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Rapid transfer of DNA from agarose gels to nylon membranes

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ABSTRACT

The unique properties of nylon membranes allow for dramatic improvement in the capillary transfer of DNA restriction fragments from agarose gels (Southern blotting). By using 0.4 M NaOH as the transfer solvent following a short pre-treatment of the gel in acid, DNA is depurinated during transfer. Fragments of all sizes are eluted and retained quantitatively by the membrane; furthermore, the alkaline solvent induces covalent fixation of DNA to the membrane. The saving in time and materials afforded by this simple modification is accompanied by a marked improvement in resolution and a ten-fold increase in sensitivity of subsequent hybridization analyses. In addition, we have found that nylon membrane completely retains native (and denatured) DNA in transfer solvents of low ionic strength (including distilled water), although quantitative elution of DNA from the gel is limited to fragments smaller than 4 Kb. This property can be utilized in the direct electrophoretic transfer of native restriction fragments from polyacrylamide gels. Exposure of DNA to ultraviolet light, either in the gel or following transfer to nylon membrane, reduces its ability to hybridize.

INTRODUCTION

The capillary transfer of DNA restriction fragments from an agarose gel to an appropriate membrane ('Southern blotting'; 1) is a technique fundamental to the analysis of genome organization and expression. In recent years, the availability of nylon membranes as matrices for binding nucleic acids has markedly enhanced the utility of both this and allied techniques in hybridization analyses. Relative to nitrocellulose, nylon membranes have greater mechanical strength, have a higher capacity for nucleic acids, are able to bind smaller oligonucleotides, and have stronger retention of bound nucleic acids permitting multiple re-probing of filters (refer to technical bulletins of suppliers). In addition, it has been reported recently that ultraviolet irradiation catalyzes the covalent attachment of nucleic acids to these membranes. They thus provide all the advantages of chemically-activated cellulose papers (2, 3) without any apparent disadvantages.

Nevertheless, it seemed to us that the full potential of nylon membranes is not realized in transfer protocols that are currently in use. These owe more to their historical succession from Southern's studies with nitrocellulose (1) than to intrinsic properties of the membranes themselves. For example, the fact that nylon membranes can be used in electrophoretic transfer argues that they bind nucleic acids efficiently in buffers of low ionic strength, in marked contrast to nitrocellulose. The use of low-salt buffers for capillary transfer would be expected to improve transfer efficiency, and would certainly be more convenient (in this context we note that Amersham, the suppliers of 'Hybond N' nylon membrane,

suggest the use of 25 mM phosphate for capillary transfer).

Indirect observations in our laboratory had suggested further that nylon membranes may be able to bind native (double-stranded) DNA, in addition to denatured (single-stranded) DNA. The potential saving in time and materials afforded by elimination of gel pre-treatment steps prompted us to investigate this question in more detail.

In the course of these studies we found that DNA is retained by nylon membrane when transferred in acid or alkaline solvents. Not only does this allow large fragments to be sheared by depurination during transfer, but alkaline solvents also promote covalent fixation of DNA to the membrane. The convenience of these direct transfer procedures is complemented by a marked improvement in the resolution and sensitivity of subsequent hybridization analyses.

MATERIALS AND METHODS

DNA Preparations.

Bovine genomic DNA was isolated from homogenates of frozen liver samples prepared in 10 volumes of ice-cold NTE (100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA); nuclei were lysed by the addition of EDTA to 0.1 M and sarcosyl (sodium N-lauroyl sarcosinate; Sigma) to 2% (w/v), followed by incubation at 65°C for 15 min. Solid CsCl was added at 1 g/ml, the suspension was made 0.7 mg/ml in ethidium bromide and centrifuged at 45,000 rpm in a Beckman Ti80 rotor at 25°C for 60 h. The band of DNA was removed by side puncture, ethidium bromide extracted with n-butanol, and the sample dialyzed exhaustively against TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). The final DNA solution was stored at 4°C over a few drops of chloroform.

Human genomic DNA was isolated similarly from the Burkitt lymphoma-derived cell lines BJAB (female; provided by Dr. Barry Gorman, QIMR) and RAJI (male; ATCC).

Plasmid pSPIB3.8 is pSP64 (4) containing a 3.8 Kb Bam HI fragment of the human X chromosome (5). The 812 bp Eco RI/Bam HI fragment of this plasmid, which contains the promoter and first intron of the X-linked gene for phosphoglycerate kinase, was sub-cloned by digestion of pSPIB3.8 with Eco RI followed by re-circularization with T4 DNA ligase and transformation into HB101 (6). The resultant recombinant (pSPIB0.8) was amplified in liquid culture and the plasmid isolated and purified according to the method of Birnboim and Doly (7), with the inclusion of a final purification by isopycnic centrifugation in CsCl and ethidium bromide (8).

Bacteriophage Lambda DNA (cI*857* Sam7) was obtained from New England BioLabs.

Salmon sperm DNA (Sigma), used as the carrier in filter hybridizations, was dissolved in 0.2 M NaOH at 10 mg/ml and sheared by heating at 100°C for 45 min. The solution was then chilled, neutralized with acetic acid and centrifuged to remove debris. DNA was recovered by ethanol precipitation and the pellet dissolved in TE and stored in small aliquots at -20°C.

Restriction Endonuclease Digestions.

Samples of DNA were digested with the appropriate restriction endonuclease(s), obtained variously from Bethesda Research Laboratories, New England BioLabs, Boehringer, and Pharmacia-PL, under conditions recommended by the suppliers.

End-Labelling of DNA Digested with Restriction Endonuclease.

Bam HI-digested genomic DNA was incubated with the large (Klenow) fragment of DNA polymerase I (New England BioLabs) with 0.2 mM dGTP and [a-^{32}P]-dATP (Amersham) at room temperature for 30 min (8).

Agarose Gel Electrophoresis.

Gels were cast and run in the Pharmacia GNA-100 mini-gel apparatus, using either 5- or 8-tooth combs that form sample wells of 10 mm x 1 mm x 7 mm and 4.5 mm x 1 mm x 7 mm respectively. The gel volume was 60 ml, usually of 1% (w/v) agarose (Sigma Type I) in TAE buffer (40 mM Tris-acetate (pH 7.8), 20 mM sodium acetate, 2 mM EDTA) containing ethidium bromide (0.5 µg/ml); the gel dimensions were 100 x 80 x 7.5 mm. Electrophoresis was conducted at 125 mA/45 V (2.8 V/cm) for 100-200 min at room temperature.

Gel Pre-Treatment.

Following electrophoresis, each gel was photographed on a medium-wavelength (302 nm) ultraviolet transilluminator (Oliphant, Adelaide) then immediately subjected to one of the following pre-treatments (for treatments (A) and (B), the free-floating gel was agitated gently and continuously at room temperature):

(A) Acid/alkali/neutralization (depurination): the gel was treated sequentially with 2 volumes of 0.25 M HCl (2 x 10 min), 2 volumes of 0.5 M NaOH/1.5 M NaCl (2 x 15 min), and 2 volumes of 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl (2 x15 min) (9).

(B) Acid: the gel was treated with 2 volumes of 0.25 M HCl (2 x 10 min).

(C) Ultraviolet irradiation: the gel was placed 1 cm below the germicidal ultraviolet strip light (254 nm, 30 W) in a biosafety cabinet. Each side of the gel was exposed for half the total time indicated in the text, with the origin placed directly beneath the light to ensure that the largest DNA fragments received the greatest exposure (10).

(D) No pre-treatment.

Capillary Transfer to Nylon Membranes.

For overnight transfer, the pre-treated gel was placed on three sheets of saturated Whatman 3MM paper supported on an inverted gel casting tray in a small plastic box. The paper was the same width as the tray and had extended wicks dipping into a reservoir of solvent in the bottom of the box. A sheet of Zeta Probe nylon membrane (Bio-Rad), cut to the same dimensions as the gel and previously wetted in distilled water, was placed on the gel surface and this in turn was covered with eight sheets of 3MM paper and a stack of paper towel to the height of the box rim. Light pressure was applied to the stack by sealing the box with a snap-seal lid.

For shorter transfer periods, the protocol adopted was similar to that described by Wahl *et al.* (9). To effect a change of solvent, a glass plate was placed on top of the transfer assembly and the assembly inverted. The pad containing transfer solvent was carefully peeled off the base of the gel and replaced with a fresh pad saturated with the new solvent, the assembly was returned to its original orientation and transfer resumed.

Fixation of Transferred DNA.

Following the transfer of native DNA, the membrane was removed from the gel and placed, DNA surface uppermost, on a pad of 3MM paper saturated with 0.5 M NaOH/1.5 M NaCl for 5 min, then placed for 5 min on a second pad saturated with 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl (11).

These membranes, together with those to which denatured DNA had been transferred, were agitated briefly in 2 x SSC (0.3 M NaCl, 0.03 M trisodium citrate) to remove possible adherent gel fragments and, in the case of alkaline transfers, to neutralize the membranes. They were then blotted dry and subjected to one of the following fixation procedures:

(a) Baked in a vacuum oven at 80°C for 2 h (1).

(b) Wrapped in Glad Wrap (equivalent to Saran Wrap) and placed, DNA surface down, on the ultraviolet transilluminator (refer above) for the times specified.

(c) Placed, DNA surface uppermost, on a clean sheet of 3MM on the work surface of the biosafety cabinet (refer above) and exposed to its ultraviolet light for the times specified. The light source was located 60 cm above the membrane.

Nick-Translation of DNA.

Probes for hybridization analyses (and, in the experiments of Figure 2, genomic DNA) were labelled with [a-^{32}P]-dCTP (Amersham) according to the principle of Rigby *et al.* (12), using a modification that ensures high efficiency of label incorporation (13).

Hybridization of DNA Bound to Nylon Membranes.

In most cases, the hybridization solution consisted of 1.5 x SSPE (0.27 M NaCl, 15 mM sodium phosphate (pH 7.7), 1.5 mM EDTA), 0.5% (w/v) BLOTTO ('Diploma' non-fat powdered milk; 14), and 1% (w/v) SDS. To ensure uniformity of hybridization conditions, all membranes prepared for a single comparative experiment were placed in a small plastic box with 1ml/4 cm^2 of hybridization solution containing heat-denatured, sheared salmon sperm DNA (0.5 mg/ml). A sheet of Glad Wrap was molded to the inside, in contact with the surface of the solution and overhanging the edges of the box, and the box closed with a snap-seal lid. Pre-hybridization was continued overnight at 68°C with continuous agitation.

The radio-labelled probe was mixed with salmon sperm DNA in 0.2 M NaOH, heated at 100°C for 10 min, chilled, and neutralized with acetic acid. The solution of denatured, sheared probe and carrier DNA (final concentration 0.5 mg/ml) was mixed with fresh hybridization solution (1 ml/8 cm^2 of membrane), the membranes were added and hybridization continued at 68°C for up to 20 h. Subsequent studies have shown that the omission of carrier DNA from prehybridization and hybridization solutions has no adverse effects on either background or sensitivity under these conditions.

When probing for single-copy fragments of the PGK gene, prehybridization was carried out at 42°C in plastic bags containing 10 ml of 4 x SSPE, 0.5% BLOTTO, 1% SDS, and 0.5 mg/ml salmon sperm DNA in 50% (v/v) formamide. Hybridization was performed under similar conditions, with the inclusion of 10% (w/v) dextran sulfate (9). It was found subsequently that in using dextran sulfate, carrier

DNA could be omitted from hybridization solutions but not from the prehybridizations.

Washing of Membranes and Autoradiography.

On completion of hybridization, the membranes were rinsed briefly in 2 x SSC/0.1% SDS then washed successively (for 15 min at room temperature with vigorous agitation) in 2 x SSC/0.1% SDS, 0.5 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS, with a final high-stringency wash in 0.1 x SSC/1% SDS at 50°C for 30 min. The membranes were blotted dry, wrapped in Glad Wrap and exposed to pre-flashed X-ray film (Fuji RX) with an intensifying screen (DuPont Cronex 'Lightning Plus') at -70°C (15).

Fixation and Drying of Gels.

On completion of transfer of radio-labelled restriction fragments, the gels were soaked in 7% (w/v) trichloroacetic acid for 30 min then dried by blotting with 3MM paper under a moderate weight (8). The dried gels and the membranes used in transfer were wrapped in Glad Wrap and autoradiographed at room temperature.

RESULTS

Retention of Native DNA by Nylon Membrane.

Initial experiments were designed simply to determine (i) whether Zeta Probe retains DNA at lower ionic strength than is recommended in standard protocols, and (ii) whether it binds native DNA with an efficiency comparable to that found with denatured DNA. Each of the 8 tracks of a gel contained

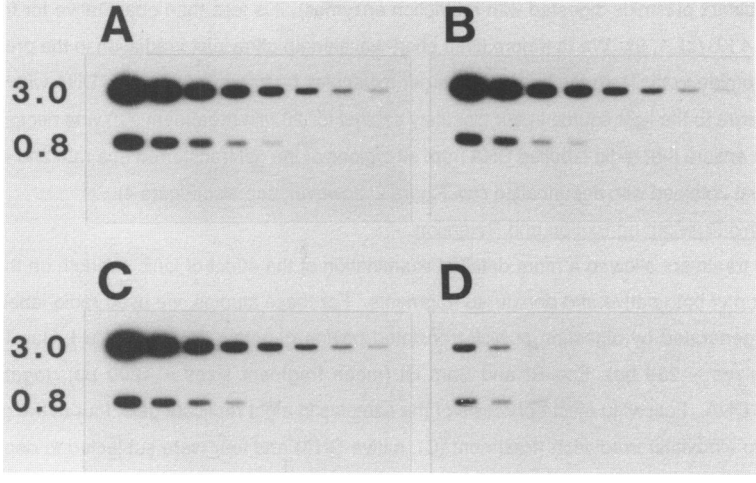

Figure 1. Transfer of native DNA to Zeta Probe. Bovine genomic DNA (0.8 µg, digested with Eco RI) was mixed with doubling dilutions of pSPIB0.8 (6.4-0.05 ng, digested with Bam HI and Eco RI) and electrophoresed on 1% agarose gels. The resolved fragments were transferred to Zeta Probe overnight, either directly ((c), (d)) or after first being subjected to acid depurination ((a), (b)); the transfer solvent was either 1 x SSC ((a), (c)) or 10 x SSC ((b), (d)). The membranes were rinsed (in (c) and (d), following denaturation), baked, and hybridized with nick-translated pSPIB0.8 (30 µCi/µg at 25 ng/ml) for 20 h, then washed and autoradiographed overnight. Numbers at the left refer to sizes (Kb) of the vector and insert fragments .

identical amounts of bovine genomic DNA (0.8 µg, digested with Eco RI), together with doubling dilutions of pSPIB0.8 (6.4-0.05 ng, digested with Bam HI and Eco RI) as the target for hybridization analysis.

The transfer and retention of depurinated DNA (treatment (A), Materials and Methods) in 1 x SSC is at least equal to that in 10 x SSC, the recommended solvent (Figs. 1a, 1b); in fact, the lower ionic strength results in a two-fold stronger hybridization signal, implying more efficient transfer. Of greater interest is the comparison of results obtained with the transfer of depurinated and native DNA in 1 x SSC (Figs. 1a, 1c). Clearly, native DNA fragments of the sizes detected (3.0 and 0.8 Kb) are transferred and bound with similar efficiency to sheared, denatured fragments. Furthermore, the bands of hybridized DNA are less diffuse, an expected consequence of eliminating the pre-treatment steps.

The hybridization signals detected following transfer of native DNA in 10 x SSC are markedly weaker than those seen with 1 x SSC (Figs. 1d, 1c). This probably reflects their less efficient transfer at high ionic strength, since the signal intensity for the 812 bp insert is similar to that for the 3 Kb vector suggesting poorer elution of larger fragments.

Extension of these studies has shown that electrophoretic transfer of native DNA fragments from polyacrylamide gels to Zeta Probe similarly occurs at high efficiency and with improved resolution and sensitivity (Klaus Matthaei, unpublished observations).

In Situ Fragmentation of Native DNA by Ultraviolet Light.

While direct capillary transfer of native DNA is useful for screening relatively small DNA molecules (*eg.* recombinant plasmids digested with restriction enzymes), it is less than quantitative for fragments larger than 4 Kb (*cf.* 1, 9). We therefore used short-wavelength ultraviolet irradiation in the presence of ethidium bromide to facilitate the transfer of larger molecules by fragmenting native DNA within the gel (10). Exposure to the light source in our biosafety cabinet for 20 min (treatment (C)) was necessary and sufficient to ensure that radio-labelled DNA from all regions of the gel transferred at a rate and efficiency similar to that obtained with depurination (*eg.* Figure 2; however, see also Figure 4).

Effect of Ionic Strength on Elution and Retention.

This treatment allowed a more detailed examination of the effect of ionic strength on the elution and retention of both native and denatured fragments. For these studies, we used radio-labelled DNA fragments generated by digestion of nick-translated bovine genomic DNA with Alu I, Hae III (mean fragment sizes ≈ 250 bp), Eco RI and Bam HI (mean fragment sizes ≈ 4200 bp), together with undigested DNA. Following electrophoresis of the samples in eight replicate gels, four of the gels were subjected to ultraviolet irradiation (treatment (C); native DNA) and four were subjected to depurination (treatment (A); denatured DNA) to fragment large molecules. DNA was then transferred for 3 h in distilled water, 1 x SSC, 6 x SSC, or 20 x SSC.

Autoradiographs of the gels and membranes revealed a slight but noticeable decrease in transfer with increasing salt concentration for both native and denatured DNA (data not included). However, retention of DNA by the membranes was quite independent of ionic strength, illustrated clearly by Figure 2 which shows the results from the transfer of native and denatured DNA in distilled water. The

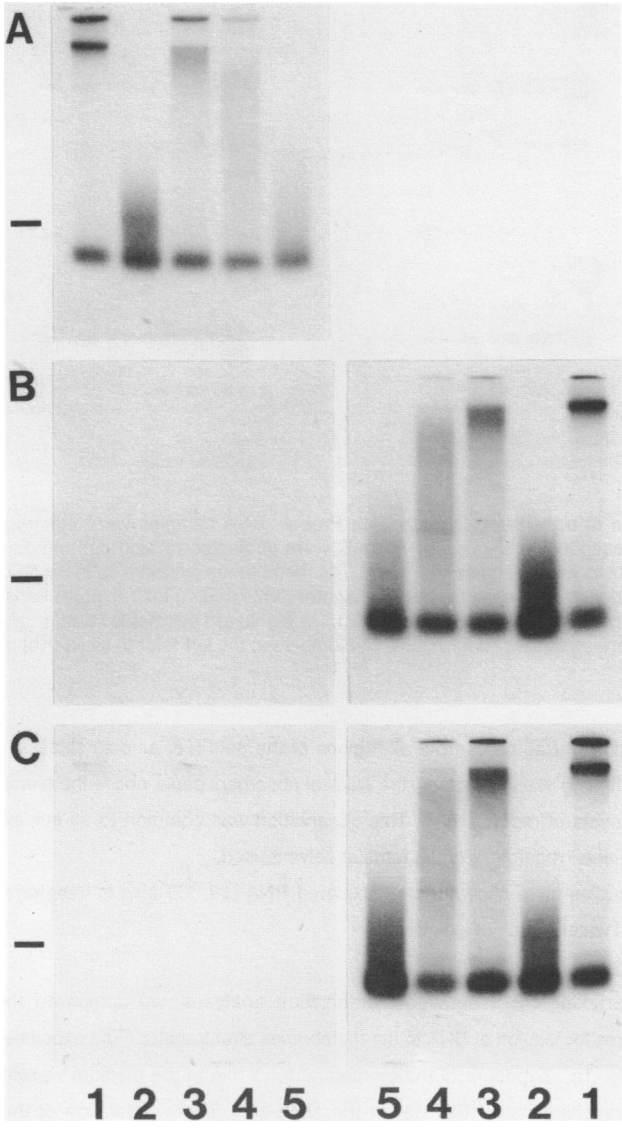

Figure 2. Transfer and retention of fragmented native and denatured DNA in distilled water. Bovine genomic DNA was labelled by nick-translation to 0.2 μCi/μg; 1 μg samples were digested with restriction enzymes as described and electrophoresed in 1% agarose gels. Replicate gels were subjected to acid depurination (b) or exposed to high-intensity ultraviolet irradiation (c), and DNA transferred to Zeta Probe for 3 h in distilled water. The membranes and dried gels were autoradiographed for 2 h at room temperature, together with an untreated (control) gel (a). Restriction enzymes used were: lane 1, none; lane 2, Alu I; lane 3, Bam HI; lane 4, Eco RI; lane 5, Hae III. In each case, the autoradiograph of the dried gel is shown on the left, the filter on the right. Marks indicate the position of bromophenol blue tracking dye.

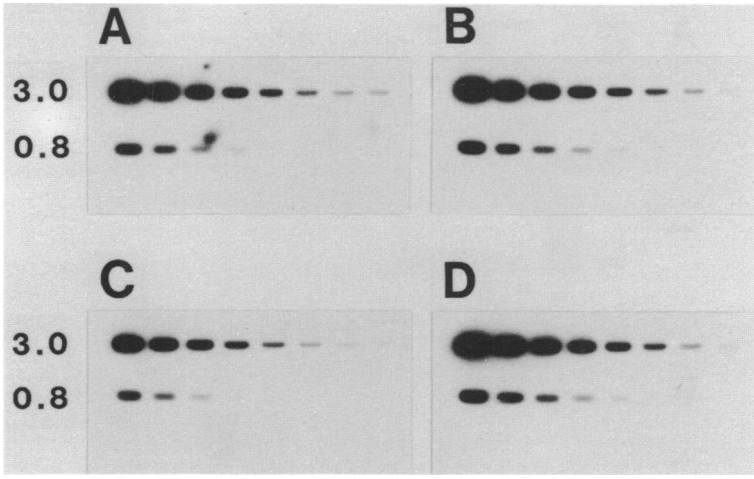

Figure 3. Fixation of transferred DNA to Zeta Probe. DNA samples were electrophoresed in 1% agarose gels as described for Figure 1. All gels were subjected to acid depurination and the DNA transferred overnight to Zeta Probe in 10 x SSC. The membranes were rinsed in 2 x SSC and subjected to the following fixation procedures: (a) transilluminator (302 nm u.v.) for 1 min; (b) transilluminator for 5 min; (c) biohazard cabinet (254 nm u.v.) for 1 min; (d) biohazard cabinet for 5 min. Hybridization and autoradiography were as described for Figure 1. Numbers at the left refer to sizes (Kb) of the vector and insert fragments.

high level of retention of fragments from all regions of the gels (*i.e.* all size classes) was confirmed qualitatively by our finding that in all cases, the stack of absorbent paper above the membrane contained barely-detectable levels of radioactivity. This observation was common to all our experiments with radio-labelled fragments, regardless of the transfer solvent used.

Additional studies have shown that glyoxalated RNA (16, 17) also is transferred and retained efficiently in distilled water (data not included).

Post-Transfer Fixation of DNA to Zeta Probe.

Before undertaking more detailed hybridization analyses, we compared the efficiency of alternative procedures for fixation of DNA to the membranes after transfer. The experimental design was similar to that of Fig. 1b. Exposure of the membrane for 1 min to the medium-wavelength ultraviolet irradiation of the transilluminator (Fig. 3a) or the short-wavelength irradiation of the source in the biosafety cabinet (Fig. 3c) was clearly inadequate. However, 5 min irradiation with either light source (Figs. 3b, 3d) was equivalent to (or better than) vacuum baking for 2 h (Fig. 1b).

Consequently, in all following experiments involving hybridization, denatured DNA was fixed to the membranes after transfer (and denaturation where appropriate) by 5 min irradiation in the biosafety cabinet. This is more efficient than vacuum baking (it is certainly much faster), and the use of an unfiltered light source avoids solarization of the expensive quartz filters on transilluminators (*cf.* 9).

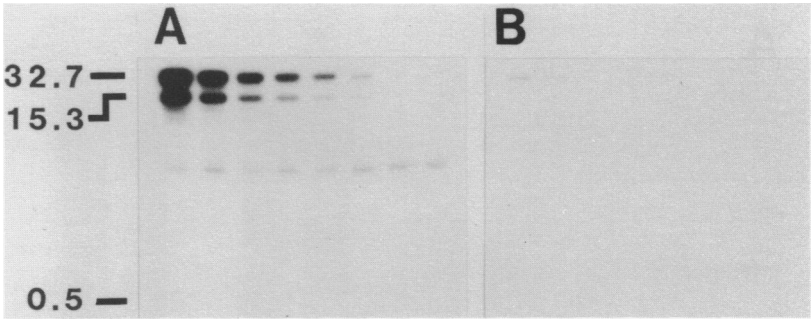

Figure 4. Ultraviolet shearing prevents hybridization. Bovine genomic DNA (0.8 µg, digested with Eco RI) was mixed with doubling dilutions of bacteriophage lambda DNA (6.4-0.05 ng, digested with Sal I) and electrophoresed on 0.6% agarose gels. The resolved fragments were subjected to acid depurination (a) or high-intensity ultraviolet irradiation for 20 min (b), then transferred to Zeta Probe for 3 h in distilled water. The filters were rinsed (in (b), following denaturation), exposed to low-intensity u.v. for 5 min, hybridized with nick-translated lambda DNA (50 µCi/µg at 20 ng/ml) for 16 h, washed and autoradiographed. Numbers at the left refer to sizes (Kb) of the lambda DNA fragments.

Ultraviolet Irradiation Inhibits Hybridization.

Using optimal parameters established in the preceding studies, a Southern blot was prepared to determine whether high-intensity u.v. irradiation in the presence of ethidium bromide has deleterious effects on the hybridization of transferred DNA. The experimental design was similar to that described in Fig. 1, with the exceptions that the target for hybridization was the large fragments generated by digestion of lambda DNA with Sal I, and native DNA was fragmented by exposure to ultraviolet light (treatment (C)). Figure 4 compares these results with those obtained from an otherwise identical experiment in which denatured fragments were transferred after depurination (treatment (A)).

High-intensity ultraviolet irradiation causes a dramatic loss of sensitivity (Figure 4), of the order of 40-fold at the intensity and duration that is required to break large molecules to a size suitable for quantitative transfer. This treatment obviously induces structural alterations that prevent DNA from forming stable hybrids; by implication, these findings impose a serious limitation on the use of ultraviolet light for post-transfer fixation.

Depurination by Transfer in Alkali.

Since acid depurination remains the method of choice for fragmenting large DNA molecules within gels, we sought to determine if the DNA-binding properties of Zeta Probe would allow a rapid modification of this procedure. Two alternatives were considered: (i) pre-treatment of the gel in acid (treatment (B)) followed by transfer in 0.25 M NaOH; (ii) no pre-treatment, using 0.25 M HCl as the transfer solvent for the first 30 min, followed by transfer in 0.25 M NaOH for a further 150 min.

Elution and retention were monitored with end-labelled fragments of bovine and human genomic DNA digested with Bam HI. After 3 h transfer, >98% of the radioactivity was bound to the membranes in both cases (Figure 5). Differences between treatments in the amount and distribution of radioactivity

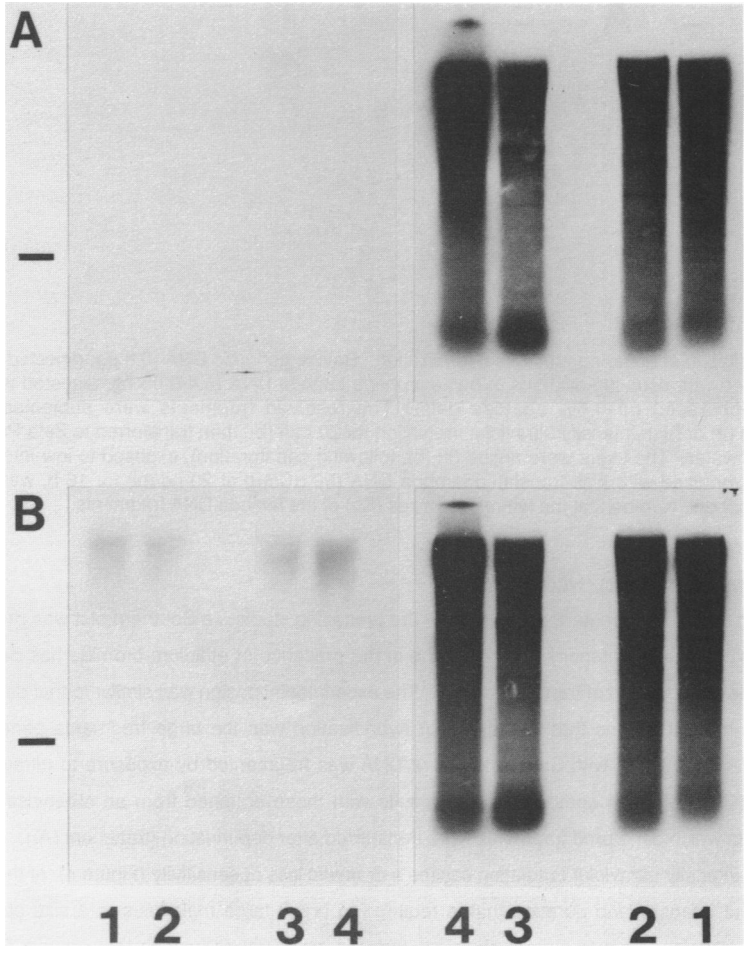

Figure 5. Transfer and retention of DNA in alkali. Samples (4 μg) of genomic DNA from human male (lane 1) and female (lane 2) cell lines, and bovine male (lane 3) and female (lane 4) liver were digested with Bam HI, end-labelled to 0.5 μCi/μg, and electrophoresed on 1% agarose gels. DNA was either transferred directly to Zeta Probe in 0.25 M HCl for 30 min followed by a change of transfer solvent to 0.25 M NaOH for 150 min (a), or first pre-treated with 0.25 M HCl then rinsed briefly in water and transferred to Zeta Probe in 0.25 M NaOH for 3 h (b). In each case, autoradiographs of the dried gels are shown on the left and transfer membranes on the right. Marks indicate the position of bromophenol blue tracking dye.

transferred to the membranes were minor; these were subsequently eliminated by the substitution of 0.4 M for 0.25 M NaOH as the transfer solvent (see below). The autoradiographs of Fig. 5 provide particularly striking evidence of the high resolution attainable with rapid transfer protocols: minor satellite bands are clearly resolved, despite being indistinct in the original gel photographs (not included).

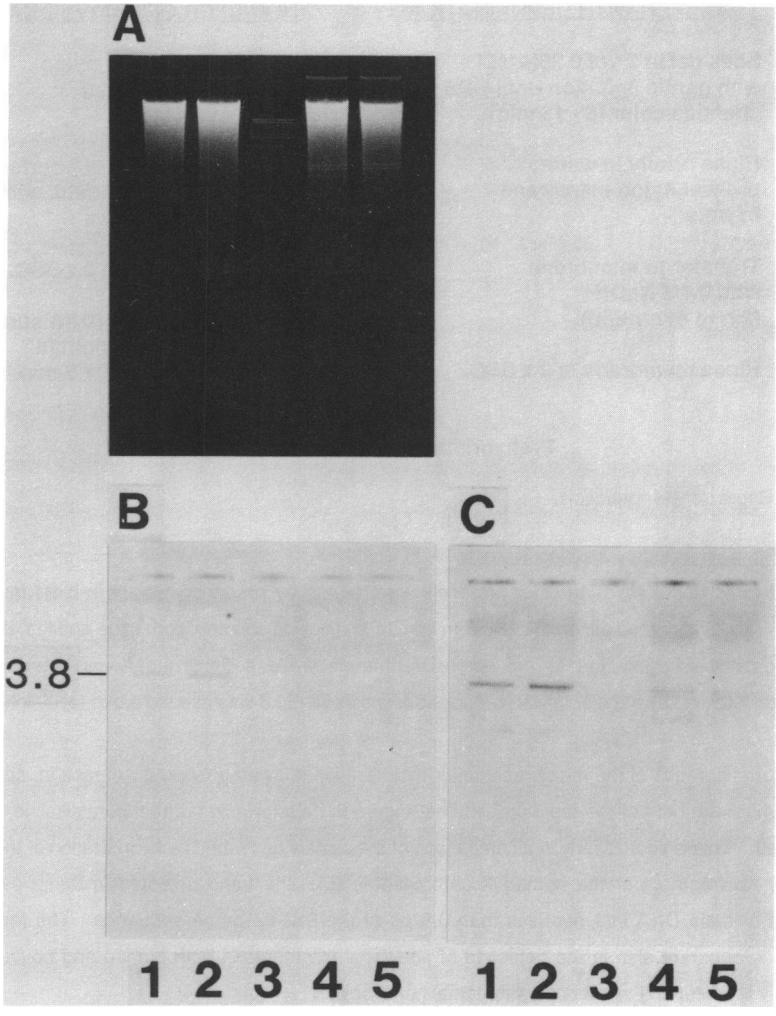

Figure 6. Detection of single-copy sequences in mammalian DNA following rapid transfer in alkali. Samples (4 μg) of genomic DNA from human male (lane 1) and female (lane 2) cell lines, and bovine male (lane 4) and female (lane 5) liver were digested with Bam HI and electrophoresed on 1% agarose gels, together with size markers of lambda DNA digested with Acc I (lane 3). After photography (a), DNA was either depurinated and transferred to Zeta Probe in distilled water for 3 h (b), or was transferred directly to Zeta Probe in 0.25 M HCl for 30 min followed by a change of transfer solvent to 0.25 M NaOH for 150 min (c). The membranes were rinsed briefly in 2 x SSC and exposed to low-intensity u.v. irradiation for 5 min, then hybridized in dextran sulphate with nick-translated pSPIB0.8 (90 μCi/μg at 16 ng/ml), washed and autoradiographed overnight. The 3.8 Kb fragment derived from the human X chromosome is indicated.

TRANSFER WITH DEPURINATION	TRANSFER OF NATIVE DNA
1. Soak gel in 2 vol 0.25M HCl with gentle agitation until BPB changes color (5 - 15 min).	1. Transfer directly to nylon membrane with water (2 h to overnight).
2. Rinse briefly in water; pre-wet nylon membrane in water.	2. Place membrane (DNA surface uppermost) on pad satd. with 0.4M NaOH for 5 min.
3. Transfer to membrane with 0.4M NaOH (2 h to overnight).	3. Rinse membrane in 2 x SSC.
4. Rinse membrane in 2 x SSC.	4. Expose membrane (DNA surface uppermost) to u.v. light in biohazard cabinet for 5 min.

Prehybridize and hybridize as required.

Figure 7. Rapid transfer protocols.

Southern Blots Prepared by Alkaline Transfer.

The rapid depurination/transfer protocols were tested by preparing Southern blots from gels containing Bam HI-digested genomic DNA from human and bovine sources, both male and female. Two identical gels were subjected to the above treatments while a third underwent standard acid depurination followed by transfer in water. Nick-translated pSPIB0.8 was used to probe for X-linked PGK fragments.

Autoradiographs of the membranes after hybridization showed a marked increase in sensitivity and resolution with both of the rapid procedures (Figure 6; data for acid pretreatment/alkaline transfer not included). This is seen clearly in a comparison of the signals generated by hybridization of the probe to the 3.8 Kb fragment of the human X chromosome (5): the band detected in these overnight exposures of male DNA includes less than 0.5 pg of the 812 bp probe sequence. The increased sensitivity is apparent also in the detection of additional fragments in both human and bovine DNA, presumably representing sequences with partial homology.

The improved resolution is to be expected; the improved sensitivity is probably due to the fact that in this procedure the DNA is totally denatured when fixed to the membrane. In contrast, the standard procedure (treatment (A)) involves neutralization of denatured DNA at high salt concentration followed by transfer under neutral conditions, during both of which some renaturation may occur.

We have found that if adequate depurination is to be achieved by sequential transfer in acid and alkali, the time required for preliminary transfer in acid (or more correctly, the volume of acid required for elution) is dependent on the strength of the electrophoresis buffer. For example, with TBE (Tris-borate-EDTA) up to 1 h is required. In this case, we find it advisable to use either a thick pad of acid-saturated 3MM paper (8 sheets) or wicks extending into a small solvent reservoir. In any event,

color change of the bromophenol blue tracking dye is a reliable index of acidification.

Additional work has shown that the transfer of large fragments is more rapid in 0.4 M NaOH than in 0.25 M NaOH, reflecting the need for high pH following acid treatment in order to achieve satisfactory depurination. Neither the retention of DNA by Zeta Probe nor hybridization sensitivity are diminished with the stronger alkali, even following continuous exposure during overnight transfer (data not included).

A surprising bonus afforded by alkaline transfer is that it promotes the covalent fixation of transferred DNA to nylon membranes. We have found that radio-labelled DNA transferred in alkali cannot be stripped from the membrane (data not included). Furthermore, signal intensities generated by hybridization to fragments transferred in alkali were more than twice as strong as those from identical samples which had been additionally subjected for 5 min to the ultraviolet light of the biohazard cabinet (experiment similar to Fig. 4; data not included). Apparently this mild exposure to ultraviolet radiation, the minimum required for fixation of DNA transferred in neutral solvents, is sufficient to render half of the transferred DNA incapable of forming hybrids.

DISCUSSION

The properties of Zeta Probe allow virtually any modification of standard protocols for the capillary transfer of DNA from agarose gels for hybridization analysis. Solvents that result in quantitative retention include distilled water, 0.25 M HCl and 0.4 M NaOH, in addition to the concentrated buffers that have been used traditionally with nitrocellulose.

Optimal transfer protocols are those in which pre-treatment of the gel is minimized (to limit diffusion and hence improve resolution) and the DNA is denatured immediately before fixation (to improve sensitivity), either by transfer in alkali or by post-transfer denaturation. We have adopted a routine procedure of acid pre-treatment followed by transfer in 0.4 M NaOH (Fig. 7).

With alkaline transfer, the need for separate fixation treatments is eliminated since the solvent itself promotes base-catalyzed fixation of DNA to the membranes. The use of ultraviolet irradiation in this context is counter-productive since it diminishes the ability of fixed DNA to form hybrids.

The sensitivity of hybridization analyses is increased approximately ten-fold by the rapidity, complete denaturation, and optimal fixation of the alkaline transfer protocols. Single-copy sequences are easily detected by hybridization to a Southern blot prepared from less than 1 μg of mammalian genomic DNA with an overnight exposure of X-ray film.

If alkali is not used, we suggest distilled water (or a low concentration of a suitable buffer) as the transfer solvent for either of native (Fig. 7) or depurinated DNA. The inverse relation between ionic strength and transfer efficiency is negligible for small molecules, but is very pronounced for fragments larger than a few hundred bases (Fig. 1). Even at low salt concentrations, molecules larger than 4 Kb are not eluted quantitatively; high-intensity ultraviolet irradiation is unsatisfactory for shearing larger native fragments since it causes a dramatic reduction in hybridization efficiency. Similar considerations apply to the transfer of RNA for the preparation of 'Northern' blots.

On completion of transfer of native DNA, the membrane must be treated briefly with alkali to denature the DNA, rinsed in buffer, and exposed to a low-intensity ultraviolet light source for 5 min. Our studies suggest the germicidal light of a sterile workstation or biohazard cabinet as the most appropriate (Fig. 7). Alternatively, the need for ultraviolet fixation could probably be eliminated by increasing the duration of alkaline denaturation.

In all applications, the time required for complete elution of DNA from an agarose gel varies from 2-4 h (depending on gel concentration and thickness; *cf.* 9), and can be gauged conveniently by the extent of transfer of tracker dye (xylene cyanol, bromophenol blue).

The ability of Zeta Probe to bind native DNA in solvents of low ionic strength finds particular application in the electrophoretic transfer of restriction fragments from polyacrylamide gels (without prior denaturation). This property could be extended to the automation of Southern blots, using an apparatus similar to that described by Beck and Pohl (18) in conjunction with short agarose gels to transfer native restriction fragments directly onto nylon membranes during electrophoresis (*cf.* 19). Such a technique could conveniently process large numbers of samples, and has obvious application in clinical diagnoses and population genetics.

The base-catalyzed fixation of DNA to Zeta Probe may have implications for the stripping of hybridization probes from membranes prior to their re-use. In our hands, the recommended procedure of heating at 42°C in 0.4 M NaOH for 2 x 30 min never succeeds in removing more than 80-90% of bound label. This residue may represent probe molecules that have been covalently attached to the membrane as a direct result of the stripping treatment, although some covalent attachment probably occurs under neutral conditions during prolonged hybridization incubations at elevated temperatures. Nevertheless, stripping treatments that depend on denaturation at neutral pH (boiling TE, hot formamide) would seem to be preferable to alkali.

A final comment is warranted in relation to our finding that the ability of DNA to form hybrids is compromised severely by its prior exposure to ultraviolet irradiation. Photography of a stained gel under ultraviolet light before transfer is clearly not desirable; accordingly, it should be eliminated (or at least minimized) whenever possible.

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