Regulation of transcription by the heterogeneous nuclear ribonucleoprotein E1B-AP5 is mediated by complex formation with the novel bromodomain-containing protein BRD7

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E1B-AP5 was initially identified as a target of the early adenovirus E1B-55 kDa protein during the course of lytic infection. E1B-AP5 belongs to the heterogeneous nuclear ribonucleoprotein family and was demonstrated to be involved in mRNA processing and transport [Gabler, Schutt, Groitl, Wolf, Shenk and Dobner (1998) J. Virol. **72**, 7960–7971]. In the present paper, we demonstrate that E1B-AP5 differentially regulates basic and ligand-dependent transcription. We found that E1B-AP5 represses basic transcription driven by several virus and cellular promoters, and mapped the repression activity to the N-terminal part of the protein. In contrast with basic repression, E1B-AP5 activated the glucocorticoid-dependent promoter in the absence of dexamethasone, but did not contribute to the dexamethasone-induced activation. Mutant analysis indicated the presence of an additional cellular factor that modulates E1B-AP5 transcriptional activity. Using yeast two-hybrid screening, we identified a novel chromatin-associated bromodomain-containing protein, BRD7, as an E1B-AP5 interaction partner. We confirmed E1B-AP5–BRD7 complex formation *in vivo* and *in vitro*. We found that, although BRD7 binds to histones H2A, H2B, H3 and H4 through its bromodomain, this domain was not necessary for the interaction with E1B-AP5. Indeed, the triple complex formation of E1B-AP5, BRD7 and histones was demonstrated. Disruption of the E1B-AP5–BRD7 complex increased E1B-AP5 repression activity for basic transcription and converted it from being an activator of the hormone-dependent promoter into being a strong repressor. We conclude that complex formation between BRD7 and E1B-AP5 links chromatin events with mRNA processing at the level of transcriptional regulation.

Key words: bromodomain, glucocorticoid, heterogeneous nuclear ribonucleoprotein (hnRNP), histone, transcription promoter, transcriptional regulation.

INTRODUCTION

E1B-AP5 was first identified as an interacting partner of an early adenovirus 5 E1B-55 kDa protein [1]. On the basis of sequence similarity, E1B-AP5 belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP) family and possesses single-strand-nucleic-acid-binding activity. hnRNP family members function at all stages of mRNA metabolism, ranging from transcriptional regulation to cytoplasmic compartmentalization and translation [2–4]. Several lines of evidence pointed to the involvement of E1B-AP5 in mRNA processing and transport. Its adenovirus partner E1B-55 kDa is required for selective accumulation of viral mRNA during the late phase of infection and, indeed, the E1B-55 kDa regions that mediate the interaction with E1B-AP5 are crucial for the host cell protein synthesis shut-off [5]. Overexpression of E1B-AP5 stimulates the export of viral transcripts and simultaneously alleviates the block of host cell mRNA export [1]. E1B-AP5 can bind to the N-terminal domain of TAP, an essential RNA export mediator, which bridges the interaction between specific ribonucleoprotein export substrates and the nuclear pore complexes [6].

Several findings indicate that the role of E1B-AP5 in the regulation of gene expression is not restricted to mRNA processing or export and that E1B-AP5 might already be involved in the regulation of gene expression at the level of transcriptional regulation. Recently, we demonstrated that E1B-AP5 forms a complex with the human arginine methyltransferase, HRMT1L1, which belongs to a novel family of transcription co-activators [7]. Another strong argument is the similarity between E1B-AP5 and hnRNP-U (or scaffold attachment factor A; SAF-A). hnRNP-U is a multifunctional RNA-binding phosphoprotein implicated in gene expression owing to its binding to chromatin through its scaffold attachment region [8] and regulation of polymerase II (Pol II)-dependent transcription [9]. The latter function was recently elucidated in detail: a subfraction of hnRNP-U was shown to be associated with Pol II holoenzyme, inhibiting Pol II elongation. The function was based on the ability of hnRNP-U to form a complex with transcription factor IIH (TFIIH) kinase and interfere with TFIIH-dependent phosphorylation of the Pol II C-terminal domain (CTD). Since hnRNP-U down-regulated several viral and cellular promoters, it was suggested that hnRNP-U functions as an inhibitor of basic transcription.

In addition to being a negative regulator of the basic transcription machinery, hnRNP-U is involved in hormone-responsive transcription. hnRNP-U is associated with the glucocorticoid receptor (GR) – a ligand-dependent transcription factor – and

Abbreviations used: aa, amino acid(s); BBS, BRD7 binding site; BD, bromodomain; CTD, C-terminal domain; FCS, foetal calf serum; GR, glucocorticoid receptor; GST, glutathione S-transferase; HA, haemagglutinin A; hnRNP, heterogeneous nuclear ribonucleoprotein; HSV-TK, herpes simplex virus thymidine kinase; IP buffer, immunoprecipitation buffer; MMTV, mouse mammary tumour virus; Pol II, polymerase II; RGG, Arg-Gly-Gly; RZPD, Deutsches Ressourcenzentrum für Genomforschung Primary Database; SV40, simian virus 40; TFIIH, transcription factor IIH.

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The nucleotide sequence data reported for human bromodomain-containing protein appears in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AJ271881.

interferes with glucocorticoid-dependent transcriptional activation [10]. Repression of GR-driven transcription by hnRNP-U requires its physical association with GR and can be squelched by high amounts of GR. This finding prompted the authors to propose a model where hnRNP-U acts as a storage site for intranuclear GRs [11].

In the present study, we analysed the role of E1B-AP5 in the regulation of transcription. Using several viral and cellular promoters, we demonstrated that E1B-AP5 inhibits general transcription in a manner similar to hnRNP-U and that the N-terminal part of the protein is responsible for this function. In contrast with hnRNP-U, E1B-AP5 did not interfere with GR-driven transcription and could activate a GR-responsive promoter in the absence of ligand-stimulation. We also identified an additional protein component involved in this activity, the bromodomain (BD)-containing protein BRD7, and were able to precipitate the E1B-AP5–BRD7 complex *in vivo*, as well as to show co-localization of both proteins in the nucleus. Deletion of the E1B-AP5 region responsible for the interaction with BRD7 converted E1B-AP5 from an activator into a strong repressor of a GR-responsive promoter and, remarkably, increased the basic E1B-AP5 transcription repression activity. Our results point to several mechanisms of transcription regulation by E1B-AP5, one of which is based on the interaction with a novel BD-containing chromatin-binding protein.

EXPERIMENTAL

Cell culture, transfection and reporter assay

H1299 cells were cultured in monolayers in Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin, streptomycin and 10 % foetal calf serum (FCS; Gibco). Cells at 50 % confluence in six-well dishes were transfected using the calcium-phosphate-precipitation technique using pUC19 as carrier DNA. The total amount of transfected DNA was 7 μg per transfection; the amounts of particular plasmids are indicated in the Figure legends. In some cases, cells were grown in serum-free medium in the presence of 0.1 μM dexamethasone (Sigma) 24 h before harvesting. At 48 h after transfection, cells were washed twice with PBS, harvested and used for the luciferase reporter assay (Promega), according to the manufacturer's protocol. The protein concentration in cell lysates was determined using the Bio-Rad protein assay.

Plasmids

Plasmids pHA–E1B-AP5wt, pHA–E1B-N-AP5 and pHA–E1B-AP5ΔRGG were described previously in [7]; pHA–E1B-AP5ΔPP was constructed by deleting amino acids (aa) 696 to 716 of E1B-AP5 using PCR-splicing. pHA–E1B-AP5wt was used as a template; fragments corresponding to haemagglutinin A (HA) fused to aa 1 to 696 and aa 716 to 869 were amplified by PCR with partially overlapping 3′ and 5′ primers for the N-terminal and C-terminal fragments, then mixed and used as a template for second-round PCR. The resulting product, representing HA-tagged E1B-AP5 lacking the polyproline-rich domain (aa 696 to 716), was cloned in the *Eco*RI/*Xho*I sites of pcDNA3 (Invitrogen). The same cloning procedure was used to construct pHA–E1B-AP5ΔBBS-1 (by the deletion of aa 456 to 497) and pHA–E1B-AP5ΔBBS-2 (by the deletion of aa 456 to 594). pHA–E1B-AP5BBS (BBS denotes BRD7 binding site) was constructed by subcloning the PCR-generated E1B-AP5 fragment corresponding to aa 454 to 611 directly after first 23 aa of the E1B-AP5 N-terminus. A short N-terminal fragment was left to optimize the mutant protein's stability. pHA–hnRNP-U was a gift from G. Dreyfuss. For the transcriptional regulation experiments, the following reporter constructs with luciferase driven by different promoters were used: pGL2control with simian virus 40 (SV40) promoter (Promega); pGL-2basic with minimal fos promoter (kindly provided by P. Groitl); pXP2 with H2A promoter [12]; pGL2basic with herpes simplex virus thymidine kinase (HSV-TK) promoter [13]; pGLbasic with E1B-AP5 promoter (kindly provided by P. Groitl); pGL2 basic with Ad5-E1B promoter [14]. The mouse mammary tumour virus (MMTV)–luciferase reporter plasmid was a gift from Dr E. Pfitzner [15]. Histone expression constructs in pGEX-2Tl (Pharmacia) and pET15b (Novagen) were generated by A. Hoffman and were kindly provided by T. Stammiger.

Yeast two-hybrid screening and cloning of full-length human BD-containing protein

Yeast two-hybrid analysis was performed according to standard protocols [16]. The central part of E1B-AP5 corresponding to aa 212 to 732 was used as bait. The yeast strain Y190 containing pASE1B-AP5-C was transformed with a HeLa cDNA library in the pACT vector (a gift of S. Elledge). Identification of the full-length cDNA was performed by using a multiple cell line Northern blot (Clontech). pSPORT–BRD7 containing full-length cDNA was obtained from RZPD (Deutsches Ressourcenzentrum für Genomforschung Primary Database) as clone DKFZp415C0669Q2. For mammalian expression, pFLAG–BRD7 was constructed by cloning FLAG-tagged full-length BRD7 between *Kpn*I and *Xba*I restriction sites of pcDNA3. pFLAG–BRD7ΔBD-1 was cloned in pcDNA3 by PCR splicing (deletion of BRD7 aa 129 to 237). For bacterial expression, full-length BRD7 or PCR-generated BRD7ΔBD-2 (deletion of BRD7 aa 1 to 375) was cloned between the *Sma*I and *Xho*I sites of pGEX-4T-1 (Pharmacia Biotech).

In vitro protein–protein interactions

Glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* strain BL21-CodonPlus-RIL (Stratagene). Protein expression and purification were performed under non-denaturing conditions as described in [17]. Proteins were eluted from GST–Sepharose in the presence of 15 mM glutathione, dialysed overnight at 4 °C against PBS and stored in aliquots at −80 °C. Amounts of GST-purified proteins and immunoprecipitated proteins were analysed by comparison with BSA standards using gel electrophoresis followed by protein staining with Gelcode Blue stain reagent (Pierce).

In vitro translation of proteins was performed according to the standard protocol (Promega) in the presence of [^{35}S]methionine. For the *in vitro* binding assay, 1 μg of GST-fused protein, or GST alone, and 5 μl of *in vitro* translation mixture were used. Binding was performed in 400 μl of DA500 buffer [1] or ELB buffer [18]. Protein competition assays were generally performed as described in [18]. Increasing amounts of *in vitro* translation mixture are indicated in the Figure legends. Binding reactions were supplemented by additional amounts of rabbit reticulocyte lysate to ensure equal concentrations corresponding to the maximal amount of *in vitro* translation mixture used. All binding reactions were performed at 4 °C for 1.5 h, washed five times in 1 ml of appropriate buffer and analysed by PAGE.

Immunoprecipitation and Western blotting

For analysis of protein–protein interactions, H1299 cells were transiently transfected with the constructs by the calcium-

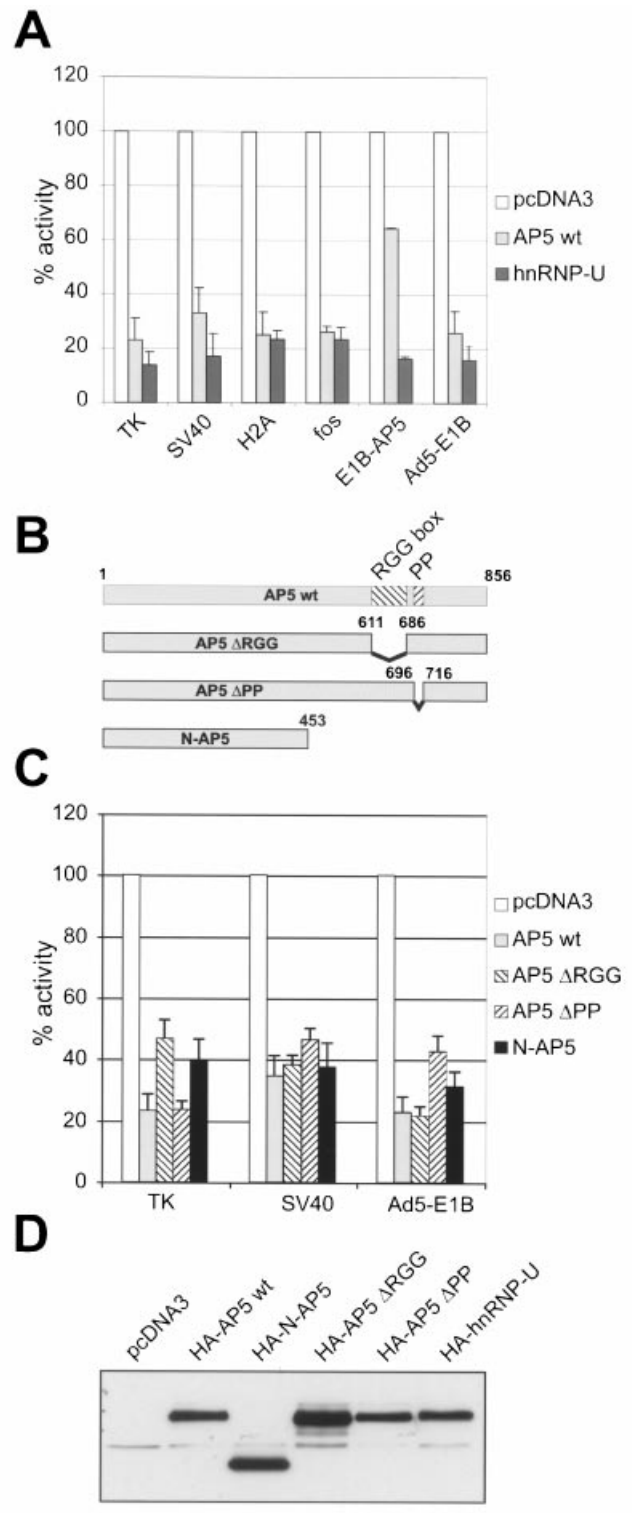


Figure 1 E1B-AP5 represses the basal transcriptional activity of different promoters

(**A**) and (**C**) Histograms of activation values obtained in co-transfection experiments. Luciferase activities are expressed as a percentage of the corresponding luciferase construct activity in the presence of empty vector (100%). Results are from at least ten independent experiments; standard deviations are indicated by the error bars. In all transfections, 0.3 μg of the reporter luciferase construct was co-transfected with 1.6 μg of the expression construct or empty vector. (**A**) Luciferase assay after co-transfection of pHA–E1B-AP5 or pHA–hnRNP-U with different reporter constructs. (**B**) Diagram of the E1B-AP5 mutants cloned with an HA tag in the pcDNA3 vector and used in the reporter assays (see the text for details). (**C**) Luciferase assay after co-transfection of pHA–E1B-AP5 mutants with different reporter constructs. (**D**) Steady-state level of proteins expressed by constructs used in the luciferase assays. H1299 cells were transfected with the indicated pcDNA3-HA expression constructs. Transfection, propagation and cell harvesting were performed under conditions identical with the reporter assay. Total cell lysates were analysed by Western blotting with anti-HA monoclonal antibody. Representative results of three independent experiments are shown. wt, wild-type; PP, polyproline region; N-AP5, E1B-AP5 lacking the entire C-terminal region.

phosphate precipitation method, harvested 48 h after transfection and lysed in immunoprecipitation (IP) buffer [50 mM Tris/HCl, pH 8.0, 150 mM NaCl and 1 % (v/v) Nonidet P40]. Cell lysates were pre-cleared with Protein G–Sepharose. Pre-cleared lysates (200 μg) were incubated overnight with Protein G coupled to the corresponding antibody in a total volume 1.5 ml, then washed five times with IP buffer and analysed by Western blotting. Primary antibodies used were anti-FLAG (Sigma) and anti-E1B-AP5 4A11 [7]; corresponding secondary antibodies were purchased from Amersham Biosciences.

Steady-state levels of E1B-AP5 mutants were analysed by Western blotting using 12CA5 (Roche) for the HA-tagged E1B-AP5 expression constructs.

Indirect immunofluorescence

H1299 cells were grown on cover slips, transiently transfected with FLAG-tagged BRD7 by calcium-phosphate precipitation, and analysed by indirect immunofluorescence 24 h after transfection. All procedures were performed as previously described in [7]. Antibodies were used at the following dilutions: 1:800 for anti-E1B-AP5 rabbit polyclonal serum [1]; 3.5 μg/ml for anti-FLAG M2 (Sigma); 1:200 for Cy3-conjugated anti-rabbit (Dianova); 1:3000 for FITC-labelled anti-mouse (Alexa). Samples were viewed with a Leica Microscope DM at a magnification of $\times$2000. Pictures were acquired with a DC500 digital camera (Leica).

RESULTS

E1B-AP5 is a negative regulator of basic transcription

Since the closest E1B-AP5 homologue, hnRNP-U, was shown to inhibit basic transcription in transient reporter assays [9], we analysed the ability of E1B-AP5 to influence transcription from different cellular and viral promoters, and compared the E1B-AP5 activity with that of hnRNP-U. Both HA-tagged E1B-AP5 and hnRNP-U were transiently expressed in H1299 cells together with a luciferase reporter construct containing promoters for HSV-TK, fos, SV40, Ad5-E1B, H2A or E1B-AP5. E1B-AP5 repressed the activity of all the promoters tested to between 25 % and 65 % of the initial level set for cells transfected with empty vector (Figure 1A). The maximum repression was observed with the HSV-TK promoter and the minimum with E1B-AP5's own promoter. E1B-AP5-dependent repression was generally less efficient then hnRNP-U-dependent repression, although equal expression levels of both proteins were confirmed by Western blotting (Figure 1D), indicating that the effect measured is due to transcriptional repression and not a toxic effect of protein overexpression.

In order to analyse the domain of E1B-AP5 involved in transcriptional repression, several deletion mutants were tested in a transient reporter assay with HSV-TK, SV40, and Ad5-E1B promoters. We deleted the Arg-Gly-Gly (RGG) domain, which is responsible for E1B-AP5 interaction with ribonucleic acid (results not shown), the polyproline region, which has been widely

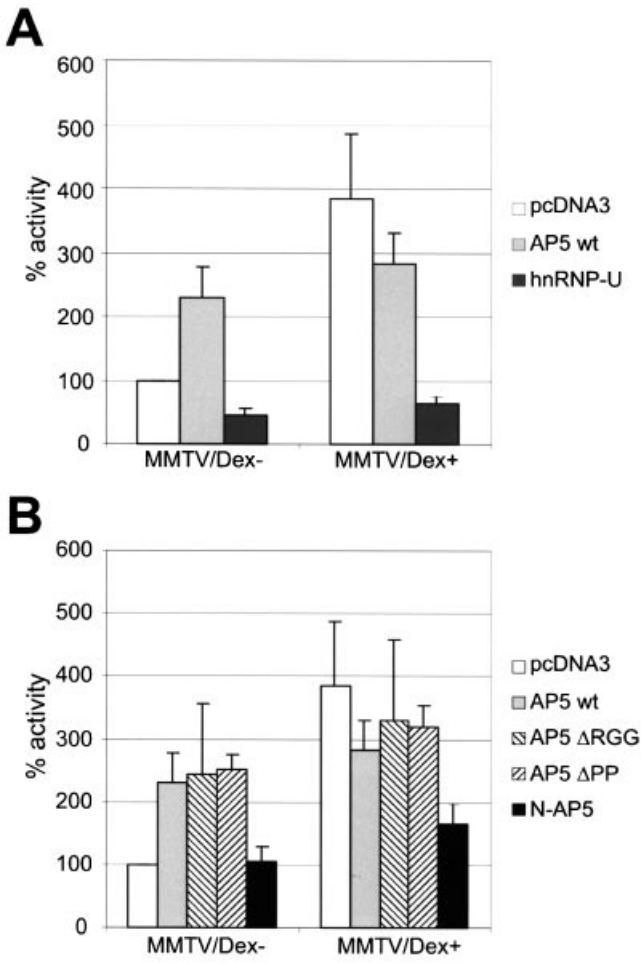


Figure 2 E1B-AP5 activates the MMTV promoter in the absence of dexamethasone

Histograms of activation values obtained in co-transfection experiments. Luciferase activities are expressed as a percentage of the MMTV promoter activity in the absence of dexamethasone and co-transfected with empty pcDNA3 vector (100%). Results are from at least 10 independent experiments; standard deviations are indicated by the error bars. In all transfections, 0.3 μg of the reporter luciferase construct was co-transfected with 1.6 μg of the expression construct or empty vector. (**A**) Luciferase assay of pHA–E1B-AP5 or pHA–hnRNP-U co-transfected with the MMTV–luciferase construct in the presence (Dex +) or absence (Dex −) of dexamethasone. (**B**) Luciferase assay of pHA–E1B-AP5 mutants co-transfected with MMTV–luciferase construct in the presence or absence of dexamethasone. See the legend to Figure 1 for details of the mutants.

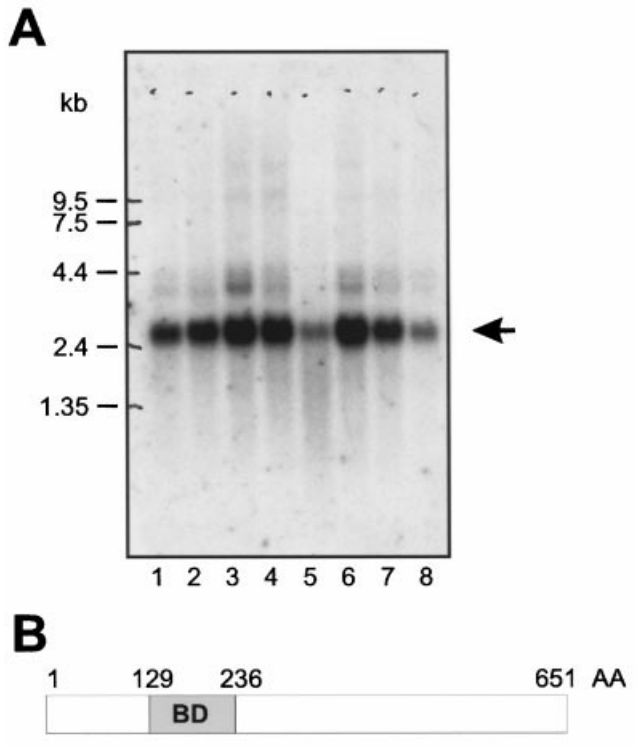


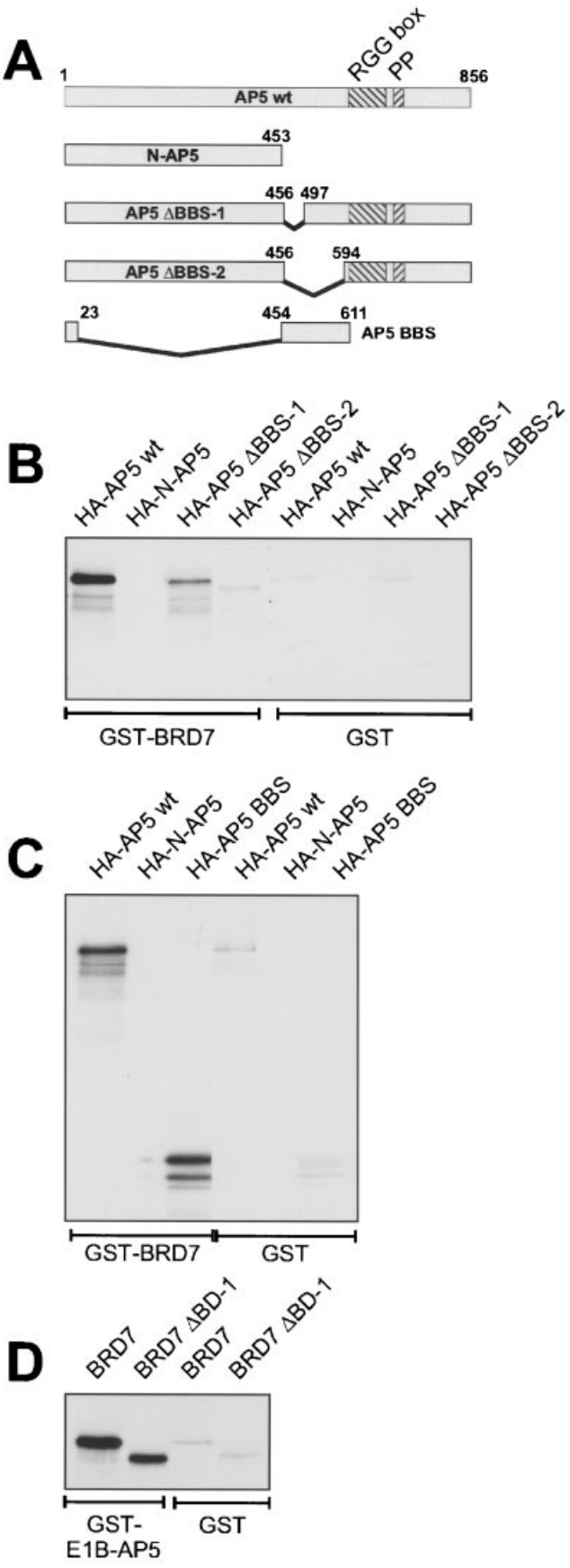
Figure 3 Cloning of E1B-AP5-interacting protein BRD7

(**A**) Multiple tissue Northern blot analysis with ^{32}P-labelled BRD7-specific probe. Lane 1, promyeloblastic leukaemia HL-60; lane 2, cervical carcinoma; lane 3, lymphoblastic leukaemia MULT-4; lane 5, Burkitt's lymphoma (Raji); lane 6, colorectal adenocarcinoma SW480; lane 7, lung carcinoma A549; lane 8, melanoma G361. The specific 2.6 kb fragment is indicated by the arrow. The mRNA loading control was performed with β-actin probe (results not shown). (**B**) Schematic representation of BRD7 protein with indicated boundaries of the BD.

demonstrated for many proteins to mediate protein–protein interactions, and the complete C-terminal part of the protein (Figure 1B). All constructs were expressed at equal levels (Figure 1D) and showed nuclear localization (results not shown). Interestingly, deletion of the RGG domain or the polyproline region modified the repression activity profile of the E1B-AP5 derivatives in a promoter-dependent manner, with the N-terminal part of E1B-AP5 retaining the most consistent E1B-AP5 repression activity (Figure 1C). We concluded that the C-terminal part of E1B-AP5 contains domains that may have a regulatory effect on basic transcription repression activity, but that the domain responsible for this repression is located within the N-terminal part of E1B-AP5.

E1B-AP5 transcriptional activity in a dexamethasone-dependent system

As demonstrated previously, hnRNP-U is not only a repressor of basic transcription, but its overexpression also interferes with glucocorticoid-induced transcription [10,11]. Using the GR-responsive MMTV promoter we analysed the ability of E1B-AP5 to interfere with dexamethasone-induced transcription. An MMTV–luciferase reporter construct was co-transfected with HA-tagged E1B-AP5 or hnRNP-U in H1299 cells. The cells were grown in FCS-free medium to ensure the absence of glucocorticoids, with dexamethasone being added 24 h before cell harvesting. In the absence of dexamethasone, E1B-AP5 enhanced MMTV-driven transcription more than 2-fold, while hnRNP-U repressed the transcription to about 30% of control levels (Figure 2A). Under dexamethasone stimulation, the activity of the MMTV promoter was about four times higher than basal levels. E1B-AP5 did not work synergistically with dexamethasone, but rather exhibited weak repression activity, while hnRNP-U retained its repression activity on the MMTV promoter in the presence of dexamethasone (Figure 2A). Therefore, E1B-AP5 may play a dual role in the regulation of MMTV-driven transcription. In the presence of dexamethasone, E1B-AP5 overexpression had the typical effect reflecting basic transcriptional repression. For HSV-TK, SV40, H2A and Ad5-E1B promoters this effect was independent of the presence or absence of dexamethasone or FCS in the culture medium (results not



shown). Apparently, however, a specific mechanism must be responsible for E1B-AP5 activation of the MMTV promoter in the absence of dexamethasone, and mutant analysis revealed that the region responsible for this activation is located between aa 453 and 611 of E1B-AP5 (Figure 2B). Based on these results, we concluded that (i) an unknown E1B-AP5-binding protein might be involved in specific activation of the MMTV promoter by E1B-AP5, and (ii) the central part of E1B-AP5 should mediate such a protein–protein interaction.

Identification of a novel BD-containing protein as an E1B-AP5-interacting protein

Yeast two-hybrid screening was performed to identify potential E1B-AP5-interacting partners. A HeLa cDNA library was screened with pASE1B-AP5 expressing the Gal4 DNA-binding domain fused to a fragment of E1B-AP5 comprising aa 213 to 732. This fragment covered the region of interest involved in regulation of transcription as well as providing the stability of the bait protein in the yeast system. After double histidine/β-galactosidase selection, 16 independent clones were obtained. The clones were sequenced and database analysis revealed that eight of these clones contained cDNA with 86 % similarity to the cDNA for the mouse BD-containing protein BP75 (GenBank® accession no. AF084259). Northern blot analysis revealed a full-length transcript approx. 2.6 kb long that was expressed in all cell lines tested (Figure 3A). Screening of a human foetal brain cDNA library (RZPD, created by Bernhard Korn) resulted in identification of full-length cDNA, which was sequenced and submitted to the database (GenBank® accession no. AJ271881). Sequence analysis revealed an open reading frame coding for a protein with a predicted molecular mass of 74 kDa. A search for consensus domains resulted in identification of a conserved BD between aa 129 and 236 (Figure 3B), a typical domain for mediating interaction with histones (reviewed in [19]). The protein was named BRD7, corresponding to its official locus name.

Analysis of E1B-AP5–BRD7 interaction *in vitro* and *in vivo*

To analyse the interaction of E1B-AP5 with the novel human BD-containing protein BRD7, we purified the GST-fused full-length BRD7 and performed *in vitro* binding assays. It was found that *in vitro* translated E1B-AP5 interacted strongly with GST–BRD7 but that the N-terminal part of E1B-AP5 did not (see Figure 4B, lanes 1 and 2), while GST alone acted as the negative control. Prediction of the domain responsible for E1B-AP5 binding to BRD7 was originally based on which E1B-AP5 mutants were capable of activating the MMTV promoter in the absence of dexamethasone (Figure 2B), leading to our guess that the binding site was located between aa 453 and 611. To actually map the E1B-AP5 region that mediates the interaction with BRD7, two E1B-AP5 mutants with deletions of aa 456 to 497 (E1B-AP5ΔBBS-1) and aa 456 to 594 (E1B-AP5ΔBBS-2) were generated and analysed by *in vitro* binding assays (Figure 4A). Both mutants showed dramatically reduced binding activity (Figure

Figure 4 ***In vitro*** **analysis of the E1B-AP5–BRD7 complex**

(**A**) Diagram of E1B-AP5 mutants used to analyse the interaction with BRD7. (**B** and **C**) Binding of *in vitro* translated wild-type E1B-AP5 (E1B-AP5wt) and various deletion mutants to bacterially expressed GST-fused BRD7. Each *in vitro* translation mixture (5 μl) was used as indicated. GST–BRD7 lanes: purified GST-fused BRD7 was used in the binding reaction; GST lanes: purified GST was used as a negative control. (**D**) Binding of *in vitro* translated BRD7wt or mutant to bacterially expressed GST–E1B-AP5. Aliquots of 5 μl of *in vitro* translated BRD7 or BRD7 lacking the BD (BRD7ΔBD-1) were used as indicated. Lanes 1 and 2, GST–E1B-AP5 was used in the binding reaction; lanes 3 and 4, GST was used as a negative control.

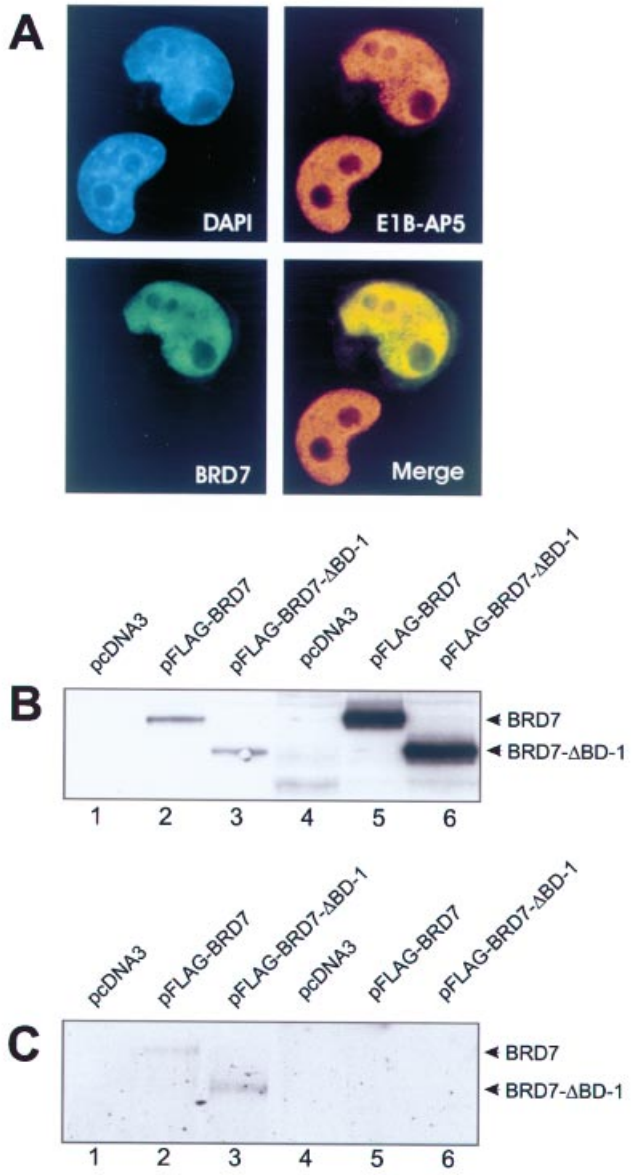


Figure 5 ***In vivo*** **interaction of E1B-AP5 with BRD7**

(**A**) Immunofluorescence analysis of protein co-localization in H1299 cells. The red colour corresponds to endogenous E1B-AP5, the green colour corresponds to the transiently transfected FLAG-tagged BRD7. The yellow colour indicates protein co-localization. (**B**) Control of expression level for the transiently transfected FLAG-tagged BRD7 or BRD7ΔBD-1. H1299 cells were transfected with empty vector (lanes 1 and 4), pFLAG–BRD7 (lanes 2 and 5) or pFLAG–BRD7ΔBD-1 (lanes 3 and 6). The amount of the FLAG-tagged protein in the soluble fraction (lanes 1–3) and pellet (lanes 4–6) after lysis in IP buffer is presented. (**C**) Co-immunoprecipitation of BRD7 with E1B-AP5. Transfection was performed as described in (**B**). Immunoprecipitation was performed with anti-E1B-AP5 4A11 monoclonal antibody (lanes 1–3) and non-specific antibody (lanes 4–6). Co-precipitated proteins were detected by Western blotting with anti-FLAG monoclonal antibody.

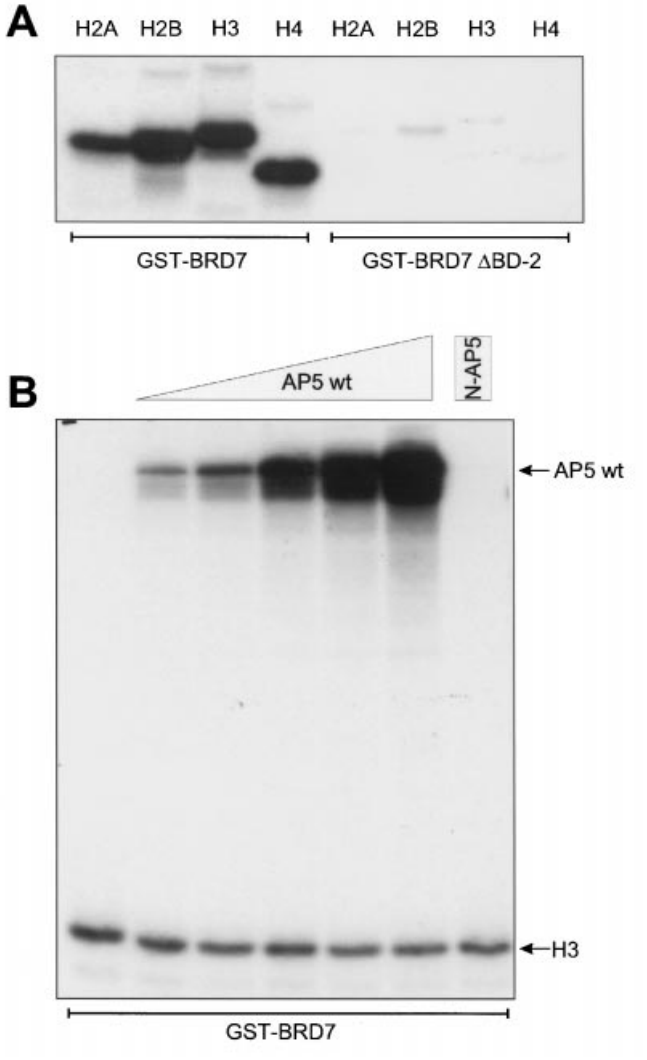


Figure 6 E1B-AP5 can bind to BRD7 simultaneously with histones

(**A**) Binding of histones to BRD7 requires its BD. *In vitro* translated histones were incubated as indicated with purified GST–BRD7 (lanes 1–4) or mutant GST–BRD7ΔBD-2 lacking the BD (lanes 5–8). (**B**) E1B-AP5 does not compete with histone for binding to GST–BRD7. Aliquots of 5 μl of *in vitro* translated histone H3 were used in each reaction. Increasing volumes of *in vitro* translated pHA–E1B-AP5wt supplemented with rabbit reticulocyte lysate to a final volume of 40 μl were used as follows: lane 2, 2 μl; lane 3, 5 μl; lane 4, 10 μl; lane 5, 20 μl; lane 6, 40 μl. Negative controls were designed as follows: lane 1, 40 μl of rabbit reticulocyte lysate; lane 7, 40 μl of *in vitro* translated pHA–E1B-N-AP5.

4B). To confirm this, we generated an E1B-AP5 mutant protein containing a short N-terminal fragment fused to the BRD7-binding site (E1B-AP5BBS, Figure 4A). The first 23 aa on the N-terminus provided equal stability for the fragment and full-length protein (results not shown). Both full-length E1B-AP5, as well as its BBS fragment, interacted strongly with GST–BRD7 (Figure 4C). Conversely, using GST-purified E1B-AP5 and *in vitro* translated BRD7 and BRD7 lacking the BD (BRD7ΔBD-1), we demonstrated that the BD of BRD7 is not involved in complex formation with E1B-AP5 (Figure 4D).

To examine further the interaction of E1B-AP5 and BRD7 *in vivo*, we analysed their co-localization in mammalian cells. H1299 cells growing on coverslips were transiently transfected with FLAG-tagged BRD7 and subjected to an indirect immunofluorescence assay. Fixed cells were incubated with a mixture of rabbit polyclonal anti-E1B-AP5 and mouse monoclonal anti-FLAG antibodies and then stained by a combination of Cy3-conjugated anti-rabbit and FITC-conjugated anti-mouse antibodies (Figure 5A). Endogenous E1B-AP5 is stained red and overexpressed FLAG-BRD7 is stained green. Overlaying identical red- and green-stained cells resulted in a yellow colour

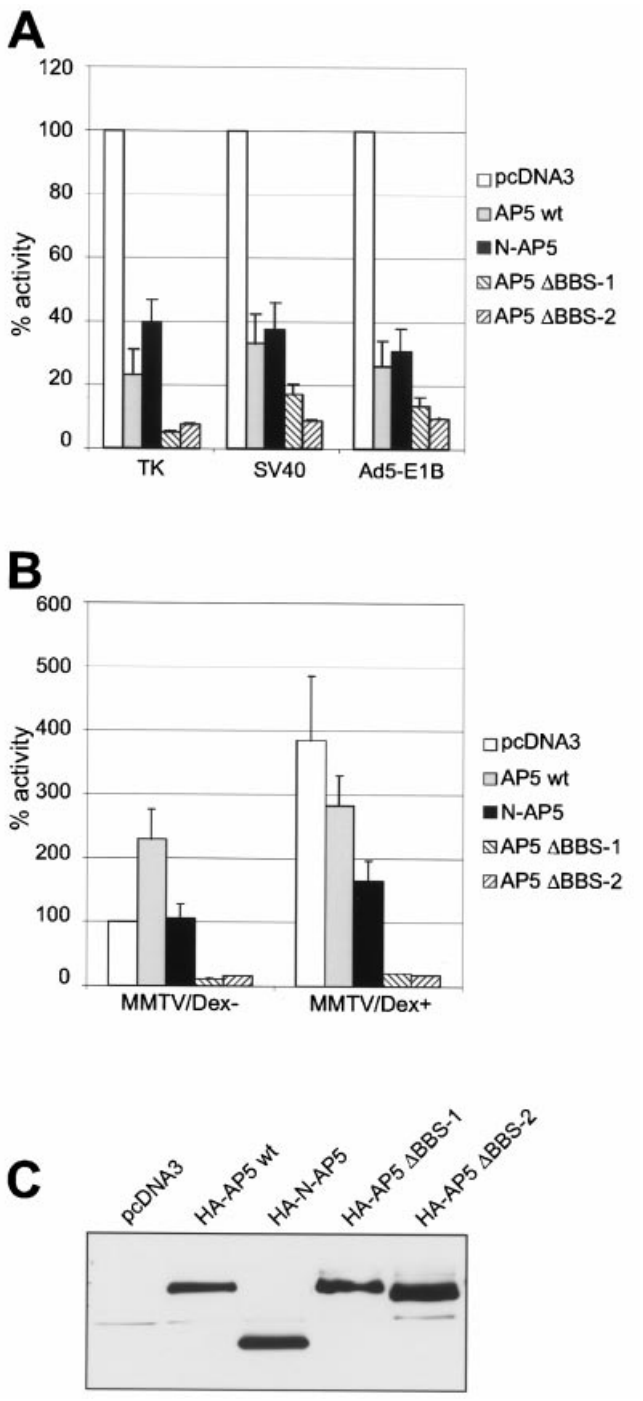


Figure 7 Deletion of the BRD7 binding site increases the transcriptional repression activity of E1B-AP5

(**A**) and (**B**) Histograms of activation values obtained in co-transfection experiments. The luciferase activity is expressed as a percentage of the respective luciferase construct activity in the presence of empty vector. Results are from at least ten independent experiments; standard deviations are indicated by the error bars. In all transfections, 0.3 μg of the reporter luciferase construct was co-transfected with 1.6 μg of the expression construct or empty vector. (**A**) Luciferase assay after co-transfection of pHA–E1B-AP5wt or mutant with the indicated reporter constructs. (**B**) Luciferase assay after co-transfection of pHA–E1B-AP5wt or mutant with the MMTV–luciferase reporter construct in the absence (Dex−) or presence (Dex+) of dexamethasone. (**C**) Steady-state protein level expressed by constructs used in the luciferase assay. H1299 cells were transfected with indicated pcDNA3-HA expression constructs. Transfection, propagation and cell harvesting were performed under conditions identical with the reporter assay. Total cell lysates were analysed by Western blotting with an anti-HA monoclonal antibody. Representative results of three independent experiments are shown.

at the sites of protein co-localization. Significant amounts of E1B-AP5 and BRD7 co-localize in the nucleus. The presence of green and red structures indicates that some portion of each protein does not form complexes with the other. We also observed that deletion of the BD changed the localization of BRD7 to being more granular, with no significant co-localization with E1B-AP5 (results not shown).

Our attempts to immunoprecipitate transiently expressed BRD7 demonstrated that more than 90% of the protein remains associated with the non-soluble fraction after lysis in the IP buffer (Figure 5B). However, a small amount of FLAG-tagged BRD7, as well as BRD7ΔBD-1, was co-precipitated with E1B-AP5 from transiently transfected H1299 cells (Figure 5C). The fact that the amount of *in vivo* co-immunoprecipitated BRD7 did not change after the deletion of the BD was consistent with our *in vitro* observation that the BD of BRD7 is not involved in complex formation with E1B-AP5.

Interaction of BRD7 with histones

A BD is present in several proteins involved in transcriptional regulation and chromatin remodelling (reviewed in [19,20]). Structural studies have revealed the role of the BD in specific recognition of histone tails [21,22]. We analysed the ability of BRD7 to form a complex with histones *in vitro* using bacterially purified GST-fused BRD7 and *in vitro* translated histones. Histones H2A, H2B, H3 and H4 were found to bind to GST–BRD7 with similar affinity and the binding was dramatically decreased after deletion of the BD (Figure 6A). An *in vitro* competition experiment was designed to investigate whether or not E1B-AP5 can compete with histones for binding to BRD7. GST-fused BRD7 was incubated with constant amounts of *in vitro* translated histone (H3) and increasing amounts of *in vitro* translated E1B-AP5. A 10-fold excess of E1B-AP5 in the reaction mix did not affect the BRD7–histone binding (Figure 6B), suggesting that E1B-AP5 does not interfere with the binding of histones to BRD7. The ability of E1B-AP5, BRD7 and histones to form a complex *in vitro* correlates with the conclusion derived from our *in vitro* binding studies suggesting that E1B-AP5 and histones use different domains of BRD7 for complex formation.

The transcriptional repression activity of E1B-AP5 is negatively regulated by binding to BRD7

Since the observed dual role of E1B-AP5 in transcriptional regulation had led to the discovery of BRD7, we wanted to analyse the role of the BRD7–E1B-AP5 complex formation in basic and dexamethasone-dependent transcription systems. E1B-AP5, and mutants E1B-AP5ΔBBS-1 and E1B-AP5ΔBBS-2 with dramatically decreased binding to BRD7, were co-transfected with luciferase reporter constructs containing SV40, HSV-TK and Ad5-E1B promoters. Deletion of the BRD7 binding site increased the ability of E1B-AP5 to repress basic transcription 2–5-fold (Figure 7A), whereas the steady-state level of E1B-AP5 was not affected (Figure 7C).

The same E1B-AP5 mutants were analysed in the dexamethasone-dependent system using the MMTV-promoter-driven luciferase construct. Deletion of the E1B-AP5 region mediating the complex formation with BRD7 converted E1B-AP5 from an MMTV-promoter activator into a potent repressor. In the absence of dexamethasone, E1B-AP5wt activated the MMTV promoter about 2.5-fold, whereas both deleted-BRD7-binding-site mutants repressed promoter activity about 10-fold (Figure 7B). The repression activity of E1B-AP5ΔBBS-1 and E1B-AP5ΔBBS-2 in the presence of dexamethasone was also

increased about 10-fold in comparison with that of E1B-AP5wt. Summarizing the results of the different reporter systems, we conclude that deletion of the region of E1B-AP5 mediating its interaction with BRD7 increased the transcriptional repression ability of E1B-AP5, and that the strength of this effect is promoter-specific.

DISCUSSION

mRNA metabolism is a complex process starting with mRNA transcription and processing in the nucleus, followed by specifically regulated transport to the cytoplasm, and subsequent translation and degradation. It had widely been assumed that the molecular machinery responsible for each step of mRNA metabolism in the nucleus functions independently. However, an increasing amount of evidence indicates that mRNA transcription, capping, polyadenylation and splicing are not distinct events, but rather are orchestrated by the CTD of Pol II (reviewed in [23]). The discovery of multiple intimate links between Pol II and other groups of nuclear proteins formed to co-ordinate such nuclear events recently evoked great interest. In particular, several hnRNPs were found to be associated with Pol II [9,24–26]. The hnRNP family comprises a wide group of proteins with RNA-binding activity and is involved in all steps of mRNA metabolism. Functional relationships between hnRNP-U and Pol II were described in detail, showing that hnRNP-U, well known as a scaffold-attachment factor [8], may inhibit transcriptional elongation by interfering with Pol II CTD phosphorylation [9]. The highly similar hnRNP protein, E1B-AP5, was first identified as a target of the early adenoviral E1B-55 kDa protein involved in modulation of the host cell's mRNA balance. E1B-AP5 was postulated to act at the step of nuclear–cytoplasmic mRNA transport [1,27]. Since a transcriptional repression function for the closely related E1B-AP5 analogue hnRNP-U was identified, we decided to investigate the role of E1B-AP5 in the regulation of transcription.

Using transient reporter assays, we demonstrated that E1B-AP5 acts as a transcriptional repressor with promoter-dependent strength, although E1B-AP5 was less efficient at repressing basal transcription than hnRNP-U. In addition, hnRNP-U specifically represses GR-driven transcription [10,11]. Interestingly, we could demonstrate that, in the case of the glucocorticoid-dependent MMTV promoter, E1B-AP5 acts as an hnRNP-U antagonist, activating the MMTV promoter in the absence of dexamethasone. The activating effects of E1B-AP5 and dexamethasone were not additive; rather, E1B-AP5 had a weak repressive effect on the dexamethasone-activated MMTV promoter, suggesting that dexamethasone and E1B-AP5 may compete for the same limiting factor to activate the MMTV promoter. The slight repressive effect of E1B-AP5 on GR-driven transcription differed dramatically from the strong hnRNP-U-dependent repression, whereas E1B-AP5 mutant analysis indicated that different E1B-AP5 domains are responsible for the basic transcription repression and MMTV-promoter activation.

To elucidate the mechanism of the latter process in more detail, our search for an E1B-AP5-interacting protein identified a novel BD-containing protein, BRD7, as an E1B-AP5-interacting partner. The E1B-AP5–BRD7 protein–protein interaction, confirmed *in vivo* by co-immunoprecipitation and co-localization experiments and *in vitro* by mutant analysis, was independent of the BD. However, *in vitro* experiments showed that BRD7 binds to histones through its BD. This agrees with the findings of other groups, demonstrated for several proteins, that the conserved BD is responsible for the interaction with transcriptionally active chromatin, and, in particular, for binding to acetylated lysine in histone tails [21,28–31].

The location of the E1B-AP5 region mediating the interaction with BRD7 in the central part of the protein correlated well with the initial observation that the E1B-AP5 central region is required for the MMTV-promoter activation in the absence of dexamethasone. In fact, deletion of the BRD7-binding region in E1B-AP5 turned E1B-AP5 from an activator of the MMTV promoter into a strong repressor, as well as increasing the repression activity of E1B-AP5 in basal transcriptional processes. The ability of hnRNP-U to bind BRD7 *in vitro* (results not shown) suggests that the formation of an E1B-AP5–BRD7 complex is necessary, but not sufficient, for the activation of the MMTV promoter, and further identification of other regulatory factors would be required to explain the antagonistic action of E1B-AP5 and hnRNP-U on the activity of glucocorticoid-dependent promoters.

Binding of hnRNPs to BD-containing proteins indicates the presence of a novel link involved in co-ordinating nuclear events. In parallel with our study, BRD7 was identified by another research group as an interacting partner of the transcription factor interferon regulatory factor 2 [32]. BRD7 (Celtix-1) was shown to be associated with active chromatin and a hyperphosphorylated form of Pol II. A model can be proposed where BRD7 recognizes the active chromatin by interacting with acetylated histones and subsequently forms a complex with hnRNPs (E1B-AP5, hnRNP-U). This complex formation then interferes with hnRNP-mediated inhibition of Pol II CTD hyperphosphorylation, allowing efficient transcription. Since E1B-AP5 and hnRNP-U are involved in further mRNA metabolism, the function of BRD7 as a factor coupling chromatin activation and mRNA processing can be proposed. The positive regulatory role of an E1B-AP5–BRD7 complex in glucocorticoid-dependent transcription and the identification of BRD7 as an interacting partner of interferon regulatory factor-2 suggest that BRD7–hnRNP complex formation may be a specifically regulated mechanism in ligand-stimulated transcription. The intriguing question would be to investigate the role of E1B-AP5 and hnRNP-U in interferon-dependent transcription.

Our study reveals new facts that complement our understanding of adenovirus–host cell interactions. The ability of adenoviruses to target crucial host cell regulatory proteins is well documented [33,34]. A number of functions for the adenovirus protein E1B-55 kDa have already been described [27]. One of these functions is transcriptional repression [35]. E1B-55 kDa has no DNA-binding domain and must therefore be targeted to the promoter to exert its repression activity [36,37]. It was proposed that this transcriptional repression requires an unknown cellular factor as well as the existence of a co-repressor that does not belong to the Pol II holoenzyme, but is associated with the Pol II complex [38]. E1B-AP5 forms a stable complex with E1B-55 kDa during the early and late phases of lytic adenovirus infection [1]. Our discovery that E1B-AP5 represses transcription implies that E1B-AP5 is one of the co-factors required for E1B-55 kDa-mediated repression of basic transcription. This is supported by the observation that E1B-AP5-mediated transcriptional repression is specifically increased in the presence of E1B-55 kDa (results not shown). Investigations into possible E1B-AP5 association with the Pol II complex will be required to support this hypothesis.

In summary, we propose that the role of E1B-AP5 is to contribute to the regulation of basic and ligand-induced transcription. E1B-AP5 is a component of highly co-ordinated nuclear processes and its complex formation with BRD7 may link chromatin events with mRNA processing. The identification of further protein complex components, as well as unravelling

the mechanism of how adenovirus taps into the cellular transcription machinery, will certainly lead to a better understanding of transcription regulation in the cell in general.

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REFERENCES

1 Gabler, S., Schutt, H., Groitl, P., Wolf, H., Shenk, T. and Dobner, T. (1998) E1B 55-kilodalton-associated protein: a cellular protein with RNA-binding activity implicated in nucleocytoplasmic transport of adenovirus and cellular mRNAs. J. Virol. **72**, 7960–7971
2 Krecic, A. M. and Swanson, M. S. (1999) hnRNP complexes: composition, structure, and function. Curr. Opin. Cell Biol. **11**, 363–371
3 Nakielny, S. and Dreyfuss, G. (1997) Nuclear export of proteins and RNAs. Curr. Opin. Cell Biol. **9**, 420–429
4 Nakielny, S., Fischer, U., Michael, W. M. and Dreyfuss, G. (1997) RNA transport. Annu. Rev. Neurosci. **20**, 269–301
5 Yew, P. R., Kao, C. C. and Berk, A. J. (1990) Dissection of functional domains in the adenovirus 2 early 1B 55K polypeptide by suppressor-linker insertional mutagenesis. Virology **179**, 795–805
6 Bachi, A., Braun, I. C., Rodrigues, J. P., Pante, N., Ribbeck, K., von Kobbe, C., Kutay, U., Wilm, M., Gorlich, D., Carmo-Fonseca, M. et al. (2000) The C-terminal domain of TAP interacts with the nuclear pore complex and promotes export of specific CTE-bearing RNA substrates. RNA **6**, 136–158
7 Kzhyshkowska, J., Schutt, H., Liss, M., Kremmer, E., Stauber, R., Wolf, H. and Dobner, T. (2001) Heterogeneous nuclear ribonucleoprotein E1B-AP5 is methylated in its Arg-Gly-Gly (RGG) box and interacts with human arginine methyltransferase HRMT1L1. Biochem. J. **358**, 305–314
8 Kipp, M., Gohring, F., Ostendorp, T., van Drunen, C. M., van Driel, R., Przybylski, M. and Fackelmayer, F. O. (2000) SAF-Box, a conserved protein domain that specifically recognizes scaffold attachment region DNA. Mol. Cell. Biol. **20**, 7480–7489
9 Kim, M. K. and Nikodem, V. M. (1999) hnRNP U inhibits carboxy-terminal domain phosphorylation by TFIIH and represses RNA polymerase II elongation. Mol. Cell. Biol. **19**, 6833–6844
10 Eggert, M., Michel, J., Schneider, S., Bornfleth, H., Baniahmad, A., Fackelmayer, F. O., Schmidt, S. and Renkawitz, R. (1997) The glucocorticoid receptor is associated with the RNA-binding nuclear matrix protein hnRNP U. J. Biol. Chem. **272**, 28471–28478
11 Eggert, H., Schulz, M., Fackelmayer, F. O., Renkawitz, R. and Eggert, M. (2001) Effects of the heterogeneous nuclear ribonucleoprotein U (hnRNP U/SAF-A) on glucocorticoid-dependent transcription in vivo. J. Steroid Biochem. Mol. Biol. **78**, 59–65
12 Oswald, F., Dobner, T. and Lipp, M. (1996) The E2F transcription factor activates a replication-dependent human H2A gene in early S phase of the cell cycle. Mol. Cell. Biol. **16**, 1889–1895
13 Nevels, M., Rubenwolf, S., Spruss, T., Wolf, H. and Dobner, T. (1997) The adenovirus E4orf6 protein can promote E1A/E1B-induced focus formation by interfering with p53 tumor suppressor function. Proc. Natl. Acad. Sci. U.S.A. **94**, 1206–1211
14 Parks, C. L., Banerjee, S. and Spector, D. J. (1988) Organization of the transcriptional control region of the E1b gene of adenovirus type 5. J. Virol. **62**, 54–67
15 Pfitzner, E., Jahne, R., Wissler, M., Stoecklin, E. and Groner, B. (1998) p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivation domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response. Mol. Endocrinol. **12**, 1582–1593
16 Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H. and Elledge, S. J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7**, 555–569
17 Rosorius, O., Heger, P., Stelz, G., Hirschmann, N., Hauber, J. and Stauber, R. H. (1999) Direct observation of nucleocytoplasmic transport by microinjection of GFP-tagged proteins in living cells. Biotechniques **27**, 350–355
18 Winkler, M., aus dem Siepen, T. and Stamminger, T. (2000) Functional interaction between pleiotropic transactivator pUL69 of human cytomegalovirus and the human homolog of yeast chromatin regulatory protein SPT6. J. Virol. **74**, 8053–8064
19 Filetici, P., Ornaghi, P. and Ballario, P. (2001) The bromodomain: a chromatin browser? Front. Biosci. **6**, D866–D876
20 Winston, F. and Allis, C. D. (1999) The bromodomain: a chromatin-targeting module? Nat. Struct. Biol. **6**, 601–604
21 Jacobson, R. H., Ladurner, A. G., King, D. S. and Tjian, R. (2000) Structure and function of a human TAFII250 double bromodomain module. Science **288**, 1422–1425
22 Ornaghi, P., Ballario, P., Lena, A. M., Gonzalez, A. and Filetici, P. (1999) The bromodomain of Gcn5p interacts *in vitro* with specific residues in the N terminus of histone H4. J. Mol. Biol. **287**, 1–7
23 Hirose, Y. and Manley, J. L. (2000) RNA polymerase II and the integration of nuclear events. Genes Dev. **14**, 1415–1429
24 Conrad, N. K., Wilson, S. M., Steinmetz, E. J., Patturajan, M., Brow, D. A., Swanson, M. S. and Corden, J. L. (2000) A yeast heterogeneous nuclear ribonucleoprotein complex associated with RNA polymerase II. Genetics **154**, 557–571
25 Lei, E. P., Krebber, H. and Silver, P. A. (2001) Messenger RNAs are recruited for nuclear export during transcription. Genes Dev. **15**, 1771–1782
26 Yoshida, T., Kokura, K., Makino, Y., Ossipow, V. and Tamura, T. (1999) Heterogeneous nuclear RNA-ribonucleoprotein F binds to DNA via an oligo(dG)-motif and is associated with RNA polymerase II. Genes Cells **4**, 707–719
27 Dobner, T. and Kzhyshkowska, J. (2001) Nuclear export of adenovirus RNA. Curr. Top. Microbiol. Immunol. **259**, 25–54
28 Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K. and Zhou, M. M. (1999) Structure and ligand of a histone acetyltransferase bromodomain. Nature (London) **399**, 491–496
29 Hudson, B. P., Martinez-Yamout, M. A., Dyson, H. J. and Wright, P. E. (2000) Solution structure and acetyl-lysine binding activity of the GCN5 bromodomain. J. Mol. Biol. **304**, 355–370
30 Owen, D. J., Ornaghi, P., Yang, J. C., Lowe, N., Evans, P. R., Ballario, P., Neuhaus, D., Filetici, P. and Travers, A. A. (2000) The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. EMBO J. **19**, 6141–6149
31 Dyson, M. H., Rose, S. and Mahadevan, L. C. (2001) Acetyllysine-binding and function of bromodomain-containing proteins in chromatin. Front. Biosci. **6**, D853–D865
32 Staal, A., Enserink, J. M., Stein, J. L., Stein, G. S. and van Wijnen, A. J. (2000) Molecular characterization of celtix-1, a bromodomain protein interacting with the transcription factor interferon regulatory factor 2. J. Cell. Physiol. **185**, 269–279
33 Gallimore, P. H. and Turnell, A. S. (2001) Adenovirus E1A: remodelling the host cell, a life or death experience. Oncogene **20**, 7824–7835
34 Shenk, T. (1996) Adenoriridae and their replication In Virology (Fields, B. N., Knipe, D. M. and Howley, P. M., eds.), pp. 2111–2148, Lippincott-Raven, New York, NY
35 Yew, P. R. and Berk, A. J. (1992) Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature (London) **357**, 82–85
36 Martin, M. E. and Berk, A. J. (1998) Adenovirus E1B 55K represses p53 activation *in vitro*. J. Virol. **72**, 3146–3154
37 Yew, P. R., Liu, X. and Berk, A. J. (1994) Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev. **8**, 190–202
38 Martin, M. E. and Berk, A. J. (1999) Corepressor required for adenovirus E1B 55,000-molecular-weight protein repression of basal transcription. Mol. Cell. Biol. **19**, 3403–3414

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