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A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*

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Abstract. We have developed a non-radioactive in situ hybridization technique for the localization of RNA in whole mount *Drosophila* embryos. After fixation, whole embryos are hybridized in situ with a DNA probe which has been labeled with digoxygenin. The hybridization products are detected by using a phosphatase-coupled antibody against digoxygenin. In parallel experiments, embryos can be treated with an antibody directed against the corresponding protein product to allow the detection of its distribution using standard immunochemical techniques. We have used this approach to compare the spatial and temporal distribution patterns of the RNA and protein products of the segmentation gene *hunchback* (*hb*) during the early stages of embryogenesis. This comparison revealed translational control of the maternally derived *hb* mRNA, which was difficult to detect by conventional techniques. The non-radioactive in situ hybridization method is as sensitive as conventional methods, but is faster and easier to perform. This may make it a useful tool for a variety of other systems.

Introduction

The segmentation gene *hunchback* (*hb*) is a key gene for the early pattern formation process in the *Drosophila* embryo (Lehmann and Nüsslein-Volhard 1984; Tautz et al. 1987). *hb* has been classified as a gap gene (Nüsslein-Volhard and Wieschaus 1980) and acts at the top of the zygotic segmentation gene hierarchy (reviewed in Akam 1987; Ingham 1988). However, there are additional expression aspects of *hb* which have separate functions (Schröder et al. 1988). Maternal expression of *hb* plays an important role in the correct formation of the abdomen (Hülskamp et al. 1989). The first zygotic expression occurs in the anterior half of the preblastoderm embryo and is under the direct control of the anterior pattern organizer gene *bicoid* (Frohnhöfer and Nüsslein-Volhard 1986; Driever and Nüsslein-Volhard 1989). Expression of *hb* in this case is functionally most directly related to its gap gene character.

Secondary zygotic expression at the mid blastoderm stage has a specialized function in the formation of the second thoracic segment and the seventh and eighth abdominal segments (Lehmann and Nüsslein-Volhard 1984; Tautz et al. 1987; Schröder et al. 1988). These expression patterns of *hb* RNA have been analyzed using radioactive probes for in situ hybridization experiments on sections of early embryos (Tautz et al. 1987; Schröder et al. 1988). This technique depends highly on the quality of the sections and on morphological markers to determine the orientation of the section relative to the whole embryo. Thus the interpretation of the spatial pattern during early stages of embryogenesis is sometimes a matter of subjective judgement. In contrast, expression pattern of the protein can be examined in whole embryos by the use of specific antibodies in combination with immuno-staining techniques. This allows an easy and very detailed analysis of preblastoderm embryos (Macdonald and Struhl 1986; Tautz 1988; Driever and Nüsslein-Volhard 1988). It therefore seemed desirable to develop a technique which would allow the RNA patterns to be examined at the same level of accuracy as the protein pattern. Here we describe a protocol for non-radioactive in situ hybridization on whole embryos. The technique is as sensitive as standard immuno-staining techniques with specific antibodies and therefore it is now possible directly to compare and analyze dynamic RNA and protein expression patterns in parallel. Using this technique for the segmentation gene *hb*, we can show that there is strict translational regulation of the maternally derived *hb* mRNA. This regulatory mechanism leads to a transient posterior gradient of the *hb* protein distribution, which may be involved in the formation of the posterior segment pattern (Hülskamp et al. 1989).

Materials and methods

All embryos in this study came from wild-type *Drosophila* flies and were collected on apple juice agar plates at intervals of 0–4 h at 25° C (Wieschaus and Nüsslein-Volhard 1986). The DNA labeling and detection kit was supplied by Boehringer (Mannheim).

Preparation of embryos. The embryos are collected in small baskets, washed with water and dechorionated in a solution of 50% commercial bleach ("Klorix", about 5% sodium hypochlorite) in water for about 2–3 min. The embryos are then washed with 0.1% Triton X-100 and fixed either with formaldehyde or paraformaldehyde.

Formaldehyde fixation. The embryos are transferred into glass scintillation vials containing 4 ml of 0.1 M Hepes, pH 6.9, 2 mM magnesium sulfate, 1 mM EGTA [ethylenglycol-bis(2-aminoethylether)-N,N-tetraacetic acid; stock-solution 0.5 M, adjusted to pH8 with NaOH]. To this 0.5 ml 37% formaldehyde solution and 5 ml heptane are added. The vial is shaken vigorously for 15–20 min to maintain an effective emulsion of the organic and the water phase.

The lower phase is then removed and 10 ml methanol are added. The embryos sink to the bottom at this stage. If not, the upper phase (heptane) is removed and more methanol added. The embryos may now be stored in methanol for some time (up to several weeks) in the refrigerator.

Paraformaldehyde fixation. This protocol is more laborious, but leads in some cases to a lower background and to better preservation of the morphology. The embryos are transferred into a glass scintillation vial containing 1.6 ml of 0.1 M Hepes, pH 6.9, 2 mM magnesium sulfate, 1 mM EGTA. Then 0.4 ml 20% paraformaldehyde (stock solution in water kept at −20° C; the paraformaldehyde can be dissolved by heating to 65° C and neutralizing with a little NaOH) and 8 ml heptane are added. The vial is shaken and methanol treated as above. Afterwards the embryos are transferred into a solution of 90% methanol and 10% 0.5 M EGTA (ME) in 1.5 ml Eppendorf tubes. The embryos are then refixed and dehydrated by passage through a series of steps consisting of ME and PP [PP, 4% paraformaldehyde in phosphate buffered saline (PBS, 130 mM NaCl, 10 mM sodium phosphate (pH 7.2)]. The first step is for 5 min in 7/3 ME/PP, the second for 5 min in 1/1 ME/PP, the third for 5 min 3/7 ME/PP and the last step for 20 min in PP alone. The embryos are then washed in PBS for 10 min and directly subjected to the subsequent steps. However they can also be stored at this point by dehydrating them in a 30%, 50% and 70% ethanol series and leaving them at −20° C. Before proceeding with the protocol they must be rehydrated.

Pretreatment. All pretreatment steps are performed in 1.5 ml Eppendorf tubes in 1 ml solution at room temperature and on a revolving wheel. Potential RNAase contamination should be avoided. The PBS for the following steps is treated with diethylpyrocarbonate (20 µl in 500 ml) and autoclaved. The embryos are first washed 3 times for 5 min each in PBT (PBS plus 0.1% Tween 20). They are then incubated for 3–5 min in 50 µg/ml Proteinase K in PBS. The digestion is stopped by incubating for 2 min in 2 mg/ml glycine in PBT and the embryos are then washed 2 times for 5 min each in PBT, refixed for 20 min with PP and finally washed 3 times for 10 min each in PBT. The proteinase digestion time is critical. If it is too short, the background is increased and sensitivity is lost. If it is too long, the embryos will burst during the subsequent steps.

Hybridization and washing. The hybridization solution (HS) consists of 50% formamide, 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium-citrate), 50 µg/ml heparin, 0.1% Tween 20 and 100 µg/ml sonicated and denatured salmon sperm DNA. (HS may be stored at −20° C). The embryos are washed for 20 min in 1/1 HS/PBT then for 20–60 min in HS. Afterward they are prehybridized for 20–60 min at 45° C in a waterbath. Most of the supernatant is then removed and the heat-denatured probe (see below) is added and thoroughly mixed. The probe is heat denatured in the presence of 10 µg sonicated salmon sperm DNA, which should effectively compete against simple sequences and thus reduce the background. The probe is used at a concentration of about 0.5 µg/ml (calculated on the basis of the concentration of the primarily labeled fragment see Probe labeling). Hybridization is overnight at 45° C in a waterbath and may be done without shaking. The embryos are then washed at room temperature for 20 min at each step. The first step is in HS, the second in 4/1 HS/PBT, the third in 3/2 HS/PBT, the fourth in 2/3 HS/PBT, the fifth in 1/4 HS/PBT and the last two 20 min each in PBT. A less extensive washing protocol may be used for probes which produce low background.

Staining. The embryos are incubated for 1 h at room temperature on a revolving wheel in 500 µl antibody-conjugate solution (supplied with the Boehringer kit, freshly diluted 1/2,000 to 1/5,000 in PBT). The antibody may be preabsorbed for 1 h with fixed embryos to reduce the background. The embryos are washed 4 times for 20 min each in PBT, then 3 times for 5 min each in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 1 mM Levamisole (a potent inhibitor of lysosomal phosphatases), 0.1% Tween 20. The embryos are brought into a small dish with 1 ml of the above solution. To this are added 4.5 µl NBT and 3.5 µl X-phosphate solution (both supplied with the Boehringer kit) with thorough mixing. The color develops in 10–60 min in the dark. Color development may however be occasionally controlled under the binocular microscope and stopped in PBT before background develops. The embryos can then be dehydrated in an ethanol series and mounted in GMM (Lawrence et al. 1986).

Probe labeling. Probes consisted of isolated DNA fragments (Tautz and Renz 1983) and were labeled according to the protocol supplied with the Boehringer Kit. In short, the DNA fragments are denatured for 10 min at 100° C and quickly cooled for 10 min in an ice/NaCl bath. Buffers, primers and enzyme are added and the mixture incubated for between 3 h and overnight at 37° C. We found no difference whether the probe was then precipitated according to the Boehringer protocol or used directly. The labeling was done by random priming (Feinberg and Vogelstein 1983), which produces fragments of 100–300 bp in length, which are optimal for in situ hybridization experiments. To determine their specific activity we tested the labeled fragments in standard blot hybridization experiments. From these we concluded that even probes with a moderate specific activity (about five times less than the claimed maximum) work very well for in situ hybridization experiments. Probes should therefore normally be no problem. Higher specificity may however be obtained with single-stranded probes. These can be synthesized by first producing a sense RNA strand for the gene in question and then using reverse transcriptase for the random priming reaction. The RNA template can then be digested and the resulting probe will no longer hybridize with itself.

Protein staining. Embryos were fixed using the above formaldehyde fixation protocol, incubated with specific antibodies against the *hb* protein (Tautz 1988) and immunostained using the horse radish peroxidase ABC Vectastain kit (Vector Laboratories) as described (Macdonald and Struhl 1986).

Results and discussion

DNA fragments from the coding regions of several segmentation genes which show spatial regulation in the early embryo were labeled using the random priming technique with a digoxygenin-labeled deoxynucleotide. These probes were

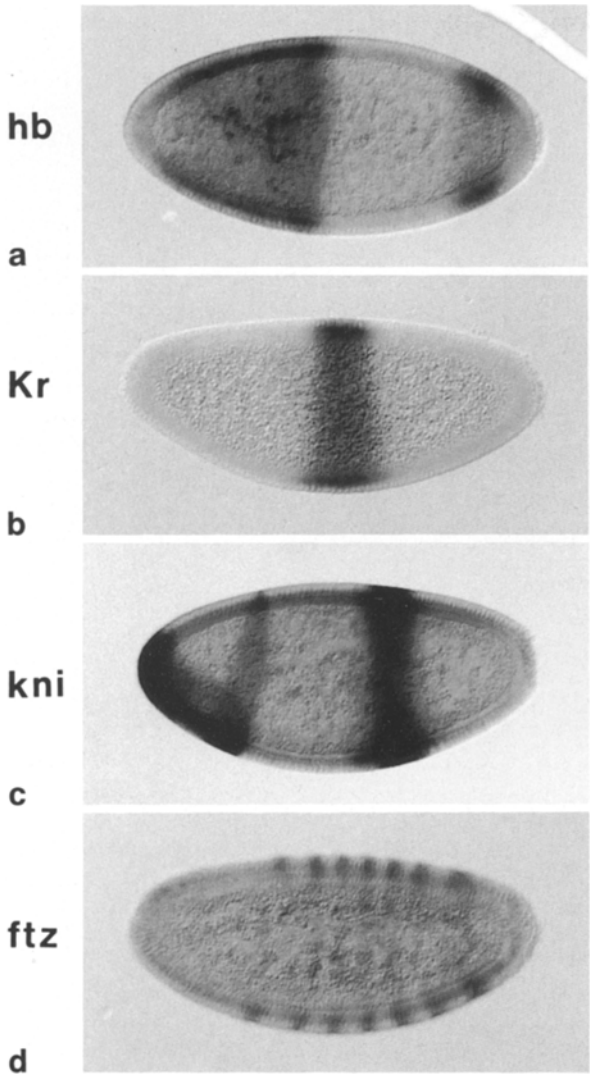

Fig. 1a–d. RNA expression patterns of several segmentation genes using non-radioactive detection. Whole embryos were hybridized with labeled fragments from the coding regions of **a** *hunchback*, **b** *Krüppel*, **c** *knirps* and **d** *fushi tarazu*. All embryos are at the beginning of formation of the cellular blastoderm

used to develop an in situ hybridization protocol for whole embryos, the details of which are described in Materials and methods. Figure 1 shows examples of the staining of the RNA products from the segmentation genes *hb* (Tautz et al. 1987), *Krüppel* (Preiss et al. 1985), *knirps* (Nauber et al. 1988) and *fushi tarazu* (Kuroiwa et al. 1984; Weiner et al. 1984). It appears that it is possible to obtain almost single cell resolution and that the products are predominantly located in the cytoplasm, as is expected for bona fide mRNAs.

We compared the RNA and protein expression patterns of *hb* in embryos of preblastoderm and blastoderm stages (Fig. 2). Figure 2a shows very early embryos prior to the formation of the pole cells, at about nuclear division cycle 6 to 7 (Foe and Alberts 1983). The *hb* RNA is homogeneously distributed throughout the whole embryo, though it appears that it may be concentrated within the cytoplasmic regions which surround the individual nuclei. At the same stage, the first expression of the *hb* protein already shows differential distribution along the anterior-posterior axis (Fig. 2a), forming a linear concentration gradient in the posterior half of the embryo (Tautz 1988). This differential protein distribution could be explained by translational regulation, differential degradation or even active redistribution of the *hb* RNA. The latter two possibilities are however not compatible with the present observation of homogeneous distribution of *hb* RNA at this stage. It is thus clear that the maternally provided *hb* RNA is under translational control. This control is exerted by the posterior maternal pattern organizer genes (Nüsslein-Volhard et al. 1987; Tautz 1988; Hülskamp et al. 1989). If one of these is mutant, the *hb* RNA is uniformly translated (Tautz 1988) and the ectopic presence of the *hb* protein in the posterior region causes suppression of abdominal segment formation (Hülskamp et al. 1989). This translational control is one of the first regulatory events in the early *Drosophila* embryo and is thus of profound importance for the whole process of pattern formation.

The first differential distribution of the maternally provided *hb* RNA can only be seen after the formation of the pole cells. It appears that there is less staining in the posterior half of the embryo, while it persists in the anterior half (Fig. 2b). This stage was difficult to analyze by conventional techniques using radioactive probes for in situ hybridizations on sectioned embryos. We have interpreted the *hb* RNA distribution at this stage as a concentration gradient along the entire anterior-posterior axis (Tautz et al. 1987; Schröder et al. 1988); others have not observed a differential distribution at all (Bender et al. 1988). Staining of whole mount embryos with the new technique shows unambiguously that the *hb* RNA is degraded in the entire posterior half of the embryo while it persists in the whole of the anterior half. The *hb* protein is completely confined to the anterior half at this stage (Fig. 2b), even though there is residual *hb* RNA in the posterior. The degradation of the *hb* RNA may thus be simple a consequence of translational repression in this region and presumably needs no additional control mechanism.

The first zygotic expression of *hb* occurs in the anterior half of the embryo around stage 12 to 13 and is fully developed at early stage 14 (Fig. 2c). Protein expression at this time is only slightly delayed. At mid stage 14 a posterior cap (Fig. 2d) and a little later a posterior stripe (Fig. 2e) of expression develops. Also at mid stage 14, RNA expression recesses from the anterior tip (Fig. 2e). These patterns are in each case closely followed by the expression of the protein. This indicates that there is no additional translational regulation effective at this time of development. It is most interesting that the posterior stripe of expression, which derives from the same promoter as the maternal *hb* RNA (Schröder et al. 1988), also does not show any signs of translational control, even though it lies in a region where the translation of the same RNA was strongly suppressed only a few nuclear cycles earlier. This demonstrates very clearly that the regulatory influence of the maternal gene products has ceased at this stage.

A similar effect is apparent shortly before gastrulation, where a very clear distinction between the RNA and the protein expression patterns can be observed (Fig. 2f, g). The RNA expression in the anterior region is replaced first by a very transient three-striped (Fig. 2f) and then a two-striped (Fig. 2g) expression pattern. This expression is derived from the P1 promoter of *hb*, which transcribes a 3.2 kb

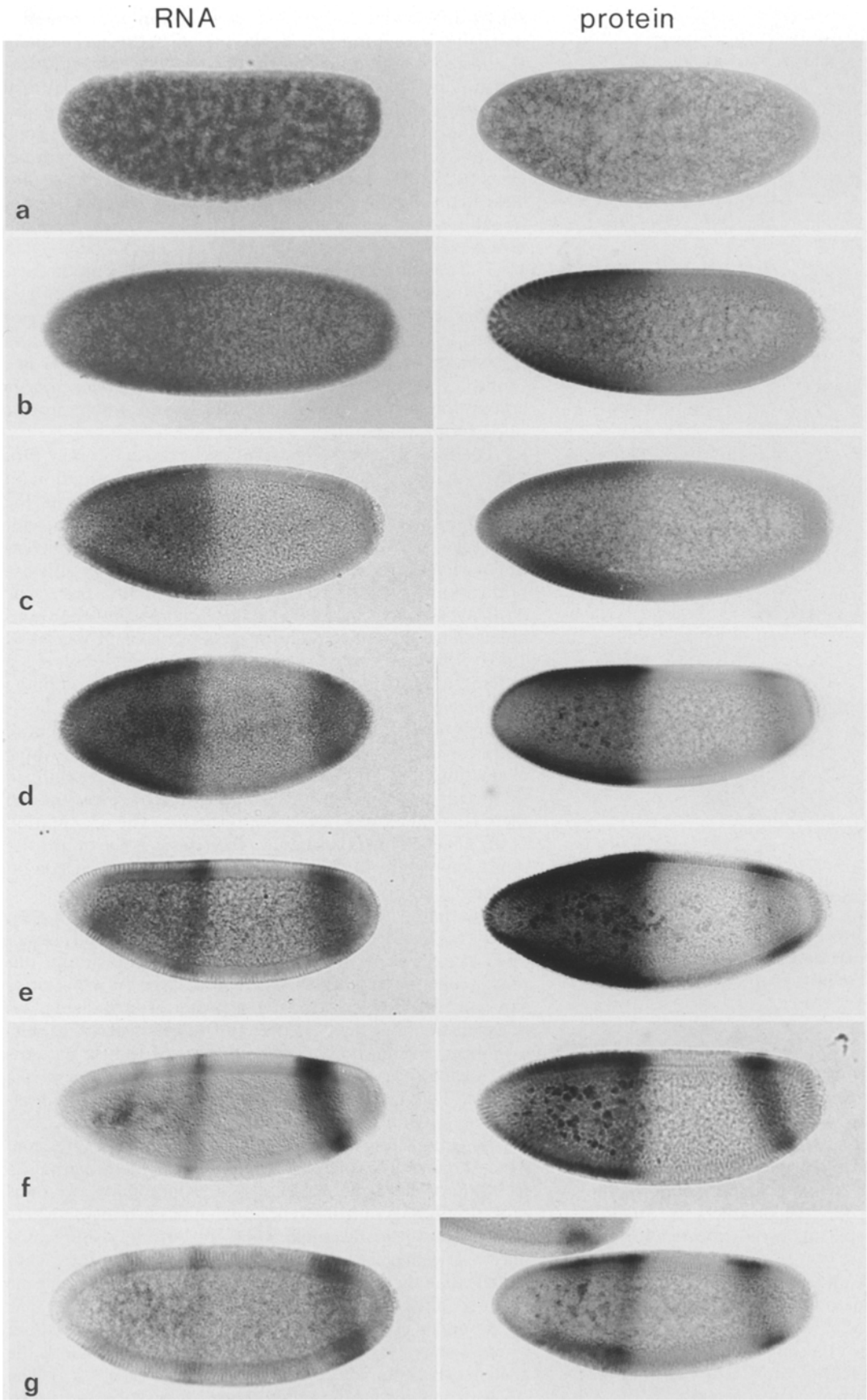

Fig. 2a–g. Comparison of the spatial and temporal expression patterns of the RNA (*left panel*) and protein (*right panel*) products of the segmentation gene *hunchback*. Equivalent stages are compared in each case. See text for further details

mRNA (Tautz et al. 1987; Schröder et al. 1988). The 2.9 kb mRNA expressed from the P2 promoter is apparently no longer present at this stage. This implies that the regulatory action of *bicoid* on *hb*, which results in the transcription of RNA from this P2 promoter (Schröder et al. 1988; Driever and Nüsslein-Volhard 1989), presumably ceases at mid stage 14, even though the *bicoid* protein is still present at this time (Driever and Nüsslein-Volhard 1988). The *hb* protein remains in the anterior half at this stage, though the staining is already weaker and the additional expression in the two anterior stripes becomes more evident (Fig. 2g).

Our high resolution in situ hybridization technique shows that there is a very clear translational regulation of the maternally provided *hb* RNA and that this regulation is effective only in preblastoderm embryos. This analysis was only possible by a direct comparison of the spatial and temporal RNA expression patterns in whole embryos. This demonstrates that it can be very useful to be able to make such a direct comparison. We think therefore that this approach may also be applicable to a variety of other systems. Needless to say, this improved in situ hybridization technique is also much faster and easier to perform than the techniques employed so far (Akam 1983; Hafen et al. 1983) and it may therefore be the preferred choice for future experiments.

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