Human and *Drosophila* Homeodomain Proteins That Enhance the DNA-Binding Activity of Serum Response Factor

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Cells with distinct developmental histories can respond differentially to identical signals, suggesting that signals are interpreted in a fashion that reflects a cell's identity. How this might occur is suggested by the observation that proteins of the homeodomain family, including a newly identified human protein, enhance the DNA-binding activity of serum response factor, a protein required for the induction of genes by growth and differentiation factors. Interaction with proteins of the serum response factor family may allow homeodomain proteins to specify the transcriptional response to inductive signals. Moreover, because the ability to enhance the binding of serum response factor to DNA resides within the homeodomain but is independent of homeodomain DNA-binding activity, this additional activity of the homeodomain may account for some of the specificity of action of homeodomain proteins in development.

In both developing and adult organisms, individual cells grow and differentiate in response to extracellular signals. A single cell must distinguish among multiple signals in its environment. It can do this in part because of differences in the intracellular signal transduction pathways activated by the receptors for each type of signaling molecule. Thus, the signal itself can have specificity and information content. In many cases, however, different cells execute specific and unrelated programs of growth and differentiation in response to an identical signal. In these cases, there is no information inherent in the signal that specifies the cellular response. Instead, the specificity resides in the receiving cell; the developmental history or identity of the cell may determine its response to a signal that is itself generic.

A simple example of a generic signal eliciting distinct responses in different cells is the pheromone response of the yeast *Saccharomyces cerevisiae*. Although each yeast cell type, **a** and α, produces its own distinctive mating pheromone and receptor, the intracellular signals generated by the pheromone receptors are identical in the two cell types (*1*). The specificity of the response of each cell type to pheromone lies in the cell type–specific pattern of gene expression established by the products of the mating type (*MAT*) locus, which are the determinants of cell identity in yeast (*2*). Thus, signals that have no specific information content are interpreted at the transcriptional level by the identity determinants encoded by *MAT*. A key partner for *MAT* is *MCM1* (*3*). The MCM1 protein cooperates with the MATα1 protein to activate α-specific genes and with the MATα2 protein to repress **a**-specific genes, thereby establishing the cell type–specific patterns of gene expression in the two haploid cell types of yeast (*4*). Furthermore, MCM1 participates in the induction of these genes by pheromone and thus provides a physical link between cell identity and signal responsiveness.

MCM1 is a member of a family of proteins defined by a region of amino acid similarity termed the MADS box (*5*). Other members of the MADS box family include proteins with roles in flower development (*6*) and three human proteins (*7, 8*). One of these human proteins, serum response factor (SRF), plays a critical role in the activation of mammalian genes by growth and differentiation factors (*9*). SRF binds to an element termed the serum response element (SRE) within the enhancer of the proto-oncogene c-*fos*. The SRE is the target for activation of c-*fos* transcription by multiple signal transduction pathways (*9*). And, like MCM1, SRF appears to function in cooperation with accessory factors that impart specific responses to different SRF-binding sites (*10*). MCM1 and SRF are 70 percent identical in amino acid sequence in the domain that comprises the DNA-binding and protein-protein interaction activities of the two proteins (*7, 11, 12*). Consequently, they bind to similar DNA sequences and are capable of interacting with some of the same accessory proteins (*12–14*).

Here we have exploited the similarities between MCM1 and SRF to devise a genetic screen in yeast for human proteins that can cooperate with MCM1 to activate a cell type–specific reporter gene. The cDNA we isolated encodes a human homeodomain protein that also interacts with SRF in vitro and in vivo to enhance the binding of SRF to the SRE. Because homeodomain proteins participate in the establishment of cell identity (*15*), the interaction of SRF with proteins of the homeodomain family suggests a simple model for how cells interpret signals in the context of cell identity. Moreover, such an interaction may contribute to the specificity of action of homeodomain proteins in development, which has been difficult to explain on the basis of their DNA-binding activities alone.

Selection of human cDNA's that activate an *MCM1*-dependent reporter gene in yeast. We constructed a reporter gene consisting of three tandem copies of a 49–base pair (bp) oligonucleotide encoding the upstream activating sequence (UAS) of the yeast *STE3* gene. This gene, which encodes the receptor for **a** pheromone, is expressed only in α cells and is up-regulated in response to pheromone (*16*). The *STE3* UAS binds the complex of MCM1 and MATα1 (*17*). The oligonucleotides were inserted 21 bp upstream of the distal TATA element of the *HIS3* gene (*18*). This construct was integrated into the genome of an MATα *his3* strain, conferring on these cells a His^+ phenotype (*19*). Upon inactivation of the *MATα1* gene, the resulting strain, MG27, became sterile and His^-. The His^- phenotype likely results because the interaction of MCM1 and MATα1 is highly cooperative; in the absence of MATα1, MCM1 does not bind to the UAS (*12, 17*). This strain permitted us to select plasmids encoding proteins that reactivate this UAS, perhaps by interacting with MCM1.

We transformed MG27 with a human glioblastoma cDNA library in a high-copy yeast expression plasmid (*20*). Transformants were selected directly for growth in the absence of histidine. About 60 His^+ colonies appeared over the course of 7 days; these were purified and tested by segregation analysis to determine whether the His^+ phenotype was plasmid dependent. From 31 plasmid-dependent colonies we recovered plasmid DNA. All plasmids retransformed MG27 to His^+. Restriction mapping indicated that the plasmids fell into five groups carrying cDNA inserts of different sizes, all derived from the same human mRNA. The cDNA's had identical 5′ ends and differed only in the length of 3′ untranslated sequence; all encoded the same protein (*21*).

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The deduced amino acid sequence of the encoded protein (*22*) (Fig. 1A) is closely related to the product of the *Drosophila* pair-rule gene *paired* (*23*) (Fig. 1B). The related sequence corresponds to the homeodomain of the *paired* protein. The human protein, which we call Phox1 (for paired-like homeobox), is 70 percent identical to *paired* protein over the 60 amino acids comprising the homeodomain. The homeodomain encodes a DNA-binding domain with a three-helix structure related to the helix-turn-helix structure of bacterial repressors (*24–27*). One difference between the sequences of Phox1 and *paired* protein is the ninth amino acid in the DNA recognition helix of the homeodomain, which is an important determinant of DNA-binding specificity (*28, 29*). In *paired* protein, this amino acid is a serine, whereas in Phox1 it is a glutamine. This difference suggests that the DNA-binding specificities of Phox1 and *paired* protein are not identical.

A

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  1  LDSPGNLDTL QAKKNFSVSH LLDLEEAGDM VAAQADENVG EAGRSLLESP   50
 51  GLTSGSDTPQ QDNDQLNSEE KKKRKQRRNR TTFNSSQLQA LERVFERTHY  100
101  PDAFVREDLA RRVNLTEARV QVWFQNRRAK FRRNERAMLA NKNASLLKSY  150
151  SGDVTAVEQP IVPRPAPRPT DYLSWGTASP YRSSSLPRCC LHEGLHNGF   199
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B

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                 ____________________  _________  ____________________
Phox1    EEKKKRKQRRNRTTFNSSQLQALERVFERTHYPDAFVREDLARRVNLTEARVQVWFQNRRAKFRRNER
prd      GIAL------C----SA---DE---A----Q---IYT--E--Q-T------I----S----RL-KQHT
gsb-p    SVQL------S----SND-ID----I-A--Q---VYT--E--QSTG----------S----RL-KQLN
gsb-d    GIPL------S----TAE--E---GA-S--Q---VYT--E--QTTA-----I----S----RL-KHSG
otd      PGVNT-----E----TRA--DV--AL-GK-R---I-M--EV-LKI--P-S------K-----C-QQLQ
gcd      NQLHC-RK--H--I-TDE--E---NL-QE-K---VGT--Q-----H-R-EK-E---K-----W--QKR
ceh-10   GKAS---K--H--I-TQY-IDE--KA-QDS----IYA--V--GKTE-Q-D-I----------W-KTEK
MIX.1    ASLVPAS---K--F-TQA--DI--QF-QTNM---IHH--E---HIYIP-S-I----------V--QGA

                 ____________________  _________  ____________________
Phox1    EEKKKRKQRRNRTTFNSSQLQALERVFERTHYPDAFVREDLARRVNLTEARVQVWFQNRRAKFRRNER
S8       GT-R-K-------------------------------E-------S--------------------
Pax-3    DLPL------S----TAE--EE---A--------IYT--E--Q-AK----------S----RW-KQAG
Pax-7    DLPL------S----TAE--EE--KA--------IYT--E--Q-TK----F-----S----RW-KQAG
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Fig. 1. (**A**) Deduced amino acid sequence of the protein encoded by the Phox1 cDNA. The DNA sequence has been deposited in the GenBank database (M95929) and is also available from the authors on request. (**B**) Comparison of the Phox1 sequence with other related homeodomain proteins (*47*). The approximate positions of the three predicted helices in the Phox1 homeodomain are overlined. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

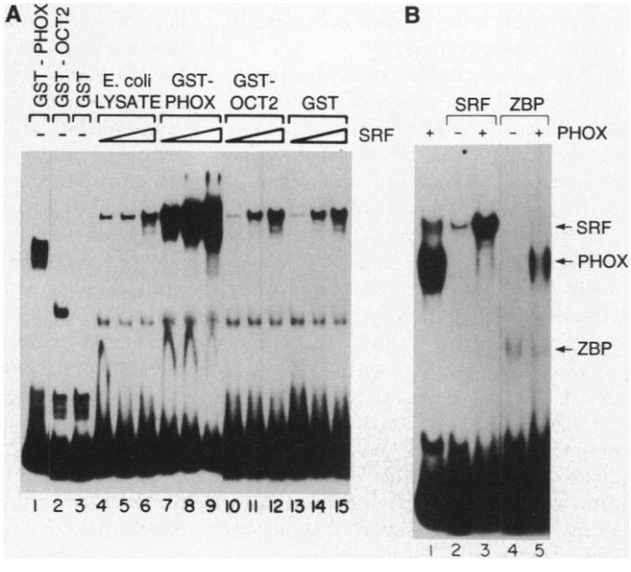

Fig. 2. Mobility-shift assays of *E. coli*–produced proteins on a c-*fos* SRE probe. Glutathione-*S*-transferase (GST) fusion proteins were affinity-purified on glutathione-agarose. SRF was supplied as a soluble *E. coli* extract approximately 0.5 percent SRF. (**A**) In lanes 4 to 15, total protein was normalized to 3 μg by the addition of a soluble extract of control *E. coli* cells. Each titration contained approximately 5, 10, and 15 ng of SRF, respectively. GST-Phox1, GST, and GST–Oct-2 were present at 5 ng. Phox1 DNA-binding activity was inhibited by *E. coli* lysate (*31*). (**B**) Mobility-shift assays of SRF and SRE-ZBP (*33*), a zinc finger–containing SRE-binding protein in the absence (−, lanes 2 and 4) and presence (+, lanes 3 and 5) of 5 ng of GST-Phox1. SRF and SRE-ZBP (the latter as a GST fusion) were present at 5 ng. Total protein in all reactions was adjusted to 1 μg with *E. coli* lysate. Arrows indicate the positions of the complexes generated by SRF, Phox1, and SRE-ZBP, respectively. Phox1 DNA-binding activity was only partially inhibited by the lower concentration of *E. coli* lysate used in this experiment.

Interaction of Phox1 with SRF. Genetic analysis of yeast transformed with the Phox1 cDNA suggested that its ability to activate the *STE3* UAS required the binding of MCM1 to the UAS (*31*). To determine whether Phox1 interacted in vitro with the related human MADS box protein, SRF, a series of mobility-shift assays with *Escherichia coli*–produced proteins and a c-*fos* SRE probe were performed (*32*). Phox1 bound directly to the SRE probe (Fig. 2A, lane 1) but with low affinity; SRF also bound to the SRE as expected (lanes 4 to 6), but its activity was enhanced in the presence of Phox1 (lanes 7 to 9). Two control proteins, including a derivative of the human homeodomain protein Oct-2, had no effect on SRF activity (lanes 10 to 15). Thus, the enhancement of SRF is specific to Phox1. Furthermore, the enhancing effect of Phox1 is exerted specifically on SRF because Phox1 had no effect on the binding of another *E. coli*–expressed SRE-binding protein, SRE-ZBP (*33*) (Fig. 2B). These observations suggest that Phox1 interacts specifically with SRF in vitro, enhancing its ability to bind to the SRE in a mobility-shift assay. This activity is consistent with the ability of Phox1 to activate the *STE3* UAS in yeast by an MCM1-dependent mechanism. Surprisingly, the presence of Phox1 did not affect the mobility of the SRF-SRE complex, and therefore Phox1 may not be a stable component of the complex visualized in this assay.

Similar observations were made in deoxyribonuclease (DNase) I footprinting assays (Fig. 3) (*34*). At low SRF concentrations, footprints over the SRE were observed only in the presence of Phox1 (compare lanes 1 to 3 and 12 to 14 with 4 to 6 and 15 to 17, respectively). The footprints were identical to those observed with SRF alone at a concentration five times higher than the highest concentration used in the SRF titration (lanes 10 and 21). When Phox1 was incubated with the SRE probe alone, no footprints were observed at the Phox1 concentration used in the SRF titrations (lanes 7 and 18). At the highest Phox1 concentration, however, a partial footprint formed on the upper strand of the probe (lane 9), and at all Phox1 concentrations a hypersensitive site appeared on the lower strand. Both the

partial Phox1 footprint and the hypersensitive site map to the AT-rich core of the SRE, which contains a TAAT motif recognized by many homeodomain proteins (Fig. 3B) (*15*). Thus, in both mobility-shift and footprinting assays, Phox1 enhances the association of SRF with the SRE.

Enhancement of SRF-binding activity was a function of the Phox1 homeodomain. A 69–amino acid fragment of Phox1 containing the homeodomain and four flanking amino acids on either side (*35*), which retained measurable DNA-binding activity (Fig. 4A, lane 2), enhanced the binding of SRF to the SRE (Fig. 4A, lanes 5 and 6). Although the apparent specific activity for both DNA binding and SRF enhancement was lower than that of the full-length protein, this 69–amino acid fragment comprising the homeodomain was sufficient for both activities (*36*).

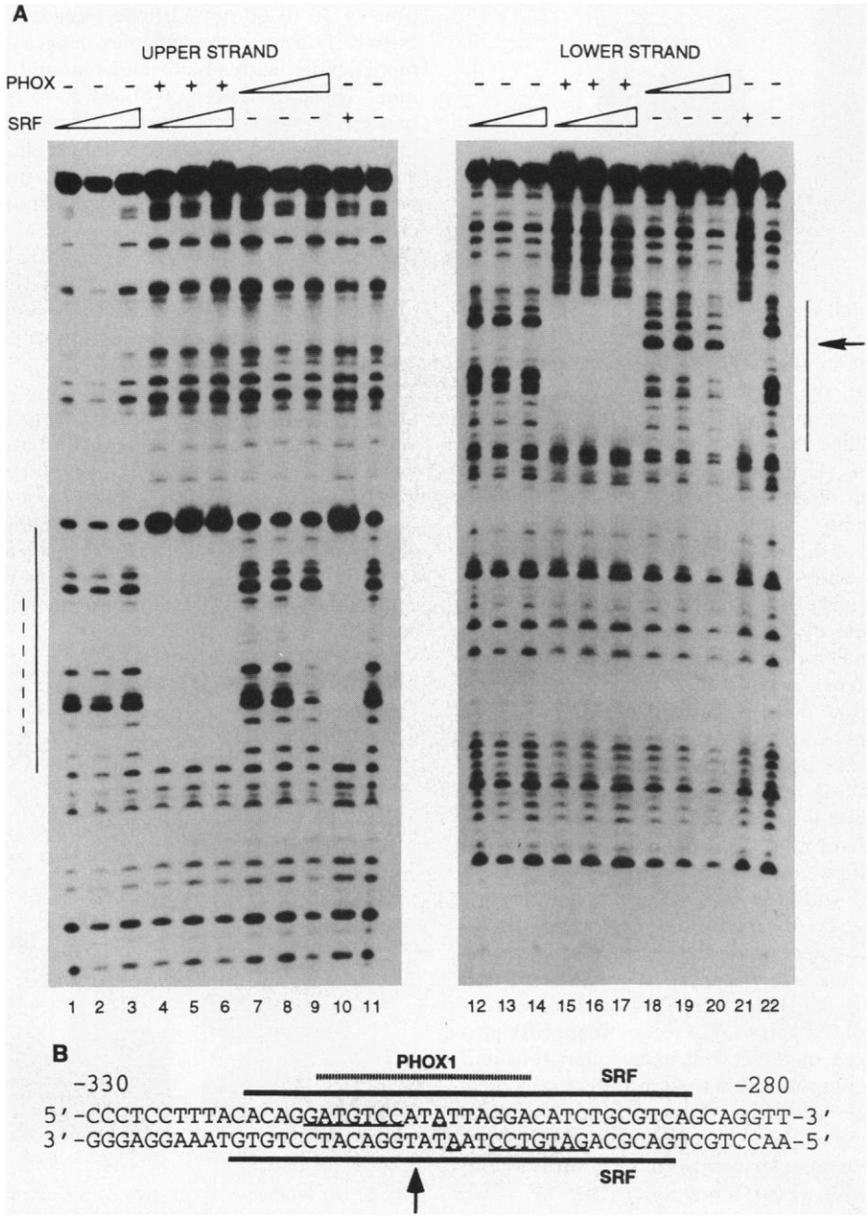

Fig. 3. DNase I footprinting assays of SRF and Phox1. (**A**) Lanes 1 to 11 show the upper strand, lanes 12 to 22 the lower strand. Binding reactions contained SRF alone (0.5, 1, and 2 μl of *E. coli* extract; lanes 1 to 3 and 12 to 14); the same concentrations of SRF in the presence of 250 ng (5 μl) of GST-Phox1 (lanes 4 to 6 and 15 to 17); and GST-Phox1 alone (250, 375, and 500 ng; lanes 7 to 9 and 18 to 20). Lanes 10 and 21 show footprints obtained with a high concentration (10 μl) of SRF alone. Lanes 11 and 22 show protein-free ladders. The solid bar marks the SRF footprint, the dashed bar marks the partial footprint observed at the highest concentration of GST-Phox1, and the arrow marks the hypersensitive site observed on the lower strand in the presence of GST-Phox1. (**B**) The data in (A) are summarized, with bars marking the footprints and the arrow marking the hypersensitive site. The dyad symmetry within the SRE is underlined.

SRF and MCM1 each contain a related domain of approximately 80 amino acids, which includes the MADS box and contains most of the DNA-binding, dimerization, and protein-protein interaction activities of the two proteins (*7, 11–13*). A fragment of SRF, termed SRF core (amino acids 92 to 222), which contains this domain, retained the ability to be enhanced by Phox1 (Fig. 4B). Thus, the interaction between Phox1 and SRF involves the evolutionarily conserved domains of these proteins.

SRF and Phox1 are also capable of interaction in mammalian cells. HeLa cells were transiently transfected with a reporter gene carrying an SRE oligonucleotide positioned upstream of the c-*fos* TATA box. Co-transfection of an expression plasmid that produced the SRF core fragment fused to the strong activation domain of the viral protein VP16 enhanced the expression of the reporter gene (Fig. 5, lane 2), indicating the association of VP16–SRF core with the reporter plasmid. Inclusion of a plasmid producing an 82–amino acid fragment of Phox1 that contains the homeodomain potentiated the activation of the SRE reporter gene by VP16–SRF core (lane 4). This observation is consistent with both the ability of Phox1 to activate an MCM1-dependent reporter gene in yeast and its ability to enhance the binding of SRF to the SRE in vitro. Expression of Phox1 alone activated the wild-type reporter (lane 3), but it did not activate a similar reporter carrying a mutant SRE that does not bind SRF (*31*). This observation may mean that Phox1 is also able to recruit endogenous transcriptional activators, such as SRF, to the SRE.

Because enhancement of SRF binding was intrinsic to the Phox1 homeodomain, related *Drosophila* homeodomain proteins were examined for this activity (Fig. 6A). Mobility-shift assays of *E. coli*–produced SRF in the presence of equivalent amounts of several different homeodomain proteins showed that SRF activity was enhanced by the closely related *Drosophila* homeodomain proteins encoded by the *paired* and *orthodenticle* genes and by a *paired* derivative, M_5, which carries a serine to glutamine substitution in position 9 of the DNA recognition helix (*29*). Although this substitution, which is present in Phox1, alters the DNA-binding specificity of the paired protein in vitro (*29*), it did not affect its ability to enhance SRF binding.

In contrast to the *paired* family proteins, the product of another *Drosophila* pair-rule gene, *fushi tarazu* (*ftz*), more distantly related in primary sequence, did not enhance SRF binding (lane 6). Both *ftz* protein and

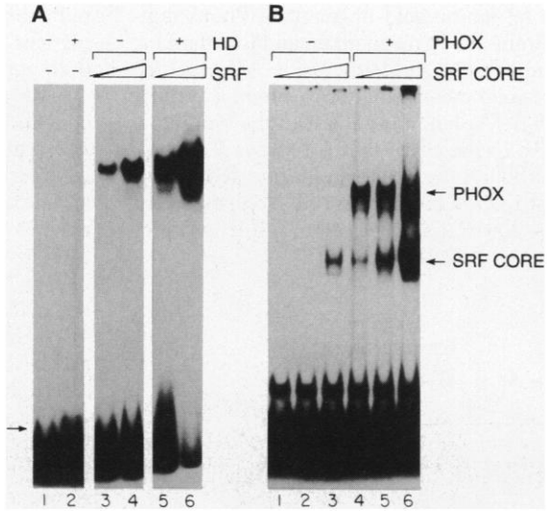

Fig. 4. (**A**) The Phox1 homeodomain is sufficient for enhancement of SRF binding. *Escherichia coli*–produced SRF, 0.5 μl (lanes 3 and 5) or 1.0 μl (lanes 4 and 6), was incubated with the SRE probe in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of 100 ng of purified Phox1 homeodomain (HD) (amino acids 71 to 139). Lane 2 shows 100 ng of Phox1 homeodomain incubated with the SRE probe alone. A band of retarded mobility is visible just above the free probe. (**B**) The SRF core is sufficient for enhancement by Phox1. Purified GST–SRF-core (10, 20, and 50 ng) was incubated with the SRE probe in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of 100 ng of purified GST-Phox1.

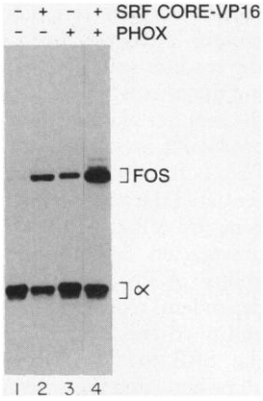

Fig. 5. HeLa cells were transfected with a reporter gene carrying a single copy of the SRE and expression vectors producing SRF core–VP16 and Phox1 as indicated at the top of the panel (*49*). Cells in lane 1 received an empty expression vector. Cytoplasmic RNA was isolated from cells 48 hours after transfection and analyzed by ribonuclease protection. "FOS" indicates the probe fragment protected by transcripts of the reporter gene; "α" indicates probe fragment protected by transcripts of the human α-globin gene transfected as an internal control. Protein expression was verified by immunoblot.

Oct-2, which also failed to enhance SRF binding, bound directly to the SRE under these conditions (Fig. 6B, lanes 5 and 6), indicating that the proteins were active. Therefore, enhancement of SRF binding to the SRE appears to be a specific property of homeodomains of the *paired* family. In addition, the ability of the homeodomain proteins that we examined to enhance the binding of SRF to the SRE showed little correlation with their ability to bind directly to the SRE.

The absence of correlation between the affinity of the homeodomain proteins for the SRE and their ability to enhance the binding of SRF suggested that the sequence-specific DNA-binding activity of the homeodomain may not be required for interaction with SRF. Therefore, we prepared a mutant derivative of Phox1 in which the invariant Asn at position 10 of the DNA recognition helix was converted to Gln (Fig. 7) (*35*). In the *engrailed* protein, the invariant Asn makes two major-groove hydrogen bonds with an adenine residue in the bound TAAT subsite (*25*). This substitution in the homologous position of the *bicoid* protein abolishes DNA-binding activity (*37*). DNA-binding activity of the mutant Phox1 protein was undetectable (Fig. 7, lane 3), but its ability to enhance the binding of SRF was retained (lane 6). Thus, DNA-binding activity of the Phox1 homeodomain is not required for enhancement of SRF binding. This interaction must occur with other conserved features of the homeodomain.

Mechanism of enhancement of SRF binding by the Phox1 homeodomain. To determine the mechanism by which Phox1 enhances the DNA-binding activity of SRF, we examined the rate of association of SRF with the SRE in a mobility-shift assay. The *E. coli*–produced SRF formed detectable complexes with the SRE slowly, taking up to 2 hours or more to go to completion (Fig. 8A, lanes 1 to 5), a result consistent with the behavior of recombinant SRF produced in insect and mammalian cells and dephosphorylated in vitro (*38*). In the presence of Phox1, however, the SRF-SRE complex formed rapidly, with nearly complete complex formation at 5 min (lanes 6 to 10). That formation of the SRF-SRE complex is slow is surprising, in that the initial encounter between SRF and the SRE should be diffusion-limited. Therefore, the SRF-SRE complex detected by our binding assays is likely not to be the initial SRF-SRE complex, but rather an isomerized form of the complex. Because this isomerization is rate-limiting for stable binding, it must be characterized by a large activation energy barrier. To enhance the rate of SRF-SRE complex formation, Phox1 must lower the activation energy for this isomerization. Furthermore, since complex formation is reversible, Phox1 must also enhance the rate of dissociation of the complex. To test this prediction, we assayed preformed SRF-SRE complexes in the absence (Fig. 8B, lanes 1 to 5) or presence (lanes 6 to 10) of Phox1 with excess unlabeled SRE-containing DNA. In the absence of Phox1, SRF-SRE complexes decayed with a half-time of 30 to 60 min. In the presence of Phox1, however, the complexes decayed more rapidly, with a half-time of around 10 min. Thus, the effect of Phox1 is to enhance the rate of exchange of SRF with its binding site, and this effect is detectable as an increased yield in complex at any time prior to equilibrium. This effect of Phox1 on the kinetics of SRF binding is similar to the effect of phosphorylation of SRF by casein kinase II (*38*), an indication that SRF exchange may be enhanced by both covalent modification and protein-protein interaction.

The mobility of the SRF-SRE complex in our assays was not altered in the presence of Phox1 and other homeodomain proteins, suggesting that the homeodomain proteins were not present in the complexes visualized in the mobility-shift assay. The effect of Phox1 on the association and dissociation of the SRF-SRE complex suggests an explanation for this phenomenon. In solu-

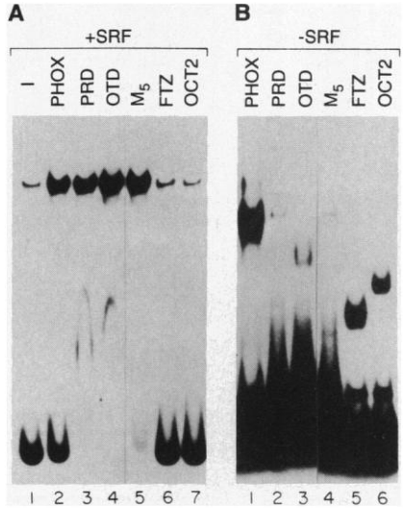

Fig. 6. (**A**) Mobility-shift assays of SRF in the presence of the indicated homeodomain proteins. *Orthodenticle*, *paired*, and the *paired* mutant M_5 were supplied as total *E. coli* lysates. Phox1, *fushi tarazu* protein, and Oct-2 were purified GST fusion proteins, but these reactions were supplemented with *E. coli* lysate to bring all reactions to the same final protein concentration. (**B**) Mobility-shift assays of the same homeodomain proteins in the absence of SRF. This panel is derived from the same gel as (A) but is a longer autoradiographic exposure.

tion, Phox1 is free to accelerate both the rate of formation and dissociation of these complexes, but once the reactions are on the mobility-shift gel, Phox1 may become separated from the larger SRF-SRE complexes by electrophoresis. In the absence of Phox1, however, the SRF-SRE complex is slow to dissociate, and these complexes become trapped and are relatively stable during electrophoresis. Thus, we believe that ternary Phox1-SRF-SRE complexes form transiently but are not stable under our assay conditions.

It is likely, however, that such ternary complexes are stable in vivo. When Phox1 was expressed in yeast, the mobility-shift complex attributable to MCM1 was quantitatively shifted to a lower mobility, indicating the formation of a stable complex between MCM1 and Phox1 on the DNA probe (*31*). With *E. coli*–produced proteins, however, these ternary complexes were not detected, suggesting that stable ternary complex formation may require posttranslational modifications of the proteins that are absent from the *E. coli*–produced material (39). Moreover, the observation that Phox1 enhanced the activity of an SRE-containing reporter gene in HeLa cells suggests that Phox1 enhances the overall occupancy of the SRE in vivo. If we assume that the interactions in transfected cells are near equilibrium, Phox1 must therefore enhance the affinity of SRF for the SRE. Presumably, the DNA-binding activity of the homeodomain contributes binding energy to the interaction, perhaps by contacting directly the AT-rich core of the SRE (*40*). In vitro, by contrast, Phox1 DNA-binding activity is dispensable because transiently formed SRF-SRE complexes are trapped by the mobility-shift gels.

MADS box proteins and the specificity of homeodomain function. A paradox in our understanding of homeodomain function is that these proteins act with a high degree of specificity in development that is not easily explained by differences in DNA-binding specificity (*15*). However, homeodomain swap experiments suggest that this domain is largely responsible for the specificity of action of these proteins in vivo (*41*). These observations indicate that factors other than simple DNA-binding specificity contribute to homeodomain function in vivo. Our data indicate that the ability of homeodomains to enhance the binding of SRF does not correlate with DNA-binding specificity and is separable from DNA-binding activity in vitro. Thus, the interaction of homeodomain proteins with proteins of the MADS box family may account for part of the functional specificity of homeodomain proteins in development.

Development of complex organisms is controlled by an interplay of intrinsic cell identity and external cues that trigger the execution of a cell's developmental fate. The interaction of homeodomain proteins, which in many cases define cell identity, with proteins of the SRF family, with roles in nuclear signal transduction, could provide a means for coordinating cell identity with response to external signals. For example, homeodomain proteins could determine where SRF-containing complexes (or complexes anchored by other MADS box proteins) are assembled in the genome. Thus, homeodomain proteins could establish cell identity at least in part by determining which genes are activated in response to an otherwise generic inductive signal. As a cell undergoes a developmental switch and produces a new homeodomain protein, MADS box proteins would be recruited to new sites, altering the cell's response to subsequent signals. The exchange function of the homeodomain would ensure that the cell reequilibrates rapidly with MADS box proteins at new sites; this function could be of cardinal importance in the early embryo where cell cycles are short and microenvironments change rapidly.

Among other examples of interactions of homeodomains with other proteins is the interaction between the yeast proteins MCM1 and MATα2, the latter a homeodomain protein (*42*). MCM1 and MATα2 interact cooperatively to bind to specific operator sequences in **a**-specific genes, repressing these genes in α cells. Although it might be expected that the SRF-Phox1 complex would be most closely related to the MCM1-MATα2 complex, there is at least one significant difference. In the MCM1-MATα2 complex, MATα2-binding sites are located on each side of the MCM1 site, whereas in the putative SRF-Phox1 complex the footprint is identical to that obtained with SRF alone, and Phox1

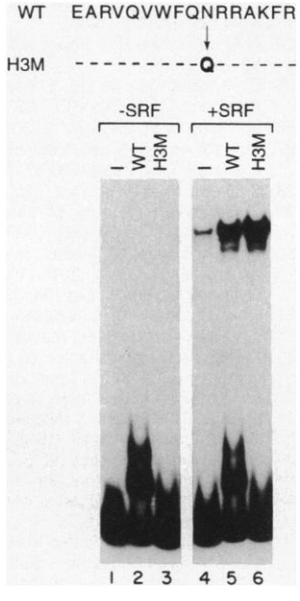

Fig. 7. Effect of a helix 3 mutation on Phox1 activity. Shown at the top is a portion of Phox1 protein sequence corresponding to the predicted third or DNA recognition helix of the homeodomain. The single amino acid substitution present in the H3M mutant is indicated with the arrow. The wild-type (WT) and mutant proteins were produced as GST fusions, purified, cleaved with thrombin, and repurified for use in this experiment. SRF was partially purified from an *E. coli* lysate by DE-52 chromatography. Mobility-shift assays with the SRE probe contained 5 ng of wild-type (lanes 2 and 5) or mutant (lanes 3 and 6) Phox1 homeodomain fragments. Lanes 4 to 6 contained 5 ng of SRF. Bovine serum albumin (5 μg) was present in all reactions.

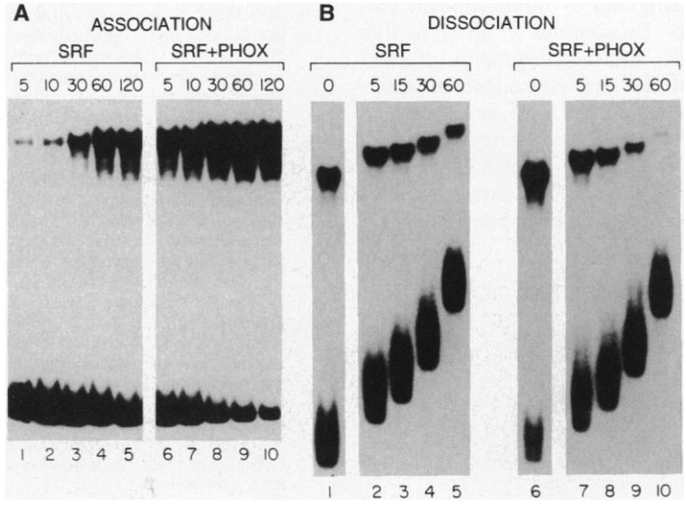

Fig. 8. (**A**) Measurement of the rate of association of SRF with the SRE in the absence (lanes 1 to 5) and presence (lanes 6 to 10) of GST-Phox1. Binding reactions were incubated at room temperature for the indicated times (in minutes). (**B**) Dissociation of SRF in the absence (lanes 1 to 5) and presence (lanes 6 to 10) of Phox1. SRF-SRE complexes were allowed to form for 30 min at room temperature; then at time 0, a 100-fold excess of wild-type SRE competitor was added to the reactions. Samples were withdrawn at the indicated times (in minutes) and placed on a running gel.

alone appears to recognize the AT core in the center of the SRF-binding site. Furthermore, interaction of MATα2 with MCM1 requires a short polypeptide sequence immediately NH_2-terminal to the homeodomain (*43*), whereas under our conditions the Phox1 homeodomain is sufficient for interaction with SRF. The second example of a homeodomain-cofactor interaction is the interaction of human Oct-1 with the viral regulatory protein VP-16, which occurs via helices 1 and 2 of the Oct-1 homeodomain and reprograms both the DNA-binding specificity and transcriptional properties of Oct-1 (*44*). Our current data suggest that the Phox1-SRF complex more closely resembles Oct-1–VP16, but more data are needed to resolve the structural organization of the Phox1-SRF complex.

Both SRF and Phox1 belong to gene families that are found in animals, plants, and unicellular eukaryotes. The role of homeodomain proteins in determining cell identity is well defined in animals, and this function appears in fungi as well. In *S. cerevisiae*, *S. pombe*, and *U. maydis*, homeodomain proteins are key regulators of cell type identity (*2*, *45*). In *S. cerevisiae*, definition of cell identity also involves MCM1. MCM1 cooperates with MATα1 and MATα2 to establish cell type–specific patterns of gene expression and with the STE12 protein, also a protein with homeodomain homology (*46*), to mediate pheromone induction of these genes (*2*).

In plants, MADS box genes participate in determining cell identity (*6*). Where the functions of these genes have been studied, they are analogous to the functions of the homeotic genes in *Drosophila*. Thus, these functions are fulfilled by MADS box proteins in plants and by homeodomain proteins in flies. Because our results show that MADS box and homeodomain proteins interact, MADS box–homeodomain interactions may be part of a conserved mechanism for establishing cell identity shared by plants, animals, and lower eukaryotes.

Note added in proof: The mouse homolog of Phox1 has recently been isolated (*47*).

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19. The starting strain for our library recipient was FY250 (*MATα ura3–52 his3Δ200 leu2Δ1 trp1Δ63*; C. Dollard and F. Winston, unpublished results). We transformed this strain with p603+3 DNA that had been linearized within the *URA3* gene by digestion with Nco I. The resulting strain, MG26 (*MATα ura3–52::p603+3 his3Δ200 leu2Δ1 trp1Δ63*), was Ura$^+$His$^+$. To disrupt the *MATα1* locus in this strain, we inserted a 1450-bp *TRP1* fragment into the Xho I site of plasmid αX113 [K. Tatchell, K. A. Nasmyth, B. D. Hall, C. Astell, M. Smith, *Cell* **27**, 25 (1981)] and transformed the strain with a PCR product amplified from this plasmid using primers flanking the *MATα1* coding sequence. The Trp$^+$ colonies were selected and tested for mating. A sterile transformant, designated MG27 (*matα1::TRP1 ura3–52::p603+3 his3Δ200 leu2Δ1 trp1Δ63*), was chosen. It was phenotypically His$^-$ and was the recipient for the library screening.
20. We screened a human glioblastoma cDNA library in the expression vector pADNS, which carries an *LEU2* marker [J. Colicelli *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2913 (1991)]. A 1-liter culture of MG27 was grown to a density of 1×10^7 cells/ml in YPD medium (yeast extract, peptone, dextrose) and transformed with 100 μg of library DNA [R. H. Schiestl and R. D. Gietz, *Curr. Genet.* **16**, 339 (1989)]. Transformed cells were plated onto 29 dishes (150 mm) containing synthetic complete (SC) medium lacking leucine and histidine. To estimate the transformation efficiency, we plated a portion of the transformed material on SC medium lacking leucine only, which selects only for transformation by library plasmids, and we estimated that we obtained 4.2×10^6 transformants. The original complexity of the cDNA library was approximately 2×10^6, but because the library had been amplified, we are uncertain of the true complexity of the DNA sample that underwent transformation. Segregation analysis of positive colonies was performed by growing cells in YPD for 24 hours at 30°C to allow cells to lose plasmids. Cultures were diluted by 10^{-5}, and 100-μl portions were plated on YPD to obtain roughly 100 colonies. The plates were incubated for 2 days at 30°C, and the colonies were replica-plated to two different SC plates, one lacking leucine and one lacking histidine. We typically found that more than 50 percent of the colonies on YPD were Leu$^-$, indicating loss of plasmid. Approximately half of the original isolates segregated the Leu$^-$ and His$^-$ phenotypes together. To recover plasmid DNA from yeast transformants, we isolated total yeast DNA from 10-ml cultures grown to near saturation in SC medium lacking leucine and histidine as described [C. S. Hoffman and F. Winston, *Gene* **57**, 267 (1987)] and transformed into *E. coli* by electroporation.
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32. Details of the construction of *E. coli* expression vectors are available upon request. Full-length human SRF (*3*) was expressed from pT7f1.A [J. Kuret, K. E. Johnson, C. Nicolette, M. J. Zoller, *J. Biol. Chem.* **263**, 9149 (1988)], a derivative of pET11c [F. W. Studier, A. H. Rosenberg, J. J. Dunn, J. W. Dubendorff, *Methods Enzymol.* **185**, 60 (1990)]. SRF was used as a crude *E. coli* lysate after passage over a DE-52 column to remove nucleic acids, as indicated in figure legends. The glutathione-*S*-transferase (GST)-Phox1 fusion plasmid used in most of the experiments produced a protein beginning at the fifth amino acid of Phox1 and ending at the natural Phox1 COOH-terminus. The GST–Oct-2 plasmid was a gift of M. Tanaka (Cold Spring Harbor). All proteins were produced in *E. coli* strain BL21(DE3). The GST fusion proteins were purified on glutathione-agarose (Sigma) as described [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. For cleavage of GST fusion proteins with thrombin [D. P. Gearing *et al.*, *BioTechnology* **7**, 1157 (1989)], washed

glutathione-agarose beads with bound fusion protein were washed and suspended in thrombin cleavage buffer (50 mM tris, pH 8, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM EDTA). Thrombin (Sigma; 1 U per 100 μg of bound protein) was added, and the reaction mixture was incubated for 12 hours at 25°C. Cleaved proteins were eluted from the beads and stored at −70°C. All proteins were analyzed by SDS–polyacrylamide gel electrophoresis prior to use, and equivalent amounts of each purified protein were used in all assays. Mobility-shift assays (20 μl) contained 10 mM tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 5 percent glycerol, 1 mM sodium phosphate, and poly (dI-dC)·(dI-dC) at 5 μg/ml. Reactions were incubated in the absence of probe for 10 min at room temperature; the probe (20,000 cpm) was added, and the reactions were incubated for an additional 10 min (except for data in Fig. 8). In reactions that included crude SRF, all reactions were supplemented with control *E. coli* lysate to the same final protein concentration, as described in the individual figure legends. In experiments with partially purified SRF, bovine serum albumin (BSA) (5 μg) was added to all reactions. Analysis was on 5 percent polyacrylamide gels (39:1 acrylamide::bis ratio) in 0.5× TBE (tris borate EDTA) buffer. Mobility-shift probes were prepared by PCR amplification of mouse c-*fos* sequences from −354 to 207 with the use of ^{32}P end-labeled primers, as described (*33*).

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35. Deletions of GST-Phox were generated by PCR and subsequent three-part ligations consisting of a GST-encoding fragment, a Phox1 PCR fragment, and Nde I, Bam HI–cleaved pET11c [R. Aurora and W. Herr, *Mol. Cell. Biol.* **12**, 455 (1992)]. The Phox1 homeodomain fragment (Fig. 4A) carried Phox1 amino acids 71 to 139 (KKKR through RAML), whereas the longer fragment used in Figs. 5 and 7 contained amino acids 58 to 139 (TPQQ through RAML). All of these proteins carried several polylinker-encoded amino acids on either end. For site-directed mutagenesis of Phox1 [T. A. Kunkel, *Proc. Natl. Acad. Sci. USA* **82**, 488 (1985)] we used the mutagenic oligonucleotide 5′-CAGGTGTGGTTTCAGCAGCGCAGAGCCAAG-3′ (the underlined triplet encodes the Asn → Gln substitution and the italicized C is a silent substitution that created an Hha I restriction site. Mutants were identified by restriction with Hha I and confirmed by DNA sequence analysis.
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49. HeLa cells on 10-cm dishes were transfected by calcium phosphate coprecipitation. The reporter plasmid carried a single copy of the c-*fos* SRE positioned upstream of a c-*fos*–CAT fusion gene containing mouse c-*fos* sequences from −56 to +109. The Phox1 expression vector contained a Phox1 cDNA fragment encoding amino acids 58 to 139 in the expression vector pCG [M. Tanaka and W. Herr, *Cell* **60**, 375 (1990)]. The SRF core–VP16 expression vector, a derivative of pCG, produced a protein consisting of an influenza hemagglutinin epitope tag [J. Field *et al.*, *Mol. Cell. Biol.* **8**, 2159 (1988)], the nuclear localization signal of SV40 T antigen, and amino acids 411 to 490 of the herpes simplex virus VP16 protein, fused at the NH_2-terminus of SRF amino acids 92 to 222 (R. M. Attar and M. Z. Gilman, unpublished). Transfection precipitates contained 2 μg of reporter, 0.8 μg of SRF core–VP16 expression plasmid, 2.4 μg of Phox1 expression plasmid, and 0.1 μg of a human α-globin plasmid, which served as an internal control. Precipitates were supplemented to a total of 20 μg of DNA with pUC119. RNA was isolated 48 hours after transfection and analyzed by ribonuclease protection assay [L. A. Berkowitz, K. T. Riabowol, M. Z. Gilman, *Mol. Cell. Biol.* **9**, 4272 (1989)].
50. We thank M. Tanaka for help with *E. coli* expression; S. Fields, W. Herr, and M. Tanaka for continued input into this project; G. Graham and A. Majid for technical assistance; and many colleagues for reagents, protocols, and advice, including K. Arndt, R. Attar, E. Chang, J. Colicelli, C. Desplan, K. Ferguson, S. Fields, R. Treisman, B. Tye, F. Winston, and M. Zoller. For comments on the manuscript, we thank M. Cleary, P. Gergen, T. Grodzicker, N. Hernandez, H. Ma, and M. Wigler. Supported by NRSA postdoctoral fellowship CA08968 (D.A.G.); fellowships from the Long Island Biological Association and the International Human Frontier Science Program Organization (C.A.); and by NIH grant CA45642, American Cancer Society grant MG-4, and the Oxnard Foundation (M.Z.G.).

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