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

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

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Communicated by Keith Yamamoto, April 23, 1992 (received for review February 4, 1992)

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 immunoprecipitated 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, NFI1) 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- and 85-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 there-
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 CaCl₂ 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 T₁ 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% NaN₃. 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). 35S-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.
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/p150Rb, and p60Cyclin-α E1A-associated proteins (31–33) were recovered in an immunoprecipitation of endogenous E1A from an 35S-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.

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 Pii-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–Pii-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.
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 p105Rb (15, 16). As expected, in vitro-translated p105Rb (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.

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.
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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.

We thank N. Dyson (pGEX-EIA 135), Q. Hu (pBSKRb), R. Aurora (pBSp1-1), M. Tanaka (pCCG-oct2), and G. Henry (pETG-Oct-1-POU) for plasmids; M. Tanaka for DNA-affinity Sepharose beads; M. Mathews for Ku antisera; S. Bell, M. Cleary, N. Hernandez, M. Tanaka, W. Tansey, and A. Wilson for critical readings of the manuscript; J. Duffy and P. Renna for artwork; and J. Reader for typing. This work was funded by National Cancer Institute Grant CA15106.