Analysis of TFAP2A mutations in Branchio-Oculo-Facial Syndrome indicates functional complexity within the AP-2α DNA-binding domain

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Multiple lines of evidence indicate that the AP-2 transcription factor family has an important regulatory function in human craniofacial development. Notably, mutations in TFAP2A, the gene encoding AP-2α, have been identified in patients with Branchio-Oculo-Facial Syndrome (BOFS). BOFS is an autosomal-dominant trait that commonly presents with facial clefting, eye defects and branchial skin anomalies. Examination of multiple cases has suggested either simple haploinsufficiency or more complex genetic causes for BOFS, especially as the clinical manifestations are variable, with no clear genotype–phenotype correlation. Mutations occur throughout TFAP2A, but mostly within conserved sequences within the DNA contact domain of AP-2α. However, the consequences of the various mutations for AP-2α protein function have not been evaluated. Therefore, it remains unclear if all BOFS mutations result in similar changes to the AP-2α protein or if they each produce specific alterations that underlie the spectrum of phenotypes. Here, we have investigated the molecular consequences of the mutations that localize to the DNA-binding region. We show that although individual mutations have different effects on DNA binding, they all demonstrate significantly reduced transcriptional activities. Moreover, all mutant derivatives have an altered nuclear:cytoplasmic distribution compared with the predominantly nuclear localization of wild-type AP-2α and several can exert a dominant-negative activity on the wild-type AP-2α protein. Overall, our data suggest that the individual TFAP2A BOFS mutations can generate null, hypomorphic or antimorphic alleles and that these differences in activity, combined with a role for AP-2α in epigenetic events, may influence the resultant pathology and the phenotypic variability.

INTRODUCTION

Craniofacial defects are among the most frequent human congenital malformations, with ‘CL/P’—clefting of the lip with or without clefting of the primary palate—particularly prevalent (reviewed in 1). About 70% of CL/P is non-syndromic, in that it is not associated with any other pathology, with the remaining 30% of CL/P being syndromic as it occurs as part of a more extensive set of defects often affecting the brain, heart or limbs (1,2). Mounting evidence has linked mutations in the genes encoding the AP-2 family of transcription factors with human craniofacial abnormalities (2–9). In particular, with respect to CL/P, human genetic mapping studies have shown that mutations in TFAP2A, the gene encoding the AP-2α protein, are associated with BOFS—Branchio-Oculo-Facial Syndrome (6,7,10–19). BOFS is a dominantly inherited birth defect in which the common features involve multiple craniofacial abnormalities, skin defects, eye defects and hearing problems (7,20). External facial defects include skull deformity, malformed nasal tip, hypertelorism, obstructed nasolacrimal duct, small teeth and malformed pinnae. There is also a spectrum of cleft lip phenotypes ranging from severe bilateral CL/P to lesser forms such as ‘pseudocleft’ (which has the appearance of a poorly repaired cleft) and mild defects of the philtrum. Skin anomalies are found particularly in the cervical region, and eye defects include microphthalmia and coloboma. Other symptoms seen less frequently include premature hair graying, ectopic thymus and kidney defects. The defects associated with the eye, face, skin,
hearing and melanocytes are also observed to varying degrees in various AP-2α mutant mouse models strengthening the causative nature of TFAP2A in human BOFS (21–29).

Despite recent progress in identifying TFAP2A as the gene mutated in BOFS, the molecular mechanisms by which such mutations cause this syndrome remain unclear. One issue is that the small number of cases that have been analyzed to date (<100 cases) has not produced a clear genotype:phenotype correlation (7,20). In addition, although penetrance may be complete, expressivity is variable and marked clinical variability has been noted within affected family members. The initial basis for identifying TFAP2A as the causative agent in BOFS was based on the identification of a large heterozygous chromosomal deletion encompassing this gene in a mildly affected family (6). Subsequently, sequence analysis indicated that the majority (~90%) of BOFS mutations occur within exons 4 and 5 of TFAP2A and cause missense mutations that alter conserved amino acid residues in the DNA-binding domain of AP-2α (7). The consequences of these mutations on DNA binding and transcriptional activation remain to be characterized. Nevertheless, in general, the phenotypes resulting from these missense mutations appear more severe than those caused by the complete deletion of one TFAP2A allele (6,7). This latter finding is suggestive of a dominant-negative effect—creating an allele that is an antimorph—although the expressivity issues noted above complicate this conclusion. In this study, we have examined how the separate BOFS mutations in the AP-2α DNA-binding domain affect the DNA binding, dimerization, transcriptional activity and cellular localization properties of the AP-2α protein. Our results indicate that the BOFS mutations frequently result in dominant-negative versions of the AP-2α protein that are capable of inhibiting wild-type AP-2 partner proteins.

RESULTS
Reduced transcriptional activity of BOFS-associated AP-2α mutants

The majority of BOFS mutations (>90%) occur in exons 4 and 5 of TFAP2A, which encode highly conserved residues associated with the DNA-binding domain of the AP-2 proteins (7). These two exons encode 118 amino acids, of which the 76 residues between 209 and 284 are believed to form the basic DNA contact region that is juxtaposed to the C-terminal dimerization domain [see Fig. 1 and (30–32)]. Within this basic region, 24 different BOFS mutations have been identified (6,7,10–19). Sixteen residues have missense mutations, and of these the amino acids at L218, R237, R254 and R255 can exhibit changes to two or three different amino acids (see Supplementary Material, Table S1). One deletion and one deletion insertion have also been uncovered. Sixteen of the mutations are sporadic or familial, whereas eight of the missense mutations occur in more than one family. The altered amino acids found within this 76-residue region are conserved between all five human AP-2 family members, with the exception of AP-2β, which has a lysine (K) at the position equivalent to Q235 (4,33). Furthermore, these amino acids are conserved between AP-2 proteins from human to Drosophila strongly suggesting that they would be critical for normal function. We began by studying the molecular properties of eight representative BOFS mutations (Fig. 1; also see Table 1), which correspond to those mutations identified in the human population when we began our studies (6,15,17,18). These represent six missense mutations (L218P, S239P, L249P, R254W, R255G, G262E), one deletion (233–236delEVQR; 233–236D), and one deletion insertion (276–281delLPAGRRinsRI; 276–281DI). However, we have since extended aspects of the analysis to all 24 mutations that have now been identified within this region, as well as to two BOFS mutations that map further C-terminal in the dimerization domain (see Supplementary Material, Table S1 and data not shown). We began by introducing the eight representative BOFS mutations into standard AP-2α expression vectors. Initially, we assessed the ability of these BOFS proteins to activate transcription of an AP-2 binding site dependent reporter plasmid in comparison with the wild-type protein. The individual expression constructs, along with an AP-2 responsive luciferase reporter plasmid and a pTK-Renilla luciferase control, were transfected into HepG2 cells, which lack endogenous AP-2α. Compared with the empty expression vector SP(RSV)NN, the wild-type AP-2α expression plasmid produced an ~18-fold
activation in relative luciferase activity (WT; Fig. 2). In contrast, all BOFS mutants generated significantly reduced relative luciferase activity (Fig. 2). L249P was the most potent, but gave only ~4-fold activation, whereas R254W, R255G and G262E produced less than a 2-fold induction. The eight BOFS mutants were also less active than the wild-type expression construct when tested in an equivalent assay in Hela cells, which contain endogenous AP-2 protein (data not shown). These findings indicate that each BOFS mutation tested is severely compromised in its ability to activate transcription.

Altered DNA-binding activity of BOFS-associated AP-2α mutants

We next examined the DNA-binding properties of in vitro translated BOFS mutants using electrophoretic mobility shift assay (EMSA) to determine whether changes in these AP-2α functions could account for the loss of transcriptional activity (Fig. 3). The ability of the mutant proteins to form a functional DNA-binding complex with a wild-type partner was also examined in this assay by co-translating each full-length construct with an N-terminal deletion, DΔN165, that we have shown previously retains normal EMSA activity with the AP-2 binding site oligonucleotide (31). Thus, co-translation of the wild-type and DΔN165 constructs produces a slow migrating wild-type form, a fast migrating DΔN165 moiety and an intermediate full-length/DΔN165 dimer (Fig. 3A). Examination of the individual BOFS proteins alone indicated that S239P, R255G and 276–281DI retained limited DNA-binding ability, whereas G262E gave binding levels equivalent to the wild-type AP-2α protein. As expected from these latter findings, the four mutant proteins also formed a productive dimeric DNA-binding complex when co-translated with DΔN165 (Fig. 3). In contrast, when translated alone, the missense mutations L218P, L249P and R254W as

Table 1. Summary of AP-2α BOFS mutant properties

<table>
<thead>
<tr>
<th>Protein</th>
<th>WT</th>
<th>L218P</th>
<th>Del EVQR</th>
<th>S239P</th>
<th>L249P</th>
<th>R254W</th>
<th>R255G</th>
<th>G262E</th>
<th>LPAGRR/RI</th>
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<tr>
<td>DNA binding alone</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>DNA binding + ΔN165</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>NT</td>
<td>NT</td>
<td>Yes</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>Dimer with ΔN165 off DNA</td>
<td>NA</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Inhibit ΔN165 binding</td>
<td>18×</td>
<td>&lt;3×</td>
<td>&lt;3×</td>
<td>&lt;4×</td>
<td>&lt;2×</td>
<td>&lt;2×</td>
<td>Yes</td>
<td>Yes</td>
<td>NT</td>
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<td>Relative Txn activation</td>
<td>Stim</td>
<td>Stim</td>
<td>Stim</td>
<td>Stim</td>
<td>Cyto</td>
<td>Cyto</td>
<td>Cyto</td>
<td>Cyto</td>
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<tr>
<td>Human BOFS occurrence</td>
<td>L218P is sporadic, but L218R occurs too</td>
<td>Sporadic</td>
<td>Single familial</td>
<td>Multiple</td>
<td>R254G and R254P occur too</td>
<td></td>
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<td>Phenotypec</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
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<td>YES</td>
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<td>YES</td>
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<tr>
<td>Clef lip, ocular defects, skin lesions, low set ears, hearing defects</td>
<td>NO</td>
<td>NOT REP</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>NOT REP</td>
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<tr>
<td>Kidney defects</td>
<td>Hair pigment defects</td>
<td>Limb, dental defects</td>
<td>Hair pigment defects</td>
<td>Foot defect</td>
<td>Dental and nail defects</td>
<td>Dental, nail and hair pigment defects</td>
<td>Dental, nail and hair pigment defects</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Other</td>
<td>WT, wild-type; Stim, stimulate; Txn, transcriptional; NT, not tested.</td>
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<td>aEquilibrium DNA binding is similar to wild-type, but off-rate is significantly faster.</td>
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<td>bProduced aberrantly migrating EMSA complexes.</td>
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<td>cPhenotype information is taken from the following references (7,15,17,18). ‘YES’ indicates that particular defects were associated with at least one patient containing the indicated mutation.</td>
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<tr>
<td>dOphthalmological examination reported as normal; ‘NO’ kidneys were included in analysis but no defects mentioned; ‘NOT REP’, not reported.</td>
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</table>
well as the 233–236 deletion did not bind in these EMSA studies. However, L249P generated a specific DNA-binding complex when co-translated with ΔN165, indicating that this BOFS mutation maintained aspects of DNA contact that were stabilized by dimerization with a wild-type protein (Fig. 3A). The other three mutations that failed to bind alone also failed to generate the intermediate form in the presence of the ΔN165 form, even though they were synthesized in similar levels to the other mutants (Fig. 3B). The inability of BOFS mutations L218P, R254W and 233–236D were also able to form complexes with the ΔN165 polypeptide (data not shown), indicating that all three of these proteins can form dimers with a wild-type AP-2α partner. However, these latter two proteins did form aberrantly migrating protein:DNA complexes in EMSA when co-translated with ΔN165, suggesting that their protein:protein interactions may be partially compromised resulting in the formation of higher order protein complexes (Fig. 3A and data not shown).

The analysis of DNA binding and dimerization suggested that the BOFS proteins could be placed into three different categories: (i) able to bind DNA alone as well as with a wild-type partner (S239P, R255G, G262E and 276–281DI); (ii) unable to bind alone, but capable with a wild-type partner (L249P); and (iii) unable to bind DNA alone or with a wild-type partner, but able to dimerize in solution (L218P, R254W and 233–236D). These observations indicate that the various BOFS mutations are not equivalent in their disruption of AP-2α function, and also suggest that they would have different effects on the binding potential of wild-type partners. We explored this possibility further by performing EMSA titration experiments, in which increasing amounts of a full-length AP-2α construct were co-translated with a fixed amount of ΔN165. When this experiment is performed using the wild-type protein, the observed complexes shift from the ΔN165 dimer and full-length/ΔN165 forms towards the full-length dimer (Fig. 5). As predicted from the DNA-binding ability of G262E (Fig. 3A), a similar result is obtained when G262E is used instead of the wild-type (Fig. 5). In contrast, increasing levels of R254W abolish any complex formation in the EMSA experiments, as the ΔN165 partners are

Figure 3. DNA-binding analysis of the AP-2α BOFS mutants. (A) EMSA performed using a full-length AP-2α wild-type form (WT), an N-terminal truncated form containing the functional DNA-binding domain (ΔN165), and the indicated BOFS mutants either translated individually or co-translated (+). Proteins were translated in vitro and incubated with a radiolabeled hMtIIa probe. The shifted complexes and the free probe are shown by arrows. A non-specific single-stranded band occurs below the free probe. NC refers to probe incubated with unprogrammed reticulocyte lysate; ‘mt’ indicates complexes containing BOFS mutant proteins. The asterisk indicates one of the aberrantly migrating species observed with L218P mutant proteins. The other three mutations maintained aspects of DNA contact that were stabilized by dimerization with a wild-type protein (Fig. 3A). The inability of BOFS mutations L218P, R254W and 233–236D to form a productive DNA complex either alone or with a wild-type partner raised the possibility that they have lost the ability to bind DNA and/or to dimerize effectively.

To assess the ability of these three BOFS mutants to form dimers, we utilized chemical cross-linking in the absence of the AP-2 DNA-binding site. The 50 kDa wild-type protein or the 30 kDa ΔN165 polypeptide yielded products of ∼100 kDa or 60 kDa, respectively, when cross-linked in the presence of glutaraldehyde, indicating that the protein exists as a dimer in solution (Fig. 4). Moreover, an additional ∼80 kDa dimeric complex formed when the full-length and ΔN165 forms were cross-linked following co-translation, but not when simply mixed after separate translation. When the full-length R254W BOFS mutation was used in place of the wild-type protein, this also generated an ∼100 kDa band when cross-linked, and gave the ∼80 kDa form when co-translated with ΔN165 (Fig. 4) demonstrating dimerization potential. L218P and R254W forms dimers in the absence of DNA-binding activity. Cross-linking performed with the full-length AP-2α (WT), a dimerization competent deletion (ΔN165) or full-length R254W protein. Protein was translated in vitro in the presence of [35S]Methionine, either left untreated (−) or cross-linked (+), separated by SDS–PAGE and visualized by autoradiography. Both the ∼50 kDa wild-type and R254W proteins are mainly converted to products of ∼100 kDa after cross-linking, and form an intermediate band of ∼80 kDa when co-translated (CO), but not mixed with ΔN165, prior to cross-linking.

Figure 4. AP-2α BOFS R254W forms dimers in the absence of DNA-binding activity. Cross-linking performed with the full-length AP-2α (WT), a dimerization competent deletion (ΔN165) or full-length R254W protein. Protein was translated in vitro in the presence of [35S]Methionine, either left untreated (−) or cross-linked (+), separated by SDS–PAGE and visualized by autoradiography.
incorporated into heterodimers that have lost the ability to bind DNA. L249P yields an intermediate result, generating a DNA-binding complex only with ΔN165.

The observation that G262E produced equivalent DNA binding to a wild-type protein in the normal EMSA equilibrium conditions but was unable to produce significant transcriptional activation prompted us to examine its interaction with DNA in greater detail. One major parameter we analyzed was the off-rate of the complex as the rates of protein:DNA dissociation can significantly influence transcriptional properties (34). Wild-type or G262E DNA complexes were allowed to reach equilibrium and then challenged with an excess of cold specific competitor DNA. Samples were taken at various time intervals and immediately loaded on a gel that was undergoing electrophoresis to stabilize formed complexes. Using this assay, we determined that the wild-type protein:DNA complex had a half-life of >20 min at room temperature (Fig. 6 and Supplementary Material, Fig. S1). The half-life of the ΔN165 homodimer and the wild-type and ΔN165 heterodimer were similar, again indicating that this truncated protein has essentially normal binding activity. In marked contrast, the half-life of the G262E homodimer with DNA was significantly less than 30 s, the time from addition of the cold competitor, to mixing, and gel loading (Fig. 6 and Supplementary Material, Fig. S1). Moreover, when G262E was tested as a complex with ΔN165 using EMSA, the half-life of this heterodimer on DNA was ~1 min, intermediate between the wild-type and mutant homodimers (Fig. 6). These findings indicate that the presence G262E protein partner can drastically alter DNA-binding properties of a wild-type partner, consistent with a dominant-negative effect.

Variable transcriptional interplay between wild-type AP-2α and BOFS-associated mutants

We next examined how the BOFS mutations might impact trans-activation driven by wild-type AP-2α in cell-based assays (Fig. 7). In these assays, L249P, R254W and G262E were chosen as representative examples of the three categories of BOFS mutations that differ in their DNA-binding potential (Fig. 7). A constant sub-threshold amount of a wild-type AP-2α expression vector, as well as AP-2-responsive and control luciferase reporter constructs, were transfected into HepG2 cells. Increasing amounts of a wild-type AP-2α expression vector, an empty expression vector or one of the three BOFS expression plasmids was also included in the transfection. As shown in Figure 7, increasing quantities of wild-type AP-2α increased reporter activity, whereas there was no major change in activity upon addition of the empty vector (NN), as expected. The individual BOFS mutations produced three different expression profiles. L249P increased relative luciferase activity, stimulating expression in combination with the wild-type protein. In contrast, increasing amounts of G262E in association with a fixed amount of wild-type plasmid did not alter relative luciferase activity, while rising R254W expression levels inhibited activation obtained with the wild-type protein (Fig. 7). These findings indicate that specific BOFS mutations can exhibit a dominant-negative effect on wild-type transcriptional activation, but that this phenomenon is not a universal property of the mutant alleles.

Altered cellular localization of BOFS-associated AP-2α mutants

The dominant-negative action of R254W on transcriptional activation would be predicted from its ability to inhibit DNA binding. However, the transcriptional readouts when L249P and G262E were tested in the presence of wild-type AP-2α were unexpected given their in vitro DNA binding and dimerization properties. These observations prompted us to investigate the behavior of the BOFS proteins in intact cells. Wild-type or BOFS mutant proteins were transfected into HepG2 cells and AP-2 protein levels assessed by western blotting using the 3B5 monoclonal antibody that recognizes an epitope N-terminal to the BOFS mutations (35). Relative nuclear and cytoplasmic

Figure 5. Different properties of AP-2α BOFS mutations in EMSA when titrated with ΔN165. EMSA was performed as in Figure 3. The analysis tested the DNA-binding ability of protein complexes made in vitro using different ratios of wild-type (WT) or BOFS mutant to a fixed amount of ΔN165 as shown.

Figure 6. The G262E BOFS mutation alters DNA-binding kinetics. Semi-log plot of the percentage of bound probe detected after the addition of an excess of cold specific competitor DNA at time 0 min. ‘G262E’ and ‘Wild Type’ are homodimers of these respective protein forms. Time points are shown by closed circles (wild-type: ΔN165 and wild-type:ΔN165), open circles (G262E) and triangles (G262E:ΔN165).
levels were also ascertained using cell fractionation (Fig. 8 and Supplementary Material, Fig. S2). The highest level of expression was observed for the wild-type construct, with similar levels obtained for R255G, G262E and 276–281DI. Expression of the other BOFS mutations was slightly reduced compared with wild-type (Fig. 8). The most noticeable finding, though, was the switch between the mainly nuclear localization of the wild-type protein to roughly equal nuclear:cytoplasmic levels for G262E, or a predominantly cytoplasmic location for the other BOFS proteins (Fig. 8 and Supplementary Material, Fig. S2). Cellular localization of the various BOFS proteins was further investigated after transfection using immunofluorescence. These experiments confirmed that the wild-type protein was mainly nuclear, but that many BOFS proteins were predominantly cytoplasmic (Fig. 9, Table 1 and data not shown). The ability of the BOFS mutations to dimerize with a wild-type partner prompted us to assess if these proteins could sequester wild-type AP-2α in the cytoplasm. In these experiments, we co-transfected a GFP tagged wild-type construct in association with an RFP-tagged BOFS expression vector. Figure 10 shows that while AP-2α:GFP alone was mainly nuclear, the addition of the R254W BOFS protein led to accumulation of the tagged wild-type protein in the cytoplasm (see also Supplementary Material, Fig. S3). Equivalent results were obtained with all eight BOFS mutants tested (Table 1 and data not shown), demonstrating that these molecules can alter the subcellular localization of the wild-type AP-2α protein. The implications of these findings for the pathogenesis of BOFS are discussed in detail below.

**DISCUSSION**

BOFS is a dominantly inherited human birth defect that is characterized by multiple abnormalities affecting the face, eyes and neck (7,20). Genetic mapping and sequence analyses have demonstrated that BOFS is associated with more than 25 different mutations affecting TFAP2A, the human gene encoding AP-2α (6,7,10–19). Some of these mutations are large chromosomal deletions or frame-shifts that would be predicted to eliminate protein function from the affected allele. However, the
As judged by EMSA, the individual mutations did not produce equivalent effects on DNA binding. Eight of the mutations, including L218P, 233–236D and R254W, completely abolished normal DNA-binding activity, either alone or when tested in conjunction with a wild-type partner (Supplementary Material, Table S1). Eight other mutations, exemplified by L249P, were also unable to bind as homodimers, but could recognize the AP-2 binding site when forming a heterodimer with a wild-type construct. Two additional mutations in the dimerization domain, E296K and H384Y, also fell into this category (Supplementary Material, Table S1). The remaining eight BOFS mutations in the DNA contact region were able to bind DNA as homodimers to varying degrees. Indeed, using EMSA, the G262E mutation showed similar equilibrium binding as the wild-type protein. However, further analysis indicated that the G262E:DNA off-rate was significantly increased compared with the wild-type protein. Subsequently, we determined that the only other BOFS mutation we examined that produced wild-type equilibrium DNA binding, A246E, also showed a similar increase in DNA off-rate (Supplementary Material, Table S1 and data not shown). Additional studies on the G262E DNA-binding complex indicated that this protein also displayed a faster on-rate and was more readily disrupted by increasing [NaCl] than the wild-type homodimer (data not shown). Our findings further showed that heterodimeric complexes formed between the wild-type and G262E displayed abnormal DNA interaction kinetics. Therefore, one commonality between all the BOFS mutations analyzed is that they have altered DNA-binding properties compared with the wild-type protein, and that such changes can extend to heterodimeric complexes. At the same time, the data clearly demonstrate that BOFS is not caused by the absence of AP-2α DNA-binding activity.

A second consistent observation was that all the BOFS mutants were severely compromised in their ability to activate transcription in transfected cells compared with the wild-type molecule (Supplementary Material, Table S1). Unexpectedly, though, various BOFS mutants behaved quite differently in the transcription assays when tested in association with the wild-type protein. The R254W mutation, which lacks DNA-binding activity either alone or with a wild-type partner, resulted in a slight reduction in transcriptional activity consistent with a modest dominant-negative effect. The G262E mutation was neutral in this assay, but L249P, which only binds DNA in association with a wild-type partner, was able to activate transcription in this context. On the one hand, these findings may indicate that the mutations that result in BOFS are unlikely to act by simply inhibiting AP-2α dependent transcriptional activation when present with the wild-type protein. One caveat to this conclusion is that these assays have so far only been performed in cell lines using transfection of artificial constructs rather than in an endogenous setting. A second consideration is that AP-2α may not simply function as an activator, but also as a repressor (36,37). Therefore, the overall effect of the BOFS mutations may reflect the sum of alterations in the binding to a range of sites required for both gene activation and repression.

Examination of other BOFS mutations indicates that, in general, those located between 218 and 242 activate transcription in the presence of the wild-type protein, whereas those between 246 and 269 tend to inhibit wild-type activity (Table 1, Supplementary Material, Table S1, and data not
shown). This property does not correlate with DNA-binding potential and instead may indicate an additional functional sub-region within the DNA contact domain. One possibility that could be examined in future is that mutants in this region may interact with and titrate out an inhibitor of wild-type AP-2α transcriptional activation, whereas BOFS mutations in the vicinity of residues 246–269 are unable to fulfill this function. Conversely, the more C-terminal mutants may titrate out an activator compared with the more N-terminal mutants. It is also noteworthy that patients listed as having nail anomalies tend to have mutations within the 254–262 amino acid region, potentially representing a genotype:phenotype correlation that deserves further examination in the human population (7).

The third property common to all the BOFS mutants in the cell transfection assays was that—while the wild-type protein was mainly nuclear—all the BOFS mutants had diminished nuclear localization capability as judged by both immunofluorescence and cellular fractionation (Supplementary Material, Table S1 and Fig. S2). Furthermore, each mutant tested was still capable of dimerizing with the wild-type protein, and indeed when co-expressed could drive the latter protein into the cytoplasm. These findings imply that there is normally a dynamic equilibrium between nuclear and cytoplasmic AP-2α that is dependent to some extent upon the DNA-binding potential of the protein, although this correlation is not absolute (Fig. 8, Table 1 and data not shown). The AP-2α protein, particularly in its stable dimeric form, would be too large to cross the nuclear membrane by passive diffusion (38) and is therefore likely to possess sequences critical for nuclear localization. One possibility is that the AP-2α DNA contact region may also contain sequences involved in nuclear import and/or export that may be affected by the BOFS mutations. Mutations that inhibited nuclear import or favored nuclear export would thus affect the nuclear to cytoplasmic AP-2α ratio. Examination of the AP-2α peptide sequence did not reveal a classical monopartite nuclear localization signal (NLS), but prediction programs indicate that the region between 254 and 285 in the DNA contact region has some similarities to a bipartite NLS (38–41). The peptide sequence within this region, between residues 269 and 274, is also predicted to possess nuclear export function via the motif LDKIGL (42). Of note, previous studies of differentiation and tumorigenesis have indicated that AP-2α nucleocytoplasmic distribution might be regulated (43–46). In this context, we have obtained preliminary data suggesting a role for active nuclear:cytoplasmic transport in the subcellular localization of the AP-2α protein. Specifically, treatment of cells with leptomycin B, an inhibitor of nuclear export, results in the accumulation of an otherwise cytoplasmic BOFS mutant protein in the nucleus (L.H., unpublished data). Therefore, the observations that the various BOFS mutations cause an altered cellular AP-2α distribution might indicate the properties of NLS/NES signal sequences may be affected alongside any changes in DNA binding. It should also be noted that this region of AP-2α may mediate protein:protein interactions with other transcriptional co-factors including Myc and may also be subject to protein modification (47–49). Therefore, an individual point mutation might result in disruption of not just DNA binding, but also nuclear localization, modification and higher order transcriptional complex formation. The ultimate phenotype caused by the mutation would then result from the summation of all these effects.

One further consideration raised by the alterations in DNA binding and subcellular localization of the BOFS mutants is that this could impact the potential of AP-2α to function as a pioneer transcription factor during development (50). Although many transcription factors dissociate from DNA during mitosis, AP-2α remains co-localized with the condensed chromosomal DNA [(51) and H.L. data not shown]. This property would enable AP-2α tobookmark critical genes for development once mitosis had been completed and the nuclear membrane reformed. In contrast, AP-2α mutants with altered DNA-binding potential and kinetics would likely fail to remain associated with mitotic chromatin and would require active nuclear localization to access chromosomal DNA following cell division. The disruption of appropriate chromatin bookmarking caused by the presence of BOFS mutant proteins would result in epigenetic events, and this property could also account for the marked variability in the BOFS phenotypes. Future studies will be required to determine whether wild-type AP-2α fulfills a pioneer function that is dominantly inhibited by the BOFS mutations.

Examination of the conserved ~75 amino acid DNA contact region in which the majority of the BOFS mutants are located reveals an overall basic charge and a propensity for alpha helix formation. It is notable that about half of the BOFS mutations result in the introduction of proline or glycine residues within this domain, and these would be predicted to act as helix breakers. Second, the majority of the remaining changes result in the alterations in the number of basic or acidic amino acids within this region. R254W, R255G and G262E would all fall into this latter class. Together, these findings imply that appropriate structure and charge distribution within this region is critical for normal AP-2α function. Our studies indicate that these changes do not create null alleles, instead generating proteins with diminished function and apparent dominant-negative activity. Therefore, we hypothesize that the more severe forms of BOFS do not result from simple haploinsufficiency of TFAP2A, but reflect that the mutations generate antimorphic alleles. This conjecture is supported by data obtained from studies in both human and mouse. First, chromosomal deletions encompassing one allele of TFAP2A often produce a milder mutant craniofacial phenotype than the missense mutations analyzed above (6,7). Second, mouse studies have shown that most animals containing one Tfap2a null allele are normal, although there is a low frequency of cranial suture defects (25). Tfap2a null animals have very severe morphological defects affecting multiple developmental systems including the face, eye, brain and trunk and die at birth (22,27–29,52). Therefore, it is unlikely in human that the BOFS mutations completely inhibit the function of the wild-type TFAP2A copy. Instead, it is probable that the BOFS alleles produce a partial loss of function that reduces the amount of normal AP-2α (and possibly of other AP-2 family members) below threshold levels for adequate performance in specific, critical regions associated with face, eye and neck development. In this context, we have previously shown that tissue-specific loss of Tfap2a in the neural crest or ectoderm can cause defects in face, eye, ear and melanocyte development consistent with human BOFS phenotypes (23,24,26). Similarly, chimeras composed of wild-type and null mutant cells often display phenotypes consistent with BOFS, including eye, face and branchial cleft defects (25). Finally, we note that the TFAP2A locus on human chromosome 6p24 is associated
not only with BOFS but also with 6p deletion syndrome (3,53), and possibly non-syndromic facial clefting (2). Moreover, mutations of an AP-2 binding site in an IRF6 enhancer are associated with orofacial clefting (2), and BCOR mutations resulting in oculo-facial-dental syndrome are accompanied by misregulation of AP-2 expression (54). Therefore, TFAP2A has a growing association with human craniofacial defects and further study will be required to understand the detailed molecular and cellular interactions responsible for its function in mammalian facial development.

**MATERIALS AND METHODS**

**Generation of expression plasmids for wild-type and BOFS AP-2α mutations**

The eight individual BOFS mutations that targeted the AP-2α DNA-binding domain were generated both in the SPRSV-AP2α and pBSal-AP2α expression plasmids (31) using the Quik-Change® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. Primers for each mutation were designed using available software (https://www.genomics.agilent.com/) and their sequences are listed in Table 2. The wild-type AP-2α N-terminal fusion to GFP was made using the primers AP2-Nhe-F and AP2-Kpn-R on linearized SP(RSV)AP-2 for PCR amplification with KOD XTreme Hot Start DNA polymerase (Novagen, UK). Standard PCR conditions were 1 cycle of 94°C for 2 min followed by 32 cycles of 98°C for 10 s, 60°C for 30 s and 68°C for 1 min 20 s, and then 1 cycle of 68°C for 10 min. The PCR products were digested at unique NheI and KpnI sites and cloned into the equivalent sites of pEGFP-N1 (gift of Lynn Heasley). GFP fusions to the BOFS mutations were generated in an equivalent manner using the SP(RSV)BOFS mutations and primers AP2-HdIIIF and AP2-Xh1-R prior to digestion with HindIII and XhoI and cloning into the equivalent sites of pcDNA3-mRFP (gift of Mary Reyland). In each instance, the 3′ primer removed the normal AP-2α stop codon and enabled an in-frame fusion with either GFP or RFP. DNA sequence analysis was employed to confirm that the appropriate wild-type sequence or BOFS mutations had been introduced into the various constructs without altering other sequences within the expression plasmid.

**Dual luciferase assay**

HepG2 cells were maintained in 12-well plates in DMEM with 10% FBS, 1-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C with 5% CO2. For analysis of activation potential of individual BOFS mutations, HepG2 cells were transfected with 0.5 μg of either the empty SPRSV-NN expression vector or the SPRSV-AP2α WT or BOFS mutant expression constructs together with 0.5 μg 3xAP2-Bluc and 0.02 μg pTK-Renilla (gifts from Helen C. Hurst) using FuGENE HD transfection reagent (Roche, Nutley, NJ, USA). For assessment of how the BOFS proteins interacted with wild-type AP-2α, each transfection contained 0.2 μg 3xAP2-Bluc and 0.02 μg pTK-Renilla along with variable amounts wild-type AP-2α or BOFS expression vectors. The amount of plasmid DNA was normalized to 1 μg per transfection by the addition of pUC118 DNA. About 48 h post-transfection, cells were washed with PBS and lysed in Passive Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions, read using a Veritas™ microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA) and imported into Microsoft Xcel. Firefly luciferase activities were normalized by Renilla luciferase and each experiment was repeated at least three times.

**In vitro transcription/translation**

The pBSal-AP2α wild-type, pBSal-AP2α mutations or pBSal-ΔN165 (31) were utilized to express proteins in vitro. In *in vitro*...
transcription/translation was performed using a TNT T7 Quick-coupled Transcription/Translation System (Promega) in a 50 μl reaction mixture containing 2 μg of plasmid, 40 μl of TNT Quick Master Mix and 2 μl of 35S-labeled methionine (Perkin Elmer, Waltham, MA, USA). The reactions were incubated at 30°C for 90 min and then terminated by the addition of 1 μl 1 mg/ml RNase A at 30°C for 15 min. A 2 μl aliquot of each reaction mixture was used directly in each gel-shift reaction. Autoradiography analysis of SDS–PAGE indicated that equivalent amounts of all proteins were made (data not shown).

EMSAs were performed essentially as described (31) with some modifications. Briefly, 2 μl of in vitro translation products were incubated in a buffer containing 10 mM Tris-C1 (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mg/ml of BSA, 5% glycerol and 40 ng/μl of poly[d(IC)] plus 2 μl DTT for 10 min on ice before the addition of 32P-labeled double-stranded AP-2 oligonucleotide. Binding reactions were allowed to proceed for an additional 30 min at RT and then were loaded on pre-electrophoresed 4% (40:1) polyacrylamide/1 × TBE gels. Gels were dried and exposed through one piece of developed blank X-ray film to at least overnight.

Nuclear and cytoplasmic extraction and western blot

HepG2 cells in six-well plates were transfected with 2 μg of SPRSV-NN, WT AP-2α or BOFS mutants. After 48 h, the cells were trypsinized, washed with PBS and divided into three aliquots. One aliquot was used for total protein extraction using RIPA lysis buffer. A second aliquot was used for the separation of cytoplasmic and nuclear fractions using the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Pierce, Rockford, IL, USA). One hundred and fifty microliters of Cytoplasmic Extraction Reagent I was used for cytoplasmic protein extraction and 75 μl Nuclear Extraction Reagent was used for nuclear protein extraction according to the manufacturer’s instructions. The final aliquot was kept in reserve. Total protein (15 μl), cytoplasmic (15 μl) and nuclear extracts (7.5 μl) from each transfection were analyzed by 10% SDS–PAGE and western blotting. Mouse monoclonal antibodies 3B5 (AP-2α) and GAPDH-71.1 (Sigma-Aldrich, St Louis, MO, USA) were used as primary antibodies, and goat anti-mouse IgG (H + L) HRP (Thermo, Rockford, IL, USA) was the secondary antibody. Western Lightning Plus Enhanced Chemiluminescence Substrate (PerkinElmer, Waltham, MA, USA) was used for signal detection. Integrated optical density representing the band intensity was quantified using a Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD, USA).

NOTE ADDED IN PROOF


SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.
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