Selective blotting of restriction DNA fragments on nitrocellulose membranes at low salt concentrations

Yoshikuni Nagamine, André Sentenac and Pierre Fromageot

Service Biochimie, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, B.P. No. 2, 91190, Gif-sur-Yvette, France

Received 3 April 1980

ABSTRACT

The pattern of restriction DNA fragments transferred from agarose gel to nitrocellulose membranes (Southern's technique) can be affected by the salt concentration of the transfer solvent.

INTRODUCTION

Since Southern developed the method (1), the transfer of restriction DNA fragments from agarose gels to nitrocellulose sheets has been a powerful technique in the analysis of cloned DNA. The faithful replica of DNA bands immobilized on the membrane can be subjected to hybridization with radioactive RNA or DNA probes to detect specific fragments. The method essentially consists in the denaturation of DNA in the gel followed by the transfer of the restriction fragments to a nitrocellulose membrane with a salt solution.

The great sensitivity, ease and low cost of the technique made it of routine use in all the laboratories concerned with DNA analysis. Similar methods have been developed for the analysis of RNA molecules (2) and proteins (3,4). The main disadvantage of the Southern's technique is that the large DNA fragments are not efficiently transferred and the fragments smaller than 0.3 ~ 0.5 Kb do not bind well to nitrocellulose. This can be improved by the introduction of a cleavage step of the DNA in situ and the use of diazobenzyl-oxymethyl paper instead of the nitrocellulose membrane (5).
Southern also pointed out that the retention of DNA fragments on the nitrocellulose membrane increases with the salt concentration of the elution buffer (1). However, because the salt effect was analyzed by a membrane filtration assay using a random population of sonicated DNA fragments, the results were only statistical. There is still no information about the comparative retention of specific DNA fragments blotted at different salt concentrations. Here we show that the pattern of restriction fragments retained on nitrocellulose is greatly altered both qualitatively and quantitatively depending on the salt concentration of the transfer solvent.

**MATERIALS AND METHODS**

**DNA.** Plasmid pYe cyc 1-9 (5.10) DNA was used throughout this work. The *E. coli* strain which carries this plasmid was given by D. Montgomery. This plasmid was prepared by inserting a yeast DNA fragment carrying the iso-1-Cyt C gene at the HindIII site of pBR322 (6). DNA was purified by the method of Elwell et al. (7).

**Enzymes.** Restriction endonuclease HindIII was purchased from Boehringer Manheim; BamHⅠ and XhoⅠ were from B.R.L.; *E. coli* alkaline phosphatase was purchased from Worthington; T4 polynucleotide kinase was from Gipep (Paris).

**Chemicals.** Agarose was type ME of Seakem. Nitrocellulose membrane (SM 113, pore size 0.45 μ) was purchased from Sartorius. [γ²³]ATP (5,000 Ci/m mole) was purchased from Amersham. Other reagents were of analytical grade.

**Preparation of ³²P-labelled DNA.** Radioactive DNA was prepared by labelling the 5' end of restricted fragments with ³²P using T4 polynucleotide kinase. After digestion of 2.5 μg of pYe cyc-1-9(5.10) DNA by restriction enzymes, the restriction fragments were treated with *E. coli* alkaline phosphatase in the presence of 0.1 % SDS (8). Then, the 5'-OH end was phosphorylated by
T4 polynucleotide kinase in the presence of 1 mM spermidine (9). Specific radioactivity of DNA was 4.0 to 2.0 x 10⁵ cpm/μg.

Agarose gel electrophoresis and DNA transfer. Labelled DNA fragments (10⁴ cpm) were subjected to electrophoresis in vertical 0.7 % agarose gels (3 mm thick) in 90 mM Tris, 90 mM borate and 2.4 mM EDTA, pH 8.2, for 4 h at 9 V/cm. Four identical gel electrophoresis were run in parallel. The DNA fragments were transferred to a nitrocellulose membrane by the method of Southern (1) but using varying salt conditions. After the electrophoresis, the gels were dipped into 25 ml of 0.5 M NaOH, 0.6 M NaCl for 15 min, and neutralized with 250 ml of 0.5 M Tris-HCl, pH 7.0, containing either 0.6 M NaCl, 0.9 M NaCl, 1.5 M NaCl or 3.0 M NaCl, for 15 min. The gels were then laid onto two sheets of Whatman 3 MM paper on a glass plate, with both ends of the paper dipping into 400 ml of transfer solvent, either 4 X SSC, 6 X SSC, 10 X SSC or 20 X SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7). Over the gels was laid the nitrocellulose membrane, a little bigger than the gel and previously wetted with 2 X SSC. Over the membrane were laid two sheets of Whatman 3 MM paper and 100 sheets of paper towels to draw the buffer by capillarity through the gel. Blotting was allowed overnight. After blotting, the nitrocellulose membranes were soaked into 200 ml of 2 X SSC for 3 min, dried at 37° and subjected to autoradiography. The radioactivity of each DNA band on nitrocellulose was estimated by scanning the autoradiogram with a Vernon spectrophotometer. The control pattern of radioactivity in the restriction fragments was obtained using a duplicate sample. In that case, the gel was directly dried without blotting onto a dialysis membrane and subjected to autoradiography.

RESULTS

In the following experiments, we used a recombinant plasmid DNA, which was contracted by the integration of a
5.1 kilobase yeast DNA fragment, containing the iso-1-cytochrome C gene within the HindIII site of pBR322 (6). The plasmid DNA was cut with HindIII and the two linear fragments were labelled with $^{32}$P at the 5'-end, separated by agarose gel electrophoresis and blotted on a nitrocellulose membrane using different transfer solvents (Fig. 1). Surprisingly, although the two fragments were of comparable size, the yeast DNA was selectively retained when the solvent was 4 X SSC or 6 X SSC (Fig. 1a - 2,3). Even after overexposure of the film for 8 days (instead of 6 h), pBR322 DNA was not detectable on the autoradiogram (Fig. 1a - 1). The recovery of the two fragments increased dramatically with 10 X SSC (Fig. 1a - 4).

Taking into account the fact that pBR322 was preferentially labelled by T4 polynucleotide kinase (Fig. 1a - 6), it can be seen that the yeast DNA fragment was always more efficiently retained, even with 20 X SSC. This is better shown in Fig. 2a where the results have been quantified.

Further cutting of the HindIII fragments with BamH1 yields two yeast fragments of 2.7 and 2.4 Kb as well as a small and large fragment originating from pBR322 (4.0 and 0.4 Kb). We compared the blotting efficiency of these DNA at different salt concentrations (Fig. 1b) and obtained similar results. The two yeast fragments were retained exclusively at 4 X SSC and 6 X SSC. The 4.0 Kb DNA from pBR322 needed 10 X SSC to be significantly adsorbed on nitrocellulose and the recovery of all fragments was increased when using 20 X SSC. The small DNA fragments of 0.4 Kb was weakly retained as expected (1). Quantification of the results is shown in Fig. 2b. These observations were repeated after cutting the yeast DNA with a different combination of restriction enzymes while leaving intact pBR322 (Fig. 1c). The selective loss of the 4.4 Kb DNA at low salt was clearly reproducible. With 4 X SSC the yeast 2.2 Kb fragment was preferentially retained (Fig. 1c - 1, 2 and Fig. 2c) (Note that the kinase labelling of that fragment was weak as compared with the 1.5 Kb DNA). The 0.9 Kb and 0.5 Kb DNA fragments were barely visible on the over exposed autoradiogram and the bands were more diffuse. With 20 X SSC the retention of the various DNA fragments was a
Figure 1: Effect of salt concentration on the blotting of restriction DNA fragments on nitrocellulose. Plasmid DNA was digested with different restriction enzymes: (a): HindIII; (b): HindIII + BamHI; (c): HindIII + XhoI. After labelling of their 5' end with $^{32}$P, the DNA restriction fragments were separated by electrophoresis on agarose gel and blotted on nitrocellulose membrane at varying salt concentrations as described under Materials and Methods. (1) and (2): 4 X SSC; (3): 6 X SSC; (4): 10 X SSC; (5): 20 X SSC. Sample (6) corresponds to control input DNA, before blotting (half of the radioactivity was loaded on the gel). Autoradiography was for 6 hours in samples (2) to (6) and for 8 days in sample (1). The DNA fragments are identified by their size in kilobases.
DISCUSSION

The property of nitrocellulose membranes to adsorb single stranded DNA was first recognized and used by Nygaard and Hall in 1963 for RNA-DNA hybridization studies (10). Their method of filter hybridization which provided the ultimate in convenience for handling many samples is therefore a long known but still empirical procedure. Little is known about the nature of the physical interaction between the DNA and the membrane. The influence of DNA size and the effect of salt concentration on DNA retention was noticed very early (11). A saline citrate solution (6 X SSC) was generally adopted to

---

**Figure 2**: The radioactivity of the restriction DNA fragments shown in Fig. 1 was estimated by scanning the autoradiogram. The results are given as percent of radioactivity in the corresponding control DNA fragment as described under Materials and Methods. The fragments are identified by their size in kilobases. Open symbols: DNA fragments derived from pBR322; closed symbols: yeast DNA fragments. The abscissa shows the salt concentration (times SSC used for blotting).
dissolve the DNA and adsorb it on the nitrocellulose membrane (11, 12). Southern, on the basis of a similar filtration assay, recommended to use a higher salt concentration (20 X SSC) to blot restriction DNA fragments, because the overall retention of sonicated DNA fragments on filters decreased with the salt concentration (1). The technique of Southern is now widely spread and subjected to a number of minor modifications in many laboratories with the implicit assumption that these will not affect qualitatively the pattern of restriction DNA fragments retained on the filter.

Here we show that the salt concentration of the eluant buffer is a critical parameter. At salt concentrations lower than 20 X SSC, the blotting pattern can be drastically altered. For instance using 6 X SSC, or 4 X SSC not only the amount of DNA retained is lower but some DNA fragments are completely lost. This may obviously lead to uncorrect conclusions in the general case where a specific DNA sequence is sought within an unknown genome. Depending on the nature of the restriction fragment where the sequence is inserted, it may be retained or not on the membrane. We noticed several examples of work done under conditions promoting the selective loss of DNA fragments using 6 X SSC (13-19). An electrophoretic technique at very low salt concentration was also introduced to speed up the DNA transfer (20, 21). It is suggested, in the light of the present results, that any modification of the original transfer solvent be accompanied with the appropriate controls to demonstrate the validity of the experiments.

REFERENCES