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.

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 (DIG)-labeled 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 et al. (7) to produce PCR fragments that could be directly used for in vitro transcription, omitting plasmid preparation steps. We applied this technique for labeling 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 (2,5,6,9) 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.

Short Technical Reports

MATERIALS AND METHODS

Production of PCR-Derived Antisense RNA Probes

PCR fragments representing base pairs 821–1493 of the Xbra coding region, base pairs 20–1012 of the Xtwi coding region, base pairs 13–1042 of the xNeuroD coding region, and base pairs 868–1512 of the Xcad-6 coding region (2,5,6,9) were produced under standard PCR conditions using *Taq* DNA polymerase (Eurogentec, Seraing, Belgium) (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 the synthesis of sense transcripts. PCR fragments were gel-purified using Quick-spin™ columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and 50 ng were subsequently used as template DNA for in vitro transcription incorporating Dig-UTP via a Dig-labeling kit (Roche Molecular Biochemicals, Mannheim, Germany). Primer sequences are shown below, whereby promoter sequences are underlined: Xbra (5'-GGATGAGGGAATCGATAGTC-3' and 5'-AATTAACCCTCACTAAAGGGCTCAGGCCAGGAAATACTG-3'), Xtwi (5'-GACACACTGTGATAGGGCTG-3' and 5'-AATTAACCCTCACTAAAGGGGTGGCAACGCATGCATGCAG-3'), xNeuroD (5'-GACCAAAATCGTATGGAGAGAATGGG-3' and 5'-AATTAACCCTCACTAAAGGGGGCACTCATGACTCTTTCATGG-3'), and Xcad-6 (5'-TAATACGACTCACTAATAGGGCCCTGTGGATTCTTCCATT-3' and 5'-AATTAACCCTCACTAAAGGGACAAATATCTCATACAACAATG-3').

Whole Mount In Situ Hybridization

Whole mount in situ hybridization was performed as described by Hollemann and Pieler (4) for PCR and conventional plasmid-derived antisense probes where the transcripts were detected via phosphatase reaction. Plas-

mid-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 Science Products, Boston, MA, USA). Additionally, the washing time after incubation with Red-FISH was extended to 48 h.

Whole Mount Immunofluorescence

Whole mount staining against fibronectin was performed using mab 6D9 (1) according to standard proto-

cols. Embryos subjected to antibody staining were post-fixed for 2 h in MEMFA (100 mM MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO₄, 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.

RESULTS AND DISCUSSION

Figure 1A shows the gel-purified PCR products resulting from PCR using the primer pairs and conditions described above. 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

Figure 1. Gel-purified PCR-products (A) containing a T3 promoter artificially introduced via the downstream primer were used to produce DIG antisense RNAs (B) corresponding to Xbra, Xtwi, and XneuroD. The lengths of the PCR products are 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 RNA polymerase. As often observed under native gel conditions, the RNA forms double bands here.

Short Technical Reports

primers did not impair the amplification of the DNA (Figure 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 Figure 1B.

The amount of the DIG-labeled transcripts generated from PCR templates 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 lin-

earized plasmid. A corresponding 644-bp 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 (Figure 1C). Subsequent whole mount *in situ* hybridizations revealed *Xcad-6* transcripts in the brain, the peripheral nervous system, and in neurogenic placodes (Figure 2G) (2). As for the marker genes described below, no differences between plasmid and PCR-derived probes were observed (data not shown). In Figure 2, whole mount *in situ* hybridizations of albino embryos using PCR- and conventional plasmid-derived probes were compared. 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* (9) shows its typical ring

Figure 2. DIG-labeled antisense RNAs produced conventionally (A, C, and E) or via the PCR approach (B, D, and F) were used for whole mount *in situ* hybridizations. Equal results were obtained for the marker genes *Xbra* (A and B), *Xtwi* (C and D), and *XneuroD* (E and F). The additional introduction of a T7 promoter 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.

Figure 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.

Short Technical Reports

of expression around the blastopore using either the PCR- (Figure 2A) or the conventional plasmid-derived (Figure 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* DNA polymerase. Equivalent results were also produced for the neural crest marker *Xtwi* (Figure 2C, PCR-derived probe; Figure 2D, plasmid-derived probe) (5) at early and late tailbud stage, as well as for the proneural marker *XneuroD* (Figure 2E, PCR-derived probe; Figure 2F, plasmid-derived probe) (6) that shows a highly specific pattern in brain and neurogenic placodes in late tailbud specimens (8). PCR-derived probes are also sensitive enough to allow fluorescence detection of mRNAs, giving the advantage of localizing transcripts and pro-

teins in the same section when high-quality microscopes are used. Figure 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. First, 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 activation domain 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. The 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 because 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 (2), *Xenopus* Reptin (3), and various novel clones isolated using the yeast two-hybrid methodology. Excellent results were also obtained when fluorescein-labeled 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* (2). The antisense probe revealed *Xcad-6* transcripts in the brain, the eye, the peripheral nervous system, and in neurogenic placodes (Figure 2G), and, as expected, no signal was observed using the corresponding sense probe (Figure 2H).

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