

Prothymosin α interacts with the CREB-binding protein and potentiates transcription

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Prothymosin α (ProT α) is a histone H1-binding protein localized in sites of active transcription in the nucleus. We report here that ProT α physically interacts with the CREB-binding protein (CBP), which is a versatile transcription co-activator. Confocal laser scanning microscopy reveals that ProT α partially colocalizes with CBP in discrete subnuclear domains. Using transient transfections, we show that ProT α synergizes with CBP and stimulates AP1- and NF- κ B-dependent transcription. Furthermore, overexpression of ProT α enhances the transactivation potential of CBP. These findings reveal a new function for ProT α in transcription activation, probably through CBP-mediated recruitment to different promoters.

INTRODUCTION

Prothymosin α (ProT α ; 109–111 amino acids, pI 3.5) is perhaps the most acidic protein existing in the cell nucleus (Haritos *et al.*, 1984; Clinton *et al.*, 1991; Manrow *et al.*, 1991). The function of ProT α is associated with cell growth (Eschenfeldt and Berger, 1986) and its gene is a target of the c-myc proto-oncoprotein (Eilers *et al.*, 1991).

The unusual chemical features of ProT α provide important clues for its function. The primary structure of ProT α contains 52 acidic residues, 31 of which are clustered in the central part of the molecule (Frangou-Lazaridis *et al.*, 1988). Similar polyacidic tracks are found in several nuclear proteins whose functions are linked to chromatin decondensation, nucleosome assembly/disassembly and establishment of chromatin structures competent for transcription (Earnshaw, 1987). Previous studies have shown that ProT α interacts specifically with linker histone H1, which is a major determinant of the folding of nucleosomal arrays into higher-order structures (Papamarcaki and Tsolas, 1994; Karetso *et al.*, 1998); under *in vitro* conditions, binding to core histones at high concentrations has also been reported

(Diaz-Jullien *et al.*, 1996). In the context of a physiological chromatin reconstitution reaction, we have shown previously that ProT α modulates the interaction of H1 with chromatin (Karetso *et al.*, 1998). Moreover, other studies have shown that cells overexpressing ProT α have increased levels of histone H1-depleted chromatin (Gomez-Marquez and Rodriguez, 1998). Taken together, these results support the notion that ProT α is involved in chromatin remodeling.

Recently, indirect immunofluorescence labeling and confocal scanning laser microscopy studies have indicated that ProT α is localized close to transcription sites and in nuclear domains that often overlap or engulf promyelocytic leukemia protein-containing bodies (PML bodies) (Vareli *et al.*, 2000). Interestingly, PML bodies contain several transcription factors, CREB-binding protein (CBP)/p300 transcription co-activators and RNA polymerase II (von Mikecz *et al.*, 2000). These morphological observations raise the interesting possibility that ProT α is implicated in transcription. In this study, we report that ProT α interacts with the transcription co-activator CBP both *in vivo* and *in vitro*, and colocalizes with it in subnuclear domains. Using transient transfections, we show that ProT α synergizes with CBP and stimulates AP1- and NF- κ B-dependent transcription. In addition, we find that overexpression of ProT α stimulates the transactivation activity of CBP. Based on these results, we propose that ProT α is a new CBP cofactor that potentiates transcription, possibly through reorganization of the chromatin structure.

RESULTS

ProT α interacts with CBP *in vivo* and *in vitro*

Recent studies have shown that ProT α is localized in sites of active transcription (Vareli *et al.*, 2000) and interacts with the

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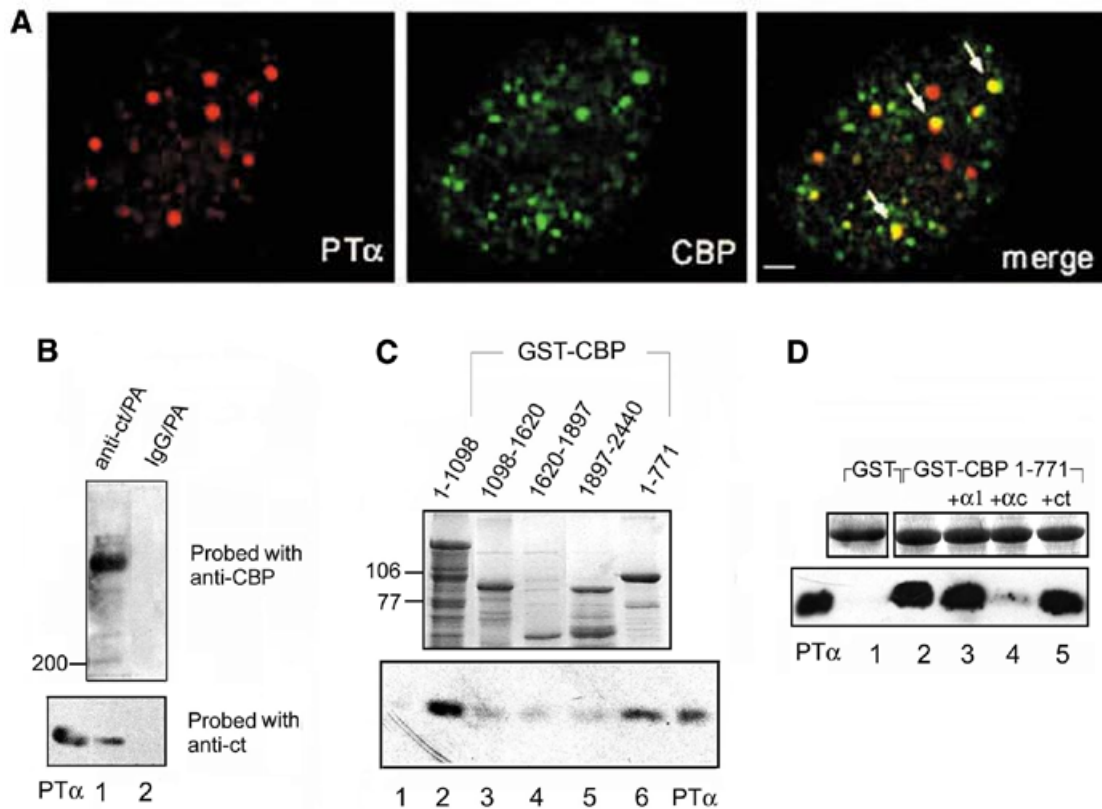


Fig. 1. ProT α binds to CBP *in vivo* and *in vitro*. (A) CBP colocalizes with ProT α . HeLa cells were double-labeled with the polyclonal anti-ProT α (anti-ct, red) and the monoclonal anti-CBP antibody C1 (green). The merge represents the overlay of ProT α and CBP staining, and arrows indicate the colocalization of the proteins. Size bar, 2 μ m. (B) *In vivo* interaction between ProT α and CBP. Whole-cell lysates from HeLa cells were immunoprecipitated using anti-ct antibody–protein A beads (lane 1) or control IgG–protein A beads (lane 2) as indicated in Methods. Immune complexes were analyzed by western blotting with the specific anti-CBP antibody (A-22) diluted 1:200 (upper panel) and the affinity-purified anti-ct antibody diluted 1:500 (lower panel). The blot for ProT α represents 1/5 of the total sample and PT α indicates 0.1 μ g of ProT α used as a positive control. Reactions were detected by enhanced chemiluminescence (ECL) (Amersham). (C) GST pull-down experiments. Purified GST (lane 1) or GST fusion proteins (2 μ g each) encoding different CBP fragments (1–1098, 1098–1620, 1620–1897, 1897–2440, 1–771, lanes 2–6) were immobilized on glutathione–agarose beads and incubated with 2 μ g of ProT α as indicated in Methods. The beads were washed and analyzed for ProT α by western blotting. PT α corresponds to 0.1 μ g of purified protein. Detection was performed by ECL. The upper panel shows Coomassie Blue staining of the gel to document equal loading. (D) Identification of the CBP-binding site within the ProT α molecule. ProT α (2 μ g) was incubated with GST (lane 1), or the CBP fragment 1–771 (4 μ g each) was immobilized on glutathione–agarose beads in the absence (lane 2) or in the presence of 6 μ g of the indicated peptides: thymosin α 1, the acidic peptide (ac) or the peptide ct (lanes 3–5, respectively) (for sequences see Methods). The beads were analyzed as indicated in (C) and 0.05 μ g of ProT α (PT α) were used as positive control. The upper panel shows Coomassie Blue staining of the relevant part of the gel to show equal loading. The insert shows the loading of GST for comparison.

histone acetyltransferase p300 (Cotter and Robertson, 2000). To gain insight into the potential role of ProT α in transcription, we performed colocalization studies for ProT α and the versatile transcription co-activator CBP, a protein highly homologous to p300, employing specific antibodies in dual labeling experiments followed by confocal microscopy. Using the monoclonal anti-CBP (C1) and the polyclonal affinity-purified anti-ProT α antibodies (anti-ct), we found that CBP staining partially overlapped with that of ProT α (Figure 1A).

To test whether the colocalization of CBP and ProT α correlates with *in vivo* complex formation of these proteins, we performed co-immunoprecipitation assays. Lysates from HeLa cells were immunoprecipitated with the anti-ct antibodies and the presence of CBP was detected by western blotting. This analysis revealed that CBP co-immunoprecipitated with ProT α (Figure 1B, lane 1).

Control IgG–protein A beads did not bind CBP and ProT α (Figure 1B, lane 2). Furthermore, no cross-reaction of the anti-ct antibodies with CBP was detected in western blots (data not shown).

Direct evidence for a physical interaction between ProT α and CBP was obtained by glutathione *S*-transferase (GST) pull-down experiments. Purified ProT α was tested for its ability to interact with different fragments of CBP fused to GST. We found that ProT α binds to the N-terminal region of CBP (lane 2) and, more precisely, within the first 771 amino acids (Figure 1C, lane 6). This region includes several functional domains of CBP, such as the binding site for nuclear hormone receptors, the zinc finger domain and the CREB binding domain (Goodman and Smolik, 2000). The binding was specific because ProT α was not retained by GST alone (lane 1), or by other regions of CBP (lanes 3–5).

In addition, the CBP fragments tested ranged in pI from 7.35 (region 1–1098) to 11.35 (region 1897–2440), which argues against non-specific binding of ProT α to basic regions of CBP.

To identify the region of ProT α responsible for the interaction with CBP, we performed additional GST pull-down assays in the presence of three peptides modeled after the published amino acid sequence of bovine thymus ProT α , thymosin α 1, the acidic peptide (ac) and the C-terminal peptide ct (residues 1–26, 52–69 and 87–109, respectively). The results shown in Figure 1D indicate that the acidic peptide inhibited strongly the *in vitro* binding of CBP fragment 1–771 with ProT α ; a peptide composed exclusively of glutamic residues also inhibited binding, suggesting that this interaction is highly dependent on the presence of polyglutamic stretches in ProT α (see Supplementary data available at *EMBO reports* Online). Similar results were obtained when CBP fragment 1–1098 was substituted for region

1–771 (data not shown). These data allow mapping of the CBP binding site within the acidic region of ProT α , which also binds to linker histone H1.

ProT α stimulates transcription

To examine the role of ProT α in gene expression, we asked whether overexpression of ProT α could affect transcription driven by the AP1 transcription factor, a well-characterized regulator of cell proliferation that is dependent on CBP (Arias *et al.*, 1994; Bannister *et al.*, 1995). HeLa cells were co-transfected with a luciferase reporter gene, 5 \times coll TRE–tata–luc based on the TPA-responsive element (coll TRE) of human collagenase gene, which binds AP1 (van Dam *et al.*, 1998) and a ProT α -expressing plasmid (pCMVPT α). As shown in Figure 2A, increasing amounts of pCMVPT α stimulated the promoter activity in a dose-dependent fashion. Conversely, the activity of a tata–luc construct, which does not contain the TPA-responsive elements, was not affected by overexpression of ProT α (Figure 2A). Since c-Jun is a major component of AP1, we also checked the effect of ProT α on the collagenase promoter after c-Jun overexpression. As expected, increased c-Jun levels in the cells activated the collagenase promoter. Co-expression of ProT α further enhanced the transcription to an extent similar to that observed in the absence of c-Jun (not shown).

Next, we examined the ability of ProT α and CBP to stimulate 5 \times coll TRE individually and in combination with each other. As depicted in Figure 2B, ProT α and CBP potentiated the promoter 2.2- and 2.7-fold, respectively. Interestingly, simultaneous addition of ProT α and CBP resulted in a synergistic activation (13-fold). We further extended these studies to two other targets of CBP: the activator NF- κ B (Gerritsen *et al.*, 1997) and the MHC class II gene promoter (Kretsovali *et al.*, 1998). ProT α and CBP could individually stimulate p65-mediated activation of an NF- κ B–luc reporter. Again, when added simultaneously, they led to a synergistic activation of the NF- κ B–luc (Figure 2B). However,

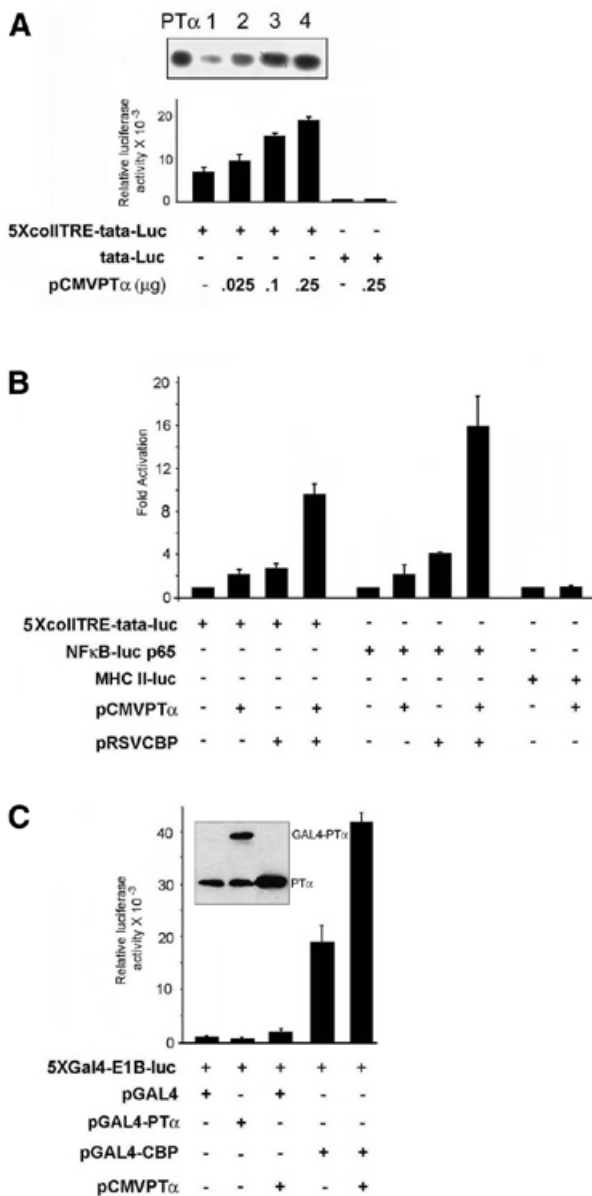


Fig. 2. ProT α potentiates transcription. (A) Overexpression of ProT α stimulates transcription from the collagenase promoter. HeLa cells were transiently transfected with the 5 \times coll TRE–tata–luc or tata–luc constructs (0.1 μ g each) in the absence or in the presence of the indicated amounts of pCMVPT α . The upper panel shows western blotting analysis giving the expression levels of ProT α in the transfection experiment described in (A). The experiments were repeated at least three times and the error bars indicate the standard deviations of triplicate values. (B) Synergism between ProT α and CBP enhances AP1- and NF- κ B-dependent transcription. HeLa cells were transfected using the calcium phosphate method with the reporter plasmids 5 \times coll TRE–luc (0.5 μ g), NF- κ B–luc and p65 expression vector (0.5 and 0.1 μ g, respectively) or MHC II–luc (0.5 μ g) in the presence of pCMVPT α and pRSVCBP (1.0 μ g each), as indicated. Error bars represent the standard deviation from three independent experiments. (C) Transactivation by CBP is stimulated by ProT α . HeLa cells were transfected with 0.1 μ g of 5 \times GAL4–E1B–luc reporter plasmid and expression vectors encoding either GAL4 alone or GAL4–ProT α or GAL4–CBP (full-length) or GAL4–CBP in the presence of pCMVPT α (0.1 μ g each), as indicated. The upper panel shows western blotting analysis giving the expression levels of ProT α and GAL4–ProT α in the transfection experiment. The experiments were repeated at least three times and the error bars indicate the standard deviations of triplicate values. In all cases, 0.1 μ g of a plasmid expressing β -galactosidase (pCMV–LacZ) was co-transfected and used as an internal standard, and luciferase values were normalized to each other based on the respective β -galactosidase activity.

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the MHC class II promoter was not activated by ProT α (Figure 2B). Therefore, ProT α collaborates with CBP to potentiate AP1- and NF- κ B-mediated transcription.

In an attempt to elucidate the mechanism of ProT α action, we fused it to the DNA-binding domain of GAL4. The fusion protein was expressed equally well as the wild-type ProT α (Figure 2C) and its overexpression activated AP1-mediated transcription (not shown). However, GAL4-ProT α could not direct transcription of a GAL4 site-dependent reporter (Figure 2C), in agreement with a previous report (Martini *et al.*, 2000). We next examined whether ProT α could stimulate the activity of CBP when the latter was tethered on DNA through fusion to the GAL4 DNA-binding domain. Indeed, co-transfection of pCMVPT α and GAL4-CBP resulted in a strong enhancement of CBP transactivation potential (Figure 2C). Taken together, these data suggest that ProT α does not act as a 'classical' transcription activator that directly provides promoter contacts with the basic transcription machinery, but rather as a dynamic component, which needs to be recruited through CBP in order to exert its function.

DISCUSSION

We have shown previously that ProT α modulates the interaction of histone H1 with chromatin and proposed a role for this polypeptide in chromatin remodeling (Karetsov *et al.*, 1998). The observations described here provide evidence that ProT α interacts with the versatile co-activator of transcription CBP both *in vivo* and *in vitro* and stimulates gene transcription. The site of interaction was mapped within the N-terminal domain of CBP (residues 1–771) and a region of ProT α composed of two polyglutamic stretches. This binding could be due to the specific sequence characteristics of this acidic domain and/or its local conformation. However, under the experimental conditions employed in the binding studies (pH 7.4 and 150 mM ionic strength), this motif is not expected to adopt any special type of secondary structure, as predicted by the program AGADIR (Munoz and Serrano, 1995). This prediction is in agreement with experimental work showing that native ProT α has a random coil conformation (Gast *et al.*, 1995; Uversky *et al.*, 1999). Therefore, we can conclude that the CBP-ProT α interaction is mediated through the extended acidic clusters of ProT α . This is further supported by the observation that polyglutamic acid (10–20 residues), predicted to be unfolded under the same experimental conditions (Munoz and Serrano, 1995), also inhibited binding.

Recently, the histone acetyltransferase p300, a protein highly homologous to CBP, has been reported to bind to ProT α through its C-terminal region (Cotter and Robertson, 2000). This discrepancy with our results could be due to the GST-ProT α used in those assays, as opposed to the use of purified native ProT α in our system. The recombinant protein might lack potentially important post-translational modifications or could adopt an altered structure due to the presence of the large GST moiety. Alternatively, p300 and CBP might differ in their properties to bind ProT α .

Confocal laser scanning microscopy shows that ProT α partially colocalizes with CBP in discrete subnuclear domains. The pattern and degree of colocalization were similar to the colocalization characteristics of CBP and RNA polymerase II, but also of transcription factors that associate with CBP (von Mikecz *et al.*, 2000), suggesting a dynamic interaction between CBP and

ProT α that is spatially and temporally regulated. Furthermore, recent work has shown that ProT α is concentrated in nuclear domains that often overlap or engulf sites of active transcription (Vareli *et al.*, 2000). In this respect, the colocalization of ProT α and CBP and their *in vivo* interaction point to a role of ProT α in gene regulation.

In agreement with this assumption, we provide experimental evidence showing that overexpression of ProT α enhances the transcription activity of CBP-dependent promoters, such as coll TRE and NF- κ B, and stimulates the transactivation activity of CBP. On the other hand, another target of CBP, the MHC class II gene promoter, was not affected by ProT α . This result implies that additional factors, such as the chromatin state of the promoters or the composition of the co-activator complex, are implicated in this mechanism. A recent report has shown that ProT α can specifically enhance transcription activation by the estrogen receptor by eliminating the estrogen repressor REA and favoring recruitment of the co-activator SRC-1 (Martini *et al.*, 2000). Since the estrogen receptor is also dependent on CBP (Wang *et al.*, 2001), the possibility that ProT α might provide the receptor with higher amounts of CBP and, consequently, SRC-1 cannot be excluded.

The precise mechanism whereby ProT α increases transcription needs to be studied further. ProT α does not bear an intrinsic transcription activity when tethered to the GAL4 DNA-binding domain (Martini *et al.*, 2000; this study). Thus, the effect of ProT α on transcription might be exerted either on the chromatin state of the promoter or on the enzymatic activity of CBP, which is directly involved in transcription activation (Martinez-Balbas *et al.*, 1998). *In vitro* HAT assays using core histones as substrates revealed that the acetyltransferase activity of CBP was not affected by ProT α (Z. Karetsov, A. Kretsovali and T. Papamarcaki, unpublished data). Therefore, our data are most consistent with the first hypothesis and point to chromatin as the possible target of ProT α 's function. We propose that ProT α might be targeted to promoter regions by CBP and subsequently promotes H1 reorganization or displacement leading to unfolding of the compact 30 nm chromatin fiber (Karetsov *et al.*, 1998). ProT α might also prevent histone H1 from rebinding, maintaining the partially H1-depleted and extended chromatin conformation necessary for the passage of RNA polymerase II.

Overall, the interaction of ProT α with the versatile co-activator of transcription CBP, together with its ability to stimulate transcription, suggest an important role for this acidic polypeptide in gene expression.

METHODS

Protein chemical procedures. ProT α was purified from calf thymus as described previously (Karetsov *et al.*, 1998). Thymosin α 1 (residues 1–26, Ac-SDAAVDTSSSEITTKDLKEKKEVEEA, pl 4.2), the acidic peptide (residues 52–69, GSGDEEEEEEGGEEEEEEEE, pl 3.0) and the ct peptide (residues 86–109, GKRAAEDDEDDDDVDTKKQKTDDEDD, pl 3.8) of bovine ProT α were synthesized by the EMBL Protein Sequencing and Peptide Synthesis Facility. Polyglutamic acid (mol. wt 1500–3000, pl 3.1–2.9) was obtained from Sigma.

Plasmids. To generate pCMVPT α , the coding region of the rat ProT α cDNA (Frangou-Lazaridis *et al.*, 1988) was amplified and subcloned into the *EcoRI*–*XhoI* sites of a cytomegalovirus (CMV)

expression cassette p163/7, where the H2 promoter was exchanged with the CMV promoter (Woodrooffe *et al.*, 1992). The construct has been verified by sequencing. pGAL4-PT α was made from pCMVPT α that was digested by *Xho*I, filled in with the Klenow fragment of DNA polymerase I, digested with *Eco*RI and cloned into the *Eco*RI-*Sma*I restriction sites of the pBXGI vector containing GAL4-DBD (1–147). The reporter plasmids 5 \times coll TRE-tata-luc, tata-luc have been described previously (van Dam *et al.*, 1998) and were obtained from A. Pintzas (National Research Institute, Athens, Greece). The mouse class II-353 Ea chloramphenicol acetyltransferase construct has been described previously (Thanos *et al.*, 1988) and was used to generate an equivalent luciferase reporter. GAL4-CBP fusion construct, 5 \times GAL4-E1B-luc reporter plasmid and p65 expression vector were kindly provided by D. Thanos (Columbia University, New York, NY). NF- κ B-luc (κ B-TK5-luc) was provided by C. Esslinger (Ludwig Institute for Cancer Research, University of Lausanne, Switzerland).

Cell culture and transient transfections. HeLa cells were grown in Dulbecco's modified Eagle's medium in the presence of 10% fetal calf serum (FCS) and antibiotics. For transient transfections, HeLa cells were grown in 6-well plates to 60–70% confluence and transfected using effectene (Qiagen) or the calcium phosphate method (Thanos *et al.*, 1988), where indicated. Cells were harvested 36–48 h later and luciferase activity was detected in the extracts using a Promega luciferase assay system according to the manufacturer's instructions. A plasmid expressing β -galactosidase (pCMV-LacZ) was co-transfected as an internal standard, and luciferase activity was normalized for transfection efficiency with the respective β -galactosidase activity. Total DNA was adjusted using empty vectors.

Indirect immunofluorescence. HeLa cells grown on coverslips were fixed with methanol at -20°C for 5 min, followed by incubation in 3.8% paraformaldehyde for 20 min at room temperature and quenched in 50 mM ammonium chloride for 15 min. Specimens were blocked in 10% FCS in phosphate-buffered saline for 1 h in a humidified chamber and antibody incubations were carried out for 1 h. Samples were then mounted in Mowiol containing 100 mg/ml DABCO (Sigma). For ProT α and CBP co-staining, primary antibodies from mouse or rabbit were used simultaneously and detected with species-specific secondary antibodies linked to fluorescein (FITC) or rhodamine (TRITC) (Dianova, Hamburg, Germany). ProT α was visualized using the affinity-purified anti-ct antibody followed by FITC-labeled donkey anti-rabbit IgG, and CBP was stained using the monoclonal C1 antibody (Santa Cruz Biotechnology) followed by rhodamine-labeled anti-mouse IgG. Specimens were visualized in a Leica TCS-SP confocal scanning microscope, equipped with an argon/krypton laser and Leica TCS software. Images were exported to Adobe Photoshop.

Immunoprecipitation and western blotting. Whole-cell extracts were prepared from HeLa cells ($\sim 24 \times 10^6$ cells) lysed in 200 μ l of buffer containing 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 5 mM EDTA, 5 mM NaF, 25 mM β -glycerolphosphate, 1% NP-40 and 1 μ g/ml each of protease inhibitors aprotinin, leupeptin and pepstatin. The extract was centrifuged at 13 000 g for 10 min and pre-cleared with 20 μ l of IgG-protein A (40 μ g of IgG) for 30 min at room temperature. The supernatant was divided into two portions and incubated with (i) 25 μ l of anti-ct-protein A beads (20 μ g of anti-ct antibody) and (ii) 25 μ l of IgG-protein A beads

for 12 h at 4°C . After incubation, the beads were washed three times with 1 ml of lysis buffer containing 300 mM NaCl, resuspended in 15 μ l of Laemmli buffer and analyzed by western blotting.

ProT α was analyzed by the anti-ct polyclonal antiserum affinity purified over a ct-Affigel (Karetsova *et al.*, 1998). CBP was detected by the rabbit polyclonal A-22 antibody (Santa Cruz Biotechnology).

GST pull-down experiments. Fragments of CBP were subcloned into pGEX vectors (Pharmacia) in frame with GST and were expressed in *Escherichia coli* DH5 α (Kretsovali *et al.*, 1998). Fusion proteins (~ 2 μ g each) were immobilized on glutathione beads and incubated with 2 μ g of purified ProT α in a buffer containing 20 mM HEPES pH 7.5, 150 mM KCl, 5 mM Mg Cl₂, 0.1% NP-40 and 0.2% bovine serum albumin (BSA), supplemented with protease inhibitors. Reactions were carried out for 5 h at 4°C and the beads were washed three times in the same buffer without BSA. Bound proteins were subjected to 15% SDS-PAGE and ProT α was detected by western blotting.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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