Fibroblast growth factor-8 (FGF8) plays a critical role in vertebrate development and is expressed normally in temporally and spatially restricted regions of the vertebrate embryo. We now report on the identification of regions of Fgf8 important for its transcriptional regulation in murine ES cell-derived embryoid bodies. Stable transfection of ES cells, using a human growth hormone reporter gene, was employed to identify regions of the Fgf8 gene with promoter/enhancer activity. A 2-kilobase 5′ region of Fgf8 was shown to contain promoter activity. A 0.8-kilobase fragment derived from the large intron of Fgf8 was found to enhance human growth hormone expressed from the Fgf8 promoter 3–4-fold in an orientation dependent manner. The intronic fragment contains DNA-binding sites for the AP2, Pbx1, and Engrailed transcription factors. Gel shift and Western blot experiments documented the presence of these transcription factors in nuclear extracts from ES cell embryoid bodies. In vitro mutagenesis of the Engrailed or Pbx1 site demonstrated that these sites modulate the activity of the intronic fragment. In addition, in vitro mutagenesis of both Engrailed and Pbx1 sites indicated that other unidentified sites are responsible for the transcriptional enhancement observed with the intronic fragment.

The fibroblast growth factors (FGFs),1 a family of secreted proteins encoded by at least 18 genes in mammals, are mitogens and morphogens for a variety of cell types and tissues (reviewed in Ref. 1). Despite the discrete FGF expression patterns, the molecular mechanisms controlling the regulation of these genes are poorly understood. Human FGF1 spans over 120 kb, contains three protein coding exons, and at least four upstream untranslated exons (2). Multiple FGF1 transcripts are generated by alternate promoter usage and tissue-specific splicing (3, 4). Murine Fgf3 contains three distinct promoters and two separate polyadenylation sites that generate different mRNA, but all of them encode the same protein (5–8). The Fgf4 gene has an enhancer element in the 3′-noncoding region of exon 3, both in mice and humans (9–12).

Fgf8 is primarily an embryonic growth factor that is expressed in several regions of vertebrate embryos: i.e. epiblast, primitive streak, surface ectoderm of branchial arches, apical ectodermal ridge of the limb bud, isthmus of midbrain-hindbrain junction, and forebrain/nasal placode (13–17). Fgf8 is also weakly expressed in adult gonads, primarily in germ cells (18, 19). This discrete temporal and spatial expression pattern of Fgf8 is important for induction and patterning of the embryo during gastrulation, limb development, and midbrain-hindbrain formation (20–23). FGF8 is pathologically expressed in some human breast cancer cell lines (24).

Currently, little is known about the Fgf8 promoter. Its 5′-flanking region is very GC-rich and it is located 5 kb from the 3′ end of Npm3, which encodes a ubiquitously expressed member of the nucleolar/nucleophosmin family (25). The transcriptional start site(s) for Fgf8 are unknown, but are inferred to be within 300 bp upstream of the start codon. In the 5′-region of Fgf8, there is no TATA box. Prior work demonstrated that the 2-kb 5′-region of Fgf8 did not direct expression of a reporter transgene in a Fgf8-like expression pattern.2 We therefore postulated that DNA elements cis to Fgf8, but not included in this 2 kb of upstream DNA sequence, might be important for Fgf8 expression. Because of the presence of the Npm3 gene 5 kb upstream of Fgf8 (25), we searched for DNA sequences downstream of the beginning of Fgf8 that were conserved between mice and humans. Small regions in the proximal portion of the large intron are conserved between murine Fgf8 and human FGF8 (26), despite the lack of coding function for this region. This large intron lies between the last exon of the cluster of first exons (exon 1D) and exon 2 (18). There is no conservation between murine Fgf8 and human FGF8 in the remainder of the large intron, and in other introns (26).

Characterization of cis-regulatory elements in Fgf8 through the usual transfection methods is complicated since Fgf8 is not normally expressed in established cell lines, and hence these cell lines may lack appropriate transcription factors that regulate Fgf8 expression. Because transgenic approaches are expensive, we sought a relevant in vitro system to analyze potential Fgf8 regulatory regions. Prior work demonstrated Fgf8 expression in murine ES cells differentiated into embryoid bodies (27). In this paper we use a stable transfection human growth hormone (hGH) reporter assay in ES cell embryoid bodies to analyze Fgf8 DNA sequences for promoter and enhancer activity. We find that the 2-kb 5′-region of Fgf8 contains promoter activity, and that a 0.8-kb fragment of the large intron enhances transcription of the reporter gene. We show co-localization of expression of the endogenous Fgf8 gene and the reporter hGH gene in embryoid bodies derived from ES cells transfected with the Fgf8-hGH reporter construct. DNA sequence analysis, in conjunction with electrophoretic mobility shift assays (EMSA), identify multiple transcription factors

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Fibroblast Growth Factor-8 Expression Is Regulated by Intrinsic Engrailed and Pbx1-binding Sites*

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1 The abbreviations used are: FGF, fibroblast growth factor; kb, kilobase(s); bp, base pair(s); hGH, human growth hormone; EMSA, electrophoretic mobility shift assay.

2 G. Martin, personal communication.
that bind to the Fgf8 promoter and enhancer. In vitro mutagenesis experiments demonstrate that although the Engrailed and Pbx1-binding sites modulate the enhancer activity of the intrinsic fragment, other sites are responsible for most of the transcriptional enhancement seen with the intrinsic fragment.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The 1.8-kb XhoI-XhoI fragment containing neomycin gene was isolated from pPNT vector (28), made blunt with Klenow fragment, and after addition of EcoRI linkers was subcloned into the EcoRI site of the promoter-less plasmid, pcGH (Nichols Institute Diagnostics). The 1.5-kb or 2-kb EcoRV-SmaI fragment containing the 0.8-kb region of murine Fgf8 gene (Fig. 1), after addition of BamHI linkers, was added in the forward orientation into the BamHI site of pcGH with neomycin gene subcloned previously. The 0.8-kb PstI-XhoI intrinsic fragment from the Fgf8 gene (Fig. 1) was made blunt with T4 DNA polymerase, and subcloned in forward and reverse orientation into the SphI site of pcGH vector downstream from the IGH gene, after adding SphI linkers. Promoter sequences and the neomycin gene were then added to the constructs as described above.

**Mutagenesis**—Mutations in the binding sites for transcription factors AP2, Pbx1, or Engrailed were introduced into the plasmid, pGEM-7Zf (+), containing the 0.8-kb Fgf8 intronic fragment subcloned into the SphI site using the Quick Change Site-directed Mutagenesis kit (Stratagene, Inc. if available or custom made by Biosynthesis (Engrailed mutant, corresponding to mutant primer above)). All mutants contain 2-bp substitutions in the middle of the binding motif for the transcription factor. Following 20-min incubation at room temperature, the binding reactions were fractionated on 4% polyacrylamide gels run in 45 mM Tris borate buffer, pH 8.0, containing 1 mM EDTA for 2 h at 200 V. The gels were subsequently dried and autoradiographed.

**DNA Sequencing**—The plasmids above were sequenced by the thermocycle technique, using the fmol kit (Promega), and a combination of plasmid and Fgf8 sequence-specific primers (26). Sequences were read with the IBI-Kodak gel reader and MacVector v5.0 software (IBI-Kodak).

**Cell Culture**—CCE ES cells (29) were routinely cultured on mitomycin C-treated STO feeder cells in medium consisting of high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 10% newborn calf serum, 0.1 mM 2-mercaptoethanol, glucose, Dulbecco’s modified Eagle’s medium supplemented with 10% (IBI-Kodak).

**RESULTS**

**Fgf8 Sequence**—We were interested in understanding the regulation of Fgf8, a developmentally regulated FGF with expression in discrete regions of the vertebrate embryo, but only minimal expression in the gonads of the adult organism (13–19). We cloned the murine Fgf8 gene in prior work (Fig. 1A) (18), and now report the sequence of the 1462 bp upstream of the translation start codon (GenBank accession number AF065607). Analysis of the sequence revealed DNA-binding sites for the transcription factors Max2 (562–568 bp), CREB/ATF (814–819 bp), Egr1 (971–979 bp and 1060–1069 bp), AP2 (1068–1075 bp), and Sp1 (1072–1077) (Fig. 1B).

As we prepared to test two different 5′-regions for promoter activity, we learned of the inability of this region to direct reporter transgene expression in a Fgf8-like pattern in mouse embryos.2 We therefore sought to identify additional Fgf8 sequence elements that might be important for regulating Fgf8 expression. We previously cloned the human FGF8 gene (26). The homology between the mouse and human genes is very high in coding regions (85–90% at nucleic acid level), but not in noncoding regions. The mouse and human noncoding sequences were analyzed for short stretches (30–60 bp) of complete identity, as we hypothesized that coding sequences preserved in mice and humans might be important in gene regulation. We identified a 767-bp region of the proximal portion of the large intron of Fgf8 that had several well conserved stretches of DNA sequence when compared with the corresponding region of human FGF8 (Fig. 1B). This sequence is now reported in GenBank (accession number AF065608). Analysis of this sequence revealed DNA-binding sites for transcrip-
Fgf8 Intron Regulates Expression

Fgf8 Intron Regulates Expression

**Fig. 1.** Fgf8-hGH reporter constructs. The murine Fgf8 gene is represented in A. An upstream repetitive element that separates Npm3 from Fgf8 is indicated by □, noncoding Fgf8 exons by □, exons coding for all isoforms by □, and alternatively spliced exons by □. The promoters, S and L, and the downstream enhancer, E, are shown in B. They are directly under their locations in the Fgf8 gene depicted in A. The DNA-binding sites in Fgf8 for AP2, CREB/ATF, Egr1, Engrailed (En), Msx2, Pbx1, and Sp1 are indicated in B. In C, the reporter constructs used in the experiments are indicated. Arrows indicate the orientation (5′→3′) of the hGH and enhancer elements relative to the promoters.

**Fig. 2.** Intronic region of Fgf8 enhances transcription from hGH reporter gene. The reporter constructs, numbered as shown in Fig. 1C, were analyzed in mouse ES cell embryoid bodies. The results are presented as nanograms of hGH (determined by radioimmunoassay) normalized to the amount of protein (in mg) of the sample. The error bars represent the standard error of the mean, based on four separate experiments with duplicate samples in each experiment.

**Temporal and Spatial Analysis of Reporter Constructs—** With the identification of potential DNA elements in Fgf8 that stimulate reporter gene expression, we sought to determine if these same elements directed expression of the reporter gene in a fashion similar to the endogenous Fgf8 gene. Previously, it was shown that steady state levels of the endogenous Fgf8 RNA could be detected by ribonuclease protection assays at days 10 and 18, but not days 0, 4, or 6 (27). We have subsequently demonstrated expression of Fgf8 at day 8 of development of embryoid bodies (data not shown). We therefore did a temporal analysis of the hGH reporter gene expression from our highest expressing construct in Fig. 2, construct 5. As for the endogenous Fgf8 gene, we did not detect reporter gene expression at day 0 and day 1 of development of embryoid bodies (Fig. 3A). We detected low level reporter hGH expression at day 3, that increased to full expression at day 8 (Fig. 2). This reporter hGH expression pattern is similar to the endogenous Fgf8 expression pattern seen in embryoid bodies (27), although expression levels of the reporter gene seem higher compared with the endogenous Fgf8 gene (see below).

We compared the spatial pattern of the hGH reporter gene expression with the endogenous Fgf8 gene by in situ hybridization at day 9 of development of embryoid bodies, using constructs 3 and 5 (Fig. 1C). There was co-localization of endogenous Fgf8 expression with reporter hGH gene expression in the less eosinophilic cells of the embryoid bodies, compared with expression in the more eosinophilic cells in construct 5.

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3 J. Gemel, C. Jacobsen, and C. A. MacArthur, unpublished results.
and the Fgf8 gene and the embryoid body. Staining cells at similar levels. In expressed in both lighter and darker bodies transfected with construct 5. The hybridization pattern of day 9 ES cell embryoid bodies transfected with construct 5. The left figure is a bright field photomicrograph, the center figure is a dark field photomicrograph of the left figure, hybridized to antisense Fgf8 cDNA probe (AS Fgf8), and the right figure is an adjacent section to the left figure, hybridized to antisense hGH probe (AS hGH). Expression of both the endogenous Fgf8 gene and the Fgf8-hGH reporter genes are primarily in lighter staining cells of the embryoid body. C, in situ hybridization pattern of day 9 ES cell embryoid bodies transfected with construct 3. Panels are the same as in B. The endogenous Fgf8 gene is expressed in lighter staining cells as in B, but the reporter hGH is expressed in both lighter and darker staining cells at similar levels. In panels B and C, bars in the upper right corner of the photomicrographs represent 10 μm. The exposure was carried out for 7 days in panel B, and for 3 days in panel C.

(Fig. 3B). In contrast, the reporter hGH gene was expressed in both the less eosinophilic and the more eosinophilic cells in construct 3 (Fig. 3C). Although there was co-localization of the reporter hGH gene with the endogenous Fgf8 gene in construct 5, the reporter gene was expressed at higher levels (note that Fgf8 probe is 3 times the length of hGH probe), possibly due to higher reporter gene copy number in the transfected cells. Despite the differences in expression levels, the time course and co-localization experiments (Fig. 3) demonstrate that the reporter gene containing the intronic fragment has expression similar to the endogenous Fgf8 gene.

**Gel Shift and Western Blot Analyses**—To determine if the identified DNA sequences in Fgf8 actually were occupied by transcription factors present in nuclei of day 9 embryoid bodies, we prepared nuclear extracts and performed EMSA. Specific gel shifts were obtained when nuclear extracts from day 9 embryoid bodies were assayed with radiolabeled oligonucleotides corresponding to the Mxx2-, CREB/ATF-, and Egr1/Sp1/AP2-binding sites in the 5′-region of Fgf8 (Fig. 4). The gel shifts were competed with non-radiolabeled authentic oligonucleotide that matched oligonucleotide (when available).

Similarly, specific gel shifts were obtained when the nuclear extracts of day 9 embryoid bodies were assayed with radiolabeled oligonucleotides corresponding to the AP2, Sp1/Pbx1, and Engrailed DNA-binding sites in Fgf8 (Fig. 5). The AP2 and Sp1/Pbx1 sites were competed with non-radiolabeled authentic, but not mutant, oligonucleotide (Fig. 5). The Engrailed site was fully competed by authentic non-radiolabeled oligonucleotide, suggesting the possibility of additional factors binding at adjacent sites in the oligonucleotide, or some ability of a transcription factor in day 9 embryoid bodies to bind to the mutated Engrailed site.

We were unsuccessful in our attempts to supershift complexes seen in EMSA assays using antisera, and thereby confirm the identity of the proteins binding to these sites in Fgf8. We therefore performed Western blots to demonstrate that En1, En2, and Pbx1 were present in day 9 embryoid extracts (Fig. 6).

**In Vitro Mutagenesis**—To determine whether the AP2, Pbx1, and/or Engrailed DNA-binding sites in the intron of Fgf8 were responsible for the enhancement of reporter hGH gene expression seen in construct 5 compared with construct 3 (Fig. 1C), we created two base mutations in these binding sites by polymerase chain reaction methods. The mutated constructs were compared with the wild type construct 5 in the ES cell stable transfection assay. Although proteins bound to the AP2 site in the intron (Fig. 1B) in a specific fashion (Fig. 5), mutation in this AP2-binding site did not significantly affect expression of the reporter gene (Fig. 7). In contrast, a mutation in the Engrailed-binding site of the intron (Fig. 1B) reduced hGH expression to 45% of the wild type construct (Fig. 7). A mutation in the Pbx1 DNA-binding site in the Fgf8 intron (Fig. 1B) reduced expression to 75% of the wild type construct (Fig. 7). Surprisingly, a double Engrailed/Pbx1 mutant gave wild type expression of the reporter gene (Fig. 7). As the intron increases expression of the reporter transgene from 26 ng of hGH/mg of protein to 89 ng of hGH/mg of protein (Fig. 2, constructs 3 and
In this paper, we identified promoter activity in the 2-kb 5′-region of Fgf8, spanning the region between the EcoRl and SmaI sites (Figs. 1 and 2). We also demonstrated that deletion of the 0.2-kb SmaI-SmaI fragment in this 5′-region of Fgf8 results in 44% reduction of hGH production (Figs. 1C and 2), reflecting a significant decrease in Fgf8 promoter activity. Sequence analysis of this region of Fgf8 identified binding sites for AP2 and Sp1 at the SmaI site, which are destroyed when the 0.2-kb SmaI-SmaI fragment is removed from longer constructs. Specific gel shifts were observed in EMSA with this region of Fgf8 (Fig. 4). These results suggest that the AP2/Sp1-Fgf8 promoter region (Fig. 1Fgf8) is important for transcriptional activity of Fgf8 in mouse ES cells.

Our data further indicate that a Fgf8 intronic fragment in the proximal portion of the large intron regulates Fgf8 expression in mouse ES cells (Figs. 1–5). Specific gel shifts were observed for the AP2-, Pbx1-, and Engrailed-binding sites of this intronic fragment, when analyzed with nuclear extracts of day 9 embryoid bodies (Fig. 5). Pbx1, En1, and En2 are present in day 9 embryoid bodies as determined by Western blotting (Fig. 6). Although, mutation of the AP2-binding site in the intronic fragment abolished competition in the gel shift assay (Fig. 5), the mutation had no effect on the Fgf8 intronic enhancing activity (Fig. 7). In contrast, mutation of the Engrailed-binding site in the intronic fragment markedly affected the ability of this region to compete with the authentic binding site, but did not abolish competition (Fig. 5). The same Engrailed mutation decreased the enhancing activity of the Fgf8 intron by 75% in mouse ES cells (Fig. 7). A mutation in the

**FIG. 4. Identification of transcription factor-binding sites in Fgf8 promoter.** EMSAs were performed on nuclear extracts from day 9 ES cell embryoid bodies, using radiolabeled oligonucleotides corresponding to the Mx2-, CREB/ATF-, and Egr1/Spi/PA2-binding sites in the Fgf8 promoter region (Fig. 1B). Solid arrows at left of gels indicate specific gel shifts that are competed with non-radiolabeled authentic oligonucleotide (self), but not with non-radiolabeled oligonucleotide mutated at the binding site indicated. Dashes (- -) in the competitor row refer to no competitor, while numbers refer to the fold excess of non-radiolabeled oligonucleotide used as competitor. Zero (0) in the extract refers to no added extract, where plus (+ +) refers to lanes with nuclear extract added.

5), the mutation in the Engrailed-binding site removes 75% of the enhancing activity of the intron. The mutation in the Pbx1-binding site removes 35% of the enhancing activity of the intron. Mutations in both Engrailed- and Pbx1-binding sites do not alter the ability of the intronic fragment to increase reporter gene expression, and indicate that additional site(s) are responsible for the transcriptional enhancement seen with the intronic fragment.

**DISCUSSION**

In this paper, we identified promoter activity in the 2-kb 5′-region of Fgf8, spanning the region between the EcoRl and SmaI sites (Figs. 1 and 2). We also demonstrated that deletion of the 0.2-kb SmaI-SmaI fragment in this 5′-region of Fgf8 results in 44% reduction of hGH production (Figs. 1C and 2), reflecting a significant decrease in Fgf8 promoter activity. Sequence analysis of this region of Fgf8 identified binding sites for AP2 and Sp1 at the SmaI site, which are destroyed when the 0.2-kb SmaI-SmaI fragment is removed from longer constructs. Specific gel shifts were observed in EMSA with this region of Fgf8 (Fig. 4). These results suggest that the AP2/Sp1-DNA-binding site in the Fgf8 5′-region is important for transcriptional activity of Fgf8 in mouse ES cells.

Our data further indicate that a Fgf8 intronic fragment in the proximal portion of the large intron regulates Fgf8 expression in mouse ES cells (Figs. 1–5). Specific gel shifts were observed for the AP2-, Pbx1-, and Engrailed-binding sites of this intronic fragment, when analyzed with nuclear extracts of day 9 embryoid bodies (Fig. 5). Pbx1, En1, and En2 are present in day 9 embryoid bodies as determined by Western blotting (Fig. 6). Although, mutation of the AP2-binding site in the intronic fragment abolished competition in the gel shift assay (Fig. 5), the mutation had no effect on the Fgf8 intronic enhancing activity (Fig. 7). In contrast, mutation of the Engrailed-binding site in the intronic fragment markedly affected the ability of this region to compete with the authentic binding site, but did not abolish competition (Fig. 5). The same Engrailed mutation decreased the enhancing activity of the Fgf8 intron by 75% in mouse ES cells (Fig. 7). A mutation in the

**FIG. 5. Identification of transcription factor-binding sites in Fgf8 enhancer.** EMSAs were performed as described in the legend to Fig. 4, with radiolabeled oligonucleotides corresponding to the AP2, Sp1/Pbx1, and Engrailed (En) DNA-binding sites in the intronic enhancer of Fgf8 (Fig. 1B). The gels are labeled as in Fig. 4. Open arrows at the left of the gels indicate specific gel shifts that are competed equally well with authentic and mutated oligonucleotides.

**FIG. 6. En1, En2, and Pbx1 transcription factors present in day 9 embryoid bodies.** Western blots of nuclear extracts, probed with antiserum against Engrailed proteins (left panel) or Pbx1 (right panel). The 55-kDa En1 and 41-kDa En2 proteins are indicated in the left blot, while the 53-kDa Pbx1 protein is indicated in the right blot. The protein molecular weight sizes are indicated in kilodaltons between the blots.

Pbx1-binding site that completely abolished competition in a gel shift assay (Fig. 5) decreased the enhancing activity of the Fgf8 intron by 35% in mouse ES cells (Fig. 7). In contrast to the decrease in expression of the reporter gene seen with either the Pbx- or Engrailed-binding sites, the Pbx1/Engrailed double mutant showed a wild type level of expression (Fig. 7).

To explain our data, we propose the following model. First, we hypothesize that there are activating sequences, presently unidentified, in the 0.8-kb intronic fragment that increase Fgf8...
expression. Second, the Pbx and En proteins modulate the activity of the intronic activator sequence(s). If only Pbx proteins, or only Engrailed proteins, are present, they inhibit the activity of the intronic fragment and decrease Fgf8 expression. If both Engrailed and Pbx proteins are present, then they interact with their binding sites and relieve the inhibition of each other. The mechanism of this interaction, based on prior studies (see below), may well involve heterodimerization of Pbx and Engrailed proteins versus homodimerization.

The Engrailed proteins are members of a homeodomain-containing protein family (37), encoded by two mammalian genes, En1 and En2 (38). Both genes are expressed in posterior portions of the midbrain-hindbrain junction and spinal cord in the mouse embryo (38). Mice lacking En1 die at birth with severe midbrain-hindbrain deletion, and rib and limb defects (39), while mice lacking En2 survive with subtle cerebellar defects (40). En2 can substitute for En1 in midbrain-hindbrain function (41). En1 is essential for ventral limb patterning, as loss of Engrailed function results in a dorsal-ventral patterning defect of the limb (42). En1 was shown responsible for limiting the ventral aspect of the apical ectodermal ridge expression of Fgf8 (42). Additionally, En1 is expressed prior to Fgf8 in the midbrain-hindbrain junction (isthmus) (15, 43, 44), and may be responsible for localizing the expression of Fgf8 to the cells of the isthmus (45).

The studies in the limb suggest that Engrailed represses Fgf8 expression in this location (42). Our results in ES cells suggest that Engrailed inhibits Fgf8 expression in the absence of Pbx1. Engrailed proteins can heterodimerize with members of the Pbx family of homeodomain proteins (46, 47). Extradenticle, a Drosophila homolog of the vertebrate Pbx proteins, has been shown to heterodimerize with homeodomain proteins and convert a transcriptional repressor homeodomain protein (as a monomer or homodimer) to a transcriptional activator (48). We identified single Engrailed- and Pbx-binding sites in the Fgf8 intron that inhibit expression of Fgf8 in mouse ES cells, but that together can allow full Fgf8 expression. We also demonstrate binding of these elements by nuclear proteins present in mouse ES cell embryoid bodies, and identify both Pbx and Engrailed proteins in day 9 mouse ES cell embryoid bodies by Western blotting. Our data support the model that Engrailed heterodimerized with Pbx might activate transcription, while Engrailed or Pbx proteins alone might repress transcription (48).

An interesting finding in our work is the weak competition of the Engrailed mutant site observed in EMSA, but not seen for any of the other sites (Figs. 4 and 5). This weak competition may occur for two reasons. First, the mutated site may still be bound by the transcription factor, albeit in a much weaker fashion. Second, other unidentified binding site(s) in this region may exist that is/are not affected by the mutation. Evidence for the second possibility exists, since one of the gel shifts observed is competed equally well by the Engrailed mutant and wild type probes ("open arrow", Fig. 5).

This report is the first example of transcriptional regulation by intron sequences in the FGF family of genes, but numerous examples of intronic regulation of transcription exist for other genes (49–52). Traditionally, enhancer sequences are thought to be position and orientation independent. The Fgf8 intron's ability to enhance transcription shows orientation dependence, activating only in the forward orientation (Figs. 1C and 2). Hprt intronic sequences that regulate transcription in ES cells also show similar orientation and position dependence (53).

Our use of stable transfection of ES cells, and the analysis of reporter genes in embryoid bodies derived from these transfected ES cells, allows one to analyze transcriptional control elements of developmentally regulated genes in an appropriate physiological context in vitro. Similar analyses can be performed in P19 cells following differentiation in vitro (54).

In summary, we have identified important DNA-binding sites in the Fgf8 gene that regulate in vitro expression of a reporter gene in embryoid bodies following transfection into ES cells. Future work will seek to identify the proteins that bind to these elements, and to generate transgenic and/or chimeric mice to test whether the identified Fgf8 regulatory elements are necessary and sufficient for Fgf8 expression in vivo.

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REFERENCES

1. Basilico, C., and Moscatelli, D. (1992) Adv. Cancer Res. 59, 115–165
2. Myers, R. L., Payson, R. A., Chotani, M. A., Deaven, L. L., and Chiu, I. M. (1993) Oncogene 8, 341–349
3. Myers, R. L., Ray, S. K., Elderidge, R., Chotani, M. A., and Chiu, I. M. (1995) J. Biol. Chem. 270, 8257–8266
4. Ray, S. K., Yang, X. Q., and Chiu, I. M. (1997) J. Biol. Chem. 272, 7546–7555
5. Mansour, S. L., and Martin, G. R. (1998) EMBO J. 7, 2035–2041
6. Smith, R., Peters, G., and Dickinson, C. (1988) EMBO J. 7, 1013–1022
7. Azland, P., Dixon, M., Peters, G., and Dickson, C. (1990) Nature 343, 662–665
8. Grinberg, D., Thurlow, J., Watson, R., Smith, R., Peters, G., and Dickinson, C. (1991) Cell Growth Diff. 2, 137–143
9. Curatola, A. M., and Basilio, C. (1990) Mol. Cell. Biol. 10, 2475–2484
10. Dailey, L., Yuan, H. B., and Basilio, C. (1994) Mol. Cell. Biol. 14, 7758–7769
11. Yuan, H. B., Corbi, N., Basilio, C., and Dailey, L. (1995) Genes Dev. 9, 2635–2645
12. Ambrosiotti, D. C., Basilio, C., and Dailey, L. (1997) Mol. Cell. Biol. 17, 6321–6329
13. Hekkinen, M., Lawshe, A., Shackleford, G. M., Wilson, D. B., and MacArthur, C. A. (1994) Mol. Cell. Biol. 14, 129–138
14. Osuchi, H., Yoshihika, H., Tanaka, A., Kawakami, Y., Nohno, T., and Noji, S. (1994) Biochem. Biophys. Res. Commun. 204, 882–888
15. Crossley, P. H., and Martin, G. R. (1995) Development 121, 439–451
16. Lorenzo, M. V., Long, J. E., Miik, T., and Auronen, S. A. (1995) Oncogene 10, 2051–2055
17. Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colposhon, K., Martin, P., Lumazed, A., Dickson, C., and Mason, I. (1995) Curr. Biol. 5, 797–806
18. MacArthur, C. A., Shankar, D. B., and Shackleford, G. M. (1995) J. Virol. 69, 2501–2507
19. Valve, E., Pentila, T. L., Paranko, J., and Harkonen, P. (1997) Biochem. Biophys. Res. Commun. 232, 173–177
20. Crossley, P. H., Minowada, G., MacArthur, C. A., and Martin, G. R. (1996) Cell Mol. Biol. 42, 127–136
21. Crossley, P. H., Martinez, S., and Martin, G. R. (1996) Nature 380, 66–68
22. Vogel, A., Rodriguez, C., and Izpisua-Belmonte, J. C. (1996) Development 122, 1737–1750
Fibroblast Growth Factor-8 Expression Is Regulated by Intronic Engrailed and Pbx1-binding Sites
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