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Detection of labelled RNA species by contact hybridization

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ABSTRACT

An improved contact hybridization technique for the analysis of labelled RNA species is presented. The method combines high sensitivity of detection with the high resolution of polyacrylamide gel electrophoresis and should be especially useful for the characterization of transient RNA precursor molecules. Its application to gene mapping is illustrated.

INTRODUCTION

Amongst the techiques which have had a great impact on nucleic acid analyses are the electrophoretic separation of RNA or DNA molecules on gels and the characterization of RNA or DNA sequences by hybridization or reannealing. Several methods have recently been published which combine electrophoretic separation of nucleic acids with hybridization, notably the transfer of DNA from agarose gels to Millipore filters(1) and of RNA to diazobenzyloxymethyl-paper(2)

Contact hybridization (3) consists of transferring and hybridizing labelled, electrophoretically resolved RNA sequences to denatured DNA bound to nitrocellulose sheets. The RNA/DNA hybrids obtained are then detected by autoradiography or fluorography. Here, we describe an improved method of the technique. We quantitate the sensitivity of the technique for various types of RNA molecules and find it to be quite high. The method is especially suited for RNA molecules of high specific activity and therefore should be particualarly useful for the

detection of pulse labelled primary transcripts in eukaryotic cells. Contact hybdridization complements the method of Alwine et al. (2) which allows the detection of small quantities of unlabelled RNA molecules, but without reference to their turn-over characteristics. An additional advantage of our techique is that it can be applied to high resolution fractionation of RNA molecules in standard polyacrylamide gels.

MATERIALS AND METHODS

1. Preparation of RNA and DNA

Histone mRNA of *Psammechinus miliaris* was pulse labelled with ^{3}H-uridine and polysomal RNA was prepared (4). Long-term labelled 18S and 28S rRNA were prepared from cultured *Xenopus laevis* kidney cells which were incubated with 100µCi ^{3}H-uridine/ml for 55 hrs, starting with 1/3 confluent monolayer cells. 2ml RNA isolation buffer containing 100mM Tris pH 7.8, 300mM NaAc and 1mg/ml Proteinase K (preincubated for 10 min at room temperature) was added to the tissue culture cells. After 1 min, the cells were lysed by addition of SDS to a final concentration of 1% and the lysate kept at 20°C for 1 hr before total cellular nucleic acids were extracted with phenol/chloroform 1:1. The RNA and the DNA were precipitated with 2 1/2 vol of ethanol, pelleted, washed extensively with 80% ethanol, then dissolved in 50mM Tris pH 7.8, 10mM $MgCl_2$. 200µg/ml RNase-free DNase I was added and the solution was incubated for 30 min at room temperature. 50µg/ml Proteinase K were then added and the RNA reextracted with phenol/chloroform. The RNA was finally stored in ethanol at -20°C.

Cloned rDNA of *Xenopus laevis* was a gift from Dr. P. Boseley. Cloned histone DNA of *Psammechinus miliaris* (λh22 Sam 7) (5)) was given by Miss A. Binkert.

2. Gel Electrophoresis

Histone mRNA was resolved on 1mm thick polyacrylamide slab gels at 35°C in 0.1 E-buffer (4). RNA of *Xenopus laevis*

was electrophoresed on 3mm thick slab gels of 1% or 2% agarose containing 5mM methylmercury-hydroxide (6). The RNA was denatured before electrophoresis by making the sample 10-25mM in methylmercury-hydroxide labelled RNA was detected by fluorography essentially as described by Laskey & Mills (7) and Bonner & Laskey (8), replacing DMSO by 100% acetic acid as PPO solvent for polyacrylamide or agarose gels (W. Schaffner, unpublished results).

3. Contact Hybridization

Histone or rDNA were loaded onto circular sheets of nitrocellulose with a diameter of 27 cm. For this a 2mm thick porous polyethylen sheet was glued with Silicone rubber at its circumferance to a Buchner funnel and a Millipore sheet was fitted onto the polyethylen sheet. Enough DNA to yield 1μg/cm^2 of λh22 Sam 7 (5), or 0.6μg/cm^2 28S rDNA and 0.9μg/cm^2 18S rDNA (9) were taken up in 0.1 x SSC and denatured with sodium hydroxide to a final concentration of 0.3N (final volume of 10ml). After standing for 20 min at room temperature the DNA solution was diluted out to two litres with 1M NaCl and 10mM Tris pH 7.8 and loaded onto Millipore filters under low suction. The filters were dried for 2 hrs at 80°C in the vacuum oven. Nitrocellulose filter strips were cut out in such a way that there was at least a 1 cm border around the RNA gel to be transferred.

Contact hybridization was performed essentially as described (3). The following changes were adopted: The RNA in the polyacrylamide gel was partially degraded by incubation of the gel in 50mM NaOH at 20°C for 5 min. The gel was then neutralized and equilibrated during 10 min in a large volume of 4 x SSC containing 200mM Tris pH 7.8. RNA fractionated in denaturing methylmercury hydroxide agarose gels was reconstituted to its original form by incubation of the gels in 0.5M NH_4Acetate for 15 min, followed by washing for 10 min in distilled water. The gel was then immersed in 50mM NaOH for 10 min and finally equilibrated in 4 x SSC and 200mM Tris pH 7.8 for 10-15 min. No attempt has been made to optimize the incubation time since they were found to give satisfactory results. However, we

consider it advisable to vary the conditions, especially if gels thicker than 3mm are to be used.

The assembly of the different parts required for contact hybridization was done at room temperature (3). As a modification to published procedure, two nylon nets were placed below the five Whatmann 3MM sheets supporting the gel to assure an even flow of buffer through the central and distal parts of the gel. The hybridization buffer (4 x SSC; 0.1% SDS) was prewarmed to hybridization temperature. 10mM NH_4Acetate was added to hybridization buffers if methylmercury hydroxide had been used in separation of the RNA molecules.

After contact hybridization the RNA remaining in the gels was detected by fluorography in order to determine the amount and resolution of the RNA remaining in the gel. The sheets of nitrocellulose were washed for 1 hr at 65°C in 2 x SSC and for 1 hr at 50°C in 0.1 x SSC. Alternatively, the hybrids were treated with RNase and the Millipore filters dried in the vacuum oven at 80°C. The nitrocellulose sheets were dipped for 5 seconds in 1% PPO dissolved in ether and exposed to preflashed X-ray films (7,8).

RESULTS AND DISCUSSION

1. Contact hybridization of labelled sea urchin histone mRNA resolved on polyacrylamide gels

The technique of contact hybridization has been used previously to detect histone messenger of *Drosophila* using the heterologous cloned histone DNA probe from the sea urchin (3). One of the problems that we encountered was that only small and variable amounts of the RNA could be eluted from the gels during contact hybridization. This problem has now been solved by partially degrading the RNA with alkali in the gel prior to contact hybridization. This treatment did not measurably diminish the power of resolution of the technique.

In order to demonstrate the sensitivity of the technique, different amounts of ^{3}H-uridine-labelled histone mRNA of

Psammechinus miliaris were separated by electrophoresis on 6% polyacrylamide slab gels and contact-hybridized to cloned histone DNA of the same species (h22 Sam 7 (4)) bound to nitrocellulose filters (Fig.1). Each histone mRNA band contained, at input, 100 counts (slot A) 500 counts (slot B) and 2500 cpm (slots C & D). Hybridization became detectable in some slots after 12 hrs fluorography by exposure of the sheet to preflashed X-ray film and was clearly visible in all slots after 4 days (Fig.1a-d). The hybridization intensity was roughly equal to the radioactivity of the RNA bands remaining in the gel after contact hybridization.

The amounts of RNA left in the polyacrylamide gels may be controlled by varying the conditions of partial degradation. It is clearly advantageous not to render the RNA too small since small RNA molecules diffuse rapidly in the gel and this could diminish the high resolution of the technique. It is useful to retain part of the RNA in the gel as this allows a

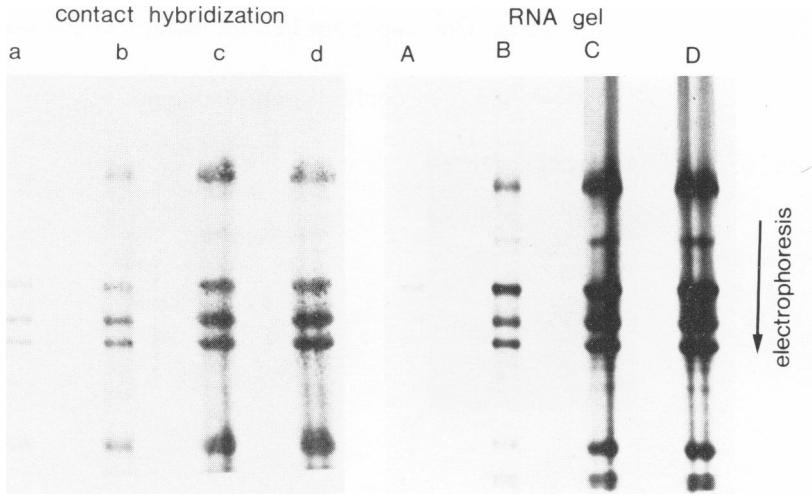

Fig. 1. Contact hybridization of sea urchin mRNAs resolved on polyacrylamide gels.
slots a - d Fluorogram of contact hybridization
slots A - B Fluorogram of RNA remaining in the gel after contact hybridization (for technical details, see text)

detailed comparison between the patterns of hybridized and electrophoretically resolved RNA species. The degradation procedure which we prescribe should be suitable in most cases.

2. Contact hybridization of labelled 18S and 28S rRNA resolved on agarose gels

To test the possibility, whether contact hybridization could also be used for RNA of high molecular weight separated on agarose gels we studied contact hybridization of the high molecular weight rRNA species. Xenopus laevis tissue culture cells were labelled for 2½ days with ^{3}H-uridine to give a 18S and 28S RNA of high specific activity (of the order 1.2 x 10^{5} cpm/µg). The purified total cellular RNA was electrophoresed on 1% agarose gels containing 5mM methylmercury-hydroxide (Fig.2 A-C) and the RNA challenged by contact hybridization with cloned rDNA. The Millipore filter was fluorographed after contact hybridization and exposed to a flashed X-ray film (Fig.2a-c). Two strong bands are visible in the slow migrating RNA sequences when stained with ethidium bromide. These bands coincide with the two hybridized bands and these

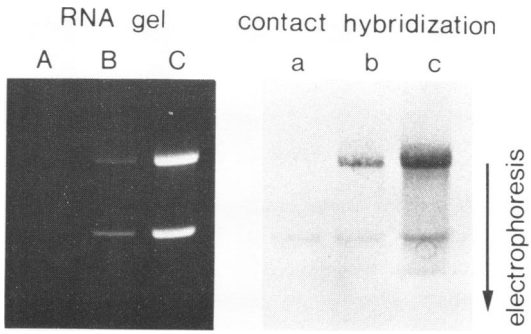

Fig. 2. Contact hybridization of labelled 18S and 28S rRNA resolved on agarose gels.

slots A - C Ethidium bromide-fluorogram of RNA species prior to contact hybridization. Slots were charged with H3-labelled rRNAs containing 10^{5}; 3 x 10^{4} and 10^{4} cmp, respectively.

slots a - c corresponding fluorograms of contact hybridization.

are identified as 18S and 28S rRNA.

3. Direct mapping of *in vivo* labelled sea urchin histone mRNA to restriction fragments of a cloned histone DNA

Labelled RNA sequences may be directly mapped onto restriction fragments of a gene unit by the use of contact hybridization in a manner similar to that of Hutchison (10) for DNA restriction mapping. In short, the DNA restriction fragments are separated on an agarose gel by electrophoresis, denatured and transferred to Millipore filters. The labelled RNA is fractionated electrophoretically on either agarose or polyacrylamide gels using wide slots for sample loading. The RNA is then transferred and hybridized to unlabelled Millipore-bound DNA placed onto the RNA gel at right angles. Spots of hybridization that are detected by autoradiography or fluorography which relate in a simple way to the distribution of complementary sequences in the resolved RNA and DNA bands.

In order to illustrate this mapping procedure we have hybridized the histone mRNAs of *Psammechinus miliaris* to the Hpa II digestion products of the homologous histone DNA repeat. There are extensive restriction and sequencing data available on this clone (11,12) so that the relationship between Hpa II restriction sites and messenger coding sequences are well understood (3). Fig.3a shows the Hpa II digest analyzed on a 1.5% agarose gel. The five labelled histone mRNAs of the sea urchin were separated on a 6% polyacrylamide gel according to the procedure of Gross et al.(4) and is shown in Fig.3b. RNA/DNA hybrids at the intersections of complementary RNA and DNA molecules are easily located and identified (Fig.3c). As predicted from the sequencing experiment of Schaffner et al. (11,12) seven spots of hybridization become evident.

Such mapping of coding regions by contact hybridization makes the time consuming purification of single DNA fragments and of mRNA sequences unnecessary. In addition, the method takes full advantage of the high resolution power of electrophoresis of both RNA and DNA molecules in polyacrylamide gels. Where one is dealing with complex digestion mixtures partial

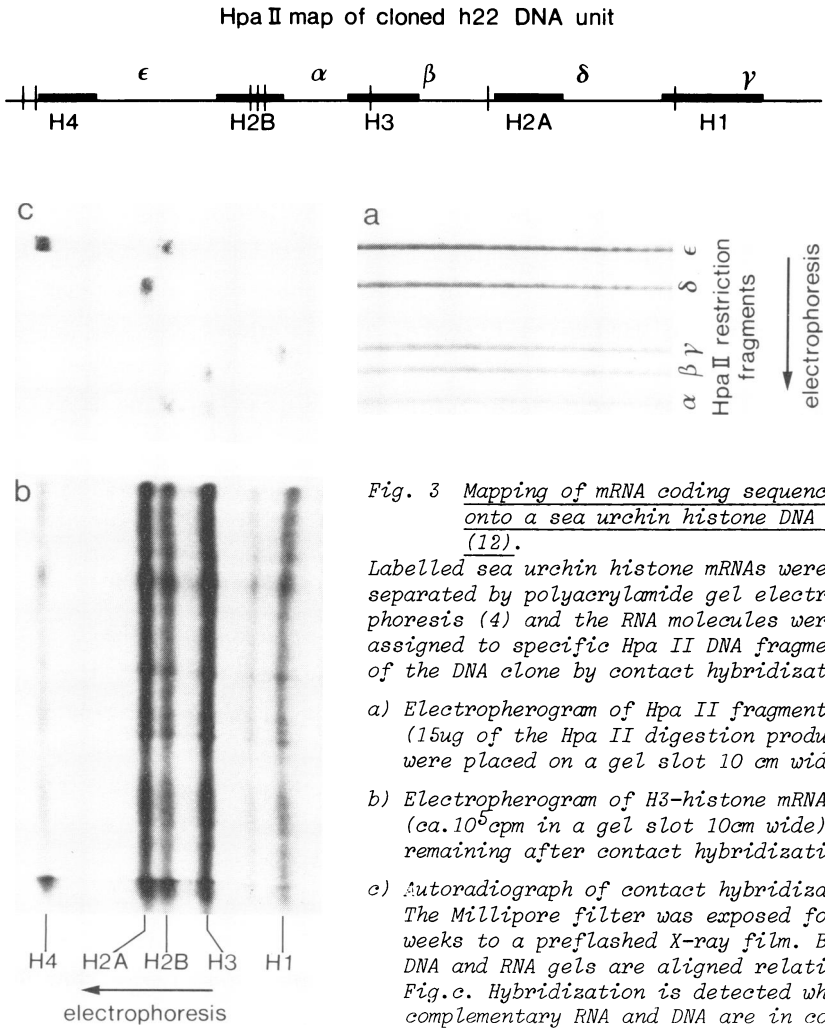

Fig. 3 *Mapping of mRNA coding sequences onto a sea urchin histone DNA clone (12).*

Labelled sea urchin histone mRNAs were separated by polyacrylamide gel electrophoresis (4) and the RNA molecules were assigned to specific Hpa II DNA fragments of the DNA clone by contact hybridization.

a) Electropherogram of Hpa II fragments (15ug of the Hpa II digestion products were placed on a gel slot 10 cm wide).

b) Electropherogram of H3-histone mRNAs (ca. 10^5cpm in a gel slot 10cm wide) remaining after contact hybridization.

c) Autoradiograph of contact hybridization. The Millipore filter was exposed for two weeks to a preflashed X-ray film. Both DNA and RNA gels are aligned relative to Fig.c. Hybridization is detected where complementary RNA and DNA are in contact with one another. The hybridization pattern is in perfect agreement with the map obtained from the sequencing experiments (12).

restriction of the DNA by an endonuclease should be avoided since this would complicate considerably the hybridization pattern obtained. However, in systems of low complexity partial restriction of the DNA can be useful since in this way the map position of neighbouring genes can be determined.

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REFERENCES

1. Southern, E.M. (1975) J.Mol.Biol. 98, 503-517
2. Alwine, J.C., Kemp, D.J. and Stark, G.R. (1977) Proc.Natl. Acad.Sci.USA 74, 5350-5354
3. Burckhardt, J. and Birnstiel, M.L. (1978) J.Mol.Biol. 118, 69-79
4. Gross, K., Probst, E., Schaffner, W. and Birnstiel, M. (1976) Cell 8, 455-469
5. Clarkson, S.G., Smith, H.O., Schaffner, W., Gross, K.W. and Birnstiel, M.L. (1976) Nucl.Acids Res. 3, 2617-2632
6. Baily, J.M. and Davidson, N. (1976) Analyt.Biochem. 70, 75-85
7. Laskey, R.A. and Mills, A.D. (1977) FEBS Letters 82, 314-316
8. Bonner, W.M. and Laskey, R.A. (1974) Europ.J.Biochem. 46, 83-88
9. Boseley, P.G., Tuyns, A. and Birnstiel, M.L. (1978) Nucl. Acids Res. 5, 1121-1137
10. Sato, S., Hutchison III, C.A. and Harris, J.I. (1977) Proc. Natl.Acad.Sci.USA 74, 542-546
11. Schaffner, W., Gross, K., Telford, J. and Birnstiel, M.L. (1976) Cell 8, 471-478
12. Schaffner, W., Kunz, G., Daetwyler, H., Telford, J., Smith, H.O. and Birnstiel, M.L. (1978) Cell 14, 655-671