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An improved *in situ* hybridization method for the detection of cellular RNAs in *Drosophila* tissue sections and its application for localizing transcripts of the homeotic *Antennapedia* gene complex

Ernst Hafen, Michael Levine, Richard L.Garber and Walter J.Gehring*

Biozentrum der Universität, Abt. Zellbiologie, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

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An improved method for the detection of cellular RNAs in tissue sections has been developed. It involves *in situ* hybridization of tritium-labeled cloned DNA probes to tissue sections and autoradiography. The method was calibrated by using a cloned DNA probe complementary to transcripts abundant in the midgut cells of *Drosophila* larvae. The improved method also permitted the detection of these transcripts in sectioned embryos where they are much less abundant. The sensitivity of the method can be approximated by quantifying the signal intensities over the hybridizing embryonic midgut cells relative to the larval midgut cells for which the number of transcripts has been estimated. Based on these calculations we estimate that the method is sensitive enough to detect ~100 complementary RNA molecules per cell after 3 days of autoradiographic exposure with a signal-to-noise ratio of 10. The method has been successfully applied to detect transcripts of the homeotic gene *Antennapedia*. Serial sections allow us to study the spatial pattern of gene expression in the course of development.

Key words: homeotic genes/*in situ* hybridization/mRNA localization/tissue sections/tissue-specific hybridization probes

Introduction

Methods have been described for the localization of specific cellular RNA species in cultured cells and serial tissue sections (Harrison *et al.*, 1973; Harding *et al.*, 1977; John *et al.*, 1977; Brahic and Haase, 1978; Brennan *et al.*, 1982; Singer and Ward, 1982; Akam and Hogness, unpublished results). Cloned DNA segments or viral genomic DNA sequences were used as hybridization probes for the detection of complementary RNA sequences contained in the cultured cells or tissue sections following fixation. DNA hybridization probes were labeled either with tritium or biotin (Singer and Ward, 1982; Brigati *et al.*, 1983). The former methods employed autoradiographic signal detection while the latter method involved detection by immunofluorescence or immunohistochemical stains. A drawback to these previous efforts is the limited sensitivity of signal detection; only abundant mRNAs that constitute between 1% and 5% of the total poly(A)$^+$ RNA population of a given target cell were visualized. Therefore, it is of considerable interest to extend the sensitivity of this method to permit the detection of developmentally important mRNAs that might be present at substantially lower concentrations. In addition, from many of the earlier studies involving the labeling of tissue culture cells, it is difficult to assess the hybridization specificity of a given DNA sequence for interaction with the complementary RNA species contained within the target cell. Here we describe an improved *in situ* hybridization method for the detection of RNA species present at cellular concentrations in the range 0.1 – 0.01% of the poly(A)$^+$ RNA population of a given target cell. Furthermore, by employing cloned DNA hybridization probes that encompass genes which specify tissue-specific cytoplasmic RNAs, we also show that this method can be used for the rapid and reproducible localization of specific RNA species contained in serial tissue sections.

The method was used to detect a midgut-specific mRNA species that is present in ~150 copies per responding presumptive midgut cell (with dimensions 6 x 6 x 6 μm) of 16 – 18 h *Drosophila* embryos.

Recently, overlapping genomic DNA segments that derive from the *Antennapedia (Antp)* locus have been cloned and cDNA clones representing some of the transcripts specified by the locus have been isolated (Garber *et al.*, in preparation). We demonstrate the localization of putative *Antp*$^+$ transcripts within serial sections of *Drosophila* embryos using cloned cDNA segments as hybridization probes.

Results

The sensitivity of localizing specific mRNA sequences within tissue sections by *in situ* hybridization principally depends upon the following parameters: (1) the degree of cellular RNA retention during preparation of the tissue sections; (2) accessibility of the target RNA species for interaction with the corresponding DNA hybridization probe; (3) the efficiency of probe hybridization to the complementary mRNA species; (4) the specific activity of the tritium-labeled hybridization probe; and (5) the efficiency and sensitivity of the autoradiographic method used for signal detection.

Retention of RNA in tissue sections of *Drosophila* embryos was monitored by acridine orange staining after each of the pretreatments required for subsequent hybridization. In this way we found that paraformaldehyde fixation of the tissue results in a more reproducible retention of RNA than does ethanol/acetic acid fixation (data not shown). This observation is in agreement with previously published data that indicate substantially better retention of *in vivo* labeled cytoplasmic RNA in glutaraldehyde-fixed tissue sections of sea urchin embryos as compared with ethanol/acetic acid-fixed sections (Angerer and Angerer, 1981).

To assess the accessibility of a specific DNA hybridization probe for efficient interaction with the corresponding target mRNA species, a cloned DNA segment that specifies an abundant, tissue-specific transcript was employed. The use of such a probe permitted rapid signal detection and provided an inherent control for the specificity and fidelity of hybridization: plasmid aDm3201 DH contains a *Drosophila* genomic DNA segment that is homologous to an abundant mRNA species present in midgut cells of third instar larvae (Carlson, 1982; Akam and Hogness, unpublished results). The finding that 4% paraformaldehyde provides better retention of cellular RNAs than does ethanol/acetic acid fixation was

*To whom reprint requests should be sent.

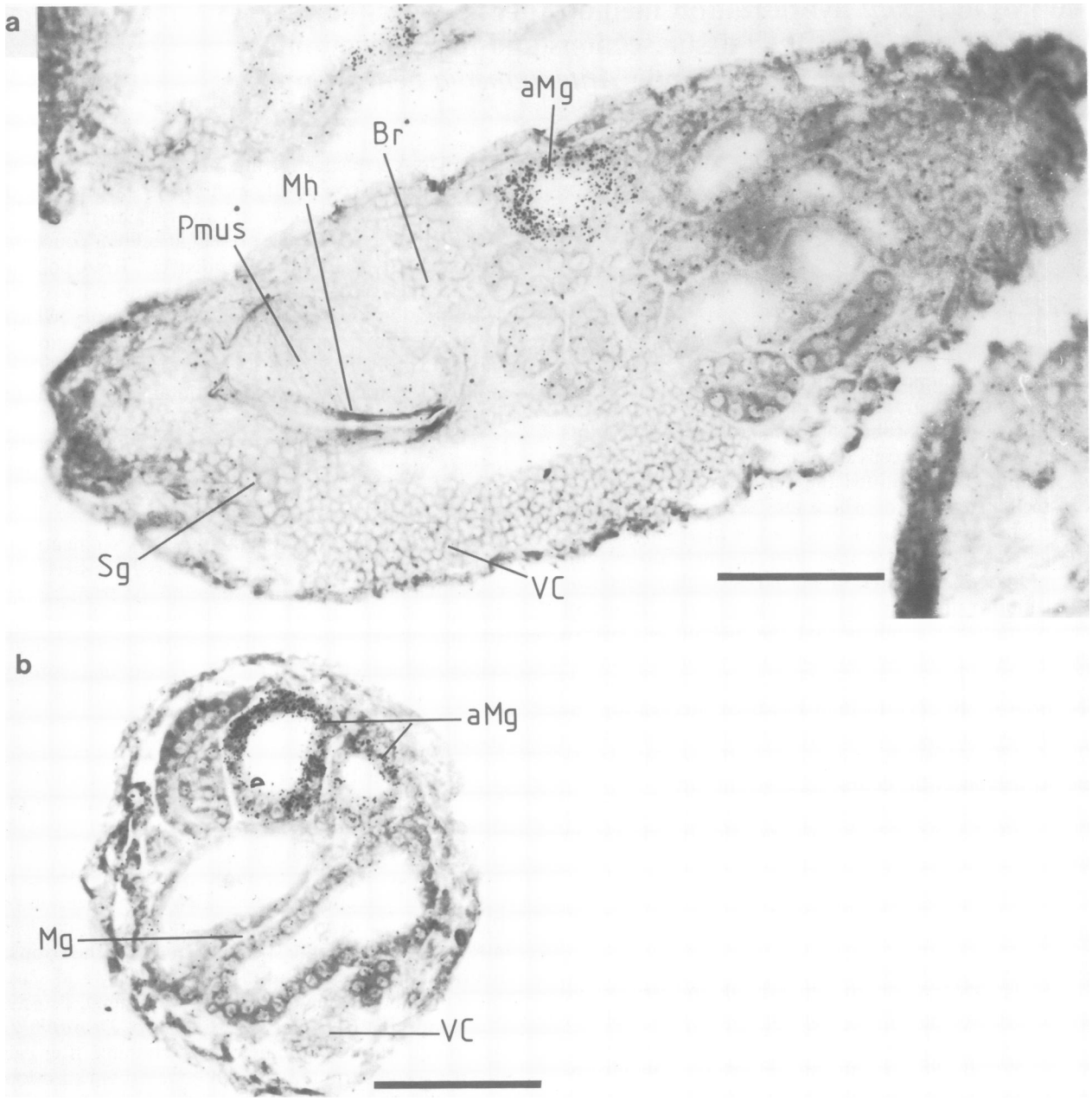

Fig. 1. Location of transcripts homologous to recombinant aDm3201 DH DNA in sectioned 18 h embryos. **(a)** A sagittal section through an 18 h embryo after 6 days of autoradiographic exposure. Hybridization was carried out as described in Materials and methods without the use of dextran sulfate. **(b)** A cross-section through an 18 h embryo. Hybridization was performed as above except that 50% dextran sulfate was added to the hybridization solution to achieve a final concentration of 10%. The section was autoradiographed for 3 days. The horizontal bar of each photomicrograph indicates a length of 0.1 mm. aMg, anterior midgut; Br, brain; Mh, mouth hooks; Pmus, pharyngeal muscles; Sg, salivary gland; VC, ventral nerve cord.

confirmed by hybridization of tritium-labeled aDm3201 DH DNA to serial sections of third-instar larvae (data not shown). The resulting autoradiographic signal intensities observed over third-instar larval midgut cells were consistently more intense when paraformaldehyde was used as fixative. The accessibility of the target RNA sequence in larval midgut cells for hybridization with the aDm3201 DH probe was found to be optimal after incubation of the sectioned tissue in a solution containing 0.25 – 0.50 mg/ml pronase as compared with incubation in lower or higher pronase concentrations or in proteinase K solutions at concentrations in the range 1 – 3 μg/ml. Immediately following pronase digestion, the tissue sections were incubated in 4% paraformaldehyde to cross-link the exposed cellular RNA to the remaining components of the cell and to the gelatin matrix of the slide (Singer and Ward, 1982). Stronger autoradiographic signals were obtained over third-instar larval midgut cells following hybridization at 35°C to aDm3201 DH probes possessing mean single-stranded lengths of 100 – 250 bases as compared with probes with mean lengths of 50 bases hybridized at either 25°C or 35°C. The use of paraformaldehyde fixation in combination with higher concentrations of protease for the permeabilization of the sectioned tissue resulted in a 2-fold increase in detection sensitivity and is in agreement with previous observations (Singer and Ward, 1982). The hybridization efficiency was extended 2-fold by increasing the aDm3201 DH probe concentration to 2 μg/ml in the hybridization solution (Singer and Ward, 1982). The use of

dextran sulfate at a final concentration of 10% in the hybridization solution in combination with performing the hybridization at 35°C rather than 25°C resulted in another 4-fold increase in signal detection sensitivity (compare Figure 1a and 1b).

Tritiated deoxyadenosine triphosphate with a specific activity of 50 – 100 Ci/mmol has recently become commercially available. Employing this deoxynucleotide for the nick-translation of cloned DNA segments to be used as hybridization probes permits labeling of DNA to specific activities of $1-1.5 \times 10^8$ c.p.m./μg. The specific activity of such a probe is approximately three times higher than those used in this study. However, we have more recently utilized the higher specific activity probes for *in situ* detection of aDm3201 DH transcripts and obtained autoradiographic signals linearly more intense (data not shown). No attempts were made to enhance the sensitivity of the autoradiographic method used for signal detection.

Using this method of detection, the aDm3201 DH hybridization probe appears to interact specifically with the homologous mRNA species present in larval midgut cells on the basis of the following criteria: (1) specific hybridization signals are observed only to those cells that have been previously shown to accumulate transcripts homologous to aDm3201 DH DNA; (2) hybridization is observed to cytoplasmic, but not nuclear, regions of the responding midgut cells; and (3) aDm3201 DH hybridization signals over midgut cells are reduced to background levels when larval tissue sections are incubated in a solution containing 2.0 mg/ml pancreatic RNase for 1 h at room temperature prior to hybridization (data not shown). As compared with the original method employed for the detection of aDm3201 DH mRNAs in serial sections of third-instar larvae (Akam and Hogness, unpublished results), the protocol presented in Materials and methods provides at least a 10-fold increase in the sensitivity of signal detection.

Cytoplasmic RNAs homologous to the aDm3201 DH probe were detected in a discrete population of the presumptive midgut cells of *Drosophila* embryos as well as in the midgut cells of third-instar larvae. A sagittal section through a 16 – 18 h embryo following hybridization to tritiated aDm3201 DH DNA after 6 days of autoradiographic exposure is shown in Figure 1a. An accumulation of silver grains is observed over the cytoplasmic regions of a population of cells within the embryonic midgut. Figure 1b shows a cross-section through an embryo of the same stage following hybridization to the same probe. The concentration of silver grains over a unit area of the responding embryonic midgut cells is ~35-fold lower (under identical conditions of hybridization and autoradiography) than the signal intensity observed over the responding midgut cells of third-instar larvae (data not shown).

The hybridization method also permitted detection of RNA species specified by the *Antennapedia* locus region in sectioned embryos. The hybridization probe (903) used in these experiments is a recombinant pBR322 plasmid containing a 2.2-kb cDNA insert that is homologous to cloned DNA sequences that are derived from the *Antennapedia* locus (Garber *et al.*, in preparation). Figure 2a shows a sagittal section through a 13 h wild-type embryo following hybridization to tritiated 903 DNA and autoradiography. Cross-sections through four different embryos of approximately the same age are shown in Figure 2b. An accumulation of silver grains is observed over a discrete population of ganglion cells associated with the ventral cord. At this stage of embryonic development the ventral cord of the central nervous system consists of 12 pairs of segmentally arrayed ganglia: the subesophageal ganglion, three thoracic ganglia and eight abdominal ganglia (Poulson, 1950). The most significant accumulation of silver grains is observed over the ganglion cells of the second thoracic segment (Figure 2). Consistent hybridization of the 903 probe to the ganglion cells of the mesothoracic segment is observed upon examination of a consecutive series of sagittal sections through a single 13 h embryo (Figure 3), indicating the reproducible labeling of these cells and further permitting a spatial localization. Finally, the same probe was found to label a group of cells at an anterior region of the embryo (Figure 3f). The identity of these cells has not been established.

Discussion

For several reasons we believe that the autoradiographic signals observed over sectioned *Drosophila* embryos after hybridization to the tritiated cloned DNAs used in this study correspond to homologous RNA species. The aDm3201 DH hybridization probe was shown to contain sequences complementary to transcripts that significantly accumulate only in midgut cells (Carlson, 1982; Akam, unpublished results) and, following hybridization of this probe to sectioned embryos, specific signals were detected exclusively over presumptive midgut cells. In addition, the autoradiographic signal observed over larval third-instar midgut cells was lost after pretreatment of the tissue sections with pancreatic RNase. The most compelling indication of faithful hybridization of probe DNA to complementary RNA is the parallel localization of aDm3201 DH and 903 autoradiographic signals to embryonic midgut cells and neural cells, respectively. Both hybridization probes included pBR322 vector sequences but were nonetheless shown to label different tissues in serial sections of embryos of approximately the same age (compare Figure 1 with Figures 2 and 3); the probes differed only in the *Drosophila* DNA sequence inserted into the same vector.

Carlson (1982) has previously estimated that the mRNA transcripts homologous to the recombinant plasmid aDm3201 DH constitute ~5% of the total poly(A)$^+$ RNA population of the third-instar larval midgut. The volume of a midgut cell of a third instar larva is roughly 2- to 5-times smaller than that of a larval fat body cell. On the basis of RNA extraction data (M.Levine, unpublished results), each larval fat body cell contains ~0.5 ng of RNA. Assuming that 1% of the RNA of a cell is polyadenylated, a 1-kb transcript that constitutes 5% of the poly(A)$^+$ RNA population is present in $\sim 5 \times 10^5$ copies per fat body cell. This corresponds to $1-3 \times 10^5$ transcripts per larval midgut cell with dimensions of ~20 x 20 x 20 μm. After adjusting for background autoradiographic levels, we obtained between 300 and 350 silver grains per unit area of 75 μm^2 specifically over the cytoplasm of each responding midgut cell of sectioned third-instar larvae after 6 days of autoradiographic exposure. With the same probe we obtained on the average of 9 grains/75 μm^2 over each presumptive midgut cell of sectioned 18 h embryos after the same period of autoradiographic exposure.

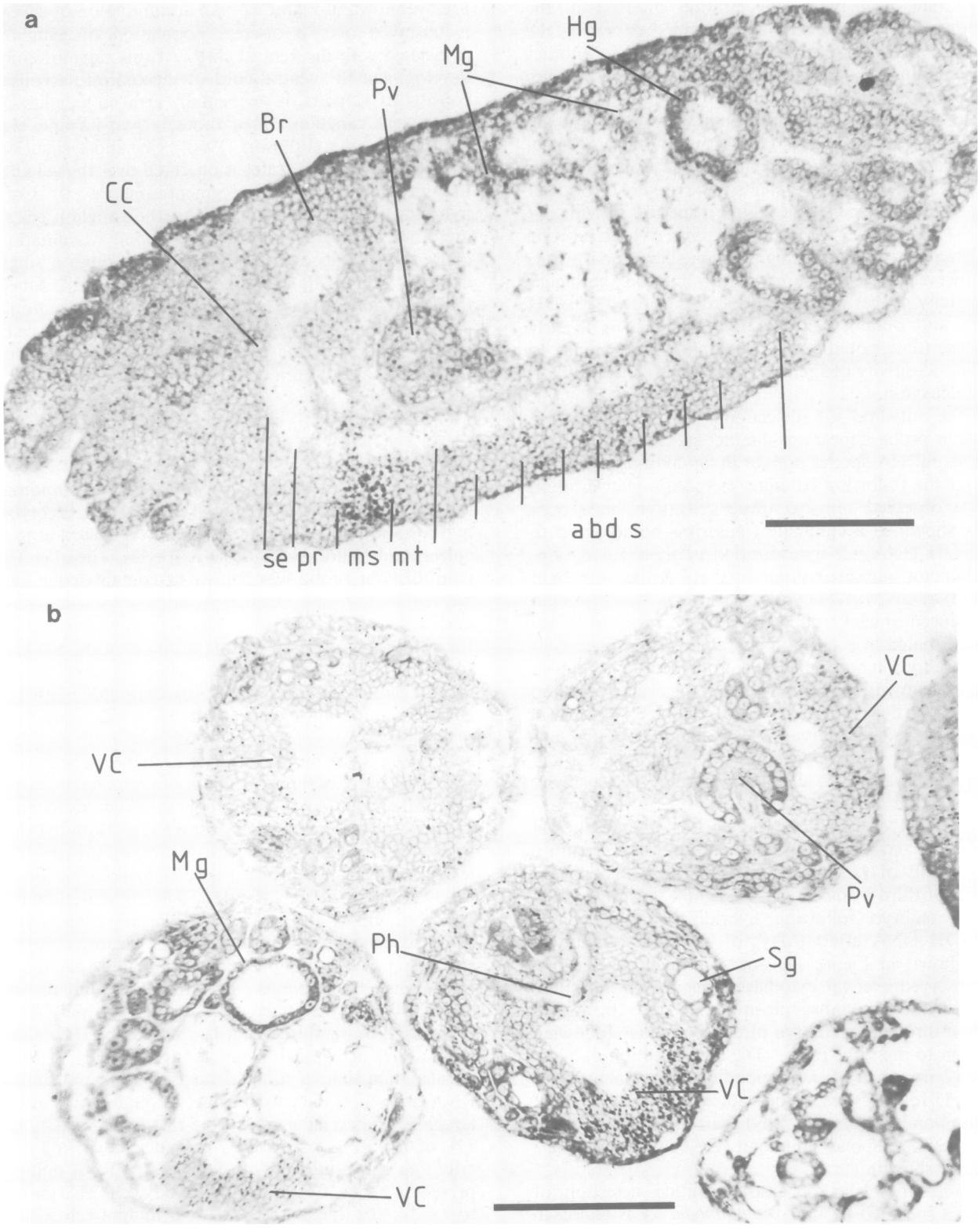

Fig. 2. Location of transcripts homologous to recombinant 903 DNA in sectioned 13 h embryos. **(a)** A sagittal section through a 13 h embryo. Autoradiographic exposure was for 12 days at 4°C. Accumulation of silver grains is observed over the ventral cord ganglion cells of the mesothoracic segment. The horizontal bar indicates a length of 0.1 mm. **(b)** Cross-sections of four different 12–14 h embryos. Autoradiographic exposure was for 5 days at 4°C. Accumulation of silver grains is observed over the ventral cord ganglion cells of one of the embryos. This particular embryo was sectioned near the anterior pole since the pharynx is clearly visible. The horizontal bar indicates a length of 0.1 mm. The abbreviations are the same as indicated for Figure 1 with the following additions: abds, abdominal segments of the ventral cord; CC, circumesophogeal commisure; Hg, hindgut; ms, mesothoracic segment of the ventral cord; mt, metathoracic segment of the ventral cord; Ph, pharynx; pr, prothoracic segment of the ventral cord; Pv, proventriculus; se, subesophogeal ganglion.

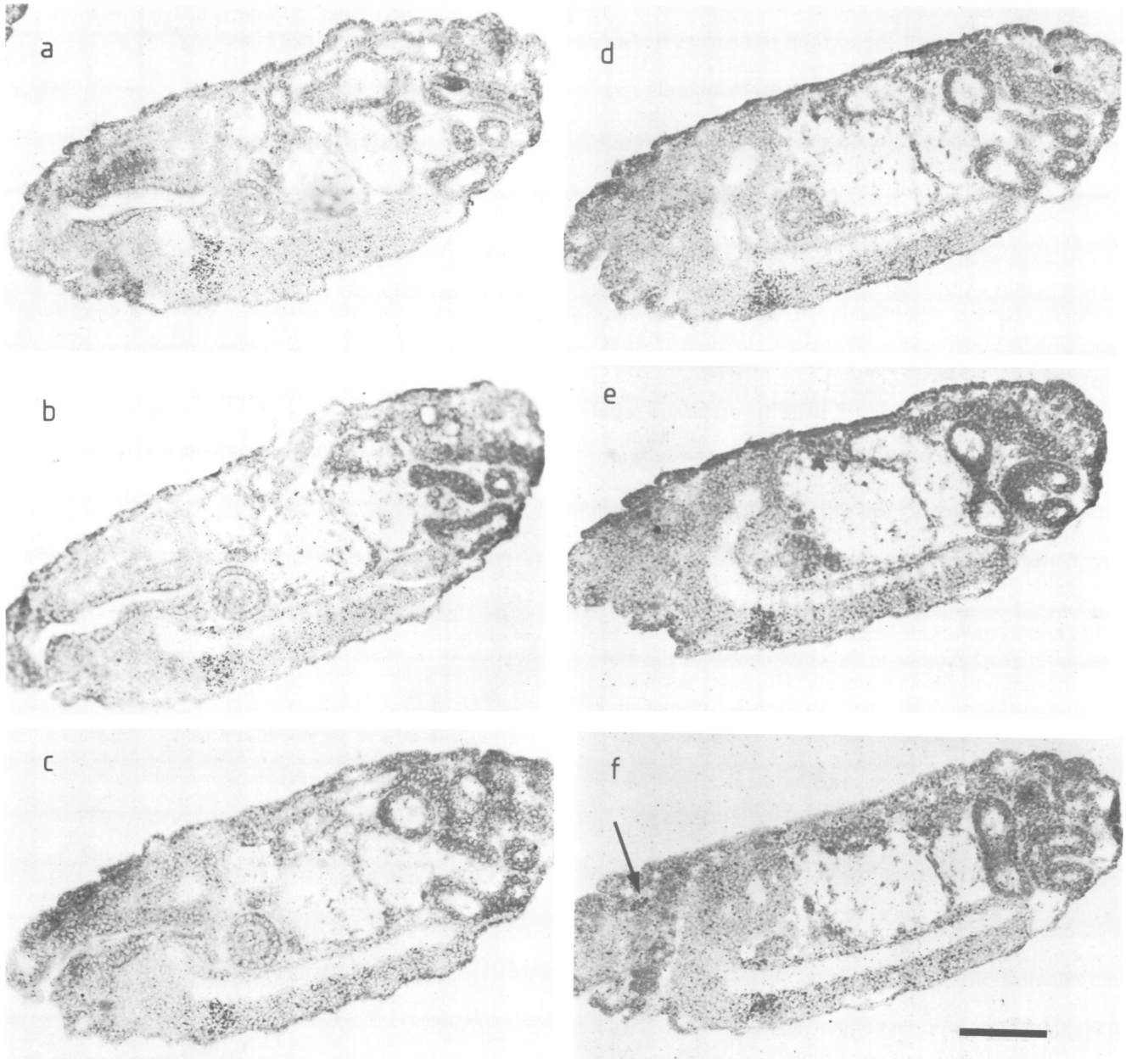

Fig. 3. Location of transcripts homologous to recombinant 903 DNA in consecutive sagittal sections of a single 13 h embryo. Following hybridization and removal of non-specifically bound probe, the sections were autoradiographed at 4°C for 12 days. An enlargement of the section shown in **d** is presented in Figure 2a. In addition to labeling of the mesothoracic ventral ganglion cells, silver grain accumulation is observed to a second site (arrow, **f**).

Hybridization of the aDm3201 DH probe to embryonic midgut cells also appears to involve primarily cytoplasmic, but not nuclear, regions of the responding cells. Since the presumptive midgut cells at this stage of development measure 6 x 6 x 6 μm, the number of transcripts present per cell is ~150. It should be emphasized that this is a rather crude basis for estimating the abundance of the aDm3201 DH transcript in embryonic midgut cells. For example, since the autoradiographic signal was observed to be so intense, the autoradiographic emulsion over the third instar larval sections used for signal quantitation might have been partially saturated. Furthermore, it is difficult to obtain accurate abundance measurements for a specific mRNA species. In any event, by using tritiated probes with specific activities of 4 x 10^7 c.p.m./μg, unambiguous detection of the embryonic midgut transcript was achieved after three days of autoradiographic exposure with a signal to noise ratio of ~14 (see Figure 1).

Approximately 5% of the cellular mass of 18 h embryos correspond to presumptive midgut cells. RNAs homologous to the aDm3201 DH probe were detected only in a specific region of the midgut representing no more than 10% of the total tissue, and furthermore, signals were not observed in tissues other than the presumptive midgut. If we assume that these observations indicate virtually no accumulation of the aDm3201 DH transcripts in cells other than the responding presumptive midgut cells, then this transcript constitutes only ~0.001% of the poly(A)$^+$ RNA obtained from total extracts

of 18 h embryos.

Further improvements of the method to increase the sensitivity of signal detection are underway. It has been reported that the use of specific, single-stranded cRNA hybridization probes substantially increased the sensitivity of detecting nascent histone transcripts on lampbrush chromosomes as compared with corresponding double-stranded DNA probes (Diaz *et al.*, 1981). However, it should be noted that double-stranded hybridization probes are, in general, much less burdensome to prepare and would permit the use of this procedure to screen numerous probes. Alternatively, the use of biotinylated DNA hybridization probes offers the advantage of employing various amplification techniques to enhance signal detection (Brigati *et al.*, 1983). Biotinylated actin DNA segments have been successfully used for the detection of actin mRNA in developing chicken myoblasts (Singer and Ward, 1982). Finally, the use of paraffin or plastic serial tissue sections might provide substantially superior cell morphology as compared with that obtained by frozen sections.

The participation of homeotic genes in the establishment of cuticular segment identity in *Drosophila* has been well documented (Garcia-Bellido, 1975; Lewis, 1978). In particular, Lewis has demonstrated the involvement of the *bithorax* gene complex in the differentiation of the thoracic and abdominal segments (Lewis, 1978), and more recently Kaufman *et al.* (1980) have suggested a similar role for the *Antennapedia* gene complex in the development of the more anterior thoracic and head segments. However, despite the proposed genetic models, the mechanism by which a homeotic gene exerts such developmental control is largely unknown.

An assessment of the tissue distribution and time of appearance of homeotic gene products during the course of *Drosophila* development is crucial for better defining the role of homeotic genes in the specification of segment identity. For example, based on genetic analyses, Struhl (1981, 1982) and Denell *et al.* (1981) have proposed that the *Antennapedia* locus encodes a product that actively promotes a mesothoracic developmental pathway and/or selectively represses antennal development in the mesothorax. A prediction of the model is that the precursor cells of the mesothorax accumulate this product in wild-type embryos.

Recombinant plasmid 903 contains a cDNA insert homologous to regions of the *Antennapedia* locus (Garber *et al.*, in preparation). The number of mRNA species homologous to 903 is not currently known. Preliminary results obtained by A.Kuroiwa and R.Garber (unpublished results) indicate that probaby several distinct *Antp* transcripts share homology with the insert cDNA sequence. Despite this uncertainty, the distribution of the transcripts within serial sections of embryos principally involved neural tissue. A large proportion of the ganglion cells of the segmented ventral cord display hybridization to the 903 probe; however, a higher proportion of the ganglion cells of the mesothoracic segment manifest hybridization and appear to possess at least three times more transcripts than that present in ganglion cells of the other segments. Although the appearance of the $Antp^+$ product in neural cells is not an obvious prediction of the genetic models, it is interesting that the mesothoracic segment of the ventral cord contains the highest level of the transcripts. The observation that at least a portion of the embryonic precursor cells of the adult mesothorax display the most salient expression of the *Antp* gene(s) is consistent with the proposal that $Antp^+$ products actively promote a mesothoracic developmental pathway.

Materials and methods

Preparation of embryo sections

Embryos were collected from a population of wild-type *Oregon-R* flies reared at 25°C on Petri plates containing standard cornmeal agar medium and live yeast. After ageing for appropriate intervals at 25°C, the embryos were removed from the plates and harvested onto a nylon mesh. The embryos were dechorionated by immersion in 3% sodium hypochlorite for 3 min at room temperature. Subsequently, the embryos were washed extensively in water and blotted dry. For fixation, a solution containing equal volumes of n-heptane and 4% paraformaldehyde in phosphate-buffered saline (PBS, 130 mM NaCl/7 mM Na_2HPO_4/3 mM $NaH_2PO_4.H_2O$) was agitated for 1 min at room temperature and the organic and aqueous phases were allowed to separate. An aliquot of heptane saturated with fixative was withdrawn from the organic upper phase, transferred to the well of a depression slide and the dechorionated embryos were gently transferred to the fixative with a brush. The embryos were incubated for 10 min at room temperature in the fixative and occasionally agitated using a Pasteur pipet. The heptane was used to facilitate perfusion of the fixative through the embryo according to the method described by Zalokar and Erk (1977). The organic fixative solution was removed from the well and replaced by a solution containing 4% paraformaldehyde in PBS. The embryos were incubated for an additional 15 min and then rinsed with PBS for a total of 30 min. The PBS solution was removed and the embryos were transferred to a drop of the O.C.T. embedding medium (Miles Laboratories) and then mounted on a cryostat specimen holder. A sufficient number of embryos was added to a drop of embedding medium in order to obtain 100–200 sections per slice (0.25 cm^2). The embedded embryos were frozen by immersing the holder in liquid nitrogen. Afterwards, the embedded embryos were kept in the cryostat at the optimal sectioning temperature of −16°C to −18°C for at least 1 h. Ribbons of serial sections measuring 8 μm were prepared, collected on subbed slides (Gall and Pardue, 1971), placed on a hot plate at 50°C for 1–2 min and dried at room temperature for 1–2 h prior to post-fixation. The tissue was fixed to the slides by immersion in a freshly prepared solution containing 4% paraformaldehyde in PBS and incubated for 20 min at room temperature. The slides were then washed once in 3 x PBS and twice in 1 x PBS for 5 min each at room temperature. The sections were next dehydrated in a graded series of ethanol as follows: 2 min in 30%, 2 min in 60%, 5 min in 80%, 2 min in 94% and 2 min in 100% ethanol. Finally, the slides were air dried. The sections can be stored in a dry chamber at −20°C for at least 1 month prior to use.

Preparation of sectioned tissue for hybridization

In order to permeabilize the tissue sections and to render them accessible to the hybridization probe the following series of treatments are necessary. The fixed sections are first incubated in 0.2 N HCl at room temperature for 20 min and afterwards incubated in water for 5 min. The slides were then immersed in a solution containing 2 x SSC (0.3 M NaCl, 0.03 M Na-citrate, pH 7.0) prewarmed to 70°C and incubated at this temperature for 30 min (Brahic and Haase, 1978). The slides were rinsed in H_2O for 5 min (room temperature), blotted dry with tissue paper and incubated in a freshly prepared solution of predigested pronase at a final concentration of 0.25 mg/ml in 50 mM Tris-HCl, pH 7.5/5 mM EDTA for 10 min at room temperature (Brigati *et al.*, 1983). Pronase (Calbiochem) was dissolved in H_2O to give a final concentration of 40 mg/ml and predigested at 37°C for 4 h in order to inactivate contaminating nucleases (Brigati *et al.*, 1983). The predigested pronase was then lyophilized and stored at −20°C. In order to block further proteolytic activity, following pronase digestion the slides were immediately blotted and immersed in a solution containing 1 x PBS/2 mg/ml glycine (Brigati *et al.*, 1983) and incubated for 30 s. The slides were then rinsed twice in 1 x PBS for 30 s each and afterwards incubated in a solution containing 4% paraformaldehyde in PBS for 20 min at room temperature. Subsequently, the slides were rinsed in PBS and dehydrated in ethanol as described in the preceding section.

Acridine orange detection of cellular RNA in tissue sections

Tissue sections adhered to subbed slides were immersed in 0.2 M glycine-HCl, pH 2.0 for 5 min at room temperature. Afterwards, the slides were incubated in the dark in a solution containing 0.2 M glycine-HCl, pH 2.0 and 0.5 mg/ml acridine orange at room temperature for 30 min. Non-specifically bound stain was removed from the tissue by washing the slides in 0.2 M glycine-HCl, pH 2.0 two times for a total of 15–30 min. The slides were examined by fluorescence microscopy using appropriate filters.

Cloned DNAs used as hybridization probes

Plasmid aDm3201 DH was prepared from a genomic DNA sequence isolated by J.Carlson (1982) and was previously used as a hybridization probe for the detection of a midgut-specific mRNA species in third-instar larvae (Akam and Hogness, unpublished results). The recombinant plasmid contains the pBR322 vector DNA sequence and a 1.8-kb *Drosophila* genomic DNA segment (Akam, unpublished results). The *Drosophila* DNA insert is homologous to a 0.9-kb midgut-specific mRNA species (Carlson, 1982). The purified plasmid DNA was kindly provided by Michael Akam.

Plasmid 903 contains a 2.2-kb cDNA sequence inserted in pBR322. The cDNA sequence was originally obtained from a recombinant λ library containing cDNA sequences that were prepared from embryonic poly(A)$^+$ RNA (Goldschmidt-Clermont, unpublished results). The cDNA insert of plasmid 903 was demonstrated to possess homology with regions of cloned genomic DNA segments that derive from the *Antennapedia* locus (Garber *et al.*, in preparation).

Preparation of ^{3}H-labeled hybridization probes

Recombinant plasmid DNAs were labeled with tritiated deoxynucleoside triphosphates by a modified nick-translation protocol using *Escherichia coli* DNA polymerase I (Rigby *et al.*, 1977). Labeled nucleotides were purchased from New England Nuclear Laboratories and possessed the following specific activities: dATP, 18 Ci/mmol; dTTP, 77.8 Ci/mmol; dCTP, 62.8 Ci/mmol; dGTP 43.3 Ci/mmol.

Aliquots containing 100 μCi of each of the four labeled nucleotides were lyophilized and resuspended in 50 μl of the following solution: 50 mM Tris-HCl, pH 8.0/5 mM $MgCl_2$/0.1 mg/ml bovine serum albumin (BSA)/12.5 mM β-mercaptoethanol/0.1 ng/ml DNase I (purchased from Sigma)/20 μg/ml recombinant plasmid DNA. After addition of 15 units of *E. coli* DNA polymerase I (purchased from New England Biolabs) the reaction mixture was incubated at room temperature for 45 min. The DNase I and polymerase I enzymes were inactivated by addition of EDTA to a final concentration of 20 mM and subsequently incubating the mixture at 65°C for 10 min. The ^{3}H-labeled DNA probe was ethanol precipitated after addition of 250 μg of sonicated herring sperm DNA carrier (per microgram probe DNA). Following precipitation, the DNA was pelleted by centrifugation in an Eppendorf microfuge for 15 min and the pellet was washed three times with 70% ethanol at room temperature. The dried DNA pellet was resuspended in the following buffer to achieve a final probe concentration of 2 μg/ml: 50% deionized formamide/0.6 M NaCl/10 mM Tris-HCl, pH 7.5/1 mM EDTA/1 x Denhardt's solution (Denhardt, 1966)/500 μg/ml yeast tRNA. For some of the experiments, 50% dextran sulfate was added to the hybridization solution to a final concentration of 10%. In addition, for those experiments involving the use of poly(A-T) tailed probe sequences, the hybridization solution was brought to a final concentration of 5 mg/ml poly(U) (purchased from Sigma).

Under the conditions described, the hybridization probes were ^{3}H-labeled to a specific activity of 4 x 10^7 c.p.m./μg. The mean single-stranded length of the labeled DNA was determined to be 100–250 bases by electrophoretic fractionation on denaturing polyacrylamide slab gels.

Hybridization of ^{3}H-labeled DNA to tissue sections

The labeled DNA was denatured by boiling the hybridization solution for 1–2 min. 20 μl of the solution were applied to each slide and covered with 18 x 18 mm coverslips. To prevent evaporation of the hybridization solution, the edges of the coverslips were sealed with a 1:1 mixture of rubber cement (Sanford brand) and petroleum ether. The slides were placed in a humid chamber and incubated at 35°C for 24–36 h. Following hybridization the rubber cement was removed from each slide with forceps and the coverslips were dislodged by floatation in a solution containing 50% formamide/0.6 M NaCl/10 mM Tris-HCl, pH 7.5/1 mM EDTA. The slides were subsequently washed in the same formamide buffer at 35°C for 18–24 h with four changes of solution. Afterwards, the slides were dehydrated by incubating twice in 70% ethanol/0.3 M ammonium acetate, pH 7.0, 5 min each, and then twice in 95% ethanol/0.3 M ammonium acetate, pH 7.0, 5 min each. The slides were air-dried and then immersed in Kodak NTB-2 emulsion diluted 1:1 with 0.6 M ammonium acetate, pH 7.0 at 45°C, placed on a metal plate pre-equilibrated to 0°C for 5 min and air-dried at room temperature for 1–2 h. Autoradiography was performed as described by Gall and Pardue (1971) at 4°C in a dry chamber. After appropriate exposure times the slides were developed in Kodak D-19 developer for 4 min and fixed in 30% sodium thiosulfate. The sections were lightly stained with Giemsa and examined by light microscopy. Photomicrographs were obtained with Kodak 2415 ester base film.

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