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TRANSFER OF SMALL DNA FRAGMENTS FROM POLYACRYLAMIDE GELS TO DIAZOBENZYLOXYMETHYL-PAPER AND DETECTION BY HYBRIDIZATION WITH DNA PROBES

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SUMMARY: A method for transferring small DNA fragments from composite polyacrylamide-agarose gels to diazobenzyloxymethyl (DBM)-paper is described. DNA fragments are separated by electrophoresis in polyacrylamide-agarose gels crosslinked with N,N'-diallyltartardiamide instead of N,N'-methylene-bis-acrylamide. The crosslinks are cleaved by treating the gel with periodic acid after electrophoresis and the DNA is denatured with alkali. After neutralization, the single stranded DNA fragments are transferred to DBM-paper and detected by hybridization with labeled DNA probes. The procedure has been used to transfer and visualize unlabeled SV40 DNA fragments in the size range 28 to 1790 base pairs.

INTRODUCTION

Specific DNA fragments within a complex mixture can be detected by transferring the DNA from agarose gels to nitrocellulose filters, followed by hybridization with labeled RNA or DNA (1,2). However, the use of nitrocellulose is limited in that small DNA fragments are retained poorly or not at all, depending on their size. We have separated small DNA fragments from restriction enzyme digests on polyacrylamide-agarose composite gels and transferred the denatured DNA to DBM-paper, and detected them by hybridization with [^{32}P]-labeled DNA probes. This procedure should be

ABBREVIATIONS
SV40: Simian virus 40. DATD: N,N'-diallyltartardiamide.
ABM: Aminobenzyloxymethyl. DBM: Diazobenzyloxymethyl.
SSC: 0.15 M sodium chloride-0.015 M sodium citrate buffer.

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useful for high resolution mapping of plasmid and viral DNAs, for detecting cloned DNA sequences within mixtures of DNA fragments protected by nucleosomes during digestion of chromatin with nucleases, and for mapping of binding sites of non-histone proteins in DNA and chromatin.

MATERIALS AND METHODS

DNA. Unlabeled and [^{32}P]-labeled SV40 DNA were prepared and purified as described previously (3,4). SV40 DNA was digested with Hinf I or Hae III restriction endonucleases (New England Bio Labs) using the conditions suggested by the supplier. [^{32}P]-labeled, nick translated, SV40 DNA (5) was the kind gift of Dr. Geoff Wahl of this department.

Gel electrophoresis. Restriction fragments were separated on composite polyacrylamide-agarose slab gels (23 x 14 x 0.15 cm) containing Tris-acetate buffer (40 mM Tris-HCl, pH 7.8, 20 mM sodium acetate, 2 mM EDTA) (6). The same buffer was used in the electrode reservoirs. To prepare the gels, 8 ml of ten times concentrated gel buffer, 59 ml of water and 560 mg of agarose (Bio Rad) were mixed and the agarose was dissolved by boiling. To the solution cooled to 50° were added an appropriate volume of 30% acrylamide stock (27.78 g of acrylamide plus 2.22 g of N,N'-diallyltartardiamide per 100 ml) (Bio Rad) and 0.25 ml of 10% ammonium persulfate. The gel was polymerized by adding 25 µl of N,N,N',N'-tetraethylenediamine (Bio Rad). DNA samples were precipitated with ethanol before electrophoresis (7), which was carried out at room temperature at 15-20 mA.

Preparation of gels for transfer. A modification of the method of Alwine, Kemp and Stark (8) was used. After electrophoresis, the gel was put into 250 ml of 2% periodic acid and rocked gently for 15 min at 37° to cleave the crosslinks (9). It was then rinsed with water and put into 250 ml of 0.5 M NaOH for 10 min at room temperature to denature the DNA. The gel was rinsed with water and neutralized in 250 ml of 0.5 M sodium phosphate buffer, pH 5.5, for 10 min at room temperature, and then put into 250 ml of ice-cold 50 mM sodium phosphate buffer, pH 5.5 until the DBM-paper was ready for transfer (no longer than 15 min).

Transfer of single stranded DNA. During preparation of the gel, an appropriate strip of aminobenzyloxymethyl (ABM)-paper was diazotized (8). The DBM-paper was washed twice for 5 min with ice-cold 50 mM sodium phosphate buffer, pH 5.5, before transfer. The transfer was done essentially as described previously (1,8), except that sodium phosphate buffer, 50 mM, pH 5.5, was used and the temperature was 4°.

Hybridization with [^{32}P]-labeled probes. Hybridization was done as described previously (8). After transfer, the paper was incubated for 24-48 hr at 42° in pre-hybridization buffer (50% formamide, 5 x SSC, 0.02% (w/v) each of bovine serum albumin, ficoll and polyvinylpyrrolidone, 1.0-2.5 mg/ml sonicated denatured calf thymus or salmon sperm DNA and 1% (w/v) glycine) to quench any remaining active groups on the paper. Hybridization was done in the above buffer minus

glycine. After hybridization the paper was washed with 50% formamide, 5 x SSC at 37°.

Kodak XR-5 X-ray film and Kodak X-Omatic intensifying screens were used for autoradiography (10). In some experiments (Table I-III) individual bands of [^{32}P]-labeled DNA fragments were detected by autoradiography, cut out of the paper or the dried gel, and quantitated by Cerenkov counting.

RESULTS AND DISCUSSION

Preparation of gels for transfer. DNA diffuses out of polyacrylamide gels very poorly. Therefore, we have used composite polyacrylamide-agarose gels containing the cleavable crosslinking reagent N,N'-diallyltartardiamide (DATD) instead of the conventional N,N'-methylene-bis-acrylamide. Upon exposure to periodic acid the crosslinks are broken efficiently (9), leaving a much more permeable agarose gel. Tris-borate gel systems should be avoided since borate reacts with cis-diols and would protect the crosslinks from cleavage. After disruption of the acrylamide matrix, the remaining agarose gel keeps the DNA in place during the preparations for transfer. We have obtained similar results with ethylenediacrylate, which is reversed by treatment with alkali (11).

Preparation of the gel for transfer leads to some loss of DNA, as shown by the following experiment. [^{32}P]-labeled SV40 DNA was cleaved with Hinf I (12) and the fragments were separated on a 5% polyacrylamide-agarose gel. The radioactivity in each fragment was determined before and after treatment of the gel, with the results shown in Table I. The larger fragments (A through E) were substantially retained in the gel, whereas the smaller fragments (F, G and H) were retained to a lesser extent. The gel crosslinks are efficiently removed after the periodic acid treatment used, as shown by the fact

Table I

Recovery of DNA after Pretreatment of a Gel for Transfer

Fragment	Base pairs[a]	DNA remaining in the gel (% of control)
A	1790	71
B	1120	78
C	760	72
D	590	76
E	490	88
F	235	50
G	110	40
H	75	32
I, J	28	(b)

[^{32}P]-labeled SV40 DNA, 2.6 µg/slot, 14,300 cpm/µg, was digested with Hinf I and electrophoresed on a 5% polyacrylamide-agarose gel at 20 mA for 12 hr. The gel was then cut into two strips. One was treated with periodic acid, NaOH and sodium phosphate buffer as described in the Methods Section and the other was untreated (control).

[a]Sizes were taken from reference 12.

[b]These fragments ran off the gel.

that the gel dissolves completely when the agarose is melted at 100°. Extending the periodic acid treatment to 60 min and the base treatment to 30 min caused even greater losses.

Transfer of DNA from the gel to DBM-paper. The procedure of transfer is essentially that of Southern (1). Alwine et al. (8) used pH 8.0 or pH 6.5 and room temperature for transfer of RNA to DBM-paper. For transfer of DNA, a lower pH (5.5) and lower temperature (4°) were better, probably because the diazonium groups on the paper were stabilized better during the relatively slow diffusion of the alkali-denatured DNA from the gel. The modified conditions also work very well for RNA transfers.

The efficiency of transfer was assessed quantitatively be measuring the DNA in the gel before and after transferring and by determining the amount bound to the paper (Table II).

Table II

Efficiency of DNA Transfer to DBM-Paper

Fragment	Base pairs	Percentage of DNA remaining in the gel after transfer	Percentage of DNA transferred
A	1790	75	11
B	1120	69	17
C	760	58	18
D	590	50	18
E	490	64	20
F	235	33	42
G	110	21	80
H	75	24	80

[^{32}P]-labeled SV40 DNA, 2.6 µg/slot, 14,300 cpm/µg, was digested with Hinf I and electrophoresed on a 5% polyacrylamide-agarose gel at 20 mA for 12 hr. The gel was pretreated and then cut into two strips. One was transferred for 32 hr at 4° as described above, and the other was used as control (100%).

The efficiency depends on the size of the fragments. Only 11% of fragment A (1790 base pairs) was bound, whereas 42 to 80% of fragments F, G and H (235 to 75 base pairs) were bound. The amounts of DNA remaining in the gel and the DNA transferred to the paper do not always add up to the input amount (60 to 95%). The losses may be due to partial renaturation of DNA, since double stranded DNA does not bind to DBM-cellulose (13). Since rates of renaturation depend on the concentrations of complementary strands, renaturation may be less of a problem when much lower concentrations of specific sequences are present, as in transfer of DNA of higher complexity.

It is interesting to note that the losses during pretreatment of the gel (Table I) and the efficiency of transfer (Table II) have opposite size optima, i.e. larger fragments are retained better during pretreatment, but are transferred less well so that the overall yield of fragments transferred

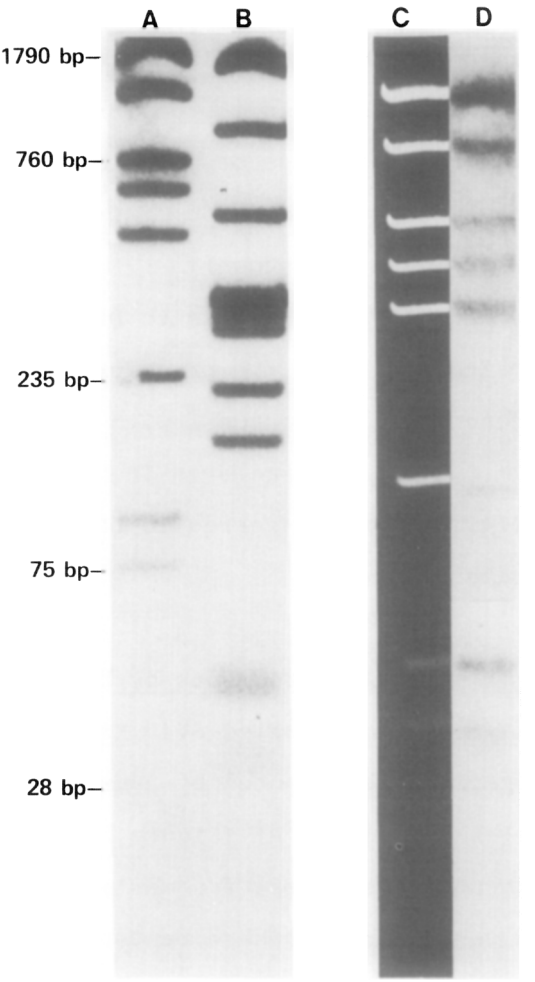

Figure 1. Transfer of SV40 DNA fragments to DBM-paper. [^{32}P]-labeled SV40 DNA, 2.3 µg/slot, 14,300 cpm/µg, was digested with Hinf I or Hae III and the fragments were separated by electrophoresis on a 7% polyacrylamide-agarose gel. Transfer was for 29 hr at 4°. (A). Hinf I digest. (B). Hae III digest. SV40 (I) DNA, 0.8 µg/slot, was digested with Hinf I, and the fragments were electrophoresed on a 5% polyacrylamide-agarose gel. One track was stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light. (C). The other track was transferred and hybridized for 22 hr at 42° with [^{32}P]-labeled, nick translated SV40 DNA ($3x10^7$ cpm/µg, $5x10^3$ cpm/cm^2). (D). After washing, the strip was autoradiographed; the upper part was exposed for 6 hr and the lower part for 70 hr at -70°.

is a relatively constant fraction of the input, with slightly better overall yields of the small fragments.

The DNA bands appear sharp after transfer (Figure 1), indicating that they do not diffuse significantly during the

whole procedure. Bands due to the small Hae III fragments are broader than bands due to the larger ones because of the low percentage of acrylamide in the gel, not because of diffusion during transfer. Use of gels containing 10 or 12% acrylamide gives improved resolution of fragments smaller than 50 base pairs (data not shown).

Transfer of large DNA fragments to DBM-paper can also be done, as an alternative to the procedure of Southern (1). DNA fragments generated from phage λ with Eco RI and electrophoresed on 0.7% agarose slab gels in 10 mM sodium phosphate buffer pH 7.0 (14), were transferred to DBM-paper by the procedure described above.

Detection of unlabeled fragments by hybridization with radioactive DNA probes. Unlabeled SV40 Hinf I fragments coupled to DBM-paper were detected by hybridization with nick-translated DNA labeled with [^{32}P] (Figure 1, D). The efficiency of hybridization to the smallest fragments I and J (28 base pairs each) was very poor and these bands were hard to detect even after long exposure times.

The stability during hybridization of DNA coupled to DBM-paper was also determined (Table III). Between 35 and 78% of the DNA remains bound to the paper after the complete procedure (prehybridization wash, hybridization and washes). A condition which causes dissociation of double stranded DNA (99% formamide at 80°), leads to slightly greater losses, but since an appreciable fraction of the DNA is still retained, the paper could be used for hybridization again, perhaps with a different probe. The nature of the unstable interaction is not clear at present.

Table III

Stability of DNA fragments bound to DBM-Paper

Fragments	Base Pairs	Percentage of DNA remaining bound to the paper	
		A	B
A	1790	71	55
B	1120	61	60
C	760	43	47
D	590	78	43
E	490	62	38
F	235	35	50
G	110	46	36
H	75	38	30

[^{32}P]-labeled SV40 DNA, 2.6 µg/slot, 14,300 cpm/µg, was digested with Hinf I and electrophoresed on a 5% polyacrylamide-agarose gel at 20 mA for 10 hr. The gel was pretreated and the DNA was transferred to DBM-paper as above. After transfer the paper was cut into three strips corresponding to individual gel tracks and dried overnight at room temperature. One strip (A) was treated ("mock hybridization") for 22 hr at 42° with prehybridization buffer and for 58 hr at 42° with hybridization buffer, finally it was washed for 12 hr at 37° with 50% formamide-5xSSC. A second strip (B) was incubated for 7 hr at 80° with 99% formamide. The third strip was used as control (100%).

CONCLUSIONS

The method described here extends the technique of Southern (1) to the detection of small DNA fragments. Fragments below the range of resolution of conventional agarose gels can be separated on polyacrylamide gels. Our results indicate that polyacrylamide-agarose composite gels can also be used for separation with high resolution and that the small fragments can be coupled to DBM-paper and detected by hybridization with labeled DNA probes. Although the overall efficiency of transfer is not particularly high, it is convenient that it is relatively constant for the size range of fragments tested.

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