Requirement of a corepressor for Dr1-mediated repression of transcription

Fred Mermelstein,^{1,5} Kam Yeung,^{1,5} Jun Cao,¹ Juan A. Inostroza,¹ Hediye Erdjument-Bromage,² Kathie Eagelson,³ David Landsman,⁴ Pat Levitt,³ Paul Tempst,² and Danny Reinberg^{1,6}

¹Howard Hughes Medical Institute, Department of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854-5635 USA; ²Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 USA; ³Department of Neurobiology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854-5653 USA; ⁴National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Maryland 20894 USA

A Dr1-associated polypeptide (DRAP1) was isolated from HeLa cells and found to function as a corepressor of transcription. Corepressor function requires an interaction between DRAP1 and Dr1. Heterodimer formation was dependent on a histone fold motif present at the amino terminus of both polypeptides. Association of DRAP1 with Dr1 results in higher stability of the Dr1-TBP-TATA motif complex and precluded the entry of TFIIA and/or TFIIB to preinitiation complexes. DRAP1 was found to be expressed in all tissues analyzed with higher levels in tissues with a low mitotic index. Analysis of DRAP1 in the developing brain of rat demonstrated undetectable levels of DRAP1 in actively dividing cells but high levels of DRAP1 expression in differentiated non dividing cells. Dr1 was immunodetected in all cells analyzed. A model for DRAP1-dependent, Dr1-mediated repression of transcription is proposed.

[**Key Words:** Dr1-mediated repression; corepression of transcription; HeLa cells; heterodimer formation]

Received January 3, 1996; revised version accepted March 7, 1996.

Studies aimed at understanding eukaryotic transcription have focused largely on identifying and characterizing RNA polymerase II and the general transcription machinery. To date, transcription of protein coding genes requires the concerted actions of six general transcription factors (GTFs) and RNA polymerase II (RNAPII) to accurately initiate RNA synthesis (Zawel and Reinberg 1993; Maldonado and Reinberg 1995). Of the six GTFs, TFIID is the only one known to exhibit sequence-specific DNA-binding activity with an affinity for the TATA element (Topol and Parker 1984; Nakajima et al. 1988; Buratowski et al. 1989). TFIID exists as a large, multisubunit complex (Pugh and Tjian 1991; Gill and Tjian 1992), yet its DNA-binding activity is intrinsic to only one polypeptide of 38 kD, namely the TATA box-binding protein (TBP) (Hoffmann et al. 1990; Kao et al. 1990; Peterson et al. 1990). The other components of the TFIID complex are referred to as TAFs for TBP-associated factors (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1992). The binding of TFIID to the promoter DNA is facilitated by TFIIA (Buratowski et al. 1989; Maldonado et al. 1990), which is thought to play an important role in the activation process (Ma et al. 1993; Liberman and Berk 1994; Ozer et al. 1994; Sun et al.

1994; Yokomori et al. 1994) as well as in antirepression by removing inhibitory factors associated with TFIID (Meisterernst and Roeder 1991; Meisterernst et al. 1991; Cortes et al. 1992; Ma et al. 1993). Once bound to the TATA motif, TFIID serves as a beacon for the entry of TFIIB (Buratowski et al. 1989; Maldonado et al. 1990). The resulting TFIID-TFIIB (DB) complex permits TFIIF to escort RNAPII to the promoter, resulting in the formation of DBPolF complex intermediate (Flores et al. 1990). TFIIE, through interactions with the various GTFs (Maxon et al. 1994), is loaded on the promoter and recruits TFIIH (Flores et al. 1992). The complete DBPolFEH complex is then competent to initiate synthesis of RNA upon addition of ribonucleoside triphosphates (for review, see Zawel and Reinberg 1995). This highly ordered process may be facilitated by regulation of the various steps leading up to the formation of a transcription-competent complex. Recent studies have suggested an alternative pathway for preinitiation complex formation involving the recruitment of a preassembled RNA polymerase II holoenzyme to the promoter (Koleske and Young 1995; Ossipow et al. 1995).

The assembly of the GTF and RNAPII on class II promoters has allowed the identification of regulatory proteins that directly interact with the GTFs (for review, see Zawel and Reinberg 1995). These include a multitude of factors known as activators, coactivators, repressors, and most recently, corepressors of transcription (Herschbach

⁵The two first authors contributed equally to this work.

⁶Corresponding author.

and Johnson 1993; Johnson 1995). It has become apparent that transcriptional repression serves as a general mechanism to regulate the expression of a variety of genes in organisms ranging from yeast to humans. Repressors have been shown to be functionally important in mediating gene expression through a variety of mechanisms (for review, see Johnson 1995; Roberts and Green 1995). Repressors have been identified that function by virtue of their sequence-specific DNA-binding properties such as the *Drosophila* even-skipped protein (Johnson and Krasnow 1992). A second class of repressors function independently of a direct interaction with DNA, but through protein-protein interactions such as the yeast Cyc8 (Ssn6) protein and Tup1, which are required for repression of genes regulated by glucose (Schultz and Carlson 1987; Trumbly 1992), oxygen (Zitomer and Lowry 1992), cell type (Mukai et al. 1991; Keleher et al. 1992), and DNA damage (Elledge et al. 1993). A second example includes *Drosophila* Groucho that upon interaction with the bHLH protein, Hairy, plays an important role during neurogenesis, segmentation, and sex determination (Paroush et al. 1994). Also, several of these regulatory proteins function both as activators and repressors. Examples include the *Drosophila* Krüppel protein (Sauer and Jackle 1993; Sauer et al. 1995), p53 (Seto et al. 1992), the Wilm's tumor gene WT1 (Maheswaran et al. 1993), the human thyroid hormone receptor, h-TR β (Baniahmad et al. 1993), and the Rel family of proteins (Lehming et al. 1994).

We have previously identified one regulator known as Dr1 that can globally repress transcription of RNA polymerase II (Inostroza et al. 1992), and RNA polymerase III-transcribed genes (White et al. 1994). The importance of Dr1 function was revealed by virtue of it being an essential gene in yeast (S. Kim, J. Na, M. Hampsey, and D. Reinberg, unpubl.). As might be expected of a general repressor of transcription, overexpression of Dr1 resulted in lethality in yeast (S. Kim, J. Na, M. Hampsey, and D. Reinberg, unpubl.). It has been shown that the ability of Dr1 to repress RNA polymerase II transcription depends on its ability to interact with TBP and preclude the subsequent entry of TFIIA and/or TFIIB into the preinitiation complex (Inostroza et al. 1992; Kim et al. 1995). This prevents the formation of an active complex, and initiation by RNAPII (Inostroza et al. 1992). Similarly, RNAPIII transcription is inhibited by Dr1 through its interaction with TBP. The RNAPIII B-related factor (BRF), which contains homology to TFIIB, is blocked from interacting with TBP by Dr1 (White et al. 1994). Interestingly, the interaction of Dr1 with TBP can be disrupted to allow active transcription. This phenomenon was shown to be mediated by the adenovirus EIA₁₂₅ product (Kraus et al. 1994). Finally, the ability of an activator to overcome Dr1-mediated repression can vary depending on the class of activator. VP16-activated transcription can completely overcome Dr1 transcriptional repression. The glutamine-rich transcriptional activator SP1 can only mildly restore Dr1-blocked transcription, whereas the proline-rich activator CTF was completely ineffective (Yeung et al. 1994).

In this paper we describe an approach taken that uncovered a unique mode of action for regulating Dr1 activity. Conventional chromatography coupled with immunoaffinity purification, using Dr1 antibodies, resulted in the purification of a repressor complex from HeLa cell extracts. We have characterized a component of this complex, termed Dr1-associated protein 1 (DRAP1). Moreover, we have isolated the cDNA representing this protein and have shown that both recombinant and native DRAP1 interact with Dr1. This interaction results in a specific increase in transcriptional repression from the adenovirus major late promoter (AdMLP). DRAP1 represents a novel corepressor of transcription.

Results

Identification of DRAP1

We have shown previously that a number of phosphorylated polypeptides coimmunoprecipitate with Dr1 from HeLa cells using antibodies directed against Dr1 (Inostroza et al. 1992). One predominant phosphorylated polypeptide migrated at ~28 kD as determined by SDS-PAGE (Fig. 1A, lane 3). Coimmunoprecipitation of p28 by Dr1

Figure 1. Coimmunoprecipitation and immunoaffinity purification of DRAPs from HeLa cell nuclear extracts. (A) Coimmunoprecipitation of Dr1 with DRAP1 from HeLa cell extracts labeled with [³²P]orthophosphate in vivo. (Lane 1) Immunoprecipitation of Dr1 from boiled extracts with protein A-agarose and Dr1 antibodies. (Lane 2) Immunoprecipitation of Dr1 with protein A-agarose and Dr1 antibodies from untreated extracts in the presence of 0.1% SDS, and (Lane 3) in extraction buffer devoid of 0.1% SDS. Arrows depict Dr1 and DRAP1. Molecular weight markers are shown at left. (B) Purification of DRAP complexes from HeLa nuclear extracts. (Lane 1) Silver stain of recombinant Dr1 (50 ng); (lane 2) immunoaffinity-purified DRAPs derived from the 0.5 M phosphocellulose fraction; (lane 3) immunoaffinity-purified complexes derived from the 1.0 M phosphocellulose step of chromatography. Arrows at right identify DRAP1 (top), ribosomal protein S9 (middle), and Dr1 (bottom). Markers are delineated at left.

antibodies was dramatically reduced in the presence of SDS or when extracts were boiled (Fig. 1; lanes 1 and 2, respectively). These results indicate that p28 is immunoprecipitated by Dr1 antibodies due to an interaction between these two polypeptides (Dr1 and p28) and not because the anti-Dr1 antibodies recognize epitopes in p28.

To isolate Dr1-associated polypeptides (DRAPs), protein fractions enriched in Dr1 were purified from HeLa cells by conventional chromatography and DRAPs were isolated on columns containing Dr1 antibodies. Dr1-immunoreactive material derived from both the 0.5 M and 1.0 M phosphocellulose steps of chromatography were bound to the Dr1 antibody column and eluted with 100 mM glycine (pH 2.6). Glycine eluates were analyzed by SDS-PAGE followed by silver stain analysis, which revealed the presence of at least two different complexes. In the phosphocellulose 0.5 M-derived fraction, two predominant polypeptides were observed on SDS-PAGE; one migrating at 18 kD identified as Dr1 by microsequencing, and a second unknown protein referred to as p28 migrating at ~28 kD (Fig. 1B, lane 2). From the phosphocellulose 1.0 M-derived fraction, several polypeptides were detected including a 30-kD protein, a doublet of ~23 kD, and a triplet of ~20 kD. Microsequencing revealed that the 23-kD polypeptides correspond to ribosomal protein S9, and the 20-kD polypeptides correspond to different forms of Dr1 (Fig. 1). The band migrating at ~30 kD was found to be immunologically related to the 28-kD polypeptide present in the 0.5 M-derived fraction (data not shown, see below). We termed the 28-kD polypeptide DRAP1.

Molecular cloning of DRAP1

Immunopurified DRAP1 was digested with trypsin, and the resulting peptides were isolated by reverse phase chromatography. Four peptides were sequenced (Fig. 2A, a–d), and degenerative oligonucleotides based on the amino acid sequence from peptide b were synthesized (see Materials and methods). Both HeLa cell and liver cell cDNA libraries were used to isolate cDNA clones for p28. The libraries were screened with the least degenerate oligonucleotide a. Two types of cDNA clones were isolated. The nucleotide sequence of the shorter cDNA clone derived from the HeLa cell library predicts an open reading frame encoding a polypeptide of 193 amino acids with a calculated M_r of 21.2. The second cDNA clone isolated from the liver cell library contains an insertion of 7 amino acids at residue 98 (Fig. 2A). This insertion appears to be attributable to alternative splicing, as analysis of the nucleotide sequence of a genomic clone demonstrates the presence of splicing acceptor and donor sites flanking the 7 amino acids (Fig. 2B). The isolated cDNA clones contained all of the DRAP1-derived peptides and each of the two clones encoded polypeptides interacting with Dr1 (see below). Analysis of the amino acid sequence of DRAP1 revealed extensive homology at the amino terminus with the yeast HAP5 protein (Fig. 2D). DRAP1 was isolated as a Dr1-binding protein, and

our previous analyses demonstrated that the amino terminus of Dr1 contained homology to HAP3 (Inostroza et al. 1992). Both HAP3 and HAP5 polypeptides are known to form a complex (McNabb et al. 1995). Our functional studies demonstrate that the HAP homology region present in Dr1 and DRAP1 are important for the interaction of these polypeptides (see below). Moreover, included within the HAP homology region are residues that form a "histone fold" (Fig. 2C) (Baxeavanis et al. 1995). The carboxyl terminus of DRAP1 was found to be rich in proline residues (Fig. 2A,D).

DRAP1 interacts directly with Dr1

To analyze whether the isolated cDNA encoded DRAP1, the open reading frame was expressed in bacteria and the recombinant protein was isolated and used to produce antibodies. DRAP1 purified from HeLa cells (see Materials and methods) migrated as a 28-kD doublet on SDS-PAGE (Fig. 3A, lane 3). The full-length recombinant polypeptide, which contained a histidine tag, migrated between the doublet (lane 4). In addition, a series of breakdown products, or premature terminated polypeptides, were also observed with the bacterially expressed protein (lane 4). Western blot analysis demonstrated that antibodies raised against the recombinant polypeptide recognized human DRAP1 (Fig. 3A, lane 1). The immunodetection was highly specific as the antibodies detected only the 28-kD polypeptide in HeLa cell nuclear extracts (data not shown).

To further scrutinize the identity of the recombinant polypeptide, HeLa cell extracts prepared from metabolically ^{32}P -radiolabeled cells were immunoprecipitated with anti-Dr1 (Fig. 3B, lane 1) or anti-p28 (Fig. 3B, lane 5) antibodies. Immunoprecipitated complexes were boiled, and a second immunoprecipitation was performed. As above (Fig. 1A), anti-Dr1 antibodies immunoprecipitated two predominant phospholabeled polypeptides, Dr1 and DRAP1 (Fig. 3B, lane 1). Similar phospholabeled proteins were immunoprecipitated by antibodies directed against the recombinant DRAP1 protein (Fig. 3B, lane 5). When boiled, immunopurified Dr1 complexes were incubated with DRAP1 antibodies, DRAP1 but not Dr1 was immunoprecipitated (Fig. 3B, lanes 2,3). Similarly, when boiled immunopurified DRAP1 complexes were incubated with Dr1 antibodies, Dr1 but not DRAP1 was immunoprecipitated (Fig. 3B, lanes 6,7). In both cases, antibodies purified from preimmune serum failed to react with either Dr1 or DRAP1 in secondary immunoprecipitations (Fig. 3B, lanes 4,8). The results demonstrate that the isolated cDNA clones encode DRAP1.

We have demonstrated previously that Dr1 isolated from HeLa cells interacts with TBP to repress transcription and that this interaction also occurs when TBP is bound to the TATA motif, as different Dr1-dependent DNA-protein complexes could be isolated using the gel mobility shift assay (Inostroza et al. 1992). Recombinant Dr1 (rDr1) could also interact with TBP. However, in the presence of DNA, rDr1 and TBP cannot form a ternary complex that could be resolved using the gel mobility

Figure 2. Structural features of human DRAP1. (A) Nucleotide sequence of a cDNA encoding DRAP1. The sequence is listed using the single-letter amino acid code. Amino acids underlined represent peptide sequence derived from microsequencing of human DRAP1 protein purified from HeLa nuclear extract. (Δ) The site of a 7-amino acid insert found in the cDNA isolated from a human liver cDNA library. (B) Partial sequence of a genomic clone of DRAP1 isolated from the HeLa genomic library. The sequence around the point of insertion is shown. Exon sequences are underlined (broken line), and the donor and two downstream acceptor sites of the intron are in *bold* face type. The additional 7 amino acids that are present in the human liver cDNA are underlined. (C) Multiple sequence alignment of several proteins containing the histone fold aligned to all four core histones. The sequences were detected with the MOST software using an alignment box of a nonredundant data set containing 268 histone sequences. The boxed region corresponds to the histone fold, and at each position, residues in agreement with any of the core histones are shown in inverse type. The *r*-values for each of the nonhistone sequences are shown. The accession numbers obtained from the histone sequence database at National Center for Biotechnology Information (NCBI) (Baxevanis 1995) for human H2A.1, human H2B, human H3.1, human H4, rat CBF-C, and yeast Hap5P are sp|P02261, sp|P33778, sp|P16106, sp|P02304, sp|U17607, and sp|Q01658, respectively. (D) Schematic representation depicting the structural features of DRAP1. Shown is the nucleotide sequence of a cDNA encoding DRAP1 derived from clones isolated from a HeLa cell cDNA library. The nucleotide sequence of DRAP1 predicts a long open reading frame encoding a polypeptide of 193 amino acids with a calculated molecular mass of 21.3 kD. The amino acid sequence was analyzed using the Wisconsin Sequence Analysis Package, Genetics Computer Group (University Research Park, Madison, WI). Analysis of the amino acid sequence of DRAP1 reveals two interesting features: (1) The shaded area at *left* shows a region homologous to HAP5/CBF-C containing a histone fold motif (see below); and (2) the shaded area at *right* indicates a proline-rich region.

shift assay, as with native Dr1. Because of the differences observed in the recombinant and native Dr1 protein, we analyzed the effect of rDRAP1 on the TBP-rDr1 complex using a DNA fragment containing the AdMLP in a gel

mobility shift assay. Under the conditions used, TBP, rDRAP1, or rDr1 failed to form a DNA-protein complex independently (Fig. 3C). Moreover, in agreement with our previous observations, we were unable to demon-

Figure 3. DRAP1 interacts with Dr1 both in vivo and in vitro. (A) Western blot and silver stain analyses of human DRAP1 and recombinant histidine-tagged DRAP1. Immunoaffinity-purified DRAP1 (lane 1) and recombinant DRAP1 (lane 2) were resolved by 12.5% SDS-PAGE, and proteins were either transferred to nitrocellulose membrane or analyzed by silver stain. Blots were developed using affinity-purified DRAP1 antibodies and developed using anti-rabbit antibodies conjugated to alkaline phosphatase with Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). (Lane 3) Silver stain analyses of immunoaffinity-purified DRAP1 and (lane 4) recombinant DRAP1. Molecular weight markers are indicated (M) at left. (B) Coimmunoprecipitation of Dr1 with DRAP1 from HeLa extracts using antibodies raised against recombinant DRAP1 and Dr1. Dr1 coimmunoprecipitates with DRAP1 from HeLa cells labeled with [32 P]orthophosphate in vivo. (Lanes 1–4) Immunoprecipitation of Dr1 and DRAP1 from whole cell extracts with protein A-agarose and Dr1 antibodies. The immunoprecipitated complexes were either untreated (lane 1) or boiled to dissociate the complexes. The boiled eluates were subsequently immunoprecipitated with protein A-agarose and DRAP1 antibodies (lane 2, 1 µg; lane 3, 5 µg) or with preimmune serum (lane 4, 5 µg). (Lanes 5–8) Immunoprecipitation of Dr1 and DRAP1 from whole cell extracts with protein A-agarose and DRAP1 antibodies. The immunoprecipitated complexes were either untreated (lane 5) or boiled to dissociate the complexes. The boiled eluates were subsequently immunoprecipitated with protein A-agarose and Dr1 antibodies (1 µg, 5 µg) (lane 6, 1 µg; lane 7, 5 µg) or with preimmune serum (lane 8, 5 µg). Arrows depict Dr1 and DRAP1. Molecular weight markers are shown at left. (C) DRAP1 is contained in TBP-Dr1 complexes and required for higher order complex formation. DNA-protein complexes were formed on the AdMLP and analyzed using the gel mobility shift assay. Binding reactions were performed using a 3'-end-labeled DNA fragment containing AdMLP (–40 to +15). hTBP-Dr1-DRAP1 complexes were formed using human histidine-tagged TBP (7.5 ng), rDr1 (25 ng), histidine-tagged DRAP1 (0.25 or 2.5 ng), or GST-DRAP1 (0.5 or 5 ng). Different complexes formed are indicated by arrows at left. Asterisks denote slower migrating complexes formed in the presence of the GST-DRAP1 fusion protein.

strate a rDr1-TBP-TATA complex. However, the addition of increasing concentrations of rDRAP1 to reactions containing rDr1 and TBP resulted in the formation of DNA-protein complexes that comigrated with complexes formed with native Dr1 (containing DRAP1) (Fig. 3C). As demonstrated previously, all of these complexes contained TBP and Dr1, as specific antibodies to TBP or Dr1 supershifted the complexes (data not shown; see Inostroza et al. 1992). Moreover, these complexes also contained DRAP1, as demonstrated by supershift experiments using anti-DRAP1 antibodies (data not shown) or a GST-DRAP1 fusion protein. The larger GST-DRAP1 fusion protein retarded the mobility of the different DNA protein complexes (Fig. 3C, cf. lanes 4 and 5 with 7 and 8). Moreover, recovery of the proteins from each of the complexes, followed by Western blot analysis, demonstrated the presence of TBP, Dr1, and DRAP1 in each complex (data not shown). Thus, DRAP1, together with

TBP and Dr1, are integral components of the different DNA-protein complexes. DRAP1 appears to stabilize the TBP-Dr1-DNA complex.

DRAP1 is a corepressor required for Dr1-mediated repression of transcription

To determine whether DRAP1 could have an effect on transcription or on Dr1-mediated repression, recombinant DRAP1 was added to transcription reactions in a system reconstituted with GTFs, RNAPII, and the AdMLP in the presence and absence of recombinant Dr1. DRAP1-enhanced, Dr1-mediated repression can be demonstrated at a concentration of Dr1 that reduces transcription from AdMLP by 10% (Fig. 4, lanes 3–5). At the highest concentration of DRAP1, ~90% of the transcription activity from AdMLP was repressed. DRAP1 in the

Figure 4. DRAP1 specifically enhances Dr1-mediated repression. (A) DRAP1 enhancement of Dr1-mediated repression in the presence of TBP. (Top) Bar graph representing transcription activity of transcription reaction mixtures containing AdMLP reconstituted using purified or recombinant general transcription factors, RNA PII is shown with increasing amounts of DRAP1 as indicated ($1 \times = 5$ ng), in the presence of rDr1 (375 ng, lanes 2–5) or absence of rDr1 (lanes 1, 6, 7, 8). (Lanes 10–12) Effect of the CMV IE2 repressor ($1 \times = 50$ ng) in transcription reactions in the presence of different amounts of DRAP1 ($1 \times = 5$ ng, lanes 13–15) or absence of DRAP1. (Lanes 1, 9) Basal transcription reactions in the absence of any repressor. Transcription activity was quantified on a PhosphorImager (Bio-Rad). (Bottom) Autoradiograph of RNA transcripts from transcription reactions quantified in the bar graph above. (B) DRAP1 enhancement of Dr1-mediated repression in the presence of TFIID. (Top) Bar graph representing transcription activity of transcription reaction mixtures containing AdMLP reconstituted using purified RNA PII, TFIID, recombinant general transcription factors TFIIB, TFIIE, and TFIIH, as described above in A, but with eTFIID (2.5 ng) in place of TBP, with rDr1 (375 ng, lanes 2–5), or without rDr1 (lanes 1, 6, 7, 8) in the presence of the different amounts of rDRAP1 ($1 \times = 5$ ng, lanes 3–8). Transcription activity was quantified on a PhosphorImager (Bio-Rad). (Bottom) Autoradiograph of RNA transcripts from transcription reactions represented in bar graph above.

absence of Dr1 had no appreciable effect on transcription (Fig. 4; data not shown; see below). DRAP1 enhancement of Dr1-mediated repression was also observed in the presence of TAFs (i.e., when TFIID replaced TBP) (Fig. 4B).

To determine the specificity of DRAP1 function, we studied its effect on the cytomegalovirus (CMV) immediate early (IE)2 repressor protein. It has been demonstrated that CMV-IE2 can repress class II promoters via a cis-acting repressor element (CRE) positioned proximal to the transcription start site (Liu et al. 1991). CMV-IE2-mediated repression has been shown in vitro using purified RNAPII, GTFs, and the Ad-MLP containing a CRE (Lee et al. 1996). Using this template, recombinant IE2 repressed transcription in a dose-dependent manner (Fig. 4A, lanes 9–12) and required the presence of a CRE site (data not shown). The repression activity of IE2 was not affected by the presence of DRAP1 (Fig. 4A, lanes 13–15) even though the same amount of DRAP1 could drasti-

cally enhance Dr1-mediated repression (Fig. 4A, lanes 2–5).

Interaction of DRAP1 with Dr1 is required for the enhancement of Dr1 repression

The primary sequence of DRAP1 revealed a region of homology to HAP5/CBF-C (CAAT-binding factor-C), and within this region a histone fold motif was identified (Fig. 2). Interestingly, the primary sequence of Dr1 revealed a region of homology to HAP3/CBF-A (Inostroza et al. 1992), and also within this region a histone fold motif is found (Fig. 2, Baxevanis et al. 1995). Because the histone fold motif is thought to be a determinant for protein-protein interaction (Baxevanis et al. 1995), and because it is known that HAP5/CBF-C interacts with HAP3/CBF-A, we analyzed the Dr1-DRAP1 interaction using recombinant proteins purified from *Escherichia coli*. The interaction of rDRAP1 with rDr1 was dose-

dependent as measured by surface plasmon resonance (Fig. 5B). After establishing the interaction of DRAP1 with Dr1, we subsequently analyzed the domains contained within DRAP1 required for this interaction. When the first amino-terminal 23 amino acids were removed from DRAP1 (N23), binding was mildly reduced (Fig. 5C). Further deletion of the amino-terminal 80 amino acids of DRAP1 (N80) completely abolished binding (Fig. 5C). Similarly, upon truncation of 92 amino acids from the carboxyl terminus of DRAP1 (C101), which contains the proline-rich region, binding was not observed (Fig. 5C).

To correlate whether the binding of DRAP1 to Dr1 was required for the enhancement of Dr1-mediated repression, the effects of different truncated mutants were assayed in the reconstituted transcription system. The result of one representative assay is shown in Figure 5 (D,E). Deletion of amino acids 144–193 (C144) had no effect on the ability of DRAP1 to enhance Dr1-mediated repression of transcription (Fig. 5D, lanes 4–6). However, removal of amino acids that are required for the interaction with Dr1 (mutant N80 or C101), completely abolished the activity of DRAP1 (Fig. 5, D, lanes 7–9, and E, lanes 7–9). Removal of the first 23 amino acids of DRAP1 (mutant N23) completely abolished its activity (Fig. 5E, lanes 4–6). However, this mutant still maintained its ability, although to a reduced level, to interact with Dr1.

The histone fold/HAP3 motif of Dr1 is required for the interaction with DRAP1

The amino terminus of Dr1 contains a histone fold/HAP3 motif; therefore, it was of interest to determine whether this region is important for interaction with DRAP1. The interaction was analyzed using Dr1 glutathione S-transferase (GST) fusion proteins (GST, GST–Dr1, and GST–Dr1Δ1–70) in a pull-down assay. The proteins were purified as described, and equal amounts of each fusion protein were immobilized on glutathione-agarose columns. The ability of different columns to retain reticulocyte lysate-transcribed/translated ³⁵S-labeled hTBP or DRAP1 was monitored by fluorography. As shown in Figure 6B, a GST–Dr1, but not a GST column, retained >20% of the DRAP1 input. In contrast, removal of the first 70 amino acids of Dr1 (Dr1Δ1–70), which includes histone fold/HAP3, completely abrogated the binding of DRAP1 to Dr1 (Fig. 6B, lane 8). The inability of the GST–Dr1Δ1–70 column to retain any DRAP1 was not attributable to an inactive protein preparation, as a considerable amount of hTBP was retained (Fig. 6B, lane 5). The TBP-binding domain is located between residues 80–100 in Dr1 (Yeung et al. 1994).

To further determine whether there is a direct correlation between binding of Dr1 to DRAP1 and repression enhancement, we studied the effect of DRAP1 on Dr1Δ1–70-mediated repression of transcription in vitro. Consistent with our previous observations, we found that rDr1 could repress transcription, although a higher concentration of recombinant polypeptide was found to

be required (Inostroza et al. 1992, Yeung et al. 1994). Under these conditions we found that the amino terminus of Dr1 is dispensable for Dr1-mediated repression of transcription (Fig. 6C, lanes 2–4, also, see Yeung et al. 1994). However, unlike its wild-type counterpart, the repression activity of Dr1Δ1–70 remained unaffected in the presence of DRAP1 (Fig. 6C, lanes 5–7). Thus, these results demonstrate further that DRAP1-mediated enhancement of repression of transcription requires direct interaction with Dr1.

The association between Dr1 and DRAP1 is required for efficient disruption of the TBP–TFIIA–TATA complex

Our previous studies have indicated that Dr1 prevented the association of TBP with TFIIA and/or TFIIB (Inostroza et al. 1992). We analyzed the effect of DRAP1 in Dr1-mediated disruption of the TBP–TFIIA (TA) complex using the gel mobility shift assay. The TA (TBP–TFIIA) complex was formed on the AdMLP under conditions where ~95% of the DNA fragment was in the complex and different combinations of Dr1/DRAP1 were added (Fig. 7A). Although the addition of DRAP1 (lane 13) or Dr1 (lane 2) to the TA complex was without an appreciable effect, the addition of both factors, DRAP1 and Dr1, resulted in displacement of the TA complex with the appearance of the characteristic TBP–Dr1–DRAP1 complexes (Fig. 7A). Effective displacement of the TA complex required both Dr1 and DRAP1 in approximately equimolar amounts (lane 8). Decreasing the amount of Dr1 (from lanes 9 to 13), or of DRAP1 (from lanes 7 to 2), resulted in the reappearance of the TA complex. Identical results were observed when the TB (TBP–TFIIB), or TAB (TBP–TFIIA–TFIIB) complexes were analyzed (data not shown).

To analyze whether a correlation between repression of transcription and displacement of TFIIA (or TFIIB) from the TA (or TB or TAB) complex exists, different DRAP1 mutants were analyzed. DRAP1 mutants that were defective in enhancing Dr1-mediated repression of transcription (N23, N80, and C101) were also defective in their ability to displace TFIIA from the TA complex (Fig. 7B). Thus, these results demonstrate that displacement of TFIIA (or TFIIB) from the TA or TAB complex correlated with the formation of Dr1–DRAP1–TBP–DNA complexes and with repression of transcription.

Interestingly, the association of Dr1 and DRAP1 with the TBP–DNA complex did not result in the formation of a single discrete complex but in a series of complexes (Figs. 3C and 7). In light of the histone fold motif present at the amino terminus of Dr1 and DRAP1, we were interested in further analyzing this phenomenon. We observed that the appearance of the different complexes correlates with the concentration of Dr1 and DRAP1 (Fig. 7C). At limiting concentrations of Dr1 (but saturating amounts of DRAP1), a faster-migrating single complex was observed (complex 1). Increasing the concentration of Dr1 resulted in the formation of the slower-mi-

Figure 5. The histone fold region of DRAP1 is required for interaction with Dr1 and enhancement of Dr1-mediated repression. (A) Schematic representation of DRAP1 showing structural features including the HAP5 homology domain (shaded bar), proline-rich region (solid bar), and the histone fold. Various truncations of both carboxy- and amino-terminal amino acids are shown below. Ability to bind Dr1 is indicated at right. (B) Sensorgram representing a dose-dependent interaction of rDRAP1 with rDr1 as detected by surface plasmon resonance. rDRAP1 (1.6, 3.3 ng, and 5.0 ng) binds to surface-immobilized rDr1 (0.6 μ g) (see Materials and methods). The net change in the baseline from before to after sample injection, in resonance units (RUs), indicates binding of proteins to the surface immobilized rDr1. (X) Resonance signals measured before and after injections. (S) Time at which 0.05% Sarkosyl washes were performed. (C) Sensorgram representing the interaction of DRAP1 mutants (N80, N23, and C101) with rDr1 by surface plasmon resonance. rDRAP1 (10 ng) is used as a positive control. Plasmon resonance experiments were performed as described in B. (D) The carboxy-terminal region of DRAP1 is dispensable for the corepression activity of DRAP1. (Top) Graphic representation of transcription activity of transcription reaction mixtures containing AdMLP reconstituted using purified or recombinant general transcription factors, RNA PII, and rDr1 (375 ng), rDRAP1 (20 ng), or different amounts of rDRAP1 mutants C144 and C101 (5, 10, and 20 ng), as indicated. Transcription activity was quantified on a PhosphorImager (Bio-Rad). (Bottom) Autoradiograph of RNA transcripts from transcription reactions represented in bar graph. (E) The histone fold/HAP5 region of DRAP1 is required for the corepression activity of DRAP1. (Top) Graphic representation of transcription activity of transcription reaction mixtures containing AdMLP reconstituted using purified or recombinant general transcription factors, RNA Polymerase II, and rDr1 (375 ng) rDRAP1 (20 ng), or different amounts (5, 10, and 20 ng) of rDRAP1 mutants N23 and N80, as indicated (1 $\times$, 2 $\times$, or 4 $\times$, where 1 $\times$ =5 ng). Transcription activity was quantified on a PhosphorImager (Bio-Rad). (Bottom) Autoradiograph of RNA transcripts from transcription reactions represented in bar graph.

grating complexes, up to four different ones (Figs. 3C and 7C). The formation of the slower-migrating complexes was also dependent on the concentration of DRAP1, as reducing the concentration of DRAP1 also reduced the amount of slower-migrating complexes. Formation of the different complexes appears to be an ordered phenomenon, where complex 1 is formed first and used to produce complex 2 and so on (Fig. 7C). The polypeptide composition in each complex was analyzed by extracting the proteins from the different complexes. This analysis

indicated that all the complexes contained Dr1, DRAP1, and TBP (data not shown). We next analyzed the DNase I footprinting pattern obtained at two different concentrations of Dr1-DRAP1 using equimolar amounts of Dr1 and DRAP1. Nucleotides extending from approximately -39 to -15 were protected from DNase I cleavage by the binding of TBP to the AdMLP-TATA motif (Fig. 7D, lane 2). Interestingly, the addition of Dr1-DRAP1 complex to the TBP-TATA complex resulted in a reduction of the area covered by TBP on the TATA motif as well as

Figure 6. The Histone Fold region of Dr1 is required for interaction with DRAP1. (A) Schematic representation of Dr1 showing regions integral for its interaction with DRAP1 (1–80), binding of TBP (80–100), as well as a region mediating Dr1 repression activity (130–150, glutamine-alanine rich region). The schematic also indicates the location of the histone fold (14–78). α -Helical regions of Dr1 are shown in the shaded areas. Numbers indicate amino acid positions in the Dr1 protein. (B) The histone fold region of Dr1 is required for interaction with DRAP1 in a GST pull-down assay. [35 S]Methionine-labeled human TBP and [35 S]methionine-labeled DRAP1 were produced by in vitro transcription/translation, and equal amounts (inputs), were incubated with either GST (1 μ g, lanes 3,6), GST–Dr1 (1 μ g, lanes 4,7), or GST–Dr1 Δ 1–70 (1 μ g, lanes 5,8) linked to glutathione-agarose beads for 1 hr, followed by extensive washing. The bound proteins were eluted in SDS-PAGE buffer, resolved by 12% SDS-PAGE, and visualized by fluorography. (C) The enhancement of Dr1 repression by DRAP1 is directed through the histone fold region of Dr1. The bar graph represents transcription activity of transcription reaction mixtures containing AdMLP reconstituted using purified or recombinant general transcription factors, RNA PII, and different amounts of DRAP1 (5, 10, and 20 ng) or Dr1 Δ 1–70 (100, 200, or 400 ng) as indicated (1 $\times$, 2 $\times$, or 4 $\times$). Transcription activity was quantified on a PhosphorImager (Bio-Rad). (D) Autoradiograph of RNA transcripts from transcription reactions represented in the bar graph in C.

an extension of protection downstream of the TATA box to approximately -5 . The upstream footprint was reduced to approximately -33 (Fig. 7C, lane 3). In addition, DNase I hypersensitive sites were observed at residues -19 and -20 . Further increase in the amount of Dr1–DRAP1 complex added to the footprinting reaction did not alter the area protected from cleavage around the TATA motif but resulted in the enhancement and production of a series of sites that were hypersensitive to DNase I cleavage (lane 4). These hypersensitive sites mapped to the upstream border of the TBP footprint at residues -32 and -33 as well as to residues downstream of the TATA motif located at -3 , -14 , -15 , -16 , -19 , and -20 . These results strongly suggest that the Dr1–DRAP1 complex not only binds to TBP and precludes the entry of TFIIA and/or TFIIB but also alters the structure of the TBP–TATA motif complex (see discussion).

DRAP1 is specifically immunodetected in differentiated nondividing cells

Northern blot analyses of DRAP1 RNA derived from different human tissues revealed a predominant RNA species of ~ 1.1 kb. This RNA was expressed at different levels in all tissues analyzed, with apparent higher levels of expression in tissues with a low mitotic index (Fig. 7A). Dr1 was also expressed at different levels in all tissues analyzed (Fig. 7A). To explore further whether the Northern blot results were of any significance, we studied DRAP1 and Dr1 in the brain of a developing rat. The brain was chosen because it contains both actively dividing and highly differentiated cells.

At day 15 before birth (E15), the cerebral wall is comprised of several zones. Mitotically active cells are confined to the ventricular and emerging subventricular zones, whereas the subplate and cortical plate are comprised of postmitotic neurons in G_0 . Mitosis does not occur in either of the latter zones (Hicks and D'Amato 1968; Levitt 1994). The intermediate zone contains mostly postmitotic, migratory neurons at this age. The spatial segregation of cell populations in the developing cerebral wall permits the identification of proteins that are present in cells that are mitotically active or have completed their terminal division. It is clear, in alternate sections stained with DRAP1, Dr1, and TBP antibodies, that there is differential immunodetection of the proteins in distinct cell populations (Fig. 8B). The ventricular/subventricular zones are devoid of DRAP1 staining (Fig. 8Ba), although most cells in this proliferative region are immunoreactive for both Dr1 (Fig. 8Bb) and TBP (Fig. 8Bc). In marked contrast to the other zones of the cerebral wall at E15, intense DRAP1 immunoreactivity is evident in postmitotic neurons that settle in the subplate/cortical plate (Fig. 8Ba); these neurons once again exhibit Dr1 immunoreactivity and continue to express TBP. The absence of DRAP1 staining in the intermediate zone suggests that this protein is not expressed immediately after cells complete their terminal division, but rather its appearance is delayed temporally, linking ex-

A

TBP + IIA

rDRAP1 (ng)	-	-	1	2	5	10	20	20	20	20	20	20	20
rDr1 (ng)	-	10	10	10	10	10	20	20	10	5	2	1	-

TA →

1 2 3 4 5 6 7 8 9 10 11 12 13

B

TBP + IIA

rDRAP1 (ng)	wt				N23				N80				C101			
	-	-	3	10	30	3	10	30	3	10	30	3	10	30		

TA →

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

C

TBP

rDRAP1 (ng)	-	-	1	2	5	10	20	20	20	20	20	20	20	20
rDr1 (ng)	-	10	10	10	10	10	20	10	5	2	1	0.5	-	-

TA →

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

D

T-D1-rDRAP1

T-D1-rDRAP1

the HeLa cell library-derived cDNA. Also, two different Dr1-containing complexes were isolated by immunoaffinity chromatography derived from the 0.5 M and 1.0 M phosphocellulose fractions. Western blot analysis of the immunoaffinity-purified complex from the 1.0 M phosphocellulose column showed that the 30-kD protein was immunologically related to DRAP1 distinguishing the existence of a modified form of DRAP1. Taken together, these results suggest that DRAP1 may represent one protein in a family of related proteins.

The discovery of DRAP1 has allowed a detailed understanding of the mechanism underlying the ability of Dr1 to repress transcription. Despite the growing number of repressors that have been identified, no precise mechanism delineating repressor function at the promoter level has been elucidated. Previous studies have shown that at least two domains in Dr1 are important for repressor function: the TBP-binding domain, and the glutamine/alanine-rich motif (Yeung et al. 1994). In the present studies we have also shown that in addition to these two domains, the amino terminus containing the histone fold/HAP3 motif of Dr1 is required for its maximal activity. Without the amino terminus of Dr1, the DRAP1 interaction is lost. Further examination of the importance of the histone fold/HAP homology region emerged from analysis of DRAP1 mutants. As expected,

Figure 8. Immunodetection of DRAP1 during development in the brain. (A) Northern blot analysis of RNA complementary to Dr1, DRAP1, and β -actin in human tissues. The entire coding sequences of Dr1, DRAP1, and β -actin were labeled by random priming and used in the analysis. As described previously, two discrete RNA species of 1.5 and 3.4 kb hybridized to the Dr1 cDNA (Inostroza et al. 1992). One discrete RNA species of 1.1 kb hybridized to the DRAP1 cDNA. Filters containing similar amounts of poly(A⁺) RNA obtained from different human tissues were obtained from Clontech and used as described in Materials and methods. Symbols representing the various tissues are as follows: (He) heart; (Br) brain; (Pl) placenta; (Lu) lung; (Li) liver; (S.M.) smooth muscle; (Ki) kidney; (Pa) pancreas. (B) Photomicrographs of coronal sections through the cerebral wall at E15 illustrating the distribution of DRAP1 (A), Dr1 (B), and TBP (C) following immunoperoxidase staining (brown). Sections are counterstained with 0.1% cresyl violet (blue). Intense DRAP1 immunoreactivity is observed only in cells situated in the cortical plate/subplate (CP). In contrast, Dr1 and TBP immunostaining is prominent in cells in the ventricular/subventricular zones (VZ) and cortical plate. Neurons migrating through the intermediate zone (IZ) do not express either DRAP1 or Dr1, but some continue to express TBP. Scale bar, 50 μ m.

the histone fold/HAP5-containing amino terminus of DRAP1 is also required for function. Without the histone fold/HAP5 region of DRAP1 (N80), the physical association between Dr1 and DRAP1 is lost. Moreover, the histone fold by itself, C101, does not interact with rDr1 or repress transcription. We have shown that the Dr1-DRAP1 complex can disrupt TA complexes. Unlike wild-type DRAP1, both N23 and C101 in combination

with Dr1 do not dissociate TFIIA from interacting with TBP. These findings demonstrate that the interaction between DRAP1 and Dr1 alone is not sufficient for activity but that the combination of proteins are required to dissociate TFIIA or TFIIB from preinitiation complexes for DRAP1 to enhance Dr1 repression of transcription.

It has become evident that a number of transcriptional repressors from eukaryotes exist in multicomponent complexes. Similar to transcriptional activators, repressor complexes can be dissected into two integral components: a specific DNA-targeting constituent that is functionally inactive, unless in the presence of a second component mediating activity. This is exemplified by the Mad-Max transcriptional repression complex, which possesses a sequence-specific DNA-binding domain but can only repress transcription when associated with the mSin3 protein (Ayer et al. 1995; Schreiber-Agus 1995). Additionally, thyroid hormone receptor and the retinoid X receptor heterodimer, which binds to the thyroid hormone response element (TRE), has been shown to repress transcription in the absence of ligand, but only in the presence of a corepressor molecule known as N-CoR/SMRT (Chen and Evans 1995; Horlein et al. 1995; Kurokawa et al. 1995). In yeast, the Cyc8 (Ssn6) and Tup1 repressor complex has been shown to be recruited to promoters via sequence-specific DNA-binding proteins (Tzamarias and Struhl 1995). For example, for repression of the *SUC2* gene, Cyc8 (Ssn6) and Tup1 interact with the specific DNA-binding protein, Mig 1 (Tzamarias and Struhl 1995). Cyc8 (Ssn6) and Tup1 can also repress the transcription of the α -specific genes by complexing with the DNA-bound MCM1 and α 2 heterodimer (Keleher et al. 1992). In the Ssn6-Tup1 complex, only Tup1 contains an identifiable repressor domain (Tzamarias and Struhl 1994). In all of these cases, the repressor domain is confined to one component of the repressor complex. This recruitment-based mechanism is similar for DRAP1 and Dr1 in that Dr1 and DRAP1 interact with the promoter via the DNA-binding protein, TBP, and that only Dr1 contains the repressor domain. It is this interaction that characterizes Dr1 and DRAP1 as general repressors of transcription and sets them apart from other repressor complexes.

The Dr1-TBP interaction is considered to compete with the ability of TFIIA and TFIIB to bind to TFIID and form a functional preinitiation complex (Inostroza et al. 1992; Kim et al. 1995). Studies using point mutations in the basic repeat region of TBP have suggested that both Dr1 and TFIIA compete directly for binding to this region (Kim et al. 1995). In the same study Dr1 was shown not to compete directly for the TFIIB-binding site on TBP, suggesting that the interaction of Dr1 with TBP results in a conformational change that disrupts the TFIIB-TFIID interaction. In our DNase I footprint analysis of the TBP-DNA complex, in the presence of Dr1-DRAP1, we observed at least four hypersensitive sites. These likely arose from changes in DNA conformation. This observation is congruent with the histone fold motif observed in both Dr1 and DRAP1. Specifically, proteins containing this motif engage in either protein-

protein interactions or protein–DNA interactions (Baxevanis 1995). High-resolution hydroxyl radical footprint analysis has shown that TFIIB requires at least 7 bp on either side of the TATA box to form a stable TFIIB–TBP–DNA complex (Lee and Hahn 1995). The structure of the TFIIB–TBP–DNA complex was subsequently confirmed by x-ray crystallography and by alanine scanning, demonstrating that core TFIIB interacts with the carboxy-terminal stirrup of TBP and contacts the backbone of the core promoter upstream and downstream of the TATA element (Nikolov et al. 1995; Tang et al. 1996). Interestingly, the hypersensitive regions that occur as a result of the binding of the DRAP1/Dr1 to the TBP–DNA complex coincide with the regions required for the production of a stable TFIIB–TBP–DNA complex. The ability of Dr1 and DRAP1 to form a stable repressor complex may also account for its action in blocking RNA polymerase III transcription. In this system Dr1 inhibits the ability of B-related factor, which contains significant homology to TFIIB, to interact with TBP and recruit RNA polymerase III (White et al. 1994).

Northern blot analysis demonstrated varied expression of DRAP1 in different tissues. Interestingly, the differences in expression appear to correlate with the overall mitotic activity of the cells. For example, DRAP1 expression was higher in tissues containing a low percentage of actively dividing cells, (i.e., heart, skeletal muscle). Because it is difficult to draw general conclusions about DRAP1 expression from tissues containing mixed populations of actively dividing, differentiated, or growth-arrested cells, immunohistochemical analyses were undertaken. The developing rat brain was chosen for our experiments as it is known to contain rapidly dividing populations of progenitor cells, as well as postmitotic, migrating, and differentiating neurons. These cell populations are segregated into different compartments of the developing cerebral cortex. Our analysis revealed striking differences in the immunoreactivity of DRAP1, Dr1, and TBP. Dr1 and TBP are expressed in the proliferative ventricular/subventricular zones in the developing brain. All three proteins were found only in the cortical plate that is restricted to differentiating postmitotic neurons. The robust colocalization to the same cell population suggests that DRAP1 may be required for enhanced repressor activity when neurons enter G_0 and begin to express phenotype-specific gene products. The immunolocalization experiments also reveal that DRAP1 protein is not detected until the postmitotic neurons reach the cortical plate and cease migrating. Despite the fact that Dr1/DRAP1 exist as general repressors *in vitro*, the ability of only certain specific classes of activators to overcome the repression mediated by Dr1–DRAP1 (Yeung et al. 1994) may be important in the expression of genes required in the differentiation process. Therefore, given to dramatic changes in patterns of gene expression that occur subsequently in these neurons, DRAP1 expression may facilitate the maintenance of a differentiated state by silencing specific genes.

In summary, we have defined a novel class of a corepressor molecule, DRAP1, that together with the TBP–

associated repressor, Dr1, can inhibit the formation of a functional preinitiation complex and the subsequent initiation of transcription. The interaction of DRAP1 with Dr1 has revealed that the activity of Dr1 as a repressor can be modulated. In the absence of DRAP1, Dr1 is a weak repressor, whereas in the presence of DRAP1, a strong repressor complex is formed. We have shown that Dr1 is expressed in neural stem cells that apparently do not express DRAP1, and therefore Dr1-mediated repression of transcription in the absence of DRAP1 in an actively dividing population cannot be expected to be as high as in nondividing, highly differentiated cells. The ability of DRAP1 to differentially affect the overall process of repression through Dr1 directly correlates with the coexpression of these proteins in mature neurons. The significance of DRAP1 as a general corepressor of transcription may prove to have profound effects during development and for determining cell fates in eukaryotic systems.

Materials and methods

Purification of DRAP1

p28 was purified from HeLa cell nuclear extracts by virtue of its tight association with Dr1. Therefore, its purification was performed as described previously for Dr1 from the 0.5 M P11 fraction (Inostroza et al. 1992), except that in the last step of purification following a gel filtration step (Superdex 200 column), a Dr1 immunoaffinity column was employed. Dr1 found in the 1.0 M P11 fraction was purified similarly, except the third step of purification (DEAE–5PW) was not performed. Affinity-purified Dr1 antibodies (2 mg) were coupled to 1 ml of recombinant protein A–agarose (Repligen) using 20 mM dimethylpimelidate as described (Harlow and Lane 1988). The resin was incubated for 90 min at 4°C with the S200 fraction (0.5 ml, 0.1 mg/ml) diluted in two volumes of buffer containing 20 mM Tris–HCl, (pH 7.9), 0.2 mM EDTA, 50 mM NaF, 100 μ M NaVO₃, 100 mM KCl, 1% NP-40 and 1 mM PMSF. Immunoabsorbed complexes were then washed with 10 ml of this buffer and eluted with 0.1M glycine–HCl (pH 2.6), or in BC100 containing 0.05% Sarkosyl to separate p28 from Dr1 and remaining bound Dr1 eluted with 0.1 M glycine–HCl (pH 2.6). Dr1 eluates derived from the immunoaffinity column steps were concentrated on a micro Mono S column. Fractions were analyzed by SDS-PAGE followed by Western blot analysis using affinity-purified Dr1 antibodies and by silver staining.

Amino acid sequencing of DRAPs

Ponceau S-stained bands were excised and processed for “internal” sequencing as described (Tempst et al. 1990), with modifications (Erdjument-Bromage et al. 1994). Briefly, *in-situ* proteolytic cleavage was done using 0.5 μ g of trypsin (Promega, Madison, WI) in 25 μ l of 100 mM NH₄HCO₃ (supplemented with 0.3% Tween 80) at 37°C for 3 hr. The resulting peptide mixture was reduced and S-alkylated with, respectively, 0.1% β -mercaptoethanol (Bio-Rad, Richmond, CA) and 0.3% 4-vinyl pyridine (Aldrich, Milwaukee, WI), and fractionated by reverse-phase HPLC. An enzyme blank was done on an equally sized strip of nitrocellulose. HPLC solvents and system configuration

were as described (Elicone et al. 1994), except that a 2.1 mm Vydac C4 (214TP54) column (The Separations Group, Hesperia, CA) was used with gradient elution at a flow rate of 100 μ l/min. Fractions were collected by hand, kept on ice for the duration of the run, and stored at -70°C before analysis. Chemical sequencing of selected peptides was done using a model 477A instrument (Applied Biosystems, Foster City, CA) with "on-line" analysis (120A HPLC system with 2.1×220 -mm PTH C18 column; Applied Biosystems). Instruments and procedures were optimized for femtomole level phenylhydantoin amino acid analysis as described (Erdjument-Bromage et al. 1994; Tempst et al. 1994).

Expression of DRAP1 in *E. coli*

A set of primers were generated bearing the amino- and carboxy-terminal ends of DRAP1-coding sequence that were flanked by *Nde*I and *Bam*HI sites [amino terminus, 5'-dGGCTGTAA-CATATGGAATTCCGGATCAAG-3'; carboxyl terminus, 5'-TCTTCTGGATCCTCAGGTGCTGAGGGGCCC-3'; the boldface sequences represent both the *Nde*I and *Bam*HI sites]. These primers were used in PCR reactions to generate a product containing coding sequences flanked by these two sites. The PCR product was digested with *Nde*I and *Bam*HI and cloned in-frame with a amino-histidine tag in the plasmid vector pET15b (Novagen). *E. coli* strain BL21 (DE3, Novagen) containing DRAP1 was grown in Luria-Bertani medium supplemented with ampicillin (100 μ g/ml) at 37°C . Cells were grown to 0.6 at OD₆₀₀ and then induced with 1 mM isopropylthiogalactoside (IPTG). After 3 hr, cells were pelleted and the histidine fusion proteins were purified as described by Hoffmann and Roeder (1991).

Construction of DRAP1 amino- and carboxy-terminal deletion mutants

For amino-terminal deletion mutants, the following oligonucleotides were designed as amino-terminal primers for PCR: (N23) 5'-dGACGGGATCCCATATGGTCATCATCTCCCGGGCG-3'; 5'-dTCCGGGATCCCATATGGACATGCAGGGG GACGGG-3' (N80). For carboxy-terminal deletion mutants, the following oligonucleotides were designed as C-terminal primers for PCR: 5'-dTGGCGATCCTCTAGAGTTGTGATGTCTCTCTTTC-3' (C144), 5'-dCCCCGGATCCTCTAGAGCTTCCGGCCCC-TGCGGGC-3' (C101). The above primers were used together with amino oligonucleotide 5'-dGGCTGTAAGGATCCCA-TATGGAATTCCGGATCAAG-3' to amplify DNA fragments from p28 cDNA cloned in pFlag-2 (Kodak, Inc.) (the boldface sequences represent the *Bam*HI and *Eco*RI sites respectively). After restriction digestion with *Eco*RI and *Bam*HI the fragments were inserted into a pFlag-2 vector (Kodak, Inc.). All mutations were verified by DNA sequencing.

Histone fold motif sequence analysis

Using the multiple alignment of the histone fold sequences (Baxeianis et al. 1995), we queried a modified SWISSPROT data base of protein sequences for possible matches to the histone fold. The data base was constructed from the data residing in the data base on August 4, 1995, to which was added the DRAP1, Hap5p, and CBF-C sequences. This data base contained 53,122 sequences and consisted of 19,162,929 total letters. We used the motif search tool (MOST) (Tatusov et al. 1994) to search this data base. An *r*-value <0.001 was used as a threshold to ensure that only highly probable events are identified. The *r*-value is

the ratio of the expected number of sequence segments with a given score, to the observed number.

Protein-binding assays by surface plasmon resonance

Protein interactions detected by surface plasmon resonance were performed using a BIAcore instrument from Pharmacia Biosensor AB. rDr1 (0.6 μ g) served as the surface-immobilized target protein and was dialyzed into PBS, coupled to a BIAcore sensor chip CM5 using *N*-hydroxysuccinimide (NHS), and *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide (EDC), and 1 M ethanolamine hydrochloride (pH 8.5). Sensor chips containing between 200 and 500 resonance units (RU) of rDr1 were used for experiments. Immobilizations were performed automatically in the instrument. HBS buffer (10 mM HEPES with 0.15 M NaCl, 3.4 mM EDTA, and 0.05% surfactant P20 at pH 7.4) was used as running buffer for injection of proteins. Different amounts of either mutant (10 ng) or wild-type DRAP1 (1.6–10 ng) were injected in a total volume of 30 μ l in HBS buffer at 5 μ l/min continuous flow. After each protein injection, two consecutive washes of Sarkosyl (0.05%) in HBS were injected in 10 μ l at 5 μ l/min continuous flow to dissociate proteins interacting with surface bound rDr1. All experiments were performed at room temperature.

Protein-binding assays using GST fusion proteins

The GST fusion proteins were expressed in *E. coli*. Approximately 20 μ l of glutathione-agarose beads containing 1 μ g of the fusion protein was incubated at 4°C in 0.5 ml of buffer containing 20 mM Tris-HCl buffer, (pH 7.5), 0.1 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 0.2% NP-40. Equal amounts of either bacterial lysate or in vitro-translated protein was added and binding was allowed to proceed for 1 hr. The beads were washed four times with the same buffer with 0.4% NP-40, and the bound proteins eluted with 30 μ l of SDS-PAGE loading buffer and resolved by electrophoresis. In vitro-translated proteins were visualized by fluorography. Bacterially produced proteins were visualized by Western blotting.

Immunocytochemistry

Immunolocalization of DRAP1, Dr1, and TBP were performed using a modification of a previously published protocol (Ferri and Levitt 1993). Brains were dissected from E15 Holtzman albino rats and immersion fixed in 4% paraformaldehyde (pH 6.5) for 2 hr and in 4% paraformaldehyde (pH 9) overnight at 4°C . Following sucrose cryoprotection, the brains were frozen, serially sectioned in the coronal plane at 14 μ m and collected in four series on chromium potassium sulfate alum-gelatin/poly-L-lysine-coated glass slides. Adjacent sections were processed for immunolocalization of each protein. Sections first were treated with 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity, followed by blocking in Blotto (4% Carnation dried milk in PBS), containing 0.3% triton X-100. Primary antibody incubation (1:4000 dilution) was overnight at room temperature, followed by extensive washing and standard biotin-streptavidin-HRP (Jackson Immuno Research Products) reaction. For DRAP1 and Dr1 staining, affinity-purified rabbit polyclonal antibodies were used (0.2 mg/ml). For TBP staining, monoclonal antibodies, SL39 (0.2 mg/ml) (gift of N. Hernandez, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were used. Sections were photographed, and counterstained with 0.1% cresyl violet and rephotographed to aid in the identification of the brain regions containing specific immunoreactivity. The terminology used to describe developing zones

of the cerebral wall is according to the Boulder Committee (1970).

Gel shift assays

Proteins and the radiolabeled AdMLP (−40 to +13, ~5000 cpm, 0.1–1 ng) were incubated in the presence of 100 µg/ml of BSA for 30–45 min at 30°C in a 20 µl volume as described previously (Maldonado et al. 1990). The reaction products were analyzed on 6% polyacrylamide gels containing 25 mM TRIS (pH 8.3) 190 mM glycine, 5 mM magnesium acetate, 2.5% (vol/vol) glycerol, and 0.5 mM DTT in running buffer containing 25 mM TRIS (pH 8.3) 190 mM glycine, and 5 mM magnesium acetate as described by Auble and Hahn (1993).

Other methods

Transcription factors were purified as described by Flores et al. (1992). RNA PII was purified as described by Lu et al. (1991). Transcription assays and DNase I footprinting analysis were performed as described by Maldonado et al. (1990). Northern blot analysis was performed as described by Ha et al. (1991). Polyclonal antibodies were produced as described previously for Dr1 by Inostroza et al. (1992).

Acknowledgments

We thank Drs. George Orphanides and Ronny Drapkin for stimulating discussions and reading the manuscript. This work was supported by a grant from the National Institutes of Health (GM48518) and Howard Hughes Medical Institute to D.R. This work was also supported, in part, by the Irma T. Hirsch Foundation and National Science Foundation grant BIR-9420123 (to P.T.), and National Cancer Institute Cancer Core grant 5P30 CA08748-29 to the Sloan-Kettering Microchemistry Facility. We thank Mary Lui for assistance with protein sequencing. We also thank Dr. Osvaldo Flores for the gift of CMV-IE2 protein and the AdMLP containing CRE.

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Genes Dev. 1996, **10**:

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