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Novel *TFAP2B* Mutations That Cause Char Syndrome Provide a Genotype-Phenotype Correlation

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To elucidate further the role, in normal development and in disease pathogenesis, of TFAP2B, a transcription factor expressed in neuroectoderm, we studied eight patients with Char syndrome and their families. Four novel mutations were identified, three residing in the basic domain, which is responsible for DNA binding, and a fourth affecting a conserved PY motif in the transactivation domain. Functional analyses of the four mutants disclosed that two, R225C and R225S, failed to bind target sequence in vitro and that all four had dominant negative effects when expressed in eukaryotic cells. Our present findings, combined with data about two previously identified *TFAP2B* mutations, show that dominant negative effects consistently appear to be involved in the etiology of Char syndrome. Affected individuals in the family with the PY motif mutation, P62R, had a high prevalence of patent ductus arteriosus but had only mild abnormalities of facial features and no apparent hand anomalies, a phenotype different from that associated with the five basic domain mutations. This genotype-phenotype correlation supports the existence of TFAP2 coactivators that have tissue specificity and are important for ductal development but less critical for craniofacial and limb development.

Introduction

Char syndrome (MIM 169100) is an autosomal dominant disorder characterized by patent ductus arteriosus (PDA), facial dysmorphism, and abnormalities of the fifth finger (Char 1978). In studies reported elsewhere, our group linked Char syndrome to chromosome 6p12-p21.1, using two large kindreds with this disorder (Satoda et al. 1999), and then identified disease-causing mutations in the transcription factor *TFAP2B* (also known as *AP-2β* [MIM 601601]) from one of those families, as well as from a second, smaller kindred (Satoda et al. 2000).

The TFAP2 transcription factors constitute a family of closely related and evolutionarily conserved sequence-specific DNA-binding proteins. *TFAP2* genes have been identified in an invertebrate (*Drosophila melanogaster*) and several vertebrates, including *Xenopus laevis*, chickens, mice, and humans (Williams et al. 1988; Winning et al. 1991; Moser et al. 1995; Chazaud et al. 1996; Williamson et al. 1996; Shen et al. 1997; Bauer et al. 1998). Although the *Drosophila* genome contains a single *TFAP2* gene, there have been evolutionary duplications, such that mice and humans have at least three *TFAP2* genes. The three murine *Tfap2* genes (*Tfap2a*, *Tfap2b*, and *Tfap2c*) are expressed in the developing limbs, epithelia, and neuroectoderm, including neural crest–derived tissues, such as the facial mesenchyme (Mitchell et al. 1991; Moser et al. 1995, 1997*b*). TFAP2 proteins form homo- and heterodimers with other TFAP2 family members. Dimers bind to palindromic GC-rich DNA-binding sequences in promoter regions of certain genes, activating their transcription.

In addition to the observation that *TFAP2B* defects cause Char syndrome, *TFAP2* gene defects have been associated with developmental anomalies in flies and mice. In *Drosophila*, several *dAP-2* mutants were identified through a mutagenesis screen (Monge et al. 2001). Null mutations resulted in reduced proboscis, shortened legs, and brain abnormalities; hypomorphic alleles caused more-modest changes in leg length. Mice with targeted disruptions of *Tfap2a* (MIM 107580) and *Tfap2b* (MIM 601602) have strikingly different phenotypes (Schorle et al. 1996; Zhang et al. 1996; Moser et al. 1997*a*), establishing that the roles of these *Tfap2* genes are not redundant. *Tfap2a*-deficient mice have severe and diffuse anomalies, including anencephaly, body-wall defects, and craniofacial defects (Schorle et

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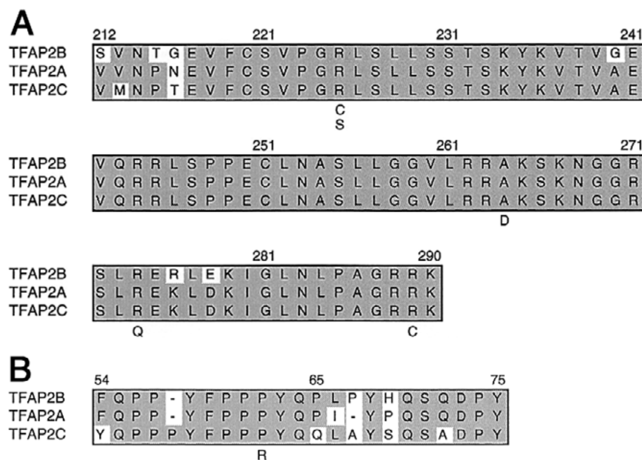

Figure 1 Clustal W alignment of human TFAP2 protein sequences. *A,* Alignment of the basic domains of the TFAP2 proteins. Numbering is according to the TFAP2B amino acid sequence. Identical amino acid residues are shaded in gray. The three novel and two previously reported mutations are indicated. *B,* Alignment of a portion of the transactivation domains that includes the PY motif. The P62R mutation is indicated.

al. 1996; Zhang et al. 1996). Loss of *Tfap2b* causes congenital polycystic kidney disease due to excessive apoptosis of renal epithelial cells (Moser et al. 1997*a*), a phenotype strikingly different from Char syndrome.

Potential adverse effects of missense defects in *TFAP2* genes can often be predicted on the basis of position, because the three functional domains that constitute the polypeptide have a completely conserved arrangement among all TFAP2 family members. The N-terminal portion of the protein contains the transactivation domain, which has an amino acid sequence that is relatively poorly conserved among the TFAP2 proteins. The basic and helix-span-helix (HSH) domains constitute the C-terminal half of the protein and are highly conserved among all TFAP2 orthologues and paralogues. The basic domain is necessary for DNA binding, and the HSH domain has DNA binding and dimerization functions (Williams and Tjian 1991). The two *TFAP2B* gene defects previously identified in families of patients with Char syndrome were missense mutations affecting the basic domain (Satoda et al. 2000). Functional analyses of these mutants disclosed that both prevent binding to TFAP2 target sequence and act in a dominant negative manner.

The purpose of the present study was to document the degree of molecular heterogeneity of *TFAP2B* mutations underlying Char syndrome, to determine whether disease-causing mutants acted consistently in a dominant negative manner and to correlate genotype with phenotype. Four novel *TFAP2B* mutations were identified among eight unrelated patients with Char syndrome and their families. Three missense mutations, observed in patients with the classic phenotype, altered highly conserved residues in the basic domain and had dominant negative effects. A family with high prevalence of PDA, mild facial anomalies, and normal hands was found to be inheriting a missense mutation affecting the conserved PY motif in the transactivation domain. This mutant bound target sequence in a normal manner but had dominant negative effects on transactivation. These data, combined with information about the two previously identified mutations, suggest that dominant negative effects, generally involving basic domain alterations, are necessary for the pathogenesis of Char syndrome. The genotype-phenotype correlation observed with the transactivation domain mutant suggests that ductal development depends critically on interactions of TFAP2B with one or more TFAP2 coactivators, which play a less important role in craniofacial and limb development.

Material and Methods

Mutation Analysis

Blood samples were obtained, with informed consent, from eight unrelated families with one or more individuals affected with Char syndrome. Genomic DNA was extracted from blood leukocytes, using the Puregene Genomic DNA Isolation kit. Sense and antisense oligonucleotide primers that correspond to intronic sequences flanking the *TFAP2B* coding exons were used to amplify those exons and their intronic junctions from genomic DNAs. Amplified DNA products were sequenced bidirectionally on an ABI 3700 DNA sequencer by cycle sequencing.

To confirm the changes identified, PCR-based mutation assays were developed. The P62R mutation introduced a *Fau*I site, and the two R225 mutations obliterated a *Hae*III site, facilitating these analyses. For the R274Q mutation, a mismatch primer that introduced an *Hha*I site in the wild-type amplimer, but not the mutant, was used. For all assays, PCR products were digested with the appropriate restriction endonuclease and were visualized directly with ethidium bromide after electrophoresis on a 2% horizontal agarose gel. More than 50 control individuals were examined for the presence of these changes, using these mutation assays.

In Vitro Transcription and Translation

Clones containing the human *TFAP2B* and *TFAP2A* cDNAs were provided by H. Hurst and T. Williams, respectively. To create mutant constructs, site-directed mutagenesis was performed using the PCR ligation method with *Pfu Taq* polymerase (Stratagene). After ligation into pSP64 Poly(A) (Promega) and sequence confirmation, in vitro transcription and translation were performed using the TnT Quick Coupled Transcription/

Translation System (Promega), according to the manufacturer's protocol. These proteins, which were labeled by incorporation of [^{35}S]-methionine, were separated by SDS-PAGE and detected by use of autoradiography.

Electromobility Shift Assays (EMSAs)

TFAP2 proteins were used in EMSAs with the *MT2A* −180 TFAP2 binding sequence (5′-GAACTG-ACCGCCCGCGGCCCGTGTGCAGAG-3′) (Promega) that had been end-labeled with ^{32}P, using the T4 polynucleotide kinase. DNA-protein binding was performed at room temperature, for 30 min, in a reaction mixture that contained 3 ml of reticulocyte lysate, 0.07 pmol ^{32}P-labeled oligonucleotide, 4% glycerol, 1.2 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)·poly(dI-dC). The products were fractionated on a 4% nondenaturing polyacrylamide gel at 4°C. Gels were dried and exposed through two blank films to attenuate the ^{35}S signal. Similar EMSAs were carried out with mutant and wild-type TFAP2B proteins that had been cotranslated with a truncated TFAP2A (ΔN165) that retained DNA binding and dimerization properties (kindly provided by T. Williams).

Cross-Linking

Ethylene glycol bis(succinimidylsuccinate) (EGS) (Sigma) was dissolved in DMSO just before use. It was incubated, at room temperature, at a final concentration of 8 mM, together with 2 ml of the reticulocyte lysate in a reaction mixture that contained 67 mM triethanolamine (pH 8.0), 1.3 mM EDTA, 3.3 mM glycerol, and 19 mM β-mercaptoethanol. After 30 min, the reaction was stopped by adding lysine to a final concentration of 77 mM. Samples were boiled briefly in SDS sample buffer containing 1% DTT and 25 mM β-mercaptoethanol and were then separated on a 10% gel for SDS-PAGE. Signals were detected by autoradiography.

Transfections and Chloramphenicol Acetyl Transferase Assays

The eukaryotic expression vector pSP72RSV, which contained wild-type *TFAP2B,* and the chloramphenicol acetyl transferase (CAT) reporter constructs, with and without the TFAP2 binding sequences (A2BCAT and BCAT, respectively), were provided by T. Williams. Mutant *TFAP2B* cDNAs were shuttled into pSP72RSV, and the *POLR2TC1* cDNA (previously known as *"PC4";* provided by M. Tainsky) (Kannan and Tainsky 1999) was transferred into the eukaryotic expression vector pcDNA3.1 (Invitrogen). NIH3T3 cells (American Type Culture Collection) were incubated at 37°C and 5% CO_2 in Dulbecco's minimal essential medium (Sigma), supplemented with 10% fetal calf serum (Gibco). Using Lipofectamine (Gibco), we transfected the cells with the DNA constructs, which were then expressed transiently. For all conditions, total transfected DNA was held constant, using an unrelated plasmid as needed.

After 60 h, the transfected cells were lysed, and relative CAT concentrations were determined in duplicate by CAT enzyme-linked immunosorbent assay (Boehringer). To normalize for transfection efficiency and cell number, a green fluorescent protein (GFP) reporter construct (pQBI25; QBI) was cotransfected, and GFP concentrations were measured in the cell lysates with a FOCI System 3 spectrofluorometer. Normalized CAT levels in cells transfected with A2BCAT alone were arbitrarily set at 1. All transfection conditions were repeated a minimum of three times. Mean normalized CAT levels were compared using the Student's t test with the significance threshold set at $P < .01$.

Results

TFAP2B *Mutations*

The coding *TFAP2B* exons and their intronic boundaries were amplified and sequenced bidirectionally from eight patients with Char syndrome and their families. Among this cohort, missense mutations were identified in four individuals.

A Palestinian boy with PDA, clinodactyly, facial features typical of Char syndrome, and a supernumerary nipple was recruited. Interestingly, his parents were first cousins, and a male first cousin, whose parents were also consanguineous, was found to have a PDA but no other features of Char syndrome. Analysis of the proband's DNA identified a coding region alteration in exon 4, a C→T transition at nucleotide (nt) 673 of the *TFAP2B* cDNA, which was present in heterozygosity. This sequence change predicted a substitution of an arginine by a cysteine at position 225 (R225C) in the TFAP2B basic domain. Because this alteration obliterated a *Hae*III site, it was readily assayed for in DNA fragments amplified from the proband's parents' and cousin's DNA samples. RFLP analysis revealed that neither the proband's parents nor his cousin harbored the R225C change. Analysis of >100 Israeli Arab control chromosomes failed to reveal this alteration. Comparison of the three human TFAP2 transcription factors (fig. 1*A*), as well as the other vertebrate and invertebrate proteins (data not shown), revealed that Arg^{225} is completely conserved.

An English family that has been described elsewhere (Sweeney et al. 2000) and that had typical facial features and hand anomalies, but no cardiovascular abnormalities, was also found to have an exon 4 mutation at nt 673. This change was a C→A transversion that was predicted to replace Arg^{225} with a serine residue (R225S). Using the same *Hae*III RFLP employed for the R225C

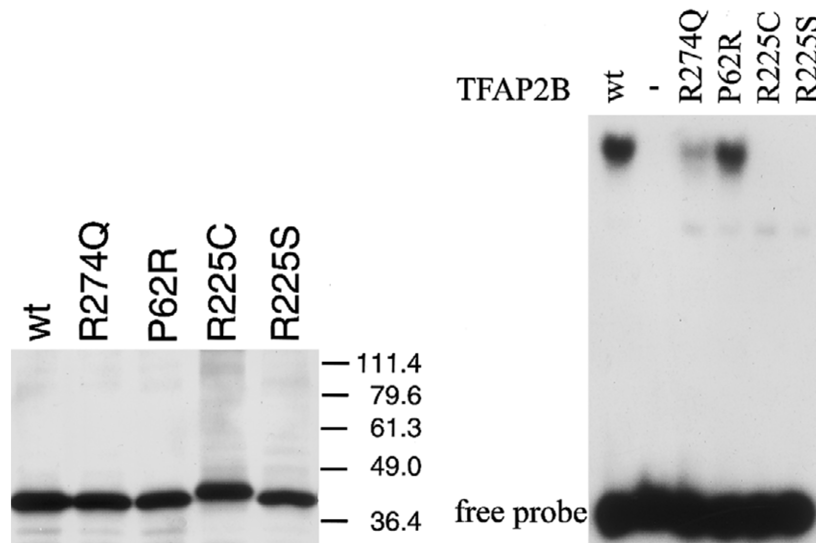

Figure 2 Expression and function of recombinant TFAP2B proteins. *Left,* Autoradiogram of an SDS-PAGE with expressed wild-type (wt) and mutant TFAP2B proteins. Mobility of proteins of varying mass are indicated at the right. *Right,* Autoradiogram of an EMSA performed using the recombinant TFAP2B proteins that had been incubated with [^{32}P]-labeled DNA with the consensus TFAP2 binding sequence. Free probe is indicated at bottom.

mutation, we confirmed that this R225S mutation was present in available affected individuals and was absent in >100 control chromosomes from white Americans.

An Australian family with four affected individuals was analyzed. All patients had a similar markedly anomalous facial appearance and PDA, but no hand anomalies were noted. A G→A transition at nt 821 in exon 5 was identified, which was predicted to change an arginine to a glutamine at position 274 (R274Q). Because this change neither created nor destroyed a restriction site, mismatch PCR was used to introduce an *Hha*I site in amplimers with the wild-type sequence. With the use of this assay, the R274Q change was confirmed in affected family members and excluded from white control individuals. Like Arg^{225}, Arg^{274} resides within the basic domain and is completely conserved among all vertebrate and invertebrate TFAP2 proteins (fig. 1*A*).

The fourth family found to have a *TFAP2B* mutation was a large kindred from Minnesota that has been described elsewhere (Sletten and Pierpont 1995) and whose disease was independently linked to 6p12-p21.1 in a genome scan (Satoda et al. 1999). The phenotype in this family is notable for a high prevalence of PDA (10 [71%] of 14 affected individuals), mildly anomalous facial features, and normal hands. In addition, one affected individual had a muscular ventricular septal defect when examined at age 8.5 years, and another died of complex cyanotic heart disease in adulthood. Analysis of exon 2 revealed a C→G transversion at nt 185, which was predicted to replace a proline with an arginine (P62R). This mutation introduced a *Fau*I site that facilitated the assay for this gene defect. Analysis of this family revealed that the P62R mutation was present in all available affected individuals but not in unaffected family members or white control individuals. Pro^{62} resides in the transactivation domain, a region that is less well conserved

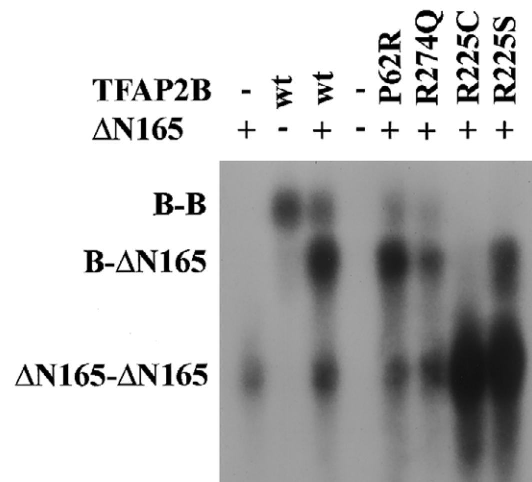

Figure 3 EMSA with cotranslated TFAP2B and truncated TFAP2A proteins. Truncated TFAP2A (ΔN165), which retains dimerization and DNA-binding properties, was cotranslated with wild-type and mutant TFAP2B. TFAP2 proteins were incubated with [^{32}P]-labeled DNA with the consensus TFAP2 binding sequence and electrophoresed. The two homodimer species (upper and lower shifted complexes) and the heterodimer (intermediate shifted complex) are indicated.

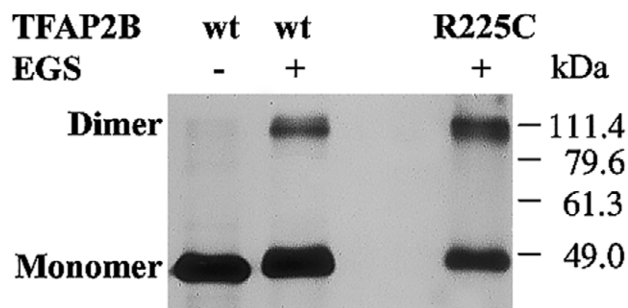

Figure 4 Chemical cross-linking of recombinant TFAP2B proteins. Autoradiography of wild-type and R225C TFAP2B proteins that were translated in vitro with incorporation of ^{35}S-Met, and then electrophoresed, under denaturing conditions, after a 30-min exposure to the chemical cross-linker EGS (+) or without exposure (−). Mobility of proteins of varying mass is indicated at the right. The expected 50- and 100-kDa masses for the TFAP2B monomers and dimers, respectively, are indicated (*left*). Untreated R225C protein (shown in left panel of fig. 2) had the same mass as the monomeric wild-type protein.

among the TFAP2 proteins. This proline residue, however, lies in a PY motif ($Y^{58}F^{59}P^{60}P^{61}P^{62}Y^{63}$); P^{62} is completely conserved among the TFAP2 proteins, and the entire motif is highly conserved (fig. 1*B*). Moreover, alteration of this motif (P60A in the context of human TFAP2A) has been shown to have adverse effects on transactivation activity (Wankhade et al. 2000).

Functional Assays of Mutant TFAP2B Binding

EMSAs were used to assess the ability of mutant TFAP2B proteins to bind TFAP2 target DNA sequence. Wild-type and mutant proteins were translated in vitro (fig. 2*A*) and incubated with the palindromic TFAP2 recognition sequence from position −180 of the human metallothionein-2A gene (*MT2A*-180). The wild-type TFAP2B produced protein-DNA complexes with retarded mobility (fig. 2*B*). As anticipated on the basis of the position of the mutation, P62R protein also shifted probe normally. The R274Q mutant weakly shifted a complex, a finding that was reproducible. The two R225 mutants did not engender shifts. Since the three mutant TFAP2B proteins with abnormal EMSA results harbored changes in the basic domain, it was likely that their dysfunction arose from a failure to bind target DNA sequence.

Next, wild-type and mutant *TFAP2B* genes were cotranslated with a truncated *TFAP2A*, ΔN165, which retains the ability to dimerize and bind DNA. Cotranslation of the two genes results in three protein species (TFAP2B homodimers, ΔN165 homodimers, and TFAP2B-ΔN165 heterodimers) that can be separated in an EMSA because of differences in mass. When the wild-type TFAP2B was used, the three shifted complexes were observed (fig. 3). As expected from the previous EMSAs, the P62R and R274Q cotranslation lysates also produced the three shifted complexes. Using the R225S cotranslation products, we noted two shifted species that corresponded to the ΔN165 homodimers and the R225S-ΔN165 heterodimers. This result documented that R225S protein was capable of dimerizing and did not have strong dominant negative effects in vitro. Similar analysis with the R225C mutant revealed only a single shifted complex, which corresponded to the ΔN165 homodimers. This experiment did not permit us to determine whether the R225C protein was failing to dimerize or was not binding target DNA, although we suspect the latter was true.

Cross-Linking

To determine formally whether the R225C protein dimerized, chemical cross-linking of wild-type and R225C mutant TFAP2B proteins was performed using the chemical cross-linker EGS. After electrophoresis of the cross-linked proteins on a denaturing gel, TFAP2B monomers with a mass of ∼50 kDa were observed in all lanes, whereas dimers of ∼100 kDa were visible only for the EGS-treated proteins (fig. 4). These results documented that R225C mutant protein was able to dimerize, establishing that it failed to bind TFAP2 target sequence per se. When this failure is combined with the EMSA data, it strongly supports the conclusion that R225C protein had strong dominant negative effects in vitro.

Transactivation in Eukaryotic Cells

To assess the ability of the mutant TFAP2B proteins to transactivate gene expression, eukaryotic expression constructs with the mutant genes were cotransfected into NIH3T3 cells along with a CAT reporter construct containing three copies of the TFAP2-binding site *MT2A* −180 (A2BCAT). As in work we reported elsewhere (Satoda et al. 2000), the TFAP2 coactivator, *POLR2TC1* (also known as *PC4*), was cotransfected to prevent transcription self-interference.

NIH3T3 cells had a low level of endogenous TFAP2 activity, as demonstrated by the small difference in CAT concentrations that depends on the presence or absence of TFAP2 binding sites in the reporter construct (A2BCAT vs. BCAT, respectively; $P < .01$) (fig. 5). Expression of wild-type TFAP2B resulted in a 5.4-fold increase of CAT concentration above the A2BCAT-only baseline. All four TFAP2B mutants produced CAT levels that were significantly less than those obtained with wild-type TFAP2B ($P < .01$). For the P62R mutant, CAT levels were modestly higher than the A2BCAT-only baseline ($P < .03$). On the basis of the EMSA results, we believe this transactivation could be attributable to mutant homodimers or heterodimers formed with endog-

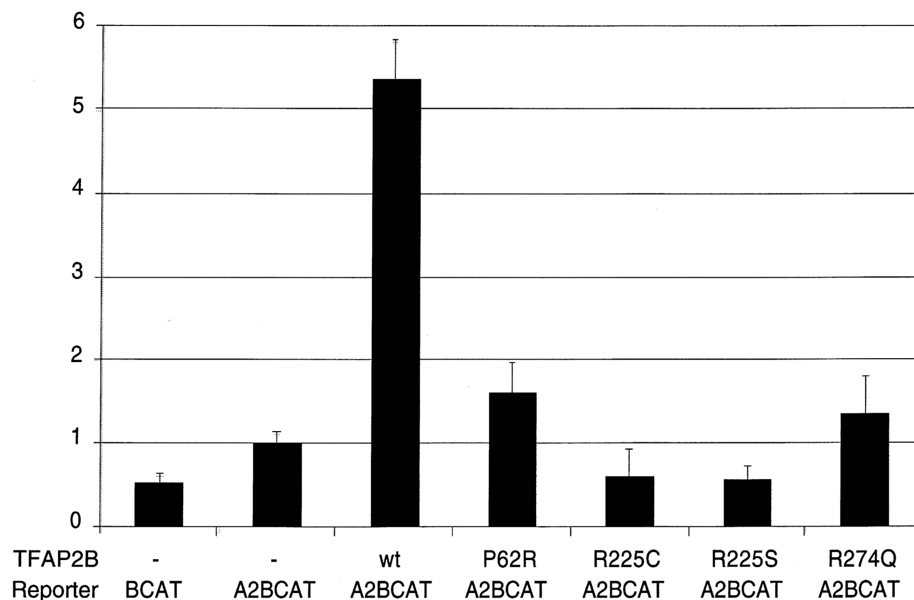

Figure 5 Transient expression of wild-type or mutant *TFAP2B* in NIH3T3 cells. Cells were transfected with 1.5 mg of the CAT reporter construct (A2BCAT) with three copies of TFAP2 binding sequence or an equivalent amount of the CAT-only construct (BCAT). To test for transactivation, 0.3 mg wild-type (wt) or mutant *TFAP2B* construct was cotransfected with A2BCAT. After 48 h, cells were lysed, and the CAT concentrations in the lysates were determined. To normalize for transfection efficiency, 0.5 mg of pQB125 was cotransfected, and GFP fluorescence was measured. The bars indicate the mean and standard errors from three independent transfections. Units are arbitrary, and the mean from the condition with only A2BCAT was set at 1.0.

enous TFAP2 proteins or both. The two R225 mutants decreased CAT levels below that obtained with A2BCAT alone ($P < .02$ for both), suggesting interference with endogenous TFAP2 activity. Furthermore, CAT levels produced by the two R225 mutants were significantly lower than those obtained with the P62R and R274Q mutants ($P < .02$).

To delineate further the effects of the TFAP2B mutants on wild-type TFAP2 proteins, mutant and wild-type *TFAP2B* constructs were cotransfected. Coexpression of each of the four mutants with wild-type *TFAP2B* produced significantly lower CAT levels than those obtained from wild-type *TFAP2B* alone ($P < .01$), despite the fact that an equal amount of the wild-type construct was used in all conditions (fig. 6). Because transcription self-interference does not cause this reduction under these conditions (Satoda et al. 2000), these results documented that all four mutants have dominant negative effects.

Differences among the four mutants were noted in the results of the cotransfection studies. As expected on the basis of the EMSA data showing that R225C mutant protein failed to bind target sequence as either a homodimer or heterodimer, coexpression of R225C with wild-type TFAP2B produced the lowest CAT levels. These levels were significantly lower than those obtained with P62R ($P < .004$) or R274Q ($P < .04$) but did not reach significance when compared with R225S ($P < .08$). These results were compatible with findings recently reported about a *Drosophila dAP-2* mutation, R243C, which altered the corresponding arginine residue (Monge et al. 2001). Flies with R243C in hemizygosity had a severe phenotype comparable to that associated with mutants that are negative for cross-reacting immunological material. When paired with hypomorphic mutations, the R243C mutation caused a more-severe phenotype, evidence of a dominant negative effect. Because the *Drosophila* genome contains a single *TFAP2* gene, these data provide clear confirmation of the functional effects of R225C protein.

CAT levels that were obtained after coexpression of R225S and wild-type *TFAP2B* were similar to those from R225C and significantly lower than those from P62R or R274Q coexpression ($P < .05$). This result suggested that R225S wild-type heterodimers were not binding TFAP2 target sequence well in the milieu of the cell, even though they bound target under EMSA conditions. This conclusion is consistent with the demonstration that R225S expression interfered with endogenous TFAP2 activity in NIH3T3 cells in the single-transfection experiment.

Coexpression of P62R and R274Q mutant proteins with wild-type TFAP2B produced roughly equal CAT concentrations. Because R274Q homodimers bound target sequence very poorly in vitro, the major source of

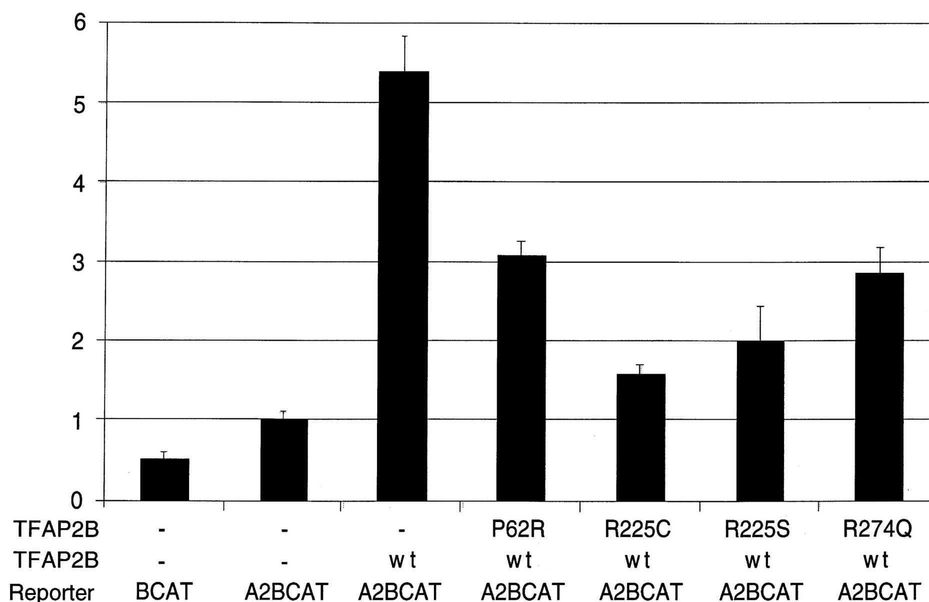

Figure 6 Transient coexpression of wild and mutant *TFAP2B* genes in NIH3T3 cells. Cotransfections and analysis were performed as described in figure 5. Total DNA used during the transfection was made equivalent for all conditions with an unrelated plasmid.

the increase in transactivation relative to the R225 mutants can be attributed to R274Q wild-type heterodimers. It can also be inferred that the P62R homodimers have little or no capacity to transactivate gene expression, a result confirming the importance of the PY motif (Wankhade et al. 2000).

Discussion

Six *TFAP2B* mutations that cause Char syndrome have now been identified among 10 patients with the disorder and their families. Several features are noteworthy. All disease-causing mutations are missense changes, and the proteins they encode have consistently dominant negative effects. This is strikingly different from the prototypical heart-hand syndrome, Holt-Oram, for which the molecular lesions to the transcription factor *TBX5* include missense, nonsense, and frameshift changes and can have dominant negative or haploinsufficient effects (Basson et al. 1999). We previously noted that the discrepancy in the phenotypes between patients with Char syndrome and mice lacking Tfap2b may be attributable to the difference in the nature of the alleles or to the variations in roles of the mouse and human *TFAP2B* genes during development (Satoda et al. 2000). The absence of haploinsufficient alleles among the enlarged cohort of patients in the present study provides evidence supporting the former explanation.

Although the entire *TFAP2B* coding region and the relevant intron boundaries were scanned for mutations by use of bidirectional sequencing, only 50% of patients and families in the present study (60% overall) had documented defects. This study did not exclude the possibility that defects in the upstream (and uncharacterized) regulatory region of the *TFAP2B* gene or in critical enhancer elements might produce the Char syndrome phenotype. Intragenic deletions also were not thoroughly excluded. Heterozygosity for *TFAP2B* intronic polymorphisms, excluding deletion of the entire gene, was noted for three of the four individuals without *TFAP2B* mutations. These two types of mutations, which would cause haploinsufficiency, seem unlikely, because a dominant negative effect would appear to be required for the pathogenesis of Char syndrome. An alternative possibility, genetic heterogeneity, could not be established with the available *TFAP2B* mutation–negative group but should be examined if an appropriate family with Char syndrome can be identified. Candidate genes would include other members of the *TFAP2* family and genes whose products are in the same developmental pathway as TFAP2B. That pathway has not been well established, although recent work with *Drosophila* documented that *dAP-2* was downstream of *Notch* during leg development (Kerber et al. 2001).

Another striking finding was that four of the six missense *TFAP2B* mutations altered arginine residues, but this amino acid constituted only 5.8% of the protein and 12.8% of the basic domain. Two putative causes of this apparently nonrandom mutation pattern seem plausible. First, arginine residues can be encoded in six ways, four CGX and two AGR codons. Since the mutation rate at CpG dinucleotides is higher than other

combinations (Holliday and Grigg 1993), we would expect a disproportionately high rate of missense mutations to affect arginine codons. In fact, all arginine residues altered in the four *TFAP2B* mutations are encoded by CGX codons. Since CGX codons encode the expected two thirds of all arginines in TFAP2B, these findings are compatible with the higher mutability mechanism. Second, arginine residues are known to play critical roles in the binding of transcription factors to their target sequences (Pabo and Sauer 1992). Specifically, they can form hydrogen bonds with bases, as well as with phosphate groups in the DNA backbone. The latter explanation seemed plausible, because all of the arginine mutations to TFAP2B resided in the DNA-binding domain. Moreover, four missense changes to Arg^{225} or to its corresponding residue in *Drosophila* have been identified (R225C and R225S in humans; R243C and R243H in flies). All lack DNA-binding activity as homodimers and have dominant negative effects, suggesting that this arginine residue plays a critical role in DNA binding.

To compare the relative importance of the two mechanisms that may underlie the overrepresentation of arginine point mutations, we reviewed databases for *CFTR* and *p53*, mutations of which cause cystic fibrosis and Li-Fraumeni syndrome, respectively. These two were selected, because the former gene encodes a structural protein and the latter encodes a transcription factor and because these mutation databases were extensive. Among 595 *CFTR* point mutations altering the coding region, 58 (9.8%; 95% confidence interval [CI] 7.5%–12.4%) altered arginine residues. Arginine residues were disproportionately represented, given that they constitute only 5.3% of the CFTR protein. In contrast, 27 of 116 *p53* point mutations (23.3%; 95% CI 15.9%–32.0%) altered arginine residues, despite the fact that the arginine content of the protein (5.1%) was nearly identical to that of CFTR. This could not be attributed to a skew in codon usage in *p53,* because 21 of 27 arginine residues were encoded by CGX codons. In addition, the distribution of the p53 arginine residues that were mutated appeared to be nonrandom. The DNA-binding region of p53 contains 18 of the 27 arginine residues in the protein but harbored 18 of 21 missense arginine mutations. This propensity to arginine mutations in the DNA-binding domain did not appear to be related merely to the alteration in charge. Seven lysine residues are present in the DNA-binding domain of p53, but only a single lysine missense mutation was reported. Thus, it appears that the disproportion of arginine missense mutations observed in TFAP2B and other transcription factors (Basson et al. 1999) can be attributed to synergism between fundamental genetic and structural mechanisms.

In the present study, a single missense mutation was identified that affected a residue in the transactivation domain. Functional analyses of this mutant, P62R, revealed that it binds DNA normally as a homo- and heterodimer, but it fails to transactivate as a homodimer and modestly interferes with transactivation from heterodimeric partners. These results confirmed the work of Wankhade and colleagues, who demonstrated that mutations of the conserved PY motif, in which Pro^{62} resides, have dramatic adverse effects on transactivation (Wankhade et al. 2000). Although the functional profile of P62R was similar to that of R274Q in the present study and to that of R289C in our previous work, the phenotype associated with P62R was strikingly different. The facial phenotype originally described by Florence Char has been consistent among affected individuals, with relatively little intra- or interfamilial variation. In the family bearing the P62R mutation, the facial dysmorphism was consistently much milder—so much so that, in the original assignments in that kindred, some individuals who did not have PDA were mistakenly thought to be unaffected. In addition, other families with multiple affected individuals have revealed some persons with abnormalities of the hands, abnormalities that range from aplasia of the middle phalanx of the fifth digit to clinodactyly. None of the 14 affected members of the family with the P62R mutation had such hand defects. Despite the mild facial and hand phenotype, the prevalence of PDA and other cardiovascular defects in this family was high. This discrepancy between the effects of P62R on cardiac development versus those on craniofacial and hand development requires explanation, particularly since two other mutants with comparable dominant negative effects are associated with the typical Char syndrome phenotype. One potential basis for this phenomenon would depend on the expression patterns of TFAP2 coactivators. The PY motif has been shown to mediate interactions between transcription factors and coactivators (Yagi et al. 1999). Thus, adverse effects of P62R could be more marked in certain tissues, in which coactivators interacting with the PY motif play a greater role in modulating transcriptional activation. Testing of this hypothesis, particularly with respect to cardiovascular development, must await identification of the relevant TFAP2B coactivators.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Cystic Fibrosis Mutation Data Base, http://www.genet.sickkids.on.ca/cftr/ (for *CFTR* mutations)

International Agency for Research on Cancer TP53 Mutation Database, http://www.iarc.fr/p53/Index.html (for *p53* mutations)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Char syndrome [MIM 169100], *TFAP2A* [MIM 107580], *TFAP2B* [MIM 601601], and *TFAP2C* [MIM 601602])

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