigations.

## Materials and methods

All solutions were prepared using deionized distilled water.

### *Dissection*

Hens' eggs were incubated at 38°C to stages 13–17 (Hamburger & Hamilton, 1951) to obtain embryos with 20–32 pairs of somites. The embryos were explanted and pinned out, ventral side uppermost, on Sylgard dishes in $Ca^{2+}$- and $Mg^{2+}$-free Tyrode's solution (CMF) at 30°C. The embryos were washed twice with CMF, immersed in double-strength CMF containing 0·1% EDTA (2×CMF/EDTA) at 30°C and the sclerotome halves dissected out from both left and right sides of the embryo. These half-sclerotomes were collected into rostral and caudal pools, and deposited on the embryo; collections from six somites at a time were transferred to Eppendorf tubes containing 200 μl CMF at 4°C. Half-sclerotomes were not collected from the most-rostral five pairs of somites (occipital) or the most-caudal five to six (epithelial) pairs of somites. The sclerotome halves were then washed with cold CMF (4°C) by centrifugation for 5 min at the 'low' speed in an MSE Micro Centaur centrifuge. The cell pellets were then resuspended in cold CMF containing 1 mM-PMSF and centrifuged at 'high' speed (11 600 *g*) for 5 min. The final pellets were fast frozen on solid $CO_2$ in 10 μl of CMF containing 1 mM-PMSF and 1 mM-EDTA and stored at −20°C until required.

### *2D-gel electrophoresis*

Apart from Trizma base (Sigma), reagents used for preparing the gels were of electrophoretic (BioRad) or isoelectrophoretic grade (NP-40, ampholines, agarose: LKB).

#### *Sample preparation*

To each cell pellet was added, at room temperature: 60 μl of 3 mM-EDTA containing 1·5 mM-PMSF, 100 μl of lysis buffer (9·5 M-urea, 10% NP-40, 1·6% ampholines pH 5–7, 0·4% ampholines pH 3·5–10, 5% mercaptoethanol) and the equivalent of 100 μl of lyophilized buffer (9·5 M-urea, 1% SDS). The cell pellet was dispersed in this solubilization buffer by three passages through a 100 μl Hamilton microsyringe. Each sample was then airfuged at 100 000 *g* (30 p.s.i.) for 1 h at room temperature. The supernatants were then assayed for protein content using a modification of the Bramhall *et al.* (1969) assay. This involved drying protein samples onto filter paper squares, staining with Coomassie blue R-250, elution into 66% methanol/1% ammonia and reading the optical density (OD) at 560 nm. A standard curve was constructed from known amounts of BSA. Supernatants were stored frozen at −20°C for not longer than one week prior to use.

For gels of neural crest cells, neural tubes were cultured on tissue culture plastic as described previously (Stern *et al.* 1986). After 24 h, the cultures were washed with CMF, the neural tube removed and the emigrated crest cells scraped off their substrate in the presence of 1 mM-PMSF. Eight neural tube cultures were used per gel.

#### *Electrophoresis*

Isofocusing gels were prepared and run as described by O'Farrell (1975) with modifications. 5 ml of 4% w/v polyacrylamide gel solution containing 2% ampholines (1·6% pH 5–7; 0·4% pH 3·5–10) were degassed *in vacuo* for 1 min, and then 10 μl of 10% ammonium persulphate and 5 μl of TEMED added. Using a 1 ml disposable syringe fitted with surgical tubing, the solution was used to fill glass tubes 130 mm long and 1 mm internal diameter sealed at their base with Nescofilm. The tubes were filled up to an 80 mm mark and then overlaid with either deionized water or overlay buffer [9 M-urea, 2% NP-40, 2% ampholines (0·8% pH 5–7: 0·2% pH 3·5–10)]. After sealing the top of each tube with Nescofilm, they were allowed to gel overnight at room temperature.

Prior to use, the overlay solution was removed and the base of the gel sealed with dialysis membrane secured by latex rings. The gels were overlaid with overlay buffer and degassed 50 mM-NaOH, and prefocused at 200 V for 1 h, with 50 mM-NaOH in the upper (−) reservoir and 10 mM-$H_3PO_4$ in the lower (+) reservoir. The overlay buffer and NaOH were then removed and up to 40 μl of sample applied to each gel, followed by fresh overlay buffer and NaOH. The sample contained 5 μg of cell protein plus 2 μl of 1× stock carbonic anhydrase (Carbamylyte) pI reference peptides. The upper reservoir was refilled with fresh degassed NaOH and the gels focused for 17 h with 400 V at room temp. On one gel per run, sample was substituted with solubilization buffer containing no protein. This gel was later divided into 1 cm lengths and each length eluted into 0·5 ml degassed deionized water for 30 min at room temperature. The pH of each elution was used to construct a pH profile.

Following removal of the gels by extrusion onto Nescofilm, the bottom (+) of each gel was marked by insertion of an insect dissection pin. All gels, except for the one for pH profile, were then dehydrated sequentially by 5 min incubations in 25% ethanol, 50% ethanol and 100% ethanol. This procedure both removed the ampholines and fixed the proteins. The dehydrated gels were stored in 100% ethanol in screw-top jars at −20°C.

The second dimension SDS–PAGE was performed on 4–15% acrylamide 0·5 mm thick minigels (9 cm × 9 cm), using the Laemmli (1970) buffer system in apparatus of the Matsudaira & Burgess (1978) design. Using a perspex trough and comb, agarose (1% IEF agarose, 0·125 M-Tris–HCl, pH 6·8) wells for the isofocused gel and molecular weight standards were made atop the minigels. The isofocused gel was equilibrated for 10 min in 10 ml of sample buffer (0·0625 M-Tris pH 6·8, 10% SDS, 10% glycerol, 0·25 mg $ml^{-1}$ DTT and 50 μg $ml^{-1}$ bromophenol blue). The equilibrated gel was placed in the agarose well so that the top (−) was adjacent to the molecular weight standard well(s) and fixed in place with more agarose (pH 6·8). 1–2 μl of standards ($^{14}C$ rainbow markers, diluted 1:3; Amersham) were placed in the smaller well(s). Electrophoresis was performed at 150 V for 90 min, or until the tracking dye was 1·5 cm above the bottom of the gel.

#### *Silver staining*

This was performed according to the method of Morrissey (1981) except that 15–20 min incubations were used.

#### *Immunoblotting*

Western blots were prepared from the 2D-gels by transfer for 90 min to Immobilon membrane (Millipore) in the continuous buffer system (39 mM-glycine, 48 mM-Tris, 0·0375% [w/v] SDS, 20% methanol) with the Novablot semidry apparatus (LKB). Transfer was checked by staining with the reversible protein stain Ponceau S (BDH). The blots were then blocked by incubation for 30 min in 0·5% Tween 20/10 mM-Tris/150 mM-NaCl (pH 7·4), washed with buffer A (50 mM-Tris/150 mM-NaCl, pH 7·4, containing 0·5 mM-$CaCl_2$ and 0·5 mM-$MnSO_4$) and then incubated in 20 μg $ml^{-1}$ of Concanavalin A (ConA, Miles) in buffer A containing 0·05% Tween 20 and 5 mg $ml^{-1}$ haemoglobin (crystalline, BDH) for 2 h at room temperature. After three washes with buffer A/Tween 20, the blots were incubated for 2 h with 20 μg $ml^{-1}$ of horseradish peroxidase (Sigma), diluted as for ConA. After three further

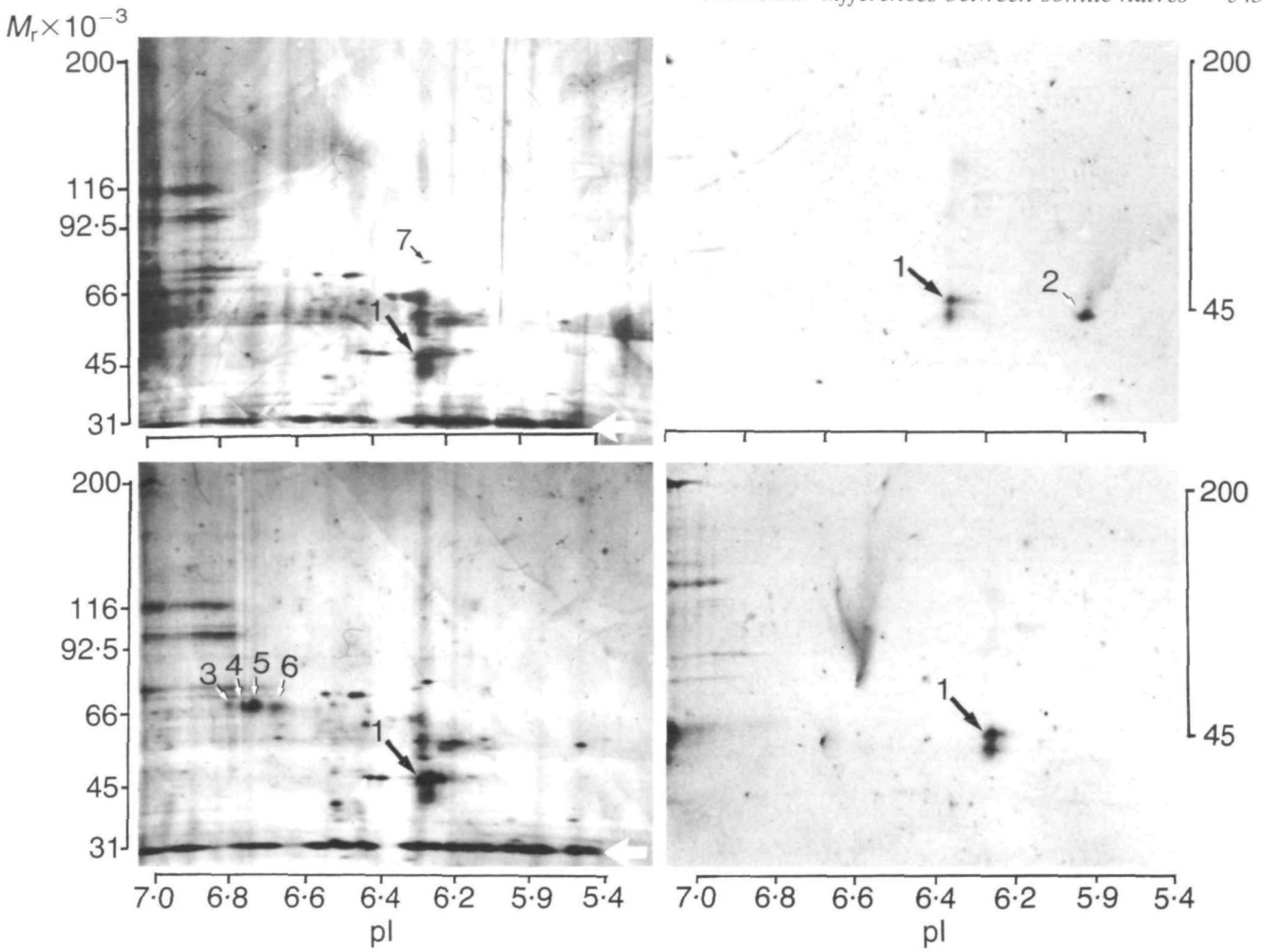


**Fig. 1.** 2-D gel analysis of rostral and caudal half-sclerotomes from chick embryos with 20 pairs of somites. Top photographs, rostral; Bottom photographs, caudal. Left photographs, silver staining; Right photographs, ConA-probed blots. The large white arrowhead marks the position of actin, and the large white arrow shows the train of pI reference proteins at $31 \times 10^3$ $M_r$. Black arrows, polypeptides present in both the rostral and caudal halves of the sclerotome. Two-tone arrows, polypeptides showing differences between the two halves (see Table 1).

washes with buffer A/Tween 20, the blots were washed once in buffer A and then incubated in 0·05% diaminobenzidine (DAB) (Aldrich) in 0·1 M-Tris–HCl (pH 7·4) for about 15 min. Cobalt enhancement was not usually necessary. The use of Tween 20 as a blocking agent rather than haemoglobin did not alter the binding pattern of ConA. All ConA binding using this system was confirmed by comparison with $^{125}$I ConA overlay on gels according to the method of Burridge (1978), using lectins iodinated using the Iodogen method described by Fraker & Speck (1978).

Although the mannose-specificity of ConA was not tested by competition with the appropriate carbohydrate, the specificity was confirmed by its binding to the molecular weight standard that contained mannose (ovalbumin; $M_r = 46 \times 10^3$). Non-carbohydrate-containing protein standards did not bind ConA.

## Results

The use of a miniaturized format for 2D-gel electrophoresis has enabled us to examine, on a single gel, the protein content of half-sclerotomes from as few as two embryos. To eliminate differences between rostral and caudal halves due to the peculiarities of individuals, we pooled dissections from six to eight embryos. These embryos had been carefully selected so that they possessed the same number of somite pairs and were at the same stage of development.

### *2D-gel analysis*

Rostral and caudal half-sclerotomes from embryos possessing 20 pairs (Fig. 1), 24 pairs (Fig. 2) and 32 pairs (Fig. 3) of somites were analysed in 2D-minigels. Each figure shows rostral (top) and caudal (bottom) halves examined for total polypeptide content by silver-staining of gels (left) and for glycoprotein content by probing of Western blots with the mannose-specific lectin ConA (right). On the silver-stained gels, a train of pI reference proteins at $31 \times 10^3$ ($M_r$) can also be seen. This train consists of the protein carbonic anhydrase and its carbamylated derivatives, and was added to each sample prior to running the first dimension. It provides an internal standard for the pH gradient, each

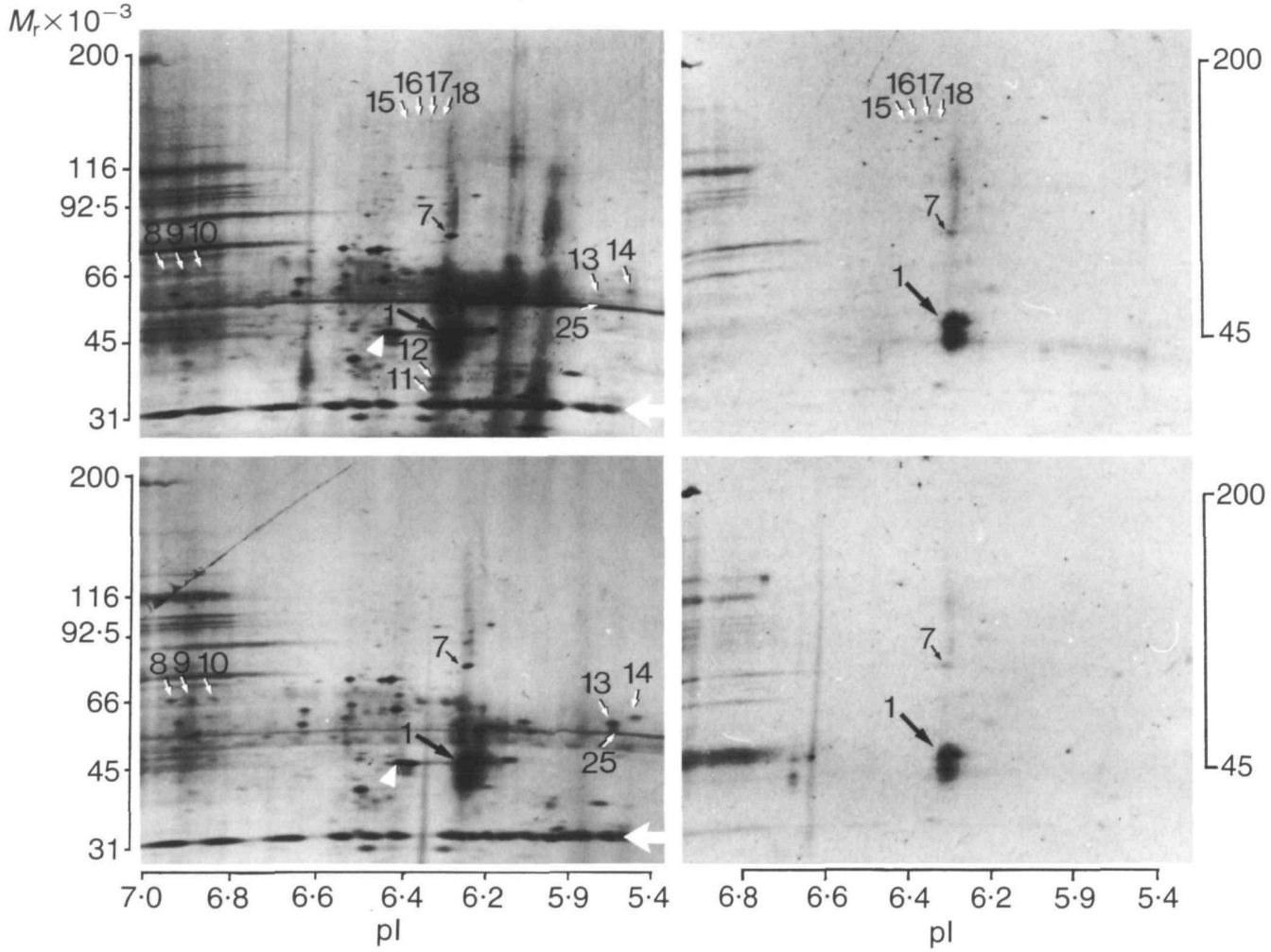


**Fig. 2.** 2-D gel analysis of rostral and caudal half-sclerotomes from chick embryos with 24 pairs of somites. Details as in Fig. 1.

spot having a unique and reproducible pI, enabling precise location and comparison of spot positions on different gels. At the basic (−) end of each gel, molecular weight markers were run in order to standardize the second dimension (not shown in Figures).

Polypeptides showing differences were numbered not with respect to $M_r$ and pI, but rather in order of their appearance from the earliest stage of development examined.

*Polypeptide differences*

Many differences between rostral and caudal half-sclerotomes could be seen in silver-stained gels. Both the number of polypeptides seen in such gels to be expressed differently in the two halves of the somite and the complexity of their distribution appeared to increase with the stage of development. A few of the variants were identified as glycoproteins on the basis of ConA binding. The changes in the polypeptide pattern are summarized in Table 1, which shows their $M_r$, pI values, rostral/caudal distribution and whether they are ConA-binding glycoproteins (*). Three glycoproteins (doublet 1a/1b and 7) that are common to both halves and are not differentially expressed at any of the stages examined are not included.

*Qualitative differences*

A number of polypeptides were expressed exclusively in one half of the sclerotome (e.g. spots 2, 11, 12, 23, 24 in the rostral portion; spots 3, 4, 5, 6, 19, 20, 21, 22 in the caudal portion). All of these appear to be expressed in a stage-specific manner. In particular, the glycoprotein train 15, 16, 17, 18 showed a complex pattern of expression, being absent from 20-somite embryos, expressed exclusively in the rostral half-sclerotome at 24 somites and present in both halves at the 32-pair stage. Expression of spots 3, 4, 5, 6 was seen only in 20-somite embryos, in which it was restricted to the caudal half of the sclerotome.

*Quantitative differences*

Other polypeptides were found to differ in amount between the two halves (e.g. 8, 9, 10, 13, 14 and 25). Again, some of these were stage specific (e.g. spot 25) but others were expressed differentially at more than one stage (e.g. 8, 9, 10, 13, 14). It may be significant that these quantitative differences occurred as reduced

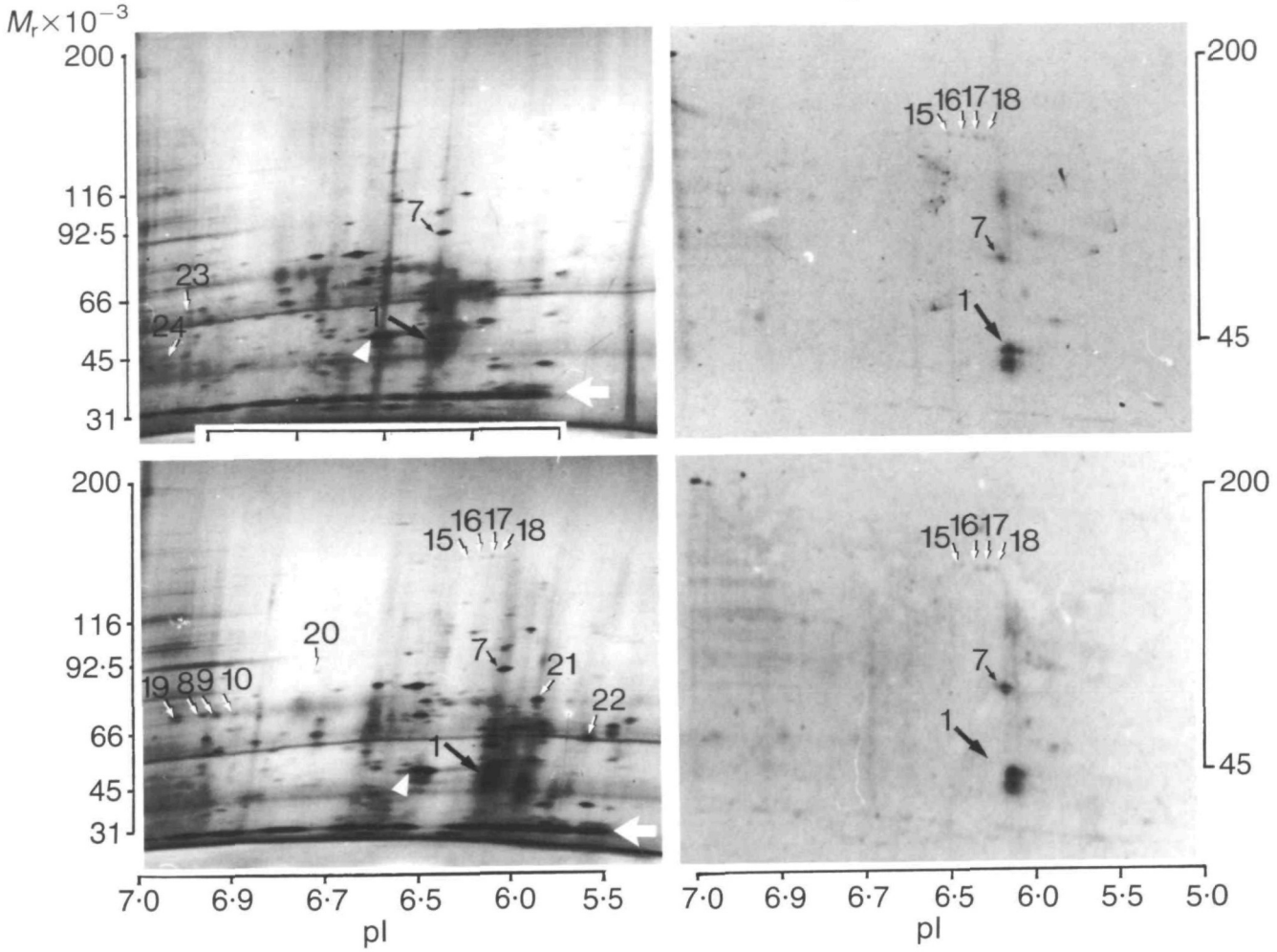


**Fig. 3.** 2-D gel analysis of rostral and caudal half-sclerotomes from chick embryos with 32 pairs of somites. Details as in Fig. 1.

amounts in the rostral half compared to the caudal half; since neural crest cells are present only in the rostral half-sclerotome (Rickmann *et al.* 1985), their presence may dilute proteins expressed by rostral half-sclerotome cells. In addition, none of these differences corresponds with spots seen in 2D gels of neural crest cells (Fig. 4). A feature of the 32-somite stage was the presence of spot 19, which was associated with the train 8, 9, 10. However, it is not clear whether this is a stage-specific quantitative change, since, in gels of earlier stage sclerotomes, the sample did not penetrate as far into the basic end of the gel. This is demonstrated by the presence of an extra spot in the carbamylyte train at the basic (−) end of the gel in Fig. 3.

## Discussion

Our results suggest that the differences in polypeptide expression between rostral and caudal half-sclerotomes are complex and that they change with the stage of development. In general, the differences between the two halves become more numerous with age between the 20- and the 32-somite stage. A large number of polypeptides were focused in the pH range 5–7 and yet only a few of these showed differences in expression between the two halves. When a polypeptide showed a *quali*tative difference in expression between the two halves of the sclerotome, the differential expression was only seen at a single stage of development: some of the polypeptides localized in one half of the sclerotome at an early stage were later found in both halves or in the opposite half. For example, the $150 \times 10^3$ glycoprotein train (15, 16, 17, 18) is not detectable in 20-somite embryos, is expressed in the rostral half at the 24-somite stage and appears in both halves at later stages (in the 32-somite embryo, the presence of this glycoprotein train in the rostral half-sclerotome was clearly demonstrable by its binding to ConA, but, for an unknown reason, it was not seen in the silver-stained gel shown in Fig. 3).

One possibility, suggested by its rostral location, is that this $150 \times 10^3$ train is neural crest derived. However, two observations argue against this: first, it is not detectable in gels of proteins extracted from cultured crest cells (Fig. 4), and second, it is subsequently expressed in the caudal half, where neural crest cells are not found (Rickmann *et al.* 1985; Teillet *et al.* 1987). It is

**Table 1.** *Summary of polypeptides showing region-specific expression in the sclerotome of chick embryos of between 20 and 32 pairs of somites*

| Spot | pI | $M_r$ ($\times 10^3$) | Rostral/caudal | Stage of difference |
|---|---|---|---|---|
| 2 | 5·8 | 44* | Rostral only | 20 pair only |
| 3 | 6·8 | 72 | Caudal only | 20 pair only |
| 4 | 6·78 | 72 | Caudal only | 20 pair only |
| 5 | 6·75 | 72 | Caudal only | 20 pair only |
| 6 | 6·7 | 72 | Caudal only | 20 pair only |
| 8 | 6·94 | 70 | Rostral < caudal | 24–32 pair |
| 9 | 6·9 | 70 | Rostral < caudal | 24–32 pair |
| 10 | 6·85 | 70 | Rostral < caudal | 24–32 pair |
| 11 | 6·3 | 37 | Rostral only | 24 pair only |
| 12 | 6·3 | 39 | Rostral only | 24 pair only |
| 13 | 5·7 | 62 | Rostral < caudal | 24 (and 32?) pair |
| 14 | 5·5 | 66 | Rostral < caudal | 24 (and 32?) pair |
| 15 | 6·5 | 150* | Rostral, then both ½ | R at 24, both at 32 |
| 16 | 6·4 | 150* | Rostral, then both ½ | R at 24, both at 32 |
| 17 | 6·3 | 150* | Rostral, then both ½ | R at 24, both at 32 |
| 18 | 6·28 | 150* | Rostral, then both ½ | R at 24, both at 32 |
| 19 | 6·98 | 70 | Caudal only | 32 pair only |
| 20 | 6·72 | 92 | Caudal only | 32 pair only |
| 21 | 5·88 | 68 | Caudal only | 32 pair only |
| 22 | 5·6 | 66 | Caudal only | 32 pair only |
| 23 | 6·84 | 58 | Rostral only | 32 pair only |
| 24 | 6·86 | 45 | Rostral only | 32 pair only |
| 25 | 5·7 | 60 | Rostral < caudal | 24–32 pair |

In the molecular weight column, an asterisk marks ConA-binding glycoproteins. In the fourth column, the regional characteristics of expression of each is summarized. The last column indicates the stage at which differential expression is seen. Polypeptides 1a, 1b and 7, although glycoproteins pointed out in the Figures, are not included in this table because they do not show tissue-specificity.

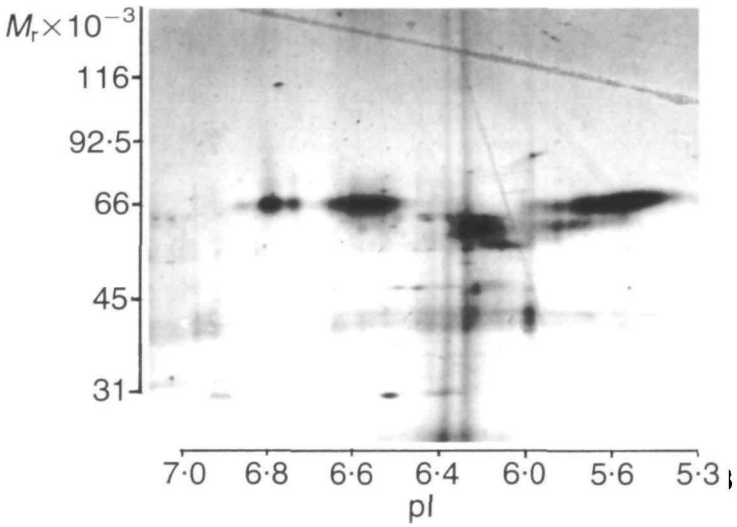


**Fig. 4.** Silver stained, 2-D gel of cultured neural crest cells.

also clear that the $150\times10^3$ glycoprotein train is not cytotactin or tenascin (Tan *et al.* 1987; Mackie *et al.* 1988; Hoffman *et al.* 1988; Faissner *et al.* 1988), for several reasons: its $M_r$ is too low, it changes in location from the rostral half to both halves (cytotactin/tenascin/J1-related molecules change in the reverse direction; Stern, Norris, Bronner-Fraser, Fraser, Carlson, Faissner, Schachner & Keynes, in preparation) and immunostaining with antibodies to these proteins on cryostat sections show that they are not present within the rostral half-sclerotome at these early stages (Tan *et al.* 1987). The expession of cytotactin/tenascin/J1-related molecules will be described in more detail elsewhere (Stern, Norris, Bronner-Fraser, Fraser, Carlson, Faissner, Schachner & Keynes, in preparation). Another macromolecule, CTB–proteoglycan, has been reported to change location within the sclerotome (Tan *et al.* 1987). However, not only is this proteoglycan considerably larger than the $150\times10^3$ polypeptide train ($280\times10^3$) but it was reported to have a homogeneous distribution within the sclerotome, subsequently becoming localized to the caudal half of the sclerotome at a later stage than those examined here. The change in the distribution of the $150\times10^3$ train occurs in the opposite manner at a much earlier stage. It is possible, nevertheless, that the homogeneous distribution of the $150\times10^3$ train in the 32-somite embryo is related to that of CTB–proteoglycan at the 34-somite stage.

In contrast with the findings made on molecules expressed differentially in a qualitative way, some of the quantitative differences observed do occur at more than one of the stages studied (e.g. 8, 9, 10). All of these show reduced expression in the rostral half and none is present in gels obtained from cultured neural crest. It is therefore unlikely that they are due to the presence of neural crest cells in the rostral half-sclerotome, although the possibility that neural crest cells express different polypeptides *in vitro* and *in vivo* cannot be excluded.

Stage-specific differences might be explained by considering that the somites included in the samples from each stage include different regions of the embryo. In the fowl, there are 15 cervical, 5 thoracic, 9–10 lumbar/sacral, and 12 caudal/coccygeal vertebrae, followed by a fused structure, the pygostyle (Robinson, 1970). In 20- and 24-somite embryos, all the somites dissected were presumptive cervical vertebrae, while the 32-somite stage included all of the cervical and thoracic regions as well as some presumptive lumbar vertebrae. Transplantation of prospective thoracic segmental plate to the cervical region gives rise to ribs (Kieny *et al.* 1972) and to a thoracic-like plumage pattern (Mauger, 1972) in the neck. These findings have been interpreted to mean that presumptive sclerotome and dermatome cells are regionally determined for their ultimate vertebral and dermal fate many hours in advance of the formation of the sclerotome, while they still reside in the segmental plate. It is therefore possible that some of the polypeptides seen as stage-specific in this study are really region-specific. A further possibility is that those polypeptides that change their pattern of expression between the different stages studied here reflect changes in the maturation of a somite and its derivatives. We are presently involved in raising monoclonal and polyclonal antibodies to some of these peptides.

The polypeptide train 3, 4, 5, 6 is probably the most interesting with regard to rostral–caudal specificity. It is

caudal-half specific, and therefore not neural crest derived, and is seen to be present at a very early stage. Preliminary data (not shown) indicate that this train is also present in the caudal halves of epithelial somites at stage 13. These polypeptides satisfy some of the criteria expected of molecules that may inhibit neural crest cells from entering into the caudal half of the sclerotome, as they are the earliest ones found to be expressed in this half prior to neural crest migration. If this is the case, however, it is puzzling that they are only seen in embryos with 20 pairs of somites. One reason for this might be that in half-sclerotomes dissected from older embryos more somites are included in the sample, which may dilute any polypeptides specific to one half but expressed only at the stage at which neural crest cells are starting to migrate segmentally through the sclerotome. One way to resolve this question is to carry out direct functional studies to establish their involvement in the decision of neural crest cells to migrate only through the rostral half of the sclerotome. Such studies are currently in progress. It will also be interesting to see if cells of the segmental plate also express any of these polypeptides, because this tissue has been found to inhibit neural crest migration (Stern & Bronner-Fraser, in preparation).

## Conclusions

Our results show that the two halves of the sclerotome of chick embryos between stage 13 and 17 express different proteins and glycoproteins, that their patterns of expression are complex, and that they vary in a stage- and region-specific manner from early stages in the development of somites. Studies using either a single existing antibody or a variety of antibodies directed against a single identified macromolecule should bear in mind this complexity before claims can be made about the involvement of such molecules in the guidance of neural crest cells or motor axons. Since all previous markers described to be specific to one or the other half of the sclerotome appear too late to be involved in the guidance of neural crest cells, we conclude that none of the sclerotome-half-specific markers described previously (peanut lectin receptors, butyrylcholinesterase activity, cytotactin, tenascin, CTB-proteoglycan) are likely to be responsible for guiding neural crest cells through the rostral half of the sclerotome. Future studies to identify suitable candidate molecules should be directed, instead, at those found to be expressed at appropriate stages of development.

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