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Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations

(POU proteins/human autoantigen Ku/immunoprecipitation/protein-affinity chromatography/contaminating DNA)

JIANN-SHIUN LAI*† AND WINSHIP HERR*

*Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; and †Genetics Program, State University of New York, Stony Brook, NY 11794

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ABSTRACT **DNA-dependent and DNA-independent associations of DNA-binding proteins are important in transcriptional regulation. The analysis of DNA-independent associations frequently relies on assaying protein interaction in the absence of target DNA sequences. We have found that contaminating DNA in protein preparations can stabilize DNA-dependent associations that may appear DNA-independent. Three cellular proteins of 70, 85, and 110 kDa coimmunoprecipitated with the octamer motif-binding protein Oct-2 because of the presence of contaminating DNA in the cell extracts. In addition, heterodimer formation between Oct-1 (or Oct-2) and Pit-1 during protein-affinity chromatography was stabilized by the contaminating DNA. In both instances, these DNA-dependent protein associations were selectively inhibited by ethidium bromide in the precipitation reaction without any evident effect on DNA-independent protein associations. Thus, ethidium bromide may serve as a simple and general indicator of DNA-dependent and DNA-independent protein associations.**

Transcriptional regulation depends not only on the interactions between sequence-specific DNA-binding proteins and their respective cis-regulatory elements but also on the interactions among these proteins and with other components of the transcriptional machinery (reviewed in refs. 1–3). The availability of tools for studying sequence-specific transcription factors (e.g., cDNA clones and antibodies) has allowed more detailed analysis of the mechanisms by which protein–protein interactions, both DNA-dependent and DNA-independent, regulate transcription. Current methods for determining DNA-independent protein–protein interactions include coimmunoprecipitation and protein-affinity chromatography. We have used coimmunoprecipitation to identify proteins that associate with the octamer motif-binding proteins Oct-1 (OTF-1, NFIII) and Oct-2 (OTF-2). These transcription factors are particularly interesting because they display the same DNA-binding specificity (4) and share very similar DNA-binding POU domains (5, 6) but display qualitatively different RNA polymerase II transcriptional activation properties. Oct-1 is an effective activator of small nuclear RNA-type promoters, whereas Oct-2 is an effective activator of mRNA-type promoters (7). Therefore, it is likely that Oct-1 and Oct-2 interact preferentially with different components of the transcriptional machinery.

We identified four cellular proteins of 68, 70, 85, and 110 kDa that coimmunoprecipitated with Oct-2 from labeled cell extracts in what appeared to be a DNA-independent manner. We noticed, however, that the 70-, 85-, and 110-kDa proteins could bind DNA on their own. We subsequently identified the 70- and 85-kDa proteins as the two heterologous subunits of the human autoantigen Ku, which possesses strong nonspecific DNA-binding properties (ref. 8 and references therein). This observation suggested that the association of these three proteins with Oct-2 might be mediated by contaminating DNA in the labeled cell extracts. Consistent with this hypothesis, these associations are sensitive to the presence of the DNA intercalator ethidium bromide (EtdBr) (9, 10), an inhibitor of DNA binding (11, 12), and are not detected following digestion with DNase. We subsequently showed that association of Oct-1 (or Oct-2) with the related POU-domain protein Pit-1 (GHF-1) during protein-affinity chromatography is also EtdBr-sensitive, suggesting that this association is also stabilized by contaminating DNA. These studies indicate that the sensitivity of protein associations to EtdBr is a good indicator of their dependence on DNA.

MATERIALS AND METHODS

Expression Constructs and Antibodies. The mammalian Oct-2 expression vector pCGoct-2 (13), and the *in vitro* translation vectors pBSoct-1, pCGoct-2, and pBSpit-1 (14) and pBSKRb (retinoblastoma gene product) (15) were as described. The T7 RNA polymerase *Escherichia coli* expression construct for the glutathione *S*-transferase (GST)–E1A fusion protein was pGEX-E1A 13S (16). The GST–Pit-1 POU domain fusion protein was made from pETG-pit-1-POU, which contains the GST coding sequences fused to the 5′ end of Pit-1 POU domain coding sequences in the *E. coli* expression vector pET11c (G. Henry and W.H., unpublished results). The two series of monoclonal antibodies raised against Oct-1 (YL series) or Oct-2 (PT series) will be described in detail elsewhere; the anti-E1A monoclonal antibody was M73 (17).

Cell Labeling, Immunoprecipitation, and DNA-Affinity Precipitation. Cells were transfected and labeled as described (13). Labeled cells were washed with phosphate-buffered saline, collected, and lysed on ice for 1 hr in 1 ml of lysis buffer containing 25 mM Hepes (pH 7.9), 200 mM KCl, 0.1% Nonidet P-40 (Sigma), 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), aprotinin (1 μg/ml), 10 mM NaF, 0.1 mM Na_3VO_4, 5 mM EDTA, and 5 mM dithiothreitol. Cell debris was removed, and the resulting lysate was precleared with normal rabbit serum and fixed and killed *Staphylococcus aureus* Cowan I (Zymed Laboratories) (18). Typically, for immunoprecipitation, 250 μl of cleared lysate was incubated with monoclonal antibody for 1 hr on ice with intermittent, gentle mixing. Immune complexes were collected with recombinant protein G-agarose beads (GIBCO/BRL) by rocking at 4°C for 1 hr, and the immunoprecipitates washed and prepared for SDS/PAGE (18).

For DNA-affinity precipitation, DNAs containing multimerized copies of the octamer and heptamer Oct-2 binding sites from the immunoglobulin heavy-chain promoter flanked by *Pvu* II sites (5′-CAGCTGCCTCATGAGTATGCAAAT-CAGCTGC-3′) covalently coupled to Sepharose beads (19)

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Abbreviations: GST, glutathione *S*-transferase; EtdBr, ethidium bromide.

were kindly provided by M. Tanaka (Cold Spring Harbor Laboratory). We used the same procedure that was used for immunoprecipitation except that the DNA Sepharose beads were used in place of protein G-agarose beads.

EtdBr, Micrococcal Nuclease, and RNase Treatment of Cleared Lysates. EtdBr was added (10–400 μg/ml) and the lysates were incubated on ice for 30 min. Precipitates were removed by centrifugation for 5 min at 4°C in a microcentrifuge and the supernatant was transferred to a fresh tube. The resulting lysate was then ready for immunoprecipitation or DNA-affinity precipitation. The original concentration of EtdBr was maintained during the washing steps.

For micrococcal nuclease treatment, the immunoprecipitates or DNA-affinity precipitates bound to the beads after four washes were suspended in 50 μl of digestion buffer (50 mM NaCl/10 mM Tris, pH 7.0/4 mM $CaCl_2$ with or without 10 mM EGTA). This order of precipitation and subsequent digestion allowed us to concentrate the sample and to transfer it to a buffer appropriate for micrococcal nuclease treatment. After incubation at 37°C for 1 hr with micrococcal nuclease (Worthington), the samples were washed twice with 1 ml of digestion buffer prior to SDS/PAGE. For RNase A and T_1 treatment, the samples were treated as for micrococcal nuclease treatment except that the RNase digestion buffer was 300 mM NaCl/10 mM Tris, pH 7.5/5 mM EDTA and the incubation was at 30°C for 1 hr.

Purification of GST Fusion Proteins from *E. coli*. The GST–E1A 13S and GST–Pit-1 POU domain fusion proteins, and GST alone were expressed in *E. coli* BL21(DE3) (20) and purified from induced cultures (21). Glutathione-agarose beads bound with GST or GST fusion protein were washed extensively with phosphate-buffered saline containing lysozyme (3 mg/ml), 0.1% Tween 20, and 0.1% Triton X-100 and were stored at 4°C in the same buffer with 1 mM phenylmethylsulfonyl fluoride and 0.02% NaN_3. The purified proteins thus treated remained stable for at least 1 month. The relative amount of fusion protein bound to the beads was determined by SDS/PAGE of proteins released from the beads and staining of the gel with Coomassie blue. Before protein-affinity chromatography, the beads were washed twice in binding buffer [50 mM KCl/20 mM Hepes, pH 7.9/2 mM EDTA/0.1% Nonidet P-40/5 mM dithiothreitol/10% (vol/vol) glycerol/0.5% nonfat milk]. Beads (150 μl) bearing 2 μg of each fusion protein were incubated on ice for 30 min in the absence or presence of EtdBr (10–400 μg/ml). ^{35}S-labeled *in vitro*-translated proteins (30 μl) were then added to the beads and incubated for 1 hr on ice with intermittent, gentle mixing. The beads were subsequently washed four times with 1 ml of the binding buffer containing the same concentration of EtdBr used in the binding reaction. Bound labeled proteins were resolved by SDS/PAGE.

RESULTS

Four Cellular Proteins of 68, 70, 85, and 110 kDa Associate with Oct-2 During Immunoprecipitation. Fig. 1 shows two series of immunoprecipitations from labeled 293 cell (human embryonic kidney cell line) extracts containing or lacking ectopically expressed Oct-2, with a panel of anti-Oct-1 and anti-Oct-2 monoclonal antibodies. Oct-2 was recovered from the Oct-2-containing extract with each of the six monoclonal antibodies directed against Oct-2 (lanes 2–7), but not in the absence of antibody (lane 1) or with an Oct-1-specific antibody (lane 8). The recovered Oct-2 molecules migrate as a doublet due to heterogeneous phosphorylation (13). In addition to Oct-2, four other proteins of 68, 70, 85, and 110 kDa were coprecipitated by five of the six Oct-2 antibodies (lanes 2–6). Except for a reduced level of the 68-kDa protein, neither Oct-2 nor the other cellular proteins were recovered from the mock-transfected cell extracts (lanes 9–16). This

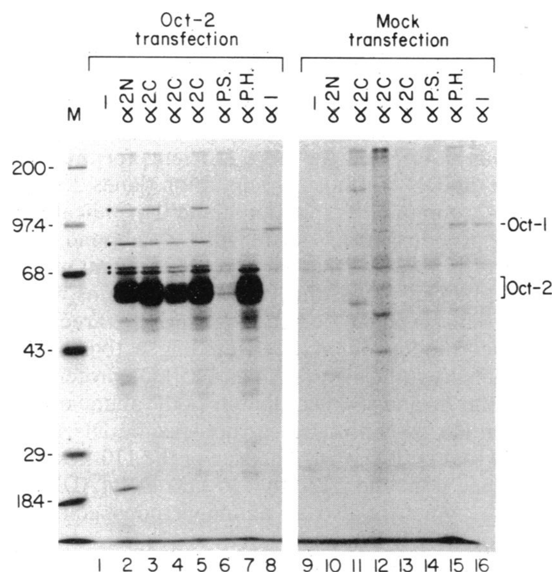

FIG. 1. Four cellular proteins of 68, 70, 85, and 110 kDa coprecipitate with ectopically expressed Oct-2 from 293 cells during immunoprecipitation. A panel of monoclonal antibodies specific for Oct-1, Oct-2, or both proteins was used in immunoprecipitation reactions with ^{35}S-labeled extracts from 293 cells transfected with an Oct-2 expression vector (lanes 1–8) or mock transfected (lanes 9–16). Immunoprecipitated proteins were separated by SDS/10% PAGE and stained for fluorography. Samples were precipitated either in the absence of antibody (lanes 1 and 9) or with monoclonal antibodies (α) directed against the N-terminal (PT1, lanes 2 and 10) or C-terminal (PT2, PT3, and PT7 in order in lanes 3–5 and 11–13) regions of Oct-2, the POU-specific regions (YL21, lanes 6 and 14) or POU homeodomains (YL123, lanes 7 and 15) of Oct-1 and Oct-2, or the unique POU-domain linker region of Oct-1 (YL15, lanes 8 and 16). The positions of Oct-1 and Oct-2 and the molecular size (kDa) of the protein standards (lane M) are indicated to the right and left, respectively. Bands corresponding to the coprecipitated 68-, 70-, 85-, and 110-kDa cellular proteins are indicated by dots. Oct-2 is less abundant in lane 6 because antibody YL21 has a low affinity for Oct-2 (unpublished results).

result, together with the ability of multiple Oct-2 antibodies to coprecipitate the four cellular proteins, indicated that the coprecipitation was due to association with Oct-2 rather than due to antibody crossreactions.

One of the four Oct-2-associated proteins (68 kDa) can be distinguished from the others because it is the only one recovered with the monoclonal antibody directed against the Oct-2 (and Oct-1) homeodomain (lane 7); high-resolution two-dimensional gel electrophoresis (22) of Oct-2 immunoprecipitates indicates that this 68-kDa protein is the heat shock protein Hsp70 (data not shown). The inability to recover the 70-, 85-, and 110-kDa proteins with the Oct-2 homeodomain antibody suggested that these proteins associate with the homeodomain. Consistent with this hypothesis, they can be recovered, albeit with reduced efficiency, when only the Oct-2 homeodomain is present (data not shown). Thus, these three proteins apparently associate with the Oct-2 DNA-binding domain.

The 70-, 85-, and 110-kDa Proteins Can Bind DNA in the Absence of Oct-2. Protein association with POU domains occurs on DNA, as in the interaction between Oct-1 and the herpes simplex virus transactivator VP16 (23–25), or in the absence of DNA, as in the interaction between the *Drosophila* POU proteins I-POU and Cfl-a (26). Indeed, in the latter case the interaction between I-POU and Cfl-a inhibits the ability of Cfl-a to bind DNA (26). To test whether the association of Oct-2 and the 70-, 85-, and 110-kDa proteins is compatible with Oct-2 DNA binding, we asked whether these

three proteins could be recovered in a DNA-affinity precipitation assay with multimerized copies of the octamer motif covalently linked to Sepharose beads. Fig. 2 shows the results of such an experiment using two different concentrations of DNA beads. The recovery of the Oct-2-associated proteins was as efficient (or more efficient, for the 110-kDa protein) in the DNA-affinity precipitation (lanes 2 and 3) as in an Oct-2 immunoprecipitation (lane 1), indicating that these three proteins can associate with Oct-2 bound to DNA. To our surprise, however, in a control experiment with a 293 cell extract lacking Oct-2, the same three proteins were also recovered (lanes 4 and 5), indicating that these three proteins possess Oct-2-independent DNA-binding activity. Consistent with this hypothesis, the 70- and 85-kDa proteins comigrate with the 70- and 86-kDa subunits of the abundant human autoantigen Ku, which possesses nonspecific DNA-binding activity (8) (data not shown); the size of the 110-kDa protein suggests that it is the 112- to 116-kDa poly(ADP-ribose) polymerase, which is also an abundant nonspecific DNA-binding protein (27, 28).

Association of the 70-, 85-, and 110-kDa Proteins with Oct-2 Is Sensitive to EtdBr and Micrococcal Nuclease Digestion. The realization that the 70-, 85-, and 110-kDa proteins can bind DNA in the absence of Oct-2 suggested that coimmunoprecipitation of these three proteins with Oct-2 may not occur directly but rather may result from binding of Oct-2 and these three proteins to contaminating DNA in the protein extracts. To test this possibility we assayed the effect of EtdBr, a DNA intercalator (9, 10) that interferes generally with protein–DNA interaction (11, 12).

To demonstrate that EtdBr does not generally affect protein–protein interactions, we tested the effect of EtdBr on associations between the adenovirus E1A proteins and cellular proteins (29, 30). In the absence of EtdBr (Fig. 3, lane 1), the p300, p107/p105^{Rb}, and p60$^{Cyclin-A}$ E1A-associated proteins (31–33) were recovered in an immunoprecipitation of endogenous E1A from an ^{35}S-labeled 293 cell extract; recovery of the E1A-associated proteins was resistant to EtdBr at 12.5 μg/ml (lane 2) or 50 μg/ml (lane 3). The same concentrations of EtdBr, however, did affect the recovery of Oct-2 and the associated proteins in a DNA-affinity precipitation assay (compare lanes 8 and 9 with lane 7), showing that EtdBr inhibits protein–DNA interactions. As expected, in an Oct-2 immunoprecipitation, addition of EtdBr did not affect the monoclonal antibody–Oct-2 interaction (lanes 4–6). Recovery of the Hsp70-like protein was not sensitive to EtdBr (lanes 4–6), but recovery of the 70-, 85-, and 110-kDa proteins was disrupted by the addition of EtdBr (compare lanes 5 and 6 with lane 4), suggesting that the association between Oct-2 and these three cellular proteins is indeed DNA-dependent.

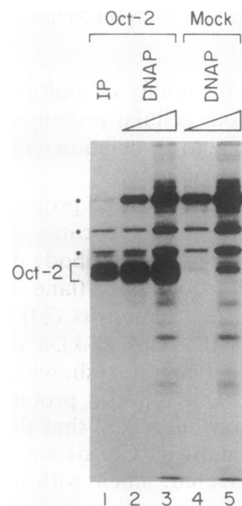

FIG. 2. The 70-, 85-, and 110-kDa Oct-2-associated proteins bind DNA in the absence of Oct-2. Extracts of ^{35}S-labeled 293 cells, either transfected with an Oct-2 expression vector (lanes 1–3) or mock transfected (lanes 4 and 5), were used for immunoprecipitation (IP) (lane 1) with the N-terminal Oct-2-specific antibody PT1 or for DNA-affinity precipitation (DNAP) (lanes 2–5) with Sepharose beads linked to multimerized copies of the immunoglobulin heavy-chain promoter octamer and heptamer motifs (see *Materials and Methods*). Two different concentrations of DNA beads were used, which could bind either 0.5 μg (lanes 2 and 4) or 1.5 μg (lanes 3 and 5) of *E. coli*-expressed Oct-2 as measured by Coomassie blue staining of bound Oct-2 after electrophoresis. Dots identify the 70-, 85-, and 110-kDa proteins.

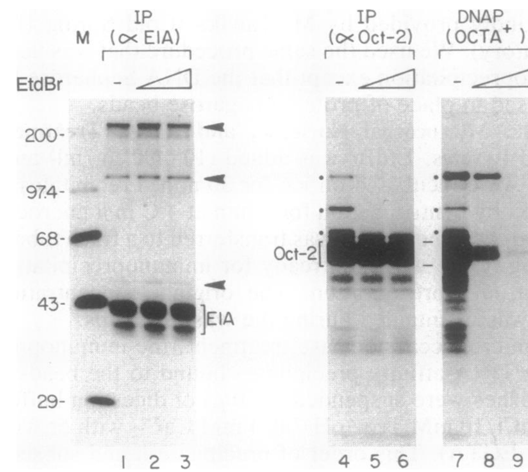

FIG. 3. Association of the 70-, 85-, and 110-kDa proteins with Oct-2 is sensitive to the presence of EtdBr. A 293 cell extract containing Oct-2 was immunoprecipitated (IP) with E1A-specific (M73, lanes 1–3) or Oct-2-specific (PT1, lanes 4–6) monoclonal antibody or used for DNA-affinity precipitation (DNAP) with multimerized octamer and heptamer motifs (lanes 7–9). The reactions were done in the absence of EtdBr (lanes 1, 4, and 7) or in the presence of EtdBr at 12.5 μg/ml (lanes 2, 5, and 8) or 50 μg/ml (lanes 3, 6, and 9). Precipitated proteins were separated by SDS/10% PAGE and visualized by fluorography. Arrowheads indicate the positions of the E1A-associated proteins p300, p107/p105^{Rb}, and p60$^{Cyclin A}$. Dots indicate the positions of the three Oct-2-associated proteins.

To confirm the DNA-dependent association of Oct-2 with the 70-, 85-, and 110-kDa proteins, we treated the Oct-2 immunoprecipitates with micrococcal nuclease or RNase after immunoprecipitation from Oct-2-containing 293 cell extracts. Micrococcal nuclease treatment (Fig. 4, lane 4) had the same deleterious effect on association of Oct-2 with the three cellular proteins as did addition of EtdBr (lane 2). The effect of micrococcal nuclease treatment was prevented by the addition of EGTA (lane 6), which inhibits micrococcal nuclease activity (34). Treatment of the immunoprecipitates with RNase had no effect on recovery of Oct-2 or the three associated proteins (lane 8), indicating that RNA could not function to tether Oct-2 and the 70-, 85-, and 110-kDa proteins.

DNA Is Also Involved in Oct-1 and Pit-1 Heterodimer Formation. The unexpected finding that contaminating DNA can stabilize associations between DNA-binding proteins during immunoprecipitation led us to test whether other DNA-binding-protein associations thought to occur in the absence of DNA might also be stabilized by contaminating DNA. For example, in the absence of any added DNA binding sites, *in vitro*-translated Oct-1 was shown to associate with a GST–Pit-1 POU domain fusion protein purified after expression in *E. coli* and bound to glutathione-agarose beads (21). We tested the sensitivity of this association to the presence of EtdBr.

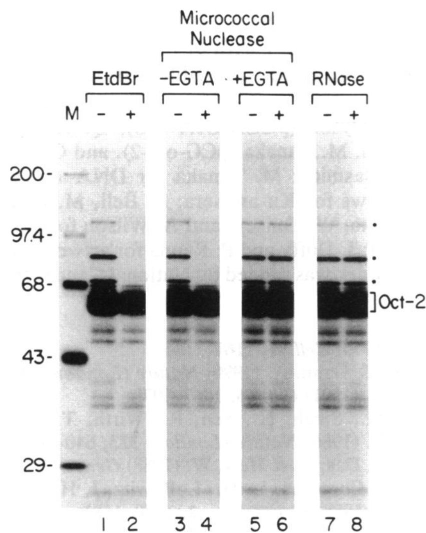

FIG. 4. Association of the 70-, 85-, and 110-kDa proteins with Oct-2 is sensitive to micrococcal nuclease but not to RNase digestion. In each sample, an ^{35}S-labeled Oct-2-containing 293 cell extract was used for immunoprecipitation with the Oct-2-specific monoclonal antibody PT1. For micrococcal nuclease treatment (lanes 3–6), the immunoprecipitates bound to protein G-agarose beads were suspended in 50 μl of digestion buffer containing 4 mM $CaCl_2$ (lanes 3 and 4) or additionally 10 mM EGTA (lanes 5 and 6) with (lanes 4 and 6) or without (lanes 3 and 5) the addition of 0.4 unit of micrococcal nuclease. For RNase treatment, the immunoprecipitates were suspended in 50 μl of RNase digestion buffer with (lane 8) or without (lane 7) RNase A (80 μg/ml) and RNase T_1 (4 μg/ml). Lanes 1 and 2, control immunoprecipitations in the absence or presence of EtdBr (400 μg/ml).

As a control to demonstrate that EtdBr does not generally affect protein association in this assay, we tested its effect on the association of a GST–E1A 13S fusion protein with *in vitro*-translated $p105^{Rb}$ (15, 16). As expected, *in vitro*-translated $p105^{Rb}$ (Fig. 5, lane 1) did not associate with GST protein alone (lane 2) but was recovered with the GST–E1A 13S fusion protein (lane 4); this recovery was insensitive to EtdBr at 200 μg/ml (lane 3). As described previously (21), *in vitro*-translated Oct-1 (lane 5) was recovered with the GST–Pit-1 POU domain fusion protein (lane 8) but not with GST alone (lane 6), showing that the Pit-1 POU domain is required for the association. However, this Oct-1–Pit-1 association was very sensitive to EtdBr (compare lanes 7 and 8), suggesting that it was dependent on contaminating DNA in either the purified GST–Pit-1 POU domain fusion protein preparation or the *in vitro*-translated Oct-1. Consistent with this result, the association of Oct-1 and the GST–Pit-1 POU domain fusion protein was also sensitive to micrococcal nuclease treatment (data not shown).

A similar association between Oct-2 and the Pit-1 POU domain (lanes 9–12) was also disrupted by EtdBr. Curiously, however, although ethidium bromide had a large effect on Pit-1 self-association, a residual level of self-association was apparently resistant to EtdBr (compare lanes 15 and 16). This low level of association in the presence of EtdBr may be significant because in a serial titration of EtdBr, the residual Pit-1 self-association was resistant to as much as 400 μg of EtdBr per ml, whereas the majority of the self-association was disrupted by 10 μg/ml (data not shown). This resistance to EtdBr suggests that Pit-1 can form homodimers in the absence of DNA.

DISCUSSION

We have described two instances in which DNA contamination apparently stabilized associations between DNA-binding proteins that might otherwise be thought to occur independently of DNA. (*i*) We found that three cellular proteins that coimmunoprecipitate with Oct-2 could bind DNA on their own. This finding led us to test the sensitivity of these associations to EtdBr, a general inhibitor of DNA binding by proteins (11, 12), and micrococcal nuclease digestion, both of which disrupted the associations. (*ii*) We found that association between the POU-domain proteins Pit-1 and Oct-1 in solution (21) was also dependent on contaminating DNA, as indicated by sensitivity to EtdBr and micrococcal nuclease digestion. Pit-1 POU domain self-association, however, revealed an EtdBr-resistant component (Fig. 5), which may reflect bona fide DNA-independent association. Oct-1 also associates with itself in solution in a protein-affinity assay similar to the one used to assay Pit-1–Oct-1 and Pit-1–Pit-1 association (35). It will be of interest to determine whether this association is EtdBr-sensitive or not.

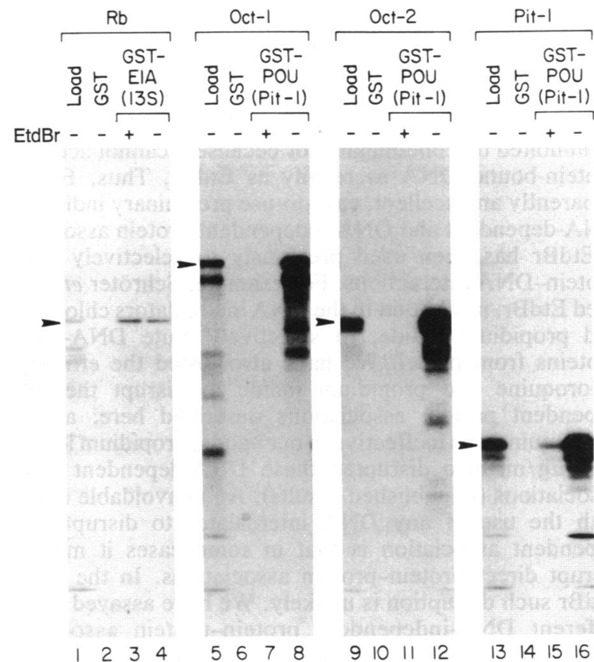

FIG. 5. Association of Oct-1, Oct-2, and Pit-1 with the Pit-1 POU domain in protein-affinity chromatography is sensitive to EtdBr. Glutathione-agarose beads bearing the GST–E1A 13S fusion protein were incubated with *in vitro*-translated ^{35}S-labeled $p105^{Rb}$ either in the presence (lane 3) or absence (lane 4) of EtdBr (200 μg/ml). Beads bearing the GST–Pit-1-POU domain fusion protein were incubated with ^{35}S-labeled Oct-1 (lanes 7 and 8), Oct-2 (lanes 11 and 12), or Pit-1 (lanes 15 and 16) in the presence (lanes 7, 11, and 15) or absence (lanes 8, 12, and 16) of EtdBr. The beads were then washed and the precipitates were eluted for SDS/10% PAGE. Lanes 2, 6, 10, and 14, eluates of beads bearing GST proteins alone after incubation with ^{35}S-labeled $p105^{Rb}$, Oct-1, Oct-2, and Pit-1, respectively. Lanes 1, 5, 9, and 13, portions (5%) of reaction mixtures containing ^{35}S-labeled protein incubated with the POU domain and control beads, loaded directly onto the gel. Positions of the major $p105^{Rb}$, Oct-1, Oct-2, and Pit-1 *in vitro* translation products are indicated by arrowheads.

Addition of EtdBr and digestion with micrococcal nuclease are complementary methods for distinguishing DNA-dependent and DNA-independent protein association. Both EtdBr (36) and micrococcal nuclease (37) interact with DNA in a relatively nonspecific manner; therefore, the ability of both reagents to disrupt protein–DNA interactions is likely to be reasonably indiscriminate. As a routine assay, however, addition of EtdBr provides several advantages over micrococcal nuclease digestion. Digestion with micrococcal nuclease requires transfer of the sample to the appropriate digestion buffer and generally involves prolonged incubation

(about 1 hr) at elevated temperature (37°C). Furthermore, unlike EtdBr, which has proved very effective for disrupting DNA-dependent protein association (Figs. 3 and 5), micrococcal nuclease digestion is often incompletely effective (unpublished observations), perhaps because the enzyme can be inhibited by contaminants or because it cannot access the protein-bound DNA as readily as EtdBr. Thus, EtdBr is apparently an excellent, easy-to-use preliminary indicator of DNA-dependent and DNA-independent protein association.

EtdBr has been used previously to selectively disrupt protein–DNA interactions. For example, Schröter *et al.* (12) used EtdBr, in addition to the DNA intercalators chloroquine and propidium iodide, to selectively elute DNA-binding proteins from nuclei. We have also tested the efficacy of chloroquine and propidium iodide to disrupt the DNA-dependent protein associations described here: although chloroquine was ineffective in our hands, propidium iodide at ≥15 μg/ml also disrupted these DNA-dependent protein associations (unpublished results). An unavoidable concern with the use of any DNA intercalator to disrupt DNA-dependent association is that in some cases it may also disrupt direct protein–protein associations. In the case of EtdBr such disruption is unlikely. We have assayed over 10 different DNA-independent protein–protein associations (e.g., E1A with its associated proteins, and numerous antibody–antigen interactions), none of which was affected by the addition of EtdBr. Nevertheless, it is imperative to check the specificity of EtdBr disruption of protein associations by also assaying the effects of micrococcal nuclease digestion or inclusion of propidium iodide.

The association of the 70-, 80-, and 110-kDa proteins with Oct-2 in the coimmunoprecipitation assay is probably enhanced by the transient ectopic expression of Oct-2. For example, when endogenous Oct-2 is immunoprecipitated from a B-cell extract, only the 110-kDa protein is readily coimmunoprecipitated (unpublished observation). Other experiments have shown that the 70-, 85-, and 110-kDa proteins also coimmunoprecipitate with transiently expressed Oct-1, Pit-1, and an Oct-1 variant carrying the Oct-3/4 POU domain (14). These associations are all dependent on the integrity of the DNA-binding domain, because they do not occur when the POU domain is deleted or with a mutant Oct-1 protein carrying three alanine substitutions in the homeodomain that disrupt DNA binding (38). In an identical assay, however, the same three cellular proteins do not associate with the serum response factor SRF, the bovine papilloma virus transcription and replication factor E2, or the TATA box-binding protein TBP (unpublished results). Further, in a protein-affinity assay similar to the Pit-1–Oct-1 assay used here, the Oct-1 POU domain does not associate with the adenovirus replication factor NFI (35). The efficiency of associations that are dependent on contaminating DNA may reflect differences in the ability of these various DNA-binding proteins to bind to the contaminating DNA or differences in bona fide protein interactions when the proteins are bound to DNA.

Many important associations between DNA-binding proteins are dependent on DNA, as in the case of Oct-1 and VP16 (39–41). Inhibition of DNA-dependent protein association by EtdBr does not discriminate between specific and nonspecific associations stabilized by DNA. For example, Voss *et al.* (21) have shown that Pit-1 and Oct-1 can form heterodimers on specific cis-regulatory targets. Thus, whereas our assays clearly show a DNA dependence for the interaction of Oct-1 and Pit-1 in solution, it is not clear whether this association is stabilized by specific protein–protein contacts between DNA-bound Oct-1 and Pit-1 molecules or simply by nonspecific tethering of the two proteins by the DNA. Our studies suggest that EtdBr can be an excellent reagent to discriminate between protein associations that are entirely independent of DNA and those that remain dependent on DNA, but further studies with other classes of DNA-binding proteins will be required to establish its general utility.

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