The Identification of Prx1 Transcription Regulatory Domains Provides a Mechanism for Unequal Compensation by the Prx1 and Prx2 Loci

Received for publication, January 10, 2001, and in revised form, May 11, 2001 Published, JBC Papers in Press, May 22, 2001, DOI 10.1074/jbc.M100239200

Russell A. Norris and Michael J. Kern†‡
From the Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, South Carolina 29425-2204

Transcription regulatory domains of the Prx1a and Prx1b homeoproteins were analyzed in transient transfection assays using artificial promoters as well as an established downstream target promoter (tenascin-c). Activation and repression domains were detected in their common amino end. In the carboxyl end of Prx1a an activation domain and an inhibition/masking region (OAR domain) were detected. The Prx1b isoform, generated by alternative splicing, does not contain these carboxyl activation or inhibition domains. Instead, the data demonstrate that the carboxyl tail of Prx1b contains a potent repressor region. This difference in the carboxyl tail accounts for a 45-fold difference observed in transcription regulatory activity between Prx1a and Prx1b. The data also support the likelihood that this difference between Prx1a and Prx1b is higher in the presence of still undetermined cofactors. DNA binding affinities of Prx1a, Prx1b, and a series of truncation mutants were also examined. The carboxyl tail of Prx1a, which inhibited transcription activation in the transfection assays, also inhibited DNA binding. These differences in biochemical function between Prx1a and Prx1b, as well as the recently described activities of Prx2, provide a mechanism for the unequal compensation between the Prx1 and Prx2 loci.

Paired type homeobox transcription factors are important regulators of morphogenetic processes and have been conserved in a diverse selection of species. The Prx1 and Prx2 genes are members of this class and thus encode proteins that contain a paired type DNA binding homeodomain but lack a second DNA binding domain (i.e., the paired domain) present in other paired related homeoproteins (1, 2). Prx1 (also known as mhoX, k2, Pnx, PRRX1) (3–6) and Prx2 (also known as S8 and PRRX2) (6–8) are 97% identical within their homeodomains and 64% identical over the entire molecule. This high degree of sequence homology as well as similar expression and genomic organization suggest that Prx1 and Prx2 are the result of a gene duplication event (3–6, 8–10).

Despite many similarities, important differences in splicing of Prx1 and Prx2 are evident. Alternative splicing of Prx1 results in two isoforms, Prx1a and Prx1b, whereas only one isoform of Prx2 is produced. The Prx1a and Prx1b proteins have identical amino acids from 1 through 199, whereas their carboxyl termini are completely different. The carboxyl tail of Prx1a (amino acids 200–245) contains an evolutionarily conserved domain (OAR domain) present in at least 40 other members of the paired class (1). This domain is not encoded in the carboxyl terminus of Prx1b (200–217), making Prx1 the only murine homeobox gene that encodes an OAR domain in one isoform (Prx1a), but not in the second isoform (Prx1b). The function of the OAR domain is not well understood; however, three proteins with an OAR domain have been examined. In the Prx2 and Pitx2 homeoproteins, the OAR domain has been defined as inhibiting transactivation (6, 11). For the Otp homeoprotein, the OAR domain may either be a transactivator or have an adjacent transactivation domain (12). Therefore, it is not clear whether this domain will function similarly in all homeoproteins.

The RNA expression of Prx1 and Prx2 has been examined extensively and is very similar between the two genes, although not identical (5, 9, 10, 13–15). Very little is known about the expression pattern of the two isoforms of Prx1. Although the Prx1a and Prx1b transcripts appear to be coexpressed in the limited mice and human tissues examined, there are dramatic tissue-specific differences in the ratio of these two mRNAs in human tissue (5, 6). The transcripts of Prx1 and Prx2 are expressed primarily in mesoderm within regions of undifferentiated mesenchyme during embryogenesis, particularly in the ectomesenchyme of the craniofacial region and branchial arches, as well as mesenchymal cells of the developing limbs and cardiac cushions. Although Prx1 and Prx2 appear to be down-regulated during chondrogenesis and osteogenesis, expression of both is retained in the perichondrium and the periosteum (9, 10, 14). Also, Prx1 transcripts are highly expressed in cardiac and skeletal muscle of mouse embryos as well as adults (3, 5, 6, 10). The function of the Prx genes was investigated by creating null alleles in embryonic stem cells. The gene-targeted mice have demonstrated the developmental importance of Prx1 and Prx2 (16–20). Prx1-gene targeted mice die perinatally with a host of craniofacial and limb malformations. In contrast, Prx2 gene-targeted mice are morphologically normal. The double mutant mice (Prx1−/− Prx2−/−) developed more severe manifestations of the Prx1−/− phenotype, as well as novel craniofacial, limb, and vascular defects indicating functional compensation between these two loci. However, this compensation is unequal. Prx1+/− Prx2−/− mice are morphologically normal demonstrating that only one allele of Prx1 is necessary to compensate for the lack of Prx2. The opposite is not true because one Prx2 allele on a Prx1 null background only decreases the severity of some malformations while completely rescuing others (16–20).
The mechanism underlying this unequal functional redundancy is not understood. A better understanding of how Prx1 and Prx2 proteins regulate transcription should provide insight into their biochemical function as well as this unequal compensation. Recently, we have identified important transcription regulatory regions in the Prx2 homeoprotein (6). To examine similarities and differences between the transcription factors encoded by the Prx1 and Prx2 loci, this study focuses on defining functional domains within the Prxl a and Prxl b homeoproteins. These data demonstrate that Prxl a and Prx2 are very similar and functionally different from Prxl b. Based on these experiments and our previous analysis of Prx2, we propose a mechanism for the unequal functional compensation between the Prxl a and Prxl b loci.

**EXPERIMENTAL PROCEDURES**

**Eukaryotic Expression and Reporter Plasmids**—All deletion mutants of the Prxl a protein were generated by polymerase chain reaction (PCR) amplification with Q-Taq polymerase (Qiagen) and full-length mouse Prxl a and Prxl b as the templates. The oligomers used to terminate at nucleotides that encoded the Prxl a amino acids at positions 1, 28, 45, 78, 167, 199, 216, and 245 within the open reading frame. The PCR-amplified products were subcloned into pcDNA3.1. His (Invitrogen) downstream and in-frame with the start and histidine (6X) codons. Prxl a OAR mutations were generated by using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s recommendations. Plasmids expressing the Gal4-Prxl a fusion proteins were generated by subcloning a PCR-amplified Gal4(1–147) DNA binding domain (DBD) into the expression plasmid pcDNA3.1.1. PCR-generated pieces of Prxl a were inserted downstream and in-frame of the Gal4 DBD. The oligomers used to make the Prxl a pieces terminated at nucleotides that encoded the Prxl a amino acids 1, 93, 153, 216, and 245. The Prxl a-Ptx2 chimeric construct was generated by PCR using primers that added 167 for Prxl a as well as 274 and 317 for Ptx2 (Swissprot P97474). The Gal4, tenascin-c, and Prx reporters were made as reported previously (6).

**Protein Production, Western Blotting, and DNA Binding Assays of Prxl a Expression Constructs**—All Prxl a expression constructs were derived from pcDNA3.1.1. His, which contains a T7 promoter upstream from the insert. Constructs were transcribed and translated in vitro with the TnT kit (Promega) according to the manufacturer’s instructions. Procedures for transcription and translation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; Pre, Prx response element; DBD, DNA binding domain. The slopes generated for each truncation were compared against the slope obtained for the 1–245 construct.

**RESULTS**

**Activation and Repression Domains of the Prxl a Proteins**—Prxl a proteins contain a number of regions that are conserved with other proteins (Fig. 1). These domains are identified solely on their amino acid sequence except for the homeodomain, which has been functionally examined in terms of DNA binding specificity (3). In addition to its homeodomain, Prxl a contains two other highly conserved regions, the Prx domain and the OAR domain. The Prxl a domain is only observed in the Prxl a, Prxl b, and Prx2 proteins, whereas the carboxyl OAR domain is present in many other paired type homeoproteins. Prxl a and Prx2 contain the OAR domain, whereas Prxl b does not. Splicing of Prxl a primary transcripts incorporates a fourth exon that codes for 18 different amino acids (200–217). This splicing also alters the alanine percentage in the carboxyl tail from 14% to 6% while not altering the high percentage of prolines and serines. Regions amino to the Prxl a homeodomain contain a high percentage of alanines, glycines, and serines (Fig. 1). Predominance of these residues is associated with transcription regulatory regions in other proteins (6, 11–22–26).

To assess the ability of specific domains contained within the Prxl a protein to mediate transcription regulation, a series of truncation mutants was generated based on the domains described above. These expression plasmids were sequenced and then analyzed in vitro to ensure that proteins of the appropriate size were generated (data not shown). Radiolabeled TnT-produced proteins were analyzed using SDS-polyacrylamide gel electrophoresis. The ability of each protein to bind a Prx consensus DNA sequence was evaluated by EMSA (data not shown). All Prxl a truncation mutants bound this DNA sequence, demonstrating that they were folded sufficiently to retain this function.

The transcription regulatory properties of each truncation mutant were examined in eukaryotic cells. Transient cotransfection assays were performed using a plasmid containing the SV40 early promoter driving luciferase expression. Three Prx-responsive elements (3X PRE) were inserted immediately upstream of the SV40 promoter. Cotransfection of this reporter and full-length Prxl a into NIH3T3 cells resulted in essentially no change of luciferase gene transcription relative to base line (Fig. 1). Similarly, as the amino end of Prxl a was removed (constructs 28–245 and 78–245), very little if any change was detectable in reporter activation. However, when the carboxyl 29 amino acids were removed (1–216), a 10-fold increase in gene activity was observed, suggesting that this region functions as an inhibition or repressor domain. The carboxyl region containing the OAR domain likely functions as an inhibitor because any construct that contains it has very low activity (1–245, 28–245, and 78–245), whereas those without it (1–216 and 1–167) have higher activity.
Prx1 Transcription Regulatory Domains

There were two other important regions identified by this assay. First, an activation domain is present in the carboxyl portion of the protein between amino acids 168 and 216 (compare 1–216 with 1–167). Second, the amino region of the Prx1a protein also activated transcription (compare 1–167 with 78–167). Finally, it should be noted that expression of full-length Prx1b functioned in a manner similar to the full-length Prx1a protein also activated transcription (compare 1–167 with 78–167). Second, the amino region of the Prx1a protein between amino acids 168 and 216 (comp-167 with 1–167). In comparing the two cell lines, activation of transcription is predominant in NIH3T3 cells, whereas repression is more prevalent in the C2C12 cells. Furthermore, there is at least one regulatory activity in the amino end (28–77) of Prx1a which is functioning in C2C12 but not NIH3T3 cells.

The Prx1b isoform was examined for its ability to regulate transcription by Prx1 truncations in NIH3T3 and C2C12 cells. Prx1a protein is shown schematically to the left of the graph. Regions conserved among the Prx1 proteins and other paired type homeoproteins are the homeodomain, Prx domain, and OAR domain. Percentages of prevalent amino acids (A, alanine; P, proline; S, serine; G, glycine) are shown. Numbers above the schematic correspond to amino acid positions. NIH3T3 fibroblasts (light gray bars) and undifferentiated C2C12 myoblasts (dark gray bars) were cotransfected with the 3X PRE-SV40-Luc construct (above the graph) and either one of the truncated Prx1 proteins listed to the left or pCDNA3.1’ His (not shown). All transfections included the pSV-βGal plasmid and were normalized by β-galactosidase activity. Results represent the average of a minimum of three transfection experiments performed in triplicate. Results are represented as fold changes relative to transfection of pCDNA3.1’ His.

**Fig. 1. Regulation of transcription by Prx1 truncations in NIH3T3 and C2C12 cells.** Prx1a protein is shown schematically to the left of the graph. Regions conserved among the Prx1 proteins and other paired type homeoproteins are the homeodomain, Prx domain, and OAR domain. Percentages of prevalent amino acids (A, alanine; P, proline; S, serine; G, glycine) are shown. Numbers above the schematic correspond to amino acid positions. NIH3T3 fibroblasts (light gray bars) and undifferentiated C2C12 myoblasts (dark gray bars) were cotransfected with the 3X PRE-SV40-Luc construct (above the graph) and either one of the truncated Prx1 proteins listed to the left or pCDNA3.1’ His (not shown). All transfections included the pSV-βGal plasmid and were normalized by β-galactosidase activity. Results represent the average of a minimum of three transfection experiments performed in triplicate. Results are represented as fold changes relative to transfection of pCDNA3.1’ His.

**Prx1 Can Activate Transcription Independent of DNA Binding**—Because other homeoproteins have been described as being able to regulate gene transcription independent of DNA binding (6, 22, 23), it is important to determine if Prx1 can also function this way. To test if DNA binding was essential, the constructs that gave the highest levels of transcription activity in NIH3T3 cells (1–216 and 1–167) were used in a cotransfection assay. These expression constructs were cotransfected with a reporter containing either Prx binding sites (3X PRE) or Gal4 binding sites (5X GAL4). Both Prx1 truncations are able to activate transcription to approximately the same level in the presence or absence of DNA-binding elements (Fig. 2).

**Prx1 Domains Assayed in the Context of a Heterologous DNA Binding Domain**—To examine further the function of Prx1 domains involved in transcription regulation, expression constructs were generated which fused the Gal4 DBD with portions of the Prx1 protein. The constructs were sequenced, and the ability of these chimeric proteins to bind a Gal4 recognition sequence was evaluated by EMSA. All of the fusion proteins bound to this element, demonstrating that they were likely folded normally (data not shown).

The chimeric Prx1 proteins were tested for their ability to regulate transcription by transient cotransfection assays in NIH3T3 cells (Fig. 3). The Gal4-Prx1 1–245 construct repressed transcription ~2-fold (data not shown). Removal of the carboxyl tail (construct 1–216) relieved this repressing effect and activated transcription 2-fold over base line. This demonstrated that the Gal4 DBD blunted the level of Prx1 transcription activation domains. However, these results were consistent with earlier data (compare with Figs. 1 and 2). Like the previous DNA-dependent transfection results, these results suggested that the carboxyl terminus contained either a repressor or a masking domain. The function of the carboxyl tail was examined further by fusing the last 29 amino acids of Prx1a to the Gal4 DBD. This chimeric protein did not activate...
or repress the reporter gene. When the entire carboxyl region of the Prx1 protein (153–245) was fused to the Gal4 DBD, luciferase activity was also maintained at basal levels. However, when the carboxyl terminus was removed (153–216), the activation doubled. Furthermore when this was trimmed to 153–199 the activation disappeared. In toto, these data indicate that not only is there an activation domain in the 200–216 region but that the 29 most terminal amino acids, a region that contains the OAR domain, masks transcription activation domains.

The carboxyl terminus of the Prx1b protein was also examined with this approach. Fusion of the last 18 amino acids (200–217) to the Gal4 DBD resulted in base-line levels of transcription activity. When a larger portion of the Prx1b carboxyl region was examined (153–217), more than 2-fold repression was observed. The 153–199 region did not alter transcription from base-line levels in this assay. Thus the repression observed here must be attributed to the last 18 amino acids of the Prx1b protein. This demonstrates that the carboxyl terminus of Prx1b is functioning as a repressor. These data suggest that sequences adjacent to the carboxyl 18 amino acids are likely necessary for proper folding and function of the carboxyl terminus of Prx1b. An alternative possibility is that the repression domain is partially contained in 153–199, and when separated it is nonfunctional. We favor the former explanation because the 153–199 region was inert in this assay, and this region is also contained in the Prx1a protein, which has very little repressive activity in the carboxyl portion in any cell type examined by any assay.

The amino region of the Prx proteins (1–78) was defined previously as containing an activation domain. This region was fused with the Gal4 DBD and gave approximately a 2-fold activation above base line. The 45–78 region demonstrated a similar level of activation, indicating that the majority, if not all, of the amino activation is found in the 45–78 region.

The majority of the chimeric proteins were also evaluated in C2C12 cells, but no activity above base line was observed for any of the constructs (data not shown). This demonstrated two interesting cell-specific differences in the transcription regulatory abilities of the amino activation domain and the Prx1b carboxyl terminus. Both domains that were active in NIH3T3 cells (45–78 and 153–217 of Prx1b) were inactive in C2C12 cells. Nevertheless, examination of Prx1 transcription regulatory domains in the context of the homeodomain as well as using the heterologous Gal4 DBD defined similar regions as being important. Although the level was different, both assays demonstrated the presence of amino and carboxyl activation domains as well as carboxyl masking and repression regions.

Cell-specific and Domain-specific Effects of Prx1 Proteins on the Tenascin-c Promoter—Recently tenascin-c has been identi-
fied as a downstream target of the Prx1 transcription factor (28). To examine how the various domains of Prx1 regulate this downstream target, a portion of the tenascin-c promoter (257 to 198) which contains a Prx1 binding site was placed in control of the luciferase gene and used as a reporter in transient cotransfection assays (Fig. 4). The full-length Prx1 protein was able to transactivate the tenascin-c promoter 9-fold in NIH3T3 cells. Removal of the carboxyl 29 amino acids, which includes the OAR domain, resulted in 370-fold activation over baseline. This is consistent with the OAR domain masking activation regions. Further carboxyl truncations (1–167 and 1–199) resulted in activation levels above base line of 119- and 114-fold, respectively. This is approximately a 3-fold decrease in activation compared with construct 1–216 and maps an important activation domain to the 200–216 region of Prx1a.

The domains within the amino end of the protein were examined both in the context of the complete carboxyl end or its removal. In the context of deletion of the carboxyl end (construct 1–167), truncations of the amino end were examined in NIH3T3 cells. Removal of the first 27 amino acids (28–167 compared with 1–167 in Fig. 4) resulted in a 3-fold decrease in activation compared with construct 1–216 and maps an important activation domain to the 200–216 region of Prx1a. Further amino truncations (1–27 and 45–78) revealed a second amino activation domain between residues 45 and 77. These amino regulatory domains were also examined in the context of full-length Prx1a (compare 1–245, 28–245, 45–245, and 78–245). The results were very similar, except the level attributed to each domain was different. In the context of the complete carboxyl end, the first 27 amino acids activated 2-fold, the 28–44 region repressed 5-fold, and the 45–77 region activated 18-fold. This more modest activation by the 1–27 region in the context of the full-length protein is consistent with the OAR domain masking the activation potential of this amino-terminal domain. Only when the OAR domain is removed can the more complete transcription activity of this domain be revealed.

The Prx1b isoform was also examined for its ability to regulate the tenascin-c promoter in NIH3T3 cells (Fig. 4). Whereas the Prx1a isoform results in a 9-fold induction of gene activity, Prx1b results in a 5-fold repression. Therefore, there is a 45-fold difference between the two isoforms in regulating transcription of the tenascin-c promoter. The best comparison to ascertain the function of the unique 18 amino acids at the carboxyl terminus of Prx1b is to examine the activity of the region that is common between the two isoforms (1–199) and compare that with Prx1b. The data demonstrate that these 18 amino acids repress transcription activity more than 500-fold.

All of the Prx1 protein truncations described above were also tested in C2C12 cells (Fig. 4). A very similar pattern of regulation was demonstrated for all of the constructs. In the amino end, one repression domain (28–44) was situated between two amino activation domains (1–27 and 45–78). In the carboxyl tail of Prx1a, an activation domain (200–216) is flanked by the carboxyl terminus, which masks activation domains. Once again, the 1–27 region demonstrated higher transcription regulatory activity when the carboxyl end was removed. The difference observed between Prx1a and Prx1b in NIH3T3 cells was also recapitulated in the C2C12 cells. However, the difference was only about 10-fold in C2C12 cells versus 45-fold in NIH3T3 cells. In fact, the difference in the level of gene activation between the two cell types was the only discordance observed. NIH3T3 cells had, on average, a 3–10-fold higher level of gene activity than was demonstrated by C2C12 cells.

FIG. 4. Regulation of the tenascin-c promoter by Prx1 truncation mutants. Truncations of the Prx1 transcription factor are shown schematically with the conserved domains and the potential transcription regulatory domains. NIH3T3 fibroblasts (panel A) and undifferentiated C2C12 myoblasts (panel B) were cotransfected with a tenascin-c reporter construct and the Prx1 truncation mutants. Results represent the average of a minimum of three transfection experiments performed in triplicate.
The Carboxyl Terminus of Prx1a Inhibits DNA Binding Activity—One mechanism for the alterations in regulatory activity from one truncation mutant to another was that DNA binding affinity was changed. The stability of protein-DNA interactions of the truncation mutants was assessed by EMSA (Fig. 5). Equimolar amounts of Prx1a and Prx1b, as well as three truncation mutants (1–216, 1–167, and 1–199), were incubated with increasing amounts of a radiolabeled Prx binding site (PRE). The values for all constructs were compared with Prx1a. Removal of the carboxyl terminus of Prx1a, or additional carboxyl truncation, resulted in an increase of 2–4-fold in DNA binding. The binding activity of Prx1b was very similar to that observed for the 1–216 construct. Thus, removal or lack of regions containing the OAR domain increases DNA binding. However, this difference alone is not sufficient to account for the changes in gene activation observed for Prx1 truncation mutants.

Mutations and a Chimera Identify the OAR Domain as Masking Prx1a Activation Domains—The carboxyl terminus of the Prx1a protein demonstrated masking of activation domains in all previous assays. The OAR domain is conserved in the carboxyl terminus of more than 40 paired type homeoproteins. To test the importance of this conserved domain on Prx1a function and the potential for it to mask activation domains, we performed site-specific mutagenesis. In the known OAR domains there are only four positions that have invariant amino acids (Fig. 6). Two of these (Arg-228 and Ser-223) for Prx1a were mutated with alanine, proline, or both. An additional site that is highly variant (Asn-226) was also mutated with a proline to examine if introduction of prolines in the OAR domain might destabilize any secondary structure. The Prx1a OAR mutations were tested in NIH3T3 and C2C12 cells (Fig. 6). Mutations in residues 223 and 226 result in no change in activity compared with wild-type Prx1a. However, both alanine and proline substitutions for arginine 228 result in roughly a 45-fold induction of gene activity in NIH3T3 cells. A 10-fold induction of gene activity is also demonstrated in C2C12 cells for the R228P construct. This difference in gene activity is not the result of alterations in DNA binding affinity because EMSA confirmed comparable binding affinities between the 228 mutations and wild-type Prx1a protein (data not shown). Therefore, the high level of transregulation of the R228A or R228P mutation strongly suggests that the OAR domain is the most critical region within the carboxyl terminus and that its function is likely to mask activation domains.

The function of the Prx1a OAR domain was also analyzed by comparing it with that of another homeoprotein. We tested whether the high level transcription regulatory activity of the Prx1a 1–216 truncation mutant, which did not contain the OAR domain, could be masked by the addition of the Pitx2 OAR domain. A chimeric expression plasmid was created in which the Pitx2 carboxyl terminus was fused to the Prx1a truncation 1–216. Sequence analysis and EMSA demonstrated that this chimeric protein expressed a functional protein (data not shown). This chimeric protein does not activate transcription like the 1–216 truncation; rather it stimulates transcription even less than the Prx1a full-length protein in NIH3T3 cells (Fig. 7). The OAR domain is the common source between the Prx1a carboxyl terminus and that of Pitx2. This suggests that the Prx1a OAR domain functions very similarly to the Pitx2 OAR domain in masking transcription activation domains.

DISCUSSION

In this study important transcription regulatory domains of the Prx1a and Prx1b homeoproteins were defined. At least some of these domains were able to regulate transcription of reporter genes as effectively in the presence or absence of DNA binding sites. Similar protein-protein interactions likely mediate both of these functions. Prx1 has been described as interacting with serum response factor regardless of the presence of DNA (13, 37, 38). Prx1 is also able to interact with the retinoblastoma gene product (39), TFII-I (38), and the oncoprotein Maf (40), all independent of DNA binding. Our data demonstrate that Prx1 can regulate transcription independent of DNA binding, either by one of these known interactions or some yet to be elucidated interaction. Critical protein-protein interactions as well as DNA-independent transcription regulation have been demonstrated previously for a number of homeodomain-containing transcription factors including Max1, Max2, Ftz, and Pbx (22, 29–33). Furthermore, homeoproteins that lack the homeodomain are still able to modulate transcription, demonstrating that DNA binding is not necessary for this regulation (34–36). All together this supports the model of Prx1 as a multifaceted transcription factor that can interact with a variety of proteins and regulate transcription in both a DNA binding-dependent and -independent manner.

Prx1a C-terminal 29 Amino Acids Mask Transactivation—The data presented in this study demonstrate that the Prx1a carboxyl region can mask transcription activation domains. For the following reasons it is clear that this region is a mask and not a repressor, i.e. a domain interacting with the transcription
complex to decrease transcription. First, all the expression constructs that incorporated this region stimulated transcription to base line levels or higher. This was observed in all three of the assays and in both cell types. Most importantly this included the Gal4 DBD fusion proteins. Second, if the OAR domain functioned as a repressor then a single amino acid change would be unlikely to activate transcription (R228P and R228A) while nearby mutations did not affect activity. Third, the Pitx2 carboxyl region has been defined as a mask of intramolecular transcription activation regions, and it was capable of replacing this function in the Prx1 OAR mutant. The R228A mutation was tested only in NIH3T3 cells. Wild-type Prx1a and truncation mutant 1–216 are shown above the mutations as a means for comparison. Results represent the average of a minimum of three transfection experiments performed in triplicate.

Carboxyl inhibitory or masking domains are common regulatory motifs observed in a number of transcription factors including PHO4, c-Myb, Pax2, Pax5, Pax8, C/EBPβ(NF-M), Nkx2.5, and Pitx2 (11, 41–45). The mechanism of action for these inhibitory domains varies depending on the transcription factor examined. The mechanism of Prx1a function most closely mimics what has been described for Pitx2. In the case of Pitx2, there is an intramolecular interaction of the OAR domain with an amino portion of this homeoprotein. This results in masking of transcription activation as well as inhibition of Pitx2 binding with its cognate DNA sequence. These suppression and inhibition effects are relieved when the OAR domain interacts with a cofactor, Pit1. Like Pitx2, the Prx1a OAR domain both inhibits DNA binding as well as masks transcription activation. Furthermore, mutation analyses of Arg-228 within the OAR domain indicate that this is an important amino acid in maintaining OAR function. This amino acid may make hydrogen bonds with basic amino acids located in the amino end of the protein (Fig. 4). As noted in our model (Fig. 8), a mutation of Arg-228 does not demonstrate the extremely high activation observed when the OAR domain is removed. This suggests that the interaction with the amino end is broken and

![Fig. 6. Substitution mutations within the OAR domain of Prx1 alter transcription regulation. Mutations were made in residues within the OAR domain of Prx1. The OAR consensus sequence is underlined and in bold. The circled X represents a highly variant residue. Arrows point to completely invariant residues in all OAR domains to date. Asterisks above the bold amino acids highlight the substitution mutation. OAR mutations were analyzed for transcription regulation of the tenascin-c promoter. NIH3T3 fibroblasts (dark gray bars) and undifferentiated C2C12 myoblasts (white bars) were cotransfected with a tenascin-c reporter construct and the Prx1 OAR mutant. The R228A mutation was tested only in NIH3T3 cells. Wild-type Prx1a and truncation mutant 1–216 are shown above the mutations as a means for comparison. Results represent the average of a minimum of three transfection experiments performed in triplicate.](http://www.jbc.org/)

![Fig. 7. Chimeric analysis of the function of the Prx1a carboxyl terminus. Truncations and a chimera of the Prx1 transcription factor are shown schematically to the left of the graph. NIH3T3 fibroblasts were cotransfected with a tenascin-c reporter construct and the expression constructs. Results represent the average of a minimum of three transfection experiments performed in triplicate.](http://www.jbc.org/)
unmasked, but the carboxyl activation domain remains hidden. The biological relevance of these data is that in vivo there are likely cofactors that interact with the OAR domain of Prx1a and unmask the activation regions (Fig. 8). Therefore, cells that contain both Prx1a and these unknown factors should stimulate transcription of target genes dramatically.

**Prx1b Carboxyl Terminus Contains a Repression Domain**—The data presented in this study demonstrate that the Prx1b carboxyl region (200–217) contains a repression domain as opposed to a mask. This region is not a mask of activation domains because all of the expression constructs that incorporated it either repressed transcription below base-line levels or remained near base-line levels. This was observed in all three of the assays and in both cell types. The analysis of just the Prx1b 200–217 region fused to the Gal4 DBD was surprising because it did not repress transcription. However, when additional amino acids were included, the protein repressed transcription; this effect is likely the result of the extra amino acids of Prx1b facilitating normal folding of this region. The repression potency of the carboxyl-terminal 18 amino acids in the context of the Prx1b protein is best defined by the comparison of the Prx1b 1–199 truncation with Prx1b 1–217 in NIH3T3 cells driving the tenascin-c promoter. There is more than a 500-fold difference in transcription activation. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference. The in vivo transcription regulatory function of Prx1b compared with Prx1a is possibly even greater than this 500-fold difference.

**Unequal Functional Redundancy of Prx Proteins Is Likely the Result of Distinct Differences in Biochemical Activities**—Prx1 and Prx2 are similar in their genomic organization, primary sequence, and expression. As expected, gene targeting experiments revealed that there is functional compensation between Prx1 and Prx2. However, as described in the Introduction and elsewhere, this compensation is unequal (16–20). Our data suggest a biochemical mechanism to explain the unequal genetic compensation between Prx1 and Prx2.

The Prx1b and Prx1a proteins have very different transcription regulatory activity. One is a repressor of transcription, and the other likely interacts with a cofactor(s) to alter it from a modest to a potent activator. Based on amino acid content, and most importantly function, the Prx2 transcription factor is...
most similar to Prx1a (6). The similarities of Prx1a and Prx2 are highlighted in Fig. 9. It should be noted, however, that the Prx1a protein appears to be a slightly better transcription activator than Prx2. Thus when the Prx2 locus is deleted it is highly likely that the Prx1a protein could entirely compensate for the loss of the Prx2 protein. Conversely, when the Prx1 locus is deleted the Prx2 protein likely compensates for most of the function of the Prx1a protein. However, Prx2 would be unable to compensate for the repressor function of Prx1b. The similar transcription regulatory properties of the Prx1a and Prx2 proteins, combined with the predicted inability of the Prx2 protein to compensate for Prx1b, is the most likely biochemical explanation for the observed unequal genetic compensation.

There were some other similarities and differences between the Prx proteins, and these are summarized in Fig. 9. Most notable of the differences is the function of the Prx domain. Our recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NIH3T3 cells (6). However, recent data for Prx2 demonstrate that it functions as an activator of tenascin-c expression in NI...
