MOLECULAR AND CELLULAR BIOLOGY, Nov. 1997, p. 6653–6662 Vol. 17, No. 11
0270-7306/97/$04.00+0

Protein and DNA Contact Surfaces That Mediate the Selective Action of the Phox1 Homeodomain at the c-*fos* Serum Response Element

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Received 3 January 1997/Returned for modification 20 February 1997/Accepted 28 July 1997

The human homeodomain protein Phox1 can impart serum-responsive transcriptional activity to the c-*fos* serum response element (SRE) by interacting with serum response factor (SRF). This activity is shared with other Paired class homeodomains but not with more distantly related homeodomains. To understand the mechanism of action of Phox1 at the SRE and the basis for the selective activity of Paired class homeodomains in this context, we performed a detailed mutagenesis of the Phox1 homeodomain. We found that amino acid residues that contact the major groove of the DNA are required for SRE activation in vivo, suggesting an in vivo requirement for major-groove DNA contact by the homeodomain. In contrast, substitution of a lysine residue in the N-terminal arm of the Phox1 homeodomain appeared to abolish DNA binding without affecting activity in vivo. Certain substitutions on the exposed surfaces of helices 1 and 2, not required for DNA binding, abolished activity in vivo, suggesting that these surfaces contact an accessory protein(s) required for this activity. We also found that transfer of a single amino acid residue from the surface of Phox1 helix 1 to the corresponding position in the distantly related Deformed (Dfd) homeodomain imparts to Dfd the ability to activate the SRE in vivo. We propose that Phox1 interacts with one or more factors at the SRE, in addition to SRF, and that the specificity of this interaction is determined by residues on the surfaces of helices 1 and 2.

As a multicellular organism develops from a single cell, individual cells become progressively committed to particular differentiation pathways. These pathways ultimately lead to fully differentiated phenotypes. This process of differentiation requires the expression and repression of specific genes in different cell types at different times during development (26, 37, 45, 47, 56). Many of these cell type-specific genes are activated in response to extracellular signals (4, 11, 43, 50). These signals act through cell surface receptors and intracellular signal transduction cascades that relay signals to the nucleus. Although the components of these signal transduction cascades are similar in different cells, specificity is regained at the level of transcription by the expression of factors that direct signals to specific genes in cells of a given identity (2, 4, 19, 38).

Cell identity can be determined by one or a few regulatory factors in the cell. For example, homeotic mutations in single genes result in the mislocalization of entire body segments in the fruit fly (10, 60). Ectopic expression of homeotic genes results in the formation of normal structures at the site of ectopic expression (20, 24, 25, 60). These experiments demonstrate that the presence of a single protein in a cell can be both necessary and sufficient to specify the fate of an entire set of cell lineages. Many of the regulatory factors that play homeotic roles in development are transcription factors (10, 29, 36, 46). These proteins are therefore likely to be activating the genes that are unique to the developmental pathways of specific cell types.

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The homeodomain is a 61-amino-acid DNA-binding domain originally identified as a conserved region in the products of *Drosophila* homeotic genes (35). Homeodomain proteins play a major role in gene regulation and development. The homeodomain consists of an N-terminal arm and three α-helices. Helix 3 makes extensive contacts in the major groove of the DNA. Helices 1 and 2 are separated by a short loop and lie above and perpendicular to helix 3, where they are in position to make contact with other proteins. The N-terminal arm is unstructured in solution, but upon binding of the homeodomain to DNA, it makes phosphate and base contacts in the minor groove (27, 28, 42, 58, 59).

Although the homeodomain has a highly conserved tertiary structure, the biological activities of homeoproteins are diverse. The homeodomain alone binds DNA with rather low affinity and sequence specificity in vitro (21, 44, 46). Nevertheless, in many cases, the homeodomain is entirely responsible for the biological specificity of the protein in which it resides (6, 13, 18, 31, 33). This suggests that the homeodomain likely interacts with other factors to either increase the complexity of its DNA recognition or cooperate discriminately with other specificity-providing factors.

One class of proteins that interacts with homeodomains is the MADS (Mcm1-Agamous-Deficiens-SRF) box family of transcription factors (7, 18, 48). Members of this family have a homologous sequence which forms part of a unique DNA-binding and dimerization domain. In plants, MADS box proteins have been shown to play a homeotic role in flower development (34, 56). In mammalian cells, the MADS box protein serum response factor (SRF) mediates the signal-responsive transcription of immediate early genes such as c-*fos*, the β-actin gene, *junB*, and others (53, 54). While SRF has a transcriptional activation domain, it is generally believed that it activates transcription by recruiting accessory proteins to the

serum response element (SRE). These accessory proteins activate transcription in response to extracellular signals such as growth and differentiation factors (16, 22, 23). Members of the MADS box family of transcription factors have been shown to interact with homeodomain-containing proteins. The yeast homeodomain protein Matα2 interacts with the yeast MADS box protein Mcm1 to repress a-specific genes (48). In mammals, the Phox1 and Nkx 2.5 homeodomains interact with SRF in vitro and in vivo (7, 18). These examples represent the crossroads of two families of proteins that play fundamental roles in development by potentially connecting cell identity to the signal transduction apparatus.

Phox1 was originally isolated in a screen to identify human proteins that could cooperate with Mcm1 to activate a cell type-specific pheromone-inducible reporter gene in yeast. Phox1 has several additional activities. It permits the serum induction of an SRE-driven reporter gene in a transient transfection assay in HeLa cells. It enhances the rate of binding of SRF to the SRE in vitro and does so in the absence of detectable Phox1 DNA binding, suggesting a direct interaction between SRF and Phox1. It also forms a stable higher-order complex with Mcm1 on the DNA when expressed in *Saccharomyces cerevisiae*. This complex cannot be reconstituted with purified Phox1 and Mcm1, suggesting that another factor is required for stable complex formation (17, 18). The homeodomain with small amounts of flanking sequence is sufficient for all known functions of Phox1. These activities are shared with other homeodomains of the Paired class, including Paired (Prd) and Orthodenticle, but not with more distantly related homeodomains.

We wished to investigate the structural basis of the specificity of homeodomain action at the SRE. We first performed fine mapping of functional sites of Phox1 by alanine scanning the exposed surfaces of the homeodomain. We also made chimeras between Phox1 and the unrelated homeodomains Dfd and fushi taratzu (Ftz). In doing so, we have identified two to three functionally distinct regions of the Phox1 homeodomain. This analysis suggests that major-groove DNA contacts by the homeodomain and protein-protein interactions mediated by helices 1 and 2 of the homeodomain are required for activation of the SRE. Finally, we wanted to determine whether it would be possible to impart to a non-Prd-class homeodomain the ability to activate the SRE in an SRF-dependent fashion. We demonstrate that the transfer of a single amino acid from helix 1 of Phox1 to the Dfd homeodomain imparts to Dfd the ability to activate the SRE.

MATERIALS AND METHODS

Plasmid constructions. For transient transfection assays with HeLa cells, the reporter plasmid contained a single copy of the c-*fos* SRE positioned upstream of a c-*fos*–chloramphenicol acetyltransferase (*c*-fos–CAT) fusion gene containing mouse c-*fos* sequence from −56 to +109 (3, 14). Fragments of Phox1 encompassing either amino acids 71 to 156 (see Fig. 1 to 3) or 76 to 156 (see Fig. 4 and 5) were expressed from the cytomegalovirus-based vector pCGN (51). These fragments include the Phox1 homeodomain, either 18 or 23 N-terminal amino acids, and 3 C-terminal amino acids. A fragment of Dfd encompassing amino acids 358 to 438 was also expressed from the pCGN vector. This fragment includes the Dfd homeodomain, 18 N-terminal amino acids, and 3 C-terminal amino acids. In addition, the vector incorporates an N-terminal extension with the sequence MASSYPYDVPDYASLGGPSRM, which includes the influenza hemagglutinin epitope tag (underlined). An analogous 82-amino-acid fragment of the Drosophila Ftz protein was produced from the same vector.

Site-directed mutagenesis of the Phox1 and Dfd cDNAs was performed with single-stranded DNA derived from the pCGN expression plasmids (30). The sequences of the oligonucleotides used for mutagenesis are available upon request. The Dfd RLHY, RLIE, RLHT, and RLIEHT mutants were constructed by substituting 107-bp *Xba*I-*Bsr*I fragments from the H21E;Y22R, I32E;E33D, H36R;T37R, and IEHT mutants, each constructed by site-directed mutagenesis, with a 107-bp *Xba*I-*Bsr*I fragment from the R10S;L14Q mutant, also generated by site-directed mutagenesis. The RLHYIEHT mutant was constructed by substituting a 145-bp *Xba*I-*Mlu*I fragment from the IEHT mutant with a 145-bp *Xba*I-*Mlu*I fragment from the RLHY mutant. The sequences encoding the E32A;D33A, R36A;R37A, and N51Q mutants of Phox1 have been described previously (18, 19). The sequences of all constructs were verified by DNA sequencing.

Transient transfection assay. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All transfections were performed using calcium phosphate coprecipitation as described previously (19). Cells were seeded on 10-cm-diameter plates and transfected at 30% confluence. Transfection cocktails contained 3 μg of reporter plasmid and 2.4 μg of Phox1 expression plasmid, unless otherwise indicated. In all cases, the total DNA concentration was adjusted to 20.8 μg with pUC119 DNA. The cells were incubated with the transfection precipitates for 16 h, washed three times with phosphate-buffered saline, and incubated for 24 h in DMEM containing 0.5% FBS to achieve serum starvation. The serum-starved cells were treated with DMEM containing 15% FBS for 8 h. After serum treatment, the cells were washed three times with phosphate-buffered saline, harvested, and resuspended in 150 μl of IP buffer (0.1% Nonidet P-40, 25 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The resuspended cells were disrupted by sonication with two 2-s pulses. A 20-μl aliquot was mixed with an equal volume of 2× sodium dodecyl sulfate loading buffer to prepare whole-cell extracts for immunoblots. The remaining sonicated cell extracts were centrifuged for 20 min to remove cell debris. The protein concentration of the extract was determined by the Bradford method (5).

CAT assays. Extract containing 50 μg of protein was assayed for 30 min for CAT activity by standard procedures and quantified with a Fuji BioImage analyzer (15). The results are displayed as the percentage of acetylated chloramphenicol for each point relative to the value for wild-type Phox1 within a single experiment (+ standard deviation). Because the Phox1 homeodomain activates the internal controls that we attempted to use for transfection efficiency, we normalized each experiment for equal expression of the homeodomain fragment by performing Western blotting (immunoblotting) against the hemagglutinin epitope tag with the 12CA5 monoclonal antibody. Each experiment has been repeated at least three and up to ten times.

DNA binding assays. We used 2 μl of the whole-cell extract for mobility shift assays of transfected cells. Assay mixtures contained 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 5% glycerol, and 50 μg of poly(dI-dC) · (dI-dC) per ml in a final volume of 20 μl. Reaction mixtures were incubated in the absence of probe for 15 min at room temperature; the probe (20,000 cpm) was added, and the reaction mixtures were incubated for an additional 15 min. Analysis was on 5% polyacrylamide gels (39:1 acrylamide/bis ratio) in 0.5× Tris-borate-EDTA buffer. The double-stranded oligonucleotide probe used for mobility shift assays was the PM58 SRE with the sequence GATCCACAGGAT GTCCTAATTAGGACATCTGCGT. The probe was prepared by filling in 5′ overhangs within the oligonucleotide with Klenow polymerase in the presence of ^{32}P-labeled nucleoside triphosphates.

RESULTS

Phox1 and Prd, another member of the Prd homeodomain class, but not more distantly related homeodomains can activate a reporter gene carrying the c-*fos* SRE in transient transfection assays in HeLa cells (19). To determine what surfaces of the Phox1 homeodomain are required for this activity, we have constructed a series of Phox1 mutants targeting amino acids that are predicted to lie on exposed surfaces of the homeodomain (Table 1). These constructs were cloned into a mammalian expression vector and transiently cotransfected into HeLa cells with a CAT reporter gene driven by a single SRE. Twenty-four hours after transfection, cells were serum starved for 16 h and then serum stimulated for 8 h. The cells were harvested, and a whole-cell lysate was prepared. We used this lysate to assay CAT activity as a measure of transcriptional activation of the SRE, for immunoblotting against an epitope tag to assess expression of the homeodomain fragments and transfection efficiency, and for gel mobility shift assays to measure the ability of mutant proteins to bind the SRE.

Major-groove interactions are required for activation of the SRE. We have previously shown that Phox1 exhibits specific sequence requirements within the core of the SRE for activity in vivo (19). Furthermore, we have also observed that a substitution in a conserved asparagine residue (N51Q) that makes major-groove DNA contacts in all known homeodomain structures abolishes Phox1 activity in vivo (18). To determine the

TABLE 1. Summary of Phox1 and Dfd mutants used in this study

Homeodomain no. and mutation name[a]	Location[b]	Transcriptional activity[c]	DNA binding[c,d]
1. Wild-type Phox1	NA	++++	++++
2. Phox1 K-1A;R2A	N-term	++++	−
3. Phox1 K-1E	N-term	++++	−
4. Phox1 R2A	N-term	++	+++
5. Phox1 R3A	N-term	+++	+++
6. Phox1 N4C	N-term	+++	+++*
7. Phox1 R5A	N-term	−	−
8. Phox1 T6A	N-term	++	++++
9. Dfd N-term/Phox1 helices 1 to 3	N-term chimera	++++	++++*
10. Wild-type Dfd	NA	−	++++*
11. Phox1 N-term/Df helices 1 to 3	N-term chimera	−	++++*
12. Phox1 E17A	Helix 1	−	++++*
13. Phox1 E17Q	Helix 1	+	++++*
14. Phox1 E21A;R22A	Helix 1	++++	++++*
15. Phox1 E21H;R22Y	Helix 1	++	ND
16. Phox1 E21H	Helix 1	+	ND
17. Phox1 R22Y	Helix 1	+++	ND
18. Phox1 H24A	Helix 1/2 loop	+	++++*
19. Phox1 Y25A	Helix 1/2 loop, major groove	−	+
20. Phox1 R31A	Major groove	−	−
21. Phox1 E32A;D33A	Helix 2	−	++++*
22. Phox1 E32A	Helix 2	++++	++++*
23. Phox1 D33A	Helix 2	++	++++*
24. Phox1 R36A;R37A	Helix 2	−	++++*
25. Phox1 R36A	Helix 2	++++	++++
26. Phox1 R36E	Helix 2	++	++++*
27. Phox1 R37A	Helix 2	++++	++++*
28. Phox1 R44A	Major groove	++++	++++
29. Phox1 Q50C	Major groove	+++	++*
30. Phox1 Q50K	Major groove	++	+*
31. Phox1 Q50S	Major groove	++++	++++*
32. Phox1 N51Q	Major groove	−	−
33. Dfd R10S;L14Q	Helix 1	−	ND
34. Dfd H21E;Y22R	Helix 1	++++	ND
35. Dfd H21E	Helix 1	−	ND
36. Dfd H21A	Helix 1	−	ND
37. Dfd Y22R	Helix 1	++	ND
38. Dfd Y22A	Helix 1	−	ND
39. Dfd I32E;E33D	Helix 2	−	ND
40. Dfd H36R;T37R	Helix 2	−	ND
41. Dfd RLHY	Helix 1	++++	ND
42. Dfd RLIE	Helces 1 and 2	−	ND
43. Dfd RLHT	Helces 1 and 2	+++	ND
44. Dfd IEHT	Helix 2	++++	ND
45. Dfd RLIEHT	Helces 1 and 2	++++++	ND
46. Dfd RLHYIEHT	Helces 1 and 2	++++++	ND

[a] The homeodomain numbering scheme (1 to 46) is that used in structural studies. Mutation names are derived from the identity and position of the wild-type residue followed by the substituted residue. N-term, N-terminal.

[b] Major groove, contact site in the DNA. Residues that contact the major groove can be in the loop between helices 1 and 2 (Helix 1/2 loop), in helix 2, or in helix three. NA, not applicable; N-term, N- terminal arm.

[c] −, no activity.

[d] ND, not done. Asterisk indicates data not shown.

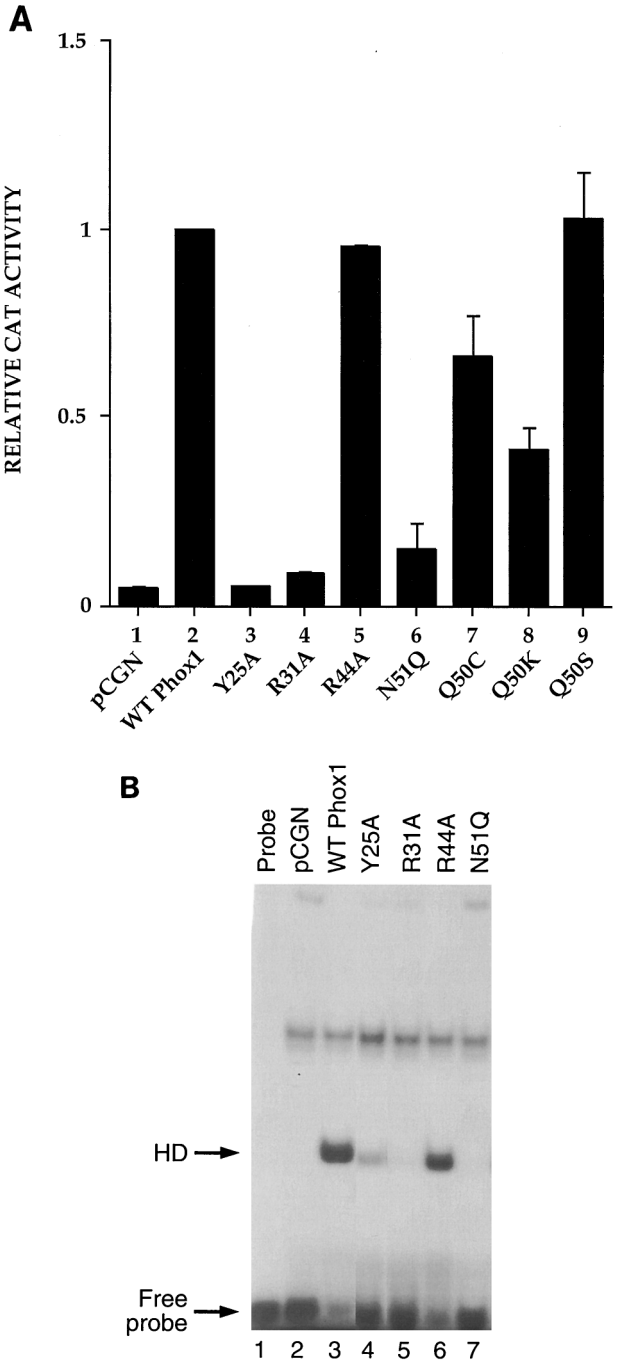

FIG. 1. Interaction with the major groove of the DNA is required for Phox1 activity at the SRE. (A) Mutants of Phox1 containing substitutions in residues that contact the major groove of the DNA were assayed for CAT activity as described in the legend to Fig. 2. Only one mutation predicted to affect DNA binding affinity, R44A (lane 5), retained the ability to activate the SRE. Mutations of Phox1 residues that were predicted to modify DNA sequence specificity (lanes 7 to 9) were also able to activate the SRE to various degrees. (B) Phox1 major-groove contact mutants were assayed for their ability to bind DNA as described in the legend to Fig. 2. Mutant R44A (lane 6) bound the probe at levels similar to that of wild-type Phox1 (lane 3). Mutants R31A and N51Q failed to bind the probe in this assay (lanes 5 and 7, respectively), while DNA binding by mutant Y25A (lane 4) was greatly reduced as compared to that of wild-type Phox1.

extent to which interaction of the Phox1 homeodomain with the major groove is required for its activity at the SRE in vivo, we examined amino acid residues that, unlike N-51, are predicted to make contacts with the phosphate backbone rather than with base pairs (27, 28, 40, 58, 59). Two putative phosphate contact mutants, Y25A and R31A, exhibited significant reductions in activity in vivo, comparable to or more significant

than the previously characterized N51Q mutant (Fig. 1A, compare lanes 3 and 4 to lane 6). As predicted, all three of these mutants exhibited undetectable or greatly reduced DNA-binding activity in vitro (Fig. 1B, lanes 4, 5, and 7). In contrast, a mutant protein carrying an alanine substitution in another putative phosphate contact residue, R44A, was essentially fully active both in vivo and in vitro. Despite the highly conserved secondary structure possessed by virtually all homeodomains, this observation suggests either that R44 does not make a phosphate contact in the Phox1-DNA complex or that this contact is dispensable for DNA binding. Clearly, however, we observe a strict correlation between DNA binding and in vivo activity for mutant proteins carrying substitutions in residues predicted to contact both the phosphate backbone and base pairs in the major groove.

Uncoupling of DNA binding activity and in vivo activity by substitution of a lysine in the N-terminal arm. The N-terminal arm of the homeodomain of several homeotic selector genes, including that of Dfd, has previously been shown to determine the specificity of action of these proteins in vivo (6, 33, 60). Because Dfd is unable to activate the SRE in vivo and the homology between the N-terminal arms of Dfd and Phox1 is only 30%, we wished to determine whether the specificity of action at the SRE in vivo resides in the Phox1 N-terminal arm. We addressed this question by constructing chimeras between Phox1 and Dfd. When we replaced amino acids 1 to 8 of the N-terminal arm of Phox1 with those of Dfd, the resulting chimera retained full activity (Fig. 2A, lane 10). The reciprocal chimera, substituting the N-terminal arm residues of Phox1 onto Dfd, was unable to activate the SRE (Fig. 2A, lane 12). Similar results were obtained with the Ftz homeodomain (data not shown). Thus, the N-terminal arm of Phox1 is neither necessary for activation of the SRE nor sufficient to impart this activity to an unrelated homeodomain.

We next generated a series of single and double alanine substitutions in residues in the Phox1 N-terminal arm. Only a substitution in the highly conserved arginine residue at position 5 (R5A) had a significant effect on the ability of Phox1 to activate the SRE (Fig. 2A, lane 8). Other tested substitutions in the N-terminal arm had little or no effect. We also measured the ability of the N-terminal arm mutants to bind DNA (Fig. 2B). We found that, as expected, the R5A mutant lacked DNA-binding activity (Fig. 2B, lane 8), but surprisingly, mutants carrying substitutions in a lysine residue at position −1 also lacked activity (Fig. 2B, lanes 4 and 5). That this residue has never been directly implicated in DNA binding in known homeodomain structures makes this observation difficult to interpret. One possibility is that this residue is indeed required for formation of a binary Phox1-DNA complex in vitro, which would imply that such an interaction is not required for in vivo activity. Alternatively, this residue may simply be required for proper folding of the N-terminal arm in the Phox1-DNA binary complex but that it is dispensable for this function when in complex with SRF. In either case, however, this observation suggests that in the higher-order complex formed on the SRE in vivo, the N-terminal arm may not be in an identical position to that observed in structures of binary complexes.

Nature of the required contacts on Phox1 helices 1 and 2. Helices 1 and 2 of the homeodomain have been shown by crystallography to face away from the DNA and are in position to contact other proteins. We had shown previously that substitutions in three neighboring pairs of amino acids predicted to lie on these surfaces abolished activity in vivo without affecting DNA binding activity in vitro. Here, we attempted to increase the resolution of this analysis and to explore the

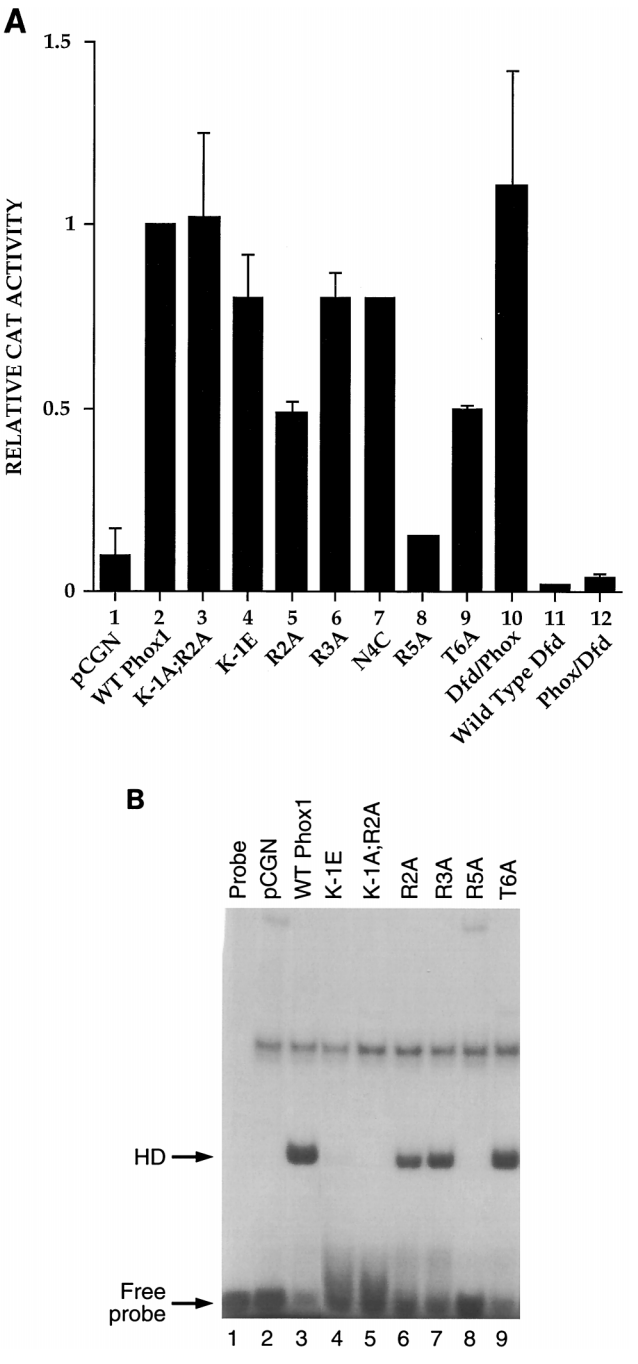

FIG. 2. Activity of Phox1 N-terminal arm mutants and chimeras between the Phox1 and Dfd homeodomains. (A) HeLa cells were cotransfected with a CAT reporter gene containing a minimal c-*fos* promoter driven by a single SRE and either an empty expression vector (lane 1) or expression vectors containing wild-type Phox1 (lane 2), wild-type Dfd (lane 11), Phox1 N-terminal arm mutants (lanes 3 to 9), Phox1 containing the N-terminal arm of Dfd (lane 10), and Dfd containing the N-terminal arm of Phox1 (lane 12). Whole-cell extracts were prepared and assayed for CAT activity. With the exception of the Phox1 R5A mutation, substitutions in the N-terminal arms of Phox1 and Dfd had little effect on their ability to activate transcription. All mutants, including R5A, which had no activity in any of our assays, were expressed at equivalent levels as determined by immunoblotting (data not shown). (B) Whole-cell extracts (10 mg of protein) from HeLa cells (lane 2), HeLa cells expressing wild-type Phox1 (lane 3), or Phox1 homeodomain N-terminal arm mutants (lanes 4 to 9) were assayed by a mobility shift assay for their ability to bind an SRE containing a high-affinity Phox1 homeodomain binding site. The ^{32}P-end-labeled probe is shown alone in lane 1. Mutants K−1E and K−1A;R2A failed to bind to the probe in this assay. These mutants did, however, activate transcription from the SRE.

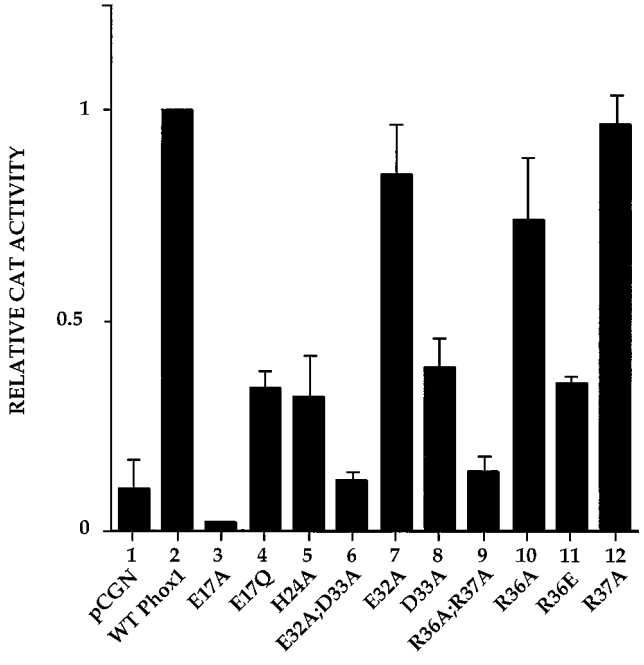

FIG. 3. Exposed surface residues are required for Phox1 activity at the SRE. Mutants of Phox1 containing substitutions in exposed surface residues were assayed for their ability to activate the SRE, as described in the legend to Fig. 2. Substitutions at position 17 of helix 1 (lanes 3 and 4), position 24 in the loop between helix 1 and 2 (lane 5), and positions 32, 33, 36, and 37 in helix 2 (lanes 6 to 12) reduced the ability of Phox1 to activate the SRE. See the text for discussion.

chemical nature of the contacts made by the exposed surfaces of the homeodomain.

A single substitution of alanine for the glutamic acid at position 17 gave the strongest phenotype of all the mutants examined (Fig. 3, lane 3), suggesting that this residue has a critical function in Phox1 activity in vivo. Changing this residue to glutamine restored partial activity (Fig. 3, lane 4), suggesting that the critical contact made by the glutamic acid residue at this position is not entirely ionic in nature.

In contrast, our data suggest that the contact(s) made by the two neighboring arginine residues (positions 36 and 37) requires a charged amino acid side chain. Whereas we had previously observed that a double alanine substitution at positions 36 and 37 led to a reduction in Phox1 activity in vivo (19) (Fig. 3, lane 9), either single substitution had no significant effect (Fig. 3, lanes 10 and 12). These observations suggested that Phox1 activity in vivo required a positively charged side chain in this vicinity, which could be provided redundantly by either arginine residue. To test this hypothesis, we replaced arginine 36 with glutamic acid, reasoning that the negative charge on this residue might locally neutralize the positive charge on arginine 37, thereby mimicking the effect of mutating both arginines to uncharged residues. Indeed, the R36E substitution was nearly as effective as the double alanine substitution at reducing Phox1 activity in vivo (Fig. 3, lane 11). Thus, we conclude that at least one ionic interaction with a putative accessory protein must be occurring on the surface of helix 2.

The third previously characterized double mutant altered two negatively charged residues, glutamic acid 32 and aspartic acid 33, on helix 2. This mutation significantly reduced Phox1 activity (19) (Fig. 3, lane 6). In this case, we observed that a single alanine substitution for glutamic acid 32 had no effect on Phox1 activity, whereas an alanine substitution for aspartic acid 33 reduced Phox1 activity by approximately 2.5-fold. Thus, in contrast to the double arginine mutant, these neighboring negatively charged residues did not apparently provide redundant charged contacts with a putative accessory protein interacting with the surface of helix 2.

Together, therefore, these data support the hypothesis that Phox1 must interact with another protein through the exposed surfaces of helices 1 and 2. Furthermore, they suggest that a critical, and probably nonionic, interaction is made by the glutamic acid side chain at position 17. They also identify aspartic acid 33 as an important contact point. Finally, they suggest the requirement for a positively charged side chain on helix 2, presumably in a salt bridge with an acidic side chain on another protein. The space-filling representation of the Prd homeodomain crystal structure shown below (see Fig. 6) demonstrates that these residues define surfaces positioned to contact other proteins in the complex and not the DNA (58).

A single amino acid substitution confers to Dfd the ability to activate the SRE. We have demonstrated that the N-terminal arm of the homeodomain of Phox1 does not play a role in specifying its action at the SRE relative to non-Prd-class homeodomains. This is in contrast to the *Drosophila* homeotic selector genes Dfd, Ubx, and Antp, whose functional specificity in vivo is determined by the N-terminal arm (6, 33). We have also found that residues on the exposed surfaces of helices 1 and 2 of the Phox1 homeodomain are required for its activity. We therefore wished to determine if these same surfaces also played a role in determining the specificity of homeodomain action at the SRE. To address this question, we transferred surface amino acids of helices 1 and 2 from Phox1 to Dfd to identify residues that might impart to Dfd the ability to activate the SRE.

We first assayed a series of substitutions where pairs of neighboring amino acids on the surface of helices 1 and 2 of Phox1 were transferred to Dfd (Fig. 4). Of these, we identified one double substitution, H21E;Y22R, that was sufficient to impart to Dfd the ability to activate the SRE to nearly the level of wild-type Phox1 (Fig. 4, compare lanes 5 and 2). This observation suggests that one or more contacts on the surface of helix 1 is sufficient to program a homeodomain for activation of the SRE. But this result was unexpected, since alanine substitutions at these positions did not affect Phox1 activity, suggesting that they were not necessary for activity in the context of the Phox1 homeodomain (Fig. 5, lane 4). Thus, we explored in greater detail the nature of the contacts at positions 21 and 22 and also addressed what other portions of the Phox1 surface might contribute to this activity.

Because alanine substitutions at positions 21 and 22 of Phox1 do not affect activity in vivo, one hypothesis to account for the gain of function observed in the Dfd H21E;Y22R mutant is that the histidine and/or tyrosine residues normally present in Dfd are inhibitory for activation of the SRE, perhaps because they prevent contact with a necessary accessory protein. To test this hypothesis, we substituted these positions in Phox1 with the corresponding Dfd residues. Indeed, this substitution reduced Phox1 activity by 60% (Fig. 5, lane 5). We then examined single substitutions at these two positions and found that a single substitution of histidine for glutamic acid at position 21 reduced Phox1 activity by more than 75% (Fig. 5, lane 6). Thus, a histidine residue, but not an alanine, at position 21 in helix 1 is strongly inhibitory for Phox1 activation of the SRE in vivo.

We next made the reciprocal single substitutions in Dfd. Replacement of histidine 21 in Dfd with either glutamic acid or alanine completely failed to activate Dfd (Fig. 5, lanes 9 and 10). In contrast, replacement of tyrosine 22 with arginine, the corresponding residue in Phox1, but not with alanine was suf-

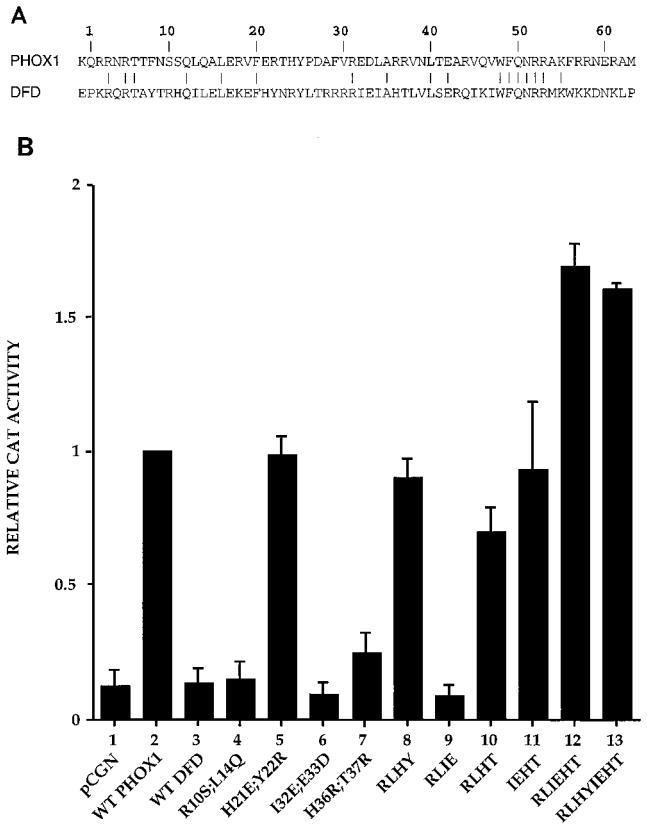

FIG. 4. (A) The amino acid sequences of the Phox1 and Dfd homeodomains are shown. The identity between these two protein domains is 30%. (B) Transferring surface amino acids from Phox1 to Dfd is sufficient to confer to Dfd the ability to activate the SRE. Substitutions were made in the Dfd homeodomain, creating mutants of Dfd with Phox1 surface amino acids. These mutants were assayed for the ability to activate the SRE as described in the legend to Fig. 2. Wild-type Phox1 (lane 2) activates the SRE, while the distantly related Dfd (lane 3) protein does not. The double mutation H21E;Y22R (lane 5) conferred to Dfd the ability to activate the SRE to levels similar to that of wild-type Phox1. The double mutations R10S;L14Q, I32E;E33D, and H36R;T37R had no effect on the ability of Dfd to activate the SRE (lanes 4, 6, and 7). When the H36R;T37R mutation was combined with the R10S;L14Q (RLHT) or the I32E;E33D (IEHT) mutation or both (RLIEHT), the resulting mutants were able to activate transcription at the SRE (lanes 10 to 12, respectively).

ficient for partial activation of Dfd (Fig. 5, lanes 11 and 12). Taken together, these data identify this small region of helix 1 as critical for determining the specificity of homeodomain action at the SRE. Furthermore, they indicate that the arginine at position 22 in Phox1 plays a positive role in imparting this activity to the homeodomain, whereas the histidine at position 21 in Dfd is strongly inhibitory for this activity.

Consistent with our finding that there are multiple points of contact between helices 1 and 2 of Phox1 with putative accessory proteins at the SRE, we found that additional modifications to the Dfd homeodomain can enhance its activity at the SRE. In particular, we observed that certain combinations of amino acid substitutions that had no activity on their own acted cooperatively to impart activity to the Dfd homeodomain. In some cases, the activity achieved by these mutants exceeded that of wild-type Phox1 (Fig. 4, lanes 10 to 13). We conclude that several contact points on the surface of helices 1 and 2 act together to specify the nature of the accessory protein interactions in homeodomain-containing complexes.

DISCUSSION

The Phox1 homeodomain forms an uncharacterized complex with SRF at the c-*fos* SRE that promotes serum-inducible transcriptional stimulatory activity in vivo. To begin to understand the organization of this complex, we have performed a systematic mutagenesis of the Phox1 homeodomain to address two key issues. The first is the role for homeodomain DNA recognition in formation of an active in vivo complex. The second is the source of the selective action of homeodomains of the Prd class in this assay.

Our studies strongly suggest that interaction of Phox1 with the DNA major groove is required for activity in vivo. While our previous work showed that mutation of a highly conserved asparagine residue that makes major-groove base-pair contacts abolished in vivo activity (19), here we have shown that phosphate contacts are also required. We observed a strong quantitative correlation between in vitro DNA binding and in vivo function in proteins carrying substitutions in these positions.

Our results with substitutions in the N-terminal arm of the Phox1 homeodomain, which interacts predominantly with the DNA minor groove, are more ambiguous. Substitution of a conserved arginine residue at position 5 abolished both DNA-binding activity in vitro and activity in vivo. This result, taken together with the observation that this residue makes a minor-groove contact in several known structures, suggests that minor-groove binding, like major-groove binding, is also required for the function of Phox1 in the in vivo complex. In contrast to this result, however, we observed that substitution of a lysine

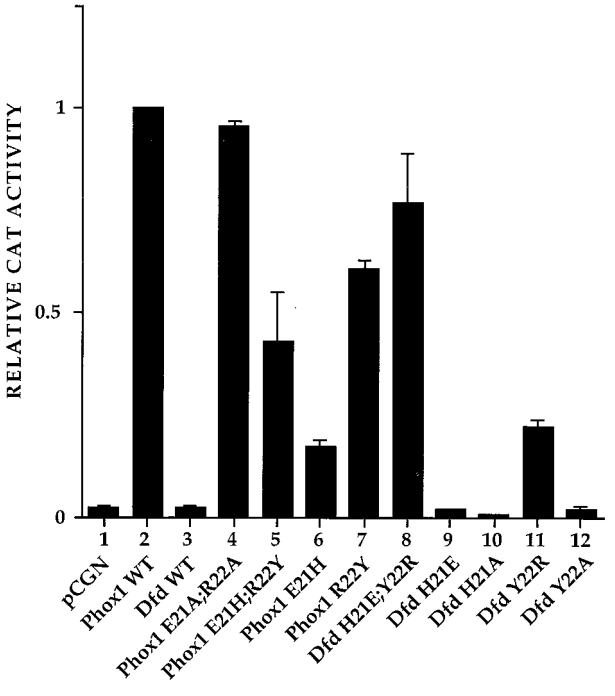

FIG. 5. A single amino acid substitution on the surface of Dfd is sufficient to impart to Dfd the ability to activate the SRE. HeLa cells were transfected with an empty expression vector (lane 1), expression vectors containing wild-type Phox1 (lane 2), mutants of Phox1 (lanes 4 to 7), wild-type Dfd (lane 3), or mutants of Dfd (lanes 8 to 12). Extracts were prepared, and CAT assays were performed as described in the legend to Fig. 2. Alanine substitutions at positions 21 and 22 of Phox1 had no affect on the ability to activate the SRE, whereas histidine and tyrosine substitutions, residues found in Dfd, reduced the ability of Phox1 to activate the SRE. Single substitutions in Phox1 demonstrate that histidine at position 21 is inhibitory to SRE activation by homeodomains. The single substitution Y22R in Dfd confers to Dfd the ability to activate the SRE.

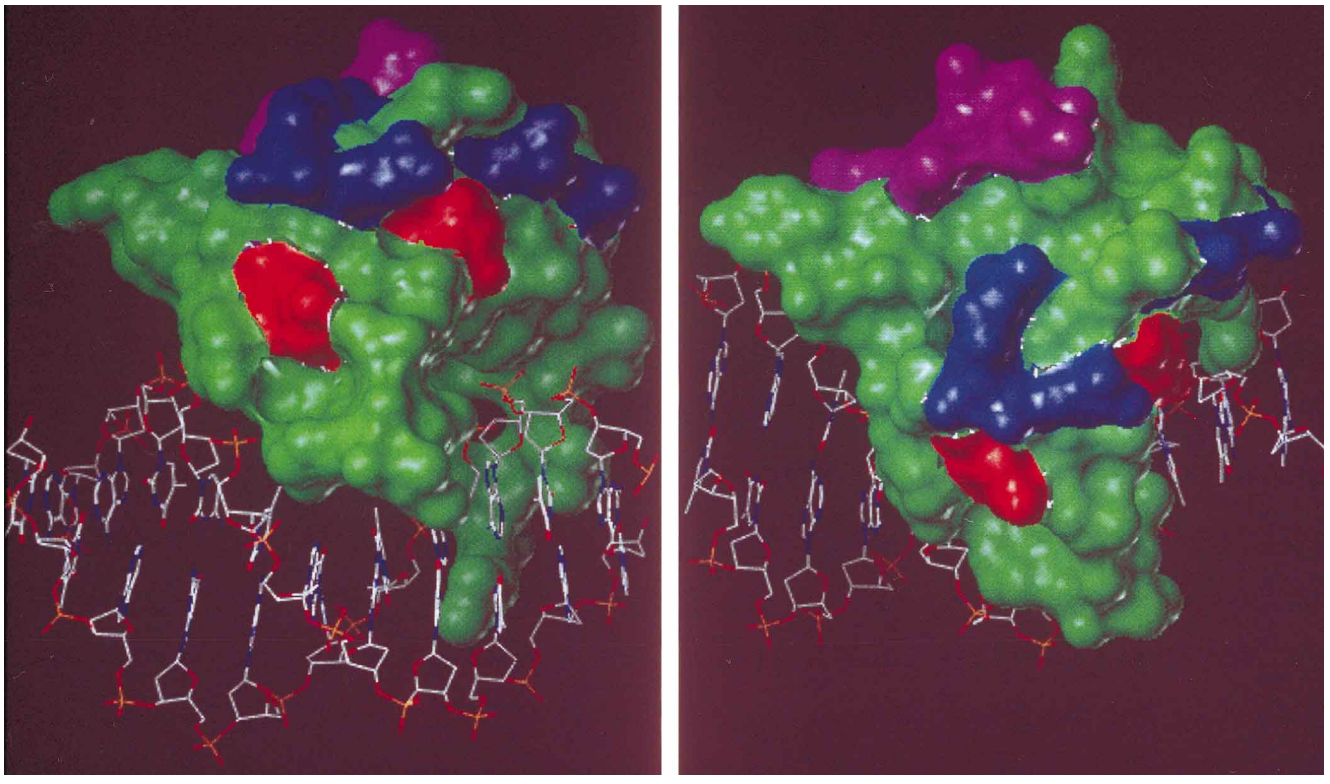

FIG. 6. Homeodomain residues shown to play a role in activation of the SRE form a contiguous exposed surface and are not in close proximity to the DNA. The crystal structure of the Prd homeodomain with a Connolly electron density surface is shown (9, 58). Residues at positions 17 and 24, required for activation of the SRE by Phox1, are shown in red. Residues at positions 10 and 14 and 21 and 22, which impart to Dfd the ability to activate the SRE, are shown in blue. Residues at positions 36 and 37 play a role in the ability of both Phox1 and Dfd to activate the SRE and are shown in purple. (A) Side view of the homeodomain-DNA complex showing that residues required for Phox1 and Dfd to activate the SRE are not in close proximity to the DNA. (B) Top view of the homeodomain-DNA complex showing that the residues required for homeodomain activity at the SRE form a contiguous surface which is exposed to solvent. We propose that this surface plays an essential role in the interaction with additional factors to form a transcriptionally competent complex at the SRE. This figure was generated by the molecular modeling program SYBYL (TRIPOS).

residue at position −1 abolished DNA-binding activity while sparing function in vivo. This separation of DNA binding and in vivo function could be interpreted to mean that minor-groove interaction, at least as observed in the published binary homeodomain-DNA structures, is not required for formation of the active complex in vivo. Resolution of these possibilities will probably require detailed structural studies.

We note that there are several other precedents for dissociation of in vitro DNA-binding activity from functional activity in vivo for proteins of the homeodomain family. Mutations that change the in vitro DNA-binding affinity or specificity of the fushi tarazu protein spare its in vivo activity in a variety of contexts (1, 12, 44, 52). In addition, a triple mutation in the yeast α2 homeodomain that severely affects its ability to bind DNA alone did not affect DNA binding of the a1/α2 complex (55). These cases all support the idea that cooperative interactions between the homeodomain and additional factors can alleviate the requirement for tight DNA binding by the homeodomain, at least as measured in simple in vitro DNA binding assays. Additionally, the type of DNA interaction observed with purified proteins in vitro might not be representative of the active complexes formed in vivo.

We have also shown that residues on the exposed surfaces of helices 1 and 2 of Phox1 are required to activate the SRE. Consistent with these results, mutations that make Dfd like Phox1 at these residues result in proteins that, unlike wild-type Dfd, are capable of activating the SRE. In particular, we find that while the histidine at position 21 in Dfd is inhibitory to SRE activation, arginine at position 22 in Phox1 plays a positive role in SRE activation. Interestingly, histidine 21 is conserved among 66% of homeodomains but is not found in any members of the Prd class. Arginine 22, on the other hand, is strictly conserved among members of the Prd class. This is consistent with the observation that only Prd class homeodomains are active at the SRE in our assays.

Position 22 also plays a role in determining the specificity of action of the Oct 1 and Oct 2 homeodomains. Oct 1, but not the highly homologous Oct 2, forms a multiprotein complex with the herpes simplex virus transcriptional activator VP16 and host cell factor (8, 49, 57). Interestingly, Oct 2 can be made to form a complex with VP16 and host cell factor if it carries the Oct 1 amino acid at position 22 (32). These observations identify this region of helix 1 as perhaps a general determinant of homeodomain specificity in vivo.

The sequence homology between the homeodomains of Phox1 and Prd is about 70%, suggesting that the structure of the Prd homeodomain is a reasonable model for that of Phox1. The crystal structure of the Prd homeodomain is shown in Fig. 6 (58). In Fig. 6, the surface amino acids that are required for Phox1 to activate the SRE and those that play a role in determining the functional specificity of Dfd at the SRE are highlighted. The structure shows a virtually continuous surface across helices 1 and 2 that is not in close proximity to the DNA. This surface is oriented such that it is likely to contact addi-

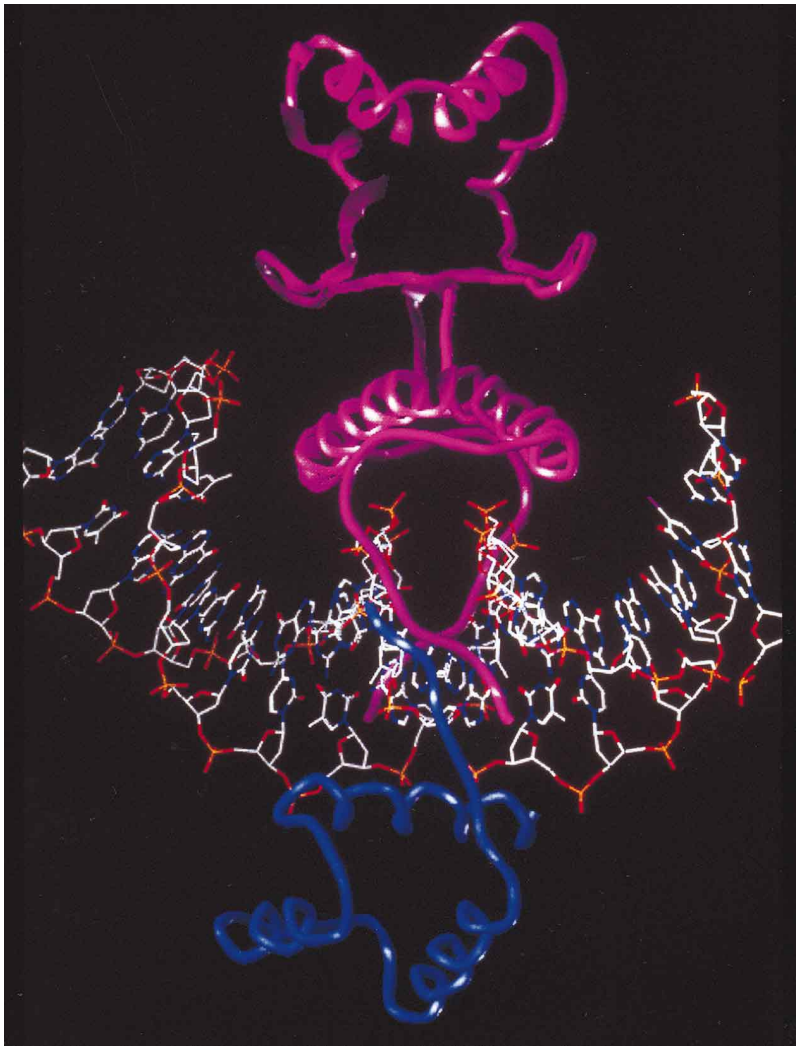

FIG. 7. Molecular model of a proposed Prd-SRF-DNA ternary complex. This model was created by superimposing the common bottom strand TAAT motif found in the DNA of the crystal structures of both SRF (41) and Prd (58). The DNA from the Prd structure was deleted, leaving the Prd protein bound to the TAAT motif of the SRF structure. The model indicates that the homeodomain and SRF are on opposite sides of the DNA. Exposed surface residues required for homeodomain activity at the SRE are positioned such that they are likely to contact one or more additional factors on the DNA. Our data suggest that the N-terminal arm of the homeodomain would not contact the minor groove of the DNA, as shown in this model. An additional 131 amino acids of SRF that were not included in the crystal structure would extend from the N-terminal extension seen contacting the minor groove of the DNA. We predict that these residues would be in close proximity to the homeodomain and might play a role in homeodomain action at the SRE. This figure was generated by the molecular modeling program SYBYL (TRIPOS).

tional factors which are required for Phox1 to form a stable complex at the SRE in vivo.

Our data are consistent with a computer model created by aligning a common TAAT motif found in the crystal structures of the Prd homeodomain and SRF (Fig. 7) (41, 58). One obvious conflict in this model is that the DNA minor groove is occupied by the N-terminal regions of each protein, as they are configured in binary protein-DNA complexes. SRF has been shown by crystallography and biochemical means to bind DNA primarily through minor-groove interactions (39, 41). In fact, a substantial contribution of the DNA-binding affinity of SRF is due to the interaction of the N-terminal arm of SRF with the minor groove of the DNA. The N-terminal arm of homeodomains, however, appears to be unordered until the protein binds to DNA. Together, these observations suggest that the N-terminal arm of Phox1 could adopt a different configuration when in complex with SRF. An alternative scenario for accommodating the N-terminal arms of both SRF and Phox1 on a single SRE is that only a single SRF monomer is present in this complex, allowing the Phox1 N-terminal arm free access to the minor groove on one half of the SRE dyad.

Our structural model also suggests that an additional protein or proteins are present in the transcriptionally competent complex at the SRE. In the model, the contact surfaces of homeodomain helices 1 and 2 are situated on the opposite side of the DNA from SRF. Furthermore, there are large unoccupied spaces where additional factors could interact with the DNA as well as both the homeodomain and/or SRF. We propose that specificity of homeodomain action at the SRE is determined by the formation of a specific complex composed of multiple proteins. Consistent with this hypothesis, we have recently identified a protein that appears to support higher-order complex formation of Phox1 and SRF on the SRE (17). We propose that this factor participates in the complex formed by Phox1 and SRF on the DNA. Formation of this complex, mediated by exposed residues on helices 1 and 2 of Phox1,

could alleviate the requirement for the N-terminal arm contacts with the minor groove of the DNA that are required for the homeodomain to bind DNA alone in vitro. It remains to be determined exactly what role the homeodomain N-terminal arm is playing at the SRE. We speculate that the protein-protein interactions between this factor and the homeodomain are mediated by helices 1 and 2 of the homeodomain. If true, then we would expect this factor to play a role in specifying which homeodomains are capable of activating the SRE. Specifically, we would predict that this factor will interact only with members of the Prd class of homeodomains.

ACKNOWLEDGMENTS

We thank Tim Richmond and John Kuriyan for providing the coordinates for the SRF and Prd crystal structures. We are grateful to Ellen Laird and Marcos Hatada for assistance with the generation of three-dimensional structural models of the Prd homeodomain and SRF. We also thank Sridaran Natesan, Roy Pollock, David Wilson, and members of the Gilman lab and the James laboratory at Cold Spring Harbor Laboratory for useful discussions.

This work was supported by Public Health Service grant CA45642 from the National Cancer Institute.

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