Oligonucleotide probes detect splicing variants *in situ* in *Drosophila* embryos

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ABSTRACT

We describe a method for the *in situ* detection of specific splicing variants. The method is based on the use of antisense oligonucleotides designed to span splice junctions labelled with digoxigenin by terminal transferase tailing. We find that the spatial patterns of *Ubx* splicing variants Ia and IIa are similar in early embryos, but differ in late embryos. Variant IVa is only detected in the CNS (ps6) at stages 16 and 17. We also present evidence indicating that the first splicing event is cotranscriptional.

INTRODUCTION

The transcripts of many eukaryotic genes show alternative splicing (1,2). In the case of the *Drosophila* homeotic genes *Antennapedia*, *Ultrabithorax*, *labial* and *proboscipedia*, alternative splicing yields a family of related homeoproteins that vary in the region immediately upstream of the DNA-binding homeodomain (3–9). These patterns of alternative splicing have been analyzed in most detail for *Antp* and *Ubx* where they have been shown to be temporally regulated. There is also evidence suggesting that alternative splicing is also tissue specific (3–6,10–12).

To date it has not been possible to detect the spatial distribution of alternatively spliced transcripts directly in the *Drosophila* embryo. The methods generally used for the *in situ* detection of RNA in the *Drosophila* embryo are based on the labelling of cDNA or genomic DNA fragments (13–15); they can only be used to recognize those splicing variants that include a unique exon in the structure of the processed transcript (e.g. transcripts arising from the alternative promoters of *Antp*) (16). Here we describe a method for the detection of specific splicing variants *in situ* even though they may contain no unique sequences. The method is based on the use of digoxigenin labelled probes (15) but in this case we use antisense oligonucleotides that span the splice sites. Under appropriate conditions, these probes hybridize only to transcripts containing the defined splice junction. The use of this technique should help to dissect the temporal and spatial pattern of expression of complex transcription units, such as those of the homeotic genes.

MATERIALS AND METHODS

Embryo preparation, fixation and pretreatment

Drosophila embryos are collected, fixed with paraformaldehyde and processed for whole mount hybridization as described previously (15) with minor modifications. The paraformaldehyde concentration of the first fixative is increased to 10%. Because specific transcript variants are rare compared to total transcripts, it is necessary to take full precautions to eliminate RNases during embryo preparations (e.g. DEPC treatment of buffers, baked glassware, etc)

Oligonucleotides

30-mer oligonucleotides were designed to be complementary to splice junctions within *Ubx* transcript variants Ia, IIa and IVa (see figure 1). These were:

Oligo Ia: 5'-CAGATCTTATCTTAC/CTGCGATAGCCA-TCC-3', the inverse complement of the sequence 1689–1703/1731–1745. Base numbering according to Fig. 2 of Kornfeld et al (6); the/indicates the splice point.

Oligo IIa: 5'-CTGAGTATCTCTTAC/CTGCGATAGCCA-TCC-3', the inverse complement of the sequence 1689–1703/1782–1796.

Oligo IVa: 5'-GCAGACCATTGTGTAC/CTGCGATAGCCA-TCC-3', the inverse complement of the sequence 1689–1703/1833–1847.

In addition, three control probes were made. Oligo A1 was designed containing the same two sections of *Ubx* sequence as probe Ia, but coupled in the reverse order, i.e., 5'-CTGCGAT-AGCCATCC/CAGATCTTATCTTAC-3', the inverse complement of the sequence 1731–1745/1689–1703. The second control probe (Oligo E5-965) is an oligo directed against the 5' exon, that should hybridize both to processed and unprocessed *Ubx* RNA: 5'-AAAGTACGAGTTCATTGCGCT-GCTGGCGGT-3', the inverse complement of the sequence 950–979. The third control oligo is required because the first six nucleotides of microexons 1 and 2 are identical. This makes it unavoidable that oligos Ia and IIa will be identical in the last 21 nucleotides, instead of only 15 nucleotides. In order to make sure that this does not result in any spurious hybridization, we designed a third control probe, oligo A2, which corresponds to the sequence 10–30/1–9 of oligo Ia. All oligonucleotides were purchased from MedProbe (Oslo, Norway).

Probe labelling

Oligonucleotides are labelled with digoxigenin by 3' tailing using terminal transferase (Boehringer Mannheim) essentially as described by Schmitz et al (17) but using a lower dATP concentration. The labelling reaction is set up by combining the following components: 35 pmol oligonucleotide; 4 μ l 5 $\times$ tailing buffer (Boehringer Mannheim); 6 μ l 5 mM CoCl₂; 2.5 μ l DIG-dUTP (1 mM stock solution, as supplied by Boehringer Mannheim); 2 μ l 50 μ M dATP; 1 μ l (25 U) terminal transferase; and water up to 20 μ l. The mixture is incubated at 37°C for 1 h and ethanol precipitated in the presence of LiCl as described (17).

Oligos labelled with higher dATP concentrations have an average tail length of 50 nucleotides, five of them digoxigenin molecules (17). These seem to give lower hybridization signals, possibly because they reduce the penetrance of the oligonucleotides into the embryos (Artero and Pérez-Alonso, unpublished observations). In our labelling protocol we estimate that the same number of digoxigenin molecules are incorporated but a much shorter tail is produced due to the limited amount of dATP in the labelling mixture.

Hybridization and washing

The embryos are processed for hybridization as described by Tautz and Pfeifle (15). The hybridization solution consists of 50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.1% Tween 20, 1 mg/ml sonicated salmon sperm DNA and 250 μ g/ml tRNA. Prehybridization is done for at least 1 h at 37°C. Hybridization is overnight at the same temperature, with a probe concentration of 0.1 to 0.3 μ g/ml. The embryos are then washed as described (15) except that the temperature is increased to 40°C.

Staining

The detection of the digoxigenin is carried out as described (15) except that the concentration of the antidigoxigenin antibody (Boehringer Mannheim) is increased to 1:1000. All other procedures are as in Tautz and Pfeifle (15). The alkaline phosphatase reaction needs to be incubated for at least 3–4 hours when using probes specific for processed RNA variants. However, in those hybridizations performed with the oligo probe for the 5' exon, the signal is clearly visible after less than 1 hour. The long incubation time necessary to obtain a clear signal when using a splicing specific probe makes it unavoidable that the endogenous background will increase, limiting the detection of the rarest variants.

RESULTS AND DISCUSSION

All *Ubx* proteins include a common amino terminal region, which is 247 amino acids long, and a common carboxy terminal region of 99 amino acids which includes the homeodomain. These two common regions are encoded by the 5' and 3' common exons. Alternative splicing of the primary transcript involves the presence or absence of three variable protein-coding elements: microexons 1 and 2 (both 51 bases), and a third element of 27 bases lying between the two alternative 5' splice sites that terminate the *Ubx* 5' exon (6). Transcript variants of class I contain both microexons; variants of class II contain only microexon 2; variants of class IV lack both microexon 1 and microexon 2. No transcripts of the potential class III (microexon 1 only) have been detected in vivo. Variants of each class are

classified as 'a' or 'b' according to the use of the first or second of the alternative 5' splicing sites in the 5' exon (6).

Only transcripts of class I are defined uniquely by the inclusion of specific sequences (in this case, microexon 1). However, all transcripts are defined by unique sequence juxtapositions at the first splice junction (see Fig. 1). Oligonucleotides spanning these splice junctions were labelled with digoxigenin by terminal transferase tailing and these labelled oligos were used as probes for whole mount in situ hybridization as described in Materials and Methods.

Oligonucleotides detect splicing variants

Figures 2A to 2E show embryos about the same developmental age (stage 11 of Campos-Ortega and Hartenstein (18)) hybridized with different oligonucleotide probes. Oligo E5 is expected to hybridize to unprocessed *Ubx* RNA as well as to all kinds of processed transcripts. This probe gives the strongest signal (Fig. 2A) revealing a transcript distribution similar to that described previously using long DNA fragments as probes (14,19). Oligos Ia and IIa are expected to hybridize to processed transcripts only. They give much weaker hybridization signals (Figs. 2B and 2C) but both reveal a transcript distribution that is similar to that observed with the common probe E5. At this stage, probe Ia shows a stronger signal than probe IIa, as expected from the temporal pattern of splicing previously described (5,6).

We have used two control oligo probes. Probe A1 contains the same 15-base sequence elements as oligo Ia, but linked in the reverse order (oligo A1, see Materials and Methods). Both halves of this oligo could hybridize to the same transcripts as oligo Ia, but the extent of base pairing in any one duplex can be only 15 bases. Embryos hybridized with this probe do not show any detectable signal at any of the stages tested (data not shown), thus confirming that the common 15-base element which is included in all three oligo probes (Ia, IIa and IVa) do not contribute to any unspecific hybridization signal. Probe A2 represents a more stringent control, it takes into account the fact that the first six nucleotides of microexon 1 and microexon 2 are identical (see Methods). Figure 2E shows an embryo hybridized with this probe where no *Ubx* pattern can be resolved.

Figure 1. Oligonucleotides used in this project and their relation with the *Ubx* transcription unit. E5' and E3' denote the 5' and 3' common exons; microexon 1 and microexon 2 indicate the positions of the two microexons in the genome.

Figure 2. (A) to (E) show embryos at stage 11 of Campos-Ortega and Hartenstein (17). The preparation in (A) was hybridized with oligo E5-965; the probes for (B), (C), (D) and (E) were Ia, IIa, IVa and A2, respectively. Note the difference in the hybridization levels between panels A and B (see text). The tissue specificity of some of the splicing variants is shown in figures F to H. Panel F: Embryo at stage 13, hybridized with probe Ia (lateral view). This probe labels principally the mesoderm and epidermis which express *Ubx* maximally at this stage. Panels G and H: Comparable views of stage 16 embryos hybridized with probes Ia (G) and IIa (H). Ventro-lateral views focussed on the CNS. Probe IIa labels the CNS strongly at this stage, whereas probe Ia labels it only very weakly (compare with earlier stages shown in panels B and C where probe Ia hybridizes the more strongly). Figures I and J show that the first splicing event is cotranscriptional. Due to the large size of the transcription unit, *Ubx* transcripts are mainly detected as nascent RNA during elongation, attached to its locus (foci of transcription, see text). Panels I and J show the epidermis and the amnioserosa from embryos about stage 11 hybridized with oligos E5-965 and Ia, respectively. We interpret the dots in j as RNA intermediates (during elongation) that have already been spliced following the Ia pattern (exon 5' joined to microexon 1). Staining times for these embryos are between 5 and 8 hours. The probe concentrations are in the range of 0.1 to 0.3 $\mu\text{g}/\text{ml}$.

The transcript distributions revealed by probes Ia and IIa at stage 11 are qualitatively similar. Probe IVa shows no signal at this stage (Fig. 2D), this result being consistent with the observation that transcripts with this splicing pattern are not produced at stage 11 (5,6). At later stages, the splicing specific probes reveal distinct patterns of labelling. Probe IIa yields strong signal in the CNS (Fig. 2H), whereas probe Ia shows much weaker labelling in the CNS (predominantly in parasegment 6, see Fig. 2G), with relatively higher levels of labelling in the somatic and visceral mesoderm (Fig. 2F). Oligonucleotide IVa hybridizes the rarest *Ubx* transcript form. With this probe IVa transcripts can only be detected in embryos at very late stages (16 and 17) as a weak hybridization signal on PS6 of the CNS, just above background (data not shown).

Our in situ data are generally consistent with the studies of the distribution of *Ubx* transcript variants in primary cultures of different cell types by S1 analysis (5), and with the distribution of *Ubx* protein variants by monoclonal antibodies (11). Both of these techniques suggest that variants of class I (Ia and Ib) are relatively more abundant in epidermal and mesodermal cells, whereas variants of class II and IV are most abundant in neural cells. The only discrepancy is that *Ubx* proteins of class I were not detected in the CNS (11), whereas we see low levels of class Ia transcripts. Low levels of class I transcripts were detected in cultured neural cells but were attributed to contamination with non-neural tissues. Our results suggest that there may also be a small but real population of class Ia transcripts in neural cells.

The first splicing event is cotranscriptional

Shermoen and O'Farrell (20) have recently shown that most of the *Ubx* RNA detectable in situ with a genomic DNA probe appears as nuclear dots (two per cell) representing the actual foci of transcription. Embryo preparations hybridized with the probe for the 5' exon reveal these nuclear dots at all stages analyzed (see Fig. 2I), as expected for an oligo detecting all initiated transcripts. In addition, probes Ia and IIa also reveal nuclear dots as the main source of hybridization signal (Fig. 2J and data not shown). This implies that the splicing of at least the first intron occurs before transcript molecules are released from the foci of transcription.

CONCLUSION

Splice-specific oligonucleotides can be designed to hybridize specifically to RNA splicing variants in situ. *Ubx* is not an abundant transcript and, additionally, the hybridization signal obtained with the splice-specific probes is much weaker than that obtained with the E5' common probe. This makes the application of our method in the case of *Ubx* limited to the most abundant splicing variants. However, this method should prove powerful in the analysis of more abundant transcripts, where splicing variants should be readily detectable.

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