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A human B-box-binding protein downregulated in adenovirus 5-transformed human cells

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Abstract Internal promoters of some genes transcribed by RNA polymerase III (e.g. tRNA genes, adenovirus VA1 RNA gene, human retroposons of the Alu family) contain a conserved sequence element, B-box, interacting with basal transcription factor TFIIIC2 which initiates assembly of the full transcription complex on the genes, and which represents the major determinant of the efficiency of their expression. In this study we have identified in human nuclear extracts a protein which interacts with VA1 B-box DNA and forms a high-affinity complex which is very stable after the addition of a large excess of competitor DNA. Unlike TFIIIC2, the B-box-binding activity of the B-box-binding protein is found to be decreased in adenovirus 5-transformed human cells. In these cells (line 293) increased transcription of VA1 and tRNA genes in vivo and in vitro was previously detected by other workers. Our results suggest that besides TFIIIC2, an additional B-box-binding protein factor may be involved in the regulation of expression of the RNA polymerase III-transcribed genes.

Key words: Class III transcription; B-box-binding protein; Adenovirus transformed human cell; Basal transcription factor TFIIIC2

1. Introduction

Some RNA-coding genes transcribed by RNA polymerase III contain an internal promoter composed of two properly spaced conserved sequence motifs, an upstream element named the A-box and a downstream element called the B-box, which are essential for efficient transcription [1,2]. The B-box of tRNA genes and of adenovirus VA1 gene is known to bind mammalian basal transcription factor TFIIIC2 [3] which was suggested to initiate assembly of the full transcription complex recruiting RNA polymerase III and containing TFIIIC1 and TFIIIB [4]. Mammalian TFIIIC2 contains five protein subunits [5,6] and the DNA-binding 20 kDa subunit of the factor (TFIIIC2a) has recently been cloned and sequenced [7,8]. Some workers [6,9] have identified two forms of TFIIIC2, one of which (TFIIIC2a) was active in an in vitro transcriptional assay, the other (TFIIIC2b) being inactive in the assay. Both forms were found to bind VA1 promoter B-box with an equal affinity [6]. An active form of the factor (TFIIIC2a) was also found to contain a distinct subunit of 110 kDa [6,9] and the presence of this subunit (TFIIICβ) in some fractions of a partially purified TFIIIC2 was found to correlate with the efficiency of RNA polymerase III-dependent transcription in the corresponding crude nuclear extracts [9].

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Human retroposons of the Alu family also contain an internal promoter for RNA polymerase III having A- and B-boxes homologous to the elements in tRNA genes [10] which were demonstrated to be important for Alu transcription in vitro [11,12]. However, Alu repeats are very poorly transcribed in vivo [13] and we have previously presented evidence for the existence of a negative control of Alu transcription mediated by an Alu-binding protein [14,15]. One apparent possibility for such control is interference of the protein with binding to the Alu B-box of TFIIIC2, and in an attempt to develop a biologically relevant assay for the binding interference we used in electrophoretic mobility shift assays an oligonucleotide covering the palindromic VA1 promoter sequence with a centered B-box identical in sequence to oligonucleotides used for the affinity purification of TFIIIC2 [5,6]. The results of the analysis of human nuclear extracts for proteins interacting with this oligonucleotide are presented in this study.

2. Materials and methods

Human cells (lung adenocarcinoma MCF7, kidney carcinoma OKP-GS and adenovirus 5-transformed embryonal kidney cells line 293) were obtained from ATCC and maintained in DMEM medium containing (if not otherwise indicated) 10% of bovine fetal serum. Nuclear extracts were prepared from the cells by a micro-method consisting of three steps: (1) after washing in PBS cells were incubated in hypotonic buffer A (20 mM HEPES buffer, pH 7.9, 10 mM $MgCl_2$, 10 mM dithiothreitol/DDT, 1 mM phenylmethylsulfonyl fluoride/PMSF) for 20 min on ice, disrupted by passing 10 times through a 24 gauge needle and the nuclear pellet collected by centrifugation for 10–20 s in an Eppendorf centrifuge; (2) the pellet was resuspended in buffer B (20 mM HEPES buffer, pH 7.9, 10 mM DTT, 0.45 M NaCl, 1 mM PMSF), incubated for 30 min on ice and extracted nuclei separated by 20 s centrifugation in an Eppendorf centrifuge; (3) the supernatant was dialysed for 2 h at 4°C against buffer D (20 mM HEPES buffer, pH 7.9, 100 mM NaCl, 10 mM DTT, 1 mM PMSF), aliquoted and stored at −80°C.

All oligonucleotides were synthesized using a Phamacia Gene Assembler and, if necessary, purified using FPLC on a column of Mono Q. The sequence of the self-complementary oligonucleotide from which the duplex VB1 substrate was prepared is (5′ to 3′ here and elsewhere) GACGACCGGGGTTGGAACCCC; top strand sequence tVADB1, GTTCGAACCCCGGATCCGGCC; bottom strand sequence bVADB1, ATCACGGCGGACGGCCGGATC; top strand sequence tAP3, GATCTGTGGAAAGTCCCA; bottom strand sequence bAP3, GATCTGGGACTTTCCACA; top strand sequence tAP1, GATCGTGACTCAGCGC and bottom strand sequence bAP1, GATCGCGCTGAGTCAC. Labeled duplex oligonucleotides for electrophoric mobility shift assays (VB1 and VADB1) were prepared in the following way. First, the top strand was labeled with ^{32}P using phage T4 polynucleotide kinase and [γ-^{32}P]ATP, the kinase was inactivated by heating, the bottom strand was added and annealed to the labeled strand, all four unlabeled dNTPs added along with Klenow fragment of *E. coli* DNA polymerase I and incubation continued for 1 h at room temperature (RT). Then duplex substrate was purified using a column of Sephadex G-25. Unlabeled competitor duplex oligonucleotides VB1 and VADB1 were prepared in the same way except

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for incubation with kinase. Unlabeled duplex AP3 and AP1 competitors were prepared by annealing of the corresponding complementary strands.

Electrophoretic mobility shift assay (EMSA) was performed by incubation for 30 min at room temperature of labeled duplex oligonucleotide (2–3 ng) in mixtures (20 μl) containing 12 mM HEPES buffer, pH 7.9, 1 mM EDTA, 100 mM NaCl, 0.2 mM DTT, 10% Ficoll, 100 μg/ml bovine serum albumin, nuclear extract (1–4 μl, 1.5–6 μg of total protein) and varying amounts of unlabeled competitor as indicated in the figure legends. Complexes were then separated in a low salt (22 mM Tris, 22 mM boric acid, 0.6 mM EDTA) 4% polyacrylamide gel, the gel being dried and radioautographed.

3. Results

In electrophoretic mobility shift assay (EMSA) experiments with the VA1 B-box oligonucleotide (VB1) and crude human nuclear extracts as a source of proteins, we expected to see a low mobility band representing the complex of TFIIIC2 (of about 600 kDa, see [5,6]) with the DNA. However, under our binding conditions with labeled VB1 EMSA substrate we observed (Fig. 1A,C, lane 1) a major complex (CB) of rather high mobility the formation of which was completely suppressed by simultaneous addition of a 100-fold molar excess of unlabeled VB1 (Fig. 1C, lane 2), and only weakly suppressed by simultaneous addition of a 100-fold molar excess of unspecific duplex oligonucleotide AP1 (Fig. 1C, lane 4). In order to confirm that the observed complex CB (Fig. 1A) is actually formed by a B-box-binding protein, we used in EMSA another VA1 promoter-derived oligonucleotide substrate (VADB1) and found (Fig. 1B) that under the same conditions this substrate forms a similar complex and a 100-fold molar excess of unlabeled VADB1 strongly suppresses the formation of a complex with labeled VB1 (Fig. 1C, lane 3). It can be seen from Fig. 2A that the VB1 and VADB1 sequences overlap only over the B-box sequence which is apparently responsible for the EMSA complex observed here (CB). The results also indicate that sequences located just

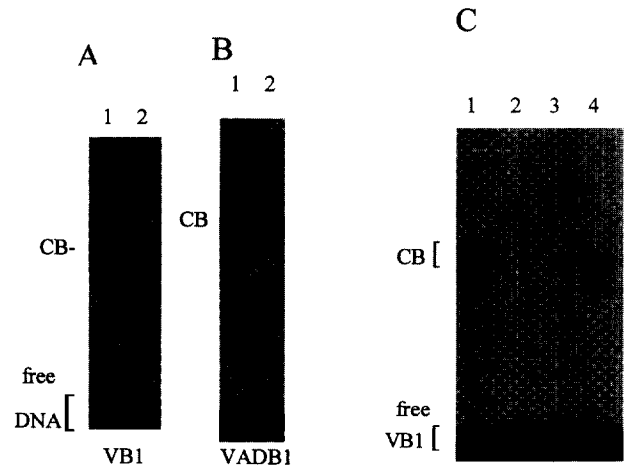

Fig. 1. Complexes formed by a protein from the nuclear extract of OKP-GS cells with the adenovirus VA1-gene B-box oligonucleotides VB1 (A,C) and VADB1 (B). All variants contained 3 ng of labeled oligonucleotide, 2 μl (3 μg of protein) of nuclear extract and other components as indicated in section 2. In (A,B) lanes 1 contained no competitor; and lanes 2, 50 ng of sonicated unlabeled bovine DNA. In (C) all lanes contained 50 ng of the indicated DNA plus 300 ng of unlabeled VB1 oligonucleotide (lane 2), 300 ng of unlabeled VADB1 (lane 3) or 300 ng of AP1 (lane 4). The major detected complex is marked as CB.

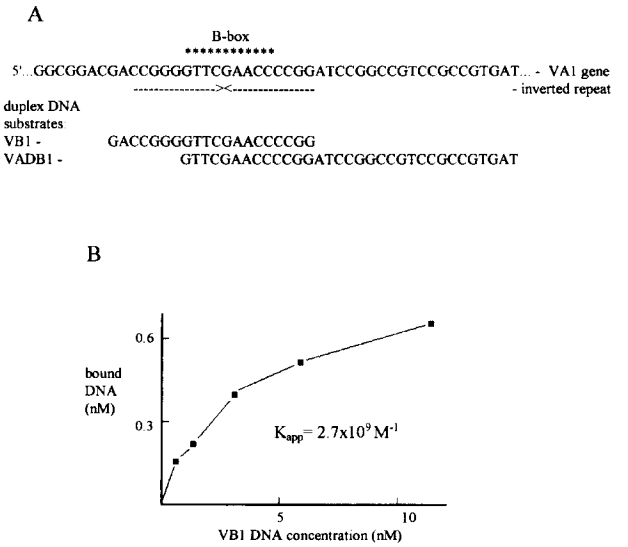

Fig. 2. (A) Adenovirus VA1 gene sequence covering promoter B-box and sequences of DNA substrates used in EMSA. (B) Measurement of the equilibrium constant of the formation of the CB complex (bound DNA, $[CD_s]$) with the VB1 binding substrate. K_{app} was calculated from the equation $[CD_s]=[C^o]\times[D_s]\times K_{app}/1+K_{app}\times[D_s]$ as described in [3], where $[D_s]$ is the concentration of unbound VB1 and $[C^o]$ is the concentration of all VB1-binding sites in the proteins added. $[C^o]$ is calculated to be 0.74 nM. EMSA was performed (see section 2 with variable amounts of the labeled VB1 substrate (0.2–4 ng per 20 μl incubation mixture) and fixed amount of nuclear extract from MCF7 cells (3 μg of protein). After separation, bands of CB and of free VB1 were located by autoradiography and radioactivity was measured in a scintillation counter.

downstream from the VA1 B-box are not very important for the formation of CB, and the incomplete suppression of CB formation by VADB1 competitor (Fig. 1C, lane 3) suggests a possible role of the VA1 sequence located just upstream of the B-box. To distinguish the observed B-box-binding activity from the TFIIIC-copurifying zinc-finger B-box-binding protein having a 55 kDa DNA binding subunit [16], we analysed whether CB formation may be suppressed by treatment of the nuclear extract with 10 mM EDTA which has been shown to inhibit the B-box-binding activity of p55 [16]. CB formation was found to be unaffected by this EDTA treatment (not shown), indicating that the corresponding protein is different from p55.

To quantitate the binding reaction observed we measured the concentrations of free and bound VB1 substrate after the addition of a fixed amount of nuclear extract and different amounts of the substrate as described for purified TFIIIC2 in [3]. Radioactive DNA contents of the EMSA bands was measured by liquid scintillation counting. Fig. 2B shows the results on CB complexes (bound DNA), and the data obtained allowed [3] us to calculate the apparent equilibrium constant of the binding reaction (K_{app}) which was found to be 2.7×10^9 M^{-1} as well as the concentration of all B-box DNA-binding sites in reactions ($[C^o]$) which was found to be 0.74 nM. The indicated K_{app} value is only one order of magnitude lower than that measured for purified TFIIIC2 [3].

Additional data on the stability of CB were obtained in the EMSA experiments in which an excess of unlabeled competitor (sonicated bovine DNA, 1 mg/ml final concentration) was added to pre-formed complex of labeled VB1 with protein and decay of the complex at room temperature was followed with

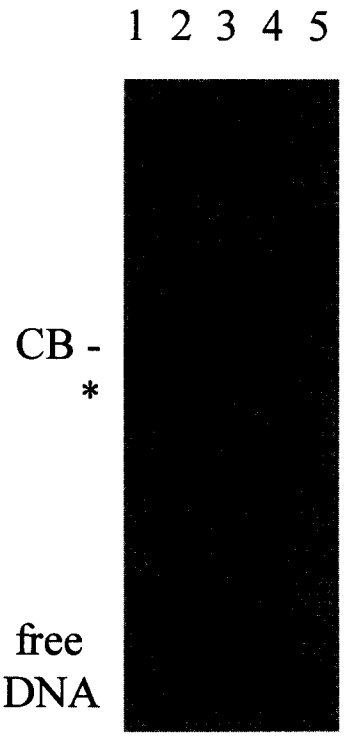

Fig. 3. Stability of the pre-formed CB complex with VB1 substrate after subsequent addition of an excess of unlabeled competitor. The incubation mixture (100 μl) contained labeled VB1 (5 ng), MCF7 nuclear extract (25 μg of protein) and other components as described in section 2. After 30 min at room temperature 10 μl (100 μg) of unlabeled sonicated bovine DNA was added, and 20-μl aliquots of the mixture were loaded onto a 4% polyacrylamide running gel after 0 min (lane 1), 20 min (lane 2), 40 min (lane 3), 80 min (lane 4) and 120 min of incubation at room temperature.

time (Fig. 3). It can be seen that CB does not dissociate even at 2 h after addition of the competitor (Fig. 3, lane 5). CB was also found to be stable for at least 1 h after addition of a 100-fold molar excess of unlabeled VB1 (not shown). Interestingly, some increases in a complex having a slightly higher mobility in EMSA than CB (Fig. 3, *) were observed during incubation with the competitor suggesting the possible conversion of CB into this complex with higher mobility. The complex of purified TFIIIC2 with full VA1 promoter was reported to decay completely within 1 h after the addition of a 100-fold molar excess of unlabeled B-box oligonucleotide [17], indicating that CB is more stable in this assay than the complex formed by purified TFIIIC2 with VA1 promoter.

To investigate the possible biologival relevance of the complex CB, we compared extracts from cultivated human embryonal kidney cells transformed with adenovirus 5 (line 293) and control non-transformed kidney cells (line OKP-GS). Under identical binding conditions and protein concentration, extracts from 293 cells were found to contain less CB-forming activity than those from control cells (Fig. 4A), indicating that protein responsible for CB formation is downregulated after adenovirus transformation. B-box-binding and transcriptional activity associated with TFIIIC2 has been reported to be increased [18] or to remain unchanged [9,19] in adenovirus-transformed cells, and transcription of all B-box-containing genes is known to be strongly stimulated after adenovirus transformation [20-22]. Therefore, suppression of formation of complex CB in EMSA experiments with crude nuclear extracts correlates with increased transcription of class III genes by RNA polymerase III. We also compared CB formation by extracts from human MCF-7 cells grown at normal 10% serum concentration (Fig. 4B, lanes 1–2) or at 0.5% serum (Fig. 4B, lanes 3–4) and found that although CB is not greatly affected by low serum concentration, stimulation of additional high- and low-mobility complexes (Fig. 4B, lanes 3–4, denoted as CX and *) takes place. Growth of cells at low serum concentration was reported to decrease RNA polymerase III-dependent transcriptional activity of nuclear extracts [9].

4. Discussion

In this study a novel B-box-binding activity has been identified in human cells and the activity is found to be downregulated after adenovirus transformation when transcription of all class III genes is known to be stimulated. This activity seems not to be associated with the well known basal transcription factor TFIIIC2 having a very large (220 kDa) DNA-binding subunit [5,6], or with B-box-binding protein p55 copurifying with TFIIIC transcriptional activity [16], and, therefore, seems to be associated with a distinct protein which we refer to here as BBP-D. This protein in vitro binds B-box with an affinity comparable to that of the purified TFIIIC2 and may affect functions of this basal complex which were suggested to be important for efficient expression of some RNA polymerase III-transcribed genes. It remains unclear why under our binding conditions the complex of VB1 with TFIIIC2 was not simultaneously detected. One possibility is that VA1 sequences located just upstream of the B-box and absent from the VB1 substrate are also important for TFIIIC2 binding in crude nuclear extracts. Corresponding DNase footprints over VA1 promoter obtained with purified TFIIIC2 or crude nuclear extract [3,23] cover a rather long (40 nucleotides) region

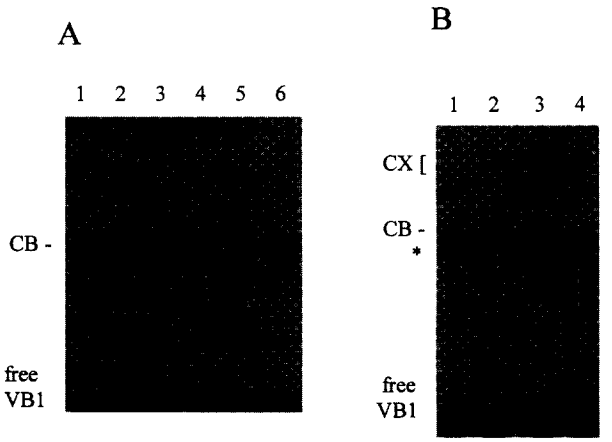

Fig. 4. Influence of adenovirus transformation (A) and of low concentration of serum (B) on formation of the CB complex with VB1 EMSA substrate. (A) Nuclear extracts were prepared from control (line OKP-GS) and adenovirus-transformed (line 293) human cells, and total protein concentration in the extracts was adjusted to 1.5 mg/ml. EMSA was performed with fixed amount of all components except variable amounts of nuclear extracts which was 1 μl (lanes 1,4), 2 μl (lanes 2,5) and 4 μl (lanes 3,6). (B) Nuclear extracts were prepared from MCF7 cells grown at 10% serum (lanes 1,2) or 0.5% serum (lanes 3,4) and 1.5 μg (lanes 1,3) or 3 μg (lanes 2,4) of protein was added to otherwise identical incubation mixtures.

with a centered B-box consistent with this view, but other explanations are also possible.

A correlation between RNA polymerase III-dependent transcriptional activity of nuclear extracts and the presence of 110 kDa β-subunit in TFIIIC2 have recently been demonstrated for human cells expressing or not expressing adenovirus E1A gene, and for cells grown at low and normal concentration of serum [9]. This observation was interpreted as evidence that TFIIIC2 activity is regulated by this subunit [9] but a potential role of other factors affected by E1A or serum was not excluded. TFIIIC2 activity in the in vitro transcription assay is known to be modulated by its phosphorylation/dephosphorylation [19,24], and adenovirus E1A expression induces a very complex response including phosphorylation-mediated activation of some transcription factors [25]. Interestingly, phosphorylation-mediated inactivation of an RNA polymerase II transcriptional repressor ERF was recently demonstrated [26]. Therefore, downregulation of BBP-D in adenovirus-transformed cells may be also important for activation of RNA polymerase III-dependent transcription and a potential role of factors different from TFIIIC2 is not excluded by the observations of others [9]. Purification of BBP-D as well as cloning and analysis of corresponding gene may be helpful to understand the mechanism of regulation of transcription of the B-box-containing genes in vivo.

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