Detection of specific DNA sequences using antibodies recognizing UV-labelled DNA

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ABSTRACT

This non-isotopic method for detection of nucleic acids is based on the in situ labelling of the nucleic acid by exposure to UV-irradiation. The different UV-induced photoproducts, mainly of the thymidine dimer type, are recognized by purified rabbit antibodies specific to the lesions introduced. The UV-labelled nucleic acids can then be visualized by conventional immunostaining procedures. A major advantage of the technique is the low cost and the ease by which the DNA is specifically labelled. The purified rabbit antibodies were shown to be specific for UV-irradiated DNA, and the method was applied for detection of specific DNA sequences hybridized to homologous target DNA on membrane support. We believe that the sensitivity of the method can be improved, and the significance of using different UV-doses, immunostaining methods and membrane types is discussed.

INTRODUCTION

There exist several non-isotopic methods for labelling of DNA. These methods circumvent most of the disadvantages associated with the use of radioisotopes (e.g. short shelf-life and safety precautions), but they are usually less sensitive (1).

The principle of some non-isotopic methods are enzymatic or chemical modification of DNA, followed by detection of modified DNA by antibodies. For example the nick-translation technique can be utilized for incorporation of biotinylated dUTP (2,3). The biotin-labelled DNA then can be detected by either enzyme conjugated avidin or streptavidin, by enzyme conjugated biotin specific antibodies, or by a complex of streptavidin and biotinylated enzymes (BluGENETM). However, the enzymatic incorporation of biotinylated nucleotides is costly and time consuming. Forster et al. (4) use photobiotin which
forms stable linkages with DNA upon irradiation with visible light (Photobiotin™). DNA can also be labelled by direct base modification, either by alkylation (5) or sulfonation (Chemiprobe™) (6).

We here describe a non-isotopic technique for labelling and detection of DNA. It is based on UV-irradiation as a means to label DNA, followed by detection of the UV-labelled DNA by immunostaining methods. A major advantage of the method resides in the very simple labelling of DNA by exposure to ultraviolet light for a few minutes. Such UV-irradiation generates several photoproducts which are stable and can be recognized by antibodies raised against UV-irradiated DNA. These antibodies have been shown to contain major specificities against photoproducts of the pyrimidine dimer type such as (6-4) photoproducts and cyclobutane thymine dimers (7,8). The bound UV-DNA specific antibodies can be visualized by different immunostaining methods.

This paper describe the development of the method and its use for detection of UV-labelled probe DNA hybridized to homologous target DNA on membrane support.

MATERIAL AND METHODS

Chemicals

Lambda DNA and the restriction enzymes HindIII, EcoRI and BstEII were from New England Biolabs. Calf thymus and salmon sperm DNA, methylated bovine serum albumin, ovalbumin, dextran sulfate, and Tween 20 were from Sigma. Formamide was from Fluka and Mixed Bed Resin AG 501.X8 from Bio-Rad. Horse radish peroxidase (HRP) conjugated anti-rabbit IgG was obtained from Dakopatts A/S, while alkaline phosphatase conjugated anti-rabbit IgG was from Cooper Biochemical. The substrate 3,3'-diaminobenzidine (DAB) was from Sigma, and nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylylphosphate (BCIP) were from BRL. Normal swine serum was from GIBCO.

Irradiation of DNA

Irradiation of DNA was performed at 254 nm using a Phillips TUV 15 W ultraviolet lamp with a dose rate of 10 W/m². DNA was
denatured by boiling on a water bath for 10 minutes followed by rapid cooling on ice. The aqueous solutions containing denatured DNA, were irradiated as 50 μl drops in a sterile petri dish placed on an ice cooled aluminium block.

**DNA probes**

Sonicated and denatured lambda DNA was UV-labelled and used as probe in slot blot hybridization.

A pBR322 derivative containing a lambda-HindIII fragment of 2322 bp was linearized by restriction enzyme EcoRI, denatured and UV-irradiated (8 kJ/m²) before use as a probe in gel blot hybridization.

**Blotting of DNA onto membrane support**

Slot blot: The slot blot equipment from Schleicher & Schüll (Minifold II, SRC 072/0), was used with both nitrocellulose membranes (Schleicher & Schüll BA 85-SB) and nylon membranes (GeneScreen™ and GeneScreenPlus™ from New England Nuclear and Hybond-N from Amersham). When using nitrocellulose membranes, the DNA samples were diluted in 1 M NH₄Ac, 0.01 M NaOH and applied to membranes saturated with the same solution. When nylon membranes were used, the DNA samples were diluted in 10 x SSC (1.5 M NaCl, 0.15 M Na-citrate•2H₂O pH 7.2) and applied to membranes soaked in 2 x SSC for 20 minutes. Shortly after application of the DNA samples to the slots, they were gently sucked onto the membranes.

Southern blot: Transfer of DNA from agarose gel to GeneScreenPlus™ membranes was performed by alkaline transfer as described by Chomczynski et al. (9).

**Hybridization procedure**

Hybridization of UV-irradiated DNA probes to target DNA immobilized onto membranes was performed according to a modified procedure of Bittner et al. (10) as outlined in the GeneScreen Instruction Manual (Catalog No. NEF-972). All the following incubation and washing steps were performed during constant agitation. Membranes containing blotted DNA were incubated in prehybridization solution for at least 6 hours at 42°C in sealed plastic bags. The prehybridization solution contained 50 % deionized formamide, 0.2 % polyvinyl-pyrrolidone (MW 40 000), 0.2 % ovalbumin, 0.2 % ficoll (MW 400 000), 0.05
M Tris-HCl pH 7.5, 1.0 M NaCl, 0.1 % Na$_2$HPO$_4$, 1 % SDS, 10% dextran sulfate (MW 500 000) and 100 µg/ml denatured salmon sperm DNA. Following prehybridization, 50 ng of UV-irradiated DNA probe per ml of hybridization solution (usually 20 ml per membrane) was introduced to the plastic bag and hybridization was carried out for 16-24 hours at 42°C. The membranes were washed 2 x 5 minutes in 100 ml 0.06 M Tris-HCl pH 8.0 containing 0.3 M NaCl and 2 mM EDTA at room temperature, then 2 x 30 minutes in 100 ml of 0.06 M Tris-HCl pH 8.0 containing 1 % SDS at 60°C, and lastly 2 x 30 minutes in 6 mM Tris-HCl pH 8.0 containing 30 mM NaCl and 0.3 mM EDTA at room temperature.

**Anti-UV-DNA antibodies**

Polyclonal antibodies specific to DNA photoproducts were produced in rabbits by immunization with UV-irradiated (50 kJ/m$^2$) denatured calf thymus DNA complexed to methylated bovine serum albumin. The anti-UV-DNA antibodies were purified by double affinity chromatography and assayed by an ELISA technique. The procedure used is described by Eggset et al. (11).

**Staining procedure**

Unspecific binding sites of the nylon membranes were blocked by incubating the membranes in blocking solution (PBS pH 7.3 containing 10% (v/v) normal swine serum, 0.1% Tween 20) for 90 minutes at 65°C. Nitrocellulose membranes were incubated in blocking solution for 60 minutes at room temperature. The membranes were then incubated with anti-UV-DNA antibodies diluted in PBS containing 0.1% Tween and 2% normal swine serum for 60-90 minutes. All incubations with antibodies or substrates were performed in sealed plastic bags at room temperature. The incubations were followed by 3 x 10 minutes washing in 100 ml PBS containing 0.1% Tween 20. All incubations and washing steps were performed with gentle agitation. After incubation with the primary antibody, the membranes were incubated for 60 minutes with enzyme conjugated secondary antibody diluted in PBS containing 0.1 % Tween 20 and 2% normal swine serum.

**Horse radish peroxidase (HRP):** After incubation with HRP-conjugated secondary antibodies, the membranes were incubated
with 0.01% H$_2$O$_2$ in a solution containing 0.05% (w/v) DAB, 0.02% (w/v) Ni$^+$ and 0.025% (w/v) Co$^+$ in 0.1 M sodium phosphate buffer pH 7.5 prepared according to Adams (12). The membranes were preincubated for 5 minutes in DAB-Ni$^+$+Co$^+$ solution without H$_2$O$_2$, and then with the same solution containing H$_2$O$_2$ until a visible precipitate had developed. The reaction was stopped by immersing the membrane in an excess of distilled water.

Alkaline phosphatase (AP): Membranes that had been incubated with AP-conjugated secondary antibodies, were washed as usual including a last washing step in 0.15 M veronal buffer pH 9.6 (0.15 M diethyl barbituric acid). They were then incubated in substrate solution for 60 minutes. 10 ml of substrate solution contained: 1 ml of 1 mg NBT per ml of 0.15 M veronal buffer, 0.1 ml of BCIP per ml of dimethylformamide, 20 μl 2 M MgCl$_2$ and 9 ml 0.15 M veronal buffer. The reaction was stopped by immersing the membrane in an excess of distilled water.

RESULTS

The specificity of the purified rabbit antibodies (anti-UV-DNA antibodies) towards UV-irradiated DNA was tested in a slot blot experiment (Figure 1). As can be seen only the UV-irradiated DNA was recognized by the anti-UV-DNA antibodies. At least 300 pg could be detected using horse radish peroxidase and DAB as substrate. The sensitivity of the detection method varies however with different immunostaining methods. It should be possible to increase detection sensitivity by using a more sensitive immunostaining method (see Discussion).

Levine et al. (13) have reported that the number of photoproducts generated by a certain dose of UV-irradiation, depends on the base composition of the DNA. The result in Figure 2 is consistent with this observation, since a higher degree of labelling is obtained for the calf thymus DNA which contains more thymidine than the lambda DNA (approx 62 % and 50 % A+T, respectively).

The sensitivity of the DNA detection depends on the UV doses used for labelling of DNA. Figure 3 indicates that UV doses up
Figure 1. Specificity of the polyclonal anti-UV-DNA antibodies. Dilution series of unirradiated and UV-irradiated (10 kJ/m²) denatured calf thymus DNA were slot blotted onto a GeneScreen™ membrane, and stained by the anti-UV-DNA antibodies and HRP.

to about 30 kJ/m² increases the amount of available antibody binding sites, and thereby the sensitivity of the immunostaining method. However, if the UV-irradiation dose is increased further, the sensitivity seems to decrease. A decrease in antibody binding capacity with high UV-doses has also been reported by others (14), but the reason for this is unknown. It is known that the number of (6-4) photoproducts increases with increasing UV-doses up to 50 kJ/m² (15). The decreasing antibody binding capacity can be a result of changes in the secondary structure of the DNA with increasing numbers of photoproducts. The binding of DNA to the membrane might also be affected by high numbers of photoproducts. This, however was not investigated further.
Figure 2. The labelling efficiency depends on base composition of the DNA to be labelled. Dilution series of UV-irradiated (10 kJ/m$^2$) lambda and calf thymus DNA (approximately 50% and 62% A+T, respectively) were slot blotted onto a Hybond-N membrane and visualized by anti-UV-DNA antibodies and alkaline phosphatase.

As shown in Figure 4, UV-irradiation can be used for labelling the probe in hybridization experiments. The results indicate that UV-irradiation affects the ability of probe to hybridize with complementary target DNA. The highest sensitivity for detection of target DNA (150 pg) was obtained when using probe labelled with the lowest UV dose (5 kJ/m$^2$). Irradiation with doses of 20 and 100 kJ/m$^2$ resulted in poorer detection sensitivity of target DNA (approximately 625 and 1250 pg, respectively). A possible explanation might be that high UV doses generate high amounts of photoproducts which interfere with the base pairing of complementary DNA sequences. For example, formation of cyclobutane pyrimidine dimers may lead to rupture of about 4 base pairs inclusive of the two directly
affected by the dimer (16). Other photoproducts such as (6-4) photoproducts and ring saturated pyrimidines also affect base pairing properties. These effects may lead to low hybridization efficiencies, especially when short probes are used. In the following experiment we therefore used a relatively low UV dose for irradiation (8-10 kJ/m$^2$). Thus, using a germicidal lamp with a typical dose rate of 10 W/m$^2$, the labelling of probe DNA is accomplished within 15 minutes.

A lambda-HindIII fragment of 2322 bp (Figure 5A) was used to study the performance of UV-labelled probes in a Southern blot hybridization. The fragment was cloned in pBR322, and the resulting plasmid was linearized by the restriction enzyme EcoRI, denatured and labelled with a UV dose of 8 kJ/m$^2$. The UV-labelled probe was hybridized to lambda-HindIII and lambda-BstEII fragments which had been separated by gel
Figure 4. The effect of UV-irradiation dose on probe hybridization efficiency. Dilution series of denatured lambda DNA was slot blotted onto GeneScreen™ membranes. Denatured lambda DNA UV-irradiated with 5, 20 and 100 kJ/m$^2$ were used as probes. Hybridized probe was detected by anti-UV-DNA antibodies and HRP.

electrophoreses (Figure 5A) and immobilized onto a membrane (Figure 5B). The lambda-BstEII fragment of 4822 bases contains the entire probe sequence but for 26 bases which are found in the larger BstEIII fragment of 7242 bases. The result of hybridization of probe to the gel blotted fragments is shown in Figure 5B. As expected, the probe detects the homologous lambda-HindIII fragment of 2322 bases. The lambda-BstEII fragment of 4822 bases is easily detected, but also the large fragment (7242 bases) containing only 26 bases homologous to the probe can be detected. The large BstEII fragment corresponds to 15% of total lambda DNA in the lanes. If one only takes into account the 26 bases which corresponds to 0.4% of the fragment, the target DNA detected in this case should be equivalent to 200-300 pg.
Figure 5. Detection of probe hybridized to Southern blotted DNA fragments. A. Different amounts (1.0, 0.5, 0.1, 0.05 μg) of lambda-HindIII and lambda-BstEII fragments were separated on an 1% agarose gel and stained with ethidium bromide. B. A duplicate gel was run in parallel with the gel in Fig.5A. The separated DNA fragments were blotted onto a GeneScreenPlus™ membrane. The pBR322 derivative containing the lambda-HindIII fragment of 2322 bp, was linearized by restriction enzyme EcoRI, UV-labelled (8 kJ/m²) and used as probe. Hybridized probe was visualized by the anti-UV-DNA antibodies and HRP.
The same probe was also used in plaque blot hybridization. We succeeded in detecting probe hybridized to M13 phages containing the lambda-HindIII fragment of 2322 bases. The control without insert could not be seen. We here used alkaline phosphatase with NBT and BCIP as substrates, and the plaques appeared as light blue spots on a pink background on a nylon membrane. These signals were difficult to demonstrate by photographic techniques, but could be registered by visual inspection. In this case the sensitivity is probably better than the one obtained in the previous experiment (Figure 5B).

DISCUSSION

We here describe a non-isotopic labelling method that can be used for detection of specific DNA sequences. There are several advantages by using the method. The labelling of DNA is very simple, rapid and cheap. Both double stranded and single stranded DNA can be labelled within minutes with a germicidal lamp, which is standard equipment in most laboratories. However, when labelling probe DNA, denaturation of probe should be performed prior to UV-irradiation to avoid UV-induced crosslinking of the complementary strands. The labelling method is inexpensive because no costly enzymes or labelled nucleotides are needed. The DNA is immediately ready for use, no purification of the labelled molecules is needed afterwards. The photoproduc ts are very stable, and photochemically modified DNA can be stored for years. If large amounts of probe DNA are available, time can be saved by labelling probe just once.

The disadvantage of this labelling method is that DNA must not be exposed to UV-irradiation before it is used as target in hybridization experiments. DNA is often subjected to UV-irradiation for example during plasmid purification or inspection of gels on UV-illuminators etc.

The sensitivity of the method can be increased by increasing the number of enzyme molecules associated with a photoproduc t. Highest sensitivity should be obtained by using an immunostaining method which relies on amplification, where preformed complexes of avidin or streptavidin and biotinylated enzymes or enzyme polymers are used (2,3). The sensitivity
also depends on the substrates used for colour development. Thus, staining is intensified by using the two-step substrate NBT & BCIP with alkaline phosphatase, or Co\(^+\) and Ni\(^+\) ions together with DAB when peroxidase is used.

The degree of labelling attained when using UV-irradiation is unfortunately dependent on the length and base composition of the DNA to be labelled (13). Labelling of very short probes with few thymidines will result in poor labelling. This problem can however be overcome by adding thymidine rich sequences to the probe, either by inserting the probe into a cloning vector adjacent to a thymidine rich sequence, or by adding such a sequence during synthesis of oligonucleotide probes. The high number of photoproducts generated in the sequence adjacent to the probe should not interfere with the base pairing of complementary sequences, but should probably contribute to an increase in detection sensitivity.

Hybridization of radiolabelled probes has to be performed with only ng quantities of probe DNA to avoid background staining of the autoradiogram. To obtain complete hybridization, the low concentration of probe must be compensated by a longer hybridization time (16-24 hours). Since background staining is a minor problem when using UV-labelled probes, larger quantities of probe may be used (1-10 \(\mu\)g) and the hybridization time can be reduced.

The type of membrane used seemed to affect the background staining and thereby the sensitivity of the detection method. Lowest background was observed when using nitrocellulose membranes, while better detection sensitivities were obtained with nylon membranes. This may be due to a more efficient transfer of DNA to the nylon membranes.

We have demonstrated that UV irradiation can be used for labelling of DNA, and that immunological methods can be used for detection of specific DNA sequences. The advantage of our method is the very simple, rapid and inexpensive labelling procedure. The method should be sensitive enough for use on Southern blots, colony- and plaque blots where multicopies of target DNA is present.

The method can be adapted to several applications.
et al. (11) used the method in studies of UV-induced DNA damage and its repair in human skin. Also, it can be used to quantify DNA in samples. The amount of DNA can be determined by densitometric scanning, or the approximate amount can be read visually by comparing with homologous DNA standards.

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