A simple and efficient method for the separation and detection of small DNA fragments by electrophoresis in formamide containing agarose gels and Southern blotting to DBM-paper

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ABSTRACT.

We show in this report that DNA fragments smaller than 300 bp are separated with high resolution by electrophoresis in concentrated (up to 7%) agarose gels containing 50 % formamide. The separated DNA fragments can subsequently be quantitatively transferred to DBM-paper [Alwine, J.C. et al., *Proc. Natl. Acad. Sci. USA* (1977) 74, 5350-54] using the Southern technique [Southern, E.M., *J. Mol. Biol.* (1975) 98, 503-517], while preserving the sharpness of the original gel pattern. Since thin (0.2-0.4 mm) and thick (up to 5 mm) agarose slab gels can be easily handled in vertical or horizontal apparatus, this method should prove to be a very useful extension of the Southern technique, applicable to a variety of analytical and preparative purposes.

INTRODUCTION.

The technique of transferring DNA fragments from agarose gels to nitrocellulose filters (1) has been used extensively for probing DNA structure. However, it cannot readily be used for small DNA fragments (<200 bp) which are poorly resolved in agarose gels and inefficiently bound to nitrocellulose. Alwine et al.(2) have described the use of DBM-paper instead of nitrocellulose filters to overcome the problem of the poor binding of small DNA fragments to nitrocellulose. Subsequently, several methods for transfer of DNA fragments from polyacrylamide gels to DBM-paper were described (3-5). However, these methods have not been extensively used, since the procedures are rather complicated and the DNA transfer is usually not quantitative.

We report here a new electrophoretic procedure for the separation of DNA fragments smaller than 500 bp, using formamide-containing agarose gels. This gel system, which results in much higher resolution than ordinary agarose gels, can be coupled to simple and efficient DNA transfer onto DBM-paper.

MATERIALS AND METHODS.

(a) DNA electrophoresis.

1. Preparation of agarose-formamide and agarose gels.

For 2 % agarose - 50 % formamide vertical slab gels (170 x 138 x 2 mm, length x width x thickness), 1.6 g of agarose (Sigma, Type II), was suspended in a solution containing 40 ml formamide (Merck GR 968), 8 ml of 10 x electrophoresis buffer (1 x buffer : 40 mM Tris-HCl, 12 mM CH_3COONa , 1 mM EDTA, pH 7.8) and 32 ml H_2O . The mixture was boiled for 1-2 minutes to dissolve the agarose. The solution was allowed to cool to 60°C while being gently stirred and then poured between two plastic Perspex plates. Solidification required about 1 hr at 4°C. Formamide gels containing higher agarose concentrations (up to 7 %) were prepared using the same procedure, except that the solutions were poured at 80-90°C. The boiling and stirring steps are necessary to obtain bubble-free gels. Since the 7 % agarose - 50 % formamide solution is very viscous the above procedure was modified when pouring these gels. The two plastic plates were sealed with a tape at the bottom, tilted, and kept apart with a small rubber cork during the pouring step and then rapidly pressed against each other and fixed with clamps. Very thin vertical 5 % agarose -50 % formamide gels (110 x 135 x 0.4 mm) were poured between glass plates using the same procedure.

Conventional 2 % agarose vertical slab gels (170 x 138 x 2 mm) were made with the same electrophoresis buffer and poured at 90°C ; 4 % agarose gels were poured as described above for 7 % agarose - 50 % formamide gels.

Vertical step gels (170 x 138 x 4 mm) (4 % agarose in the lower half, 2 % in the upper half) were made as follows : 4 % agarose (or 4 % agarose - 40 % formamide) solution was poured as described above. After the gel was solidified, the upper half of the gel (7.3 cm) was cut out and refilled with a 2 % agarose, or 2 % agarose - 40 % formamide solution.

2. Electrophoresis.

DNA fragments were dissolved in 1 x electrophoresis buffer (see above) containing 25 % sucrose. The electrophoresis buffer for formamide gels contained 50 % formamide and could be re-used several times. Formamide can be omitted from the electrophoresis buffer in the case of thin (0.2-1mm) agarose-formamide gels. Electrophoresis was carried out at approximately 100 volts at 4°C or 22°C, and bromophenol

blue (0.01 %) was used as marker.

(b) Transfer of DNA to DBM-paper, hybridization and autoradiography.

DBM-paper was prepared from commercial NPBC (Chemalog) according to the procedure of Alwine et al. (2, 6), except that acetone was used instead of benzene to wash the NPBC-paper (2 times for 5 min) after the heating step at 135°C.

After electrophoresis in agarose-formamide, 2 or 4 mm thick gels were briefly washed with water, stained with ethidium bromide and photographed under UV illumination. After treatment with 0.5 N NaOH-1M NaCl for 10 minutes at 0°C, the gel was rinsed several times with water to remove the ammonia produced by the alkaline treatment, and then washed twice in transfer buffer (5 and 20 min for 2 and 4 mm thick gels, respectively). The DNA transfer was then as described by Alwine et al. (2, 6), except that 50 mM sodium borate was replaced by 1M CH₃COONa, pH 6.1. The same transfer technique was used for agarose gels without formamide, except that the water rinse after the NaOH treatment was omitted. Thin agarose-formamide gels were transferred as described above, except that the 0.5 N NaOH treatment was shortened to 1-2 min. In all experiments, a sheet of DE 81-filter was laid onto the DBM-paper during the transfer step to detect any DNA fragments which failed to bind to the DBM-paper. After the transfer step the DBM-paper was washed, hybridized with nick-translated DNA in the presence of dextran-sulfate, and autoradiographed as described in Bellard et al. (7).

(c) Preparation of DNA samples.

An HpaII digest of pBR322 was prepared and used either unlabeled or labeled at the 5'-end of the fragments with [³²P-γ]ATP and T4 polynucleotide-kinase. A pBR322 probe for hybridization was obtained by nick-translation of pBR322 DNA with DNA polymerase I in the presence of a [³²P-α]-deoxyribonucleoside triphosphate (8). Nuclei from chicken erythrocytes were prepared and digested with micrococcal nuclease, and the DNA purified as previously described (7).

RESULTS.

High resolution agarose-formamide gels.

Excellent resolution of mixtures of small (<500 bp) DNA fragments was achieved by electrophoresis through agarose-formamide gels (Fig. 1, lane 2). To compare the resolving capacity of agarose gels with and without formamide, the same DNA fragments were also electrophoresed

Figure 1 : Resolving capacity of agarose gels with or without 50 % formamide. HpaII fragments of pBR322 DNA (0.8 μ g) were electrophoretically separated as described in Materials and Methods on a 2 mm thick gel until the dye marker was within 4 cm of the bottom of the gel, and then stained with ethidium bromide. The fragment lengths were obtained from sequence data (12). Lane 1: 2 % agarose gel ; lane 2 : 2 % agarose-50 % formamide gel ; lane 3 : 7 % agarose-50 % formamide gel.

on 2 % agarose gels without formamide (lane 1). Electrophoresis run length was adjusted so that the migration of fragment l (122 bp) was about the same in both gels. It is clear that the 2 % agarose - formamide gel provides much better resolution than the conventional agarose gel for DNA fragments shorter than 240 bp. For instance DNA fragments n, o and p, which are 90, 76 and 67 bp long, respectively, were well separated in the formamide-containing gel (see also Fig. 4). In addition, it is apparent from the data shown in Fig 2 that the linear relationship between the migration distance of the DNA fragments and the log of their lengths has a broader range for formamide-containing gels than for conventional gels.

Addition of formamide not only results in high resolution gels, but also allows one to prepare agarose gels of higher concentration, because it slows down the rate of solidification and the gels can be handled like polyacrylamide gels. For instance, it is much easier to prepare a 4 % agarose - 50 % formamide gel, than a conventional 2 % agarose gel. In Fig. 1, lane 3, we show the resolution obtained with a 7 % agarose -50 % formamide gel in which the two DNA fragments of band e

Figure 2 : Relationship between electrophoretic migration (in cm) and length of DNA fragments (in bp) in 2 % agarose ($\blacktriangle$) and 2 % agarose - 50 % formamide gels ($\bullet$). The DNA fragments a-p correspond to lanes 1 and 2 in Fig. 1, respectively. Short vertical lines on the ($\blacktriangle$) curve indicate DNA fragments which were not separated on the conventional 2 % agarose gel.

(242 and 238 bp, respectively) are separated.

We have compared the resolving capacity of agarose and agarose-formamide gels (4 mm thick) with the DNA from micrococcal nuclease digested chicken erythrocyte nuclei. Fig. 3 shows ethidium bromide stained step agarose gels (2 % and 4 %) with and without 40 % formamide. The oligonucleosome DNA fragments larger than dimers were separated in the 2 % agarose gel, whereas the DNA fragments of monomer size and smaller were separated in the 4 % agarose gel. Subnucleosomal fragments are better resolved in the agarose-formamide gel (compare Fig. 3 A and B). Band "a" of the conventional normal gel (Fig. 3 A), was separated into three bands in the agarose-formamide gel (Fig. 3 B), and band "b" into two. In addition, it was clear on the original gel that band "c" was resolved into two bands in the agarose-formamide system. This high resolving capacity for DNA fragments of subnucleosomal length indicates that agarose-formamide gel electrophoresis followed by blotting (see below) can be used to study the nucleosomal and subnucleosomal chromatin structure of specific genes. Results from such studies will be published elsewhere (Sun Y.L. et al., in preparation).

We have found that the resolution of agarose-formamide gels

Figure 3 : Comparison of the resolving capacity of agarose and agarose-formamide gels on the DNA from micrococcal nuclease digested chicken erythrocyte nuclei. Chicken erythrocyte nuclei (1 mg DNA) were digested with 100 U micrococcal nuclease for 15 min (lane 1) and 30 min (lane 2), respectively, in 1 ml of 15 mM Tris (pH 7.5), 60 mM KCl, 15 mM NaCl, 0.34 M sucrose, 15 mM 2-mercaptoethanol and 1 mM CaCl₂. DNA was extracted and electrophoresed (70 µg DNA per track) on step agarose gels (Materials and Methods). Gels were stained with ethidium bromide. (A) 2 % - 4 % agarose gel, (B) 2 % - 4 % agarose gel containing 40 % formamide.

depends on the concentration of formamide (results not shown). The best separation is achieved with 40 to 50 % formamide. The presence of formamide is not absolutely required in the electrophoresis buffer. However, when high concentration of agarose and thick gels (over 4 % agarose and 2 mm thickness) were used, we have obtained better results when the buffer contained the same concentration of formamide as the gel.

DNA transfer from agarose-formamide gel to DBM-paper and hybridization to labeled probes.

The results shown in Fig. 4 indicate that DNA transfer from agarose-formamide gels onto DBM-paper is efficient. A HpaII digest of pBR322 (2 µg DNA), labeled with T₄ polynucleotide kinase and [³²P-γ]ATP, was mixed with unlabeled DNA fragments (w/w = 1:500) and electrophoresed on a 2 mm thick 3.5 % agarose -50 % formamide gel. Fig. 4A, lane 1, shows a photograph of the gel stained with ethidium bromide. The autoradiogram of the same gel (5 hrs exposure) is shown in Fig. 2A, lane 2. A series of bands corresponding to those stained in lane 1 are clearly visible. After autoradiography, the DNA fragments were transferred onto DBM-paper as described in the Materials and Methods. After a 8 hrs

Figure 4 : DNA transfer from agarose-formamide gels to DBM-paper. 2 mm thick 3.5 % agarose-50 % formamide gels of pBR322 HpaII fragments a-p (Fig. 1) (A) Lane 1 is a photograph of the ethidium bromide stained gel on which a mixture of labeled and unlabeled DNA fragments (Materials and Methods) was electrophoresed ; lanes 2 and 3 are autoradiograms of the gel before and after 8 hours of transfer, respectively ; lane 4 is the autoradiogram of the DBM-paper after the transfer. (B) Lane 1 is as lane 1 of panel A, but contains unlabeled DNA only ; the DNA was transferred to DBM-paper and then hybridized with a labeled pBR322 DNA probe (Materials and Methods) and autoradiographed for shorter (lane 2) and longer (lane 3) exposures.

transfer, both the gel and DBM-paper were autoradiographed for 5 hrs (lanes 3 and 4, respectively. Almost no radioactive DNA fragments smaller than 400 bp remained in the gel (lane 3), whereas the relative distribution of radioactivity on DBM-paper (lane 4) was very similar to that obtained with the gel before transfer (lane 2). A sheet of DE-81 filter was laid onto the DBM-paper to absorb any DNA which failed to bind to the DBM-paper during the transfer. No radioactivity was found on this filter after the transfer (not shown). The results suggest that all of the DNA fragments smaller than 400 bp have been quantitatively transferred to the DBM-paper, irrespective of their size. The actual efficiency of DNA transfer was determined on gels run in parallel and in triplicates by measuring the radioactivity of the individual DNA fragments in the gel before and after transfer, and in the DBM-paper. The agarose slices and the DBM-paper bands (located by autoradiography) were counted by Cerenkov radiation in a scintillation counter. The average efficiency of transfer measured for fragments a and b was 50 and 75 % respectively. Fragments c to f were transferred with 90 % efficiency, and fragments g to p with 100 % efficiency (results not shown).

To show that DNA fragments transferred from agarose-formamide gels to DBM-paper can be efficiently hybridized, HpaII fragments of pBR322 DNA were electrophoresed on 3.5 % agarose-50 % formamide gels, transferred onto DBM-paper and hybridized to nick-translated [^{32}P]-labeled pBR322 DNA (Materials and Methods). Fig. 4B, lanes 2 and 3, show the autoradiograms at two different exposures. It is clear that all DNA fragments become hybridized, and that the extent of hybridization corresponds to the relative amount of DNA in each band as revealed by the ethidium stained gel (lane 1).

Since agarose-formamide gels are not fragile and can be handled like polyacrylamide gels, it is possible to make thin (0.4 mm) gels. Such gels are firm enough to be carried through all the blotting manipulations. Fig. 5A, lane 1, shows the ethidium bromide photography of 0.3 μg HpaII fragments of pBR322 DNA electrophoresed on a 5 % agarose - 50 % formamide thin gel (0.4 mm). Since the gel is thin, pre-blotting and blotting treatments can be simplified. Gel treatment with 0.5 N NaOH-1M NaCl before transfer can be shortened to 2 min. and can even be omitted when the DNA fragments contain staggered ends. Washing with

Figure 5 : DNA transfer from thin agarose-formamide gels to DBM-paper. 0.3 μg DNA of a HpaII digest of pBR322 was subjected to electrophoresis in a 5 % agarose - 50 % formamide gel (0.4 mm thick). (A) lane 1 : ethidium bromide stained gel on which a mixture of labeled and unlabeled DNA fragments was electrophoresed; lanes 2 and 3 : autoradiograms of the gel before and after a 2 hrs transfer to DBM-paper, respectively ; lane 4 : autoradiogram of the DBM-paper after transfer. B) lane 1 : ethidium bromide staining of unlabeled DNA fragments ; lane 2 : autoradiogram of the DNA fragments after transfer onto DBM-paper and hybridization to a [^{32}P]-labeled pBR322 DNA probe.

transfer buffer can be reduced to 2 min., and the transfer period to only 2 hrs. The autoradiograms of the gel before and after transfer, and of the DBM-paper after transfer are shown in lanes 2-4. It is clear that all of the DNA bands which are seen by ethidium bromide staining are also revealed by autoradiography of the DBM-paper. Fig. 5B, lane 2, shows the autoradiogram of DBM-paper onto which unlabeled HpaII fragments were transferred and hybridized to a [32 -P]-labeled pBR322 DNA probe. Again the DNA bands seen on the gel by ethidium bromide staining (lane 1) are also revealed by hybridization. Other experiments (not shown) have indicated that the DNA transfer was quantitative for DNA fragments ≤ 400 bp. Thin gels can be easily dried on glass plates after washing with water, and stored. After rehydration they can be stained again with ethidium bromide to visualize the DNA bands (not shown).

DISCUSSION.

The Southern technique (1) is an extremely powerful tool used to detect specific DNA fragments within a complex mixture by transferring the DNA from agarose gels to nitrocellulose filters and hybridizing it to specific labelled probes. Unfortunately this method cannot be used for small DNA fragments, which are poorly resolved in agarose gels and inefficiently retained on nitrocellulose filters. The use of polyacrylamide or polyacrylamide-agarose composite gels solves the fragment resolution problem, and the use of DBM-paper solves the inefficient retention problem. However, DNA fragments are not efficiently transferred out of the concentrated polyacrylamide gels which are required to achieve high resolution of DNA fragments smaller than 300 bp. Several methods have been proposed to circumvent this problem. A procedure involving polyacrylamide-agarose composite gels with a cleavable cross-linking reagent was described by Reiser et al. (9) and Alwine et al. (6). Unfortunately, the efficiency of DNA transfer from composite gels onto DBM-paper was low (20 % for a 490 bp DNA fragment and 42 to 80 % for 235 to 75 bp fragments). Levy et al. (3) used step gels made of a short concentrated polyacrylamide gel (8 %) above a long gel of relatively low polyacrylamide concentration (4 %), in order to improve the DNA transfer efficiency to DBM-paper. However not more than 30 % of DNA applied to non-denaturing gels could be transferred to DBM-paper, even in a 36 hrs transfer. Electrophoretic transfer of DNA fragments from polyacrylamide gels onto DBM-paper was proposed in order to solve the problem of

slow diffusion of DNA fragments through polyacrylamide gels (4, 5). However this method requires a special transfer equipment and the efficiency of transfer of DNA fragment smaller than 300 bp was only about 60 % (5).

The ideal procedure would be one which combines the high resolution of polyacrylamide gels and the efficient transfer of DNA from agarose gels. In transferring small DNA fragments from formamide-containing agarose gel to DBM-paper, the present technique closely approximates this ideal by extending the usefulness of the Southern transfer method to small DNA fragments below the resolution range of conventional agarose gels. The transfer procedure, which preserves the sharpness of the original gel pattern, is both simple and rapid and does not require any special equipment.

In addition to the properties of the formamide-containing agarose gels described in the Result section we have noticed several points which are worth mentioning. Depending on the amount of DNA to be analyzed, gels of almost any thickness (from 0.2 mm to 5 mm) can be made and readily handled. The resolution is identical with vertical and horizontal electrophoresis equipment. Gels can be prepared in advance and stored at 4°C for several weeks. DNA fragments can be readily recovered from the agarose gels by overnight diffusion in Tris-HCl pH 7.5 - 1 mM EDTA (4°C or 22°C) or by the technique of Dretzen et al. (10). If quantitative transfer is not important, the DNA fragments can also be transferred to nitrocellulose filters. Using the technique of Thomas (11), T. Reudelhuber (personal communication) found that pBR322 HpaII DNA fragments a to p could be transferred to nitrocellulose filters and detected by hybridization with [³²p] labelled nick-translated probes. Gels can be run at +4°C or at room temperature, but care should be taken to avoid heating during the electrophoretic step which may result in DNA denaturation. We do not know at the present time whether running formamide-containing gels at elevated temperature could be used for DNA strand separation.

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