THE PRESENCE OF PHOSPHORYLATED SUBUNITS IN YEAST RNA POLYMERASES A AND B

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1. Introduction

Covalent modification of enzymes giving rise to interconvertible forms of functionally distinct proteins has been recognized as an important mechanism of regulation [1]. Structural changes of RNA polymerase from bacterial cells occur during T_4 or T_7 phage infection. All the subunits of the prokaryotic RNA polymerase are susceptible to alterations. ADP-ribosylation of α subunit [2–4], phosphorylation of σ [2], modifications of β' and β subunits [5,6], have been described in detail, although the in vivo significance of all these structural changes has not always been clearly established [6–8].

Our interest in yeast RNA polymerases led us to investigate whether these enzymes could be subjected to this type of regulatory mechanism. The following report describes the presence of phosphorylated subunits in yeast RNA polymerases A and B.

2. Materials and methods

2.1. *Proteins*

Homogeneous unlabeled RNA polymerase A and anti-RNA polymerase A purified γ-globulins were obtained as previously described by Huet et al. [9]. ^{32}P-labeled yeast cells were grown in low phosphate medium [10] containing 10 μCi/ml.

2.2. *Immunoprecipitation of RNA polymerase A*

The $^{32}P\,O_4$-labeled cells from 200 ml of culture were broken with an Eaton press and RNA polymerase A was adsorbed batchwise on phosphocellulose as previously described by Buhler et al. [11]. After concentration by ammonium sulfate precipitation, the pellet was dissolved into 0.2 ml buffer A containing 50 mM sodium phosphate pH 7.5, 1% Triton X 100 (v/v), 0.2 M ammonium sulfate and 0.125 mM phenyl methyl sulfonyl fluoride. Immunoprecipitation was carried out for 2 h at 4°C with an excess of purified rabbit anti-RNA polymerase A γ-globulins. The immunoprecipitates were washed twice with buffer A and RNA polymerase subunits were separated by sodium dodecyl-sulfate-polyacrylamide slab-gel electrophoresis by the method of Laemmli [12]. The gel slab was stained with Coomassie blue, dried and autoradiographed.

3. Results

3.1. *Isolation of ^{32}P-labeled RNA polymerase A by polyacrylamide gel electrophoresis*

Analytical purification of RNA polymerase can be rapidly achieved by polyacrylamide gel electrophoresis after phosphocellulose batch adsorption of the enzyme from the crude extract. The rationale for this method is that mostly basic proteins are retained by phosphocellulose and therefore do not migrate in the gel under the conditions commonly used for separation of acidic proteins. After electrophoresis, RNA polymerase A and A* [9] are the major protein bands seen in the gel. Since a preliminary experiment indicated that the polymerase bands were labeled with $^{32}F\,O_4$, a two-dimensional electrophoresis was used to investigate whether the incorporated radioactivity corresponded to any subunit of the enzyme. The autoradiogram of

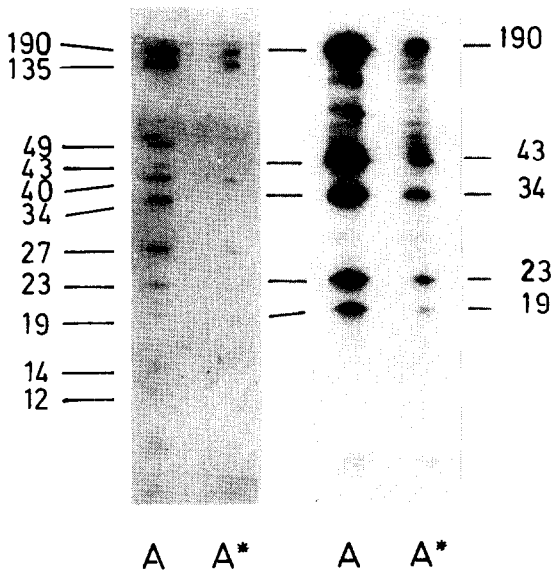

Fig.1. In vivo phosphorylation of RNA polymerase A as seen by two-dimensional gel electrophoresis. RNA polymerase A was purified from ^{32}P-labeled yeast cells by electrophoresis on 5% acrylamide gel which resolves A and A*. RNA polymerase subunits were separated by a second-dimension electrophoresis with sodium dodecyl sulfate as described under Materials and methods. On the left is the Coomassie blue staining of the gel; on the right, the autogradiogram thereof. Subunits of RNA polymerases A and B are indicated by arrows and designated by A or B with a subscript corresponding to their molecular weight (in dalton × 10^{-3}). The term subunit is referred to all polypeptides which are interacting with the enzyme molecule [9].

the gel is shown in fig.1. The ^{32}P-label migrated essentially at the level of the following 5 polypeptides: A_{190}, A_{43}, $A_{34.5}$, A_{23} and A_{19}. Radioactivity was found both at the level of A and A* subunits.

3.2. *Antibody precipitation of phosphorylated RNA polymerase A*

To confirm the above observations, a specific and rapid isolation of RNA polymerase A was achieved by precipitation with purified antibodies against pure enzyme A. Analysis of the immunoprecipitate by sodium dodecylsulfate gel electrophoresis again showed that the incorporated ^{32}P radioactivity coincided with the five polypeptide chains A_{190}, A_{43}, $A_{34.5}$, A_{23} and A_{19} (fig.2). Some radioactivity also remained at the interface between the upper and lower gels but no Coomassie blue stain was noted at this level.

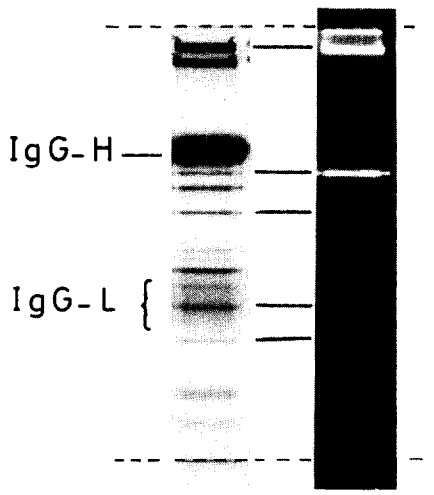

Fig.2. Analysis of RNA polymerase immunoprecipitates by sodium dodecylsulfate gel electrophoresis. RNA polymerase A was purified by immunoprecipitation as described under Materials and methods. Left, stained gel; right, autoradiogram thereof. Gel frontier (top) and migration front (bottom) are indicated by dashed lines.

3.3. *Mapping of phosphorylated polypeptides*

RNA polymerase A was again purified in a different way, from ^{32}P-labeled cells, using a microscale adaptation of the original procedure of Buhler et al. [11]. The ^{32}P-label was found at the level of the same subunit bands and was insensitive to DNAase and RNAase attack. Treatment with alkaline phosphatase at 37°C only slightly decreased the labeling of A_{190} and A_{23} subunits. Acid hydrolysis of the labeled enzyme as well as each of the five labeled subunits yielded phosphoserine, phosphothreonine and inorganic phosphate (result not shown). Further identification of the phosphorylated polypeptides was achieved by two-dimensional mapping in the presence of carrier unlabeled enzyme A (II). Staining and autoradiography of the gel showed that the ^{32}P O_4 label again comigrated with subunits A_{190}, A_{43}, $A_{34.5}$, A_{23} and A_{19} (fig.3). Heterogeneity in the migration of some subunits could indicate various extents of phosphorylation.

3.4. *Effect of cycloheximide on phosphorylation of RNA polymerase A*

Phosphorylation of RNA polymerase A was carried out in the absence of protein synthesis. Yeast cells

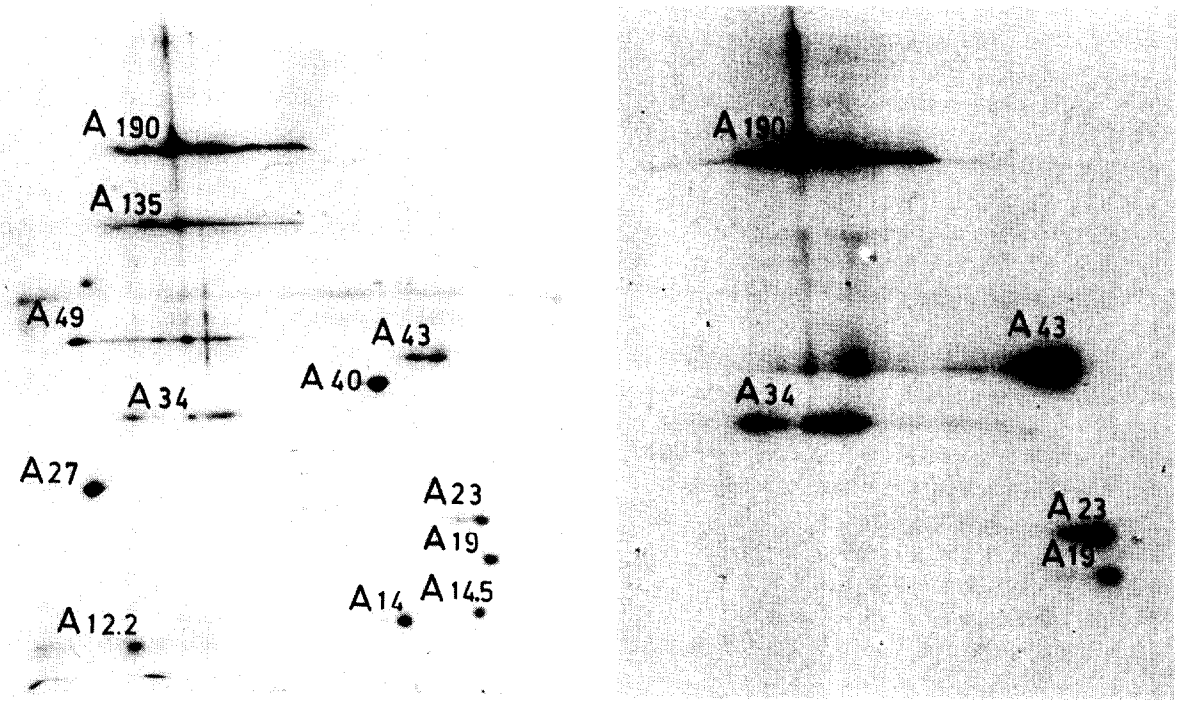

Fig.3. Subunit mapping of ^{32}P-labeled RNA polymerase A. RNA polymerase A, purified by a microscale adaptation of the original technique [11] was subjected to two-dimensional subunit mapping in the presence of carrier enzyme [13]. The gel-slab was stained (left half) and subjected to autoradiography (right half).

were treated with cycloheximide for 5 min then pulse labeled with [^{32}P]orthophosphate. The phosphorylation pattern of RNA polymerases A under these conditions is compared with that of an untreated control in fig.4. Scanning of the Coomassie blue stain revealed that recovery of RNA polymerase A by the immunoprecipitation technique was 25 percent lower after cycloheximide treatment which corresponded well

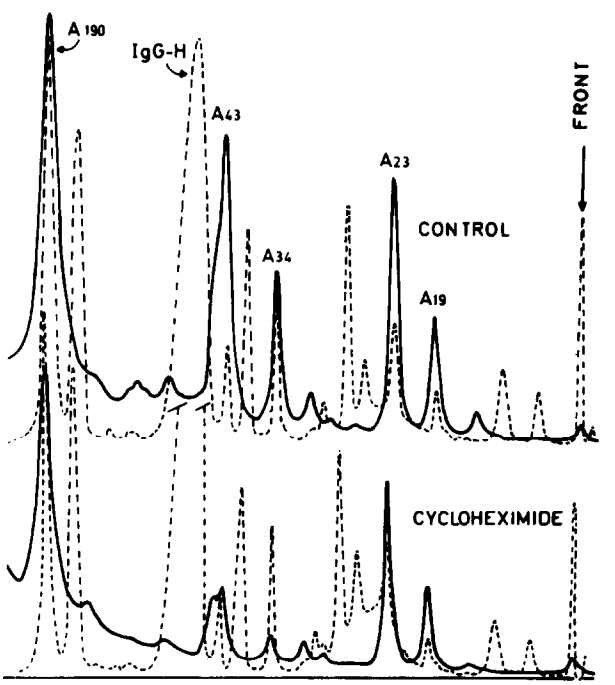

Fig.4. Effect of cycloheximide on RNA Polymerase A phosphorylation. One culture (400 ml; A_{600}, 0.35) was separated in two parts. One was supplemented with cycloheximide (100 μg/ml) and the other received none. After 5 min incubation each culture received ^{32}P O_4 (10 Ci/ml) and the cells were collected 30 min later in the cold. RNA polymerase A was isolated by immunoprecipitation and analyzed by sodium dodecyl sulfate gel electrophoresis. Autoradiograph (–) and Coomassie blue stain of protein (– – –) were scanned. The specific radioactivity of protein bands was obtained, in arbitrary units, by computing the ratio of the two corresponding peaks.

to the inhibition of cell growth by the drug. With cycloheximide, phosphorylation was markedly reduced on subunits A_{190}, A_{43} and $A_{34.5}$. The specific radioactivity of these polypeptides amounted to 50% (A_{190}), 22% (A_{43}) and 17% ($A_{34.5}$) of the untreated control. In contrast, interestingly, phosphorylation of subunits A_{23} and A_{19} was unaffected by cycloheximide (102 and 90% of control, respectively). Unbalanced phosphorylation of RNA polymerase A therefore occurred in the presence of the inhibitor.

3.5. *Phosphorylation of yeast RNA polymerase B*

The presence of phosphorylated polypeptides in yeast RNA polymerase B was also detected by gel electrophoresis as shown in fig.5. The enzyme was purified on microscale from ^{32}P-labeled cells and subjected to electrophoresis on polyacrylamide gel, with dodecylsulfate, in the presence of carrier unlabeled RNA polymerase B (exp. 1 and 2). One of the common subunits of RNA polymerases [14], the B_{23} subunits, was strongly labeled with ^{32}P, as was its A_{23} counter part. A strong band of radioactivity was also found above subunit B_{185}, which could well correspond to the largest non-proteolyzed subunit of enzyme B recently described by Dezelee et al. [15]. In contrast, the proteolyzed polypeptides B_{185} was only faintly labeled (exp. 1). Other minor bands of radioactivity were also observed, one of them at the level of B_{44} subunit. Therefore, two-dimensional gel electrophoresis was used to remove radioactive contaminants and to separate enzyme B_I from the proteolyzed form B_{II} [15]. As seen in fig.5 (exp. 4), the presence of two spots of ^{32}P-labeled subunit B_{23} reflected the separation of the two forms of enzyme. On the other hand, the unlabelled carrier RNA of polymerase B consisted mostly of the proteolyzed B_{II} form, with only a small amount of B_I (fig.5, exp. 3). Again a highly labeled band migrated at the level of subunit B_{220} of enzyme B_I, whereas the proteolyzed subunit B_{185} of enzyme B_{II} was only faintly labeled. A small amount of ^{32}P-radioactive material was also associated with subunit B_{44} in B_I enzyme only. The fact that

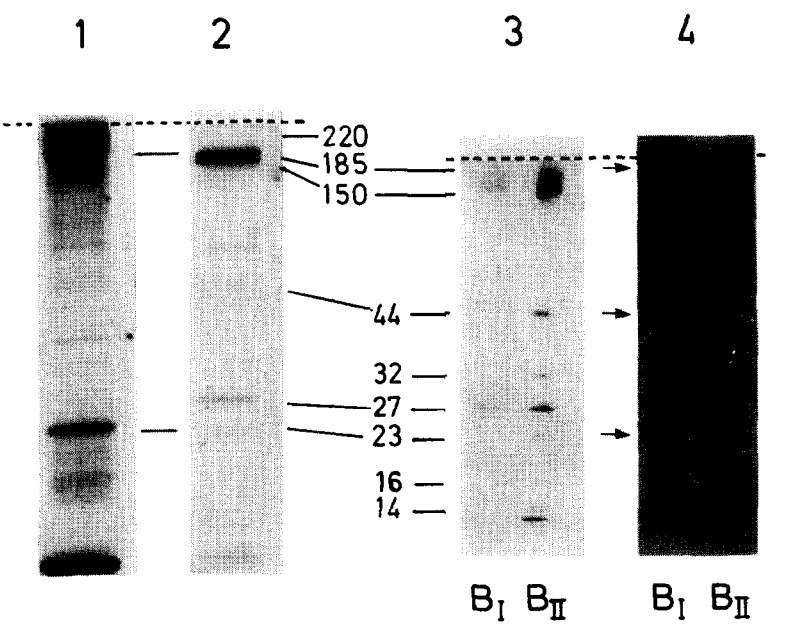

Fig.5. In vivo phosphorylation of RNA polymerase B. *Left,* yeast RNA polymerase B was purified on micro scale (J. M. Buhler, unpublished) from ^{32}P-labeled log phase cells and analysed directly by sodium dodecyl sulfate gel electrophoresis with carrier enzyme. 2. Stained gel; 1. autoradiogram; thereof. *Right,* a sample of the same RNA polymerase B preparation was further purified with added carrier enzyme by electrophoresis under non-denaturing conditions which resolves B_I and B_{II} enzymes [15], then subjected to a second dimension gel electrophoresis with sodium dodecylsulfate. 3. stained gel; 4. autoradiogram thereof. The dashed line indicates the uppper and lower gel frontier.

enzyme B_{11} was practically only phosphorylated on B_{23} subunit could be correlated with the observation that B_{220} and B_{44} subunits are the most susceptible to protease action [15].

4. Discussion

Three different purification procedures were used to isolate phosphorylated RNA Polymerases A and B from actively growing ^{32}P-labeled yeast cells. Phosphorylation occurs at the level of five subunits in RNA polymerase A. One of the phosphorylated subunits is common to both enzymes (A_{23} and B_{23}) [14]. RNA polymerase B contains several phosphorylated polypeptides, B_{23}, B_{220} and $B_{44.5}$. However, only B_{23} subunit is phosphorylated in both forms of enzymes, B_1 and B_{11}.

The normal labeling of A_{23} and A_{19} subunits in the presence of cycloheximide indicated that their phosphorylation can occur in absence of protein synthesis. This is suggestive of an enzymatic phosphorylation–de-phosphorylation process which could modulate RNA polymerase activity or its interaction with regulatory factors or chromatin. Alteration in the phosphorylation pattern of yeast ribosomal proteins in the presence of cycloheximide was also observed by Zinker and Warner [16]. Kinetics of phosphorylation also showed that subunit A_{23} and A_{19} were much more rapidly labeled than A_{190}, A_{44} and $A_{34.5}$, which further suggests different mechanisms of phosphorylation (J. M. Buhler, unpublished).

Using partially purified enzymes, Martelo and Hirsch [17] and Jungmann et al. [18] have previously described in vitro stimulation of animal RNA polymerase by homologous protein kinases. There is therefore the possibility that phosphorylation is the general rule for eukaryotic RNA polymerases, with protein kinases playing an important role in regulation of gene expression. In view of the structural complexity of RNA polymerases it is also possible that some of these phosphorylated polypeptides correspond to non-histone chromatin phosphoproteins interacting with the RNA polymerase molecule. Since completion of this work, a paper by Bell, Valenzuela and Rutter [19] has appeared which reports an experiment on the isolation of phosphorylated RNA polymerase A (I) from yeast cells and the in vitro phosphorylation of the enzyme by a yeast protein kinase preparation. Martelo and Hirsh [20] also independently observed the in vivo phosphorylation of animal RNA polymerase I.

Acknowledgements

This paper was originally submitted to Jacques Monod, shortly before his death. We would like to dedicate this work to his memory. We also express our warm thanks to Madeleine Brunerie. We are indebted to Dr Miriam Slifkin for her invaluable help in correcting the English text.

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