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Sensitive non-radioactive dot-blot hybridization using DNA probes labelled with chelate group substituted psoralen and quantitative detection by europium ion fluorescence

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ABSTRACT

A new labelling method for cloned DNA probes used in hybridization assays is described. The DNA insert of recombinant plasmid DNA was made partially single-stranded for the labelling reaction by a restriction enzyme digest, followed by a controlled exonuclease III incubation. A thiol-containing psoralen derivative was covalently bound through irradiation with UV-light to the remaining double-stranded region of the plasmid DNA. The psoralen-SH groups were labelled with a large number of metal chelators (diethylentriamine pentaacetic acid, DTPA) using poly-L-lysine as a macromolecular carrier. The main advantage of the labelling procedure is that a high degree of labelling is achieved without modification of the single-stranded DNA hybridizing sequences. The specific hybrids were labelled after filter hybridization with europium ions through the chelating groups of DTPA. The europium ions were quantitatively detected by time-resolved fluorometry. The sensitivity of the assay for target DNA detection was in the low picogram range, comparable to radioactively labelled DNA probes.

INTRODUCTION

Nucleic acid hybridization is an essential technique in both basic research and clinical applications for the detection of specific nucleotide sequences in DNA and RNA.

Methods with radiolabelled DNA probes for hybridization are well established (1,2). Non-radioactive detection systems include either direct labelling of the DNA probe by fluorochromes (3-6) and enzymes (7,8) or indirect detection after attaching a reporter group to the DNA probe. The most common reporter group is biotin, which can be linked to the DNA probe in different ways (9-17).

All methods have disadvantages: Radiolabelled DNA probes may cause problems with stability, safety and detection. Fluorochrome-labelled DNA probes are not sensitive enough. Enzymes may be inactivated by the harsh hybridization conditions. Immunodetectable reporter groups, attached to the hybridizing sequences, may hinder the hybridi-

zation reaction. Indirect detection systems may also produce high background due to unspecific binding of antibodies (avidine/streptavidine in the case of biotin) (18).

Recently published reports on high-level conjugation of chelating agents onto monoclonal antibodies (19) and on time-resolved fluorometry for the indirect detection of DNA hybrids by highly fluorescent complexes of europium (20) led to the idea of attaching a great number of europium chelating groups to a DNA probe via a macromolecular carrier.

The described labelling procedure comprises four steps. In the first step, the insert of a recombinant plasmid DNA is made partially single-stranded, leaving the remainder of the plasmid DNA double-stranded. In the second step, reactive thiol groups are covalently introduced into the double-stranded vector part by a synthesized psoralen derivative. In the third step, poly-L-lysine, which is labelled with the metal chelator diethylentriamine pentaacetic acid (DTPA), is linked to the DNA-bound psoralen-thiol groups. In the fourth step europium ions are attached through the chelator DTPA after DNA hybridization. Europium ions are finally quantitated by time-resolved fluorometry (TRF).

MATERIALS AND METHODS

Materials

The HBV specific pKKHBs34 probe was derived from cloning the large BglII-fragment of the hepatitis B virus genome into a modified pBR322 (21). Plasmid preparation was performed by standard procedures (22). Hpa I restriction endonuclease and exonuclease III were purchased from Boehringer Mannheim. Poly-L-lysine hydrobromide (M_r 17,000), 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester and cyclic anhydride of DTPA (caDTPA) was obtained from Sigma Chemical Co. (Deisenhofen, FRG), europium chloride and N,N-bis-diaminopropyl-aminomethane were from Aldrich (Steinheim,FRG), 7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin was from Molecular Probes (Eugene, OR, USA), 4'-Hydroxymethyl-4,5',8-trimethylpsoralen (1 in Fig. 2) from Calbiochem (Frankfurt, FRG), and N-succinimidyl 3-(2-pyridyldithio)propionate from Pharmacia (Freiburg,FRG). $SOCl_2$, plates for thin layer chromatography (silica gel $60F_{254}$, Al_2O_3 $60F_{254}$ neutral) and column chromatography media (Al_2O_3 90 active, 70-230 mesh) were obtained from Merck (Darmstadt, FRG), diethylaminoethyl cellulose (DEAE-DE52) was from Whatman (Maidstone,UK) and NENSORB 20 nucleic acid purification cartridges from DuPont-New England Nuclear

(Dreieich, FRG). "Enhancement solution" for time-resolved fluorometry measurements was a gift from LKB (Freiburg, FRG).

Synthesis of thiol-containing psoralen (PSH)

Psoralen-SH **5** was prepared similar to a procedure described by Saffran et al. (23). All reactions were carried out in the dark. The individual steps of the synthesis are shown in Fig. 2.

Preparation of 4'-Chloromethyl-4,5',8-trimethylpsoralen **2**

A solution of 2% $SOCl_2$ in dry chloroform (40 ml) was added dropwise to 4'-hydroxymethyl-4,5',8-trimethylpsoralen (15 mg, $5.8*10^{-5}$ mol) within 5 minutes while stirring at room temperature (rt). After additional stirring for 3 h at rt, thin layer chromatography (TLC) on silica gel indicated a complete reaction. The solvent and the gaseous components were removed under reduced pressure. The product **2** with an R_F=0.41 on silica gel TLC (1:1, petrol ether - ethyl acetate) was used without further purification.

Preparation of 4'-(9''-Amino-2'',6''-diazanonyl)-4,5',8-trimethylpsoralen **3**

A solution of **2** (16 mg, $5.8*10^{-5}$mol) and N,N-bis-(diaminopropyl)-aminomethane (150 mg, 180 µl, $9.6*10^{-4}$mol) in dry toluene (10 ml) was heated under reflux for 8 h. The solvent was removed under reduced pressure, and the residue was chromatographed on aluminum oxide (7:2:1, ethyl acetate - methanol - water) to give product **3** (7.3 mg, 33%) as an orange-brown oil with an R_f=0.16 on aluminum oxide TLC (7:2:1, ethyl acetate - methanol - water). ^{1}H-NMR (90 MHz, $CDCl_3$) δ 7.62 (1H, s, phenyl), 6.23 (1H, s, lactone), 3.86 (2H, s, Ar-CH_2-NHR), 2.4-2.55 (14H, m, 8H RR'N-CH_2-, 3H Ar-CH_3, 3H furan-CH_3, 3H pyrone-CH_3), 2.18 (3H, s, N-CH_3), 1.70 (4H, m, -CH_2-CH_2-CH_2-).

Preparation of 4'-(9''-(3'''-(2''''-Pyridyldithio)propionamido)-2'',6''-diazanonyl)-4,5',8-trimethylpsoralen **4**

A solution of N-succinimidyl 3-(2-pyridyldithio)propionate (6mg, $1.9*10^{-5}$ mol) in dry methylene chloride (100 µl) was added to **3** (7.4 mg, $1.9*10^{-5}$ mol) in dry methylene chloride (100 µl). After stirring for 30 min at rt the solvent was evaporated under reduced pressure, and the residue was chromatographed on aluminum oxide (methanol) to give product **4** (4.35 mg, 40%) with R_F=0.79 (aluminum oxide TLC, 7:2:1, ethyl acetate - methanol - water) as a white amorphous solid. ^{1}H-NMR (90 MHz, $CDCl_3$) δ 8.45 (1H, m, pyridine **3H**), 7.6-7.7 (3H, m, 1 phenyl-**H**, pyridine **5,6H**), 7.11 (1H, m, pyridine **4H**), 6.52 (1H, s, lactone), 3.9 (2H, s, Ar-CH_2-NHR), 3.11 (2H, s, $COCH_2$), 2.84 (2H, s, -CH_2-S-), 2.45-2.55 (14H, m, 8H R,R'N-CH_2, 3H Ar-CH_3), 3H furan-CH_3, 3H pyrone-CH_3), 2.17 (3H, s, N-CH_3), 1.70 (4H, m, -CH_2-CH_2-CH_2-). UV (methanol) λ_{max} (ε) 285 (5,600) 330 (1,900) nm.

Preparation of 4'-(9''-(3'''-Mercaptopropionamido)-2'',6''-diazanyl)-4,5',8-trimethylpsoralen **5** (PSH)

PSH (**5**) was freshly prepared for each labelling reaction. 24 µl of a 1.7mM stock solution of **4** in ethanol, 40 µl 100mM dithioerythritol (DTE) and 16 µl H_2O were mixed in an Eppendorf tube and incubated for 1 h at rt in the dark.

Interaction of PSH with DNA in the dark. Calculation of the binding constant

The fluorescence of a compound in a ligand-receptor-complex is frequently quenched proportional to the strength of ligand binding. This effect was used to measure the binding constant of PSH to DNA.

Sonicated salmon sperm DNA (2.5, 5, 10, 15, and 20 µg, from a 2.5 mg/ml stock solution) was added stepwise to a solution of 3µM PSH in 500 µl 10mM Tris-HCl, 1mM EDTA (pH 7.15). At the beginning and after each addition of DNA the fluorescence was measured in a LS-5 spectrofluorometer (Perkin-Elmer, Friedrichshafen, FRG; Ex 300 nm, Em 450 nm).

Data were plotted according to the equation described by Kelly et al. (24):

$$\frac{1}{\Delta F} = \frac{1}{K[N]\Delta F_\infty} + \frac{1}{\Delta F_\infty}$$

where ΔF= decrease in fluorescence intensity at emission maximum in the presence of concentration N of DNA (expressed as molarity of phosphate groups) and ΔF_∞= decrease in fluorescence intensity at infinite DNA concentration. The association constant K was obtained from the slope of the straight line when $1/\Delta F$ was plotted against $1/[N]$.

Modification of cloned DNA

pKKHBs34 (9 µg) was incubated with Hpa I (10 U) in 25 µl 10mM Tris-HCl, 50mM KCl, 10mM $MgCl_2$, 5mM mercaptoethanol (pH 7.5) for 1 h at 37°C to linearize the plasmid. The reaction was stopped by addition of 2.5 µl 100mM EDTA. The DNA was precipitated by ethanol, washed with 70% ethanol and resuspended in 36 µl H_2O. Exonuclease III (90 U) in 4 µl 10X Exo III buffer (660mM Tris-HCl, 6.6mM $MgCl_2$, 10mM mercaptoethanol, pH 7.6) was added and the solution was incubated for 30 min at 37°C to make the plasmid partially single-stranded. The reaction was stopped by addition of 5 µl 100mM EDTA. The partially single-stranded pKKHBs34 (hereafter assigned pKKHBs34') was separated from enzyme, nucleotides and buffer using NENSORB 20 nucleic acid purification cartridges, according to the manufacturer's instructions.

Labelling of DNA probe with thiol-containing psoralen PSH

Psoralen molecules intercalate into ds DNA and undergo photo-

cycloaddition with DNA thymidine residues by long wavelength ultraviolet (UV) light irradiation (25). Interstrand crosslinks between two thymidine residues are readily produced at (T-A)-sequences. ss DNA remains essentially unmodified (25).

For the photo-labelling reaction, 20 µl of freshly prepared psoralen compound PSH (0.51 mM in 50mM DTE) were added to a solution of 7.5 µg pKKHBs34' in 180 µl 10 mM Tris-HCl, 1 mM EDTA (pH 7.15). This corresponded to a ratio of psoralen to base pairs of approximately 1:1. The solution was incubated for 15 min at rt in the dark to achieve intercalation and irradiated for 2 h at 0°C in a 3mm liquid layer with UV light between 300 and 400 nm (HBO 200 W high pressure mercury lamp, Zeiss, Oberkochen, FRG; UG1 filter, Schott, Mainz, FRG). Addition of new PSH and irradiation were repeated twice to achieve a higher labelling ratio. Excess free psoralen and photobreakdown products were removed by extracting the reaction mixture once with 1 vol of phenol and once with 1 vol of chloroform - isoamylalcohol (24:1). Finally, the DNA was ethanol precipitated and washed with 70% ethanol.

The covalent crosslinking of the complementary strands in the DNA was shown by an ethidium bromide fluorescence assay (26).

Calculation of the PSH labelling ratio of the DNA

The amount of PSH covalently linked to the DNA was determined by reacting the SH-groups with the thiol-specific fluorescent dye, 7-diethylamino-3-(4'-maleimidophenyl)-4-methylcoumarin (CPM). The fluorescence of CPM is substantially increased when covalently bound to thiol groups. SH-labelled pKKHBs34' (7.5 µg) was dissolved in 50 µl 50 mM phosphate buffer (pH 7.8) containing 200 µM CPM and 1 mM EDTA. The mixture was shaken for 2 h at rt. Then 10 µl of the sample was added to 990 µl 1% Triton X-100 (v/v in water) and the fluorescence was measured with the LS-5 spectrofluorometer (Ex 390 nm, Em 465 nm). A fluorescence calibration curve with N-acetyl cysteine at concentrations between 0.1 and 250 µM was established.

Control reactions of labelled DNA in the presence of $HgCl_2$ (100 µM) and of unmodified DNA (which was treated like labelled DNA except for the photoreaction) gave background fluorescence.

Preparation of DTPA-linked poly-L-lysine

Cyclic anhydride of DTPA (caDTPA, 2.1 mg, 5.88 µmol) was added in aliquots to a solution of poly-L-lysine (PLL, M_r 17,000, 0.5 mg, 29.4 nmol) in 50 µl 100 mM $KHCO_3$ (pH 8.2) under vigorous agitation. Sufficient 3N NaOH was added to maintain the pH between 7 and 8. No precipitation of high molecular weight adducts was observed. Staining with ninhydrin showed less but still remaining free amino groups in the sample.

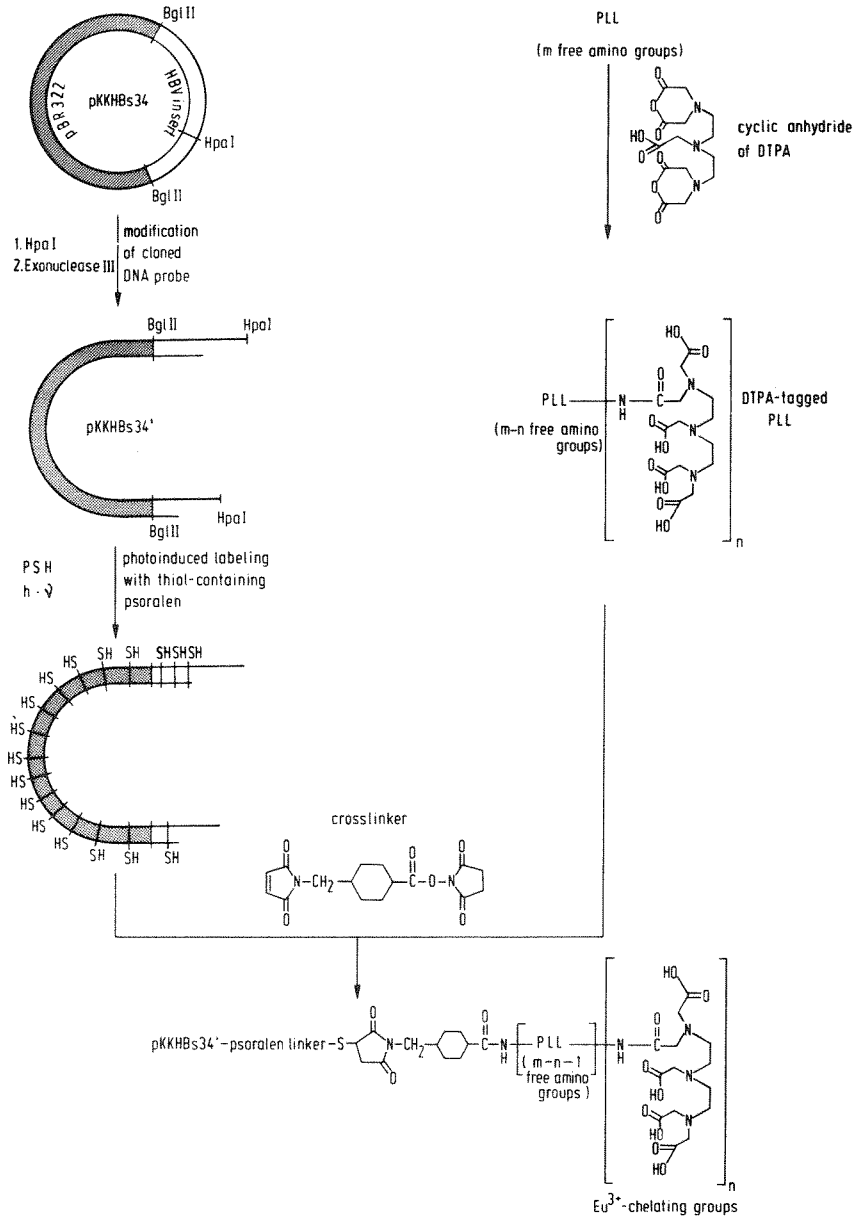

Figure 1. Preparation of DTPA-PLL-pKKHBs34'-conjugates for DNA hybridization. The pBR322 vector part of the plasmid is dotted. Note that only the double-stranded part of the plasmid is labelled and crosslinked by the psoralen molecule, while the single-stranded hybridization region remains unmodified. In our preparation of DTPA-tagged PLL, m is 82 and n approximately 16. The europium ions are added after completion of the hybridization reaction.

DTPA content in PLL was assessed by equilibrium dialysis. DTPA-PLL-conjugate (20 µg) was incubated for 2 h with 500 µM $EuCl_3$, 500 µM EDTA in 200 µl 50 mM sodium citrate buffer (pH 6.0), and was then

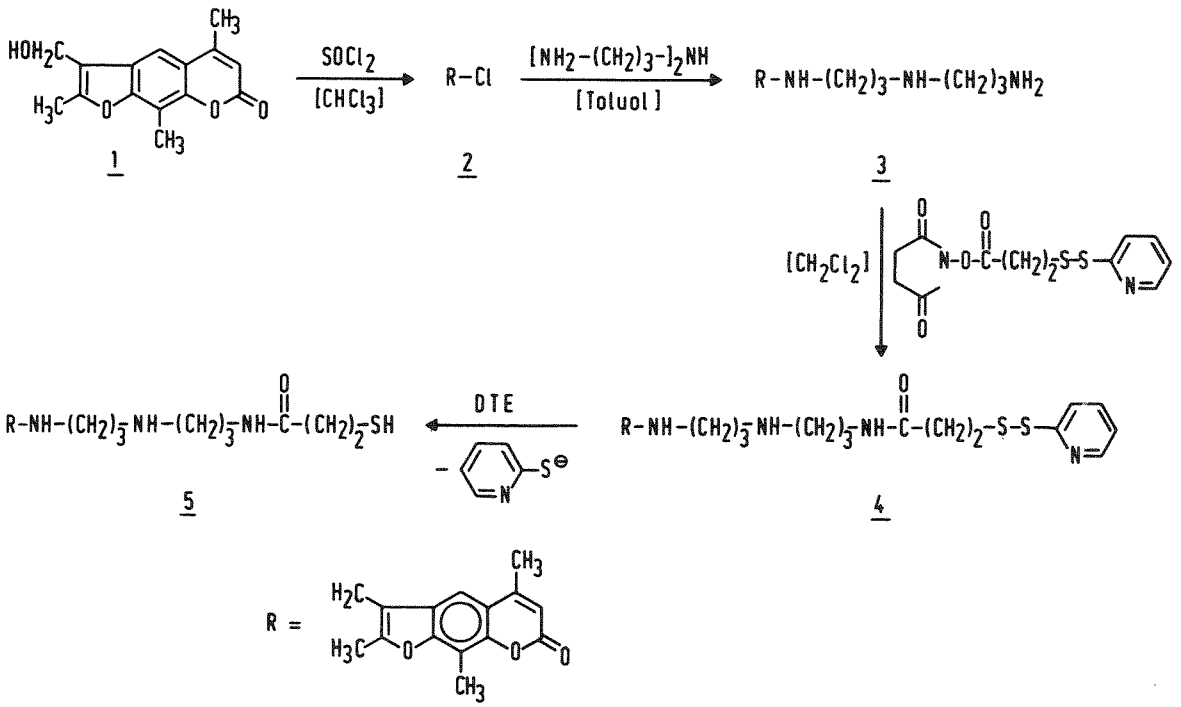

Figure 2. Reaction scheme for the synthesis of psoralen-SH (PSH).

dialyzed to equilibrium against 5 mM sodium acetate, 10 mM NaCl (pH 5.5). Europium (and therefore DTPA) content was determined by time-resolved fluorometry.

Preparation of DTPA-PLL-pKKHBs34'-conjugate

DTPA-linked PLL (100 µg, 6 nmol) in 100 µl 100mM $KHCO_3$ (pH 8.2) was cooled on ice. The heterobifunctional crosslinking agent 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (10 µg, 30 nmol, from a 1mg/ml solution in dry dimethyl formamide) was added slowly while shaking. The solution was shaken for further 30 min at rt and, after adjusting the pH to 6.5 with 0.6 N HCl,incubated with SH-labelled pKKHBs34' (7.5 µg) overnight at rt. The resulting conjugate was isolated by DEAE chromatography (Fig. 4).

Dot-blot hybridization with DTPA-PLL-pKKHBs34'

pKKHBs34 as target DNA in various amounts and pBR322 and calf thymus DNA as controls were immobilized and denatured by standard methods (27) on nitrocellulose filter disks (BA 85 NC filters, 4-5 mm diameter, Schleicher and Schuell). The filters were placed in individual polystyrene microtiter wells and prehybridized for 4 h at 42°C in 50% deionised formamide, 5X SSC (pH 7.0, 1X SSC= 150 mM NaCl, 15 mM sodium citrate), 1% sarkosyl, 0.1% bovine serum albumin, 0.1% Ficoll

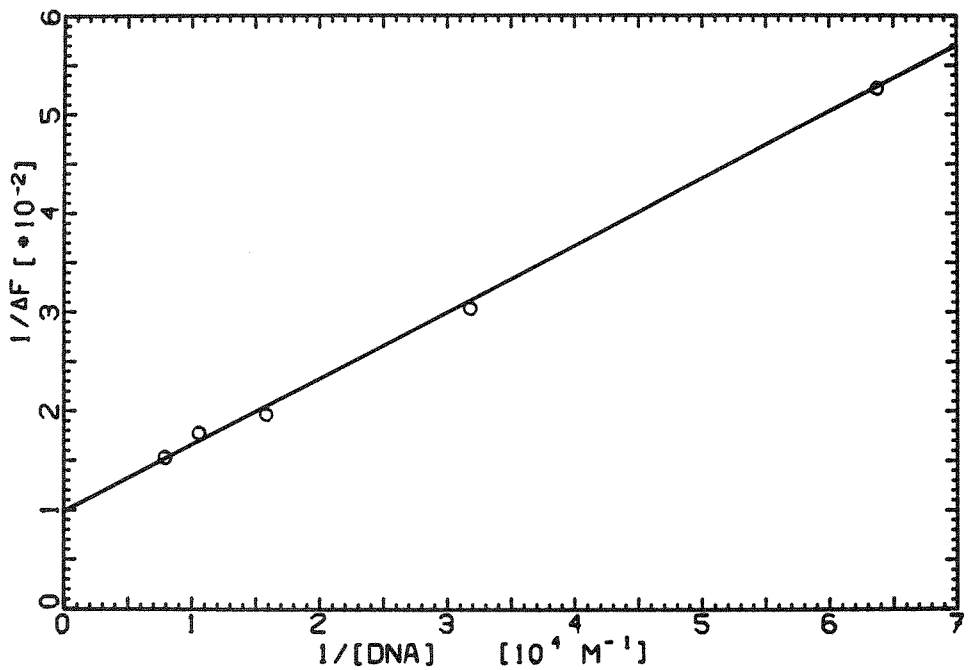

Figure 3. Double reciprocal plot for the calculation of binding constant of PSH to DNA. Salmon sperm DNA (2.5, 5, 10, 15, and 20 µg) were added to 5 µM PSH in 10 mM Tris-HCl, 1 mM EDTA (pH 7.15). The data were evaluated as described under materials and methods. A binding constant of $1.5*10^4 M^{-1}$ was determined.

400, 0.1% polyvinylpyrrolidone, and 250 µg/ml denatured, sonicated salmon sperm DNA. Hybridization was performed for 16 h at 42°C with the DTPA-labelled pKKHBs34' probe (500 ng/ml in prehybridization solution containing 10% dextran sulphate). The filters were washed twice for 10 min at rt in 2X SSC, 0.5% sarkosyl and three times for 20 min at 50°C in 0.1X SSC, 0.1% sarkosyl.

Chelation of europium and quantitation by time-resolved fluorometry

Chelation of the hybrid-bound DTPA with europium ions was achieved by incubating the filters in 100 µM $EuCl_3$, 100 µM EDTA, 1X SSC (pH 7.0, 200 µl per well) for 2 h at rt. The filters were washed at least six times with 2X SSC and finally agitated for 15 mir in 1 ml "enhancement solution" (0.1 M acetate-phthalate buffer, pH 3.2, 0.1% Triton X-100 (v/v), 1.5 µM 2-naphtoyltrifluoroacetone, 50 µM tri-n-octylphosphinoxid) in polystyrene tubes to remove DTPA-bound Eu^{3+}. The supernatants were decanted and the fluorescence measured by an "Arcus 1230" time-resolved fluorometer (LKB-Wallac, Turku, Finnland). Excitation and emission were 340 and 613 nm, respectively; delay time and counting time were both set to 400 µs (Fig.5).

RESULTS

Preparation of chelator-labelled DNA probe conjugate

The individual steps of the preparation of DTPA-PLL-pKKHBs34' conjugates is schematically shown in Fig. 1.

Modification of cloned DNA prior to PSH labelling. The pKKHBs34 plasmid DNA was enzymatically modified for the PSH labelling reaction to obtain single-stranded (ss) DNA sequences at the cloned insert which is flanked by heterologous double-stranded (ds) pBR322 vector DNA. This was achieved by a single Hpa I cleavage in the central part of the inserted HBV sequences and a controlled exonuclease III digest. The exonuclease III reaction was standardized to remove approximately 500 nucleotides which was sufficient for secific hybridization but prevented cross-hybridization by ss pBR322 vector DNA (see below).

Synthesis of the DNA-labelling reagent PSH. A thiol-containing psoralen derivative was synthesized (Fig.2) to introduce a reactive, functional group into the nucleic acid.

The reduction of the disulfide bond in **4** was followed spectrophotometrically in a test assay by the increase of absorption of the releasing group 2-mercaptopyridine (ε_{343}= 8080). This indicated a quantitative reduction and a stoichiometry of 2-pyridyldisulfide to psoralen of 1:1. Compound **4** is the storage form of the oxidation sensitive labelling reagent. There was no deterioration of **4** within one year, either in ethanol solution or as a solid in the dark at 4°C.

Photoinduced labelling of DNA with thiol-containing psoralen PSH.

The binding constant for the noncovalent interaction of PSH towards native DNA (salmon sperm DNA) was $1.5*10^4 M^{-1}$ as determined by the fluorescence quenching technique (Fig.3).

Since the psoralen molecule photodecays in solution, the labelling ratio was improved by fresh additions of the compound during UV irradiation (data not shown). One reactive SH-group per 20 base pairs was finally incorporated in pKKHBs34' DNA as determined by quantitative coupling to the thiol-specific dye 7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin.

Preparation of DTPA-PLL and conjugation to thiol-containing pKKHBs34'. Poly-L-lysine (M_r 17,000, degree of polymerization 81) was used as an intermediate carrier for the metal chelator diethylentriamine pentaacetic acid (DTPA). DTPA was coupled to the carrier via its cyclic anhydride (ca) to form stable amide bonds. Double distilled water and pure reagents were important for this reaction and for further procedures to avoid the occupation of chelating groups with trace metals competing with the subsequent chelation of europium ions.

The labelling ratio of DTPA to amino groups of PLL was determined

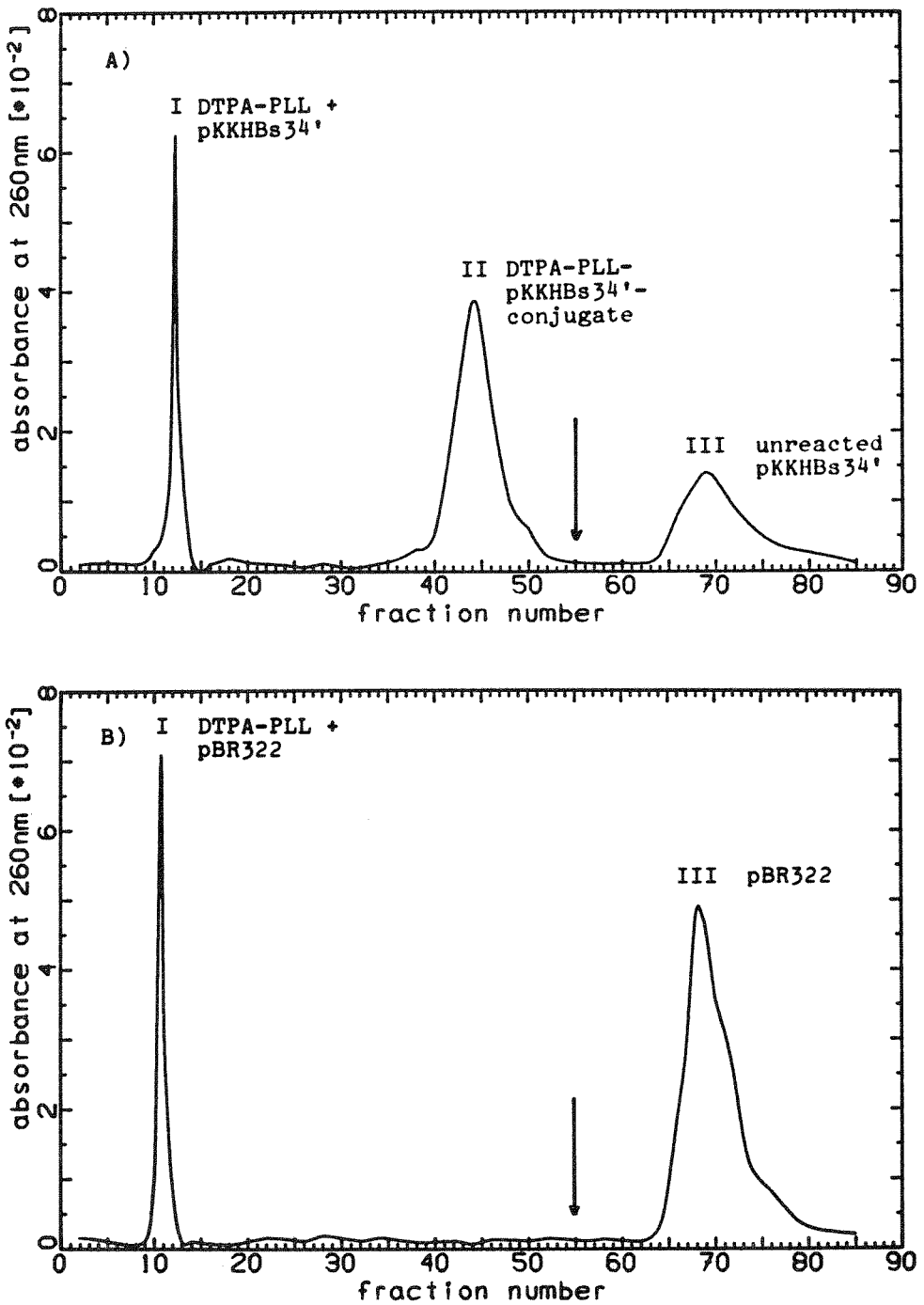

Figure 4. Elution profile on a DEAE column (A) of a reaction mixture of thiol-labelled pKKHBs34' DNA probe with DTPA-tagged PLL, which carries the crosslinker, and (B) with a mixture of pBR322 (7.5 µg) and DTPA-PLL (100 µg). The columns (0.5 x 20 cm) were equilibrated with 50 mM sodium phosphate buffer (pH 6.5) and the samples eluted with the same buffer. Each fraction contained 500 µl. The front peak was ninhydrin-positive. After elution of the conjugate, the elution buffer was changed to 100mM sodium phosphate, 100 mM NaCl (pH 6.5) as indicated by the arrow. This eluted unconjugated pKKHBs34' in A and pBR322 in B.

as 1:4. Though higher labelling ratios should be achievable by adding higher amounts of caDTPA to a more concentrated solution of PLL, a decreased level of conjugation to the DNA probe may result due to electrostatic repulsion and steric hindrance.

The DTPA-tagged PLL was coupled to the thiol-containing pKKHBs34' via the heterobifunctional crosslinking reagent 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester. The stable adduct was isolated by DEAE chromatography (Fig.4A). Fraction II contained the adduct. The presence of metal chelating groups in this fraction was shown by equilibrium dialysis after complexation of europium ions. Fraction I contained a portion of unreacted pKKHBs34' in form of a non-covalent ion conjugate with DTPA-PLL. This was demonstrated by reapplying fraction I to the DEAE-column. The resulting elution profile was identical to a elution profile of a mixture of unmodified DNA with DTPA-PLL (Fig.4B).

Quantitative detection of chelator-conjugated DNA probes by time-resolved fluorometry after hybridization to target DNA

The specific hybrids on the filter were labelled with Eu^{3+} after hybridization, because the chelates are not stable under hybridization conditions (data not shown). Eu^{3+} was added as its EDTA complex thus eliminating unspecific Eu^{3+}-adhesion to filters and phosphate groups of nucleic acids and avoiding metal ion colloid formation at neutral pH. Because the metal ion is more tightly bound to DTPA than to EDTA (the formation constants (log K) for Eu-EDTA and Eu-DTPA are 17 and 23, respectively), it is specifically exchanged to the carrier-DTPA. After chelation and removal of excess Eu-EDTA, Eu^{3+} had to be released from the hybrid-bound DTPA-chelates into a special medium ("enhancement solution") to get optimal fluorescence signals in the time-resolved fluorometry (TRF) measurement . The TRF measurement uses the long fluorescence decay times for europium complexes to eliminate background fluorescence (Fig. 5).

Amounts of target DNA (pKKHBs34) above 5 pg could be clearly detected by the hybridization assay against a background signal of a one million fold higher amount of heterologous DNA (Fig.6). This corresponds to $6*10^5$ molecules (appr. 1 attomol) and to 2 pg actual target sequences which is an equal or even higher sensitivity as compared to other detection systems. The dose-response curve was linear in this range on double logarithmic plots.

pBR322 as a target gave only background signal (2600 cps), which shows, that the crosslinking of the vector region of the pKKHBs34' probe by the psoralen molecule prevents formation of hybrids with complementary pBR322-sequences.

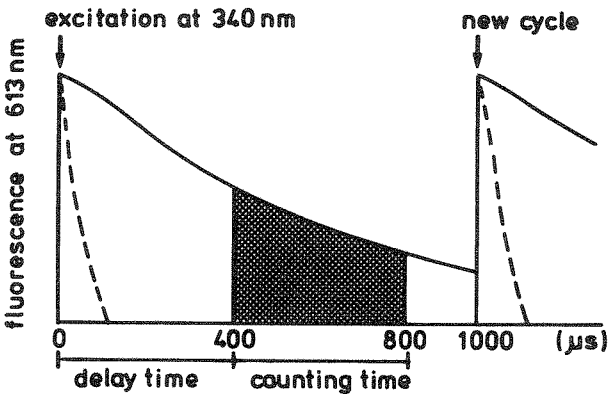

Figure 5. Principle of time-resolved fluorometry (28).The europium chelates in the sample are excited 1000 times in a single measurement, lasting only one second. The emitted photons of the europium fluorescence (——) are counted after a delay from the light pulse, when the shortlived background fluorescence from the sample (---) has decayed. This results in a very high signal to noise ratio (29). Eu^{3+}-concentrations down to 10^{-15}M can be detected in this way (29).

DISCUSSION

Several advantageous features of the described method seem to be important.

The thiol-containing psoralen compound 5 (PSH), but also its synthetic progenitor, the NH_2-containing compound 3, serve as versatile reagents to introduce reactive groups into cloned nucleic acid probes. The sulfhydryl group instead of a NH_2-group was chosen to avoid formation of crosslinked, high molecular weight adducts on conjugation of the psoralen with DTPA-carrying poly-L-lysine. The polyamine linker between the psoralen ring moiety and the thiol group contains a positively charged tertiary amino group and provides: i) higher solubility in water, ii) better accessibility of the thiol group for further reactions, and iii) higher affinity of the labelling reagent to nucleic acids by electrostatic attraction. The latter was demonstrated by the high association constant of $1.5*10^4 M^{-1}$ as compared to other psoralen derivatives (25).

Other reporter groups like biotin, fluorescent dyes and enzymes instead of chelators may be coupled to the DNA via psoralen in a similar way.

The use of a psoralen derivative to label a cloned plasmid DNA was achieved by making the inserted sequences partly single-stranded and keeping the vector region double-stranded. Other approaches to obtain ss DNA inserts use M13 clones by making them ds in the vector region (30, 31). This is more time-consuming. Furthermore, initial subcloning

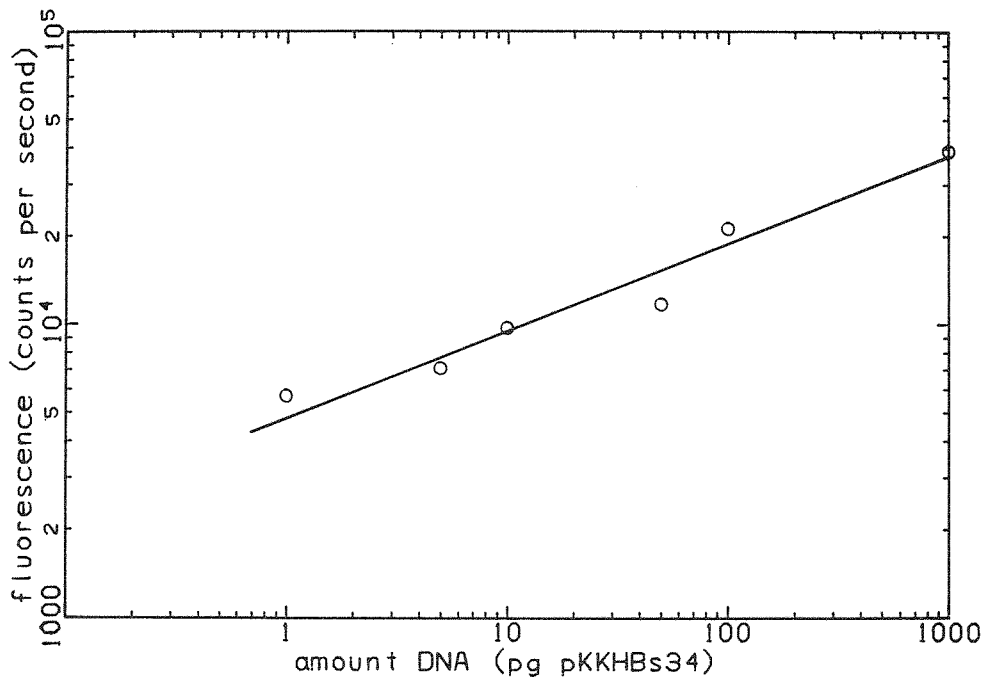

Figure 6. Hybridization of DTPA-labelled pKKHBs34' DNA probe to HBV target DNA and detection by Eu - time-resolved fluorometry. Increasing amounts of pKKHBs34 target DNA (1-1000 pg, 10^5-10^8 molecules) were immobilized on filter disks and hybridized to the DTPA-PLL-DNA probe conjugate. The specific hybrids were labelled with europium ions after hybridization and finally detected by TRF. 5 µg calf thymus DNA and 1.5 µg pBR322 plasmid DNA as negative controls gave fluorescence of 7000 and 2600 cps, respectively. The background signals from the enhancement solution alone (appr. 1500 cps) are not substracted.

into M13 is required since DNAs are generally cloned in ds plasmid vectors.

Psoralen-mediated labelling of cloned DNA probes with metal chelators has several advantages over ds probes labelled by nick translation or by unspecific chemical methods:

- The hybridizing region of the DNA probe remains unmodified by the labelling procedure which assures full hybridization efficiency.
- The single-stranded hybridizing sequences eliminate the necessity for denaturation and avoids DNA reassociation which competes with the hybridization reaction when ds DNA probes are used.
- Crosslinking of the ds vector region prevents unspecific cross-hybridization with the sample DNA and false positive hybridization signals caused by the existence of plasmids in the sample (32). This avoids isolation of the cloned insert prior to labelling or control hybridization studies with a vector DNA probe alone which is necessary in other hybridization assays.

- The degree of labelling is independent of insert size as the labelling comprises the vector part of the plasmid. This increases detection sensitivity over nick translations especially in cases where the insert is short. The sensitivity may be further increased by using several plasmid subclones with short hybridization sequences to span a whole gene in one hybridization reaction. Sensitivity may also be improved by using larger cloning vectors or larger carrier molecules, both leading to higher incorporation of chelating groups into the probe.
- DNA samples can be re-probed on the same filter by DNA probes specific for other genes in the sample (27). This is impossible if hapten/biotin-labelled DNA probes are used, where the cytochemical visualization reactions lead to precipitation of insoluble material which cannot be removed.
- Finally, carrier-mediated conjugation of chelators to DNA probes allow the potential applications of several other metal ions for different approaches, taking advantage of their specific characteristics. Besides Eu^{3+} for TRF, Gd^{3+} may be used for NMR analysis, high electron dense elements such as Os or U ions for electron microscopy, or highly radioactive metal isotopes (e.g. ions of ^{90}Yt, ^{212}Bi, or ^{57}Co) may be an attractive alternative to traditional radiolabels. Though our intention was to circumvent radioactivity in hybridization assays, the use of chelators in combination with radioactive metals for hybrid detection provides several advantages over conventional radioactive methods: the DNA probe itself is chemically and physically stable, the radioactivity is used only for a short time after the hybridization reaction and the detection time can be substantially decreased to a few minutes instead of several hours due to the very high specific activity of the metal isotopes. These metal isotopes are easily accessible in hospital radiology departments.

The described non-radioactive system avoids immunological detection procedures which are time-consuming and may produce high backgrounds (18). Time-resolved fluorometry as detection method is very sensitive and quantitative results are obtained within a few seconds. The fact that a 10fold increase in fluorescence requires a 1000fold increase in DNA (Fig. 6) is presently not understood. One possible explanation is that the DTPA-chelators in the filter-bound hybrids are buried, at increasing amounts of DNA, under strong ionic conjugates of nucleic acid strands with the positively charged carrier. The highly negatively charged DNA molecules may then form a barrier for the Eu-EDTA complexes to reach the DTPA-complexes in the chelation step.

The high signal to noise ratio obtained in TRF makes the described non-radioactive labelling system advisable for dot-blot hybridization assays in routine viral and bacterial diagnosis of crude biological material (blood, stool, urine). DTPA-labelled DNA probes can be prepared and stored frozen in large quantities.

The described TRF detection system is not yet applicable for southern/ northern, colony or in situ hybridization analysis, because the europium ions have to be released from the DNA probe for TRF measurements. However, direct detection seems possible when radioactive metal ions are used instead of Eu^{3+}. Chelator-tagged DNA probes are then suitable for all hybridization techniques.

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