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"Run-Off" Synthesis and Application of Defined Single-Stranded DNA Hybridization Probes

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A simple and efficient method for synthesizing radioactively labeled single-stranded DNA hybridization probes with *Thermus aquaticus* (*Taq*) DNA polymerase is described. This is done in a "run-off" polymerization with repeated cycles of denaturation, annealing, and extension. It leads to high yields of a single-stranded DNA of defined length (up to 5000 nt), which is labeled to a high specific activity (1.3×10^8 cpm/μg DNA). These hybridization probes are equally sensitive as nick-translated DNA probes, but strand specific. This was tested by slot blot hybridization with *in vitro*-transcribed target RNAs and by Northern blotting. The use of single-stranded DNA hybridization probes combines the benefits of DNA stability and single-strand RNA probes. © 1990 Academic Press, Inc.

Radiolabeled probes are an important tool in molecular biology to decipher gene structure and gene expression. So far, DNA probes have usually been generated by nick-translation (1) and by random priming with oligonucleotides (2,3), and RNA probes synthesized by *in vitro* transcription (4). DNA probes are easy to handle, but they require fragment isolation and lack strand specificity when synthesized with the above-mentioned protocols. *In vitro*-transcribed RNA probes are strand specific. However, the sequence which is used as a template must be cloned in special transcription vectors. Handling of these probes requires RNase free conditions which makes work more cumbersome. Recently asymmetric primer addition to the polymerase chain reaction (PCR)[1] was described as a method for the synthesis of a high fraction of single-stranded DNA which can be used for sequencing reactions (5). It was suggested that this method could also be used for the synthesis of probes, but these probes would not be strand specific, because a high amount of labeled double-stranded DNA would still be synthesized using the conditions described. Schowalter and Sommer (6) showed the generation of double-stranded DNA probes by including a radioactive triphosphate during polymerase chain reaction. Here we report on the synthesis of a completely single-stranded DNA hybridization probe with *Thermus aquaticus* (*Taq*) DNA polymerase (7). This method requires only an appropriate restriction site of the template and one primer binding at a defined distance from this site. The template is cut and in a "run-off" polymerization of repeated cycles of denaturation, annealing, and extension single-stranded DNA can be synthesized with very high yield. In many cases the highly sequence-specific sequencing primers can be used which allow the synthesis of a single-stranded probe of defined length up to 5000 nt. We optimized the conditions to label this single-stranded DNA to a high specific activity. By a slot blot we show that these probes are absolutely strand specific. The general application and the sensitivity of these probes are examined by Northern blotting. These probes are at least as sensitive as nick-translated probes. They can be handled under the same conditions as nick-translated DNA and offer the benefits of single-strand RNA probes and the stability of DNA probes.

MATERIALS AND METHODS

Vector Constructions

pBC-sis. A 700-bp *Bam*HI/*Pst*I fragment coding for the fifth exon of human c-*sis* (PDGF β-chain) (8–10) is cloned between the *Bam*HI and the *Pst*I restriction sites present in the multiple cloning site (mcs) of pBS (+) (Stratagene). Cutting pBC-sis with *Bam*HI and starting transcription from the T3 promoter give rise to the antisense strand of c-*sis*. If the vector is cut with *Hin*dIII

[1] Abbreviations used: PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; mcs, multiple cloning site; DMEM, Dulbecco's minimum essential medium; BAE, bovine aortic endothelial; TCA, trichloroacetic acid.

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and transcripts are made with T7 RNA polymerase, the sense strand of c-*sis* is produced.

pSPT18-12Δ350. The construction of the vector pSPT18-12Δ350 will be published elsewhere in more detail (Hohenadl *et al.*). In brief, a 7127-bp *Eco*RI/*Bam*HI fragment coding for the full-length reverse-transcribed cDNA of coxsackie B3 virus (11) except a 350-bp deletion at the 3′ end is inserted between the *Bam*HI and the *Eco*RI restriction sites in the mcs of pSPT18 (Boehringer). The orientation of the coxsackie B3 virus fragment is such that transcription from the T7 promoter gives rise to the positive strand of the virus.

Cell Culture

Bovine aortic endothelial (BAE) cells were maintained at 37°C and 10% CO_2. They were feed with DMEM supplemented with 5% fetal bovine serum and antibiotics.

Human fibroblasts were maintained at 37°C and 5% CO_2. They were feed with DMEM supplemented with 10% fetal bovine serum and antibiotics.

Run-Off Polymerization with Taq DNA Polymerase

The T3 primer had the sequence 5′ ATTAACCCTCACTAAAG 3′.

The T7 primer had the sequence 5′ AATACGACTCACTATAG 3′.

Run-off polymerization was carried out with 200 pmol of primer (2 μM), 200 μM each of dATP, dGTP, dTTP, and dCTP, and template DNA in 100 μl of a solution of 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin in 0.5 ml Eppendorf tubes. (Changes of dCTP concentration are mentioned in the text. Labeled [α-^{32}P]dCTP with a sp act of 3000 Ci/mmol was purchased from Amersham.) *Taq* DNA polymerase (4.2 U, Amersham) was added and the solution overlayed with paraffin oil. The polymerization reaction was performed in a DNA thermal cycler (Perkin–Elmer Cetus) where the tubes were kept for 1 min at 37°C and then incubated for 40 cycles as follows: 1 min at 94°C, 2 min at 37°C, and 3 min at 72°C. Finally the extension step was continued for 7 min at 72°C and the reaction stopped by decreasing the temperature to 4°C. The 10-μl reactions were scaled down from the 100-μl reaction. Unincorporated nucleotides were separated by NH_4Ac/EtOH precipitation. The size of the polymerization product was determined by alkaline agarose gel electrophoresis as described (1).

RNA Preparation and Northern Blot Analysis

Cells were harvested at 90% confluence by trypsination and total cellular RNA was isolated as described by Chirgwin *et al.* (12). The RNA was fractionated on a vertical 1% agarose/6% formaldehyde gel and transferred to GeneScreen plus nylon membrane (NEN) by a vacuum blotting device (Millipore). The RNA was subsequently fixed on the membrane by 5 min of uv irradiation. The amounts of RNA transferred were estimated by staining the membrane with methylene blue. The filters were prehybridized and hybridized in 50% formamide, 5× SSC, 10% dextran sulfate, 1% Sarkosyl NL-30, 100 μg/ml sonicated salmon sperm DNA, and 250 μg/ml tRNA at 42°C. The final washing of the filters was at 50°C in 0.1× SSC, 0.1% Sarkosyl NL-30. Filters were exposed to Hyperfilm-MP (Amersham) at −70°C.

The procedure for RNA slot blots using formamide/formaldehyde was as described in the instruction manual for usage of the GeneScreen plus membrane (NEN). Prehybridization, hybridization, and final wash were done as described above.

In Vitro Transcription

In vitro transcripts were synthesized using a transcription kit from Stratagene as recommended by the supplier.

Nick-Translation

Nick-translated probes were prepared as described by Maniatis *et al.* (1).

RESULTS

Radiolabeling of a Defined Single-Stranded DNA by Run-Off Polymerization

To establish the synthesis of radiolabeled single-stranded DNA hybridization probes and to show their application we examined the expression of the PDGF β-chain in BAE cells and in human fibroblasts. For probe synthesis the vector pBC-sis was used (see Materials and Methods). Cutting the vector pBC-sis with *Bam*HI and using the T3 primer a 700 nt long, single-stranded c-*sis* antisense DNA can be generated with high yield in a run-off reaction with *Taq* DNA polymerase (data not shown). During this reaction all deoxynucleotides are used in equal amounts (200 μM).

In order to radioactively label this single-stranded DNA in full length to a high specific activity we set up nine reactions. Each tube contained the reagents at the same molar ratio as described under Materials and Methods except that the concentration of unlabeled dCTP was gradually reduced from 1 to 0 mM. In an autoradiogram of an alkaline agarose gel it can be seen that down to a concentration of 12.5 μM of unlabeled dCTP a radioactively labeled, full-length, single-stranded DNA is synthesized (Fig. 1A). If only labeled dCTP at a concentration of 0.16 μM is present (Fig. 1A, lane 9), the

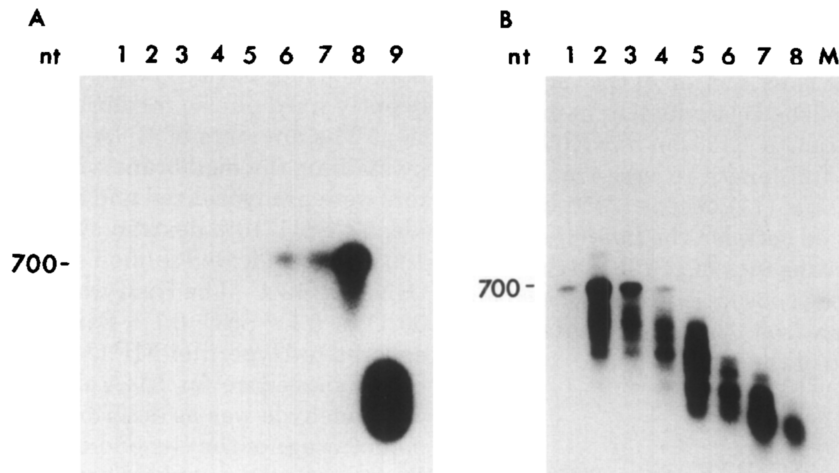

FIG. 1. Radiolabeling of single-stranded DNA. In 10-μl polymerization reactions with 5 μCi of [α-^{32}P]dCTP and 20 ng template DNA the amount of unlabeled dCTP was gradually decreased. After 40 cycles in the thermal cycler the reaction was stopped. The DNA was precipitated, redissolved in 15 μl of sample buffer, and applied on a 1.5% alkaline agarose gel. After electrophoresis the gel was dried and subjected to autoradiography for 20 min. (A) Lane 1, 1 mM dCTP; lane 2, 500 μM dCTP; lane 3, 200 μM dCTP; lane 4, 150 μM dCTP; lane 5, 100 μM dCTP; lane 6, 50 μM dCTP; lane 7, 25 μM dCTP; lane 8, 12.5 μM dCTP; lane 9, no dCTP. In lanes 1–5 defined bands at 700 nt are detected at longer exposure times (1 h). (B) Lane 1, 10 μM dCTP; lane 2, 8.3 μM dCTP; lane 3, 6.25 μM dCTP; lane 4, 3.125 μM dCTP; lane 5, 1.56 μM dCTP; lane 6, 0.78 μM dCTP; lane 7, 0.39 μM dCTP; lane 8, 0.19 μM dCTP; lane M, marker.

reaction is terminated before the full-length strand is polymerized.

To determine the exact dCTP concentration at which full-length strands are labeled to a high specific activity another assay was performed with decreasing amounts of unlabeled dCTP from 10 to 0.19 μM. At a concentration of 6.25 μM of unlabeled dCTP the full-length strand is still the main reaction product (Fig. 1B, lane 3). At lower concentrations the polymerization time is to short to obtain full-length DNA strands and prematurely terminated strands are the main products of the reaction (Fig. 1B, lanes 4–8). The measurement of the TCA-precipitable material showed that the incorporation of radionucleotides reached a plateau at concentrations less than 6.25 μM of nonlabeled dCTP (data not shown). This result is consistent with the data from the alkaline agarose gel electrophoresis and therefore a concentration of 6.25 μM of nonlabeled dCTP was used in all further experiments. The total concentration of dCTP in the reaction including the labeled nucleotide was 6.41 μM.

To determine how many cycles are required to obtain a high yield of single-stranded probe, seven identical reactions of 10 μl each with the above-determined optimal nonlabeled dCTP concentration and with 5 μCi of labeled dCTP were set up. After the indicated intervals the TCA-precipitable radioactivity was determined. There is a constant increase of the incorporated radioactivity up to 40 cycles (data not shown). Neither the concentration of deoxynucleotides nor the enzyme is limiting the reaction.

The amount of single-stranded DNA, which is synthesized in 100 μl with 6.41 μM dCTP after 40 cycles from 200 ng vector DNA, was determined. After 40 cycles in the DNA thermal cycler the reaction was stopped and nonincorporated nucleotides were removed. The absorption at 260 nm was measured and the value for the control tube (not incubated) was subtracted from the value for the test reaction. The calculated yield was 306 ng of single-stranded DNA. By the conditions used only 18% (i.e., 700 bp of c-*sis* fragment out of a total of 3904 bp of pBC-sis) of the total vector sequences serve as a template for the polymerization. Therefore, 18 ng of single-stranded template out of 200 ng of double-stranded vector gave rise to 306 ng of single-stranded probe. This means that the template had been amplified 17 times.

Length of Taq Polymerase-Synthesized Single-Stranded DNA

To test the maximal length of single-stranded DNA which *Taq* polymerase can synthesize, we used the vector pSPT18-12Δ350 (see Materials and Methods). This vector was restricted at various distances from the primer binding site to get templates of different length for polymerization. The data in Fig. 2 show that single-stranded DNA is synthesized from 700 up to 5000 bases in length. A 3′ protruding terminus (*Sac*I) of the template DNA did not cause unspecific initiation as often occurs by *in vitro* transcription (Fig. 2A, lane 1).

Sensitivity and Strand Specificity of Single-Stranded DNA Probes

The sensitivity and strand specificity of the probe were determined by a slot blot with *in vitro*-transcribed

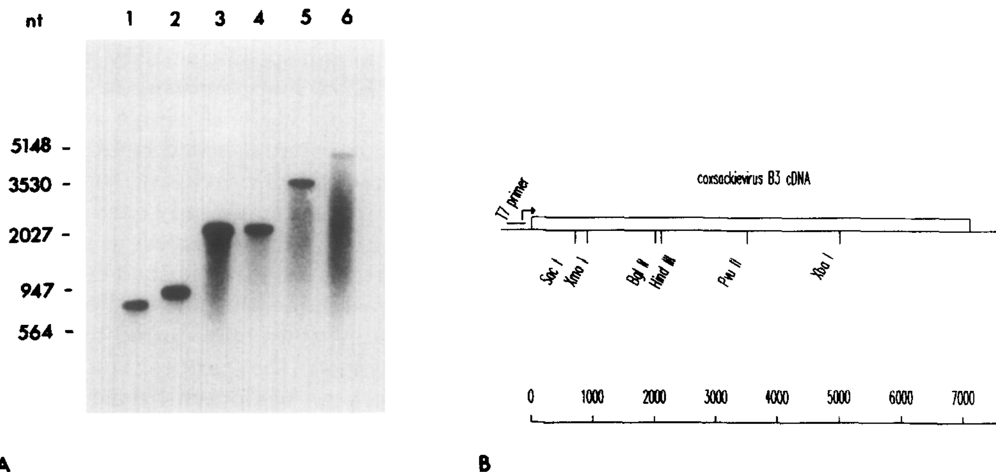

FIG. 2. Synthesis of very long single-stranded DNA. The vector pSPT18-12Δ350 was digested with each restriction enzyme indicated in B and the linearized DNAs were used as template in six separate polymerization reactions. The reaction volume was 10 μl with equal molar concentrations of the different length DNA templates (2.1 nM), 5 μCi [α-^{32}P]dCTP, and 6.25 μM unlabeled dCTP. The "run off" synthesis was for 20 cycles in the thermal cycler with an extension time of 10 min/cycle. After the reaction the DNA was precipitated and dissolved in 70 μl of sample buffer and 30 μl of the solution loaded in a 1% alkaline agarose gel. After electrophoresis the gel was dried and subjected to autoradiography for 30 min. (A) Lane 1, *Sac*I; lane 2, *Xma*I; lane 3, *Bgl*II; lane 4, *Hin*dIII; lane 5, *Pvu*II; lane 6, *Xba*I.

sense and antisense RNA of c-*sis* as a target. The results were compared with the results obtained with a nick-translated probe. c-*sis* sense RNA was applied onto two separate filters from 5 ng down to 5 pg. Two other filters were prepared with three different amounts of antisense RNA. The *Taq* DNA polymerase-synthesized single-stranded DNA probe (sp act of 1.3×10^8 cpm/μg DNA) hybridized only to the sense RNA of c-*sis*. No cross-reaction could be detected with the antisense strand. As little as 5 pg of RNA could be detected after a 12-h exposure. With the nick-translated probe (sp act of 5.3×10^8 cpm/μg of DNA) 5 pg of RNA also could be detected but both target RNA strands gave a signal (Fig. 3).

Northern Blotting with Single-stranded DNA Probes

In order to determine whether single-stranded DNA probes can be used for general applications they were tested in a Northern blot. It is known that in contrast to fibroblasts, BAE cells express the PDGF β-chain (13). Total RNA of these cells was used as a positive control and total human fibroblast RNA was used as a negative control. Staining the membrane with methylene blue showed that equal amounts of RNA were used (data not shown). After a 2-h exposure a band with the expected size of the PDGF β mRNA (4.2 kb) was detected in the track containing BAE RNA. No band could be detected in fibroblast RNA (Fig. 4). A comparison of slots 1 and 3 in Fig. 4 shows that the single-stranded DNA probe gives a signal more intense than that of the nick-translated probe for the same exposure times.

DISCUSSION

The synthesis of hybridization probes with *Taq* DNA polymerase is a novel approach in molecular biology. Gyllensten and Erlich (5) suggested the synthesis of PCR-synthesized hybridization probes. Schowalter and Sommer (6) showed the synthesis of double-stranded hybridization probes with the PCR. We show the synthesis and application of a single-stranded, strand-specific DNA hybridization probe. The probe was synthesized in a high yield by a run-off polymerization with *Taq* DNA polymerase. Using only one primer and running the reaction for 40 cycles in the DNA thermal cycler lead to a single-stranded DNA with defined length. By optimizing the conditions for the incorporation of [α-^{32}P]-dCTP we labeled this single-stranded DNA for the use as a hybridization probe to a high specific activity. The probe turned out to be absolutely strand specific. The sensitivity was comparable to nick-translated probes. Until now only *in vitro*-transcribed RNA probes have the advantage of strand specificity. However, preparation and handling of RNA probes are tedious and cumbersome. The RNA must be phenolized after the *in vitro* transcription in order to deactivate the RNases. The templates must be carefully purified. Moreover, the synthesis of RNA probes requires template fragments cloned into special transcription vectors. With our method no special cloning or fragment isolation is necessary to obtain a highly sensitive and strand-specific probe. The synthesis of these probes requires only one primer and a restriction site which can be cleaved to terminate the run-off synthesis. For many of the generally

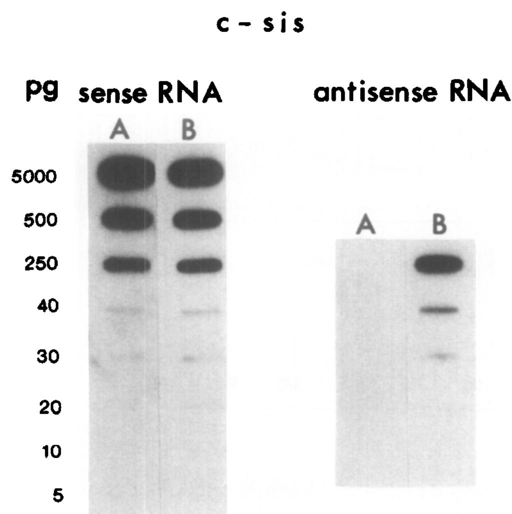

FIG. 3. Slot blot to compare the strand specificity and sensitivity of a *Taq* DNA polymerase-synthesized hybridization probe with a nick-translated probe. Sense and antisense RNA of the c-*sis* fragment was synthesized by *in vitro* transcription and fixed on a GeneScreen plus membrane in decreasing amounts (sense RNA, 5 ng and 500, 250, 40, 30, 20, 10, and 5 pg; antisense RNA, 250, 40, and 30 pg). The single-stranded DNA probe which was specific for the sense strand of the c-*sis* fragment was synthesized in a 100-μl reaction with 200 ng vector DNA, 50 μCi [α-^{32}P]dCTP, and 6.25 μM unlabeled dCTP within 40 cycles. The calculated specific activity was 1.3×10^8 cpm/μg DNA. During hybridization either 2×10^6 cpm/ml of the single-stranded *Taq* DNA polymerase-synthesized hybridization probe (A) or 2×10^6 cpm/ml of a c-*sis* fragment which was labeled by nick-translation (sp act of 5.3×10^8 cpm/μg of DNA) (B) was added. The exposure time was 12 h.

used cloning vectors like pUC (14) and pBR322 (15) there are primers commercially available, which are provided for sequencing of the inserted fragments. These primers also can be used for the synthesis of single-stranded DNA probes from the inserted fragments.

Single-stranded DNA probes with defined lengths are useful for many different applications in molecular biology. For *in situ* hybridization the optimal length of the probe is defined between 100 and 400 nt (16). By labeling with nick-translation a tedious standardization of the DNase concentration is necessary to obtain the correct size of the probe. By synthesizing the probe by *in vitro* transcription, transcripts of a defined length can be made, providing there is a restriction site at the correct distance from the promoter. Often this is not the case and longer transcripts must be degraded by alkaline hydrolysis (17). A *Taq* DNA polymerase-synthesized single-stranded DNA probe of a defined length is much easier to synthesize by using a primer which is at the correct distance from a restriction site. Single-stranded DNA probes give, like strand-specific RNA probes, the opportunity for a negative control by probing with the complementary strand.

Single-stranded DNA probes with defined length and labeled to a high specific activity will also offer an alternative for RNA mapping studies. This is normally done by RNase mapping with single-stranded RNA probes, because *in vitro*-transcribed RNA probes can be labeled to a higher specific activity than end-labeled DNA probes which are commonly used for the S1 protection assay (18,19). The high specific activity of the single-stranded DNA probes described here makes S1 mapping again a reasonable alternative. For S1 mapping studies it might sometimes be required to synthesize very long single-stranded DNA probes. Under the conditions described here we could synthesize DNA probes up to a length of 5000 nt.

Furthermore in *in vitro* transcription a 3′ protruding terminus often causes nonspecific initiation with T7 and T3 RNA polymerases. This can be sometimes circumvented by increasing the NaCl concentration in the transcription buffer, but this always causes a drastical decrease of the transcription efficiency. We showed that nonspecific initiation does not occur during the synthesis of single-stranded DNA probes with *Taq* DNA polymerase.

Lo *et al.* (20) showed that biotinylated dUTP is a substrate for *Taq* DNA polymerase which can be incorporated into DNA during polymerization. So finally, we suggest that it will also be possible to synthesize nonradioactively labeled single-stranded DNA hybridization probes using the method described here.

The synthesis of single-stranded DNA probes is a useful tool in molecular biology, because the advantages of double-stranded DNA probes and single-stranded RNA probes are combined in one system.

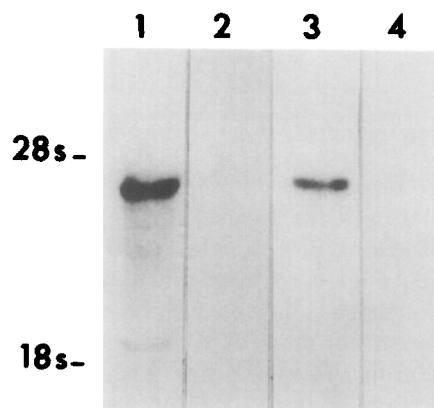

FIG. 4. Northern blot with a c-*sis*-specific single-stranded DNA hybridization probe and with a nick-translated c-*sis* fragment. Each lane contains 30 μg of total RNA. Lanes 1 and 3, BAE cells; lanes 2 and 4, human fibroblasts. Lanes 1 and 2 were hybridized with 2×10^6 cpm/ml of a single-stranded antisense *Taq* DNA polymerase-synthesized hybridization probe specific for c-*sis* and lanes 3 and 4 were hybridized with 2×10^6 cpm/ml of a c-*sis* fragment which was labeled by nick-translation. The exposure time was 2 h.

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