STUDIES ON THE PHOSPHORYLATION OF NUCLEOLAR PROTEINS

Comparison of the phosphorylation patterns of nuclear and nucleolar proteins labeled in vivo and in vitro

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1. Introduction

Evidence has been accumulated which suggests that the control of transcription may involve the phosphorylation of nuclear proteins, in particular non-histone proteins [1–3]. It was therefore interesting to compare the phosphoproteins of nuclear and nucleolar chromatins which show remarkable differences in template activity.

In the preceding paper [4] we demonstrated the presence of a protein kinase activity in purified rat liver nucleoli. In this study the proteins of whole nuclei and isolated nucleoli were fractionated into saline-soluble and chromosomal proteins and the distribution of phosphoproteins was analyzed. Since up to now very little is known about the specificity of the phosphorylation reactions in vitro we compared the distribution of nuclear and nucleolar proteins labelled both in vivo and in vitro. [10].

2. Materials and methods

For in vivo labeling of phosphoproteins rats were starved overnight and then injected intraperitoneally with 2 mCi/100 g of rat body weight neutralized, carrier-free ^{32}P-orthophosphoric acid (The Radiochemical Centre, Amersham). The animals were killed after 90 min and livers were removed for isolation of nuclei and nucleoli.

The preparation of nuclei and nucleoli was carried out as described previously [5]. The incubation conditions for the in vitro phosphorylation were as indicated in the preceding paper [4] except that the total volume of the samples was 10 ml and the mixture contained 10^{-4} PMSF (phenylmethanesulfonyl fluoride) to inhibit protease activity. The incubation time was 15 min.

Chromatin was isolated from ^{32}P-labeled nuclei or nucleoli as follows: The cell organelles were washed once in phosphorylation buffer (0.25 M sucrose; 100 mM Tris–HCl, pH 7.5; 20 mM $MgCl_2$; 100 mM NaCl; 0.06 mM ATP; 0.1 mM PMSF; 0.2% Triton X-100), then homogenized in saline–EDTA (0.075 M NaCl; 0.024 M EDTA, 0.05 M Na_2SO_3, pH 6.0) and centrifuged at 3500 *g* for 10 min. The pellet was washed once more in the saline–EDTA buffer and twice with 0.14 M NaCl, 0.01 M Tris–HCl, pH 7.5. The supernatants of the washing steps were combined, made to 0.25 N HCl and dialyzed against cold 0.25 N HCl for 2 hr. The precipitated RNA was removed by centrifugation and discarded. The proteins of the supernatant were precipitated by adding 6 vol acetone. This fraction contains the soluble proteins.

The chromatin pellet remaining after the washing steps was dissolved in 2 M NaCl, 5 M urea, 0.05 M Na_2SO_3 and centrifuged for 38 hr at 100 000 *g*. After dialysis against 0.05 M Na_2SO_3 the chromosomal proteins were precipitated with acetone. The precipitate was washed twice with acetone:water (6:1 v/v), twice with acetone and dried. The chromosomal proteins were dissolved in 0.065 M Tris–HCl, pH 6.8, 3% SDS;

Table 1
Comparison of the specific activities of nuclear and nucleolar proteins labeled either in vivo or in vitro

Fraction	Specific activity of labeling ($^{32}PO_4$-cpm • 10^{-3}/mg protein)			
	Nucleus		Nucleolus	
	In vivo	In vitro	In vivo	In vitro
Total protein	39.2	38.6	44.3	156.2
EDTA–saline soluble proteins	46.8	105.0	48.4	410.0
Chromosomal proteins	37.1	36.2	41.5	146.3

The labeling of the nuclear and nucleolar proteins in vivo and in vitro as well as the fractionation into EDTA–saline-soluble and chromosomal proteins was as described in Materials and methods.

5% mercaptoethanol, boiled for 2 min and subjected to acrylamide gel electrophoresis in 10% gels containing 0.1% SDS according to Laemmli [10]. Protein was determined as described by Heil et al. [11].

3. Results and discussion

As has been shown in another paper [8] purified nucleoli activily incorporate labeled phosphate from [γ-^{32}P] ATP into protein. The same holds true for whole nuclei. To investigate to what extent the phosphorylation of chromosomal and soluble proteins is comparable in in vivo and in vitro experiments we compared the specific activities of proteins from whole nuclei or purified nucleoli. Table 1 shows the specific activities after in vivo or in vitro labeling. The specific activities of nuclear and nucleolar proteins phosphorylated in vivo are very similar.

The phosphorylation activity of isolated nucleoli, however, is about 4-fold higher than that of nuclei. Furthermore, the EDTA–saline-soluble proteins, which are normally discarded during chromatin preparation were phosphorylated to a considerable extent. This is especially true for the soluble proteins phosphorylated in vitro. An analysis of these highly labeled soluble proteins was made by electrophoresis in SDS-polyacrylamide gels (fig. 1a + 1b). Most of the soluble proteins were common to both the nucleus and the nucleolus though significant differences in the quantitative distribution of several protein bands can be seen. Some additional bands were found in the nuclear fraction. The phosphorylated polypeptides, on the other hand, show a considerable similarity in the distribution of the labeled peaks irrespective of whether they were of nuclear or nucleolar origin. Furthermore, there are no discernible qualitative differences between the radioactivity pattern of proteins labeled either in vivo or in vitro, though some quantitative differences in the relative labeling intensities of various bands are evident.

Up to now little information is available regarding the nature of these saline-soluble proteins. It has been reported that the nucleolus contains a large pool of ribosomal proteins [8, 9]. Furthermore, it is well established that ribosomal proteins derived from cytoplasmic ribosomes can be phosphorylated both in vivo and in vitro [10, 11]. Whether, however, such a phosphorylation of ribosomal proteins may already occur in the nucleolus, is still unknown. Also our labeling studies do not permit a definite answer to this question. Indeed, the most extensive phosphorylation is found in polypeptides which are larger than those present in mature ribosomes. These highly phosphorylated proteins (slices no. 20–30) may represent the 'nucleolus-specific' proteins described by Soeiro et al. [9, 12]. However, less extensively phosphorylated proteins show electrophoretic identity with many ribosomal peptides (slices 30–90). The protein pattern, however, is too complex and the resolution is not good enough to decide whether or not ribosomal proteins are already phosphorylated before they enter the cytoplasm. In fig. 2a and 2b the electrophoretic separation of nuclear and nucleolar chromosomal proteins is shown. A comparison of the relative staining intensities of the nonhistone and histone fractions reveals that the histones are qualitatively as well as quantitatively identical in the nuclear and nucleolar fractions. In contrast, the amount of nonhistones is much higher in total nucleolar chromatin than in the nuclear chromatin. The radioactivity profile, moreover, reveals differences between the labeling behaviour of the nonhistone phosphoproteins. Slow migrating bands of the nucleolar chromosomal proteins are much

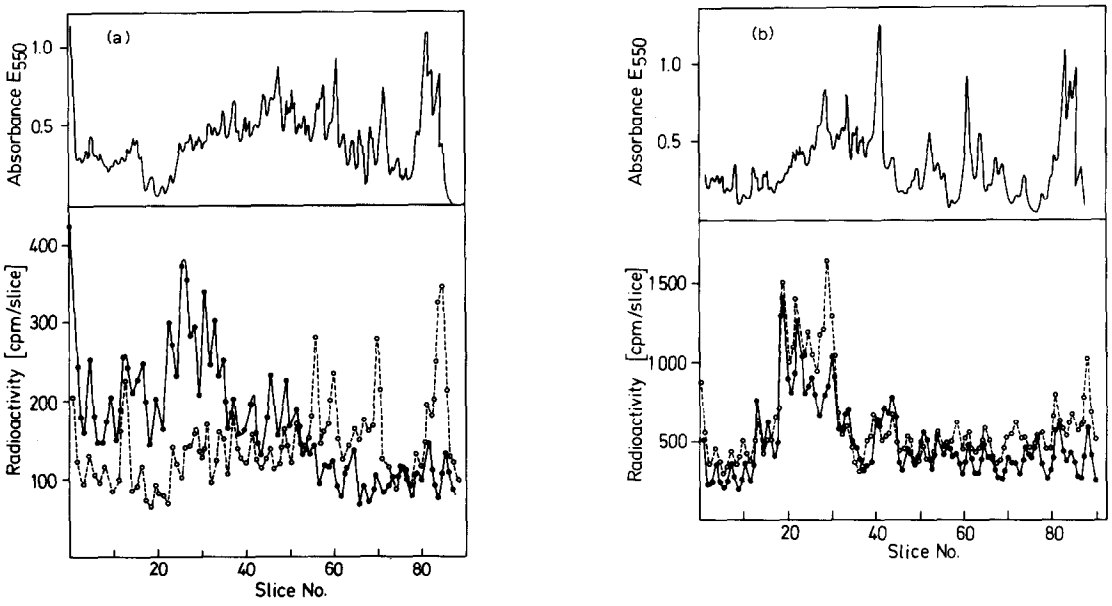

Fig. 1. a) SDS-polyacrylamide gel electrophoresis profile of ^{32}P-labeled nuclear EDTA–saline-soluble proteins. (•——•——•) in vivo labeled, (○– –○– –○) in vitro labeled; b) SDS-polyacrylamide gel electrophoresis profile of nucleolar EDTA–saline soluble proteins. (•——•——•) in vivo labeld, (○– –○– –○) in vitro labeled.

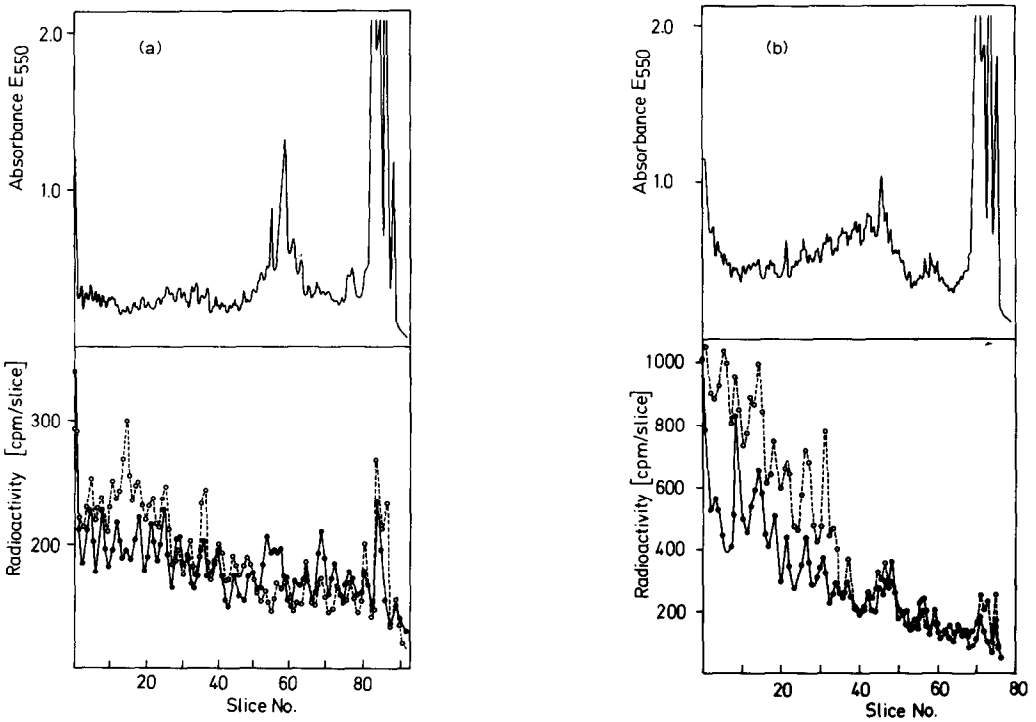

Fig. 2. a) SDS-polyacrylamide gel analysis of ^{32}P-labeled nuclear chromosomal proteins. (•——•——•) in vivo labeled. (○– –○– –○) in vitro labeled; b) SDS-polyacrylamide gel analysis of ^{32}P-labeled nucleolar chromosomal proteins. (•——•——•) in vivo labeled. (○– –○– –○) in vitro labeled.

heavier labeled than the fast moving fractions, whereas the nuclear phosphoproteins were more uniformly labeled. The radioactivity patterns of the proteins labeled either in vivo or in in vitro are remarkably similar. Therefore the phosphorylation process studied reflects a high degree of accuracy regarding its substrate. Of course, a more improved resolution of these very complex protein fractions will be necessary to get final conclusions about the specificity of in vitro phosphorylation of nuclear and nucleolar proteins. More refined techniques, such as two dimensional gel electrophoresis, should be applied to confirm the accuracy of the in vitro phosphorylation reaction.

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