Fibroblast growth factor receptors (FGFRs) have been implicated in a multitude of proliferative functions, and FGFR4 is expressed differentially in normal and neoplastic pituitary. Human pituitary tumors express a truncated FGFR4 isoform (ptd-FGFR4) for which transcription is initiated from a downstream alternative site. Analysis of FGFR4 intronic sequences predicted a possible promoter within intron 4 (In4) including a classic TATA box with a possible transcriptional start site in intron 5. We show here that the human In4 sequence can direct luciferase reporter activity in transfected pituitary GH4 cells. Four overlapping fragments (A1, A2, B1, and B2) of this intron were examined by electromobility shift assay using nuclear extracts from rat pituitary tumors. Of these, fragment B2 formed complexes with nuclear rat pituitary GH4 extracts that were competed specifically by wild type but not mutant oligonucleotides for the neural crest cell lineage-derived activating transcription factor AP-2. Conversely, an AP-2 consensus sequence probe was competed by the In4 B2 oligonucleotide but not by other fragments of the same intron. The In4 B2 complex was competed partially by NFκB, supershifted by an AP-2 specific antibody, and co-migrated with the same probe incubated with recombinant AP-2α protein. We also examined the ability of primary human pituitary tumor extracts to interact with the In4 B2 fragment. Pituitary tumor-In4 B2 complexes were competed specifically by wild type AP-2 but not mutant AP-2 oligonucleotides. Western blotting revealed higher levels of AP-2α expression in primary human pituitary tumors than in nontumorous tissue. Mutagenesis of the putative AP-2 binding site in In4 B2 resulted in a marked loss of promoter activity in a luciferase assay. AP-2α transefction in the presence of the histone deacetylase inhibitor trichostatin A resulted in enhanced expression of endogenous ptd-FGFR4. These data indicate that a cryptic promoter within intron 4 binds AP-2α. AP-2α and chromatin changes may contribute to the utilization of an alternative transcription start site leading to the genesis of the tumorigenic ptd-FGFR4 isoform.

The pituitary gland is the site of synthesis and the target of several growth factors that modulate hormone production and cell proliferation (1). Of these, members of the fibroblast growth factor (FGF) family have been shown to be overexpressed in human pituitary tumors (2). FGF signaling is mediated through one of four FGF receptors (FGFRs), a complex family of transmembrane receptor tyrosine kinases (3). Each prototypic receptor is composed of three Ig-like extracellular domains, a single transmembrane domain, a split tyrosine kinase, and a carboxyl-terminal tail with multiple autophosphorylation sites (3). Multiple cell-bound or secreted forms of FGFR1, -2, and -3 have been characterized as resulting from alternative initiation sites, alternative splicing, exon switching, or variable polyadenylation. Recently, we found that pituitary tumors express a cytoplasmic form of FGFR4 lacking the signal peptide and the first two extracellular Ig-like domains (4).

Full-length FGFR4 has been reported to be expressed mainly in adult lung, liver, kidney, pancreas, muscle, and spleen (5, 6). It initially was considered not to have a significant role in tumorigenesis. However, FGFR4 has been shown to mediate membrane ruffling in breast carcinoma cells (7) and to modulate erythroid cell proliferation (8). Moreover, mutational analyses of the kinase domains of FGFR1, FGFR3, and FGFR4 revealed that all three receptor domains have comparable transforming properties (9). We have shown that targeted expression of a human pituitary tumor-derived FGFR4 isoform (ptd-FGFR4) recapitulates pituitary tumorigenesis (4). This truncated form of FGFR4 is a result of transcription initiation in intron 5 and a translation start site in exon 6 (4). In this report, we investigate an alternative FGFR4 promoter that may be implicated in the genesis of ptd-FGFR4. Sequence analysis of the human FGFR4 gene predicts a possible promoter within intron 4 including a classic TATA box with a possible transcription start site in intron 5 (4, 10). We thus focused on intron 4 and the surrounding region and determined the relevant cis-DNA-binding elements in pituitary cells that may contribute to utilization of this site.

MATERIALS AND METHODS

Cell Culture and Tissue—HEK 293 and the rat pituitary tumorderived GH4 cell lines were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with high glucose supplemented with 10% fetal bovine serum (Sigma), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C with 5% CO2. 24 h before transfection, cells were plated with Dulbecco’s modified Eagle’s medium containing 10% serum. Primary human pituitary tumors were collected at the time of trans-sphenoidal pituitary surgery and snap frozen.

Plasmids—Promoter analysis of the human FGFR4 gene was per-
Triton X-100, and 1 mM dithiothreitol. Luciferase activity was measured.

Five buffer (10 mM HEPES, pH 7.9, 1 mM dithiothreitol, 1 mM EDTA, 60 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and lysed with three cycles of freeze/thaw. The cell lysates or nuclear fractions were solubilized in 2 M Tris for electrophoresis. The DNA sequence extending 397 bp upstream from the alternative transcription start site of ptd-FGFR4 (4) is shown. Intron-exon junctions were marked by brackets. The alternative transcription start site is indicated by a bent arrow (bottom right). Several specific binding sites of transcription factors are underlined and italicized. Predicted binding sites were identified using the TRANSFAC search software data base.

The first six nucleotides of exon 5, thereby including the entire AP-2 consensus binding site, 5'-GAAACACCGGTCAATTCAGGCCCTTCCCTGCCCCAAGCCTT-3' (Fig. 1). 

Competitor double-stranded oligonucleotides containing transcription binding sites were as follows: AP-2, sense 5'-CCACACACCCGCTGGGCGGCTG-3'; (commercially obtained from Geneka Biotech Inc., Carlsbad, CA) and its complementary strand; the mutant oligonucleotide for mAP-2, sense 5'-CCACACACACGGCGAGTGCGCGCTG-3'; and its complementary strand; AP-1, 5'-CCGGTGATGACCGGGAAGTTTG-3' and its complementary strand; AP-4, 5'-GTCGGAGCGCTGCGGCGGACG-3' and its complementary strand; and NFkappaB, sense 5'-GAAGGAGGACCTCCACAACAGC-3' and its complementary strand (synthesized by Sigma). Complementary strands were annealed in a buffer of 10 mM Tris, pH 8.0, 50 mM NaCl, and 1 mM EDTA before being used as probes or competitors.

Gel shift probes were purified with a Sephadex G-50 spin column after labeling. For competition assay, 10, 20, 50, or 100 M excess of unlabeled fragment was added as competitor DNA. Samples were electrophoresed on 4% polyacrylamide non-denatured gels containing 0.5% Triton X-100 binding buffer and 2% glycerol. Gels were dried under a vacuum and autoradiographed.

Western Blot Analysis—Protein concentrations were determined by the Bio-Rad protein assay. Equal amounts of protein (50 μg) from whole cell lysates or nuclear fractions were solubilized in 2× SDS-sample buffer, separated on 8.5% polyacrylamide gels, and transferred to nitrocellulose. Blots were incubated with polyclonal antisera raised against the carboxy terminus of AP-2a (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or MRE11 (1:1000; Santa Cruz Biotechnology), or a monoclonal antibody against actin (1:500). The specificity of the detected bands was confirmed by preabsorption of antisera with purified peptide.

RESULTS

Sequence analysis of the upstream fragments in intron 4 revealed putative binding sites for the transcription factors AP-2, Ikaros (Ik), NFkappaB, AP-4, and OCT-1 as depicted in Fig. 1. Intron 4 of FGFR4 Contains Binding Sites for AP-2a—To determine which factors potentially may interact with this putative intronic region, we screened potential candidate genes using EMSA. EMSA probes were used to screen nuclear extracts from exponentially growing rat pituitary tumor GH4 cells or from primary human pituitary tumors as described previously (13). The overlapping fragments consisted of 30–40 bp of intron 4 that were designated as A1, A2, B1, and B2. These fragments

![Fig. 1](https://example.com/fig1.png)

**FIG. 1.** Nucleotide sequence and predicted binding sites in intron 4, exon 5, and intron 5 of the human FGFR4 gene. The DNA sequence extending 397 bp from the alternative transcription start site of ptd-FGFR4 (4) shown. Intron-exon junctions are marked by brackets. The alternative transcription start site is indicated by a bent arrow (bottom right). Several specific binding sites of transcription factors are underlined and italicized. Predicted binding sites were identified using the TRANSFAC search software data base.
FIG. 2. Characterization of transcription factor-binding elements in intron 4 of the human FGFR4 gene. The 110-bp region spanning the human FGFR4 intron 4 was divided into four overlapping fragments (A1, A2, B1, and B2) that were used in EMSAs with nuclear extracts from rat GH4 pituitary cells as detailed under “Materials and Methods.”

A, In4 A1 formed nonspecific complexes that could not be competed by a 100-fold molar excess of cold oligonucleotides for any of a number of putative transcription factors. B, incubation of the same pituitary nuclear extracts with the In4 B2 fragment yields slow migrating distinct complexes that are competed specifically by a molar excess of wild type but not mutant AP-2 oligonucleotides. The complexes are competed partially by NFκB. C, extension of the In4 B2 probe to include the first six nucleotides of exon 5 (In4 B2ex) yields more distinct complexes with the same pattern of competition. The slower migrating complex is supershifted by an AP-2α-specific antibody (AP2αAb). D, an AP-2 consensus probe (left) forms the same slow migrating complexes with GH4 pituitary cells as with human MCF-7 breast cancer cells that are known to express AP-2α abundantly. The slow complexes are competed effectively by the In4 B2 fragment but not by an adjacent fragment (In4 A2) from the same promoter. The In4 B2 probe forms complexes with the recombinant GST-AP-2α fusion protein; those complexes are competed by wild type AP-2 oligonucleotides (right). E, mutation of the putative AP-2 binding site in the In4 B2ex fragment (mIn4 B2ex) as a probe yields complexes that are not competed by AP-2 but are still amenable to partial competition by NFκB. F, NFκB probe forms complexes with GH4 nuclear extracts that are competed by In4 B2 but not by the adjacent In4 A2 fragment from the same intron.
were labeled and used as probes for the assessment of specific binding. Fig. 2A depicts an example of an EMSA using GH4 rat pituitary tumor nuclear extract incubated with the In4 A1 probe. We found no evidence of specific complexes that could be competed by putative transcription factors using this In4 A1 fragment. Similar nonspecific findings were obtained with the In4 A2 and In4 B1 fragments. In contrast, incubation of the same nuclear extracts with the In4 B2 fragment yielded distinct complexes that were competed specifically by wild type AP-2 but not mutant (mAP2) oligonucleotides. B, Western blotting of primary human pituitary tumors confirms the expression of a ~50-kDa protein that is recognized by an AP-2-specific antibody and comigrates with lysates from HEK 293 cells transfected with AP-2. Expression of AP-2α (adjusted for actin) is found to be relatively higher in pituitary tumors (PT) than in nontumorous pituitary (NP) samples. The actin-loading control is shown immediately below.

To determine further the specificity of the putative intron 4–specific antibody and comigrates with lysates from HEK 293 cells transfected with AP-2α. Expression of AP-2α (adjusted for actin) is found to be relatively higher in pituitary tumors (PT) than in nontumorous pituitary (NP) samples. The actin-loading control is shown immediately below.

were labeled and used as probes for the assessment of specific binding. Fig. 2A depicts an example of an EMSA using GH4 rat pituitary tumor nuclear extract incubated with the In4 A1 probe. We found no evidence of specific complexes that could be competed by putative transcription factors using this In4 A1 fragment. Similar nonspecific findings were obtained with the In4 A2 and In4 B1 fragments. In contrast, incubation of the same nuclear extracts with the In4 B2 fragment yielded distinct complexes that were competed specifically by wild type AP-2 but not mutant AP-2 oligonucleotides (Fig. 2B). This complex was not competed by oligonucleotides for predicted binding sites for AP-4, OCT-1, Ik, or STAT but was competed partially with an oligonucleotide for NFκB. The putative AP-2 binding site is situated on the intron-exon junction (Fig. 1). Extension of the probe by 6 nucleotides into exon 5 (In4 B2ex) to complete the AP-2 consensus binding site resulted in a more distinct complex that was supershifted by AP-2α antibody (Fig. 2C).

To determine further the specificity of the putative intron 4 interaction with AP-2, we examined the ability of an AP-2 consensus probe to form complexes with GH4 nuclear extracts. This complex was competed specifically by the In4 B2 fragment but not by other fragments from the same intron (Fig. 2D). Moreover, the In4 B2 probe formed specific complexes with the recombinant GST-AP-2α fusion protein (Fig. 2D). Mutation of the putative AP-2 binding site in the B2 fragment resulted in a complex that was not competed by wild type AP-2 (Fig. 2E), consistent with the presence of a single AP-2 binding site in this fragment of intron 4. This complex, however, was competed partially by NFκB (Fig. 2E). Consistent with these findings, an NFκB probe also formed complexes with GH4 nuclear extracts that were competed specifically by In4 B2 but not by the adjacent In4 A2 sequence (Fig. 2F). These results indicate that the region of the cryptic promoter contained within In4 B2 contains adjacent consensus binding sites for AP-2 and NFκB. Furthermore, GH4 cells express nuclear factors that bind to these sites, and this multiprotein-DNA complex is supershifted with antibody to AP-2α, thus confirming that AP-2α is one factor that targets the region encompassing In4 B2.

**Primary Human Pituitary Tumors Express AP-2α**—Given our previous findings of a human pituitary tumor-derived FGFR4 isoform, we sought evidence for a potential role of the AP-2 binding site in intron 4 in primary human pituitary tumors. Nuclear proteins from primary human pituitary tumors formed the same slow migrating complexes with the In4 B2 probe (Fig. 3A). These complexes were competed specifically by an oligonucleotide for wild type AP-2 but not by mutant AP-2.

To confirm that primary human pituitary tumors express the AP-2α protein, we examined by Western blotting extracts from primary human pituitary adenomas characterized as expressing the truncated ptd-FGFR4 (4). We identified a protein of ~50 kDa using an AP-2α-specific antibody in nontumorous pituitary tissue and in pituitary adenomas (Fig. 3B). This product comigrated with protein derived from HEK 293 cells trans-
ected with an expression vector for AP-2α. When adjusted for actin levels, AP-2α protein was more abundant in the pituitary tumors than in nontumorous pituitary tissue.

**Functional Contribution of AP-2 in Regulation of FGFR4**—To determine the potential functional contribution of the AP-2 binding site in intron 4 (P313-Luc) on promoter activity, we examined the effect of mutation of this site on promoter activity. Disruption of the AP-2 site in the B2 fragment resulted in marked reduction in luciferase activity (Fig. 4). In addition, mutation of the NFκB site also resulted in a loss of reporter activity but to a lesser extent than that noted with the loss of the core AP-2 binding site. Conversely, inclusion of exon 5 and intron 5 did not enhance promoter activity (data not shown).

Transfection of AP-2α in GH4 cells treated with trichostatin-A resulted in enhanced expression of ptd-FGFR4 (Fig. 5). Taken together, these results suggest that AP-2α in pituitary tumors, coupled with chromatin changes such as HDAC inhibition, result in enhanced utilization of a cryptic promoter within intron 4, contributing to the genesis of the truncated receptor ptd-FGFR4.

**DISCUSSION**

Recognizing the importance of a tumor-derived form of FGFR4 in which transcription is initiated from an alternative downstream site (4), we set out to examine functionally important cis-DNA elements that can be implicated in the control of a downstream cryptic promoter.

Two earlier reports identified the human and murine FGFR4 promoter regions and their transcription start sites (10, 11). Neither study, however, addressed characterization of putative transcription factor binding or transcriptional activity. Our analysis of the 5′ FGFR4 promoter revealed a region 115 bp upstream of the start site that we found to be essential for reporter activity in pituitary cells (13). Overlapping 40–50-bp fragments of this minimal functional promoter were examined by EMSAs. Specific DNA-protein complexes were noted with fragments that contained multiple binding sites for Sp1. This was consistent with the role of Sp1 in the regulation of other FGFR promoters lacking TATA and CAAT sequence motifs including FGFR1 (14), FGFR2, and FGFR3 (15). For each of these FGFRs, −100 bp of sequence 5′ to the transcription initiation site appear to confer transcriptional activity to the promoters (16). We further characterized fragment C (−65 to −25) of FGFR4, as it demonstrated consistently strong binding with nuclear extracts from rat pituitary cells. This fragment contains predicted sites for binding the zinc finger-containing protein Iκ, flanked by two sites for Sp1 and Ets-type factors (13). These studies led to the localization of Iκ in the pituitary and to identifying it as an important contributor to cell-specific FGFR4 transcriptional regulation.

The current studies point to AP-2α as an important factor that recognizes a downstream cryptic promoter in intron 4 of FGFR4. The AP family of transcription factors consists of four different members known as AP-2α, AP-2β, AP-2γ, and AP-2δ (12, 17). The carboxyl-terminal half of these proteins contains a basic domain and a highly conserved helix-span-helix motif that mediates DNA binding and dimerization. In particular, it has been suggested that AP-2α is important in regulating cell proliferation through control of a number of genes responsible for terminal differentiation as well as apoptosis (17–19).

Our current findings also point to an accompanying role for NFκB in binding and regulating intron 4. In the pituitary, NFκB has been implicated in mediating a number of hormonal signals, including responsiveness to inhibition by dopamine in prolactin-producing pituitary cells (20). Interestingly, regulation of the cholecystokinin (21) and serum amyloid A1 (22) gene promoters also involves partially overlapping consensus sites for AP-2 and NFκB. These studies demonstrated a complex functional interaction between these two transcription factors. Our promoter analysis similarly indicates that both NFκB and AP-2α are involved coordinately in the regulation of this cryptic promoter. The close proximity of these two sites further suggests a potential interaction between these two distinct factors to regulate coordinately FGFR4 gene expression.

Several pituitary hormone genes, including proopiomelanocortin (23), prolactin (24), the corticopherin-releasing hormone receptor (25), and somatostatin receptors (26), have been described as containing AP-2 binding sites. The presumed functional role of AP-2α in transcriptional regulation of many of these putative target genes, however, remains to be determined.

The presence of AP-2 binding sites in intronic sequences has been identified only rarely. DNase I footprinting experiments using intron 1 of the peripheral gene revealed at least two protected regions (In 1 A and In 1 B). In 1 A encompasses an AP-2-like binding site that interacts with both neuroblast and fibroblast nuclear proteins as well as with recombinant AP-2α protein. Gel shift experiments, however, suggested that the interacting nuclear factors are distinct from AP-2α but nonetheless probably belong to the AP-2 family (27). Analysis of the laminin receptor precursor promoter similarly revealed that the first intron contains enhancer activity. This region was shown to include a functional AP-2 cis-acting element that is repressed by p53 (28). Our current findings provide evidence for AP-2α as a distinct factor that is expressed in pituitary tumors, is able to recognize the distal region of an intronic sequence, and is a participant in the regulation of a cryptic FGFR4 promoter.

The mechanisms of FGFR action in endocrine cell development and tumorigenesis remain to be elucidated (1). Our previous identification of a novel human pituitary tumor-derived truncated FGFR4 isoform with distinct properties from wild type FGFR4 (4) has provided a unique opportunity to examine the effects of alternative transcription initiation on FGFR4 function. Our current findings on the recognition of an intronic sequence of FGFR4 by AP-2α and the strong expression of this factor by pituitary tumors begin to address the mechanisms that may favor alternative promoter utilization in transformed cells.

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