Rieger syndrome is an autosomal-dominant developmental disorder that includes glaucoma and mild craniofacial dysmorphism in humans. Mutations in the Pitx2 homeobox gene have been linked to Rieger syndrome. We have characterized wild type and mutant Pitx2 activities using electrophoretic mobility shift assays, protein binding, and transient transfection assays. Pitx2 preferentially binds the bicoid homeodomain binding site and transactivates reporter genes containing this site. The combination of Pitx2 and another homeodomain protein, Pit-1, yielded a synergistic 55-fold activation of the prolactin promoter in transfection assays. Addition of Pit-1 increased Pitx2 binding to the bicoid element in electrophoretic mobility shift assays. Furthermore, we demonstrate specific binding of Pit-1 to Pitx2 in vitro. Thus, wild type Pitx2 DNA binding activity is modulated by protein-protein interactions. We next studied two Rieger mutants. A threonine to proline mutation (T68P) in the second helix of the homeodomain retained DNA binding activity with the same apparent \( K_d \) and only about a 2-fold reduction in the \( B_{max} \). However, this mutant did not transactivate reporter genes containing the bicoid site. The mutant Pitx2 protein binds Pit-1, but there was no detectable synergism on the prolactin promoter. A second mutation (L54Q) in a highly conserved residue in helix 1 of the homeodomain yielded an unstable protein. Our results provide insights into the potential mechanisms underlying the developmental defects in Rieger syndrome.

Rieger syndrome was defined as a genetic disorder in 1935 (1, 2). It is an autosomal-dominant human disorder characterized by ocular anterior chamber anomalies causing glaucoma, dental hypoplasia, mild craniofacial dysmorphism, and umbilical stump abnormalities. Other features associated with Rieger syndrome include abnormal cardiac, limb, and pituitary developments. Murray and co-workers (2) used a positional cloning strategy to find the Pitx2 gene and determined that it was a member of the homeobox gene family. The homeobox transcription gene family has been extensively studied and plays a fundamental role in development and evolution. Homeobox genes are involved in the genetic control of development, in particular in the specification of the body plan, pattern formation, and determination of cell fate (3–10). In patients with Rieger syndrome five mutations were found to affect the homeobox region. Three of these mutations were missense causing nonconservative amino acid changes in the homeodomain and two splicing mutations in the intron dividing the homeobox sequence (2).

The Pitx2 gene is a member of the Bicoid-like homeobox family (2). The defining characteristic is the lysine residue at position 9 of the third helix (11–13). The homeodomain of Pitx2 has a high degree of homology to P-OTX/Ptx1 and to a lesser extent to unc-30, Otx-1, Otx-2, and goosecoid (2). The Drosophila Bicoid (Bcd) protein binds to the DNA core sequence 5’-TAAATC-3’ (14). Other investigators cloned the mouse Pitx2 gene from an adult pituitary and whole embryo cDNA library and termed the gene Pitx2 and Otx2, respectively (15, 16). We now refer to this gene as Pitx2 as the recommended gene symbol (15).

We are studying the transcriptional regulation of Pitx2, its interaction with other factors and the role Pitx2 plays in vertebrate development. In this report, we have determined the DNA binding and transcriptional properties of Pitx2 and two Pitx2 homeodomain mutations found in Rieger syndrome patients. Interactions with another transcription factor, Pit-1, that is important in pituitary development are examined with both the wild type and mutant proteins. The results support a model in which Pitx2 DNA binding and transactivation activities are modulated by protein-protein interactions. Thus, the Rieger mutations may affect these interactions during development. We provide evidence for the molecular/biochemical basis of Rieger syndrome.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Glutathione S-Transferase (GST)-Pitx2 Fusion Proteins—The Pitx2 gene was PCR amplified from a cDNA clone provided by Drs. Elena Semina and Jeff Murray (Dept. of Pediatrics, University of Iowa). The 5′ primer contained the initiation codon and a unique SauI site (5′-CGTCCGTCGACTAAGGGGAAATGGA-3′), whereas the 3′ antisense primer contained Pitx2 sequences downstream of the stop codon and a unique NsiI site (5′-GTACTGCGATGGCCGCCGACAGTTTCCAGTC-3′), to facilitate cloning into pGex2P-2 GST vector (Amersham Pharmacia Biotech). The resulting plasmid pGST-Pitx2 was confirmed by DNA sequencing. To make plasmid pGST-Pitx2 T68P, which has a point mutation at position 68, a threonine was changed to a proline by a two-step megaprimer PCR technique. The first PCR reaction to generate the megaprimer used the SauI site (5′-CGATTTCTCTTCGTTGGGGACAGATGCTCGGGTAGC-3′) where the point mutation is underlined. The NsiI antisense primer described above and the megaprimer were used to generate the mutant Pitx2 cDNA. Plasmid pGST-Pitx2 L54Q, which has a point mutation at position 54 (leucine to glutamine) was also made using the megaprimer method described above. The antisense primer for the generation of the L54Q mutation was 5′-TGGGCTTCCTCT...
GCTGCTGGAGCTGC-3'). The PCR profiles were 94 °C, 2 min, 60 °C, 2 min, 72 °C, 3 min for 30 cycles using Pfu DNA polymerase (Stratagene). Protein was isolated from transformed BL 21 cells as described (17) with some modifications. Cultures (500 ml) were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. The bacteria were lysed in 10 ml of 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5% low fat milk, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, 100 µg/ml phenylmethylsulfonyl fluoride, 5 mM diithiothreitol, by sonication (4 bursts for 45 s each). In later experiments, protease inhibitor mixture (Sigma) was used and 4% Triton was added prior to removal of debris. The supernatant was aliquoted and stored at −80 °C in 10% glycerol. The cleaved proteins were analyzed on an SDS-polyacrylamide gel and quantitated by the Bradford protein assay (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)—Complementary oligonucleotides containing a Drosophila bicoid site (14) with flanking partial BamHI ends were annealed and filled with Klenow polymerase to generate ^32P-labeled probes for EMSAs, as described (18). For standard binding assays, the oligonucleotide (1.0 pmol) was incubated in a 20-µl reaction-containing binding buffer (20 mM Hepes, pH 7.5, 5 mM glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM diithiothreitol, 0.1 µg of poly(dIDC), 80–180 ng of Pitx2, or 160–285 ng of Pitx2 mutants on ice for 15 min. DNA binding experiments for the Scatchard plots were performed without poly(dIDC). However, we did not measure a difference in DNA binding activity with or without poly(dIDC). For competition assays, unlabeled double-stranded end-filled oligonucleotides were preincubated with the protein for 15 min on ice prior to addition of the probe. Sequences of the bicoid probe and competitor oligonucleotides, all with flanking partial BamHI ends (in lowercase), are given in the figures. Other competitor oligonucleotides used are: 5'-gatcTTGTCTTTCTCTCTTATGAAATAAG-3' and 5'-gatcTTGTCTTCCTTAGCATGCGTGGAATAAG-3'. 200 ng of Pit-1 (Santa Cruz Biotechnology, Inc.) was added to the EMSA experiments prior to addition of the probe. 1 µl of Pit-1 polyclonal antibody was added after Pit-1 and before or after probe. The samples were electrophoresed for 2 h at 250 V in an 8% polyacrylamide gel with 0.25× TBE (1×, 22.5 mM Tris-HCl, pH 8.5, 25 mM boric acid, 0.7 mM EDTA) at 4 °C following pre-electrophoresis of the gels for 1 h at 200 V. The dried gels were visualized by exposure to autoradiographic film. For quantitative analyses to establish binding constants and relative competitions, the amount of bound and free radioactive probe was measured from dried gels using an InstantImager (Packard Instrument Co.). For determination of the amount of binding competition, the ratio of bound to free probe was normalized to the absence of competitor DNA.

In Vitro Pit-1 Binding and Western Blot Assays—Imobilized GST fusion proteins were prepared as described above and suspended in binding buffer (20 mM Hepes, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1% dithiothreitol, 1% milk, and 400 µg/ml ethidium bromide). 40 ng of Pit-1 was added to 5–10 µl of immobilized GST fusion proteins or GST in a total volume of 100 µl and incubated for 30 min at 4 °C. The beads were pelleted and washed four times with 200 µl of binding buffer. The bound Pit-1 was eluted by boiling in SDS-sample buffer and separated on a 12.5% SDS-polyacrylamide gel. Following SDS gel electrophoresis, the proteins were transferred to polyvinylidene difluoride filters (Millipore), immunoblotted, and detected using Pit-1 antibody and ECL reagents from Amersham Pharmacia Biotech.

Expression and Reporter Constructs—Expression plasmids containing the cytomegalovirus (CMV) promoter linked to the Pitx2, Pitx2 bicoid-TK-luc, Pitx2 T68P, and Pitx2 L54Q DNA were constructed in pcDNA 3.1 MycHisC (CLONTECH) was cotransfected in all experiments as a control for transfection efficiency.

In Vitro Pit-1 Binding and Western Blot Assays—Imobilized GST fusion proteins were prepared as described above and suspended in binding buffer (20 mM Hepes, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1% dithiothreitol, 1% milk, and 400 µg/ml ethidium bromide). 40 ng of Pit-1 was added to 5–10 µl of immobilized GST fusion proteins or GST in a total volume of 100 µl and incubated for 30 min at 4 °C. The beads were pelleted and washed four times with 200 µl of binding buffer. The bound Pit-1 was eluted by boiling in SDS-sample buffer and separated on a 12.5% SDS-polyacrylamide gel. Following SDS gel electrophoresis, the proteins were transferred to polyvinylidene difluoride filters (Millipore), immunoblotted, and detected using Pit-1 antibody and ECL reagents from Amersham Pharmacia Biotech.
Hepes, pH 7.1) and 125 mM calcium chloride were added to the medium and allowed to precipitate overnight, followed by fresh medium for 4 h before harvest. Cells were incubated for 24 h then lysed and assayed for reporter activities and protein content by Bradford assay (Bio-Rad). Luciferase activity was measured using reagents from Promega. β-Galactosidase activity was measured using the Galacto-Light Plus reagents (Tropix Inc.). Pitx2 proteins transiently expressed in COS-7 cells were detected by immunoblotting using a c-Myc monoclonal antibody (9E10, Santa Cruz), as described above.

RESULTS

Pitx2 and the Pitx2 T68P Mutant Bind the Bicoid Site—To examine Pitx2 DNA binding activity, Pitx2, Pitx2 L54Q, and Pitx2 T68P were expressed in bacteria as GST fusion proteins and the GST moiety was cleaved from the protein (Fig. 1, A and B). Approximately 5 μg of cleaved proteins were resolved on an SDS-polyacrylamide gel and detected by silver staining (Fig. 1B). The Pitx2 L54Q mutant was apparently unstable and mostly degraded in the preparations (Fig. 1B). EMSAs demonstrated binding of Pitx2 and Pitx2 T68P to the bicoid element (Fig. 1C). To obtain comparable binding activities about twice as much Pitx2 T68P as wild type protein was used in the EMSA experiments. Even though the Pitx2 L54Q protein was mostly degraded, we still attempted to detect binding by using up to 400 ng in an EMSA. Binding could not be detected, although the cause is most likely because of protein instability (Fig. 1C).

Identification of Pitx2 DNA Binding Specificity—To determine the specificity of Pitx2 binding, competition analyses were performed using nonspecific oligonucleotides, and oligonucleotides matching known homeobox binding sites. Addition of excess unlabeled DNA-containing sites for the Nkx, Prd, or ftz classes caused less than 20% competition for Pitx2 binding to the bicoid probe (Fig. 2A). The Idx class and nonspecific oligonucleotides (see “Experimental Procedures”), also failed to compete for Pitx2 binding (data not shown). Partial competition (34%) was seen with the Pit-1 DNA binding element. In contrast, greater than 85% of the Pitx2-bicoid DNA complex could be competed using unlabeled bicoid DNA at a 50-fold molar excess (Fig. 2B).

To ascertain the binding affinity of Pitx2 for the bicoid element, an EMSA DNA titration experiment was performed. We analyzed several concentrations of Pitx2 protein binding to the bicoid sequence and determined an apparent KD of 50 nM by Scatchard plots (Fig. 2C). These experiments demonstrate that Pitx2 binds the DNA bicoid sequence with a reasonable affinity that compares well but is somewhat lower than the reported KD values of 10⁻⁸ to 10⁻⁹ for other homeodomain proteins (7, 23–26).

The T68P Point Mutation Reduces the DNA Binding Specificity of Pitx2—Since the Pitx2 T68P mutant retained bicoid DNA binding, we next asked whether the mutation affected binding specificity. Competition analyses were performed using the same panel of oligonucleotides as described for the wild type Pitx2 protein EMSA experiments. In contrast to wild type,
the mutant Pitx2 T68P protein demonstrated an overall increase in binding to all of the competitor DNAs (Fig. 3, A and B). The ability of these nonbicoid DNA competitors to bind Pitx2 T68P demonstrates a slight loss of binding specificity by this mutant. Since 2-fold more Pitx2 T68P protein was required to obtain similar binding activities as seen with wild type protein, we measured the DNA binding parameters of Pitx2 T68P. The Scatchard plot of Pitx2 T68P binding to the bicoid element demonstrates that the binding capacity ($B_{\text{max}}$) of Pitx2 T68P is 2-fold less than wild type (Fig. 3 C). Note that in this experiment 2-fold more mutant protein was used to generate the Scatchard plot compared with wild type (Fig. 2 C), hence the similar $x$ intercept ($B_{\text{max}}$). However, the dissociation constant of the mutant protein was not significantly altered compared with wild type, $K_D = 58 \text{ nM}$. Thus, the threonine to proline mutant still binds the bicoid element, although with a quantitative decrease in binding specificity and capacity compared with wild type.

**Pitx2 T68P and L54Q Mutants Do Not Transactivate a Promoter Containing the 5' TAATCC-3' Sequence**—We performed transient transfections of a CMV-Pitx2 expression vector with a TK-luciferase reporter containing 4 bicoid elements. Transient transfection of a prolactin promoter reporter with Pitx2 in COS-7 cells resulted in a 5-fold increase in luciferase activity (Fig. 5). Similar results were seen in HeLa cells (data not shown). Pitx2 T68P did not transactivate the prolactin promoter, similar to the results with the bicoid-TK reporter (Fig. 5). Since Pit-1 and Pitx2 are co-expressed during pituitary development, we asked if Pitx2 could transactivate a pituitary-specific gene. The prolactin gene 5'-flanking region is a well studied target DNA, which contains multiple bicoid elements. Transient transfection of a prolactin promoter reporter with Pitx2 in COS-7 cells resulted in a 5-fold increase in luciferase activity (Fig. 5). Similar results were seen in HeLa cells (data not shown). Pitx2 T68P did not transactivate the prolactin promoter, similar to the results with the bicoid-TK reporter (Fig. 5). As expected, since Pitx2 L54Q was not detected in the cell lysates, it did not transactivate the prolactin reporter.

Since Pit-1 and Pitx2 are co-expressed during pituitary de-
development (2, 27), we cotransfected Pit-1 and Pitx2 with the prolactin-luciferase reporter. Pit-1 alone caused about a 3-fold activation of the prolactin promoter under our conditions (Fig. 5). The combination of Pitx2 and Pit-1 yielded a synergistic 55-fold activation of luciferase activity (Fig. 5). In contrast, addition of Pit-1 with Pitx2 did not further increase transcription from the bicoid-TK reporter (data not shown). The Pitx2 T68P mutant did not synergize with Pit-1 to stimulate transcription activity. These results demonstrate that Pitx2 and Pit-1 act in vivo to synergistically transactivate the prolactin gene promoter.

**Pit-1 Interacts with Pitx2 to increase Its Binding Capacity**—The transient transfections demonstrated a synergistic effect of Pitx2 and Pit-1 on transactivation of the prolactin promoter. We next asked if Pit-1 could directly interact with Pitx2 to affect its DNA binding characteristics. Addition of Pit-1 increased the amount of Pitx2-DNA bound complex approximately 2–3-fold (Fig. 6). Interestingly, Pit-1 caused the formation of apparent Pitx2 dimers as shown in Fig. 6. Pit-1 by itself did not bind the bicoid probe, and the Pit-1-enhanced binding of Pitx2 was competed by unlabeled bicoid DNA (Fig. 6). These results demonstrate that the enhanced binding of Pitx2 by
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Pit-1 is specific for the bicoid element and that this enhanced binding is not because of Pit-1 binding to the bicoid probe. Pit-1 antibody, added after the protein-DNA complex was assembled, did not cause a supershift or inhibit the enhanced binding, suggesting that the Pit-1 contribution is transient or that the complex is not maintained during gel electrophoresis (data not shown). Pit-1 also had a comparable 2–3-fold stimulatory effect on the binding of the mutant Pitx2 T68P protein (Fig. 6). As a control we added 1 μg of bovine serum albumin and 10% glycerol and saw no effect on protein binding (Fig. 6).

To further analyze the interaction of Pit-1 with Pitx2, we performed in vitro solution binding assays using immobilized GST-Pitx2 and GST-Pitx2 T68P proteins. After incubating the immobilized proteins with Pit-1 we measured Pit-1 binding by Western blot. Pit-1 bound to wild type GST-Pitx2 and GST-Pitx2 T68P but not to GST (Fig. 7). For comparison, the same amount of Pit-1 used in the binding assay was immunoblotted (Fig. 7). Thus, Pit-1 physically interacts with Pitx2 to facilitate DNA binding and apparently to increase transcriptional activity.

**DISCUSSION**

This study represents a molecular and biochemical characterization of the newly discovered human homeodomain transcription factor, Pitx2. We compare the activities of wild type Pitx2 to homeodomain mutations in Pitx2 that cause Rieger syndrome. Pitx2 has a lysine residue at position 9 of helix 3 in the homeodomain, which is known to selectively recognize the 3′-CC dinucleotide adjacent to the TAAT core sequence (7). Consistent with this phylogenetic relationship, we have demonstrated that Pitx2 can bind the DNA sequence 5′-TAATCC-3′, which is recognized by Bicoid protein (14).

Recently, a Pitx2-related murine gene product was identified by two independent researchers and termed Ptx1 and P-OTX (Pitx1 in new nomenclature) (28, 29). The expression patterns of Pitx1 and Pitx2 are very similar, and expression in Rathke’s pouch suggests that this new family may play an important role in pituitary gland development. Our transactivation data are similar to that reported for the P-OTX/Ptx1 activation of the prolactin promoter when transfected into nonpituitary cell lines (28, 29). The POU homeodomain protein Pit-1 also binds to specific sites on the prolactin promoter to activate prolactin transcription. Pit-1 is an important transcription factor that regulates pituitary cell differentiation and expression of thyroid-stimulating hormone, growth hormone, and prolactin (27). Co-expression of Pit-1 and Pitx2 gave a 55-fold synergistic transactivation of the prolactin promoter. Consistent with this synergism, we demonstrated a direct interaction between Pitx2 and Pit-1 in vitro. These results establish that Pitx2 transactivation activity is enhanced by interaction with another transcription factor.

We demonstrate that binding of Pitx2 to the bicoid element is enhanced upon interacting with Pit-1. There is precedence for proteins interacting together to increase their binding activity (30–32). It has been shown that Pbx can increase the binding of Hox proteins to their optimal DNA binding site (33, 34). Pbx can also interact with the Drosophila homebox protein, Engrailed, to stimulate cooperative DNA binding (34). The transcription factor Prospero, has been shown to increase the DNA binding of Dfd and Hoxa-5 (35). They report that Prospero is not part of the Dfd-DNA complex and that Prospero interacts with Dfd to modify its DNA binding capacity. This enhancement of DNA binding activity without detectable ternary complexes has been reported previously with other homeodomain proteins (36). This is similar to our finding that Pit-1 and Pitx2 physically interact, yet Pit-1 is apparently not part of the Pitx2-bicoid DNA complex. These results demonstrate that Pit-1 and Pitx2 interact to increase the binding capacity of Pitx2, which may facilitate transactivation of the prolactin promoter.

In Rieger syndrome the T68P point mutation lies in helix 2 at position 30 of the homeodomain, whereas the L54Q point mutation is in helix 1 at position 16 of the homeodomain. To our knowledge there are no reports of amino acids in these positions affecting DNA binding specificity in homeodomain proteins. Comparison of the amino acid sequence of over 300 homeobox proteins reveals that position 30 of the homeodomain is not conserved, and several amino acids can be located at this position (37). Although, we have found no other proteins with a proline at position 30 it appears that this position can
accommodate changes in amino acid identity without affecting DNA binding activity. In contrast, the amino acid residue at position 16 of the homeodomain is highly conserved (37). In the approximately 300 homeobox proteins analyzed, the residue at this position is a leucine, except for EgHbx4, ap, and LH-2, which contain a methionine, and Lmx-1, which has a phenylalanine (37). This strong conservation suggests that the leucine residue plays a fundamental role in the homeodomain. Our results support this prediction by demonstrating that a mutation of the leucine to a glutamine (L54Q) is detrimental for Pitx2 activity. Since this mutant protein could not be detected in transfected mammalian cells, this suggests that the leucine at position 16 is important for stability of the homeodomain.

The binding specificity of homeodomains is dictated mostly by residues in the recognition helix and the N-terminal arm (11,38–41). The side chains of amino acids at positions 25, 28, and 31 of the Antp homeodomain contact phosphate groups of the 5′-TAAT-3′ DNA binding element (7). The amino acids located in positions 6 and 10 in helix 3 of the Drosophila Bicoid homeodomain have been shown to recognize the TAAT core sequence (42). It has been demonstrated that the amino acid at position 50 of the Bicoid homeodomain is critical for recognizing the 3′-CC dinucleotide of the DNA binding sequence (7,39). Recently, the molecular basis of Boston-type craniosynostosis was determined to involve a point mutation in the N-terminal arm of the MSX2 homeodomain (43). Similar to our results a mutation in the MSX2 homeodomain did not abolish DNA binding as one might expect. Overexpression of the wild type Msx2 gene can also produce craniosynostosis, therefore enhanced binding by the MSX2 mutant is implicated as the cause for this disorder. Pitx2 T68P binds DNA but this mutation results in slightly reduced binding specificity and capacity. This reduction in binding specificity might account for the loss of Pitx2 transactivation activity. However, although we cannot rule out this possibility, it seems unlikely that these changes are sufficient to yield no detectable transacting activity especially in the presence of Pit-1. A more plausible explanation for the mutant effect is that the point mutation affects a transactivation activity. We are currently mapping the transactivating and protein-protein interaction domains to further elucidate the biochemical activities of Pitx2.

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