RFX1, a Single DNA-binding Protein with a Split Dimerization Domain, Generates Alternative Complexes*

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The transcription of various viral and cellular genes is regulated by palindromic and nonpalindromic DNA sites resembling the EP element of the hepatitis B virus enhancer, which generate similar DNA-protein complexes. The upper EP complex contains homodimers of the transcription regulator RFX1. We show that RFX1 possesses a split, extended dimerization domain composed of several evolutionarily conserved boxes, one of which was previously shown to mediate dimerization. Such an unusually long and complex dimerization domain could potentially serve for generating multiple complexes. In addition to the previously characterized complex, RFX1 generated a novel DNA-protein complex of extremely low mobility, formed only with palindromic DNA sites. Different deletions within the dimerization domain altered the relative abundance of the two complexes, suggesting an interplay between them. Formation of the low mobility complex correlated with transcriptional repression, in that both activities were mediated by several portions of the conserved region. Our results propose a mechanism by which the extended dimerization domain mediates the formation of alternative homodimeric complexes, which differ in the nature of the intersubunit interaction. By participating in different types of interactions, this domain may regulate the relative abundance of the different complexes, thus affecting transcriptional activity.

EP is a regulatory element found in several viral enhancers, such as those of the hepatitis B virus (HBV), 1 polyomavirus, and equine infectious anemia virus (1–5). EP-homologous sites are also present in cellular genes, including the MIF-1 binding element in the mouse rpL30 ribosomal protein gene promoter (8, 9), the α element in the mouse MHC class II promoters (8, 9), the α element in the mouse rpL30 ribosomal protein gene promoter (10, 11), and a binding site in the proliferating cell nuclear antigen promoter (12, 13). These different sites can be divided into two major groups. One group includes palindromic or partially palindromic sites, such as the EP elements of the HBV and polyomavirus enhancers. Members of the other group, e.g., the MHC promoter X box, are nonpalindromic and contain only a single EP-homologous half-site. While the EP elements of the HBV and polyomavirus enhancers, as well as the X box and rpL30α element, are positively acting sites within their natural DNA context (3, 4, 8–10, 14–17), a multimerized EP site cannot stimulate transcription significantly (4). Moreover, EP and MIE multimers can silence transcription (Refs. 18–20), demonstrating that the activity of EP is context-dependent.

The EP element is bound by a ubiquitous nuclear protein complex, generating a typical pattern of several slowly migrating bands in gel shift essays (1–4). These EP complexes were independently characterized by several groups studying seemingly unrelated DNA-binding factors, which were later found to represent the same nuclear complex, referred to as EP, EF-C, MDBP, MIF, or NF-X (5, 7, 8, 19, 21–23). The EP complex contains homo- and heterodimers of RFX1, RFX2, and RFX3, which belong to a novel protein family, highly conserved in evolution from yeast to humans (9, 24–28). The RFX family members share several conserved regions, including a centrally located DNA-binding domain (DBD) and the B, C, and D regions found in the C-terminal part of these proteins (25–29). Region D has been characterized as the RFX1 dimerization domain, and it shows no significant homology to any known protein outside the RFX family (25). The dimerization of RFX proteins is very stable; hence, most RFX1 molecules found in cellular extracts are in the form of dimers (9, 17). Interestingly, the DBD and the dimerization domain of RFX proteins are functionally independent, so that DNA-binding can occur in the absence of the dimerization domain, an uncommon situation in dimeric transcription factors (25). The upper EP complex generated in cellular extracts, designated complex a, contains homodimers of RFX1 (9, 26, 30). Complex a, like the other cellular EP complexes, is similarly generated with both the palindromic EP sites of the HBV and polyomavirus enhancers and the nonpalindromic X box half-site (8, 17, 26), yet the mode of binding is different for the two types of sites (2, 3, 17, 25). The binding to the viral EP sites is symmetrical, each one of the two half-sites being contacted by a subunit of the RFX1 dimer. By contrast, the X box shows a half-site binding, where the single EP-homologous half-site is contacted by a subunit of the RFX1 dimer.

RFX1, the first cloned member of the RFX family (24), is a ubiquitously expressed protein (26). RFX1 was shown to increase transcription from the HBV enhancer and from NREy, another EP-homologous site present in the HBV genome, and to play a role in the induction of MHC class II genes by interferon-γ (9, 25, 31, 32). Previously, we have characterized an activation domain, containing a glutamine-rich region, at the N-terminal part of RFX1 and a C-terminal repression domain, overlapping the dimerization domain (33). These functional

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The abbreviations used are: HBV, hepatitis B virus; MIE, myc intron element; MHC, major histocompatibility complex; DBD, DNA-binding domain; HA, hemagglutinin; WT, wild-type; EDD, extended dimerization domain.

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regions can mutually neutralize each other’s effect, resulting in a nearly inactive transcription factor. The extreme N terminus of RFX1 contains a proline-rich region, which mediates an interaction with another EP-associated protein, the nuclear tyrosine kinase c-Abl, leading to the activation of c-Abl (34–36). Although multiple activities have been assigned for RFX1, its physiological role has not been clearly determined. Since no RFX1-deficient cell line is presently available, analyses of its transcriptional activity have been performed by overexpression or introduction of antisense DNA and may not truly reflect the full activity of the endogenous RFX1. The context-dependent transcriptional activity of its binding site suggests that RFX1 does not function merely as a classical activator and that a more complex mechanism, perhaps involving functional interactions with other DNA-bound proteins, determines its activity.

In order to gain more insight into the function of RFX1, we have analyzed DNA-protein complexes generated in cell extracts, thus identifying a novel EP complex. This complex, designated a*, contained RFX1 but was distinguished from those previously described by its extremely low mobility and its generation only with palindromic DNA sites. The formation of complex a* depended on the conserved B-C-D region, yet partial B-C-D deletions increased the relative abundance of complex a*. Complex a* formation correlated with transcriptional repression, both activities being performed by several B-C-D subregions. The B-C-D region also mediated the dimerization of complexes a and a*, yet our results suggest that the nature of the intersubunit interaction is different in each complex. Thus, by participating in different types of homodimeric interactions, the dimerization domain may determine complex formation, in this way affecting the activity of RFX1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—Cells were cultured in Dulbecco’s modified Eagle’s minimal essential medium (Life Technologies, Inc.) containing 100 units/ml penicillin and 100 μg/ml streptomycin, supplemented with 5% fetal bovine serum. Transfection, by the calcium phosphate precipitation method, and analysis of luciferase activity were performed as described previously (33). For luciferase assays, 6-cm plates were transfected with 5–6 μg of DNA using 1 μg of a luciferase reporter plasmid, 1 μg of the SV2-β-galactosidase internal control plasmid, and an expression plasmid. The amount of SV2 elements and the total amount of DNA was kept constant in each experiment. The normalized luciferase activity of each plate was calculated by dividing the total amount of DNA was kept constant in each experiment.

**Plasmid Constructions**—All RFX1 derivative expression plasmids are based on pSG5RFX1, which expresses the RFX1 cDNA under the control of SV2 (9). HA-RFX1 derivative plasmids express proteins tagged at their N termini with the HA epitope, and their structure has been described (33). FL-RFX1 was constructed by replacing the HA epitope of HA-RFX1 with the FLAG epitope. The GAL4-RFX1 expression plasmids and the EP4-luciferase and TATA-luciferase reporters have been described (33). The mdm2-derived reporter plasmids contain an insertion of four HBV EP sites (M-EP4-Luc) or five GAL4 binding sites (M-G5-Luc) inside the mouse mdm2 gene promoter, between the p35 response element and the core promoter region (37).

**Gel Shift Analysis**—Whole-cell extracts were prepared by lysing 6-cm plates with 100 μl of buffer A (33). Subcellular fractionation was performed as described (33), except that nuclei were extracted in buffer A, HeLa cells were fractionated into nuclear (n) and cytoplasmic (c) fractions. Gel shift analysis was performed using the EP probe and either fraction, a mixture of these fractions (nc), or a whole-cell extract (t). The arrowheads indicate the position of complex a. Complex a* is marked by an asterisk. s indicates complexes supershifted by αRFX1. The free probe was allowed to run out of the gel in order to obtain a high resolution.

**RESULTS**

**Identification of a Novel DNA-Protein Complex**—To analyze the cellular EP complexes, we incubated freshly prepared cell extracts with two DNA probes: the palindromic HBV EP site and the MHC promoter X box, which contains only one EP-homologous half-site. Several slowly migrating bands were generated with both probes, as formerly observed (Fig. 1, lanes 1–3 and 5). The upper and most prominent one comigrated with the previously characterized complex a of HeLa cells nuclear extract (34) (data not shown), containing RFX1 homodimers (9, 26, 30). Interestingly, we also detected a previously uncharacterized low mobility complex, which migrated more slowly than complex a and appeared only with the EP probe (Fig. 1, lane 1). Extracts from several human and murine cell lines were tested, and the novel low mobility complex, which we designated a*, was generated in all cases (Figs. 1–3, 5, and 6, and data not shown). Complex a* was supershifted by an αRFX1 antiserum, as was complex a, while no supershift was obtained with a preimmune serum (Fig. 1, lanes 3 and 4), indicating that RFX1 is a component of both complexes. Complex a* appeared in the nuclear, but not in the cytoplasmic, fraction, as did complex a (Fig. 1, lanes 7–10). Taken together, these results point to the identification of a novel EP complex, which contains RFX1 but differs from complex a in its extremely low mobility and in its appearance only with the EP probe and not with the X box.

**DNA Binding Characteristics of Complex a**—The DNA binding kinetics of complex a*, as compared with those of complex a, were examined by performing on-rate and off-rate
experiments with the EP probe. In these analyses, complex a* behaved similarly to complex a (Fig. 2, A and B). In the on-rate experiment, both complexes were already detected 10 s after probe addition, suggesting that they exist as two distinct protein complexes in the extract prior to the addition of the DNA probe. The binding characteristics of complex a* were further examined by performing competition experiments, in which an excess of unlabeled competitor DNA (either EP or X box) was added to the binding reaction prior to the addition of the labeled EP probe (Fig. 2C). Surprisingly, the X box competed efficiently for the formation of both complexes a and a* (lanes 6–9), similar to the EP competitor (lanes 2–5), while a heterologous competitor DNA did not affect either complex (lane 10). The ability of the X box to compete for the formation of both complexes was also observed in off-rate experiments, in which the competitor was added after DNA-protein complexes with the labeled probe had been formed (Fig. 2B). These results suggest that, although the a* band cannot be detected with the X box probe (Fig. 1, lanes 1–5), the a* protein complex may interact with the X box DNA in solution; the resulting X box-bound a* is likely to be an unstable complex, which disintegrates or loses its low mobility form either in solution or during the electrophoresis.

Complex a* Formation by Overexpressed RFX1—Complex a* was further analyzed by overexpressing in cells the wild-type (WT) RFX1 protein, tagged at its N terminus with the HA epitope. The overexpressed RFX1 formed with the EP probe both complexes a and a* (Fig. 3B, lane 5) and with the X box probe only complex a (lane 7). The binding reaction, two supershifted bands appeared with the EP probe, corresponding to complexes a and a* (lane 6). Interestingly, the pattern of supershifted bands obtained with the X box probe was the same and included the upper supershifted complex a* (lane 8), indicating that the antibody allowed complex a* formation with the X box probe. This finding lends further support to the notion that a* generates an unstable complex with the X box DNA. αHA appears to stabilize this complex, allowing its detection with the X box probe as a supershifted band.

The Role of the B-C-D Region in Complex Formation—To delineate the RFX1 regions involved in the formation of complex a*, different deletions were introduced into the C-terminal part of RFX1, which contains the evolutionarily conserved regions B, C, and D, the last characterized as the dimerization domain (Fig. 3A). While the WT RFX1 formed both complexes a and a* with the EP probe and with MIE, a partially palindromic EP-homologous site found in the human c-myc gene (Fig. 3C, lanes 2 and 7), two RFX1 mutants harboring deletions within region D (ΔDI and ΔDII) generated only the upper complex a* (lanes 3, 4, 8, and 9). When the deletion was extended to include the whole B-C-D region, the resulting mutant (ΔBCD) generated a complex that was slightly lower than the endogenous complex a (lanes 5 and 10). The above complexes were then compared with those formed by the corresponding recombinant bacterially expressed proteins (Fig. 3D). The recombinant WT RFX1 formed with the EP probe a complex similar in mobility to complex a but did not generate the low mobility complex a* (lane 5), while the transfected WT RFX1 formed both complexes (lane 4). Therefore, complexes similar to those formed by recombinant RFX1 proteins can be characterized as belonging to the a type and not the a* type. The recombinant ΔDI mutant generated a similar complex a* (lane 7), in contrast to the transfected ΔDI, which generated only complex a* (lane 6). The recombinant ΔBCD mutant also formed complex a, yet here the complexes generated by the recombinant and transfected protein were similar (lanes 8 and 9), indicating that the complex formed by the ΔBCD mutant in cellular extracts belongs to the a type.

The ΔDI mutant, which generated only complex a* with the EP probe (Fig. 3B, lane 9), generated a smeary complex with the X box probe (lane 11); complex a was not detected with either probe. The above results suggested that the smeary complex bound to the X box is an unstable form of complex a*. αHA supershifted the complexes generated by ΔDI with the EP and X box probes to the same height, consistent with this sugges-
were transfected with expression plasmids of the wild-type HA-RFX1 (wt) extracts were analyzed with the EP, X box (consensus binding site for RFX1 homodimers (29) is shown below. Nucleotides matching the consensus sequence are myc sites used as probes are shown: the EP element of the HBV enhancer, the MIE site of the c-intronic gene, and the X box of the HLA-DRA gene. The consensus binding site for RFX1 homodimers (29) is shown below. Nucleotides matching the consensus sequence are boxed. B and C, HepSK1 cells were transfected with expression plasmids of the wild-type HA-RFX1 (wt) or the indicated deletion mutants or mock-transfected (mock). Cellular extracts were analyzed with the EP, X box (X), or MIE probe. αHA was added where indicated by a plus sign. D, the EP or X box probe was incubated with either HepSK1 cellular extracts containing transfected HA-RFX1 derivatives (c) or the corresponding recombinant bacterially expressed proteins (r). The position of complex a* is indicated by an asterisk. The black and gray arrowheads indicate the position of complex a generated by the WT RFX1 and ΔBCD, respectively. d, dimer. m, monomer.

Fig. 3. Gel shift analysis of EP complex formation by RFX1 derivatives. A (top), the structure of the HA-RFX1 and its deletion mutants lacking the indicated amino acids. The DBD and the conserved regions B, C, and D are shown. Bottom, the sequences of the EP-homologous binding sites used as probes are shown: the EP element of the HBV enhancer, the MIE site of the c-myc gene, and the X box of the HLA-DRA gene. The consensus binding site for RFX1 homodimers (29) is shown below. Nucleotides matching the consensus sequence are boxed. B and C, HepSK1 cells were transfected with expression plasmids of the wild-type HA-RFX1 (wt) or the indicated deletion mutants or mock-transfected (mock). Cellular extracts were analyzed with the EP, X box (X), or MIE probe. αHA was added where indicated by a plus sign. D, the EP or X box probe was incubated with either HepSK1 cellular extracts containing transfected HA-RFX1 derivatives (c) or the corresponding recombinant bacterially expressed proteins (r). The position of complex a* is indicated by an asterisk. The black and gray arrowheads indicate the position of complex a generated by the WT RFX1 and ΔBCD, respectively. d, dimer. m, monomer.

In Vivo Dimerization of RFX1—Region D has been previously characterized as the RFX1 dimerization domain by the use of in vitro translated proteins (25). To examine whether this region also mediates the interaction between RFX1 molecules in cellular extracts, we performed coimmunoprecipitation experiments using transfected RFX1 derivatives (Fig. 4A). The WT RFX1, either tagged with the FLAG epitope (FL-RFX1) or untagged, was cotransfected with various HA-tagged RFX1 derivatives (HA-RFX1). The latter were immunoprecipitated using αHA, and the coprecipitation of FL-RFX1 or RFX1 was examined by Western analysis with an αFLAG or an αRFX1 antibody, respectively. The WT HA-RFX1 efficiently coprecipitated FL-RFX1, while HA-tagged ΔDI did not (lanes 1–3). These results indicate that the interaction between RFX1 proteins in cellular extracts depends on region D, consistent with the results of the in vitro studies (25). The N-terminally deleted N2 also showed a strong interaction with RFX1 (lane 6). By contrast, with the ΔBC mutant, which lacks the B-C region but contains region D, only a faint band of the coprecipitated RFX1 was observed, demonstrating a relatively weak interaction between these two proteins (lane 5). These results indicate that region D is required but not sufficient for efficient dimerization in cellular extracts.

The interaction between RFX1 derivatives was also examined by transfecting into cells the WT RFX1 together with different portions of RFX1 fused to the GAL4 DBD (Fig. 4B). Immunoprecipitations were performed with an αGAL4 anti-
Fig. 4. Coimmunoprecipitation analysis of the interaction between RFX1 derivatives. A, 293T cells were transfected with HA-tagged, FLAG-tagged (FL), or untagged RFX1 derivatives, bearing the indicated deletions. Whole-cell extracts were subjected to immunoprecipitation (IP) with αHA, followed by Western analysis with αFLAG, αRFX1, or αHA. The black arrowheads indicate the position of the WT RFX1 and FL-RFX1. The HA-tagged RFX1 derivatives are marked by gray arrowheads. B, 293T cells were transfected with the WT RFX1 together with various GAL4-RFX1 fusions, containing the RFX1 residues shown in parentheses. The last construct is a derivative of G4-RFXC2, bearing the indicated deletion. Whole-cell extracts were subjected to immunoprecipitation with αGAL4, followed by Western analysis with αRFX1 or αGAL4. The black and gray arrowheads indicate the positions of the WT RFX1 and the GAL4-RFX1 fusions, respectively. The immunoglobulin heavy chain is marked by an open arrow. Molecular mass markers in kDa are shown. A black box represents the GAL4 DBD.

body, followed by Western analysis with αRFX1. A GAL4 fusion containing the whole C-terminal part of RFX1 (G4-RFXC) interacted strongly with RFX1, while G4-RFXC2, lacking regions B and C, showed little or no interaction (lanes 1 and 2). A deleted derivative of the latter fusion (G4-RFXC2Δ766–812) did not interact with RFX1 (lane 3). Thus, while region D is essential for full dimerization in cellular extracts, additional sequences, which apparently lie within the B-C region, are also required.

The Role of the Dimerization Domain in Complexes a and a*—We next examined the role of the B-C-D region in the formation of the EP DNA-protein complexes. The analysis of a-type complexes was performed by mixing together two different RFX1 derivatives, both of which either contained or lacked the B-C-D region (Fig. 5A). The two proteins formed a-type complexes with different mobilities; therefore, mixed complexes would be of intermediate mobility. The mixing was done either in vitro, i.e. by cotransfection of the two proteins, or in vivo, i.e. by performing separate transfections and mixing the cellular extracts. When two C-terminally intact RFX1 derivatives (ΔN1 and ΔN3) were mixed in vitro, a single complex of intermediate mobility appeared with both the EP and the X box probes (lanes 1–8). By contrast, no intermediate complex appeared upon in vitro mixing (lanes 9 and 10). These results indicate that complex a generated in cellular extracts by C-terminally intact RFX1 derivatives contains stably linked RFX1 dimers.

A similar analysis was performed with two RFX1 mutants lacking the B-C-D region (ΔN2BCD and ΔN3BCD, lanes 11–18). Here a single intermediate complex was generated with the EP probe upon both in vivo and in vitro mixing (lanes 15 and 17), indicating that these EP-bound complexes contain RFX1 dimers, yet the dimer subunits are not stably linked to each other. No intermediate complex was observed with the X box probe (lanes 16 and 18), indicating that the X box-bound complexes contain monomers of RFX1. Similar results with the EP probe are also shown in Fig. 5B (lanes 4–7). Taken together, these findings indicate that the B-C-D region mediates a stable
interaction between the two RFX1 subunits of complex a in vivo, consistent with the results of the in vitro studies (25).

This type of analysis could not be performed for complex a*, since such complexes formed by deleted RFX1 derivatives retain their low mobility (Fig. 3 and data not shown). Therefore, in order to examine the role of the dimerization domain in complex a, we tried to generate hybrid a/a* complexes. To this end, we mixed together two RFX1 derivatives, one that generates complex a and another that generates complex a* (Fig. 5B). The a-forming protein was ΔBCD, which lacks the dimerization domain and is therefore found in the form of free monomers. The a* forming proteins were the WT RFX1 and ΔDI. The formation of an intermediate a/a* complex with ΔBCD would require free monomers of a*; therefore, the ability to generate such a complex would indicate of the dimerization status of the a* forming protein. The ΔDI mutant generated an intermediate a/a* complex with ΔBCD, upon either in vivo or in vitro mixing (lanes 8 and 9), while the WT RFX1 formed no such complex (lanes 10 and 11). These results indicate that a* forming ΔDI proteins are in the form of free monomers, while a* forming WT RFX1 proteins exist as stably linked dimers (or higher order oligomers), which cannot form a hybrid a/a* complex with ΔBCD monomers. Similar results were obtained when ΔN2BCD was used as the a-forming protein, yet here the hybrid a/a* complex was more diffuse, apparently due to the N-terminal deletion of ΔN2BCD (lanes 12–15). This analysis indicates that the dimerization domain mediates a stable interaction between the RFX1 subunits of complex a*, as it does in complex a.

The Effect of Dimerization on DNA-binding Stability—In order to examine the effect of the dimerization domain on the stability of binding to EP DNA, off-rate experiments were performed with the transfected WT RFX1 and the dimerization-deficient mutants ΔDI and ΔBCD (Fig. 6). Both complexes a and a* generated by the WT RFX1 showed a relatively stable binding to the EP probe. The decay in the intensity of the complexes generated by the RFX1 mutants was significantly faster for both complex a formed by ΔBCD and complex a* formed by ΔDI. Therefore, the dimerization domain of RFX1 stabilizes the binding of complexes a and a* to EP DNA.

Complex a* Formation Correlates with Transcriptional Repression—A possible transcriptional effect of complex a* was examined by overexpressing RFX1 derivatives in transient cotransfections with a luciferase reporter plasmid containing four tandem copies of the HBV EP element. A similar reporter plasmid lacking the EP sites served as a control for the EP specificity of the observed effects. The WT RFX1 induced a very mild EP-dependent transcriptional activation (Fig. 7A). By contrast, the ΔBCD mutant, which was unable to generate complex a*, induced a substantial activation, consistent with previous observations (33). These results suggest that the activation function of RFX1 is counteracted by a transcriptional inhibitory activity of the conserved B-C-D region, which mediates complex a* formation. To examine the effect of this inhibitory region on the activation induced by another transcription factor, cotransfections were performed with a reporter plasmid containing an insertion of four HBV EP elements inside the p53-responsive mouse mdm2 gene promoter, between the p53 response element and the core promoter region (37). The normalized luciferase activity is presented relative to the baseline activity in the absence of a transfected RFX1 derivative. The results shown are the mean and S.D. of three independent experiments.

DISCUSSION

RFX1 belongs to a novel family of DNA-binding proteins, highly conserved in evolution from yeast to humans (28). Mutational analyses of RFX1 binding sites in viral and cellular genes (3, 4, 8–10, 14–17) have implicated RFX1 in transcriptional regulation. Overexpression and antisense experiments (9, 25, 31, 32), as well as the identification of independent activation and repression domains (33), have also suggested that RFX1 can function as a transcriptional regulator. However, since RFX1 is ubiquitously expressed in all tissues, and since no RFX1-deficient cells are presently available, direct evidence as to its physiological role is lacking and difficult to obtain. The evolutionarily conserved regions of RFX1, some of which have already been functionally characterized (25, 29,
The evolutionarily conserved regions of RFX1 include the DBD as well as regions B, C, and D (Fig. 8), which overlap the repression domain (33). Region D was characterized as a dimerization domain by the use of in vitro translated proteins (25). By using RFX1 derivatives expressed in cells, we show that region D is required for in vivo dimerization; however, efficient dimerization in vivo required additional sequences within the B-C region (Fig. 4). Therefore, we propose to designate the in vitro defined dimerization domain, or region D, the “minimal dimerization domain,” while the region sufficient for full dimerization in vivo will be termed the “extended dimerization domain” (EDD). The N-terminal boundary of the EDD is yet to be defined, but dimerization is likely to depend on the conserved sequences of regions B or C, implying that RFX proteins possess a split dimerization domain. Region B appears in RFX proteins from Saccharomyces cerevisiae, Schizosaccharomyces pombe, and higher organisms up to humans, while regions C and D appear together in RFX proteins from S. pombe to humans but not in the S. cerevisiae protein (Fig. 8). The distance between regions C and D is the same in RFX1 and the S. pombe RFX homologue Sak1, suggesting these two regions function together, while the distance between regions B and C is different in the two proteins. Therefore, regions C and D are likely to constitute the EDD.

The novel complex a* has several unique properties, distinguishing it from the previously characterized EP complexes. One apparent feature of complex a* is its extremely low mobility, which positions it above complex a. This altered mobility and the inability of the bacterially expressed RFX1 to generate complex a* (Fig. 3D) suggest that additional molecules besides RFX1 may be present in this complex. On the other hand, the fact that the relative abundance of complex a* is maintained when RFX1 is highly overexpressed indicates that this complex does not contain an additional component whose concentration is limiting for complex a* formation. By labeling cells with [35S]methionine and immunoprecipitating the overexpressed HA-RFX1 with αHA, we have not been able to detect any protein that stably interacts with RFX1 (data not shown), arguing against the existence of such a protein as an essential component of complex a*. While such negative data cannot completely rule out the possibility of additional components, it is more likely that the difference between complexes a and a* lies in RFX1 itself, which may oligomerize or assume a different form in complex a*. An altered form of RFX1 is consistent with the different DNA binding characteristics of complexes a and a* and with the effect of a specific antibody on complex a* formation with the X box probe. If this is indeed the case, the inability of the bacterial RFX1 to form complex a* is probably due to the requirement for a specific modification or conformation of RFX1, which occurs only in eukaryotic cells.

Another unique property of complex a* is its formation only with the palindromic EP and MIE probes and not with the X box half-site, in contrast to all of the other cellular EP complexes, which are clearly detected with both types of probes. However, the ability of the unlabeled X box to compete efficiently for complex a* formation (Fig. 2, B and C) and the generation of complex a* with the X box probe in the presence of αHA (Fig. 3B) suggest that the a* protein complex may interact with the X box DNA, generating a relatively unstable X box-bound a*, which disintegrates or loses its low mobility form during the electrophoresis. Since αHA is a monoclonal antibody, which can bind a single epitope on each HA-tagged subunit of an RFX1 dimer, this antibody may stabilize complex a* by bridging between its two subunits, as observed with the dimerization-deficient ABCD mutant (Fig. 3B). Thus, palindromic DNA sites and αHA may stabilize complex a* in a similar way, by interacting simultaneously with the two RFX1 subunits. This interaction could increase the association between the subunits or orient them in a certain position with respect to each other, resulting in a stabilized complex.

In cell extracts, RFX1 molecules are mainly found in the form of stably linked dimers, and in both complexes a and a*, this interaction is mediated by the EDD (Fig. 5). By contrast, the bacterial RFX1 cannot dimerize efficiently (Fig. 3D), suggesting that dimerization is not a simple process but rather depends on a specific folding or modification of RFX1. In most dimeric transcription regulators, the dimerization domain is found adjacent to the DBD, and dimerization is essential for binding DNA. RFX proteins are an exception, since their EDD is not required for binding either the EP element (Figs. 3, 5, and 6) or the X box (25), suggesting that it may have an alternative role. Our off-rate experiments demonstrate the effect of the EDD in stabilizing the binding of complexes a and a* to EP DNA (Fig. 6), yet it may have additional functions. This unusually long and complex dimerization domain could serve to generate multiple complexes via its different subregions. An analysis of RFX1 deletion mutants demonstrated that the B-C-D region, containing the EDD, is essential for complex a* formation. Partial deletions within the B-C-D region appeared to affect complex a*, in that the low mobility band often assumed a diffuse appearance, yet only deletion of the whole region abolished this complex (Fig. 3 and 6 and data not shown), suggesting that several partially redundant sequences within the B-C-D region can support a* formation. Interestingly, partial B-C-D deletions resulted in the exclusive formation of the low mobility complex a*. The contrasting effects of the different deletions suggest a dual role for the B-C-D region, which on one hand is essential for complex a* formation and on the other hand seems to exert an inhibitory effect on the formation of this complex. Thus, an interplay between complexes a and a*, mediated by the EDD, may exist, according to the model shown in Fig. 9.

This model takes into account the two properties found in the B-C-D region: dimerization (which requires an intact EDD) and the ability to support complex a* formation (for which parts of the EDD are sufficient). Each of the EP complexes, a and a*, contains two RFX1 molecules dimerized via the EDD, yet in each type of complex the EDD is found in a different form or conformation. In the previously characterized complex a, it is
the strongly dimerized complex a to the more weakly dimerized complex a\(a\)\(n\), or vice versa, may occur upon modification of RFX1 or interactions with regulatory molecules. A possible regulator is the c-Abl tyrosine kinase, shown to interact with RFX1 (36); however, complex a\(a\)\(n\) was detected in extracts of mouse fibroblast c-Abl knockout cells (data not shown), indicating that c-Abl is not an essential component of this complex. Complex formation may also be determined by interactions with proteins bound to adjacent DNA sites, consistent with the context-dependent transcriptional activity of the EP element (4, 18, 19, 38).

The formation of two different types of interactions between the subunits of a protein dimer has been shown for heterodimers of the retinoic acid receptor and retinoic X receptor, where the interactions, formed by the two DBDs, determine cooperative binding to specific DNA sites (39, 40). Dimerization is also an important determinant for the function of the transcription regulator Kruppel, which activates transcription in the form of a monomer but acts as a repressor when present as a homodimer (41, 42). This functionally important switch in dimerization status is distinct from that proposed for RFX1, since both complexes a and a\(a\)\(n\) seem to contain RFX1 dimers, the difference between them being in the nature or strength of the intersubunit interaction. The ability of a transcription regulator to generate different DNA-protein complexes is likely to affect its activity. In the case of Kruppel, the transcriptional repression function is mediated by its dimerization domain, which, upon homodimerization, interacts with the basal transcription machinery to inhibit transcription (41, 42). Similarly, the transcriptional repression domain of RFX1 overlaps the EDD (33). The involvement of the EDD in both transcriptional repression (Fig. 7) and complex a\(a\)\(n\) formation (Fig. 3) suggests a functional link between these two activities. Transcriptional repression, like complex a\(a\)\(n\) formation, seems to be mediated by several B-C-D subregions in a partially redundant manner. Moreover, both activities were also observed by examination of chimeric proteins composed of different B-C-D portions fused to the GAL4 DBD (Ref. 33 and data not shown). Thus, the B-C-D region appears to be composed of several partially redundant subregions, each of which can support complex a\(a\)\(n\) formation and transcriptional repression independently of the others, while full activity requires their combined action. The colocalization of the regions mediating complex a\(a\)\(n\) formation and transcriptional repression supports the notion that the transcriptional inhibitory activity of RFX1 is mediated by the formation of the low mobility complex. Since these activities are performed by evolutionarily conserved regions, such a phenomenon may not be unique to RFX1 but rather a common property of RFX proteins involved in different biological systems.

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REFERENCES
1. Ostapchuk, P., Diffley, J. F., Bruder, J. T., Stillman, B., Levine, A. J., and Hearing, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8550–8554
2. Ben-Levy, E., Faktor, O., Berger, I., and Shaul, Y. (1989) Mol. Cell. Biol. 9, 1804–1809
3. Ostapchuk, P., Scheirle, G., and Hearing, P. (1989) Mol. Cell. Biol. 9, 2787–2797
4. Dikstein, R., Faktor, O., Ben-Levy, R., and Shaul, Y. (1990) Mol. Cell. Biol. 10, 3682–3689
5. Zajac-Kaye, V., Mantovani, R. M., Canedoia, S. M., Dorn, A., Staub, A., Lisowska-Groszperre, B., Griselli, C., Bensist, C. O., and Mathis, D. J. (1991) J. Immunol. 146, 3197–3204
6. Siegrist, C. A., Durand, B., Emery, P., David, E., Hearing, P., Mach, B., and Reith, W. (1993) Mol. Cell. Biol. 13, 6375–6384
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10. Safrany, G., and Perry, R. P. (1993) Gene 132, 279–283
11. Safrany, G., and Perry, R. P. (1995) Eur. J. Biochem. 230, 1066–1072
12. Labrie, C., Lee, B. H., and Mathews, M. B. (1995) Nucleic Acids Res. 23, 3732–3741
13. Lee, B. H., Liu, M., and Mathews, M. B. (1998) J. Virol. 72, 1138–1145
14. Tsang, S. Y., Nakanishi, M., and Peterlin, B. M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8598–8602
15. Tsang, S. Y., Nakanishi, M., and Peterlin, B. M. (1990) Mol. Cell. Biol. 10, 711–719
16. Bolwig, G. M., and Hearing, P. (1991) J. Virol. 65, 1884–1892
17. David, E., Garcia, A. D., and Hearing, P. (1995) J. Biol. Chem. 270, 8353–8360
18. Weissinger, G., Remmers, E. F., Hearing, P., and Marcu, K. B. (1988) Oncogene 3, 635–646
19. Reinhold, W., Emens, L., Itkes, A., Blake, M., Ichinose, I., and Zajac-Kaye, M. (1995) Mol. Cell. Biol. 15, 3041–3048
20. Blake, M., Niklinski, J., and Zajac-Kaye, M. (1996) J. Virol. 70, 6060–6066
21. Supakar, P. C., Zhang, X. Y., Githens, S., Khan, R., Ehrlich, K. C., and Ehrlich, M. (1999) Nucleic Acids Res. 17, 8611–8629
22. Garcia, A. D., Ostapchuk, P., and Hearing, P. (1991) Virology 182, 857–860
23. Zhang, X. Y., Jabrane-Ferrat, N., Asiedu, C. K., Samac, S., Peterlin, B. M., and Ehrlich, M. (1999) Mol. Cell. Biol. 13, 6810–6818
24. Reith, W., Barras, E., Satola, S., Kehr, M., Reinhart, D., Herrero-Sanchez, C., and Mach, B. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4200–4204
25. Reith, W., Herrero-Sanchez, C., Kehr, M., Silacci, P., Berte, C., Barras, E., Fey, S., and Mach, B. (1990) Genes Dev. 4, 1528–1540
26. Reith, W., Ucla, C., Barras, E., Gaud, A., Durand, B., Herrero-Sanchez, C., Kehr, M., and Mach, B. (1994) Mol. Cell. Biol. 14, 1230–1244
27. Wu, S. Y., and McLeod, M. (1995) Mol. Cell. Biol. 15, 1479–1488
28. Emery, P., Durand, B., Mach, B., and Reith, W. (1996) Nucleic Acids Res. 24, 803–807
29. Emery, P., Strubin, M., Hofmann, K., Bucher, P., Mach, B., and Reith, W. (1996) Mol. Cell. Biol. 16, 4486–4494
30. Herrero-Sanchez, C., Reith, W., Silacci, P., and Mach, B. (1992) Mol. Cell. Biol. 12, 4576–4583
31. Siegrist, C. A., and Mach, B. (1993) Eur. J. Immunol. 23, 2903–2908
32. Buckwold, V. E., Chen, M., and Ou, J. H. (1997) Virology 227, 515–518
33. Katari, Y., Agami, R., and Shaul, Y. (1997) Nucleic Acids Res. 25, 3621–3628
34. Diukstein, R., Heftetz, D., Ben-Neriah, Y., and Shaul, Y. (1992) Cell 69, 753–757
35. Diukstein, R., Agami, R., Heftetz, D., and Shaul, Y. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2387–2391
36. Agami, R., and Shaul, Y. (1998) Oncogene 17, 1779–1788
37. Ori, A., Zauberman, A., Deitsh, G., Paran, N., Oren, M., and Shaul, Y. (1998) EMBO J. 17, 544–553
38. Garcia, A. D., Ostapchuk, P., and Hearing, P. (1993) J. Virol. 67, 3940–3950
39. Zechel, C., Shen, X. Q., Chambon, P., and Gronemeyer, H. (1994) EMBO J. 13, 1414–1424
40. Zechel, C., Shen, X. Q., Chen, J. Y., Chen, Z. P., Chambon, P., and Gronemeyer, H. (1994) EMBO J. 13, 1425–1433
41. Sauer, F., and Jackle, H. (1993) Nature 364, 454–457
42. Sauer, F., Fondell, J. D., Okkumsu, Y., Roeder, R. G., and Jackle, H. (1995) Nature 375, 162–164
43. Steinle, V., Durand, B., Barras, E., Zufferey, M., Hadam, M. R., Mach, B., and Reith, W. (1995) Genes Dev. 9, 1021–1032