Eur. J. Biochem. *171*, 565 – 569 (1988)
© FEBS 1988

RNA-binding properties of hnRNP proteins

Johannes SCHENKEL [1,2], Constantin E. SEKERIS [3], Angel ALONSO [4] and Ekkehard K. F. BAUTZ [1]

[1] Molekulare Genetik der Universität, Heidelberg
[2] Institut für Immunologie der Universität, Heidelberg
[3] The National Hellenic Research Foundation, Biological Research Center, Athens
[4] Institut für Experimentelle Pathologie, Deutsches Krebsforschungszentrum, Heidelberg

(Received July 3/October 29, 1987) – EJB 87 0769

The RNA-binding properties of the hnRNP monoparticle proteins were examined using a renaturing blotting procedure. All 'core' proteins are able to bind single-stranded nucleic acids, probably not sequence-specific. The core proteins C_1 and, in one case A_2 and B_2, are able to bind nucleic acids which are double-stranded or which show a high degree of base-paired regions, among them U1 snRNA, whereas A_1, B_1 and C_2 are unable to bind base-paired nucleic acids. The characteristics of C_1 in binding base-paired nucleic acids are especially interesting, since the involvement of C_1 in the splicing process has been described.

The role of hnRNA-associated proteins in the post-transcriptional modification of RNA is beginning to be understood at the molecular level. Many components, RNAs as well as proteins, are involved in the intricate splicing process. Crucial in understanding these complicated mechanisms is the knowledge of the associations between the participating components and the three-dimensional structure of the hnRNP particles.

hnRNPs can be isolated in the monomeric form of about 40S, composed of nascent RNA with a length of about 800 nucleotides and of proteins of which the following six 'core' proteins are most prominent: A_1 (32 kDa), A_2 (34 kDa), B_1 (36 kDa), B_2 (37 kDa); C_1 (42 kDa) and C_2 (44 kDa) [1]. All core proteins are basic, except one (acidic) protein of group C.

The nature of the association between core proteins and the RNA has been the subject of previous reports [1 – 12]. In those studies, however, the binding properties of a mixture of all core proteins were investigated. In this report we have observed the binding characteristics of individual monoparticle proteins to hnRNA and various other RNA types by separating the proteins using SDS/polyacrylamide gel electrophoresis and evaluating their binding properties to radioactively labelled RNA by a blotting procedure.

MATERIALS AND METHODS

HeLa S-3 cells were grown in Belco-spinner culture in minimal essential medium containing 8% fetal calf serum up to a density of $(0.5 - 1) \times 10^6$ cells/ml [13].

Correspondence to J. Schenkel, Institut für Immunologie der Universität, Im Neuenheimer Feld 305, D-6900 Heidelberg, Federal Republic of Germany

Abbreviations. hnRNA, heterogenous nuclear ribonucleic acid; hnRNP, heterogenous nuclear ribonucleoprotein particle; snRNA, small nuclear ribonucleic acid; NEPHGE, non equilibrium pH gradient electrophoresis.

Enzymes. Polynucleotide kinase (EC 2.7.1.78); RNA polymerase (EC 2.7.7.6); DNA polymerase (EC 2.7.7.7); DNase I (EC 3.1.21.1); RNase A (EC 3.1.27.5).

Isolation of hnRNP particles

1×10^9 HeLa cells were resuspended in 30 ml Tris/Mg/K buffer (50 mM Tris/HCl pH 7.0, 10 mM $MgCl_2$, 25 mM KCl) containing 250 mM sucrose (RNase-free) and 1 mM phenylmethylsulfonyl fluoride and homogenized with 20 strokes in a Potter homogenizer (1000 rpm, 4°C). Nuclei and cytoplasm were separated at $2600 \times g$. The nuclear pellet was dissolved in 250 ml Tris/Mg/Triton buffer (20 mM Tris/HCl pH 7.0, 1.5 mM $MgCl_2$, 0.02% Triton X-100), homogenized twice in a Potter homogenizer and pelleted by a $400 \times g$ centrifugation step. Finally the nuclei were dissolved in ice-cold bidistilled water and sedimented at $650 \times g$.

The nuclei were allowed to stand for 45 min in 3 ml ice-cold pH 8 buffer solution (10 mM Tris/HCl pH 8, 140 mM NaCl, 1 mM $MgCl_2$). The hnRNP were extracted by three sonications (5 s each). The nuclear debris was removed by centrifugation at $16500 \times g$ and the supernatant incubated for 30 min at 37°C (to activate endogenous nucleases). The 40S hnRNP particles were isolated following centrifugation on a 10 – 40% sucrose gradient, $\omega^2 t = 5.4 \times 10^{11}$ rad^2 s^{-1}. The gradients were fractionated with an ISCO density gradient fractionator, the peak fractions containing the 40S hnRNP pelleted at $\omega^2 t = 1 \times 10^{12}$ rad^2 s^{-1} [14, 15].

hnRNA was isolated by phenol extraction of the sonicated supernatant of the pH 8 buffer extract, prior to incubation at 37°C; hnRNP-RNA was isolated by phenol extraction of the 40S peak fractions.

Radioactive labelling

The nucleic acids were radiolabelled with ^{32}P by well-known procedures, i.e. nick translation [16], radiolabelling at the 5′ end [17], *in vitro* transcription (Deuschle, personal communication) [2, 18 – 20].

Double-stranded RNA was prepared using a murine H1° cDNA clone and phage T_3 and T_7 RNA polymerases. Both RNAs were taken up together in 2 × NaCl/Cit (60 mM sodium citrate pH 7.0, 600 mM NaCl), incubated at 65°C and digested with 30 µg/ml RNase A. The undigested fragment was

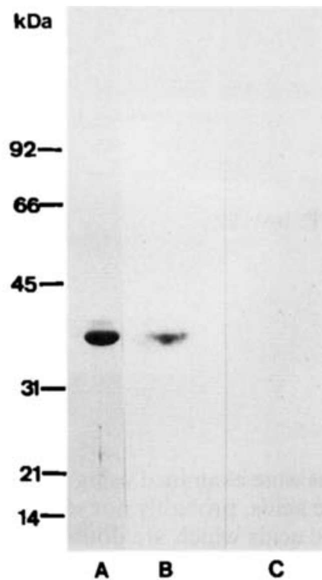

Fig. 1. *RNA binding properties of the gene-32 protein of phage T_4.* Lane A shows the gene-32 protein of phage T_4, separated in SDS-PAGE and stained with Coomassie blue. The same protein was renatured, transferred to nitrocellulose as described in Material and Methods and afterwards incubated with at the 5′-end-radiolabelled hnRNA (B) and ribosomal RNA (C)

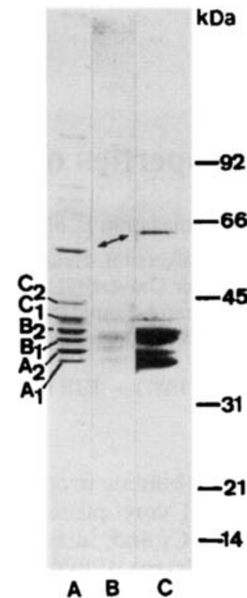

Fig. 2. *Nuclear-RNA-binding properties of hnRNP proteins.* Lane A shows hnRNP proteins isolated from HeLa cells, separated in an 8–15% SDS-PAGE and stained with Coomassie blue. The same proteins were renatured, transferred to nitrocellulose as described in Materials and Methods and afterwards incubated with radiolabelled hnRNP-RNA (B) and hnRNA (C)

separated from the oligonucleotides using Sephadex G-75 chromatography.

Protein blotting procedure

Proteins were separated on 8–15% SDS/polyacrylamide gradient gels as described by Laemmli [21]. The two-dimensional non-equilibrium pH gradient electrophoresis (NEPHGE)/SDS gel system was published by O'Farell et al. [22]. The proteins were renatured by gently shaking the gel for 3–5 h in a buffer containing 4 M urea, 10 mM Tris/HCl pH 7.0, 2 mM EDTA, 0.1 mM dithiothreitol and 50 mM NaCl [23]. The treated proteins were transferred overnight onto nitrocellulose in a 25 mM sodium-phosphate-containing buffer, pH 6.8.

Each of the radiolabelled nucleic acid probes (2 µg, 5×10^6 cpm) was dissolved in a small volume of standard binding buffer (10 mM Tris/HCl pH 7.0, 0.02% Ficoll 400, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 50 mM NaCl, 1 mM EDTA) and incubated with the protein blot overnight at room temperature. The blots were washed in the same buffer for 1 h. The dried nitrocellulose sheets were exposed for several hours.

In preincubation experiments the blots were, prior to the use of radioactive labelled RNAs, incubated with 20 µg/ml non-labelled nucleic acids in standard binding buffer for several hours and washed twice in the same buffer.

RESULTS

To examine the nucleic-acid-binding properties of SDS-PAGE-separated hnRNP proteins of HeLa cells, we used the blotting procedure of Towbin et al. [24]. The protein were renatured in a urea-containing buffer according to Bowen et al. [23] and transferred to nitrocellulose in a phosphate-buffered electroblotting system.

Standardization of the blotting procedure

In a first series of experiments we standardized the blotting procedure. A quantitative transfer of all proteins was achieved only when gradient gels were used. Amido black staining of the nitrocellulose sheet after the transfer demonstrated a satisfactory transfer of all hnRNP proteins, independent of their molecular size.

The incubation and washing steps were performed according to Bowen et al. [23]. Other methods used (for example [25]) did not yield satisfactory results.

To test the fidelity of the blotting procedure for the RNA-binding properties of proteins, we followed the binding of the well-characterized gene-32 protein of phage T_4, which only binds single-stranded RNA [26–28]. The protein was subjected to SDS-PAGE, renatured and transferred to nitrocellulose as described above. The blotted protein was incubated with radiolabelled hnRNA or rRNA. As shown in Fig. 1B and C, the gene-32 product was able to bind to hnRNA but not to rRNA, the latter being an RNA species which is characterized by its high content of base-paired regions.

hnRNP proteins bind hnRNA

Having standardized the experimental system, we checked the binding properties of hnRNP proteins to radiolabelled RNAs, which were obtained from two different nuclear sources. In one case the RNA was purified from isolated hnRNPs following *in vitro* transcription of HeLa nuclei by the method of Manley et al. [19], modified by Economides and Pederson [2] (Fig. 2B). The other RNA was extracted from the hnRNP-containing supernatant of purified nuclei with 0.14 M NaCl and radiolabelled at the 5′ end according to Richardson [17] (Fig. 2C). The results from such experiments showed that all core proteins were able to bind these RNAs. Additional proteins in the 90-kDa region were also able to

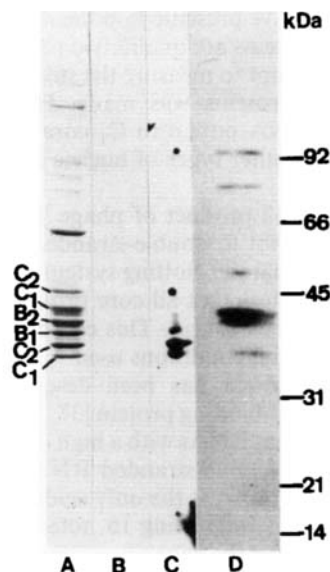

Fig. 3. *DNA-binding properties of hnRNP proteins using presaturation experiments.* Lane A shows hnRNP proteins isolated from HeLa cells, separated in an 8–15% SDS-PAGE and stained with Coomassie blue. The same proteins were renatured, transferred to nitrocellulose as described in Materials and Methods and afterwards incubated with at the 5'-end-radiolabelled hnRNA after preincubation with single-stranded DNA (B) and double-stranded DNA (C). Lane D shows the hnRNP proteins incubated with nick-translated pBR325 plasmid DNA without presaturation

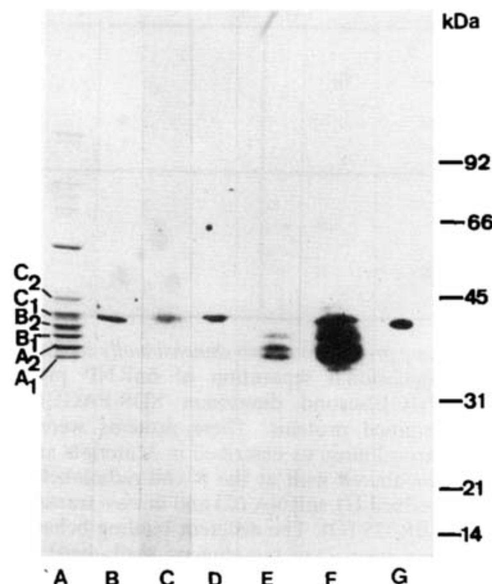

Fig. 4. *Binding properties of hnRNP proteins to different RNA classes.* Lane A shows hnRNP proteins isolated from HeLa cells separated in an 8–15% SDS-PAGE and stained with Comassie blue. The same proteins were renatured, transferred to nitrocellulose as described in Materials and Methods and afterwards incubated with ribosomal RNA radiolabelled at the 5' end (B), transfer RNA radiolabelled at the 5' end (C), *in vitro* transcribed U1 snRNA (D), RNA of a murine H1° clone transcribed by phage T_7 RNA polymerase (short-time-exposed E, overexposed F) and the double-stranded, annealed and RNase-A-treated RNA of this clone transcribed by the phage T_3 and T_7 RNA polymerases (G)

bind to hnRNP-derived RNA, but not to the *in vitro* transcription product. This might reflect differences in the RNA composition of the two RNA preparations.

One group C core protein binds double-stranded DNA

In an attempt to detect specific binding sites of hnRNA to hnRNP proteins we presaturated the protein blots either with single-stranded or double-stranded DNA prior to incubation with radiolabelled hnRNA. As shown in Fig. 3B, no binding of hnRNA to hnRNP proteins was observed when presaturation with single-stranded DNA was performed.

However, when presaturation with double-stranded DNA was applied instead, the group A and B core proteins were able to bind with hnRNA (Fig. 3C).

As an extension to these experiments we incubated the hnRNP proteins with nick-translated plasmid pBR325 DNA. Two of the larger core proteins showed binding: C_1 and (probably) B_2 (Fig. 3D). This is in accord with the results obtained from double-stranded-DNA-presaturation experiments described above, and show that group C core proteins can bind base-paired nucleic acids whereas all core proteins are able to bind single-stranded nucleic acids.

Core protein C_1 is able to bind base-paired RNA

To characterize further the binding properties of core proteins to nucleic acids we performed experiments with other types of RNA, such as transfer RNA, 18S ribosomal RNA and U1 small nuclear (sn)RNA, which all possess a high percentage of base-paired regions. As U1 snRNA we used the transcription product of a *Drosophila melanogaster* U1 snRNA clone [29].

The results of these experiments are shown in Fig. 4. Only one core protein, C_1, showed high levels of binding to ribosomal RNA (Fig. 4B), transfer RNA (Fig. 4C) and U1 snRNA (Fig. 4D).

Another approach was used to verify these results: *in-vitro*-transcribed single-stranded RNA (using RNA polymerase of phage T_7) and double-stranded RNA (after annealing and RNase A treatment of the transcripts using the RNA polymerases of phage T_3 and phage T_7) of an H1° clone were incubated with the protein blots. Whereas the single-stranded RNA binds all core proteins with different strength (as shown by short-time-exposed and overexposed blots Fig. 4E and F), the double-stranded RNA is only bound by C_1 (Fig. 4G).

To establish more precisely which of the core proteins bind to the various types of nucleic acids used in the binding experiments, we separated the hnRNP proteins in two-dimensional gel electrophoresis prior to the binding experiments (Fig. 5). Using hnRNA we confirmed that all core proteins were able to bind this RNA, except for one isoelectric form of C_1 (Fig. 5B).

U1 snRNA was bound mainly by one isoelectric form of C_1 (Fig. 5C). Furthermore, binding could also be observed to a lesser extent with core proteins A_2 nd B_2. The discrepancies in the data of one-dimensional and two-dimensional gel electrophoresis will be discussed later.

Lastly we used nucleic acids with a known lack of secondary structure for the binding experiments. According to a computer check, such an RNA can be found in the *Bam*HI fragment of clone F1 of *D. melanogaster* transcribed *in vitro* [30], which codes for the elongation factor EF1 (Hovemann, personal communication).

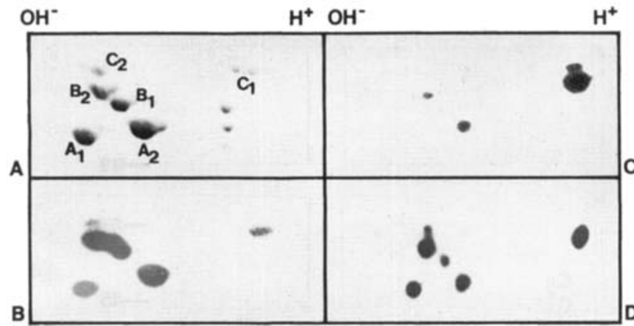

Fig. 5. *RNA-binding properties of two-dimensionally separated hnRNP proteins.* Two-dimensional separation of hnRNP proteins (first dimension NEPHGE/second dimension SDS-PAGE). (A) The Coomassie-blue-stained proteins. These proteins were renatured, transferred to nitrocellulose as described in Materials and Methods and afterwards incubated with at the 5′-end-radiolabelled hnRNA (B), *in vitro* transcribed U1 snRNA (C) and *in vitro* transcribed RNA of the plasmid pBR325 (D). The different binding behaviour of U1 snRNA to one-dimensional and two-dimensionally separated hnRNP proteins should be due to the use of urea and ampholines

The binding behaviour of hnRNP proteins to this RNP, tested in a two-dimensional electrophoretic system (see Fig. 5D with regards to the RNA of plasmid pBR325), is similar to the binding properties to hnRNA or the T_7 RNA polymerase transcript.

DISCUSSION

With the results shown above no firm evidence for a sequence-specific binding between hnRNA and core proteins could be found. This is in accord with Pulman and Martin [7] and Wilk et al. [12], who were able to reconstitute hnRNP-like particles using the core proteins and any type of single-stranded nucleic acid. It was impossible, however, to reconstitute these particles with double-stranded nucleic acids. Beyer et al. [14, 31] concluded from electron-microscopic observations that these proteins bind to the primary transcript at varying distances which are constant for the same transcript.

In this paper we examined the RNA-binding properties of the individual hnRNP proteins in an attempt to obtain more information on the structure of the hnRNP particles.

Our approach was to separate the hnRNP proteins by SDS/polyacrylamide gel electrophoresis and to transfer the separated proteins onto nitrocellulose. After studying the properties of the blotting system and considering the relevant publications [23, 32–35] we concluded that a renaturation of the proteins was necessary and also a prerequisite for the binding studies. Using the well-described western blotting system it was impossible to renature the proteins after the transfer. Also, none of the described blotting procedures, including diffusion blotting [23] or vacuum blotting [36], gave us satisfactory results.

Only the renaturation procedure [23] in combination with electrotransfer in a phosphate buffer proved satisfactory and reproducible.

The major advantage of our experimental approach lies in the renaturation step. Although there is no possibility of measuring the degree of renaturation of the proteins in SDS gels, the reproducibly clear results suggest extensive renaturation. Without this renaturation step it was impossible to detect any binding capacity of the denatured proteins.

The results we have presented on the nucleic-acid-binding capacity of core proteins are qualitative rather than quantitative. Hence, no attempt to measure the stoichiometry of binding to individual proteins was made. However, the large amount of U1 snRNA bound to C_1 core protein (Fig. 5C), compared with the other types of nucleic acids used, is noteworthy.

Using the gene-32 product of phage T_4, which binds to single-stranded but not to double-stranded nucleic acids [27, 28], we could show that the blotting system works as expected. Thus our findings show that all core proteins bind any type of single-stranded nucleic acid. This can be considered valid under the experimental conditions used.

The A_1 core protein has been described as a single-stranded-nucleic-acid-binding protein [37, 38]. In addition, we demonstrated here that RNAs with a high degree of secondary structure as well as double-stranded RNAs bind to C_1 and partly B_2 core proteins. C_1 is the only acidic one of the group C core proteins. It is interesting to note that not all of its isomers appear to have the capacity to bind RNA.

In some cases, i.e. in experiments involving core proteins separated by two-dimensional gel electrophoresis, we observed binding of A_2 core protein to RNA of high secondary structure. This difference is not yet understood, but could be due to the effects of ampholines and/or urea during the preparation of the samples and the pH-gradient gel electrophoresis. In this context it is interesting that differences in the molecular mass of hnRNA protein in the presence and absence of urea during the electrophoretic separation were detected by Wilk et al. [39].

Regarding the binding of U1 snRNA, it is interesting to note that it binds to the same core protein which binds base-paired nucleic acids (C_1). U1 snRNA has internal double-stranded regions but can also form base-paired structures with hnRNA [40, 41]. Such structures have been identified as splice sites [42].

Choi et al. [43] are able to inhibit splicing with an antibody against C_1. The hn/snRNA hybrids have been described as being RNase-resistant [44].

Considering the evidence cited above, it is probable that if there is a direct contact of a hnRNP protein with the double-stranded hn/snRNA region, this protein is most likely to be C_1.

Further studies concerning the sites of interaction of C_1 core proteins with U1 and other snRNAs with the aid of nuclease digestion and ultraviolet cross-linking experiments are currently underway in our laboratory.

We thank Dr F. A. Bautz, for providing us with the HeLa cells and Dr A. Guialis for her helpful disussions and her excellent cooperation. The work was supported by a grant of the *Stiftung Volkswagenwerk*.

REFERENCES

1. Beyer, A. L., Christensen, M. E., Walker, B. W. & Le Stourgeon, W. M. (1977) *Cell 11*, 127–138.
2. Economides, I. V. & Pederson, T. (1982) *Proc. Natl Acad. Sci. USA 79*, 1469–1473.
3. Economides, I. V. & Pederson, T. (1983) *Proc. Natl Acad. Sci. USA 80*, 1599–1602.
4. van Eekelen, C. A. G. & van Venrooij, W. J. (1981) *J. Cell Biol. 88*, 554–563.
5. Guialis, A., Arvanitopoulou, A., Patrinou-Georgoula, M. & Sekeris, C. E. (1983) *FEBS Lett. 151*, 127–133.

6. Jacob, M., Devilliers, G., Fuchs, J. P., Gallinaro, H., Gattoni, R., Judes, C. & Stevenin, J. (1981) in *The cell nucleus*, vol. 8 (Bush, H., ed.) pp. 194–246, Academic Press, New York.
7. Pullman, J. M. & Martin, T. E. (1983) *J. Cell Biol. 97*, 99–111.
8. Samarina, O. P. & Krichevskaya, A. A. (1981) in *The cell nucleus*, vol. 9 (Bush, H., ed.) pp. 2–48, Academic Press, New York.
9. Sekeris, C. E. & Niessing, J. (1975) *Biochem. Biophys. Res. Commun. 62*, 642–650.
10. Sekeris, C. E. & Guialis, A. (1981) in *The cell nucleus*, vol. 8 (Bush, H., ed.) pp. 247–259, Academic Press, New York.
11. Le Stourgeon, W. M., Lothstein, L., Walker, B. W. & Beyer, A. L. (1981) in *The cell nucleus*, vol. 9 (Bush, H., ed.) pp. 49–87, Academic Press, New York.
12. Wilk, H. E., Angeli, G. & Schäfer, K. P. (1983) *Biochemistry 22*, 4592–4600.
13. Hügle, B., Guldner, H., Bautz, F. A. & Alonso, A. (1982) *Exp. Cell Res. 142*, 119–126.
14. Beyer, A. L., Bouton, A. H. & Miller, O. L. (1981) *Cell 26*, 155–165.
15. Louis, C. & Sekeris, C. E. (1976) *Exp. Cell Res. 102*, 317–328.
16. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol. 113*, 237–251.
17. Richardson, C. C. (1965) *Proc. Natl Acad. Sci. USA 54*, 158–165.
18. Green, M. R., Maniatis, T. & Melton, D. A. (1983) *Cell 32*, 681–694.
19. Manley, J. L., Sharp, P. A. & Geftler, M. L. (1979) *J. Mol. Biol. 135*, 171–197.
20. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nuleic Acids Res. 12*, 7035–7056.
21. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680–685.
22. O'Farell, P. Z., Goodman, H. M. & O'Farell, P. H. (1977) *Cell 12*, 1133–1142.
23. Bowen, B., Steinberg, J., Laemmli, U. K. & Weintraub, H. (1980) *Nucleic Acids Res. 8*, 1–20.
24. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl Acad. Sci. USA 76*, 4350–4354.
25. Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
26. Alberts, B. M. & Frey, L. (1970) *Nature (Lond.) 227*, 1313–1318.
27. Delius, H., Westphal, H. & Axelrod, N. (1973) *J. Mol. Biol. 74*, 677–687.
28. Wu, M., Roberts, R. J. & Davidson, N. (1977) *J. Virol. 21*, 766–777.
29. Alonso, A., Jorcano, J. L., Beck, E., Hovemann, B. & Schmidt, T. (1984) *J. Mol. Biol. 180*, 825–836.
30. Walldorf, U., Hovemann, B. & Bautz, E. K. F. (1985) *Proc. Natl Acad. Sci. USA 82*, 5795–5799.
31. Beyer, A. L., Miller, O. L. & McKnight, S. L. (1980) *Cell 20*, 75–84.
32. Karawaya, E. & Wilson, S. (1982) *J. Biol. Chem. 257*, 13129–13134.
33. Karawaya, E., Swack, J. & Wilson, S. (1983) *Anal. Biochem. 135*, 318–325.
34. Rosenthal, A. L. & Lacks, S. A. (1977) *Anal. Biochem. 80*, 76–90.
35. Weber, K. & Kuter, D. J. (1971) *J. Biol. Chem. 246*, 4504–4509.
36. Peferoen, M., Huybrechts, R. & De Loof, A. (1982) *FEBS Lett. 145*, 369–372.
37. Pandolfo, M., Valentini, O., Biamonti, G., Morandi, C. & Riva, S. (1985) *Nucleic Acids Res. 13*, 6577–6590.
38. Riva, S., Morandi, P., Tsoulfsas, P., Biamonti, G., Werr, H., Henrich, B., Melchers, K. & Schäfer, K. P. (1986) in *RNA processing*, p. 142, Cold Spring Harbour, New York.
39. Wilk, H. E., Werr, H., Friedrich, D., Kiltz, H. H. & Schäfer, K. P. (1985) *Eur. J. Biochem. 146*, 71–81.
40. Deimel, B., Louis, C. & Sekeris, C. E. (1977) *FEBS Lett. 73*, 80–84.
41. Flytzanis, C., Alonso, A., Louis, C., Krieg, L. & Sekeris, C. E. (1978) *FEBS Lett. 96*, 201–206.
42. Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L. & Steitz, J. A. (1980) *Nature (Lond.) 283*, 220–224.
43. Choi, Y. D., Grabowsky, P. J., Sharp, P. A. & Dreyfuss, G. (1986) *Science (Wash. DC) 231*, 1534–1539.
44. Molnar, J. & Samarina, O. P. (1975) *Mol. Biol. Rep. 2*, 1–10.