DETECTION OF NUCLEASES DEGRADING DOUBLE HELICAL RNA AND OF NUCLEIC ACID-BINDING PROTEINS FOLLOWING SDS–GEL ELECTROPHORESIS

Janine HUET, André SENTENAC and Pierre FROMAGEOT
Service de Biochimie, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, BP no. 2, 91190 Gif-sur-Yvette, France

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1. Introduction

Recently Rosenthal and Lacks showed that nucleases can be revealed following their electrophoretic separation in sodium dodecylsulfate–polyacrylamide slab gels containing RNA and DNA [1]. Upon removal of the detergent, the nucleases renature and digest the substrate incorporated in the gel. Bands of degradation are revealed by staining the surrounding nucleic acid with ethidium bromide [1]. However this procedure does not allow the simultaneous staining of the proteins responsible for degradation of the nucleic acids. Furthermore, it remains limited to cases where the substrate is available in large quantities. In the present work, instead of using ethidium bromide, the nucleic acids are revealed by autoradiography. This allows the detection of specific nucleases using only trace amounts of radioactive polymer. The second advantage is that the protein bands can be stained with Coomassie blue before the gel is subjected to autoradiography. Finally, on the same slab gel, nucleic acid-binding proteins which promote a local concentration of labelled polymer during incubation, appear as dark bands on the autoradiogram. The technique was used to screen ribonucleases degrading RNA·DNA hybrids [2] or double-stranded RNA [3] in *Saccharomyces cerevisiae* and *Escherichia coli* crude extracts.

2. Materials and methods

2.1. *Nucleic acids*

^{32}P-Labelled RNAs were synthesized in vitro with *E. coli* or yeast RNA polymerases and the appropriate synthetic template, as in [4,5], followed by hydrolysis of the template by pancreatic DNase when necessary (fig.3). Other substrates were prepared as in [5].

2.2. *Cell extracts*

Saccharomyces cerevisiae (4094 Bα Ad_2 Ur_1) were suspended in 5 ml of one of the following extraction buffers and disrupted as a frozen paste by a single passage through an Eaton press. The crude extract was prepared by centrifugation for 60 min at 106 000 × *g* in a 50 Ti rotor of a Beckman ultracentrifuge. Extraction buffers were:

(a) 0.05 M Tris–maleate, pH 6.5, 5 mM $MgCl_2$, 10% glycerol;
(b) As (a) plus 75 mM KCl;
(c) 0.05 M Tris–HCl, pH 8, 5 mM $MgCl_2$, 10% glycerol;
(d) 0.02 M Tris–HCl, pH 8, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.3 M ammonium sulfate and 4 mM PMSF (phenylmethansulfonylfluorid).

Log phase *E. coli* MRE 600 (RNase 1$^-$) were disrupted with a Manton-Gaulin homogenizer in a buffer containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 M dithiothreitol, 0.25 M KCl, 1 mM PMSF and 5% glycerol. The extract was treated with DNase I as in [6] and cell debris and ribosomes were removed in one step by 90 min centrifugation at 106 000 × *g* in a 50 Ti rotor.

2.3. *Electrophoresis*

Polyacrylamide slab gels (130 × 80 × 1 mm) were prepared as in [7]. The ^{32}P-labelled polymer was

incorporated into the running gel mixture before polymerisation. Usually 300 000–400 000 cpm (~1 nmol ribonucleoside) were added/20 ml running gel. With this amount of radioactivity the subsequent autoradiography can be completed in 1 day. Protein samples (30–70 μg proteins in 25 μl) were prepared in Laemmli sample buffer which contains 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue and 0.0625 M Tris–HCl, pH 6.8. Except when indicated, the protein sample was not heated prior to electrophoresis which was run for 4 h, at room temperature, at constant 20 mA.

2.4. *Detection of nucleases in the gel*

Upon completion of the electrophoresis, the gel was subjected to a preliminary washing at room temperature with two changes of 300 ml water for 90 min, with gentle shaking, whereafter it was incubated with 300 ml appropriate buffer.

After 1 h at room temperature, the solution was replaced by 300 ml fresh buffer and incubation was continued, always with gentle shaking, at 37°C, for 18–40 h. Standard incubation buffer contained 40 mM Tris–HCl, pH 8, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM dithiotreitol, 75 mM NaCl. Modifications of these conditions are specified in the figure legends. After incubation, the gel was soaked in a mixture of methanol/acetic acid/water (5 : 1 : 5) for 30 min at room temperature, and the protein bands then revealed by staining with 0.1% Coomassie brilliant blue dissolved in the above methanol/acetic acid/water mixture. Excess dye was removed by shaking the gel with a suspension of resin AGI X 2 (Cl^-) in methanol/acetic acid/water (30 : 7.5 : 62.5). The gel was dried and subjected to autoradiography on a Kodirex film (Kodak). Nucleases appear as clear bands and nucleic acid binding proteins as dark bands on the autoradiogram. Molecular weights were estimated using the subunits of yeast RNA polymerase A (see fig.3) as markers [8].

3. Results

3.1. *Ribonucleases acting on RNA·DNA hybrids*

Figure 1 shows the resolution of proteins from crude extracts of *S. cerevisiae* or *E. coli* in a 12.5% polyacrylamide gel containing a synthetic ^{32}P-labelled RNA·DNA hybrid. The clear bands on the corresponding autoradiogram reveal the presence of nucleases. *E. coli* crude extracts displayed a main band of degradation of 26 000 daltons (26 kD), together with two other bands of 19 kD and 17 kD. Identical patterns of degradation were obtained with ^{32}P-labelled $(rA)_n.(dT)_n$ or ^{32}P-labelled $(rG)_n.(dC)_n$. Yeast extracts showed two strong bands of 28 kD and 31 kD or only the 28 kD band depending on the pH used to prepare the crude extract. The differential solubilization of yeast nucleases degrading ^{32}P-labelled $(rA)_n.(dT)_n$ is illustrated in fig.2. Both the pH and ionic strength of the extraction buffer influences the pattern of nucleases observed on the gel. Interestingly, a good resolution of proteins of large molecular weight was obtained by heating the extract in sample buffer. This treatment was not detrimental to most of the nuclease activities.

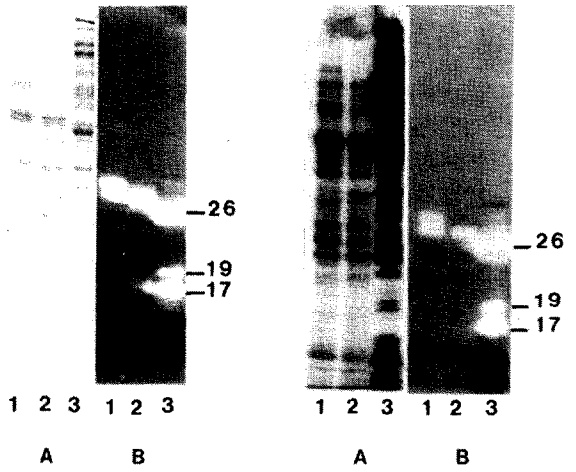

Fig.1. Detection of nucleases degrading RNA·DNA hybrids. Yeast and *E. coli* extracts were subjected to electrophoresis in a 12.5% SDS–polyacrylamide gel containing ^{32}P-labelled $(rA)_n.(dT)_n$ as substrate (left panel), or ^{32}P-labelled $(rG)_n.(dC)_n$ (right panel). The gel slab was washed and incubated as in section 2. Some molecular weights are indicated in the figure (× 10^{-3}). Wells contained: (1) yeast crude extract (buffer a, pH 6.5); (2) yeast crude extract (buffer c, pH 8); (3) *E. coli* extract. (A) stained with Coomassie blue; (B) autoradiogram thereof.

3.2. *Ribonucleases acting on* $(rA)_n$

The specificity of hybrid degradation was investi-

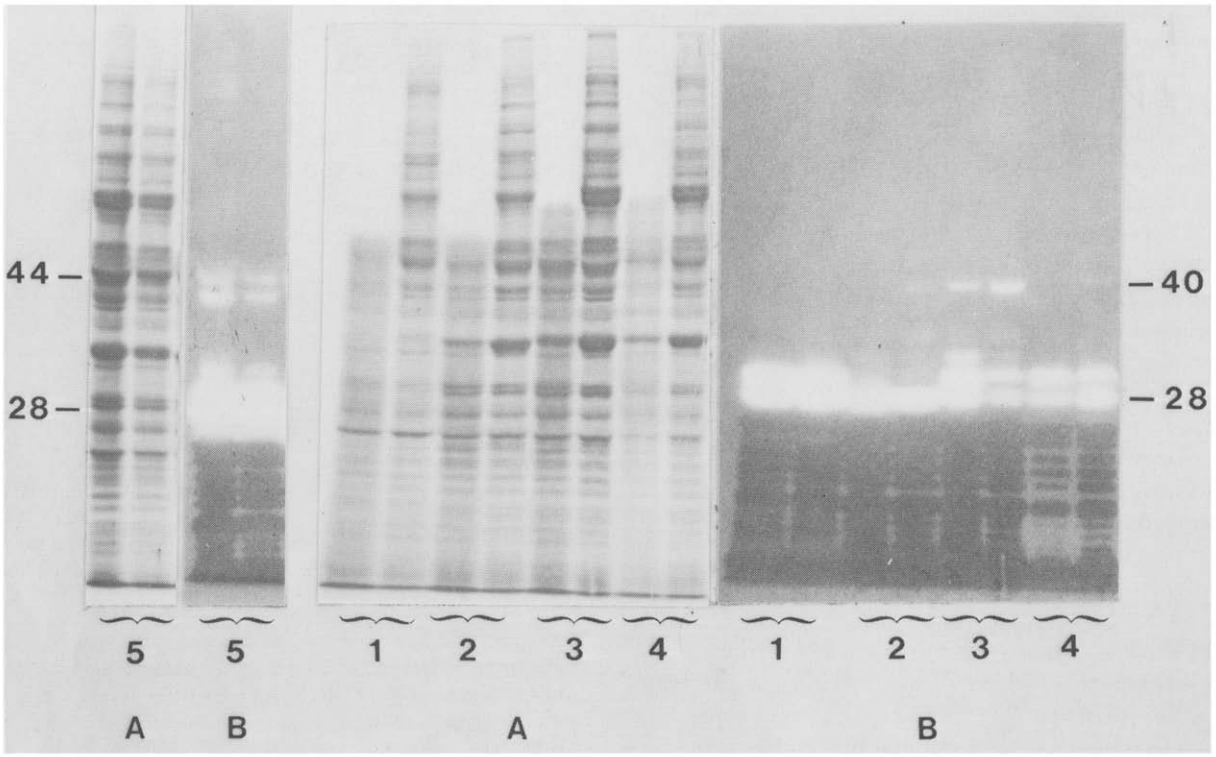

Fig.2. Differential solubilization of yeast nucleases degrading $(rA)_n \cdot (dT)_n$. Electrophoresis of yeast extracts in a 10% polyacrylamide gel containing ^{32}P-labelled $(rA)_n \cdot (dT)_n$ was conducted as in fig.1. Yeast crude extracts were prepared as in section 2, using different extraction buffers: (1) buffer d (pH 8, high salt); (2) buffer c (pH 8, low salt); (3) buffer a (pH 6.5, low salt); (4) buffer b (pH 6.5, 75 mM KCl); (5) buffer a (pH 6.5; low salt). The gel corresponding to sample 5 was incubated for 17 h at 37°C, in a buffer containing 0.01 M Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM $MnCl_2$ and 75 mM NaCl. All extracts were run in duplicate, that on the right of each pair having been heated in sample buffer for 15 s at 90°C prior to electrophoresis. (A) stained gel; (B) autoradiogram thereof. Certain molecular weights (× 10^{-3}) are presented.

gated using ^{32}P-labelled $(rA)_n$ as substrate. Surprisingly, no band of degradation was observed with this substrate in yeast crude extracts (fig.3). This suggested that the bands detected with the hybrid $(rA)_n \cdot (dT)_n$ corresponded to true RNase H. (This conclusion was further suggested by the results obtained with double-stranded RNA substrates.) On the other hand, *E. coli* extracts displayed one strong and several minor bands with the same $(rA)_n$ substrate (fig.3). The strong one, of 26 kD, was observed in $(rA)_n \cdot (dT)_n$ and $(rG)_n \cdot (dC)_n$ gels. The two small nucleases (19 kD and 17 kD) active on the hybrids were absent; instead, additional bands were found, of relatively large molecular weight (68–54 kD), which had remained unnoticed with the hybrid substrates. Therefore the technique allowed the detection of at least two distinct forms of RNase H in *E. coli* crude extracts, together with several $(rA)_n$ hydrolases, and a very active but nonspecific ribonuclease (with respect to template) which probably corresponds to the single-stranded RNase of 26 kD observed [1].

3.3. *Ribonucleases acting on double-stranded RNA*

In gels containing ^{32}P-labelled $(rG)_n \cdot (rC)_n$, a strong activity was detected in *E. coli* crude extracts, probably corresponding to the non-specific nucleases of 26 kD [1]. Several minor or diffuse bands of higher molecular weight were also weakly visible (fig.4). The same observations were made with gels containing the double-stranded copolymer ^{32}P-labelled r(A–U)

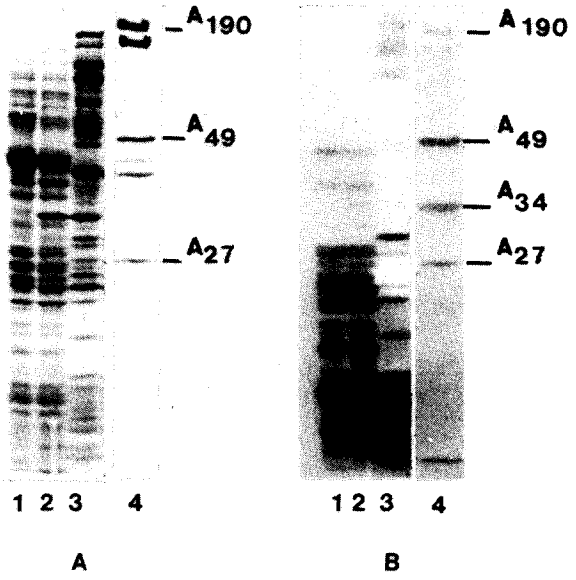

Fig.3. Electrophoresis in polyacrylamide gel containing ^{32}P-labelled $(rA)_n$. Electrophoresis of yeast and *E. coli* protein samples was carried out as in fig.1, in a gel slab containing ^{32}P-labelled $(rA)_n$. This polymer was obtained by digestion of the hybrid ^{32}P-labelled $(rA)_n.(dT)_n$ by pancreatic DNase I. Wells 1–3 contained the same samples as in fig.1, well 4, yeast RNA polymerase A. (A) stained gel; (B) autoradiogram thereof. Some subunits of yeast RNA polymerase A are indicated in the margin, as molecular weight markers.

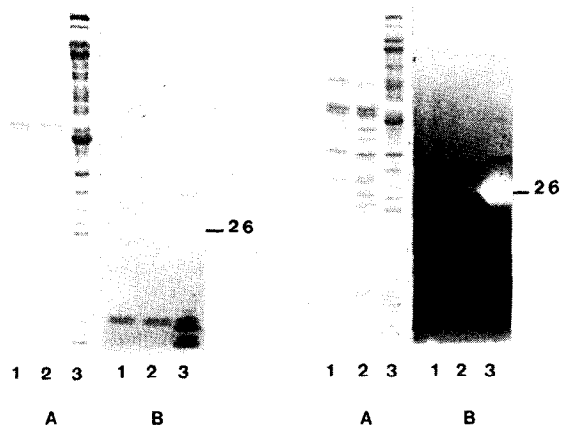

Fig.4. Nucleases degrading double-stranded RNA. *E. coli* and yeast extracts were analyzed as described in fig.1 except that the gel contained ^{32}P-labelled $(rG)_n.(rC)_n$ (left panel) or ^{32}P-labelled $r(A-U)_n$ (right panel). Wells 1–3 contained the same extracts as in fig.1. (A) stained gel; (B) autoradiogram thereof.

(fig.4). It can be seen that, except for the band of 26 kD, the activities degrading the synthetic hybrids do not attack double-stranded RNA polymers. This is in favor of the specificity of the assay especially when one compares the results with $(rG)_n.(dC)_n$ and $(rG)_n.(rC)_n$ polymers which only differ by the sugar residue.

In yeast crude extracts only one nuclease was active against ^{32}P-labelled $(rG)_n.(rC)_n$. It was 26 kD (fig.4). This band of degradation was not found with the $r(A-U)_n$ copolymer, and was clearly distinct from the activities detected with $(dC)_n.(rG)_n$.

3.4. *Detection of nucleic acid binding proteins*

During the course of these experiments it was observed that the radioactive nucleic acid is retained at the level of certain protein bands (fig.1–3). This was interpreted as the binding of the nucleic acid by the proteins, thus preventing its leakage from the gel during the long incubation period. Actually, the dark bands were not observed when the gel was autoradiographed just after completion of the electrophoresis. Each dark radioactive band always corresponded to a defined protein band but the amount of bound radioactivity was not necessarily related to the amount of protein as estimated by Coomassie blue staining. Nucleic acid binding proteins were very clearly detected using gels with ^{32}P-labelled $(rA)_n$ (fig.3). In this case, one could visualize the label at the level of the largest subunit of RNA polymerase A (A_{190} subunit) as well as A_{49}, $A_{34.5}$ and A_{27}. Other polymers such as $(rC)_n.(rG)_n$ were much less efficient in revealing binding proteins (fig.4). This could reflect the simple fact that some polymers are more stably trapped in the gel than others. This is likely to depend on the chain length of the nucleic acids. However there was also a definite selectivity of interaction as evidenced by comparing the band pattern obtained with the same protein sample on different polymers (compare wells 1 and 2 in fig.3 and 4).

4. Discussion

The main interest of the method resides in its

high resolution power, inherent in the SDS–gel electrophoresis, and in its specificity. However, it is only a qualitative method since band intensity obviously depends on a number of parameters other than enzyme concentration which would be too lengthy to discuss here. One interesting fact derived from this work is the demonstration of the multiplicity of enzymes acting on RNA·DNA hybrids in yeast. Their differential solubilization suggest that most of them are different forms of the enzymes. It is not clear whether the two specific RNase H found in *E. coli* extracts correspond to the enzymes described [6,9]. Another interesting observation is the discovery of a nuclease in yeast extracts degrading specifically the double-stranded RNA $(rG)_n.(rC)_n$. This enzyme could correspond to the bacterial RNase III [3].

Nucleic acid-binding proteins appear on the autoradiogram as dark bands where the labelled nucleic acid was retained. These preliminary observations suggest a selectivity of interaction depending on the polymer used. Several subunits of yeast RNA polymerase A are revealed in this way (A_{190}, A_{48}, A_{34}, A_{27}). The binding of $(rA)_n$ by A_{190} subunit was particularly interesting. This subunit is not basic (isoelectric point 7.5), its affinity for $(rA)_n$ is not mainly due to ionic interactions, and suggests that this subunit could be part of the RNA product site. The binding of pyridoxal phosphate to subunits A_{190}, A_{48} and $A_{34.5}$ was prevented by addition of DNA [10]. This experiment suggests that these subunits are involved in nucleic acid binding.

The application of the present methodology will certainly extend beyond the nuclease field. One is the search for primer-dependent or -independent RNA or DNA polymerases which are made of a single polypeptide chain. Another very important one would be the detection of protein–ligand interactions.

Acknowledgement

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