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The nuclear matrix protein p255 is a highly phosphorylated form of RNA polymerase II largest subunit which associates with spliceosomes

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ABSTRACT

The monoclonal antibody CC-3 recognizes a phosphodependent epitope on a 255 kDa nuclear matrix protein (p255) recently shown to associate with splicing complexes as part of the [U4/U6.U5] tri-snRNP particle [Chabot *et al.* (1995) *Nucleic Acids Res.* 23, 3206–3213]. In mouse and *Drosophila* cultured cells the electrophoretic mobility of p255, faster in the latter species, was identical to that of the hyperphosphorylated form of RNA polymerase II largest subunit (IIo). The CC-3 immunoreactivity of p255 was abolished by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, which is known to cause the dephosphorylation of the C-terminal domain of subunit IIo by inhibiting the TFIIH-associated kinase. The identity of p255 was confirmed by showing that CC-3-immunoprecipitated p255 was recognized by POL3/3 and 8WG16, two antibodies specific to RNA polymerase II largest subunit. Lastly, the recovery of RNA polymerase II largest subunit from HeLa splicing mixtures was compromised by EDTA, which prevents the interaction of p255 with splicing complexes and inhibits splicing. Our results indicate that p255 represents a highly phosphorylated form of RNA polymerase II largest subunit physically associated with spliceosomes and possibly involved in coupling transcription to RNA processing.

INTRODUCTION

RNA polymerase II (RNAP II) is a large, multisubunit enzyme composed of a dozen different polypeptides. It is found in two forms within eukaryotic cells, RNAP IIA and RNAP IIO, according to the level of phosphorylation of its largest subunit, designated either IIa (unphosphorylated; apparent M_r 210 kDa) or IIo (multiphosphorylated; 240 kDa) (1–2). The phosphorylation sites of the largest subunit are located in its C-terminal domain (CTD) composed of multiple heptapeptide repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. This domain is conserved in evolution but its length seems to be related to the complexity of organisms (3). The CTD is essential *in vivo* and may play a role in the regulation of transcription or in the linkage of transcription to other nuclear processes (3–6). Various studies suggest that the unphosphorylated CTD of RNAP IIA interacts with the promoter to form a stable preinitiation complex and that the entry into initiation of transcription is accompanied by the phosphorylation of the CTD (7–11). A multiplicity of CTD kinases have been identified, including both serine/threonine and tyrosine kinases, suggesting that the heterogeneity of CTD phosphorylation could be extensive (10).

A subpopulation of subunit IIo has recently been shown to colocalize with splicing protein SC-35 in nuclear speckled domains, and to be highly resistant to extraction by detergents (12). During states of transcriptional inhibition, both subunit IIo and SC-35 coredistributed to enlarged unconnected speckled domains which are thought to be storage and/or assembly sites for splicing factors (12). In this connection, previous work in our laboratories has shown that monoclonal antibody CC-3 reacts with a phosphodependent epitope borne by a 255 kDa nuclear matrix protein (p255) which colocalizes with splicing factor SC-35 at every stage of the cell cycle and after the cells were heat shocked (13–14). Furthermore, this nuclear phosphoprotein was recently shown to stably associate with spliceosomal complexes formed *in vitro* via an association with the [U4/U6.U5] tri-snRNP complex (15). We show here that p255 corresponds to a subset of subunit IIo carrying a particular phosphorylated epitope specifically recognized by monoclonal antibody CC-3. This finding signifies that a subpopulation of the largest subunit of RNAP II is physically associated with spliceosomes. During the preparation of this manuscript, Mortillaro *et al.* (33) reached the same

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conclusion using a different monoclonal antibody to a hyperphosphorylated form of RNAP II large subunit.

MATERIALS AND METHODS

Cell cultures

HeLa cells were grown in 75 cm^2 culture flasks at 37°C with 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS). Mouse NIH 3T3 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and *Drosophila* SL2 cells were grown in D-22 medium supplemented with 10% decomplemented FBS. In some experiments, medium was supplemented with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), prepared from 100 mM stock solution in dimethyl sulfoxide.

Antibodies

MAb CC-3 was obtained after immunization of a Balb/C mouse with pharyngeal regions isolated from 72 h chick embryos (16). The CC-3 hybridoma cell line was grown in IMDM supplemented with 10% FBS and the culture supernatant was used undiluted as a source of CC-3 antibody for immunoblotting experiments whereas ascitic fluid was used for immunoprecipitations. The mouse monoclonal antibody POL3/3 recognizes RNAP II largest subunit in a conserved region located outside the CTD and was kindly provided by E.K.F. Bautz (17). The mouse monoclonal antibody 8WG16 (Promega, Madison, WI) binds to a peptide epitope located in the CTD (18).

Immunoprecipitations

HeLa cells were washed with PBS and liquid nitrogen was poured over the cell layer to reduce protease and phosphatase activities during the solubilization step. After evaporation, the cells were solubilized with 1 ml TD buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 1 mM PMSF and 2 mM sodium orthovanadate) at 4°C during 15 min with vigorous agitation at every 5 min. The suspension was then passed through a 28G1/2 needle repetitively before centrifugation at 13 000 *g* for 30 min at 4°C for clarification. A volume of supernatant equivalent to 10^6 cells was incubated with either 10 µl CC-3 ascite or 2 µl monoclonal antibody 8WG16 for 1 h at room temperature and then anti-mouse IgG–agarose beads (Sigma) were added for 2 h at room temperature. Irrelevant primary antibodies were used to control the non-specific binding to the agarose beads. Beads were washed with TD buffer and solubilized directly in electrophoresis sample buffer. Preparations of nuclear splicing extracts (19) were immunoprecipitated as described previously (15).

Electrophoresis and immunoblotting

The samples were solubilized in electrophoretic sample buffer (20) and heated for 3 min at 95°C before loading on 5–15% polyacrylamide gradient gels (acrylamide:bisacrylamide, 30:0.15) using a mini-protean II apparatus (BioRad). The proteins were then transferred to nitrocellulose membranes and the position of high molecular weight markers (myosin heavy chain, 200 kDa; human spectrin doublet, 220 and 240 kDa) was revealed by Ponceau S staining. The membranes were saturated with PBS containing 5% skimmed milk powder (Blotto) for 1 h at 37°C prior to incubation with monoclonal antibodies CC-3, POL3/3 or 8WG16 for 45 min at 37°C. The membranes were then extensively washed with PBS containing 0.05% Tween-20 and incubated for 1 h with ^{125}I-labelled goat immunoglobulins against mouse IgG heavy chains or with peroxidase conjugated anti-mouse IgG antibody (Promega) diluted in Blotto. Washed immunoblots were exposed directly to Fuji RX film or immunodetection was performed using the ECL reaction (Amersham, Oakville, Ont). In some experiments (Figs 1 and 2), the procedure used was described previously (21).

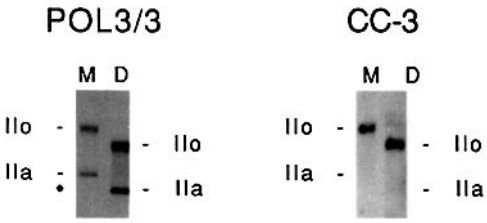

Figure 1. The CC-3 immunoreactive band (p255) comigrates with subunit IIo in mouse and *Drosophila*. Comparison of the electrophoretic mobilities of RNAP II large subunit and p255 in total homogenates from mouse NIH 3T3 cells (M) and *Drosophila* SL2 cells (D) immunoblotted respectively with POL3/3 and CC-3 monoclonal antibodies. The asterisk indicates the position of the 200 kDa molecular weight marker.

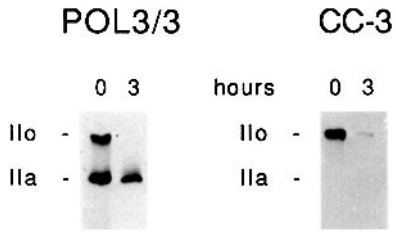

Figure 2. DRB abolishes subunit IIo and p255 immunoreactivities. Comparison of the levels of dephosphorylation of RNAP II large subunit and p255 in DRB-treated NIH 3T3 cells (100 µM, 3 h). The cell lysates were immunoblotted with POL3/3 and CC-3 monoclonal antibodies.

RESULTS AND DISCUSSION

Previous studies have shown that monoclonal antibody CC-3 stained nuclear speckles and nucleoplasmic material in vertebrate cells and immunoblotted a 255 kDa protein whose reactivity was mediated by a phosphodependent epitope (13,14). Based on its resistance to extraction with non-ionic detergents, nucleases and high ionic strength buffers, p255 was defined as a nuclear matrix protein. Its distribution at different stages of the cell cycle and after the cells were heat shocked was identical to that of splicing factor SC-35 (14). This behaviour was reminiscent of that of cytostellin, a phosphoprotein identified with monoclonal antibody H5, shown to distribute to nuclear regions enriched with splicing factors (22,23). Unlike CC-3 however, H5 reactivity with intranuclear speckles appeared to fluctuate markedly during the cell cycle (22) so that *a priori* a parallel between both antigens appeared fragile. Recently, however, H5 variable immunoreactivity was shown to originate from a masking effect since an extraction with non-ionic detergents prior to fixation and H5

staining resulted in the appearance of a speckled pattern in nearly all nuclei (12). The immunopurification and microsequence determination of cytostellin allowed its identification as RNAP II largest subunit (12), demonstrating at the same time that a subpopulation of the RNAP II large subunit was located in the speckled domains and was associated with a solid phase nuclear structure.

To see if monoclonal antibody CC-3 also reacted with a subset of RNAP II large subunit, we first verified the mobility of its antigen in immunoblotted cellular extracts from *Drosophila* cells as the heptapeptide sequence of the CTD is repeated 43 times in this species, compared with 52 times in the mouse (24). As shown in Figure 1, in murine and *Drosophila* cell lysates, antibody POL3/3 recognized two bands migrating above the 200 kDa molecular weight marker. These two bands corresponded to the two forms of RNAP II largest subunit (17,21). The murine polypeptides exhibited a slower electrophoretic mobility, as expected from their longer CTD. In both cell types, monoclonal CC-3 immunoblotted a single peptide band comigrating with subunit IIo (Fig. 1). We then compared the levels of phosphorylation of the phosphodependent CC-3 antigenic determinant with that of subunit IIo upon treatment of NIH 3T3 cells with the inhibitor of transcription DRB, which is known to promote dephosphorylation of the CTD by inhibiting the TFIIH-associated kinase (21). After incubation of the cells for 3 h in the presence of 100 µM DRB, the IIo band almost completely disappeared when analyzed by Western blot using antibody POL3/3 (Fig. 2). When probed with CC-3, the intensity of p255 was also dramatically decreased in DRB-treated cells (Fig. 2). Thus, under different circumstances p255 behaved as RNAP II largest subunit IIo. In addition, unlike subunit IIa, p255 and most of subunit IIo remained associated with nuclear matrix preparations following detergent extraction (data not shown).

To confirm the identity of both proteins, p255 was immunoprecipitated with CC-3 from HeLa cellular extracts. Both the pellet and the immunodepleted supernatant were submitted to Western blot analysis using CC-3 and antibodies to RNAP large subunit POL3/3 and 8WG16. As shown in Figure 3, CC-3-immunoprecipitated p255 was recognized by POL3/3 and 8WG16 (lanes 3). A CC-3-immunoreactive 180 kDa band was also found in the immunoprecipitated fraction. It is unclear whether this band represents a p255 breakdown product or an unrelated peptide carrying the CC-3 epitope, but it was not reacting with antibodies to RNAP large subunit. Although p255 was completely depleted from the cell extract according to the CC-3 immunoblot (Fig. 3, lane 2), some RNAP II large subunit unphosphorylated or intermediate forms, visualized by antibodies POL3/3 and 8WG16, could not be precipitated by antibody CC-3 (Fig. 3, lanes 2). Conversely monoclonal antibody 8WG16, which recognizes a non-phosphodependent epitope on the CTD, immunoprecipitated mostly hypophosphorylated forms of RNAP II large subunit as revealed by immunoblotting with POL3/3 (Fig. 4, right panel). The CC-3-reactive form could not be immunoprecipitated by 8WG16 (presumably because the epitope is not accessible) and was entirely recovered in the supernatant fraction (Fig. 4, left panel). We conclude from these experiments that p255 is a highly phosphorylated form of RNAP II largest subunit.

Recently, the association of p255 with splicing complexes was indicated by the finding that antibody CC-3 could inhibit *in vitro* splicing and immunoprecipitate pre-mRNA, splicing intermediates and products (15). Immunoprecipitations of HeLa nuclear

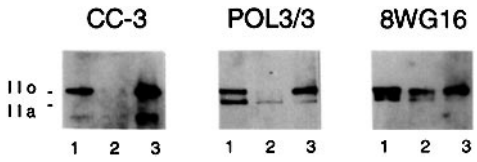

Figure 3. p255 is recognized by antibodies to RNAP II large subunit. Immunoblots of CC-3-immunoprecipitated p255 from HeLa cells with monoclonal antibodies CC-3, POL3/3 and 8WG16. Lanes 1, solubilized cell extract before immunoprecipitation; lanes 2, immunodepleted supernatant; lanes 3, immunoprecipitated pellet. The wells of lanes 3 were loaded with 6× cell equivalent aliquots. CC-3-immunoprecipitated p255 is reacting with POL3/3 and 8WG16 antibodies. The p255-depleted supernatants (lanes 2) contain intermediate and hypophosphorylated forms of RNAP II largest subunit.

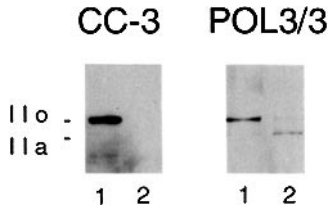

Figure 4. Immunoblots of 8WG16-immunoprecipitated RNAP II large subunit with monoclonal antibodies CC-3 and POL3/3. Lanes 1, supernatant of the immunoprecipitation reaction; lanes 2, immunoprecipitated pellet. The wells corresponding to lanes 2 were loaded with 6× cell equivalent aliquots. Monoclonal antibody 8WG16 does not immunoprecipitate p255 but hypophosphorylated forms of RNAP II largest subunit.

extracts with CC-3 revealed that p255 was stably associated with the U5 snRNP and was a component of the [U4/U6.U5] tri-snRNP complex (15). RNase protection assays showed that antibody CC-3 immunoprecipitated fragments containing branch site and 3′ splice site sequences. The recovery of branch site protected fragments required U2 snRNP and was inhibited by EDTA (15). Whereas inhibition of splicing by EDTA (25) does not prevent the entry of the [U4/U6.U5] tri-snRNP into spliceosomes, EDTA promotes aberrant U4 and U6 interactions with the pre-mRNA and U2 snRNA (26).

To extend these observations, nuclear extracts incubated under splicing conditions (60 min at 30°C) were immunoprecipitated with CC-3 and immunoblotted with POL3/3. Subunit IIo was recovered in the immunoprecipitated fraction (Fig. 5, lane 1), but less phosphorylated forms were also visible presumably because some dephosphorylation occurred during and after precipitation. When nuclear extracts containing 2.5 mM EDTA were used, only traces of RNAP II large subunit were found in the CC-3 immunoprecipitated pellet (Fig. 5, lane 2). The disappearance or masking of the CC-3 epitope in the presence of EDTA is likely responsible for the failure of the CC-3 antibody to immunoprecipitate spliceosomes under these conditions (15). Future experiments will examine the intriguing possibility that RNA polymerase II plays a role in correctly positioning the tri-snRNP in the spliceosome.

In retrospect to our previous studies, the identification of p255 as subunit IIo indicates that the speckled domains are not only enriched with spliceosomal proteins but also with a particular subspecies of RNAP II largest subunit which associates with splicing complexes as part of the [U4/U6.U5] tri-snRNP particle.

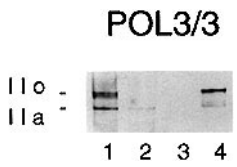

Figure 5. Monoclonal antibody CC-3 immunoprecipitates RNAP II large subunit from nuclear extracts under splicing conditions. HeLa cell nuclear extracts were immunoprecipitated with antibody CC-3 after a 60 min incubation at 30°C in splicing conditions (lane 1) or in the presence of 2.5 mM EDTA (lane 2) and the immunoprecipitated pellets were blotted with antibody POL3/3. The use of an irrelevant monoclonal antibody to immunoprecipitate gave negative results (lane 3). Lane 4 shows the immunoreactive pattern of a total homogenate from HeLa cells.

These results are consistent with those of Bregman *et al.* (12) who showed that splicing factors and RNAP II large subunit co-localize in the nuclear speckles. This subpopulation of subunit IIo carries one or more phosphorylated sites specifically recognized by monoclonal antibody CC-3. The *in vitro* phosphorylation of various mutated or truncated forms of recombinant CTD with cdc2 kinase allowed the localization of the CC-3 phosphoepitope at the C-terminal end of the CTD (Dubois *et al.*, unpublished results). In agreement with its DRB-sensitivity, the CC-3 phosphoepitope could also be generated by the TFIIH-associated kinase *in vitro*. Moreover, monoclonal antibody CC-3 can discriminate between IIo subunit phosphorylated by TFIIH kinase from that phosphorylated by a stress-activated kinase in heat shocked cells, demonstrating that different phosphorylated forms of the large subunit may coexist in the cells (Dubois *et al.*, submitted).

Altogether, these findings are consistent with the existence of a coupling mechanism between transcription and RNA processing. Greenleaf (27) presented a model proposing that certain splicing factors containing positively charged serine/arginine-rich (SR) regions may interact with the negatively charged tail of elongating RNAP II. Incidentally, p255 could be immunoprecipitated with SC-35 using anti-SC-35 antibody (Bisotto and Vincent, unpublished results). Moreover, interactions between the CTD and SR-like proteins have recently been uncovered using the yeast two-hybrid system (28). In heat-shocked cells, the decreased activity of TFIIH-kinase correlates with the disappearance of CC-3 epitope but the overall IIo subunit phosphorylation increases, presumably by the compensation effect of stress-activated MAP kinases ensuring transcription of heat-shock genes (Dubois *et al.*, submitted). Heat shock of cells is also associated with an inhibition of splicing of pre-mRNAs (29). It is possible that the disappearance of the CC-3 epitope caused by heat-shock inactivation of TFIIH kinase prevents the interaction between RNAP II large subunit and the tri-snRNP complex and causes the interruption of splicing. Interestingly, heat shock disrupts the [U4/U6.U5] tri-snRNP complex *in vivo* and *in vitro* (30–32), and antibody CC-3 could not immunoprecipitate U4, U5 and U6 snRNAs from heat-shocked extracts (B.C. and M.V., unpublished results). Since the expression of the major heat-shock genes does not require splicing, it is conceivable that inhibition of pre-mRNA splicing provides a mechanism to differentially regulate the expression of these two classes of pre-mRNAs.

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