PEPTIDE MAPPING OF IN VIVO AND IN VITRO PHOSPHORYLATED SITES OF PROTEINS FROM HeLa hnRNP

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1. Introduction

The structural complexity of the nuclear ribonucleoprotein particles containing the heterogenous nuclear RNA (hnRNP) is now well established in a wide variety of eucaryotic cells including HeLa cells [1–5]. However little is known about the function of associated proteins although it can be speculated that they serve some role in the processing of hnRNA to mRNA and its transport to the cytoplasm.

In a search for a criterion which might be used as an assay for the functional integrity of these hnRNP, we have recently described an endogenous reaction of protein phosphorylation–dephosphorylation independent of cyclic AMP [6–8]. When supplied with [γ-^{32}P]ATP, purified hnRNP from HeLa cells incorporate radioactive phosphate as phosphoserine and phosphothreonine into two discrete polypeptide species of mol. wt 28 000 and 37 000 M_r [7]. These same species, along with 2 other ones of 30 000 and 52 000 M_r, are also phosphorylated in vivo after exposure of cells to [^{32}P]orthophosphate [9,10]. Further evidence towards showing that purified hnRNP have retained the native character and specific phosphorylation properties which they exhibited in vivo should come from the demonstration that the same amino acid sites are involved in both cases. To this aim, we made use of the fingerprinting technique on SDS–acrylamide gels [11] to show that the same peptides produced by proteolytic digestion of 28 000 and 37 000 M_r species are phosphorylated in vivo and in vitro.

This result supports the validity of using the autophosphorylation reaction as a first step towards the definition of hnRNP in functional terms.

Abbreviations: hnRNP, heterogenous nuclear ribonucleoprotein particle; hnRNA, heterogenous nuclear RNA; M_r, app. mol. wt proteins in SDS

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2. Materials and methods

Procedures of growth in suspension culture, collection and washing of HeLa cells (S 3 strain) have been described in [2]. Pellets of non-radioactive cells were kept frozen at −20°C until use. hnRNP were extracted from sonicated nuclei and purified as in [7] using a slight modification of the procedure in [12].

For in vivo labeling with ^{32}P, cells were resuspended to a concentration of 3×10^5/ml in 4 l medium containing 0.05 mM phosphate and exposed to 20 mCi carrier-free $H_3{}^{32}PO_4$ (CEA, Saclay, France) for 36 h. hnRNP were immediately prepared from fresh labeled cells as above except that centrifugation through a 15–30% (w/v) sucrose gradient was used for the final purification in place of the triple sucrose layer (fig.1).

In vitro labeling was carried out as in [7] in standard reaction mixtures which contained in final vol. 0.2 ml: 10 mM Tris–HCl, pH 8.3 (25°C), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 80 μg hnRNP expressed as protein. Reaction was initiated by adding 10 μl [γ-^{32}P]ATP (195 μM, 3.8×10^5 cpm/μl) prepared as in [13]. After 10 min incubation at 30°C, two identical mixtures were pooled, adjusted to 10%

with cold trichloracetic acid and the resulting precipitate collected by centrifugation. Pooled sucrose gradient fractions of in vivo labeled hnRNP (fig.1) were precipitated in the same way. Processing of precipitates and conditions for their analysis by electrophoresis on 10% acrylamide slab gels containing 0.1% SDS have been described [7]. At the end of the run, the unfixed wet gel was covered with parafilm and autoradiographed for 3 h. Gel slices corresponding to radioactive bands of 28 000 and 37 000 M_r were carefully excised so as to avoid contamination with other labeled species.

These gel slices were then directly digested with proteases and electrophoresed on a second acrylamide gel (15%) as in [11]. Following electrophoresis, gels were fixed, dried and autoradiographed as in [7]. Although 2 proteases were used, *Staphylococcus aureus* V8 protease 5 μg/sample well (Miles) and TLCK-treated chymotrypsin 10 μg/sample well (Worthington), only the results obtained with the latter will be presented.

3. Results and discussion

Centrifugation in a sucrose density gradient of in vivo labeled hnRNP as a final step of purification yielded the typical profile of fig.1. The heaviest fractions which contained the highest proportion of phosphoproteins (hot trichloracetic acid-resistant radioactivity) were pooled as indicated by arrows and precipitated by cold trichloracetic acid. This material,

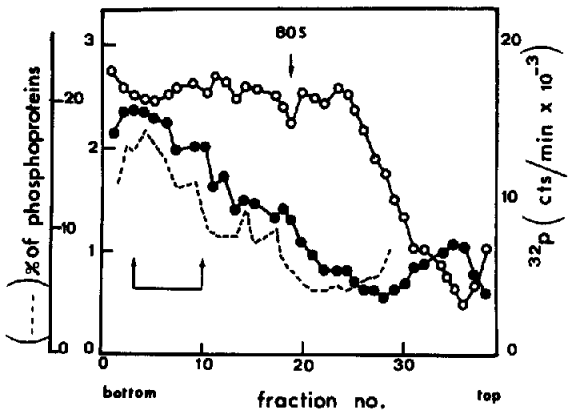

Fig.1. Sucrose gradient analysis of hnRNP from cells labeled in vivo with ^{32}P. The post-nucleolar supernatant from sonicated nuclei [7] was layered on top of a 36 ml 15–30% (w/v) sucrose gradient in 10 mM Tris–HCl, pH 7.4 (25°C); 10 mM NaCl; 1.5 mM $MgCl_2$ and centrifuged at 17 000 rev./min in the SW-27 rotor of the spinco L5-75 ultracentrifuge for 14 h. 1 ml fractions were collected and 2 separate 10 μl aliquots of each fraction were precipitated with trichloracetic acid and analysed for total and phosphoprotein ^{32}P counts. (o—o) Cold trichloracetic acid precipitable counts (total counts); (•—•) Hot trichloracetic acid precipitable counts (phosphoproteins). The ratio of phosphoprotein (hot trichloracetic acid-resistant) to RNA counts (hot trichloracetic acid-labile) × 100 is plotted as a dotted line.

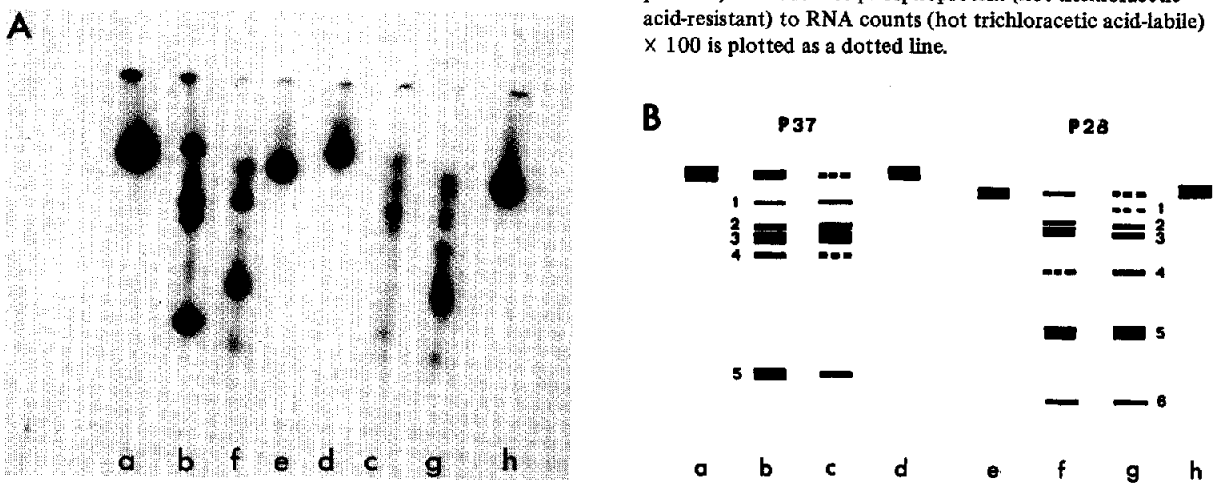

Fig.2. Chymotryptic digests of 37 000 and 28 000 M_r species labeled in vivo and in vitro. (A) Autoradiograph. (B) Schematic diagram. Tracks a, d, e, h: Non-digested control samples of 37 000 M_r labeled in vivo (d) and in vitro (a) and 28 000 M_r labeled in vivo (h) and in vitro (e). Tracks b, c, f, g: Samples digested with 10 μg chymotrypsin of 37 000 M_r labeled in vivo (c) and in vitro (b) and 28 000 M_r labeled in vivo (g) and in vitro (f).

in parallel with in vitro phosphorylated hnRNP, was run on a one-dimensional 10% acrylamide gel. Typical autoradiographs, similar to those in [7,10] were obtained (not shown). The most heavily labeled bands migrating as 28 000 and 37 000 M_r species were cut out of the gel and separately subjected to the peptide mapping technique in [11] on a second 15% acrylamide gel.

Figure 2 shows the comparison of chymotryptic digests of 37 000 and 28 000 M_r species labeled both in vivo and in vitro. Respectively 5 and 6 phosphorylated peptides could be reproducibly obtained from in vivo labeled 37 000 M_r and 28 000 M_r species (fig.2A,B). All of these bands were also phosphorylated in vitro although some of them with a different relative intensity. Among the most heavily labeled under both conditions were band 5 from 28 000 M_r (compare tracks f and g) and band 3 from 37 000 M_r (compare tracks b and c). Some other bands were more intensely labeled in vitro: bands 4 and 5 from 37 000 M_r (compare tracks b and c) and band 3 from 28 000 M_r (compare tracks f and g). These quantitative discrepancies could be explained by a differential turn-over of phosphate groups at different sites. The hnRNP associated phosphoprotein phosphatase activity in [8] could account for such a phenomenon, should it remove different phosphate groups at unequal rates.

The absolute numbers of phosphate groups incorporated in vivo and in vitro per mg hnRNP proteins as can be derived from specific activity calculations fall within the same order of magnitude (between 5×10^{-8} and 5×10^{-2} μmol P/mg protein). This shows that the endogenous phosphorylation reaction carried out in vitro by purified hnRNP is not only qualitatively but also quantitatively comparable to that effected in vivo at the cellular level.

We would therefore like to suggest that this endogenous phosphorylation reaction be used as an indication for the integrity of hnRNP.

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