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'Run-off' polymerization with digoxigenin labelled nucleotides creates highly sensitive and strand specific DNA hybridization probes: synthesis and application

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In this paper the *in vitro* synthesis and application of non-radioactively labelled strand specific DNA probes is described. The probe is labelled by incorporation of nucleotides with the hapten digoxigenin into single-stranded DNA during a 'run-off' reaction catalyzed by *Thermus aquaticus* (*Taq*) DNA-polymerase. The 'run-off' reaction requires a linearized plasmid template and one primer binding site at a defined distance from the restriction site. Single-stranded DNA can be synthesized during repeated cycles of denaturation, annealing, and extension. The conditions for the incorporation of digoxigenin-11-dUTP (dig-11-dUTP) during polymerization were optimized to generate strand specific DNA hybridization probes up to a length of 5000 nt. The strand specificity is demonstrated by a dot-blot, with *in vitro*-transcribed target RNA of c-sis. The sensitivity of the probe was tested in a Northern blot, and found to be identical to a probe radiolabelled by nick-translation (specific activity $6{\cdot}5 \times 10^8$ cpm μg^{-1}). The resolution of the signals and speed of development was even superior compared to the radiolabelled probe.

KEYWORDS: Polymerase chain reaction, run-off reaction, single-stranded DNA, strand-specific DNA hybridization probes, digoxigenin, Northern blot.

INTRODUCTION

Nowadays much effort is made to replace radioactive hybridization probes with non-radioactive probes. Non-radioactive hybridization probes reveal many advantages compared to radioactive probes. They are (i) non-hazardous, (ii) stable for a long time, (iii) allow reduction of the detection time, and (iv) give a higher resolution. So far, one major drawback is their reduced sensitivity compared to radioactive probes.

Non-radioactive probes are mostly synthesized by incorporation of biotin-11-dUTP or digoxigenin-11-dUTP (dig-11-dUTP) during polymerase chain reaction (PCR),[1,2] by random priming or nick-translation (Boehringer: DNA labelling and detection non-radioactive; applications manual). These labelling reactions produce double-stranded hybridization probes, which lack strand specificity in Northern blotting or *in situ* hybridization. Furthermore, the amount of specific strand is reduced by reannealing to the complementary strand during hybridization, causing a reduction of sensitivity.[3]

Recently, non-radioactively labelled *in vitro*-transcribed RNA probes have been introduced.[4] These probes are strand specific and sensitive. However, the handling of DNA probes is more cumbersome than that of RNA probes. One disadvantage is that *in vitro* transcription requires sequences cloned into special transcription vectors with promoters specific for the bacteriophage SP6, T7 and T3 RNA-polymerases.[5] Also a restriction site is not always present at the required distance from the promoter to terminate

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transcription. Probes of a defined length are required for *in situ* hybridization[3] and RNA mapping studies.[5] Finally diethylpyrocarbonate has to be used to inactivate RNases.[4] This reagent is very toxic and therefore imposes a possible hazard.

To overcome all these limitations, we introduced the synthesis of single-stranded DNA hybridization probes with *Taq* DNA-polymerase in a 'run-off' reaction.[6] In this procedure, a linearized DNA template with one primer binding site at a defined distance from a restriction site on the plasmid is used to generate the probe by PCR.[6]

In this paper the application of 'run-off' polymerization is described for the synthesis of non-radioactive hybridization probes. The conditions for the incorporation of digoxigenin-11-dUTP during the 'run-off' reaction were optimized to produce non-radioactively labelled strand specific DNA probes with defined length. In order to show that the labelling procedure is independent of the sequence, three different templates were used for probe synthesis (c-sis, β-actin and coxsackie B3 virus). Additionally, we investigated the optimal conditions for the use of these probes in dot-blot and Northern blot hybridization. In a dot-blot with *in vitro*-transcribed target RNAs it is shown that these probes are strand specific. The sensitivity of the probe is demonstrated in a Northern blot by direct comparison with a nick-translated radiolabelled probe.

MATERIALS AND METHODS

Materials

Reagents

Nucleotides including dig-11-dUTP, restriction enzymes, blocking reagent, Dig-AP (polyclonal sheep anti-digoxigenin-antibody (Fab):alkaline phosphatase-conjugate [750 units ml^{-1}]), BCIP (5bromo-4chloro-3indolylphosphate) and NBT (nitroblue tetrazolium salt) were from Boehringer Mannheim. [α-^{32}P]-Deoxycytosine-5'-triphosphate, triethylammonium salt ([α-^{32}P]dCTP; *c.* 3000 Ci $mmol^{-1}$), *Taq* DNA-polymerase [5 units μl^{-1}] and hyperfilm-MP X-ray films were from Amersham Buchler. Nitrocellulose BA85 membrane was from Schleicher and Schuell, GeneScreen hybridization membrane was from NEN. The thermal cycler used, has been described by Collasius *et al.*[7]

Primers

Primers were synthesized with a 380B DNA Synthetisizer from Applied Biosystems. After synthesis, the primers are kept for 12 h at 56°C to remove protective groups, dried in a speed-vac, precipitated twice with NaAc/EtOH and dissolved in H_2O.

The sequences of the primers are:

T3 primer: 5'ATTAACCCTCACTAAAG 3'
T7 primer: 5'AATACGACTCACTATAG 3'
SP6 primer: 5'ATTTAGGTGACACTATA 3'

Vector Constructions

(i) 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 inserted between the *Bam* HI and the *Pst* I restriction sites in the multiple cloning site (mcs) of pBS (+) (Stratagene). Cutting pBC-sis with *Bam* HI and starting transcription from the T3 promoter gives rise to the antisense strand of c-sis. By cutting the vector with *Hind* dIII and transcribing with T7 RNA-polymerase, the RNA-sense strand of c-sis is produced.

(ii) pCVB3-R1: A 7127-bp *Eco* RI/*Bam* HI fragment coding for the full-length reverse-transcribed cDNA of coxsackie B3 virus, except a 350-bp deletion at the 3' end, is inserted between the *Bam* HI and the *Eco* RI restriction sites in the multiple cloning site of pSPT18.[11] Transcription from the T_7 promoter gives rise to the positive strand of coxsackie B3 virus.

(iii) pGEM-β-Actin: A 450 bp *Bam* HI/*Eco* RI fragment coding for part of the β-actin cDNA is inserted into the vector pGEM-3 (Promega).[12] Cutting the vector with *Bam* HI and starting transcription from the T_7 promoter gives rise to the sense strand of β-actin. Cutting with *Eco* RI and starting the polymerization from the Sp6 site gives rise to the antisense strand.

Cell Culture

Human fibroblast cultures were established from human skin biopsies as described by Roth *et al.*[13] Cell cultures were propagated at 37°C under 5% CO_2, in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Methods

Labelling of single-stranded DNA with digoxigenin

'Run-off' polymerization was carried out with 200 pmol of primer (2 μM), 200 μM each of dATP, dGTP, dCTP, 50 μM each of dTTP, dig-11-dUTP and 180 ng of DNA template 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. *Taq* DNA-polymerase (4·2 U) was added and the solution overlayed with mineral oil. The polymerization reaction was performed for 30 cycles as follows: 1 min at 94°C, 30 s at 37°C, and 30 s at 72°C. Finally, the extension step was continued for 2 min at 72°C and the reaction was stopped by decreasing the temperature to 4°C. Unincorporated nucleotides were separated by NH_4Ac/EtOH precipitation.

Alkaline agarose gel electrophoresis and blotting of digoxigenin labelled single-stranded DNA

Alkaline agarose gel electrophoresis was done as described by Maniatis *et al.*[14] After electrophoresis, the DNA was transferred to a GeneScreen membrane and stained by the alkaline phosphatase reaction according to the protocols for digoxigenin labelling (Boehringer). In short, the membrane was washed briefly with buffer 1 (100 mM Tris-HCl, 15 mM NaCl, pH 7·5). Blocking of non-specific binding sites was done for 30 min in buffer 2 (buffer 1, 0·5% blocking reagent). Thereafter, the membrane was rinsed in buffer 1 and the DIG-AP was applied for 45 min with a concentration of 750 mU ml^{-1} in buffer 1. To remove unbound antibody the membrane was washed twice for 15 min in buffer 1. Finally the membrane was equilibrated for 2 min in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9·5) and then incubated with 10 ml freshly prepared AP substrate solution (335 μg NBT ml^{-1}, 175 μg BCIP ml^{-1} in buffer 3). The colour reaction was stopped by washing the membrane in buffer 4 (10 mM Tris-HCl, 1 mM EDTA, pH 8·0).

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.*[15] The RNA was fractionated on a vertical 1·5% agarose/6% formaldehyde gel and transferred to a BA 85 nitrocellulose membrane by a vacuum blotting device (Millipore). The RNA was subsequently fixed to the membrane by 2 h backing in a vacuum oven. The filters were prehybridized and hybridized in 50% formamide, 5 × SSC, 5% blocking reagent, 1% Sarcosyl NL-30, 250 μg ml^{-1} sonicated salmon sperm DNA and 500 μg ml^{-1} tRNA at 42°C if a digoxigenin labelled probe was used. If radioactive probes were used, prehybridization and hybridization was done as described previously.[6] The final washing of the filters was at 50°C in 0·1 × SSC, 0·1% Sarkosyl NL-30. Thereafter, the digoxigenin labelled probe was detected by immunostaining with alkaline phosphatase. If radiolabelled probes were used, the filters were exposed to Hyperfilm-MP at −70°C.

For RNA slot-blots using formamide/formaldehyde, we followed the instructions in Maniatis *et al.*[14] Prehybridization, hybridization and final washing were performed 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.*[14]

RESULTS

Incorporation of dig-11-dUTP during the 'run-off' reaction

For synthesis of single-stranded DNA hybridization probes, a plasmid template DNA was linearized and one primer, binding at a defined distance from the restriction cut, was added to a *Taq* DNA-polymerase reaction mix. After repeated cycles of denaturation, annealing and extension in a thermal cycler a single-stranded DNA was synthesized in a 'run-off' reaction. If dig-11-dUTP was added during the reaction a non-radioactive labelled single-stranded DNA hybridization probe was produced (Fig. 1).

Probe synthesis was optimized to synthesize full length single-stranded DNA probes during the 'run-off' reaction with the highest possible sensitivity in hybridization experiments. To achieve this, the optimal ratio between dTTP and its labelled analogon dig-11-dUTP was determined. The total concentration of both nucleotides was 100 μM. We set up five reactions using *Bam* HI digested pBC-sis and the T_3 primer starting with 12·5 μM dig-11-dUTP (87·5 μM dTTP) up to 100 μM dig-11-dUTP (no dTTP). Alkaline agarose gel electrophoresis shows that a full length strand is produced in all reactions (Fig. 2a). The migration rate of the single-stranded DNA is reduced, the more dig-11-dUTP is incorporated. Due to a less efficient incorporation of dig-11-dUTP by *Taq* DNA-polymerase, the yield of single-stranded DNA is decreased if more than 50 μM of dig-11-dUTP are used (Fig. 2a, lanes 4 and 5). The probe in lane 5 is only

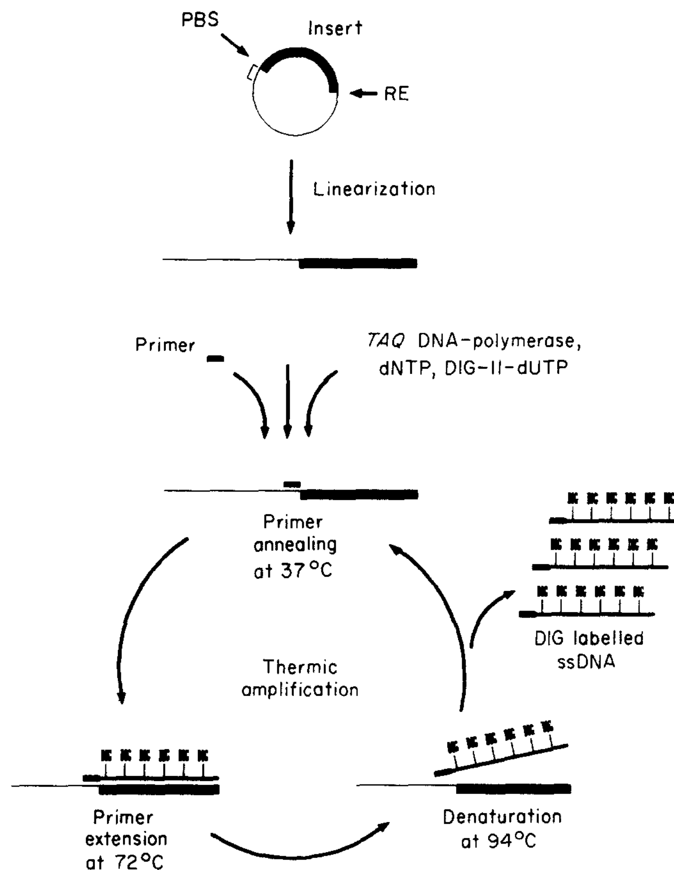

Fig. 1. Schematic presentation of the synthesis of digoxigenin labelled single-stranded DNA probes by 'run-off' polymerization. PBS = primer binding site, RE = restriction site.

visible after longer development of the colour reaction (Fig. 2a, lane 5).

The effect of the molar ratio of dig-11-dUTP incorporation on the sensitivity of the probes was examined in a dot-blot (Fig. 2b). Equal amounts of each reaction were removed and hybridized to the sense RNA of c-sis synthesized by *in vitro* transcription. The amount of target RNA applied onto the filter was from 300 pg down to 10 pg. All probes could detect 10 pg, but the highest intensity of the signal with the lowest background staining was achieved by using 50 μM of dig-11-dUTP and 50 μM of dTTP (Fig. 2b, lane 3). These concentrations were maintained in all further reactions. The data described above show, that the ratio of dig-11-dUTP and dTTP used during the 'run-off' reaction is very important for the synthesis of highly sensitive digoxigenin labelled probes with high yield.

The number of cycles required for maximum yield of probe was examined with identical reactions which were incubated for 5, 10, 20, 30 and 40 cycles. The relative yield of single-stranded DNA was defined by alkaline agarose gel electrophoresis and ethidium bromide staining. The amount of synthesized full length probe increased up to 30 cycles (data not shown). After more than 30 cycles primarily shorter molecules were produced. Alkaline agarose gel electrophoresis and subsequent ethidium bromide staining showed that this is due to thermic degradation of the template DNA (data not shown).

The absolute yield of single-stranded DNA after 30 cycles was measured by incorporation of α-P^{32}-dCTP during the reaction. One microgram of pBC-sis template in the reaction yielded 1·25 μg of single-stranded DNA. Therefore, 90 ng of single-stranded template DNA (only 700 bp of c-sis fragment out of 3904 bp of pBC-sis served as a template) were amplified up to 1·25 μg during the reaction (13–14-fold increase).

In order to test the maximal length of single-stranded DNA which can be synthesized during the reaction, we used the vector pCVB3-R1. This vector was digested at various distances from the primer binding site to get templates of different length for polymerization (Fig. 3b). The data in Fig. 3a showed that single-stranded DNA was synthesized up to 5000 bases in length, but for probes longer than 3530 nt the yield was significantly reduced.

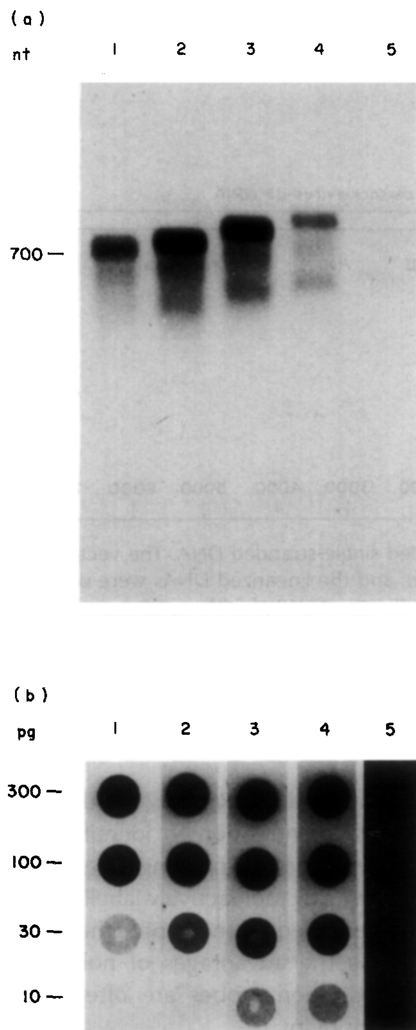

Fig. 2. Titration of dig-11-dUTP/dTTP ratio during the 'run-off' reaction. (a) Alkaline agarose gel electrophoresis of digoxigenin labelled single-stranded DNA. *Bam* HI linearized pBC-sis and the T3 primer were used for the reaction which was done as described in Materials and Methods. The total concentration of dig-11-dUTP and dTTP was 100 μM. The ratio of both nucleotides was varied between the reactions. Lane 1, 12·5 μM; lane 2, 25 μM; lane 3, 50 μM; lane 4, 75 μM; lane 5, 100 μM (dig-11-dUTP). A volume, 0·5 μl, of each reaction was applied on a 1% alkaline agarose gel transferred to a GeneScreen membrane and stained by the alkaline phosphatase reaction according to the protocols for digoxigenin labelling. (b) Dot-blot to determine the influence of dig-11-dUTP incorporation ratio on probe sensitivity. Lane 1, 12·5 μM; lane 2, 25 μM; lane 3, 50 μM; lane 4, 75 μM; lane 5, 100 μM (dig-11-dUTP). A volume, 50 μl, of the different reactions described in (a) were removed and the single-stranded DNA was used for hybridization with *in vitro* transcribed target RNAs, coding for the sense strand of c-sis. The target RNAs were applied in decreasing amounts onto nitrocellulose filters (300 pg, 100 pg, 30 pg, 10 pg). Filters were prehybridized and hybridized as described. After the final washing of the filters, the probe was detected by immunostaining with alkaline phosphatase.

Dot-blot to determine the hybridization conditions and strand specificity of digoxigenin labelled 'run-off' probes

A dot-blot with *in vitro*-transcribed sense and antisense RNA of c-sis as a target was chosen to standardize the optimal hybridization conditions and to demonstrate the strand specificity of digoxigenin labelled single-stranded DNA probes. We tested seven different hybridization membranes. Best for low background hybridization were nitrocellulose and uncharged nylon membranes. Positively charged membranes result in a very high background if the phosphatase conjugated antibody used for probe detection was applied in a concentration of 750 mU ml^{-1}, which is required for maximum sensitivity (data not shown). The amount of probe required for highest sensitivity was 200–300 ng ml^{-1}. A higher probe concentration increased the background without amplification of signal intensity (data not shown). Under these conditions we could detect down to 3 pg of c-sis RNA (Fig. 4, lane 1). No cross-reactivity occurred with the antisense strand (Fig. 4, lane 2).

Sensitivity of digoxigenin labelled 'run-off' probes in Northern blotting

Northern blot experiments were used to determine the suitability of digoxigenin labelled single-stranded DNA probes for general applications. The detection of β-actin mRNA in decreasing amounts of total human fibroblast RNA was used as a marker for the sensitivity of digoxigenin labelled 'run-off' probes (Fig. 5a).

For sensitivity control a probe of the same sequence which was labelled radioactively by nick-translation to a specific activity of $6{\cdot}5 \times 10^8$ cpm μg^{-1} DNA was used (Fig. 5b). With each probe β-actin mRNA could be detected down to 0·03 μg of total RNA. Detection time of the digoxigenin probe was much faster than the time needed with the radio-labelled probe to achieve the same sensitivity (18 h for the non-radioactive probe, 72 h for the radioactive probe).

DISCUSSION

With the polymerase chain reaction (PCR), a sensitive technique is available to detect very rare sequences.[16] Nevertheless, sensitive hybridization probes are still required to study the structure of genes in Southern blotting, to determine the molecu-

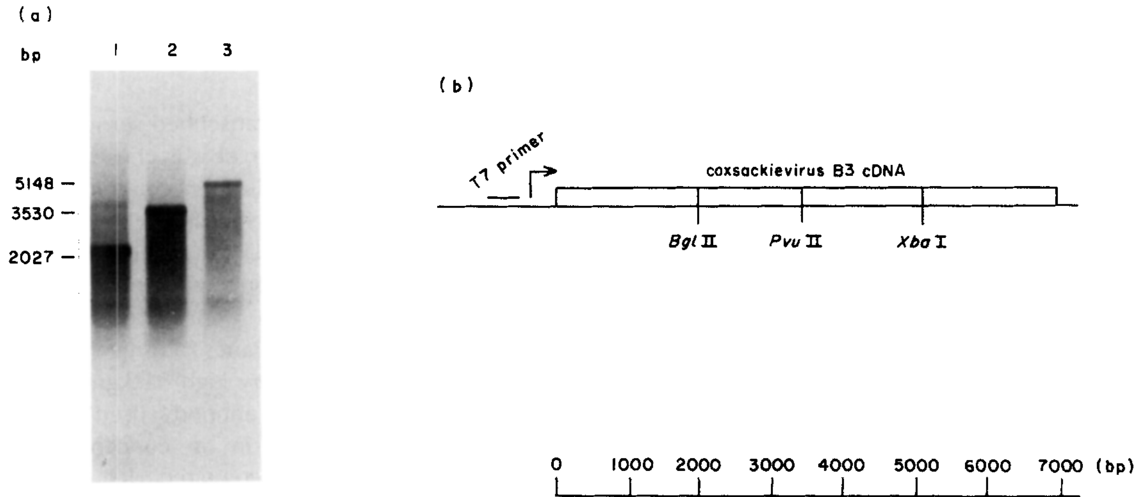

Fig. 3. Determination of maximal probe length of digoxigenin labelled single-stranded DNA. The vector pCVB3-R1 was digested with each restriction enzyme indicated in (b), and the linearized DNAs were used as templates in three separate polymerization reactions. The reaction volume was 10 µl with equal amounts of the different length DNA templates (18 ng). The conditions for probe synthesis are down-scaled from the 100 µl reactions described in Materials and Methods. The only difference is that the reaction was run for 20 cycles with an extension time of 10 min cycle^{-1}. After the reaction, the DNA was precipitated, dissolved in sample buffer and loaded on a 1% alkaline agarose gel. After electrophoresis, the single-stranded DNA was transferred to a GeneScreen membrane and stained by the alkaline phosphatase reaction.

lar weight of a specific mRNA by Northern blotting and for *in situ* hybridization to identify which cell type in a given tissue is expressing a certain gene.

Up to now, mostly radioactively labelled hybridization probes were used for the approaches mentioned above because the advantages of non-isotopically labelled hybridization probes are often limited by their lower sensitivity.

We describe the synthesis and application of digoxigenin labelled strand-specific DNA hybridization probes with very high sensitivity and low background. The composition of the reaction mix with respect to nucleotide, primer and template concentration was optimized. In addition, we established an optimal dig-dUTP/dTTP ratio and standardized the optimal time intervals and numbers of cycles in the thermal cycler. This resulted in full length probe

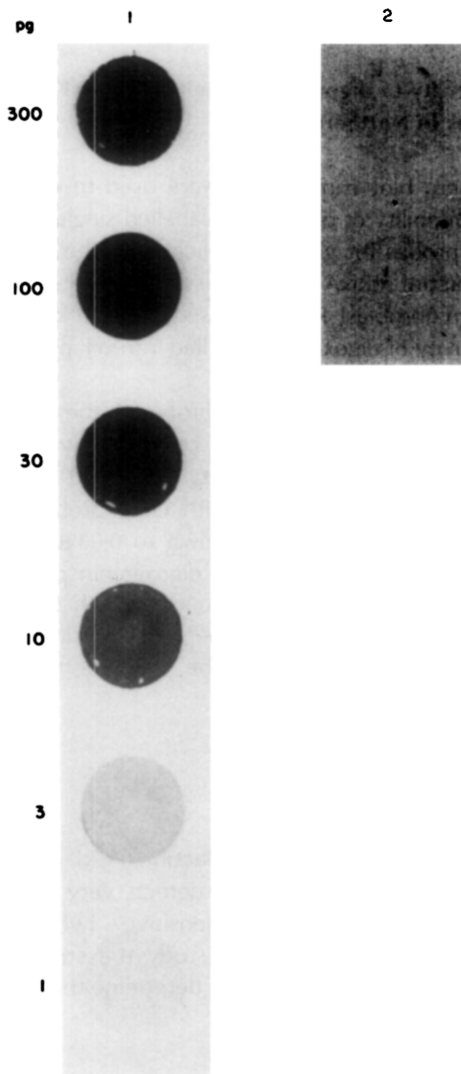

Fig. 4. Dot-blot to demonstrate the strand specificity and sensitivity of digoxigenin labelled single-stranded hybridization probes. Sense and antisense RNA of the c-sis fragment was synthesized by *in vitro*-transcription and fixed on a nitrocellulose membrane in decreasing amounts (lane 1, sense RNA, 300, 100, 30, 10, 3, 1 pg; lane 2, antisense RNA, 300 and 100 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 as described. The probe concentration used for hybridization was 300 ng ml^{-1}. The concentration of the alkaline phosphatase conjugated anti-digoxigenin-antibody was 750 mU ml^{-1}. The colour reaction was carried out for 3 days.

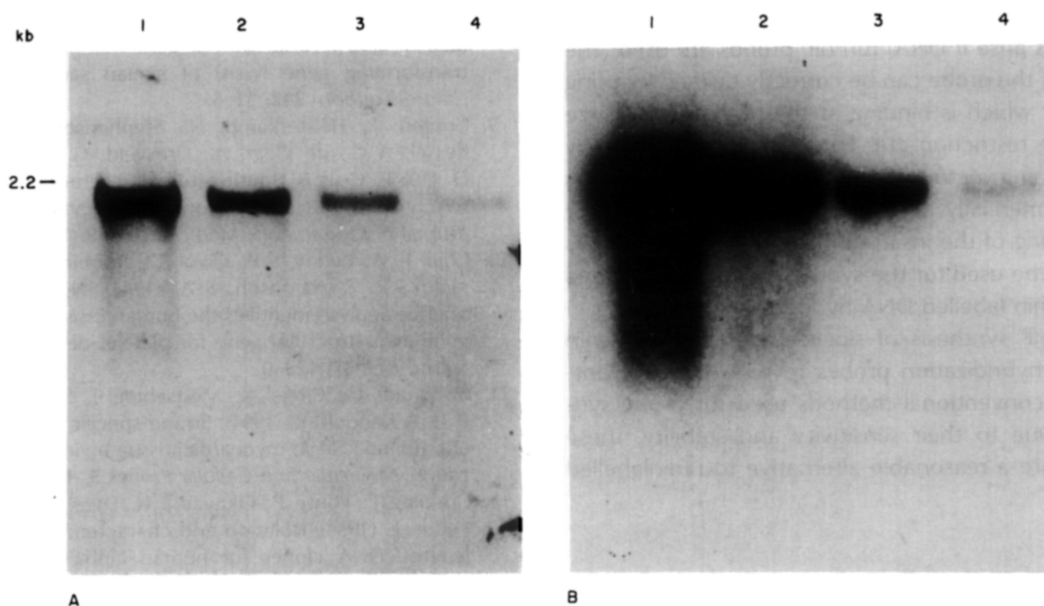

Fig. 5. Northern blot with a non-isotopically labelled single-stranded β-actin DNA (a) and with a nick-translated radiolabelled β-actin fragment (b). Decreasing amounts (1 μg, 0·3 μg, 0·1 μg and 0·03 μg) of total RNA isolated from human fibroblast cells were blotted on nitrocellulose membranes. The probe concentration for the non-isotopic probe was 300 ng ml^{-1}. From the nick-translated probe (specific activity $6{\cdot}5 \times 10^8$ cpm μg^{-1} of DNA) 2×10^6 cpm ml^{-1} were used for hybridization. The development of the non-radioactive staining reaction carried out for 18 h. The exposure time in autoradiographie was 72 h.

synthesis up to 5000 nt and a high yield (13–14-fold template amplification). Digoxigenin is linked to dUTP by an alkali stable spacer[4] and therefore could be used in alkaline agarose gel electrophoresis. We showed that alkaline agarose gel electrophoresis and subsequent blotting to hybridization membranes are easy and fast methods to measure the result of the synthesis reaction.

Membrane type and hybridization conditions were chosen to obtain maximal sensitivity in dot-blot and Northern blot. We tested seven different membranes from different suppliers and found that nitrocellulose and uncharged GeneScreen membranes revealed highest sensitivity and lowest background. For blotting from alkaline agarose gels, GeneScreen membrane was more suitable, due to a higher resistance against alkali treatment. Titration of the probe concentration showed that 200–300 ng of probe per ml of hybridization solution allowed the detection of β-actin mRNA out of only 0·03 μg of total human fibroblast RNA in a Northern blot. This was equally sensitive but with higher signal resolution and much faster (18 h compared to 72 h) than detection with a control probe, labelled radioactively by nick-translation (specific activity $6{\cdot}5 \times 10^8$ cpm μg^{-1} DNA). Recently, we could show that probes radiolabelled by nick-translation are sensitive enough to detect low expressed genes in Northern blotting and to examine single copy genes in Southern blotting.[6,17] Compared to digoxigenin labelled RNA probes[4] which can detect β-actin mRNA from 0·1 μg of total RNA, digoxigenin labelled DNA 'run-off' probes are three times more sensitive.

In addition to increased sensitivity, single-stranded 'run-off' DNA-probes reveal the benefit of increased stability compared to RNA probes. As described elsewhere,[4] a great effort has to be made to inhibit RNase in the blocking reagent if RNA probes are used. No special treatment is required for digoxigenin labelled DNA 'run-off' probes.

The strand specificity which is demonstrated in a dot-blot and the fact that their length can be easily defined, makes single-stranded 'run-off' probes extremely useful for *in situ* hybridization. Use of single-stranded DNA probes gives, like strand specific RNA probes, the opportunity for a negative control by probing with the complementary strand. For *in situ* hybridization the optimal length of the probe was defined as between 100 and 400 nt.[18] Synthesizing the probe by *in vitro* transcription sometimes makes it difficult to achieve the appropriate length of the transcript, if an adequate restriction site is missing at the correct distance of the promoter. This often requires a time consuming recloning of the sequence into an appropriate transcription vector. Alkaline degradation of longer transcripts to the correct size is an alternative, but requires a careful standardization. Moreover non-radioactive RNA probes often reveal a lower sensitivity in *in situ* hybridization because of degradation of the RNA by the conditions required

for the alkaline phosphatase reaction.[19] None of these problems arise if DNA 'run-off' probes are used. The length of the probe can be correctly defined by using a primer which is binding at the preferred distance from the restriction cut. For many of the generally used cloning vectors like pUC[20] and pBR322,[21] primers are commercially available, which are provided for sequencing of the inserted fragments. These primers also can be used for the synthesis of single-stranded digoxigenin labelled DNA hybridization probes.

'Run-off' synthesis of single-stranded digoxigenin labelled hybridization probes reveals many advantages to conventional methods used for probe synthesis. Due to their sensitivity and stability, these probes are a reasonable alternative to radiolabelled probes.

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REFERENCES

1. Lo, Y-M.D., Mehal, W.Z. & Fleming, K. A. (1988). Rapid production of vector-free biotinylated probes using the polymerase chain reaction. *Nucleic Acids Research* **16**, 8719.
2. Lion, T. & Haas, O. A. (1990). Nonradioactive labelling of probe with digoxigenin by polymerase chain reaction. *Analytical Biochemistry* **188**, 335–7.
3. Cox, K. H., DeLeon, D. V., Angerer, L. M. & Angerer, R. C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymetric RNA probes. *Developmental Biology* **101**, 485–502.
4. Höltke, H.-J. & Kessler, C. (1990). Non-radioactive labeling of RNA transcripts in vitro with hapten digoxigenin (DIG); hybridization and ELISA-based detection. *Nucleic Acids Research* **18**, 5843–51.
5. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Research* **12**, 7035–56.
6. Stürzl, M. & Roth, W. K. (1990). 'Run-off' synthesis and application of defined single-stranded DNA hybridization probes. *Analytical Biochemistry* **185**, 164–9.
7. Collasius, M., Falk, H., Ciesler, C. & Valet, G. (1989). How to build an inexpensive cyclotherm instrument for automated polymerase chain reaction. *Analytical Biochemistry* **181**, 163–66.
8. Dalla Favera, R., Gelmann, E. P., Gallo, R. C. & Wong-Staal, F. (1981). A human onc gene homologous to the transforming gene (v-sis) of simian sarcoma virus. *Nature (London)* **292**, 31–5.
9. Groffen, J., Heisterkamp, N., Stephenson, J. R., van Kessel, A. G., de Klein, A., Grosveld, G. & Bootsma, D. (1983). C-sis is translocated from chromosome 22 to chromosome 9 in chronic myelocytic leukemia. *Journal of Experimental Medicine* **158**, 9–15.
10. Chiu, I.-M., Reddy, E. P., Givol, D., Robbins, K. C., Tronick, S. R. & Aaronson, S. A. (1984). Nucleotide sequence analysis identifies the human c-sis proto-oncogene as a structural gene for platelet-derived growth factor. *Cell* **37**, 123–9.
11. Hohenadl, C., Klingel, K., Mertsching, J., Hofschneider, P. H. & Kandolf, R. (1991). Strand-specific detection of enteroviral RNA in myocardial tissue by in situ hybridization. *Molecular and Cellular Probes* **5**, 11–20.
12. Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H. & Kedes, L. (1983). Isolation and characterization of full-length cDNA clones for human alpha-, beta-, and gamma-actin mRNAs: Skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Molecular and Cellular Biology* **3**, 787–95.
13. Roth, W. K., Werner, S., Risau, W., Remberger, K. & Hofschneider, P. H. (1988). Cultured, AIDS-related Kaposi's Sarcoma cells express endothelial cell markers and are weakly malignant *in vitro*. *International Journal of Cancer* **42**, 767–73.
14. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.
15. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–9.
16. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–91.
17. Werner, S., Hofschneider, P. H. & Roth, W. K. (1989). Cells derived from sporadic and AIDS-related Kaposi's Sarcoma reveal identical cytochemical and molecular properties *in vitro*. *International Journal of Cancer* **43**, 1137–44.
18. Haase, A., Brahic, M., Stowring, L. & Blum, H. (1984). Detection of viral nucleic acids by in situ hybridization. *Methods in Virology* **7**, 189–226.
19. Ozden, S., Aubert, C., Gonzales-Dunia, D. & Brahic, M. (1990). Simultaneous in situ detection of two mRNAs in the same cell using riboprobes labeled with biotin and ^{35}S. *The Journal of Histochemistry and Cytochemistry* **38**, 917–22.
20. Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with syntetic universal primers. *Gene* **19**, 259–68.
21. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, H. J. & Falkow, S. (1977). Construction and characterization of new cloning vehicles, II. A multi-purpose cloning system. *Gene* **2**, 95–113.