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The *achaete-scute* complex is expressed in neurogenic regions of *Drosophila* embryos

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The *achaete-scute* gene complex (AS-C) of *Drosophila melanogaster* is involved in the development of the embryonic central nervous system and the cuticular sensory organs of both larva and adult. We have determined the embryonic spatial distributions of three transcripts encoded in the *achaete, scute* α and *lethal of scute* regions of the complex. The RNAs are present mainly between late blastoderm/early gastrula and the stage of germ band shortening. The patterns of expression are complex and evolve rapidly, affecting most or all of the known neurogenic regions. Gene expression precedes and is concomitant with the histological appearance of precursors of neural cells. These results support a role for the AS-C in determination and early differentiation of embryonic neural cells.

Key words: achaete-scute complex/*Drosophila*/pattern formation/neurogenesis/gene expression

Introduction

Chaetae and sensilla are sensory organs distributed according to species-specific patterns on the epidermis of both larvae and adults of insects. In *Drosophila*, the differentiation of these organs depends on the activity of the *achaete-scute* gene complex (AS-C) which is located at the tip of the X chromosome. Genetic analysis has allowed its subdivision in four functional regions (distally to proximally): *achaete* (*ac*), *scute* (*sc*) α, *lethal of scute* (*l'sc*) and *sc* β (García-Bellido, 1979). Recently, the existence of a fifth region, proximal to *sc* β and named *sc* γ, has been proposed (Dambly-Chaudière and Ghysen, 1987; Jiménez and Campos-Ortega, 1987).

In the adult, mutations at the *ac* region cause the loss of microchaetae (hairs); mutations at the *sc* α or *sc* β regions remove subsets of macrochaetae (bristles) from allele-specific positions of the fly. Deletion of both *ac* and *sc* α regions causes the almost complete absence of chaetae and sensilla. Gain of function mutations that promote the differentiation of supernumerary chaetae and sensilla (*Hairy-wing* mutations, García-Alonso and García-Bellido, 1986; Campuzano *et al.*, 1986), also map in the AS-C suggesting a key role of this complex on sensory organ determination and/or differentiation (García-Bellido and Santamaría, 1978).

The cuticle of the *Drosophila* larva also displays a set of sensory organs composed of bristles, sensilla of different types, Keilin organs and chordotonal organs (Campos-Ortega and Hartenstein, 1985; Dambly-Chaudière and Ghysen, 1986; Ghysen *et al.*, 1986). These organs, and their associated innervating neurones, are found on each segment at characteristic positions, according to one of five different patterns. In all segments, sensory organs appear in groups located on ventral, ventrolateral, lateral and dorsal positions. Deletions of the *ac, sc* β or *sc* γ regions remove different subsets of sensory organs and their associated neurones, while the deletion of *sc* α has little effect on the patterns (Dambly-Chaudière and Ghysen, 1987). Deletion of the *l'sc* region (but not those of other regions of the complex) causes cell death in the developing embryonic central nervous system (CNS). This leads to the absence of many neurones and gross anatomical malformation of the neural ventral cord (Jiménez and Campos-Ortega, 1979, 1987). Taken together, these phenotypes, and those observed in the imago, indicate that the AS-C plays an important role in the differentiation of the embryonic CNS and the peripheral nervous system (PNS) of both larva and adult.

The cloning of the AS-C DNA has revealed a molecular correlate to the genetically defined regions of the complex (Campuzano *et al.*, 1985). Three poly(A)$^+$ RNAs, one transcribed from each of the *ac, sc* α and *l'sc* regions, have been identified and considered to correspond to the ac, sc and l'sc functions. They have been named T5, T4 and T3 RNAs respectively. We try, in this work, to understand the implication of these RNAs in the differentiation of the embryonic nervous system. With this aim, we have determined the spatial and temporal patterns of distribution of these transcripts. The patterns suggest an important role for the AS-C in the commitment and early differentiation of neural cell precursors.

Results

Blastoderm

We have observed a transient expression of the T3, T4 and T5 genes in syncitial blastoderms. Expression starts after the nuclei have migrated to the periphery. The label is uniformly distributed in the periplasm, including the presumptive mesodermal region. In some sections it is more abundant in the vicinity of nuclei (Figure 1). The transcripts become undetectable around the time of the 13th nuclear division and remain so until nuclei have elongated or shortly thereafter.

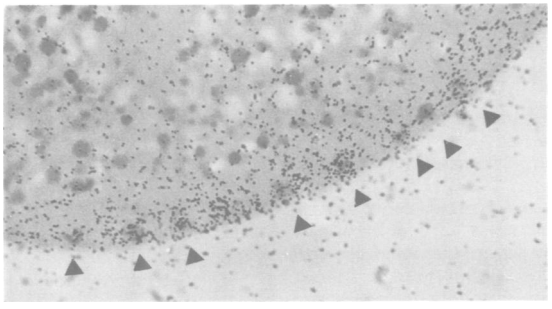

Fig. 1. Localization of T3 RNA in early syncitial blastoderm. Arrows point to the positions of visible nuclei on the periphery of the blastoderm.

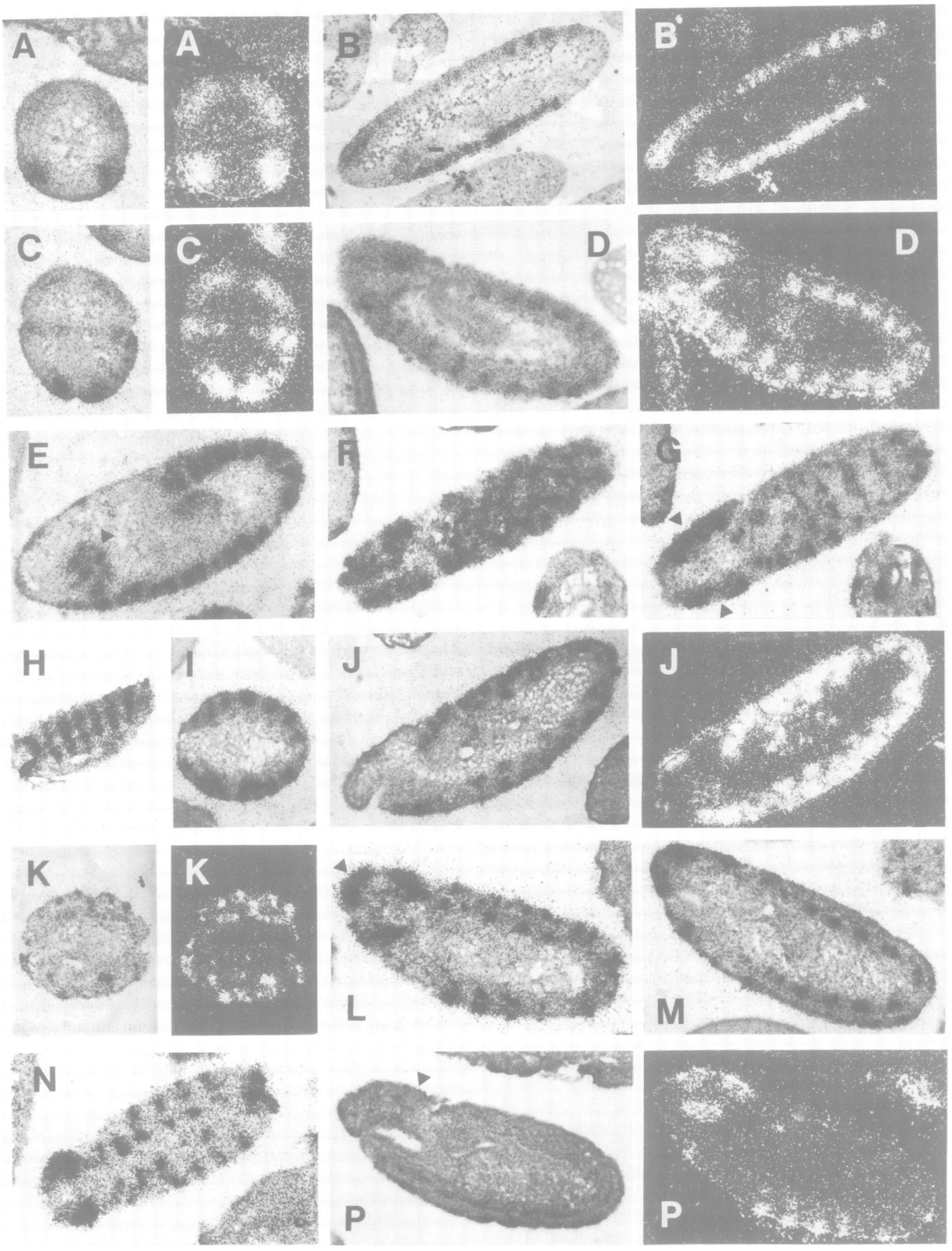

Fig. 2. Patterns of T3 gene expression. Photographs with the same letter identify light- and dark-field microscopic views of the same section. In all pertinent cases, embryos are oriented with cephalic region to the left and dorsal side on top. (**A**) Transversal section of late blastoderm. (**B**) and (**C**) Parasagittal and transverse sections of gastrulae; the second one cuts through the cephalic furrows (labeled). (**D**) Parasagittal section of stage 9 embryo. (**E**) and (**H**) Parasagittal and tangential-parasagittal sections of the same stage 8 embryo. (**F**) and (**G**) Horizontal sections of the same stage 10 embryo. F corresponds to a tangential dorsal section and G corresponds to the second section after F. The section in between F and G (not shown) displayed an intermediate pattern between those of F and G. (**I**) and (**K**), Transverse sections of approximately stage 9 and early stage 11 embryos. (**J**) sagittal section of stage 10 embryo. (**L**), (**M**) and (**N**) two parasagittal sections and one horizontal (ventral) section of early stage 11 embryos. **P**, sagittal section of stage 11 embryo. Arrows point to the following labeled structures: gnathal region (E), procephalic neurogenic region (G), optic lobe (P), and clypeolabrum (L).

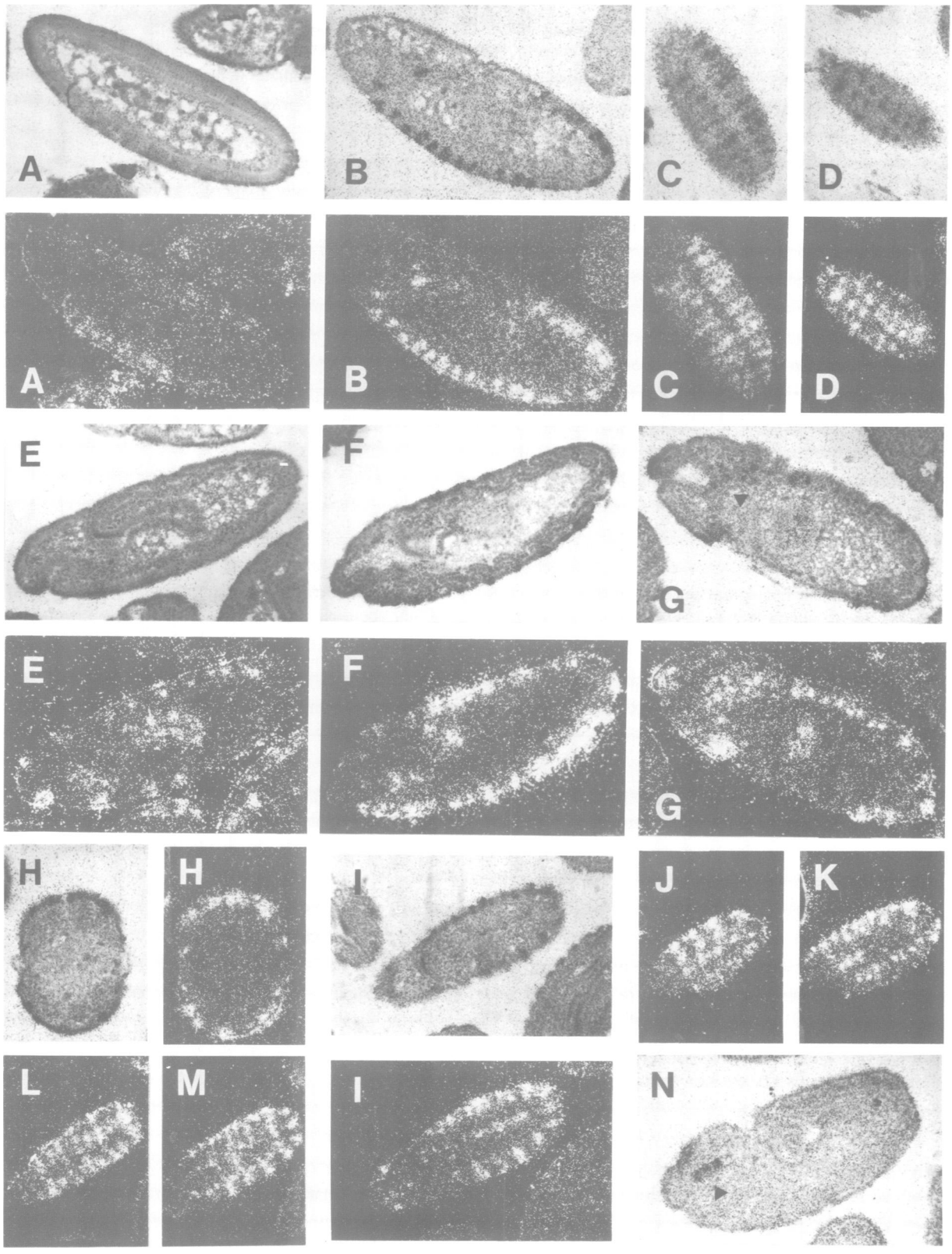

Fig. 3. Patterns of T4 gene expression. Symbolism and conventions are as in Figure 2. (**A**) Parasagittal section of late blastoderm. (**B**), (**C**) and (**D**) Parasagittal, tangential-parasagittal and tangential-ventral sections of stage 8 embryos. (**E**) Sagittal section of stage 10 embryo. (**F**) and (**H**) Parasagittal and transverse sections of early stage 11 embryos. (**G**), (**I**), (**L**) and (**M**) Horizontal sections of the same early stage 11 embryo (L, M and I are three consecutive sections, L being the most tangential; G has been photographed in a different orientation). (**J**) and (**K**) Two consecutive tangential parasagittal sections of an early stage 11 embryo. (**N**) Oblicuous sagittal section of stage 11 embryo. Arrows point to the following labeled structures: roof of stomodeum (N) and back of stomodeum (G).

Germ band

The changing patterns of expression observed on the germ band will be referred to the embryonic stages defined by Campos-Ortega and Hartenstein (1985) (see legend to Figure 5).

T3 RNA. In late blastoderms, this RNA is located in two pairs of longitudinal bands situated ventro- and dorsolaterally (Figure 2A). At gastrulation, following the mesoderm invagination, the more intensely labeled ventrolateral bands are displaced to more medial and ventral positions (Figure 2C). This labeled region includes the anlage of the ventral CNS (Poulson, 1950; Campos-Ortega and Hartenstein, 1985). Parasagittal sections of early gastrulae show the longitudinal extent of the bands (Figure 2B). Note in the figure the clumps of label, especially on the dorsolateral band.

During germ band extension, an increase in T3 gene expression takes place in a large number of clusters of cells over most of the ectoderm (Figure 2E,H,I). The disposition of these clusters varies between consecutive sections (8 μm thick) indicating complicated spatial patterns of gene expression. Some parasagittal sections of stage 9 embryos show clusters of label with a periodicity corresponding in width to segments (Figure 2D), but other sections of the same embryos still display patterns resembling that of Figure 2E (not shown). In early stage 10 embryos, some clusters of label are internalized and found very near the mesodermal layer (Figure 2J). The complexity of the patterns of expression is evident in horizontal sections of this stage embryos (Figure 2F,G). By early stage 11, expression has become more spatially restricted. Segmental periodicity is now prevalent, most clusters of label being located either on external ectodermic cells or internally near the region of contact between ectodermal and mesodermal layers (Figure 2K,L,M,N). Internal and external clusters are generally in register (Figure 2L,N). Laterally, clusters of labeled cells are found very close to the amnioserosa, that is, on precursor cells of the latero-dorsal region; they also appear in the midline (Figure 2K). By late stage 11 or stage 12, most T3 RNA expression in the germ band becomes undetectable.

T4 RNA. T4 and T5 transcripts are found in less cells than the T3 RNA, the T4 RNA probably being the least abundant. Both T4 and T5 RNAs show very similar patterns, except in the stage 9 embryo.

In late blastoderms, the T4 RNA is detectable in the neurogenic region; it already shows hints of clustering (Figure 3A). In the gastrula, clusters of label are also found on lateral regions (not shown). In stage 8 embryos, before completion of germ band elongation, relatively abundant label appears in clusters on the ectodermal layer (Figure 3B,D), even on the most lateral regions (Figure 3C). At stage 9, the transcript becomes difficult to detect in the germ band, but stage 10 embryos show small internal clusters of label with, roughly, a segmental periodicity (Figure 3E). Parasagittal tangential sections of this stage embryos also show longitudinal rows of clusters running along the dorsal ectoderm, similar to those seen with the T5 RNA. In late stage 10 and/or early stage 11, a second maximum of T4 RNA abundance occurs. Many small clusters of cells are present near to or on the most external cell layer (Figure 3F,G). Complicated patterns are observed in tangential horizontal (Figure 3L,M,I) and parasagittal sections (Figures 3J,K). Transverse sections show that T4 RNA-expressing clusters of cells are found in ventral, lateral and dorsal regions of the germ band (Figure 3H). By late stage 11 or stage 12, expression in the germ band becomes undetectable (Figure 3N).

T5 RNA. We have not detected the T5 RNA in late blastoderm. From the early gastrula to stage 8 embryo T5 and T4 RNAs have very similar distributions (see as examples Figure 4A,B). In stage 9, the label is internalized in compact clusters, which are located on the ectodermal layer, near to the mesoderm (Figure 4C,D). Tangential horizontal (Figure 4E) and transverse (Figure 4I) sections indicate that there are at this stage four longitudinal rows of clusters. Comparing serial sagittal sections, we estimate that each longitudinal row contains 25 or 26 clusters (not counting the prominent cluster on the gnathal region, Figure 4C,D). In stage 10, most of this label has disappeared, leaving in its place a pattern of small clusters with segmental periodicity, very similar to that observed with the T4 RNA at the same stage (compare Figures 3E and 4F,K).

Parasagittal, but more tangential sections, show longitudinal rows of clusters with approximately half segmental periodicity (Figure 4G). They are located on the dorsal region of the germ band, that is, adjacent to the amnioserosa (Figure 4H). Late stage 10 or stage 11 embryos again show patterns similar to those of the T4 RNA at the same stage (Figure 4L). By the start of germ band shortening T5 RNA in the germ band is undetectable.

Cephalic region

The three genes are expressed in the cephalic region. T3 RNA is found in more cells than T5 and T4 RNAs, the last one being the most spatially restricted. The regions labeled, as well as the stages of the corresponding embryos, are summarized in Table I. Only regions showing a clear label have been indicated.

The three transcripts are found in or near the gnathal neurogenic region (cephalic furrow and some of the rudiments of gnathal metamera), the clypeolabrum, and the stomatogastric neurogenic region [roof of the stomodeum; note the three clusters of label (Figure 3N) that may correspond with the three invaginations that originate ganglia of this system (Campos-Ortega and Hartenstein, 1985)]. In contrast, the T3 RNA is unique in being found in a large dorsal and lateral region of the procephalon comprising the dorsolateral procephalic neurogenic region. (The T5 RNA is restricted to a much smaller lateral area of this region.) Only two RNAs have been detected on the back of the stomodeum, which probably comprises the primordium of the anterior midgut (T3 and T4 RNAs), and on the primordia of the optic lobe (T3 and T5 RNAs).

The gut

The three genes are expressed in the posterior midgut rudiment (Figures 2D,E,J, 3E,F,G, 4B,D,F). Expression starts approximately at stage 8 and ends at early (T3 and T5 genes) and late (T4 gene) stage 11. T5 RNA is also found in some precursor cells of the Malpighian tubules (not shown).

Discussion

The three AS-C genes studied have early transient expression leading to a relatively homogenous distribution of their RNAs in the periplasm of cleavage blastoderms. We do not know how this generalized early expression, which includes the presumptive mesodermal region, is related to the regionally restricted transcription that occurs later. The early expression approximately coincides in time with the first zygotic activation of many genes. (McKnight and Miller, 1979). Perhaps the AS-C genes respond to a general activation mechanism before being repressed by more specific controls.

Expression of the three genes is resumed in the late blastoderm or early gastrula and ends at the time of germ band shortening.

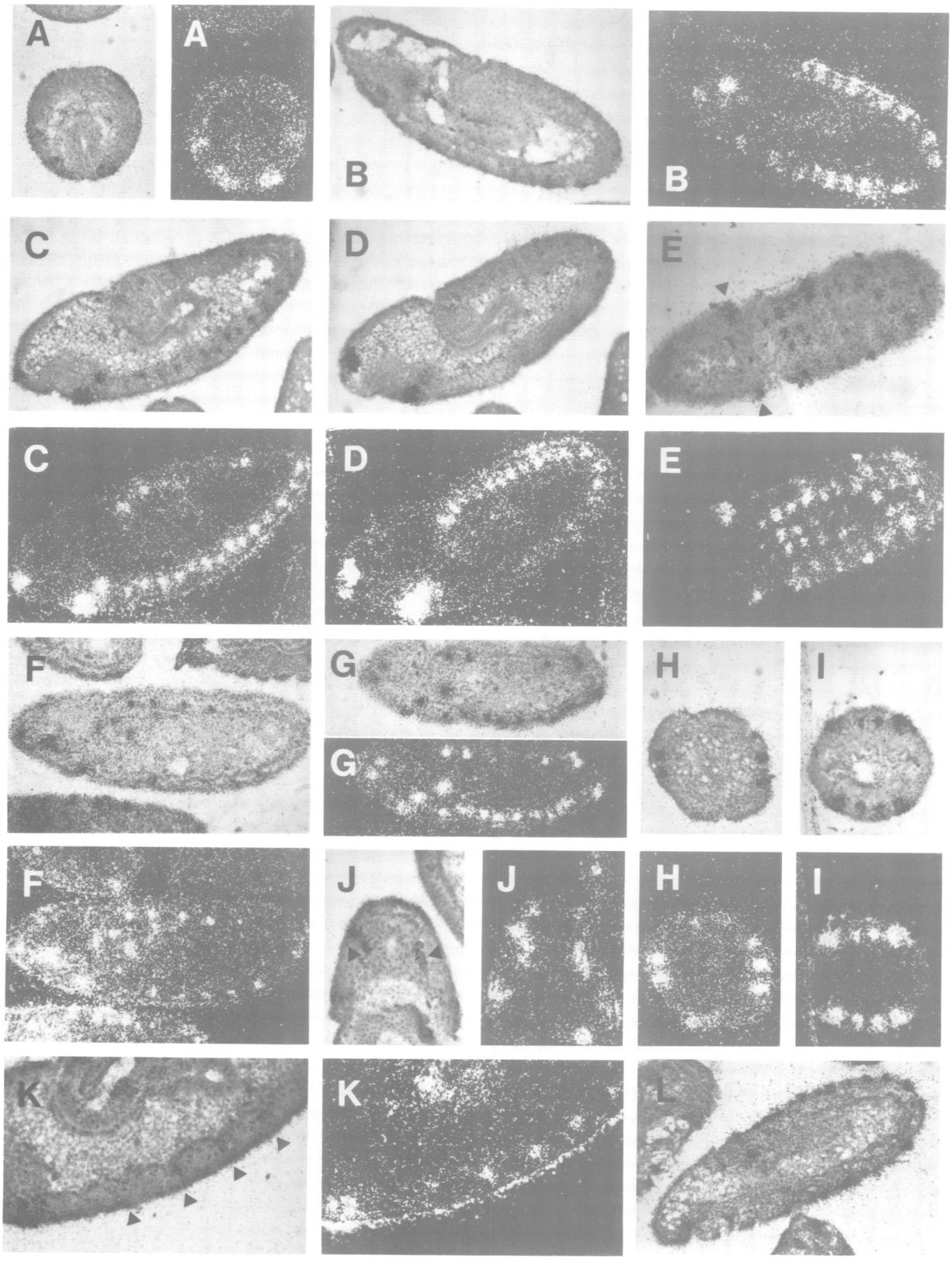

Fig. 4. Patterns of T5 gene expression. Symbolism and conventions are as in Figure 2. (**A**) Transverse section of gastrula. (**B**) Sagittal section of stage 8 embryo. (**C**), (**D**) and (**E**). Two consecutive parasagittal sections and one horizontal (dorsal) section of a stage 9 embryo (arrows point to the optic lobes). (**F**) and (**G**) Two parasagittal tilted sections of same stage 10 embryo. (**H**) and (**I**) Transverse sections of stage 10 and stage 9, embryos. (**J**) Partial view of horizontal section of stage 10 embryo showing the cephalic region and labeled cell rows (arrows). (**K**) Partial view of sagittal section of stage 10 embryo showing the thoracic region of the germ band. Arrows point to positions of small clusters of label. (**L**) Parasagittal section of early stage 11 embryo.

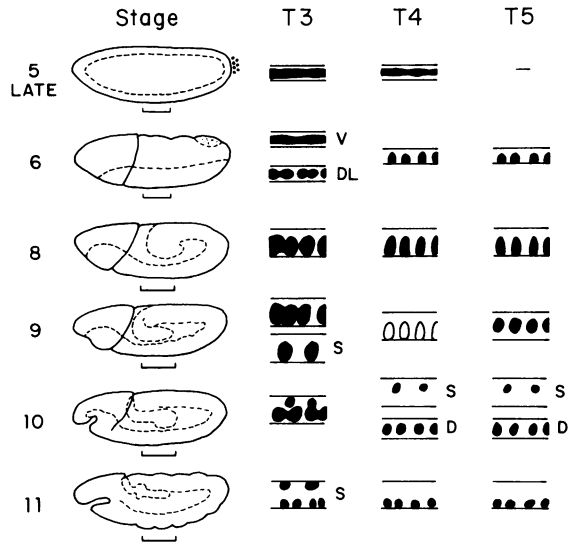

Fig. 5. Schematic representation of the patterns of AS-C gene expression in late blastula, gastrula and germ band of different stage embryos. Except where indicated, drawings of patterns correspond to a short fragment of the thoracic/abdominal region (bracketed), as observed in sagittal sections of the germ band. When pertinent, patterns of two different sections of the same stage embryo are represented. Empty symbols indicate very weak expression. V, ventral band of labelling; DL, dorsolateral band of labelling; S, pattern with segmental periodicity; D, pattern in parasagittal tangential section through dorsal region. Outlines of embryos and the corresponding approximated developmental times (indicated below) are taken from Campos-Ortega and Hartenstein (1985). Times after fertilization: late blastoderm (stage 5), 2:30–2:50 h; gastrula (stage 6), 2:50–3:00 h; stage 8, 3:10–3:40 h; stage 9, 3:40–4:20 h; stage 10, 4:20–5:20 h; stage 11, 5:20–7:20 h.

During this interval, the transcripts show related but clearly distinct spatial patterns which evolve rapidly, both in the cephalic region and the germ band. The patterns of expression in the germ band are summarized in Figure 5. The main distinctive features are the following. T3 RNA is found in more cells than T4 and T5 RNAs and its pattern is the most different. T4 and T5 RNAs have very similar patterns, except in stage 9 embryos; T5 RNA is then abundantly transcribed in four longitudinal rows of clusters, while T4 RNA is almost undetectable in the same region. The expression of the T3 gene, which in the early stages occurs in more or less continuous bands or partially overlapping clusters, becomes spatially restricted with time and, at the late stages, it occurs in well-separated clusters of cells. In contrast, expression of the T4 and T5 genes takes place in discrete clusters much earlier (stage 6). Moreover, the second round of expression of these two genes, which begins in late stage 10 embryos, already starts in many individualized clusters of cells. These results suggest that the early activity of the AS-C genes is controlled by spatial cues previously laid down in the embryo. Later, interactions between AS-C expressing cells may also play a role in the evolution (spatial restriction) of the patterns, specially with the T3 gene (see below).

AS-C expression in neurogenic regions

Although the study of the spatial distribution of transcripts in *Drosophila* embryos by hybridizations *in situ* has the methodological limitation that it makes it difficult to identify morphologically the precise type of labeled cells, it seems clear that the three AS-C genes, either singly or in combination, are expressed in most or all of the known neurogenic regions. In general, in a given region, expression starts before the appearance of neuroblasts or other anatomically recognizable structures of the nervous system. Thus, in the late blastoderm or early gastrula, the three transcripts are found in the anlage of the ventral CNS, which is located at both sides of the ventral furrow. They are present before the morphological changes which define the neurogenic ectoderm take place. Histological observations indicate that such changes start in the gastrula with the enlargement of the cells that will form this region (J.A.Campos-Ortega, personal communication). The neurogenic ectoderm extends laterally halfway to the amnioserosa and will give rise to neuroblasts that segregate from the ectodermal layer and migrate to more internal positions (Hartenstein and Campos-Ortega, 1984) between stage 9 and 11. Neuroblast segregation leads to the formation of rapidly changing clustered patterns. We observe that the AS-C genes are expressed in clusters of cells in the ventral neurogenic region and that this occurs before neuroblasts segregate. Expression continues during the time of neuroblast migration and, remarkably, we observe a simultaneous internalization of the label, which is very apparent with both T3 and T5 RNAs (Figures 2D,J, 4C,D and 5). Thus, at least part of the internalized label may be due to T3 and/or T5 RNA-transcribing neuroblasts. During early stage 10, both T4 and T5 genes are expressed in small internal clusters of cells (Figures 3E and 4K) which are located near parasegmental (Martínez-Arias and Lawrence, 1985) boundaries (Figure 4K). On the midline, these are approximately the sites where median neuroblasts originate (J.A.Campos-Ortega, personal communication). More laterally, the label could correspond to a subset of lateral row neuroblasts.

The three genes are also expressed in the dorsolateral ectoderm (stages 8 and 10; examples in Figures 2H,I, 3C and 4G,H) and around the periphery of the germ band (late stage 10–stage 11 embryos; Figures 2K and 3H). PNS elements differentiate from the ventral, ventrolateral, lateral and latero-dorsal ectoderm (Campos-Ortega and Hartenstein, 1985). These locations topologically correspond with observed sites of AS-C gene expression. The later expression precedes the development of thoracic and abdominal PNS precursor cells (Hartenstein and Campos-Ortega, 1986).

As early as stage 9, the pattern of expression of the T3 gene discloses a periodicity which corresponds in width to segments (Figure 2D). It is unknown how the periodic clusters of label are positioned with respect to parasegmental borders (Martínez-Arias and Lawrence, 1985). However, it is clear that the T3 gene is one of the genes whose expression shows that the metameric divisions of the germ band are already laid down at this stage. Assuming that a large proportion of the label corresponds to neural precursor cells, the segmental clusters suggest that the subdivision of the ventral cord in neuromeres is already outlined. The complexity of the pattern of T3 gene expression, with approximately half segmental periodicities in other parts of the CNS rudiment (Figure 5), may reflect additional subdivisions in the spatial organization of the CNS precursors. The same considerations apply to the patterns observed with the other AS-C transcripts. Specially striking are the four rows of 25 or 26 clusters seen with the T5 transcript in stage 9 embryos. Perhaps this RNA labels segmental pairs of subsets of CNS precursors corresponding to anterior and posterior compartments. This number of clusters accounts for only 13 parasegments. Some additional clusters may not be apparent due to fusions in the caudal region or to the complicated structure of the cephalic parasegments. Alternatively, the observed periodicity may correspond to a metameric organization different from compartments.

Table I. Embryonic stages of AS-C expression in cephalic regions and stages of appearance of morphologically recognizable neural precursor cells

Region	Gene expression			Appearance of neural precursor cells
	T3	T4	T5	
Gnathal	6 – ≥ 12 (2B,C,D,**E**,L)	7 – 11 (3B,E,F)	6 – 11 (4B,C,D,J)	9
PNR	6 – ≥ 12 (2B,C,D,F,**G**,P)	–	8 – 10	9
Optic lobe	≤ 9 – ≥ 12 (2D,F,L,**P**)	–	9 – 10 (4**E**)	11 late
Clypeolabrum	9 – ≥ 12 (2**L**,M)	9 – 11 late (3E,G)	9 – 11 late (4C,D,F,J)	10
Roof of stomodeum (SNS)	11 – ≥ 12 (2P)	11 – ≥ 12 (3**N**)	11 – ≥ 12	11 late
Back of stomodeum (AMG)	10 (2J)	8 – 11 late (3F,**G**)	–	
Lateral cell rows		10	10 (4**J**)	

For each region, figures indicate stages in which expression of the corresponding gene has been detected. Numbers and letters in parenthesis refer to photographs of Figures 2, 3 and 4 where examples of the corresponding expression can be seen. Bold type refers to photographs where the specified labeled region has been indicated by arrows. 'Lateral cell rows' refers to a pair of longitudinally oriented rows of cells which are located at both sides of the stomodeal invagination and are intensely stainable (Figure 5J). Stages of appearance of neural precursor cells are taken from Campos-Ortega and Hartenstein (1985). Expression of T5 gene in PNR (procephalic neurogenic region) is limited to two small lateral areas. Other abbreviations: SNS, stomatogastric nervous system; AMG, anterior midgut.

In the cephalic region, AS-C gene expression also coincides spatially (and precedes temporally) with the location of primordia of neural structures (Table I). As in the germ band, the T3 gene is expressed in many more cells than the T4 and T5 genes. T3 RNA is the only one found in most of the procephalic neurogenic region. The transcripts are also present in parts of the cephalic region whose significance for nervous system development is unknown to us (Table I). The three transcripts are also found in the posterior midgut rudiment. Neuronal bodies have been observed in this region (J.A.Campos-Ortega, personal communication).

The above discussed anatomical and histological correlations, together with the phenotypes of loss of neural elements caused by partial deletions of the AS-C (see Introduction), suggest that the AS-C genes are expressed in neural precursor cells and that they play a role in their determination. This role has been proposed, on genetic and developmental grounds, for the determination of adult sensory organs (García-Bellido and Santamaría, 1978). These genes would be first expressed in groups of cells followed by restriction to individual cells as a result of gene interactions between neighbours (lateral inhibition) (Wigglesworth, 1940; Ghysen and Richelle, 1979; Moscoso del Prado and García-Bellido, 1984b). The continuing expression observed here of the AS-C genes in the germ band and in the cephalic region during neuroblast differentiation and internalization further indicates that they may also be involved in the actual differentiation of neural precursors. The observed degeneration and death of developing neural cells in the *l'sc* deletion supports this view (Jiménez and Campos-Ortega, 1979). The similar, but distinct, patterns of expression of the AS-C genes further suggest that different sets of cells, or clusters of cells, may express combinations or amounts of AS-C products characteristic for each set. Such differential expression of AS-C genes might be important to define the basic topology of the pattern of sensory organs, both in the larva (Dambly-Chaudière and Ghysen, 1987) and the adult (García-Bellido, 1979).

The AS-C partial deletions

We have shown that the AS-C genes are expressed in most or all of the known neurogenic regions. The T3 gene is the most generally expressed, followed by the T5 and, lastly, by the T4 gene. This relative order grossly matches the phenotypic effect of the partial deletions of the AS-C which remove each of these genes (see Introduction). However, on closer examination one finds no strict correlation between expression sites and sites of the nervous system affected by the deletions. The genes are apparently expressed in many more sites than those which could correspond to the cellular precursors of the CNS neurons or sensory organs affected. Thus, the *sc* α deletion, which removes the T4 gene, does not cause a detectable effect, and the *ac* deletion, which removes the T5 gene, only suppresses differentiation of a subset of peripheral sensory organs (Dambly-Chaudière and Ghysen, 1987; Jiménez and Campos-Ortega, 1987). Both of these genes are expressed in CNS and PNS neurogenic regions. Similarly, only a slight effect has been found for the *l'sc* deletion (T3 gene removal) on the cephalic nervous system (F.Jiménez and J.A.Campos-Ortega, personal communication), although this gene is abundantly expressed in the corresponding neurogenic region. The related patterns of expression of the AS-C genes indicate that their products may be simultaneously present in many clusters of cells of the neurogenic regions. Assuming that the products actually co-exist in cells and that AS-C functions are cell autonomous during embryonic development, three possibilities, or a combination of them, can explain this paradox.

The first one is based on the postulated mutual replacement of AS-C functions by one another (Dambly-Chaudière and Ghysen, 1987). This hypothesis arises from the observed synergistic effects of the removal of two or more of the functional regions of the complex (Dambly-Chaudière and Ghysen, 1987; Jiménez and Campos-Ortega, 1987) and the lack of specificity of the mutations that cause excess of function of the complex (García-Alonso and García-Bellido, 1986; M.Ruiz and J.Modolell, unpublished data). It is also supported by the exten-

sive homologies found among the putative protein products encoded by the three AS-C RNAs (R.Villares, F.González, J.Modolell and C.V.Cabrera, unpublished data). Moreover, partial functional replacement in the CNS may also occur with other genes that promote neural development and are located outside the AS-C (White, 1980; White *et al.*, 1983; Jiménez and Campos-Ortega, 1987).

A second possibility is that the absence of one gene might promote overexpression of the remaining AS-C genes (L.García-Alonso, 1986) through *cis*-effects within the AS-C and/or interactions with the *trans*-regulatory genes of the complex (Moscoso del Prado and García-Bellido, 1984a,b).

A third possibility is that the products may act combinatorially and that the absence of one of them does not necessarily cause, in many cases, a detectable effect.

On the other hand, if determination and differentiation of neural precursors involves cellular interactions (Doe and Goodman, 1985; Technau and Campos-Ortega, 1986), the absence of an AS-C product may be compensated by the activity of the remaining ones in neighbouring cells, according to any of the above hypotheses. We are currently investigating the pattern of expression of AS-C genes in AS-C mutant embryos to help distinguish among these possibilities.

Materials and methods

Embryo sections

Eggs were collected from population cages of wild-type Oregon R flies and incubated at 25°C to reach the desired stage of development. Chorion removal and prefixation were performed as described previously (Hafen *et al.*, 1983; Akam and Martínez-Arias, 1985; Ingham *et al.*, 1985). Vitelline membrane was removed in an interphase methanol/heptane (1/1) at room temperature. The embryos were rehydrated and postfixated for a further 20 min, dehydrated and embedded in paraffin wax (Ingham *et al.*, 1985). Sections (3, 6 or 8 μm) were collected on microscope slides coated with poly-L-lysine, wax was removed with xylene and the sections were rehydrated and pretreated for hybridization (Hafen *et al.*, 1983; Akam and Martínez-Arias, 1985; Ingham *et al.*, 1985).

Labeled probes

RNA probes were synthesized in a 20 μl reaction mixture containing: 40 mM Tris−HCl pH 8.0, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 20 μg/ml bovine serum albumin, 0.1% Triton X-100, 1 U/μl human placental RNase inhibitor (Amersham), 60 μg/ml linearized template DNA, 0.5 mM ATP, GTP and CTP, 4 μM [^{35}S]UTP (1200 Ci/mmol), 0.1 U/μl SP6 RNA polymerase. Templates for the above reaction were nearly full-length cDNAs subcloned into pSP65 or pGEM-1 vectors. Incubation at 30°C for 4 h was followed by addition of DNase I (25 μg/ml) and further incubation at 37°C for 10 min. Probes were size reduced to an average of 50−100 bases by alkaline hydrolysis [pH 10.4 60°C, 30(T3 and T5 RNA) and 35 (T4 RNA) min; Cox *et al.*, 1984]. Final specific activities were 1×10^9 d.p.m./μg.

Hybridizations in situ

Hybridization, using probes at a final concentration of 0.2 ng/μl, and washing of the sections were performed as described by Ingham *et al.* (1985), except that the temperature was 42°C. After dehydration, the slides were coated with Kodak NTB-2 emulsion, dried and exposed at 4°C for 5−7 (T3), 10−15 (T5) and 22−25 (T4 RNA) days. They were developed with Kodak D-19 developer at 20°C, and stained with the haematoxylin−eosin progressive method (Humason, 1972). Controls using sense-strand RNA probes showed that the signals were specific.

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