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PCR-based RNA probes, a quick and sensitive method to improve whole mount embryo *in situ* hybridizations

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Abstract

We have developed a PCR-based technique for the preparation of RNA-probes that can be used for whole mount ***in situ*** hybridization on embryos. T3 or T7 RNA polymerase promoters were introduced at the 5`-end of gene-specific oligonucleotide primers enabling direct ***in vitro*** transcription of purified PCR-fragments. We show for various marker genes in ***Xenopus*** embryos that this method provides equivalent results as compared to conventional vector based probe preparation even when fluorescence detection (FISH) is applied. This method offers a rapid and useful means to prepare gene-specific ***in situ*** probes predominantly for expression screens or detection of splice variants that previously required time-consuming cloning steps.

1. Introduction

Whole mount ***in situ*** hybridization has become one of the standard techniques in modern biosciences. It is often used for studying endogenous spatial and temporal expression patterns of novel isolated genes. It also serves as a powerful tool in experiments when altered gene expression is examined upon genetic and epigenetic manipulations. Commonly, digoxigenin-labelled cRNAs are used for whole mount ***in situ*** hybridizations whereby the preparation of these probes involves an initial time-consuming cloning step using a bacteriophage promoter carrying vector. Specifically designed oligonucleotide primers containing T3 or T7 RNA polymerase promoters at their 5`-ends were successfully used by Logel (ref. 7) to produce PCR-fragments that could be directly used for ***in vitro*** transcription, omitting plasmid preparation steps. We applied this technique for labelling of whole mount ***in situ*** probes and compared the results with conventional vector derived probes. We demonstrate that equal results are obtained for the expression of the marker genes Xbra, Xtwi, XneuroD and Xcad-6 (refs. 9; 5; 6; 2) in ***Xenopus*** embryos. We show that the high sensitivity of the PCR-probes also allows fluorescence signal detection. In comparison to conventional vector based probe preparation, this PCR-approach offers convenience and saves time.

2. Material and Methods

2.1 Production of PCR-derived antisense-RNA-probes

PCR-fragments representing bp 821 to 1493 of the Xbra-, bp 20 to 1012 of the Xtwi-, bp 13 to 1042 of the xNeuroD- and bp 868 to 1512 of the Xcad-6 coding regions (refs. 9; 5; 6; 2) were produced under standard PCR conditions using Taq-Polymerase (35 cycles and 57 °C annealing temperature). Plasmid-DNA (10 ng) containing the full-length cDNAs served as PCR-templates. In each case, the gene-specific downstream-primer contained an artificially introduced T3-promoter at its 5`-end to enable synthesis of antisense transcripts. In the case of Xcad-6 the upstream primer additionally contained a T7-promoter at its 5`-end for synthesis of sense transcripts. PCR-fragments were gel-purified

using Quick-spin columns (Qiagen), and subsequently, 50 ng were used as template-DNA for ***in vitro*** transcription incorporating Dig-UTP via a Dig-labelling kit (Boehringer-Mannheim). Primer-sequences are shown below, whereby promoter-sequences are underlined:

Xbra: 5` GGATGAGGGAATCGATAGTC 3`
5` AATTAACCCTCACTAAAGGGCTCAGGCCCAGGAAATACTG 3`
Xtwi: 5` GACACACTGTGATAGGGCTG 3`
5` AATTAACCCTCACTAAAGGGGTGGCAACGCATGCATGCAG 3`
xNeuroD: 5` GACCAAATCGTATGGAGAGAATGGG 3`
5` AATTAACCCTCACTAAAGGGGGCACTCATGACTCTTTCATGG 3`
Xcad-6: 5` TAATACGACT CACTATAGGGCCCTGTGGAT TCTTCCATT 3`
5` AATTAACCCTCACTAAAGGGACAAATATCTCATACAACATG 3`

2.2. Whole mount in situ hybridization

Whole mount ***in situ*** hybridization was performed as described by Hollemann (ref. 4) for PCR- as well as conventional plasmid-derived antisense probes where the transcripts were detected via phosphatase reaction. Plasmid-derived probes in each of the three marker-genes corresponded to the full length gene bank sequences. Fluorescence detection of transcripts shown for the Xtwi PCR-derived probe was carried out essentially as described in http://www.people.virginia.edu/~lad4x/fluor_insitu/protocol.html. Alterations were made concerning the following points: peroxidase inactivation was omitted after antibody washes and the color substrate was diluted 1:50 in amplification diluent delivered with the TSA-kit (NEN Life sciences). Additionally, the washing time after incubation with Red-FISH was extended to 48 h.

2.3. Whole mount immunofluorescence

Whole mount staining against fibronectin was performed using mab 6D9 (ref. 1) according to standard protocols. Embryos subjected to antibody staining were postfixed for 2 h in MEMFA (100 mM MOPS, pH 7,4; 2mM EGTA; 1mM $MgSO_4$; 4% Formaldehyde) after completion of the fluorescence-***in situ*** procedure described above. Subsequently, vibratome sections were prepared that enabled simultaneous chemiluminescent detection of transcripts and protein immunostaining using confocal laser microscopy.

3. Results and discussion

The gel-purified PCR-products resulting from PCR using the primer-pairs and conditions described above are shown in Fig. 1A. Bands of the expected size were obtained for all three of the marker-genes (Xbra, Xtwi and XneuroD) showing that the addition of the T3-promoter-sequence to the primers did not impair the amplification of the DNA (Fig. 1A). Thus, template-DNAs were generated that could be directly used for ***in vitro*** transcription incorporating Dig-UTP in the transcripts. Aliquots of the corresponding samples are shown in Fig. 1B. The amount of the Dig-labelled transcripts generated from PCR-tem-

plates was equivalent to those derived from a linearized plasmid. To demonstrate this, a 644 bp fragment of Xcad-6 was subcloned into pBKS, and an antisense probe was transcribed from the linearized plasmid. A corresponding 644 base-pair PCR-product was generated in parallel by introducing the T3-promoter in the downstream primer thereby enabling transcription from the PCR-product directly. As expected either approach generated Dig-RNA-molecules of the same length and equivalent amounts (Fig. 1C). Subsequent whole mount ***in situ*** hybridizations revealed Xcad-6-transcripts in the brain, the peripheral nervous system and in neurogenic placodes (Fig. 2G; ref. 2). As for the marker genes described below, no differences between plasmid and PCR-derived probes were observed (Data not shown). In Fig. 2 whole mount ***in situ*** hybridizations of albino embryos using PCR- and conventional plasmid-derived probes were compared. In order to demonstrate the application of this novel technique over a wide spectrum of developmental stages, various well described markers were chosen so that gastrula to tailbud embryos were included. The early mesodermal marker Xbra (ref. 9) shows its typical ring of expression around the blastopore using either the PCR- (Fig. 2A) or the conventional plasmid-derived (Fig. 2B) probe. No alterations during the entire ***in situ*** procedure were necessary to obtain the same signal intensity despite the much shorter length of the PCR-template as compared to the full length cDNA and potential base-exchanges due to Taq-polymerase. Equivalent results were also produced for the neural crest marker Xtwi (ref. 5; Fig. 2C, PCR-derived probe; Fig. 2D, plasmid-derived probe) at early and late tailbud stage, as well as for the proneural marker XneuroD (ref. 6; Fig. 2E, PCR-derived probe; Fig. 2F, plasmid-derived probe) that shows a highly specific pattern in brain and neurogenic placodes in late tailbud specimens (ref. 8). PCR-derived probes are also sensitive enough to allow fluorescence detection of mRNAs giving the advantage of localizing transcripts and proteins in the same section when high quality microscopes are used. Fig. 3 shows Xtwi ***in situ*** hybridization (red) in combination with antibody staining against fibronectin (green) as an example of this technique.

The novel technique of PCR-based ***in situ*** probes described here provides a useful tool in a number of aspects. Firstly, many plasmids do not offer the appropriate bacteriophage-promoters flanking the cloned cDNA. This is the case for many TA-cloning vectors used for rapid cloning of PCR-products and for two-hybrid AD-fusion vectors. Conventionally, the isolated cDNA in such screening-vectors has to be subcloned into a standard vector to allow transcription of ***in situ*** probes. Selection of interesting clones can be dramatically accelerated by avoiding cloning and plasmid preparation steps using the PCR-approach. Even crude DNA can be used as a template as long as sufficient sequence information is available to design the appropriate PCR-primer pair. A further application is the generation of highly specific probes from full length clones, when the gene of interest belongs to a family of closely related members or when splice-variants are found. Here, the PCR approach facilitates the generation of highly specific probes by amplifying the sequence regions of lowest similarity. However, the minimal length of the PCR-fragment

should be at least 300 bp as shorter probes are less sensitive. We have successfully applied the PCR-based approach to a number of cDNAs including our recently published ***Xenopus*** Cadherin-6 (Xcad-6) clones (ref. 2), ***Xenopus*** Reptin (ref. 3) as well as various novel clones isolated using the yeast two-hybrid-methodology. Excellent results were also obtained when fluoresceine labelled probes were used for hybridization and when transcription was performed via a T7 promoter introduced in the forward primer. The latter approach enabled simultaneous synthesis of antisense and sense probes from the same PCR-template, which is shown for Xcad-6 (ref. 2). The antisense probe revealed Xcad-6-transcripts in the brain, the eye, the peripheral nervous system and in neurogenic placodes (Fig. 2G) and as expected no signal was observed using the corresponding sense probe (Fig. 2H).

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Fig. 1: Gel-purified PCR-products (A) containing a T3-promoter artificially introduced via the downstream primer were used to produce Digoxigenin-antisense RNAs (B) corresponding to Xbra, Xtwi and XneuroD. The lengths of the PCR-products is indicated. C: Comparison of the transcription efficiencies using an identical 644 bp fragment of Xcad-6 either as a PCR product or contained in a linearized pBKS-vector. Equivalent amounts of DIG-antisense RNA were obtained using T3-Polymerase. As often observed under native gel-conditions the RNA forms double-bands here.

Fig. 2: Digoxigenin-labelled antisense RNAs produced conventionally (A, C, E) or via PCR-approach (B, D, F) were used for whole mount ***in situ*** hybridizations. Equal results were obtained for the marker genes Xbra (A, B), Xtwi (C, D) and XneuroD (E, F). The additional introduction of a T7-promotor in the upstream primer allows for production of antisense (G) and sense (H) probes from the same PCR-template as shown for Xcad-6. Sense probes show no signal.

Fig. 3: Fluorescence detection of Xtwi-mRNA via PCR-derived antisense probe (red) in combination with antibody staining against fibronectin (green). b: brain; e: eye anlage; s: somite; n: notochord; en: endoderm; nc: Xtwi positive neural crest cells of the mandibular crest segment; *: subpopulation of single migratory Xtwi positive cells.

Fig. 1

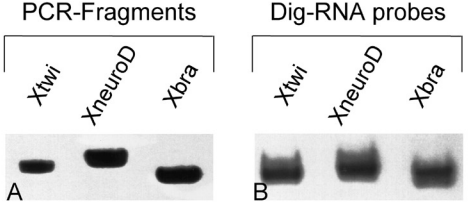

Fig. 2

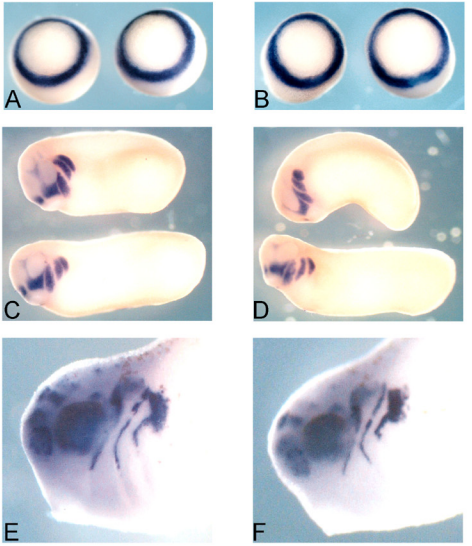

Fig. 3