AN IMPROVED METHOD OF SOUTHERN TRANSFER OF LARGE MOLECULAR MASS RNA

A. THERWATH and F. STRAUSS

Institut de Recherche en Biologie Moléculaire, Centre National de la Recherche Scientifique, Université Paris VII, 75251-Paris Cedex 05, France

Received 20 July 1981

1. Introduction

Transfer of denatured, electrophoretically separated RNA or DNA to solid substrates such as nitrocellulose paper [1] or to chemically activated diazobenzyloxymethyl-paper (DBM) [2] for hybridization to highly radioactive probes, is used in very many laboratories to-day. The technique serves to detect, isolate and quantitate specific nucleic acid molecules [3–5]. The method, though extensively used, has certain limitations. One of the drawbacks is the limited sensitivity, which when compared to hybridization in liquid phase is apparently quite inferior, and the other, the impossibility of adequate quantitation. The second problem is related to the fact that, the efficiency of transfer of RNA or DNA from the gel being inversely proportional to size, transfer of large DNA fragments or RNA molecules such as viral genomic RNA or high-M_r nuclear RNA, is poor compared to small-M_r ones. We were thus interested in improving the sensitivity of the technique and also in devising methods for complete transfer of large nucleic acid molecules. Indeed in projects involving pre-mRNA of relatively rare sequence in a heterogenous population of high-M_r nuclear RNAs, uniformity of transfer and highest sensitivity are essential.

The techniques employed are the classical electrophoresis of denatured RNA in agarose gel, transfer to DBM-paper or to nitrocellulose sheets, hybridization with nick-translated radioactive probes of high specific activity, and visualization of radioactivity by autoradiography. Controlled in situ cleavage of the RNA after electrophoresis was adopted to reduce the size of the molecules, and thus to favour rapid and quantitative transfer from the gel to the nitrocellulose sheets. We describe here in detail the procedure employed and the observations made, and discuss the advantage of this technique in relation to others actually employed.

2. Materials and methods

Chick globin 9 S mRNA was prepared from immature red blood cells of acetylphenylhydrazine-treated, anemic birds. The β-globin mRNA was isolated after hybridizing the total 9 S mRNA to cloned β-globin DNA covalently linked to DBM-paper as in [4]. Recombinant β-globin DNA was labelled by nick translation with the use of all 4 nucleotide precursors containing an α-^{32}P-label, as in [6]. Electrophoresis of denatured RNA on methyl-mercury agarose gels was carried out under a hood at room temperature. The gel was processed for transfer as in [2]. Preparation of the gel and glyoxalation of the RNA sample were essentially as in [7] except that glyoxalation was done at 65°C for 10 min. Electrophoresis was carried out in a cold chamber. Constant recirculation of the buffer allowed the maintenance of the pH at 7. At the end of the electrophoresis the RNA was directly transferred as above, but without any further treatment of the gel as necessary when transferring on DBM-paper.

Composition of the prehybridization and hybridization buffer as well as the transfer procedure were as in [1] except that prehybridization was done twice for 14 h each with fresh buffer. In order to reduce background, 250 μg poly(rA)/ml and 250 μg sonicated salmon sperm DNA/ml was included in the hybridization buffer. The nick translated probe (spec. act. 4×10^8 cpm/μg) was heat-denatured and hybridized (2.8×10^5 cpm/cm^2) to the paper-immobilized

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

RNA for 24 h (50 μl/cm^2). All subsequent steps of washing and autoradiography were as in [1,2].

Quantitative transfer of glyoxalated RNA from the gel without destruction of the glyoxal-RNA adduct was achieved as follows. After electrophoresis, the glyoxal-gel was rinsed rapidly, twice, in 200 ml buffer containing 10 mM NaCl, 10 mM Tris–HCl (pH 7.0), then transferred to 100 ml buffer containing 1 mM $CaCl_2$ (final conc.) and 0.1 g micrococcal nuclease/ml (spec. act. 15 000 units/mg). The gel was left in contact with the nuclease for 3 min at 4°C after which the enzyme was inhibited by the addition of the chelating agent EDTA at 3 mM final conc. The gel was then quickly washed with 3 changes of fresh buffer containing EDTA, as before, and then set up for overnight transfer. The transfer buffer also contained 3 mM EDTA as a precautionary measure against any nuclease activity remaining behind. To control efficiency of transfer the shrunken gel was allowed to swell for ~1 h in the electrophoresis buffer containing ethidium bromide in order to detect the presence of any RNase still remaining behind in the gel. The gel was then photographed under ultraviolet illumination. The different species of RNA that were used in this experiment were chick ribosomal 18 S and 28 S RNAs, avian sarcoma virus (ASV) 35 S RNA and chicken globin 9 S mRNA. The ASV 35 S RNA was subsequently hybridized to a ^{32}P-labelled ASV-cDNA probe and visualized after autoradiography.

3. Results and discussion

Here we opted for globin mRNA as the material of convenience since our current projects are centered around the globin genes and their products. Gel electrophoresis either in the presence of methylmercury or glyoxal results in elimination of secondary structures of the RNAs which then separate as a function of their length [7,8]. Glyoxalation for 10 min at 65°C was found to be as satisfactory as 1 h at 50°C (not shown). After electrophoresis the RNA from the

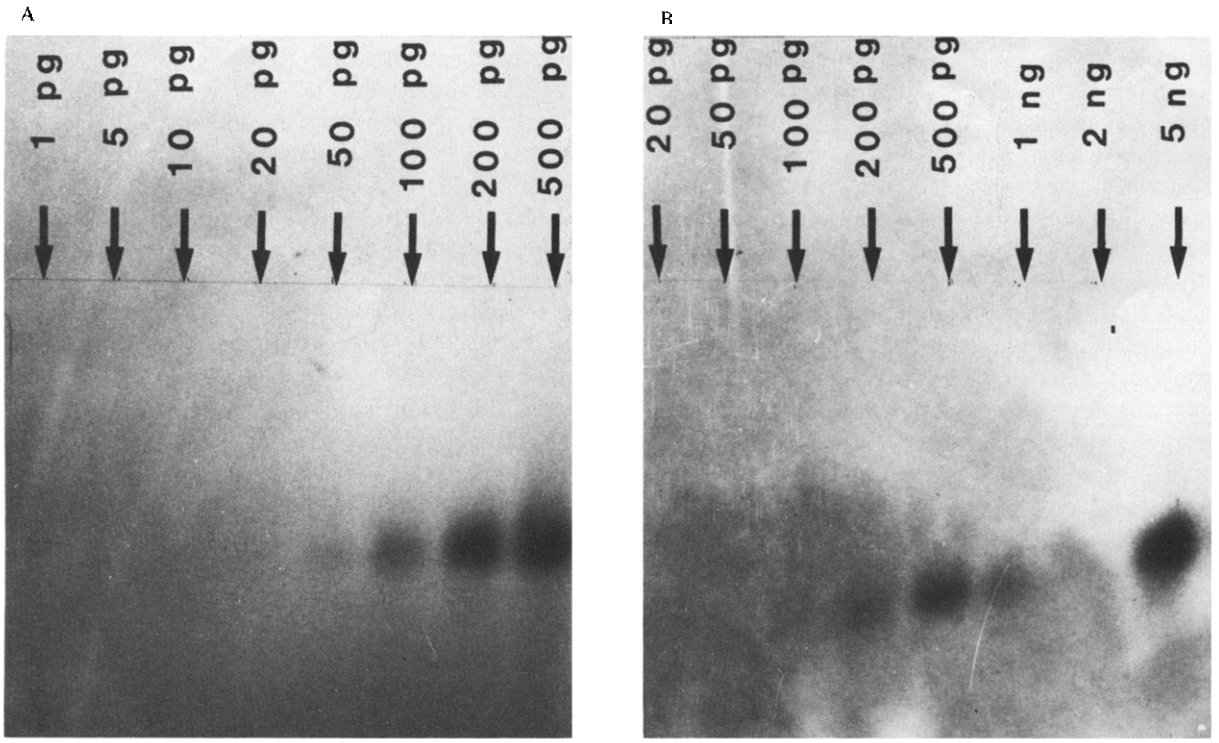

Fig.1. (A) β-Globin mRNA (1–500 pg) was denatured with glyoxal-DMSO, electrophoresed on agarose gels and transferred to nitrocellulose sheets. The RNA was revealed after hybridization with a β-globin DNA probe and autoradiography. In the photographic reproduction of the autoradiogram one can observe a hybridization signal for 50 pg RNA. (B) shows that at least 10–20-fold more RNA is required to obtain a similar signal to that in (A) when the transfer substrate employed was DBM-paper.

gels was blotted to either nitrocellulose sheets or to activated DBM-paper.

For reasons of sensitivity and ease of handling we used nitrocellulose for nucleic acid transfers. Indeed our results confirm the observations [1] that, for RNA blotting and subsequent hybridization, the easy-to-handle nitrocellulose is undoubtably superior in sensitivity to the complicated and time-consuming DBM-paper. As little as 20 pg (and certainly 50 pg can be seen in a photograph) of globin mRNA can be detected after transfer and hybridization within a reasonable autoradiographic exposure time of 72 h (fig.1A).

To obtain a similar signal using blots prepared with DBM-paper, a maximum of 200 pg of RNA is required (fig.1B). Thus, contrary to what one might expect, we find that the use of nitrocellulose for blotting results in a 10-fold increase in sensitivity. Furthermore the background obtained is low and more uniform as compared to DBM-paper. The main disadvantage however to the use of nitrocellulose paper is its fragility and brittle consistency. Handling requires added care, especially if already-used sheets are being processed for subsequent and repeated hybridization.

Among the various chemical agents available for denaturing RNA, the choice is based, on the one hand, on its ability to break all hydrogen bonds and to render the nucleic acid molecule free of any secondary structure, and on the other, the ease and safety with which it can be routinely handled in the laboratory. The choice of glyoxal as a denaturing agent satisfies these two important criteria simultaneously.

Having thus decided to use nitrocellulose instead of DBM-paper for nucleic acid transfer and glyoxal as the denaturing agent, we were confronted with the problem of getting out of the gel, by diffusion, RNA molecules of up to 10 kilobases. This problem is normally solved when transfer is done to DBM-paper since the preparation of the gel prior to transfer involves a treatment with alkali and hence cleavage of large RNA molecules. A similar alkali treatment of glyoxalated RNA would reduce the size of the RNA, permitting its easy exit from the gel, but would also destroy the RNA–glyoxal adduct [1] and thus considerably lower its binding efficiency to nitrocellulose [1]. The solution to this problem was found in the use of an easily-inactivated nuclease for cleaving the RNA, and whose base specificity is such that it does not interfere with the stable guanosine–glyoxal adduct [9]. Micrococcal nuclease, whose activity is dependent on the presence of Ca^{2+} and which has a preference for bases A, T and U [10], was thus successfully employed for cleaving RNA in situ prior to transfer. Once the enzyme has accomplished its limited cleavage of RNA, any further enzymatic action could easily be blocked by the addition of the chelating agent EDTA. Furthermore, the repeated washing of the gel removes most of the enzyme. After transferring the RNA, baking the nitrocellulose at 80°C for 2 h under vacuum appeared not to allow any residual enzyme activity.

Fig.2A shows RNAs of known M_r, visualized after ethidium bromide treatment. The lack of sharpness of the bands was probably due to diffusion resulting from long treatment of the gel (2–3 h) at pH 8.3, in order to reverse the glyoxalation and to permit intercalation of ethidium bromide. Substantial amount of 35 S ASV RNA was seen not to have left the gel even after a 24-h transfer. Fig.2B shows that the 35 S RNA (10 kilobases) after micrococcal nuclease treatment transferred completely and that this RNA bound efficiently to the nitrocellulose, as revealed by the autoradiographic signal obtained after hybridization with a ^{32}P-labelled ASV-specific probe.

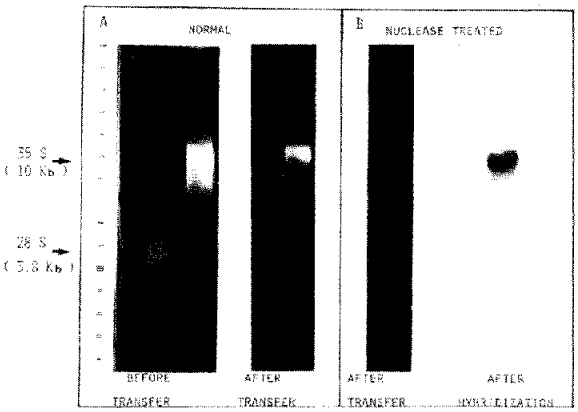

Fig.2. (A) Left panel: Glyoxalated 28 S ribosomal and 35 S viral RNA was fractionated on agarose gels. The gel was washed in a buffer at pH 8.3 for ~3 h, stained with ethidium bromide and photographed under ultraviolet illumination. This figure shows that after a 24 h transfer substantial amounts of 35 S RNA remains in the gel. (B) Exposure of the gel to micrococcal nuclease allowed complete exit of the RNA and no trace of any RNA could be detected by ethidium bromide staining after transfer. The nitrocellulose-bound RNA was detected after hybridization with a radioactive probe and autoradiography, as seen in the extreme right panel.

We have shown here that we can detect as little as 20 pg RNA after glyoxal denaturation, electrophoresis and Southern transfer, provided the solid substrate employed for immobilising the RNA is nitrocellulose, replacing DBM-paper. Detection of rare sequences, or those of low abundance in a mixed population of RNA requires that all molecules be transferred from the gel prior to hybridization. We have shown here that complete transfer of high-M_r RNA (and DNA) is possible if RNA molecules are cleaved a few times in situ by the action of micrococcal nuclease. When compared with other techniques where electrophoretic separation of RNAs on formamide–acrylamide or methylmercury–agarose gels are performed prior to Southern transfer, this method is not only equally satisfactory but it also combines efficiency with ease, in particular when rare but large-M_r RNAs are being studied.

Acknowledgements

Our thanks are due to Dr K. Scherrer, in whose laboratory this work was carried out, for his critical evaluation of the manuscript. Financial support from the French CNRS, DGRST, INSERM and the Fondation pour la Recherche Médicale is acknowledged.

References

[1] Thomas, P. S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
[2] Alwine, J. C., Kemp, D. J. and Stark, G. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5350–5354.
[3] Alwine, J. C., Kemp, D. J., Parker, B. A., Reiser, J., Renart, J., Stark, G. R. and Wahl, G. M. (1979) Methods Enzymol. 68, 220–242.
[4] Stark, G. J. and Williams, J. G. (1979) Nucleic Acids Res. 6, 195–203.
[5] Kafatos, F. C., Jones, C. W. and Efstratiadis, A. (1979) Nucleic Acids Res. 7, 1541–1552.
[6] Rigby, P. W., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237–251.
[7] Carmichael, G. G. and McMaster, G. K. (1980) Methods Enzymol. 65, 380–391.
[8] Bailey, J. M. and Davidson, N. (1976) Anal. Biochem. 70, 75–85.
[9] Shapiro, R., Cohen, B. I. and Clagett, D. C. (1970) J. Biol. Chem. 245, 2633–2639.
[10] Rushizky, G. W., Knight, C. A., Roberts, W. K. and Dekker, C. A. (1962) Biochim. Biophys. Acta 55, 674–682.