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Prothymosin α Interacts with Free Core Histones in the Nucleus of Dividing Cells

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The acidic protein prothymosin α (ProTα), with a broad presence in mammalian cells, has been widely considered to have a role in cell division, through an unrevealed mechanism in which histones may be involved in view of their ability to interact with ProTα *in vitro*. Results of co-immunoprecipitation experiments presented here demonstrate that ProTα interacts *in vivo* with core histones in proliferating B-lymphocytes (NC-37 cells). This interaction occurs with histones H3, H2A, H2B and H4 located free in the nucleoplasm, whereas no interaction was detected with histone H1, mono-nucleosome particles or chromatin. Moreover, the core histones form part of a nuclear multiprotein complex of about 700 kDa separated by ProTα-Sepharose affinity, with components including H3 and H4 acetyltranferases, H3 methyltransferases, hnRNP isotypes A3, A2/B1 and R, ATP-dependent and independent DNA helicases II, β-actin and vimentin, all co-purifying by gel filtration. This indicates that the interaction of ProTα with core histones in the nucleus may be related to the structural modification of histones H3 and H4, and hence to chromatin activity, raising the possibility that the other proteins in the nuclear complex may play a role in this process.

Key words: histone-binding proteins, proliferation, prothymosin.

Abbreviations: CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; DMP, dimethyl pimelimidate; DSP, dithiobis (succinimidyl propionate); HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; hnRNP, heterogeneous nuclear ribonucleoprotein; NCP, nucleosome core particle; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; ProTα, prothymosin α; $T\alpha_1$, thymosin α_1.

Prothymosin α (ProTα) is a protein of 109–111 amino acids with a broad presence in mammalian cells, especially in lymphoid tissues (*1*, *2*). The primary structure of ProTα is highly conserved and shows a number of important features: the first 28 amino acids of its sequence correspond to those of thymosin α_1 ($T\alpha_1$), it has a central acidic region (residues 41–85) comprised of glutamic and aspartic residues, and it has a karyophylic signal at its C-terminus (*3*–*6*). Neither type of secondary structure has been detected under physiological conditions (*7*). Early research to elucidate the biological function of ProTα demonstrated that it plays an essential role in cell division. Observations implicating ProTα in this process include activation of its transcription by c-myc (*8*), which promotes high cellular levels of ProTα gene expression throughout the cell cycle, with a moderate increase (2–4 fold) at S-phase in mitogen-activated cells (*9*) or tumors (*10*). In addition, cell division activity is abolished by ProTα mRNA antisense oligonucleotides (*11*). Concomitantly with cell division ProTα undergoes phosphorylation at Thr residues (*12*) by a cytoplasmic protein kinase which has been detected but not yet characterized (*13*). Its post-translational modification also includes proteolytic processing by a lysosomal asparaginyl endopeptidase, giving rise to significant cytoplasmic concentrations of $T\alpha_1$ (*14*).

Despite the extensive information available on the structural characteristics of ProTα, the significance of its post-translational modifications and the mechanisms of its involvement in cell division remain to be elucidated. A recent report has shown that, apart from its role in cell division, ProTα has antiapoptotic activity in the cytoplasm, attributable to its capacity to inhibit the activation of caspase 3 by the apoptosome (*15*). This suggests that ProTα is a multifunctional protein. With regard to its involvement in cell division, current experimental data suggest that ProTα occurs in the nucleus, to which it is targeted by a nuclear import signal (*16*), and that its nuclear role is related to chromatin activity. Thus, in recent years results mostly derived from protein-protein interaction analyses have demonstrated that ProTα can influence transcription activity through interactions with transcription factors such as Epstein-Barr virus antigen (*17*, *18*), CREB-binding protein (*19*) or estrogen receptor (*20*), and may also be related with the nuclear function of the histones, in view of reports showing that ProTα interacts *in vitro* with these proteins. However, data about ProTα-histone interactions are controversial: notably, it remains unclear which histones are able to interact with ProTα, possibly reflecting the diverse experimental techniques used. For example, authors using ligand blotting assays (*21*) or an immunoaffinity approach (*22*) have reported that ProTα can interact with histone H1. In our laboratory we have found that ProTα shows preferential affinity for core histones in commercial mixtures of H1, H3, H2A, H2B and H4 (*23*), as well

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in diverse cell extracts, when chromatographed on ProTα-bound Sepharose (*23*, *24*). Moreover, other reports support an involvement of ProTα in chromatin organization through interaction with histones, including studies showing the ability of ProTα to cooperate in nucleosome assembly *in vitro* (*23*) and to alter chromosome structure when it is over-expressed in HL-60 cells (*25*).

Nevertheless, no definitive proof of the interaction of ProTα with histones *in vivo* has so far been reported. By reciprocal immunoprecipitation experiments, in this paper we demonstrate an interaction between ProTα and free core histones in the nucleoplasm of proliferating lymphocytes. On the basis of ProTα affinity we also identify a nuclear complex containing the four core histones and a various other proteins including histone-modifying enzymes, RNA/DNA-binding proteins and cytoskeletal proteins, suggesting that the nuclear role of ProTα may involve these proteins.

MATERIALS AND METHODS

Cells and Subcellular Fractionation—Transformed human B lymphocytes (NC-37 cells) were cultured in RPMI 1640 and subcellular fractionation was carried out as described (*24*). Briefly, nuclei and cytosol were separated from homogenized cells (about 5×10^7 cells/ml) by differential centrifugation at $2,000 \times g$ (nuclei) and $100,000 \times g$ (cytosolic fraction). The pelleted nuclei were suspended (about 2×10^8 nuclei/ml) in 10 mM Tris-HCl pH 7.4 buffer containing 10% sucrose (w/v), 0.1 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF, supplemented with 80 μg/ml DNase I and 10 μg/ml RNase A, incubated at 0°C for 30 min, and then centrifuged to yield the nucleoplasmic extract. Both the nucleoplasmic and cytosol extracts were dialyzed against 50 mM Tris-HCl pH 7.5 buffer containing 5% glycerol (v/v), 1 mM DTT, 0.5 mM PMSF.

Cross-Linking of NC-37 Cells—The procedure was essentially as described by Liu *et al.* (*26*): cells washed and suspended in PBS (3.5×10^7 cells/ml) were treated with dithiobis (succinimidyl propionate) (DSP) (Pierce) to a final concentration of 2.5 mM, for 30 min at room temperature with occasional agitation. The reaction was stopped by the addition of 1 M Tris pH 8 (final concentration 50 mM). After 10 min cells were collected by centrifugation, washed twice with PBS, resuspended in lysis buffer at 10^8 cells/ml and homogenized to obtain nucleoplasmic and cytosol fractions as above.

Antibodies, Immunoprecipitation and Western Blotting Analyses—Antibodies against the N-terminal region (residues 2–20) and C-terminal region (residues 95–109) in the ProTα-sequence were custom-produced by NeoMPS (Strasbourg, France). Histone antibodies used were the anti-histone monoclonal MAB052 (Chemicon International), the anti–histone-H4 polyclonal SC-10810 (Santa Cruz Biotechnology), the anti–acetyl-histone-H4 polyclonal 06-866 (Upstate), the anti–histone-H3 polyclonal 06-755 (Upstate) and the anti–dimethyl-histone H3 polyclonal 07-212 (Upstate).

Best yield in immunoprecipitations was obtained (i) when anti-ProTα and anti-histone monoclonal antibodies were previously crosslinked to protein A–Sepharose, using the bifunctional crosslinker DMP (dimethyl pimelimidate) under the conditions described (*27*), and (ii) when the rabbit IgG TrueBlot™ Set (eBioscience) was used for precipitation of the anti-histone antibodies.

Immunoprecipitations with anti-ProTα and anti-histone monoclonal antibodies were carried out by incubation of cytosolic extracts (500 μg) or nucleoplasmic extracts [500 μg supplemented or not with 7 μg of ProTα purified from calf thymus (*13*)] with protein A–antibody complexes, previously prepared from 15 μg of immunoaffinity-purified anti–N-terminal, anti–C-terminal ProTα antibodies, or 5 μl of anti-histone monoclonal (rabbit non-immune IgG was used as control) in immunoprecipitation buffer (20 mM Tris-HCl pH 7.6, 75 mM NaCl, 0.5% NP-40, 2% CHAPS, 1 mM EDTA, and 1 μg/ml of leupeptin, pepstatin and aprotinin) overnight at 4°C. Immunoprecipitations with anti-histone antibodies were performed after preclearing the cytosolic (750 μg) or nucleoplasmic (750 μg supplemented or not with 15 μg of ProTα) extracts in immunoprecipitation buffer, with 50 μl of anti–rabbit IgG beads (eBioscience) followed by anti–histone-H4 (5 μg), anti–acetyl-histone-H4 (10 μl), anti–histone-H3 (5 μg), or anti–dimethyl-histone-H3 (5 μl) antibodies or rabbit preimmune serum (10 μl), and, after incubation overnight at 4°C, immunocomplexes were separated by a new addition of anti–rabbit IgG beads (50 μl). Immunoprecipitates, washed three times with immunoprecipitation buffer, were suspended in 20 μl of Laemmli sample buffer, boiled for 5 min and separated by SDS-PAGE either to be stained with Coomassie Blue or to be analysed by Western blotting. Western blotting was carried out with 0.22 μm nitrocellulose membranes activated with glutaraldehyde as described (*22*) for detection of ProTα, or with PVDF membranes for detection of other proteins.

Preparation of nNucleosome Core Particles and Analysis of the Interaction with Protα—Trimmed mouse splenocyte core particles were prepared according to the procedure described (*28*) with some modifications. Briefly, splenocytes were lysed in 5 volumes of buffer A (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM $NaHSO_3$, 0.5% NP-40, 5 mM sodium butyrate and 0.5 mM PMSF); nuclei were pelleted by centrifugation, then resuspended in buffer B (0.34 M sucrose, 15 mM Tris-HCl pH 7.4, 15 mM NaCl, 15 mM 2-mercaptoethanol, 60 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 1 mM $CaCl_2$, 0.1 mM PMSF, 10 mM $NaHSO_3$) at a DNA concentration of 4 mg/ml. The nuclear suspension was preincubated for 15 min at 37°C before adding the micrococcal nuclease (100 U/ml); the mixture was then further incubated for 15 min at 37°C with occasional agitation. After digestion, the sample was adjusted to 0.55 M NaCl and left for 10 min on ice. The precipitate formed was removed by centrifugation, to eliminate any residual histone H1. The trimmed core particles were finally purified by gel filtration on a Sepharose 4B column preequilibrated with 0.55 M NaCl, 20 mM Tris-HCl pH 7.6, 10 mM EDTA, 10 mM $NaHSO_3$, and 0.02% Na_3N (*29*), then dialyzed against 0.1× TBE buffer (1× TBE: 90 mM Tris-borate, pH 8.2 mM EDTA). DNA and protein contents in the purified nucleosome core particles were determined by agarose gel electrophoresis and SDS-PAGE respectively. The particles were routinely found to contain 146 bp of DNA and to be essentially devoid of histone H1.

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To analyse nucleosome–ProTα interactions, core particles were diluted with TBE to a protein concentration of 1 mg/ml, and incubated for 10 min at room temperature with different concentrations of ProTα. After addition of 5% glycerol, samples were analysed by electrophoresis on TBE nondenaturing gels (*30*). Gels were stained with Coomassie Blue. To determine the protein composition of the nucleosomes incubated with ProTα after their separation by this electrophoretic procedure, the nucleosome spots were excised from the gel, washed for 20 h in 10% glycerol, incubated in Laemmli sample buffer for 10 min and then analysed by SDS-PAGE.

Isolation of Nucleoplasmic Proteins with Affinity for ProTα-Sepharose—Nuclear proteins that interact with ProTα were obtained by affinity chromatography on ProTα-Sepharose columns of NC-37 cell nucleoplasmic extracts under the conditions previously described (*24*). Briefly, aliquots of 15 mg of nuclear extract in binding buffer (50 mM Tris-HCl pH 7.5, 75 mM NaCl, 1 mM DTT, 0.5 mM PMSF) were incubated with about 0.8 mg of calf ProTα or BSA coupled to 1 ml of Sepharose for 2 h at 4°C. This mixture was then loaded onto the column, which was washed with binding buffer containing 0.15 M NaCl. Finally, ProTα-retained proteins were eluted with binding buffer containing 1 M NaCl.

Mass Spectrometry—Protein bands separated by SDS-PAGE were excised from the gels, digested with trypsin and analysed by MALDI-TOF or by LS-ESI-IT dynamic exclusion mass spectrometry in the Proteomics Service of the "Severo Ochoa" Centre for Molecular Biology (Universidad Autónoma, Madrid, Spain).

HMT, HAT and HDAC Activity Assays—HMT assays were performed as previously described (*31*) in 40-μl reaction mixtures containing aliquots of nuclear proteins, 0.4 mM *S*-adenosyl-[methyl-^{3}H]-L-methionine (70 mCi/mmol; Amersham) and, as substrates, 3 μg of each of histones H1, H2A, H2B, H3 and H4 (Boehringer) or 3 μg of synthetic biotinylated N-terminal fragment (residues 1 to 24) of human histone H3 (NeoMPS, Strasbourg, France). After incubation for 1 h at 37°C, incorporation of ^{3}H-methyl to whole histones was determined by scintillation counting of aliquots of the reaction mixtures spotted onto P-81 cationic exchange paper (Whatman) washed in carbonate buffer (*32*). Incorporation of ^{3}H-methyl into individual histones was determined by counting radioactivity in bands excised from the stained gels in which components of the HMT reaction mixture had been separated by SDS-PAGE. When the N-terminal fragment of biotinylated-histone H3 was used as substrate, incorporation of ^{3}H-methyl was assayed by determining the radioactivity incorporated into the histone fragment separated by avidin-agarose.

HAT assays were carried out essentially as described (*33*), in 100-μl reaction mixtures containing aliquots of nuclear proteins, 0.6 mM ^{3}H-acetyl CoA (15 Ci/mmol, ACR Inc.) and, as substrates, 3 μg each of H1 and the core histones, 3 μg of the synthetic biotinylated fragment of N-terminal H3 (residues 1–20) or 3 μg of the synthetic biotinylated fragment of N-terminal H4 (residues 2–24). After incubation for 4 h at 37°C, incorporation of ^{3}H-acetyl into whole or individual histones and into the N-terminal fragment of H3 was determined as indicated for HMT activity.

HDAC activity was assayed using [^{3}H]acetylated biotinylated N-terminal fragments of H3 from the HDAC assay kit (Upstate), determining the release of ^{3}H-acetate produced by aliquots of nuclear proteins (20 μg), as per the manufacturer's protocol.

Gel Filtration—Aliquots of 800 μg of ProTα-retained proteins were chromatographed on a Superdex 200 10/300 GL column (Amersham Bioscences) in 20 mM phosphate buffer pH 7.6 containing 75 mM NaCl and 0.1% CHAPS, at a flow rate of 0.5 ml/min. Fractions were collected every 0.5 min.

RESULTS

Immunological Analysis and Subcellular Distribution of ProTα in Cytosolic and Nuclear Extracts—Research to characterize mechanisms of biochemical function through analyses of protein–protein interactions presents special difficulties in the case of ProTα, because of the unusual properties of this protein. First, immunological analysis is difficult, due to the incapacity of ProTα to bind the various types of membrane used in Western blotting techniques, and the weak immunogenicity of its sequence (*34*). Second, ProTα tends to leak from the nucleus when cells are disrupted, altering the concentration of ProTα in both cytosolic and nucleoplasmic extracts, as we (unpublished results) and others (*16*) have repeatedly found in the analysis of subcellular fractions from diverse cell types. To improve the immunological analysis, we prepared polyclonal antibodies raised against the more immunogenic regions in the ProTα sequence, *i.e.* the N-terminal (2–20 residues) and C-terminal (95–109 residues) regions; in addition, glutaraldehyde-activated membranes were used to favour interaction with ProTα (see "MATERIALS AND METHODS"). Moreover, and in order to obtain a rough quantification of nuclear leaking of ProTα, we determined the concentration of ProTα in cytosolic and nuclear extracts of human lymphoma B lymphocytes (NC-37 cells) treated or not treated with a reversible cross-linker (DSP). Results of the Western blotting analysis of the diverse NC-37 cell extracts (Fig. 1A) confirm nuclear leaking of ProTα, with its nuclear concentration markedly higher in cross-linker treated cells. Quantification of ProTα concentration (Fig. 1B) indicated that at least 70% of nuclear ProTα is leaked to the cytosol during the preparation of isotonic cell extracts; indeed, the proportion leaked from the nucleus may be even higher, since we cannot be sure that our cross-linking procedure is 100% effective for retaining ProTα. In any case, these results support the view that ProTα is present in both the cytosol and the nucleoplasm of proliferating lymphocytes. Furthermore, the reduced concentration of ProTα in nucleoplasmic extracts (relative to *in vivo* concentrations) means that in experimental designs to invesigate protein-protein interactions it is necessary to artificially supplement ProTα concentration in the extract (see next subsection). The data in Figure 1 also allow quantification of ProTα in NC-37 cells. The values obtained, in the order of 1.3 μg per 10^6 cells, are within the range reported from lymphoid tissues (*35*). These results confirm the reliability of the immunological quantification of ProTα: ProTα concentration estimates were similar regardless of whether anti–C-terminal or anti–N-terminal antibodies were used. Under our experimental conditions,

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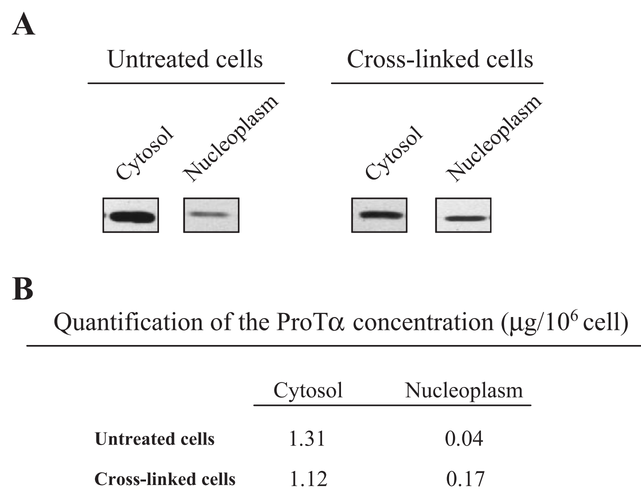

Quantification of the ProTα concentration (μg/10^6 cell)

	Cytosol	Nucleoplasm
Untreated cells	1.31	0.04
Cross-linked cells	1.12	0.17

Fig. 1. **Immunological evaluation of the subcellular distribution of ProTα in NC-37 cells treated or not treated with the cross-linker DSP.** (A) Aliquots of cytosolic (30 μg) and nucleoplasmic (30 μg) NC-37 cell extracts, either untreated or crosslinked with DSP, were separated by SDS-PAGE and transferred to glutaraldehyde-activated nitrocellulose membranes for probing with anti–ProTα-C-terminal antibodies. (B) Quantification of ProTα concentration in the NC-37 subcellular fractions. Data correspond to the densitometric analysis in A, referred to purified calf ProTα standards run in parallel (means of three experiments).

ProTα was not immunodetected unless the blotting membranes (0.22 μm or 0.45 μm pore size) were glutaraldehyde-treated (data not shown).

ProTα Interacts with Free Core Histones in Nucleoplasm of Dividing Lymphocytes—In view of the existing data on the *in vitro* interaction of ProTα with histones, we performed experiments to investigate possible *in vivo* interactions of these proteins, by studying the ability of ProTα to bind free histones or histones integrated within the chromatin structure. Interaction with free histones was investigated by reciprocal immunoprecipitation in cytosolic and nucleoplasm extracts of NC-37 cells. Due to the previous observation of the nuclear leaking of ProTα, immunoprecipitation experiments in nucleoplasmic extracts were performed in conditions in which the effect of this loss was compensated by adding extra ProTα. The diverse immunoprecipitates obtained with anti–N- and anti–C-terminal ProTα antibodies were separated by SDS-PAGE, stained with Coomassie Blue or subjected to Western blotting analysis for histones (using the anti-histone monoclonal antibodies which recognize H1 and core histones) and ProTα (using anti–N- and anti–C-terminal antibodies).

Results indicated that no histone was co-immunoprecipitated with ProTα in cytosolic extracts (Fig. 2A, top), under conditions in which both types of ProTα antibodies efficiently immunoprecipitated ProTα (Fig. 2A, bottom). By contrast, in nucleoplasmic extracts core histones were co-immunoprecipitated with ProTα by anti–C-terminal antibodies but not with anti–N-terminal antibodies, as indicated by Coomassie staining (Fig. 2A, top) and by the Western blotting analysis (Fig. 2A, bottom). However, anti–N-terminal ProTα antibodies can immunoprecipitate nuclear ProTα (Fig. 2A, bottom). It should be noted that ProTα cannot be detected in these Coomassie-stained SDS-PAGE gels, since concentrations under 2 μg are scarcely stained. Staining difficulties increased when core histones were present, since the electrophoretic mobility of ProTα coincides with that of H3.

We next investigated reciprocal immunoprecipitation of ProTα-histone complexes in the cytosolic and nucleoplasmic extracts using anti-histone antibodies. As with the anti-ProTα antibodies, no co-immunoprecipitation of ProTα and histones was observed in cytosolic extracts with any of the anti-histone antibodies used (anti-histone monoclonal, anti–histone-H3, anti–dimethyl-histone-H3, anti–histone-H4 or anti–acetyl-histone-H4), and these anti-histone antibodies likewise did not immunoprecipitate cytosolic histones. Figure 2B shows the results of the immunoblotting analysis of cytosolic immunoprecipitates obtained with anti-histone monoclonal, anti–histone-H4 and anti–acetyl-histone-H4. These were the only anti-histone antibodies effective for immunoprecipitating the ProTα-histone complexes from nucleoplasmic extracts (Fig. 2B), confirming the direct interaction of ProTα with core histones and apparently with the acetylated form of H4. Note, though, that immunoprecipitates with the anti-histone antibodies were less abundant than those obtained with anti-ProTα antibodies, with their components hardly detectable by Coomassie staining. Moreover, when nuclear extracts were not supplemented with extra ProTα (data not shown), we observed a decrease in the concentration of histone-ProTα complexes immunoprecipitated with anti-histone or anti-ProTα antibodies, confirming the nuclear leaking of this protein.

These results indicate that ProTα interacts with core histones H3, H2A, H2B and H4 exclusively in the nucleoplasm. To judge by the densitometric analysis from the nucleoplasmic immunoprecipitates in Fig. 2A (data not shown), interaction between ProTα and core histones ranged ratios of 1:1. However, it can not be discerned if the interaction of ProTα with core histones occurs through association with monomeric, dimeric or octameric forms of these histones in the nucleoplasmic extracts. The results obtained with the different ProTα and histone antibodies used suggest that the ProTα-histone interaction may block epitope domains in the N-terminal region of the ProTα sequence, and likewise may block epitopes in the histone structures. Another important conclusion is that neither by direct staining nor by immunoblot analysis did we detect any interaction of ProTα with histone H1 in either the cytosolic or nucleoplasm extracts.

To gain insight into the characteristics of the ProTα-histone interaction, we next investigated the affinity of ProTα for core histones integrated in the chromatin structure. To this end, we analysed the capacity of ProTα to interact with core histones included in nucleosomes. Different concentrations of ProTα were incubated with purified mononucleosomes at ratios in which we have previously demonstrated that in solution ProTα show similar affinity for the core histones (*23*). The analysis of the diverse reaction mixtures by electrophoresis on non-denaturing gels showed that incubation of ProTα with mononucleosomes did not affect its electrophoretic mobility (Fig. 3A), indicating that it does not form a stable association with nucleosomes. To rule out the possibility that incubation of ProTα with nucleosomes may result in alterations

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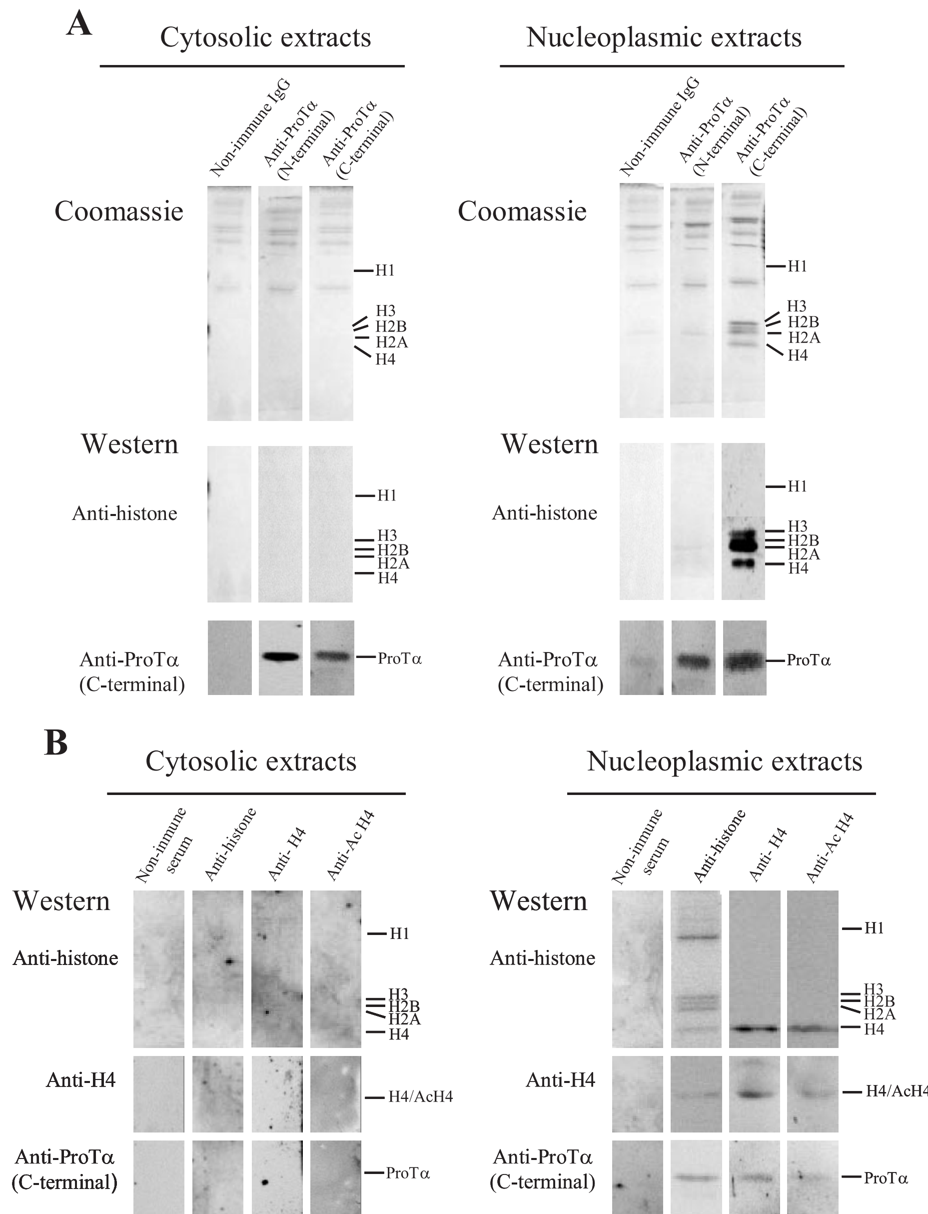

Fig. 2. **Reciprocal immunoprecipitation of core histones and ProTα.** (A) Immunoprecipitates obtained with anti–ProTα-N-terminal antibodies, anti–ProTα-C-terminal antibodies or non-immune IgG (15 μg each, crosslinked to protein A) from cytosolic (500 μg) or nucleoplasmic (500 μg plus 7 μg of ProTα) NC-37 cell extracts were separated by SDS-PAGE, then stained with Coomassie Blue (top of panel) or Western-blotted for detection of histones or ProTα (bottom of panel). (B) Immunoprecipitates obtained with anti-histone monoclonal (5 μl), anti–histone-H4 (5 μg) or anti–acetyl-histone-H4 (10 μl) antibodies, or non-immune serum (10 μl) from cytosolic (750 μg) or nucleoplasmic (750 μg plus 15 μg of ProTα) NC-37 cell extracts were separated by SDS-PAGE and Western-blotted for detection of ProTα, H1 plus core histones and histone H4.

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in the core histone content of the nucleosome, mononucleosome bands electrophoretically separated in the non-denaturing gels from the diverse reaction mixtures were excised and analysed by SDS-PAGE. As indicated in Fig. 3B, core histone content remained unchanged after incubation with ProTα. Similarly, mononucleosomes did not show affinity for ProTα-bound Sepharose (data not shown).

These results are in agreement with data showing the inability of ProTα to interact with *in vitro* reconstituted chromatin (*22*). In the other hand, experiments of chromatin immunoprecipitation which we performed using anti-histone monoclonal antibodies indicated that no ProTα was present in the immunoprecipitates which contained genomic DNA (unpublished data). All these data suggest that ProTα is unable to interact with chromatin *in vivo*.

Core Histones Form Part of a Nuclear Multiprotein Complex Separated by Affinity to ProTα—The observed interaction of ProTα with core histones extends our previous results showing that these histones are among the components of diverse cell extracts which are specifically separated by affinity to ProTα-Sepharose (*23*, *24*). Especially abundant was the multiprotein complex that shows specific affinity for ProTα-Sepharose in nucleoplasmic extracts of proliferating lymphocytes (*24*). As shown in Fig. 4A, core histones are the main components in this complex that interact with ProTα. Analysis of the proteins separated by affinity for a BSA-Sepharose column (Fig. 4A), together with our previous data showing that interaction of the nuclear proteins with ProTα-Sepharose was abolished when affinity chromatography was done in the presence of ProTα (*24*), corroborate the specificity of the interaction of ProTα with core histones and the other nuclear components. Thus, the ability of the various proteins in the complex to interact with ProTα *in vitro* may be indicative of a common involvement in the nuclear activity of ProTα

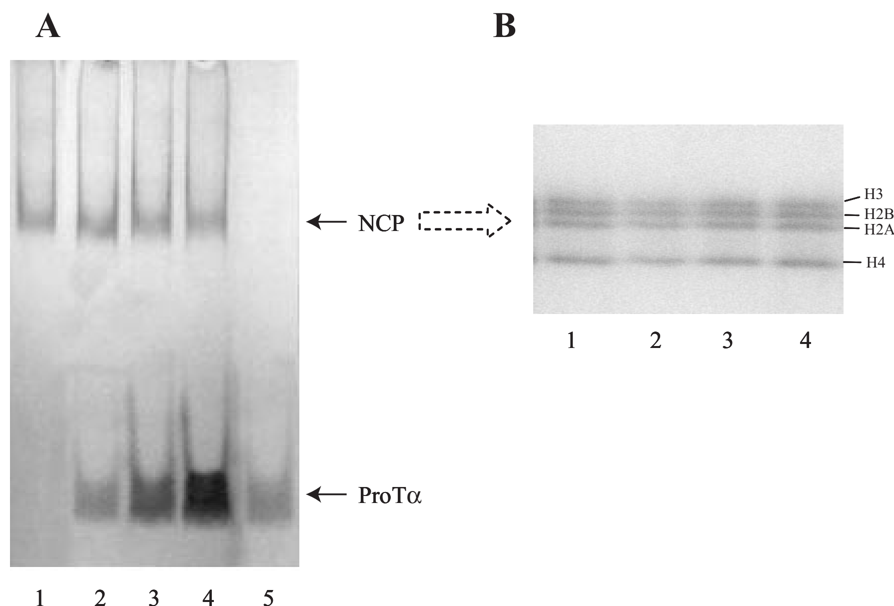

Fig. 3. **Analysis of ProTα-nucleosome interactions.** (A) Electrophoretic analysis on non-denaturing gels of ProTα–nucleosome mixtures. Mixtures of nucleosome core particles (50 μg) and ProTα at various molar ratios were analysed by electrophoresis in a non-denaturing gel, as indicated in "MATERIALS AND METHODS." Lanes 1, 2, 3 and 4 are analyses of mixtures with nucleosome:ProTα molar ratios of 1:0, 1:0.2, 1:1 and 1:2, respectively. Lane 5 corresponds to ProTα alone (30 μg). The positions of the nucleosome core particles (NCP) and of ProTα are marked to the right of the panel. (B) SDS-PAGE analysis under reducing conditions of nucleosomes after incubation with ProTα. Nucleosomes separated from the different nucleosome/ProTα mixtures by electrophoresis as in Panel A were excised from the gel, treated with denaturing buffer and analysed by reducing SDS-PAGE. Lanes 1 to 4 show the respective analyses of nucleosome core particles from mixtures of nucleosome and ProTα at molar ratios of 1:0, 1:0.2, 1:1 and 1:2, respectively.

and/or the histones. To investigate this hypothesis, we performed a structural characterization of the non-histone components of this complex obtained from NC-37 cells. Structural analysis of the main non-histone components by mass spectrometry is summarized in Fig. 4B. According to this analysis, a group of RNA/DNA-binding proteins, especially hnRNP isotypes A2/B1, A3, R and L and DNA helicases II and A, are the main components among the nuclear proteins showing affinity for ProTα, together with the cytoskeletal proteins β-actin and vimentin. In view of the presence of core histones in the complex and as a first approach to identifying the functional significance of their interaction with ProTα, we investigated the presence of enzymatic activities involved in the modification of histones, especially those related to chromatin activity, namely histone methyltransferase (HMT), histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities. These activities were assayed, using added core histones and histone H1 as substrates, in the nuclear multiprotein complex separated from nucleoplasmic extracts by affinity to ProTα and compared with those assayed in the fractions that did not bind ProTα (flow-through fractions). Results of these analyses indicate that a considerable proportion of HAT activity (Fig. 5A, top) and HMT activity (Fig. 5B, top) is separated by affinity to ProTα, while the HDAC activity found in the complex was minimal (data not shown).

In order to establish the specificities of these histone-modifying enzymes within the ProTα-bound complexes, we analysed the incorporation of tritium-labeled acetyl and methyl groups to the different histones used as substrates in the reaction mixtures after separation by SDS-PAGE. Results obtained indicate that the HAT activity corresponds to H3- and H4-acetyltransferases or to an enzyme with both activities, with predominance of the H3-acetyltransferase activity (Fig. 5A, bottom), whereas the HMT activity is due to H3-methyltransferases (Fig. 5B, bottom).

To explore possible relationships of these enzyme activities with other components in the multiprotein complex, we investigated specific interactions among the components of this complex by separation of these proteins using gel filtration, analysing protein composition and HAT and HMT activities in the different fractions. The results of the protein composition analysis (Fig. 6A) show that components in the complex have a tendency to associate as multimers: notably, the main components of the complex form part of an oligomeric association that shows HAT and HMT activities (Fig. 6B) and that ranges from 600 to 700 kDa in size. However, the HAT activity peak is slightly displaced with respect to the fractions with the maximum core histone content and maximum HMT activity; note that although Fig. 6B shows HAT activity with H3 as substrate, the elution pattern with H4 as substrate (data not shown) was similar. Since we found that HAT activity on H3 and H4 in the nuclear multiprotein complex was inhibited when the concentration of core histones in the reaction mixtures exceeded 5 μg (data not shown), it is possible that the displaced HAT activity peak may simply reflect the high concentration of substrate (core histones) in the fractions eluted to 8.5–9 ml (Fig. 6A). The major components of this oligomeric association, identified by mass spectrometry, are indicated in Figure 6C, and they included all the non-histone protein

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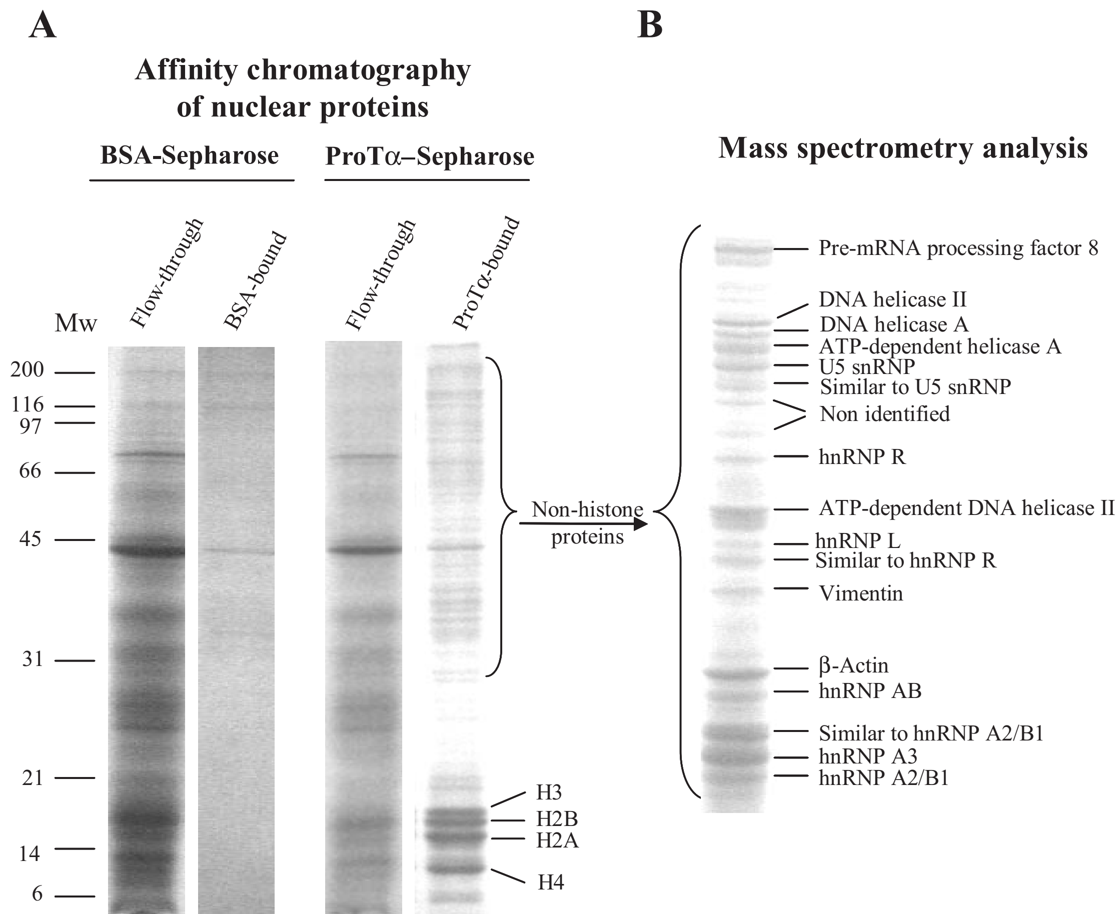

Fig. 4. **Affinity chromatography and analysis of NC-37 nuclear proteins separated by ProTα-Sepharose affinity.** (A) Analysis by SDS-PAGE (12% polyacrylamide gels) of the nuclear proteins in the flow-through fractions from BSA-Sepharose (50 μg) and from ProTα-Sepharose (50 μg) columns; and that in components bound to BSA-Sepharose (the whole products: 13 μg) and bound to ProTα-Sepharose (50 μg from 450 μg). Positions of core histones are indicated. (B) Mass spectrometry analyses of the main non-histone proteins among the nuclear ProTα-bound proteins, previously separated by SDS-PAGE in 8% polyacrylamide gels (50 μg). Results of structural analyses of the marked proteins in the gel are indicated.

components in the nuclear multiprotein complex separated by affinity to ProTα-Sepharose, except DNA-helicase A and hnRNP L. Spectrometric analysis of the main components in the multimers with diverse molecular size separated by gel filtration of the nuclear complex, specially those in the range of 150 kDa to 50 kDa, indicated that they correspond to aggregates of hnRNPs isotypes L, A3 and A2/B1 (data not shown).

On the basis of these findings, it seems possible that the interaction of ProTα with core histones in nucleoplasm may be related to the process of structural modification of histones, and hence to the control of chromatin activity, raising the possibility that hnRNPs and other proteins in the multinuclear complex separated by affinity to ProTα play a role in this process, or in some other process in which ProTα is involved in nuclear metabolism.

DISCUSSION

The structural and functional properties of ProTα already described and reported in this paper strongly suggest that this protein forms part of the "histone-binding protein" group of multitasking acidic proteins, with functional roles in both nucleus and cytoplasm, related to chromatin activity and in some cases apoptosis. Other members of this group include nucleoplasmin (*36*) and N1 (*37*) in *Xenopus*, and NAP1/NAP2 (*38*, *39*), CAF-1 (40), SET (*41*) and pp32 (*41*, *42*) in mammalian cells: these proteins, all with long acidic histone-binding domains, have been implicated in transcription regulation, DNA replication and nucleosome assembly, and in the cases of SET and pp32 in apoptosis (*15*, *43*).

To judge from the analysis of the immunoprecipitates with the diverse ProTα antibodies, ProTα did not interact with histone H1 in extracts of NC-37 cells. This is in contradiction with previous results indicating a ProTα–H1 interaction on the basis of immune absorption experiments (*21*, *22*) and Western blotting with antibodies against ProTα (*17*). However, these previous studies did not carry out reciprocal immunoprecipitation experiments, or analyses of other histones among the components with affinity for ProTα, so that they do not constitute evidence against an interaction of ProTα with other histones (as indicated by the present results). We have detected

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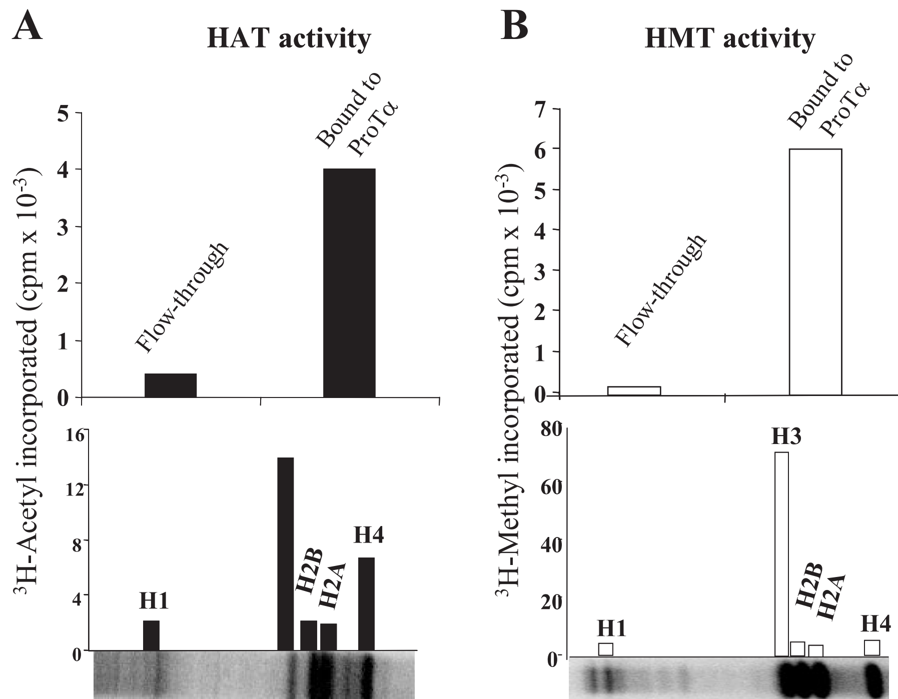

Fig. 5. **Determination of HAT and HMT activities in the nuclear multiprotein complex separated by ProT α-Sepharose affinity.** Nuclear extracts (15 mg) were separated by ProTα-Sepharose affinity chromatography and HAT and HMT activities assayed in the flow-through fraction and the fraction eluted with 1 M NaCl, using core histones and histone H1 as substrates. (A) Analyses of HAT activity. Bars at the top of the panel indicate net incorporation of ^{3}H-acetyl to the histone substrates induced by aliquots of 20 μg of flow-through or ProTα-bound fractions, as determined by scintillation counting of the P-81 cation-exchange paper to which aliquots of the HAT assay were spotted. The bottom of the panel shows the results of analysis of the specificity of the HAT activity bound to ProTα-Sepharose, evaluated by counting the radioactivity incorporated into each histone, after separating the HAT assay reaction mixture by SDS-PAGE and excising the corresponding bands from the stained gel (indicated at the bottom of the panel). (B) Analyses of HMT activity. Bars at the top indicate net incorporation of ^{3}H-methyl to the histone substrates induced by aliquots of 20 μg of flow-through or ProTα-bound fractions, as determined by scintillation counting of the P-81 cation-exchange paper, as above. The bottom of the panel shows the results of the analysis of HTM specificity, evaluated as above.

affinity of ProTα for H1 using a commercial mixture of H1 and core histones separated by ProTα-Sepharose, but in these experiments affinity for H1 was markedly lower than for the core histones (*23*). Instead, when this affinity separation was carried out with different cell extracts, H1 was not detected in the components separated by affinity to ProTα (*23*, *24*).

Structurally, the interaction of ProTα with histones can be explained by the presence of a highly acidic domain in its structure (aspartate/glutamate between residues 41 and 85), as characteristic of core-histone–binding proteins including nucleoplasmin, N1, CAF-1, SET, NAPs and pp32. Moreover, we have previously reported (*23*) that the peptide corresponding to the first 28 aminoacids in the ProTα sequence, *i.e.* thymosin α_1 (Tα_1), can also bind core histones *in vitro* with high affinity. Thus, it is possible that the interaction of ProTα with histones *in vivo* may involve binding of the Tα_1 region of ProTα.

The ability of ProTα to interact with histones is probably facilitated by its rather high intracellular concentration, around 1 pg per cell in mammalian tissues, which is in the order of that of a single histone. This high concentration of ProTα, together with its apparent ability to move between the nucleus and the cytoplasm attributable to its karyophylic signal, is in line with a multi-tasking functional role involving interaction with various nuclear and cytosolic targets. Evidence of this sort of functional role is provided by the ability of ProTα-Sepharose to separate cytoplasmic and nucleoplasmic multiprotein complexes with affinity for ProTα. For example, in the cytoplasmic complexes we have detected a protein kinase responsible for the phosphorylation of ProTα *in vivo* (*13*) and proteins related to its nuclear transport (*24*). The nuclear multiprotein complex described in the present study has the important characteristic of containing the core histones together with DNA/RNA-binding and cytoskeletal proteins, and histone-modifying enzymes, all sharing affinity for ProTα. Although further investigation is needed in order to assess the function of the diverse components of this complex, the presence of HMT and HAT activities among these proteins suggests a possible role of ProTα in the structural modification of H3 and H4, the most significant histones in the control of chromatin activity. Reports showing the ability of ProTα to interact with proteins that bind HAT activities, such as Epstein-Barr virus nuclear antigen (*17*, *18*) and CREB-binding protein (*19*), both with affinity for the acetyltransferase p300, also support this view. However, the specificity of the HAT activity in the nuclear ProTα-binding protein complex (*i.e.* specific for H3 and H4), differs from that of p300, which shows a broad specificity for the four core histones (*44*). HAT activity in the nuclear ProTα-binding complex is rather

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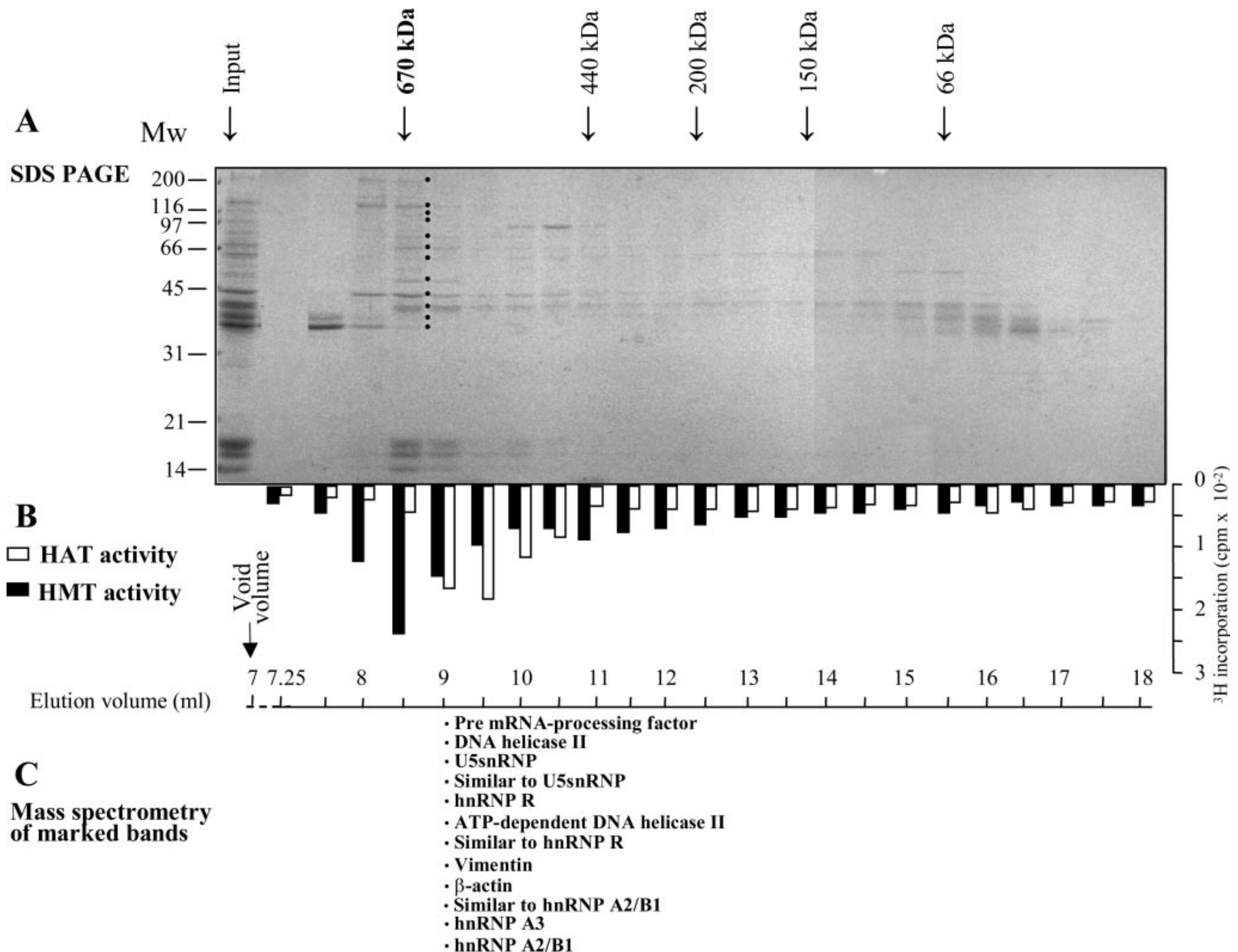

Fig. 6. **Gel filtration analysis of the nuclear protein complex with specific affinity for ProTα** The nuclear complex (800 μg) was separated by gel filtration, and aliquots of the indicated fractions were analysed for: (A) protein composition in aliquots of 50 μl separated by SDS-PAGE and Coomassie Blue staining. Input corresponds to 50 μg of nuclear complex with affinity for ProTα, run in parallel; (B) HAT and HMT activities, in aliquots of 30 μl, using a biotinylated N-terminal fragment of histone H3 as substrate; (C) structural analysis by mass spectrometry of the components (proteins marked with dots in the SDS-PAGE analysis in A, and assigned on the basis of mobility in the gel). Similar results to A and B were obtained in two independent experiments.

similar to that of PCAF, the p300/CBP-associated factor that belongs to the GNAT family of histone acetyl transferases (*44*). However, we failed to detect this protein in our Western blotting analysis of the nuclear complex (data not shown).

Of interest is the prominent presence of hnRNPs in the ProTα-bound nuclear complex detected in this study. Due to the properties of the hnRNPs, especially those derived from their ability to induce protein aggregations and to recognize discrete DNA and RNA sequence elements (*45*), their presence in this nuclear complex may indicate a role of these proteins in HAT and HMT activities and/or other aspects of the involvement of ProTα in chromatin activity. In fact, experiments in our laboratory have demonstrated direct interaction of ProTα with the hnRNP isotypes A2/B1 and A3 (unpublished results).

Also noteworthy is the variety of properties that ProTα shares with the acidic histone-binding proteins SET and pp32, both in nuclear and cytosol-located processes, as indicated by both present and previous results. Notably, and besides the shared properties of interaction with histones and involvement in nucleosome assembly (*41*), SET and pp32 modulate the nuclear function of HuR, an RNA-binding protein implicated in the export and stability of mRNAs (*46*) that has recently been reported to be involved in the translation and cytosolic concentration of ProTα mRNA (*47*). Moreover, SET and pp32 are also involved with HAT activities, since they have inhibitory effects on the HAT activities of p300/CBP and PCAF (*41*). Note, though, that we did not detect a similar influence of ProTα on the HAT and HMT activities present in the ProTα-bound nuclear complex (data not shown).

The behavior of ProTα resembles that of SET and pp32 in terms of cytoplasm-located processes. For example ProTα, pp32 and SET have all been reported to function as key regulators of apoptosis. ProTα and pp32 both regulate apoptosis through effects on apoptosome activity, though

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these effects are opposite: pp32 accelerates apoptosis by cooperation in the activation of caspase 3, while ProTα seems to be involved, by an unknown mechanism, in the inhibition of caspase 3 activation by the apoptosome (*15*). The antiapoptotic effect of SET is exercised by inhibition of a granzyme A–activated DNase (*43*). Since proteolysis plays an important role in the apoptotic effects of SET (*43*), this might reflect another relationship of this protein with ProTα, which is quantitatively processed by a lysosomal protease to yield Tα_1 (*14*), although the biological significance of this process is still unknown. The reported proteolysis of ProTα by caspase 7 and the suggestion that this may be related to the influence of ProTα on apoptosis (*48*, *49*) is in our view not supported by the fact that this proteolysis was only described in HeLa cells, hours after initiation of the apoptotic process (*48*), and we have not observed any such proteolysis in lymphocytes (unpublished data). On the other hand, the reported presence of SET among components of HeLa cell extracts separated by affinity to anti-ProTα antibodies (*50*) may reflect a combined interaction with other proteins, perhaps histones, since in that study the authors were unable to demonstrate a direct interaction between ProTα and SET in these extracts.

In conclusion, the data reported in this paper are in line with the view that the role of ProTα in nuclear metabolism is related to its interaction with free core histones in the nucleoplasm, which may serve as a basis for attraction of proteins involved in the modification of histones and/or other steps in the control of chromatin activity. Further functional characterization of these ProTα-binding proteins may contribute to a more detailed understanding of the nuclear role of ProTα.

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