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Transcriptional regulation of the murine *Elf3* gene in embryonal carcinoma cells and their differentiated counterparts: requirement for a novel upstream regulatory region

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Abstract

The transcription factor Elf3, which is one of over 25 Ets family members, is expressed in a wide variety of carcinomas and has been shown to promote the transcription of many genes implicated in cancer. To understand how the *Elf3* gene is regulated at the transcriptional level, we probed its 5′-flanking region, and we report here the identification of both proximal and distal regions that regulate murine *Elf3* promoter activity. In addition to mapping the transcription start site of the *Elf3* gene, the work described in this study identifies four *cis*-regulatory elements in the proximal promoter region of the gene. These include a *cis*-regulatory element previously designated ESE, a κB site, a POU motif, and a CCAAT box. In addition, we demonstrate that a novel 94 bp region 2 kb upstream of the transcription start site significantly elevates *Elf3* promoter activity in F9-differentiated cells, but not in the parental F9 embryonal carcinoma (EC) cells. This region appears to be largely responsible for the increase in *Elf3* promoter activity that accompanies the differentiation of embryonal carcinoma cells.

Keywords: Ets proteins; Differentiation; Gene regulation; Distal enhancer; ESX; ESE1; ERT; Jen

1. Introduction

Transcription factors belonging to the Ets family play critical roles in normal growth and development, as well as human malignancy (Dittmer and Nordheim, 1998). Since the discovery of the first Ets family member, over 50 Ets genes have been identified, and at least 25 are expressed in mammals (Laudet et al., 1999). One Ets family member, Elf3 (also known as ESX, ESE-1, ERT, and JEN), is expressed ubiquitously by epithelial cells (Chang et al., 1997; Oettgen et al., 1997; Tymms et al., 1997), but it also can be induced in other cell types under pathological conditions (Grall et al., 2003). The roles of Elf3 have been examined recently in both cell culture and animal models. Inactivation of both alleles of *Elf3* by gene targeting leads to fetal lethality (Ng et al., 2002). Approximately 30% of the null fetuses die around embryonic day 11.5. The 70% that survive to birth exhibit severe alterations in the cellular architecture of the small intestine, including poor villus formation and defective terminal differentiation of the mucus-secreting goblet cells and the absorptive enterocytes. Moreover, enterocytes from Elf3 null animals express significantly reduced levels of type II TGF-β-receptor (TβR-II) at both the RNA and the protein level. Similarly, reduction of Elf3 in a colon cancer cell line by use of an *Elf3* antisense retroviral construct

Abbreviations: EC, embryonal carcinoma; RA, retinoic acid; TβR-II, type II TGF-β-receptor; 5′-RACE, rapid amplification of cDNA ends; EMSA, electrophoretic mobility shift assay.

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also reduced *TβR-II* expression (Lee et al., 2003). Other studies have linked Elf3 directly to *TβR-II* transcription in epithelial cells by its binding to two essential ets-sites in the *TβR-II* promoter (Choi et al., 1998; Kim et al., 2002; Kopp et al., 2004). In addition to the *TβR-II* gene, Elf3 has been implicated in the regulation of multiple genes, including *endo A*, *SPRR2A*, *SPRRIB*, and *nitric-oxide synthase* (Oettgen et al., 1997; Rudders et al., 2001; Reddy et al., 2003).

Elf3 also appears to play important roles in cancer, and it is aberrantly expressed in cancers of the lung and breast (Chang et al., 1997). In the lung, elevated Elf3 expression has been implicated in the expression of the *SPRRIB* gene, a marker for early metaplastic alterations in bronchial epithelium (Reddy et al., 2003). In breast cancer, the 1q32 chromosomal region containing *Elf3* (Chang et al., 1997) is amplified in 50% of early tumors (Isola et al., 1995). However, in the case of Hs578t breast cancer cells, Elf3 is expressed at very low levels. Elevating the expression of Elf3 in Hs578t cells dramatically elevates the expression of *TβR-II* and decreases the tumorigenicity of these cells (Chang et al., 2000).

Given the important roles of Elf3 in normal cellular physiology and cancer, it is important to determine how transcription of the *Elf3* gene is regulated. Sequence analyses of the *Elf3* gene have identified several potential transcription factor binding sites in its promoter region, including ets, POU, USF, and κB sites, as well as GC, CCAAT, and TATA boxes (Neve et al., 1998; Oettgen et al., 1999). Thus far, only the κB site and a novel site, termed an ESE element, in the human *ELF3* gene have been shown to be functional (Rudders et al., 2001; Park et al., 2001). Importantly, other regions of the *Elf3* gene have not been examined for their effects on its transcription.

The aim of this study was to identify and characterize *cis*-acting regions of the murine *Elf3* gene that control its transcription in early embryonic tumor cells. This work was conducted using murine embryonal carcinoma (EC) cells and their retinoic acid (RA) induced differentiated counterparts, because the steady-state levels of *Elf3* mRNA increase at least sixfold when F9 EC cells differentiate (Kim et al., 2002). Currently, it is unknown whether increases in Elf3 expression influence the differentiation of EC cells, but it is believed to play an important role in the increase in transcription of the *TβR-II* gene after EC cells differentiate (Kim et al., 2002).

In this study, we have identified two regions of the *Elf3* gene that influence the activity of its promoter. Initially, we mapped the transcription start site of the gene in F9-differentiated cells and tested the function of six potential *cis*-regulatory elements in the proximal promoter region of the murine *Elf3* gene. Importantly, we also demonstrate that differentiation substantially increases the activity of the *Elf3* promoter and that this increase in promoter activity is due in large measure to a novel regulatory region located approximately 2 kb upstream of the transcription start site.

2. Materials and methods

2.1. Cell culture, transfection and luciferase assay

F9 EC cells were cultured on gelatinized dishes in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and antibiotics (Kim et al., 2002; Nowling et al., 2003). Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO). F9 EC cells were transfected on the day after being seeded at 20,000 cells/well in 24-well plates. For transfection of F9-differentiated cells, F9 EC cells were seeded at 4000 cells/well in 24-well plates and treated with 5 μM RA to induce differentiation. The differentiated cells were transfected 3 days later. Transfections were performed with Lipofectamine and the Plus transfection reagent (Invitrogen) according to the manufacturer's instructions using 400 ng of *Elf3* promoter-luciferase plasmids and 200 ng of pRL-TK internal control vector (Promega, Madison, WI). Cells were harvested 24 h after transfection, luciferase activity was measured using a Dual-luciferase reporter assay kit (Promega) with a Luminoskan RS plate-reading luminometer (MTX Lab Systems, Vienna, VA), and activities were normalized to the activity of an internal control vector, pRL-TK. Values are presented as the mean ± standard error of the mean (S.E.M.) of duplicate or triplicate samples.

2.2. Sequencing

The LI-COR and ABI Sequencers at the Genomics Core Research Facility at the University of Nebraska at Lincoln were used along with primer walking to sequence the 5′-flanking region of the mouse *Elf3* gene. Both strands were sequenced. The ABI sequencer was also used to verify critical sequences in various plasmids.

2.3. Mapping the transcription start site by rapid amplification of cDNA ends (5′-RACE)

The transcription start site of the murine *Elf3* gene was determined using 5′-RACE. Two internal primers, 5′-TCTCCCAGAGGTCCAAAGACTAGC-3′ (nucleotide position 378 to 401) and 5′-AGCTCCTCCAGCGCA-CAGCTGCAGAGGGTGGCTCC-3′ (nucleotide position 340 to 374), were designed based on the *Elf3* cDNA sequence (GenBank NM-007921). These two primers were used in conjunction with the RLM 5′-RACE adapter primer (Ambion, Austin, TX) to capture the 5′ end of the *Elf3* mRNA. The resulting fragments were cloned into the pSP72 vector (Promega). The inserts of 17 recombinant clones were sequenced.

2.4. Generation of promoter/reporter gene constructs

The *Elf3* promoter region −338 to +122 was amplified by PCR from genomic DNA of F9-EC cells. Restriction sites *Nhe*I and *Bgl*II were incorporated at the ends of the PCR primers to allow insertion into the pGL3-luciferase reporter vector (Promega). PCR primers were designed based on the promoter region sequence from Neve et al. (1998) 5′-GTAGCTAGCGCCAGGCCCCCAGGAAGA-3′ (sense) and the cDNA sequence of Tymms et al. (1997) 5′-GTAAGATCTGGTGTGGCAGGCGGGTGA-3′ (antisense). To mutate potential *cis*-regulatory elements within this sequence, site-directed mutagenesis of the *Elf3* promoter Luciferase construct was performed using the "quick change method", as described previously (Kim et al., 2002; Nowling et al., 2003). The following primers were used along with their reverse complements: 5′-CAGATTCTGACAATCATTAA**AACTCG**AGGCCTCTGATTTCCAG-3′ for ESE_{mut}; 5′-CAGGAAATCCCCAGCATC**TCGAG**AGCCACCAGCTCAGGTTAC-3′ for $CCAAT_{mut}$; 5′-AAGTGGCACGGAATATG**AATTTC**ACCTGGGACAGGGAGCCCAG-3′ for POU_{mut}; and 5′-GGGAGCCCAGTCTGAAGGCCAG**TTAATTAA**-CAGCATCCAATGAGC-3′ for κB_{mut}. The bolded nucleotides are the mutations introduced into the sequence. All promoter sequences were verified by DNA sequencing.

The region of the mouse *Elf3* gene from −2898 to +703 was inserted into the pGL3-Basic vector between the *Mlu*I and *Hin*dIII sites to generate the promoter/reporter construct, *mElf3* −2898/+703. Additional promoter/reporter gene constructs lacking portions of this sequence were constructed by digestion with pairs of restriction enzymes, blunting with klenow, and religation. Thus, *mElf3* −2437/+703 was generated using *Mlu*I and *Kpn*I, *mElf3* −2347/+703 using *Mlu*I and *Bgl*II, *mElf3* −2012/+703 using *Mlu*I and *Eco*RI, and $mElf3-2898/+703_{\Delta-2343/-2013}$ using *Bgl*II and *Eco*RI.

To generate *mElf3* −338/+703, the promoter region upstream of +32 was removed from *mElf3* −2898/+703 and the fragment −338/+32 was obtained from *mElf3* −338/+122 by digestion with *Kpn*I (at an upstream polylinker site) and *Msc*I. The −338/+32 fragment was then inserted into what was left of *mElf3* −2898/+703. Additional promoter/reporter constructs were made by inserting various sequences between the *Kpn*I and *Nhe*I restriction sites upstream of the promoter in *mElf3* −338/+703. Three constructs made in this manner using sequences generated by PCR from *mElf3* −2898/−703 were $mElf3-2347/+703_{\Delta-2012/-339}$, $mElf3-2347/+703_{\Delta-2211/-339}$, and $mElf3-2236/+703_{\Delta-2142/-339}$. Another series of constructs made in this manner included various mutations of the *Elf3* sequence from −2236 to −2143. These mutations were substitutions of 'CCCGGG' in each of 15 positions in the sequence (or 'CGG' in place of the first three bases). In these cases, the sequences to be inserted were obtained by synthesis of smaller overlapping oligonucleotides, which were extended using taq polymerase. All promoter sequences were verified by DNA sequencing.

The promoter/reporter construct, $mElf3-2347/+703_{\Delta-2012/-339}$, was used in turn to generate two additional constructs. $mElf3-2347/+703_{\Delta-2012/-339}$ was digested with pairs of restriction enzymes, *Kpn*I and *Eco*RI or *Eco*RI and *Nhe*I, to produce $mElf3-2093/+703_{\Delta-2012/-339}$ and $mElf3-2347/+703_{\Delta-2089/-339}$, respectively, after blunting with klenow and religation. All promoter sequences were verified by DNA sequencing.

3. Results

3.1. Sequence of the murine Elf3 5′-flanking region

The primary objective of this study was to identify regions of the *Elf3* gene required for its transcription. To initiate this study, the murine *Elf3* gene from −2898 to +703 was sequenced. This sequence has been assigned the GenBank accession number AY456682. To facilitate comparison with previous studies, we have designated the nucleotide positions in this report according to the numerical system of Neve et al. (1998), who presented an alignment of the human *ELF3* and murine *Elf3* promoters between −347 and +50 of the murine sequence.

3.2. Mapping the transcription start site of the murine Elf3 gene

The only published report concerning the transcription start site of the murine *Elf3* gene places it 50 bp downstream from a consensus TATA box (Tymms et al., 1997). However, functional TATA boxes typically direct transcriptional initiation to locations 30±2 bp downstream (Bucher, 1990). Therefore, we mapped the location of the transcription start site of the murine *Elf3* gene in F9-differentiated cells. For this purpose, 5′-RACE was used. Seventeen clones were isolated and sequenced, and a dominant transcriptional initiation site (16 out of 17 clones) was identified at nucleotide +32 (Fig. 1). This site is 30 bp downstream of the putative TATA box located at +2. The sequence of the 17th clone in our study corresponds to a start site at nucleotide +33, and it could have resulted from premature termination during the polymerization step. Thus, it appears that one major start site is employed in F9-differentiated cells. Interestingly, sequence analysis of our 17 clones identified both splice variants of *Elf3* mRNA, Elf3a and Elf3b, observed previously in the adult lung of mice (Tymms et al., 1997). Thus, both Elf3a and Elf3b, which differ by 20 amino acids, may be expressed in F9-differentiated cells.

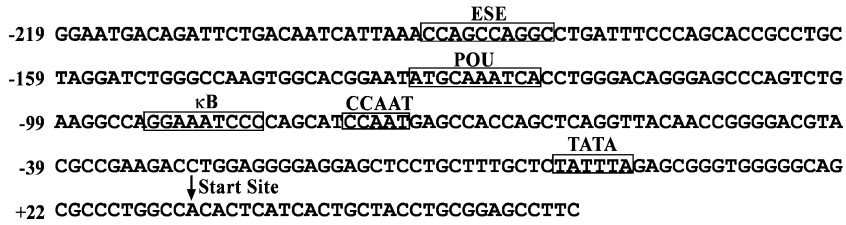

Fig. 1. Nucleotide sequence of the region containing the murine *Elf3* transcription start site. The transcription start site of murine *Elf3* in F9-differentiated cells was determined by 5′-RACE as described in Materials and methods. The major transcription start site is indicated by an arrow. Other sequences of interest are boxed, including potential ESE, POU, and κB elements and CCAAT and TATA boxes.

3.3. The proximal promoter region of Elf3 contains at least four cis-regulatory elements

To identify essential *cis*-regulatory elements in the murine *Elf3* gene, we inserted the region between −338 and +122 of the *Elf3* gene into the promoterless luciferase reporter gene construct, pGL3 Basic, to create *mElf3* −338/+122. This construct exhibited 100-fold higher luciferase activity than the parent pGL3 Basic vector (data not shown). Previous analysis of this region (Neve et al., 1998) identified several potential *cis*-regulatory elements, including a GC box, a USF site, a POU motif, a κB site and a CCAAT box. Of these, only the κB site has been shown to be functional in the human *ELF3* gene (Rudders et al., 2001). In addition, a novel *cis*-regulatory element referred to as ESE was identified recently in the human *ELF3* gene and shown to act as a positive *cis*-regulatory element in a human gastric cancer cell line (Park et al., 2001). To determine whether any of these potential regulatory sequences are functional in the murine *Elf3* gene, each of these sites were disrupted individually in the *Elf3* promoter/reporter gene construct *mElf3* −338/+122. Disruption of the ESE element and the κB site reduced promoter activity by 48% and 43%, respectively (Fig. 2). Larger reductions in promoter activity were observed when the POU motif and CCAAT box were mutated, 65% and 71%, respectively. However, disruption of the two GC boxes or the USF site did not reduce *Elf3* promoter activity (data not shown). Thus, the proximal promoter region of the *Elf3* gene contains at least four *cis*-regulatory elements that influence its activity, a CCAAT box, a κB site, a POU motif and ESE.

3.4. Differentiation of F9 EC cells elevates Elf3 promoter activity

Previous studies demonstrated that differentiation of F9 EC cells leads to significant increases in the steady-state levels of *Elf3* mRNA (Kim et al., 2002). Therefore, we examined whether the activity of the *Elf3* promoter increases when EC cells differentiate. Initially, this was examined by transiently transfecting F9 EC cells with *mELF3* −338/+122. Five hours after transfecting the cells, the medium was changed and RA was added to one set of F9 EC cells to induce differentiation. In comparison to the untreated F9 EC cells, *Elf3* promoter activity increased approximately twofold after a 3-day exposure to RA (Fig. 3A). This was significantly lower than the sixfold increase in *Elf3* mRNA observed previously when F9 EC cells differentiate (Kim et al., 2002). This raised the possibility that other regions of the *Elf3* gene are required for full promoter activity in F9-differentiated cells. To examine this possibility, we generated a murine *Elf3* promoter/reporter gene construct that contains a larger region of the *Elf3* gene.

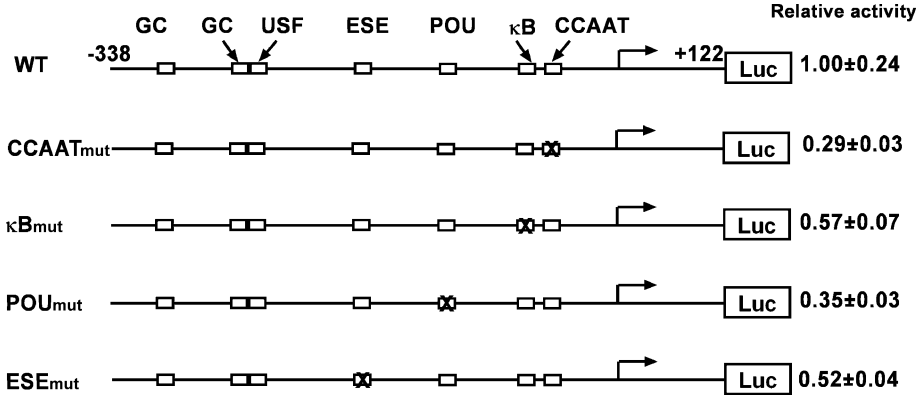

Fig. 2. Murine *Elf3* promoter/reporter activity in F9-differentiated cells. Promoter/reporter constructs with potential *cis*-regulatory sites mutated (as indicated with an "X") were transiently transfected into F9-differentiated cells and assayed for luciferase activity after 48 h as described in Materials and methods. Results were normalized with an internal control and expressed relative to the activity of the unmutated, wild-type, construct *mElf3* −338/+122. Values shown are averages ± S.E.M. for duplicate samples in a representative experiment. This experiment was performed three times with similar results.

Specifically, we inserted the region −2898 to +703 into the promoterless vector pGL3 Basic to generate *mElf3* −2898/+703. When this construct was transfected into F9 EC cells, we observed a fivefold increase in promoter activity for F9 cells exposed to RA for 3 days (Fig. 3A). To confirm and extend these findings, we directly compared the activity of *mElf3* −2898/+703 to that of *mELF3* −338/+122. There was no significant difference between the activity of the larger and the smaller *Elf3* promoter/reporter gene constructs when they were transiently transfected into F9 EC cells (Fig. 3B). In contrast, when F9 EC cells were transfected after 3 days of RA-induced differentiation, the activity of the larger construct was fivefold higher than the activity of the smaller *mElf3* −338/+122 construct (Fig. 3B). Together, these findings argue that at least one region outside the proximal promoter exerts a strong influence on *Elf3* promoter activity in F9-differentiated cells.

3.5. An upstream regulatory region elevates Elf3 promoter activity

To identify the sequences responsible for elevated promoter activity, we examined the sequences between −2898 and −339 and between +122 and +703 for their contribution to *Elf3* promoter activity. Initially, we created the promoter/reporter gene construct, *mElf3* −338/+703 and compared its activity to that of *mElf3* −338/+122. In F9-differentiated cells, the activity of *mElf3* −338/+703 was only slightly higher (approximately twofold) than that of *mElf3* −338/+122 (data not shown). Therefore, we focused our attention on the region between −2898 and −339. Additional studies in the future will be needed to identify regulatory sequences located between +123 and +703.

To identify sequences upstream of −338 that influence *Elf3* promoter activity, progressively larger amounts of the *Elf3* gene between −2898 and −2012 were deleted from 5′ end of the promoter/reporter gene construct *mElf3* −2898/+703. Removal of the region between −2898 and −2347 did not reduce *Elf3* promoter activity (Fig. 4A). However, deletion of the region upstream of −2012 reduced *Elf3* promoter activity (Fig. 4A). This suggested that the region of the *Elf3* gene between −2347 and −2012 contained one or more *cis*-regulatory elements that influence its promoter activity in F9-differentiated cells. This possibility was tested by deleting the region −2343 to −2013 to generate the construct *mElf3* $-2898/+703_{\Delta -2343/-2013}$. When this construct was transfected into F9-differentiated cells, the luciferase activity generated was approximately fourfold lower than that observed with the parent, *mElf3* −2898/+703, construct (Fig. 4A).

The region between −2347 and −2012 was examined further using five additional promoter/reporter gene constructs, which incorporated portions of this region inserted into *mElf3* −338/+703 upstream of the proximal promoter (Fig. 4B). Importantly, *mElf3* $-2236/+703_{\Delta -2142/-339}$, incorporating the 94 bp sequence from −2236 to −2143, exhibited nearly fourfold greater activity than *mElf3* −338/+703. To verify the importance of the 94 bp region in the upregulation of the *Elf3* promoter when F9 EC cells undergo differentiation, we compared the activity of *mElf3* $-2236/+703_{\Delta -2142/-339}$ to that of *mElf3* −338/+703 in F9 EC cells and F9-differentiated cells. As expected, there was no difference in the activity of these two constructs in F9 EC cells, whereas *mElf3* $-2236/+703_{\Delta -2142/-339}$ exhibited greater than sixfold higher activity in F9-differentiated cells (Fig. 4C). Hence, we focused the remainder of our efforts

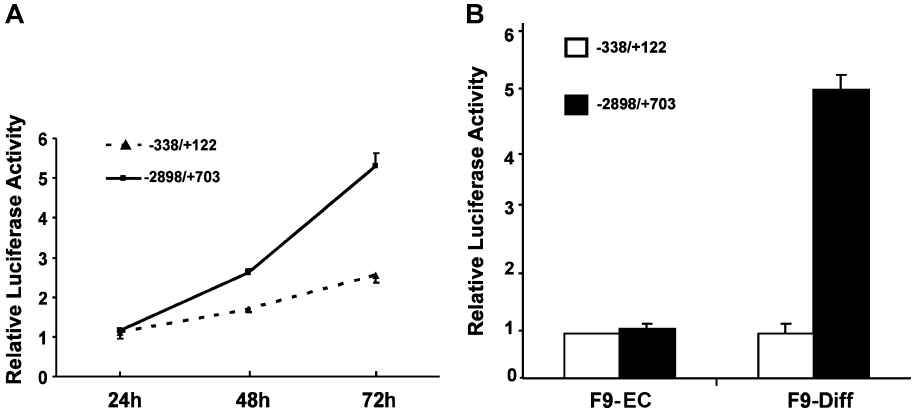

Fig. 3. An upstream distal enhancer significantly increases *Elf3* promoter activity in F9-differentiated cells. F9 EC cells were transfected with either a short −338/+122 or a long −2898/+703 *Elf3* promoter/reporter construct as described in Materials and methods. (A) F9 EC cells were transfected with the *Elf3* promoter/reporter gene constructs shown. Five hours after transfection, half of the cells were induced to differentiate with 5 μM RA. Luciferase assays were conducted 24, 48 or 72 h after transfection as described in Materials and methods. Results from duplicate F9 EC samples were averaged and used with individual differentiated cell samples to generate ratios of luciferase activity (F9-differentiated: F9 EC). The average ± S.E.M. of these ratios is plotted. This experiment was repeated with triplicate samples with similar results. (B) F9 EC and F9-differentiated cells were transfected independently. Averages ± S.E.M. are plotted for representative experiments, which were repeated multiple times with similar results.

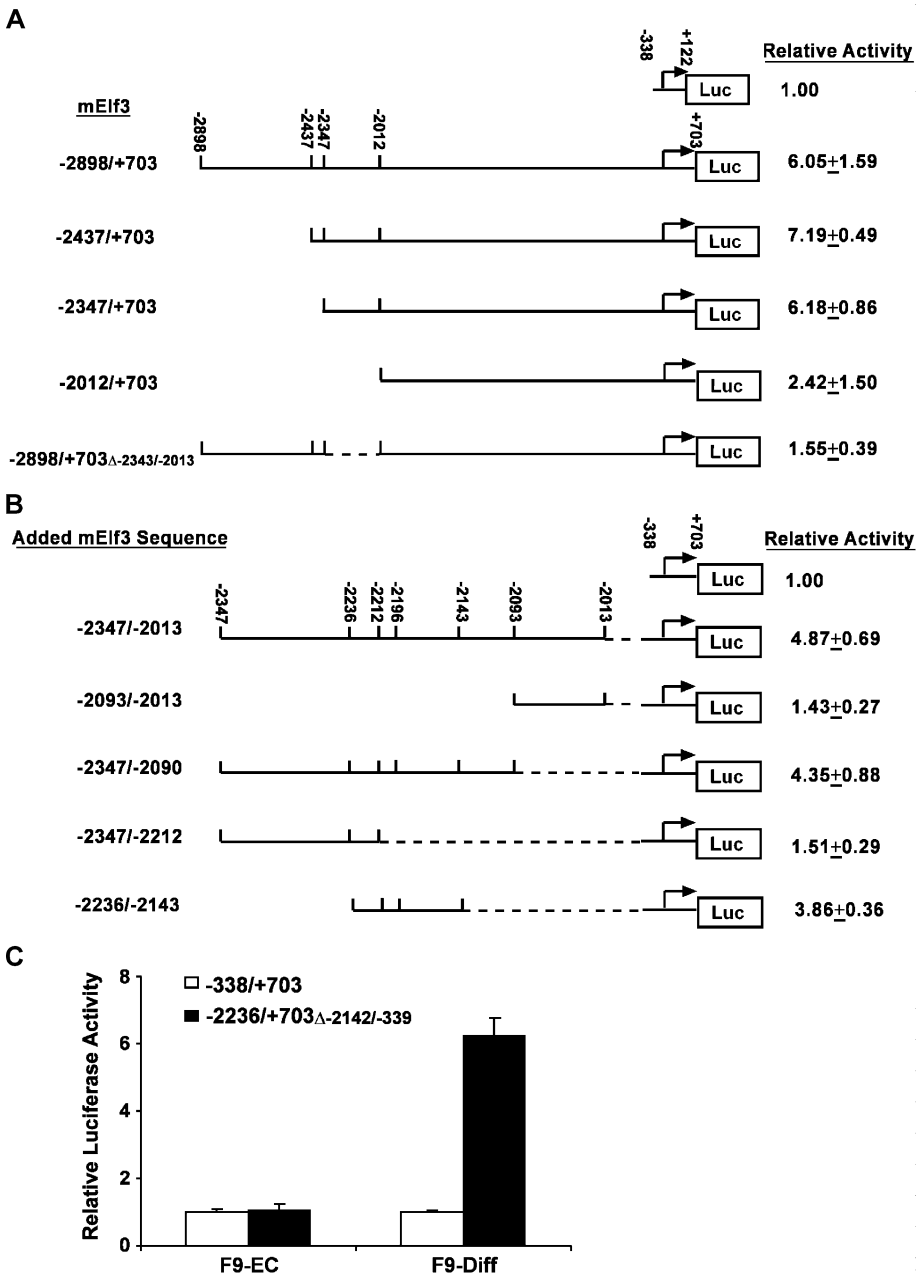

Fig. 4. Progressive deletion analysis of the *Elf3* upstream flanking region. Promoter/reporter constructs containing the indicated regions of upstream flanking sequence were transfected into F9-differentiated cells and assayed for luciferase activity as described in Materials and methods. (A) Means from three similar experiments, each with triplicate samples expressed relative to *mElf3* − 338/ + 122, were used to generate averages ± S.E.M. (B) Means from five similar experiments, each with triplicate samples expressed relative to *mElf3* − 338/ + 703, were used to generate averages ± SEM. (C) F9 EC and F9-differentiated cells were transfected independently with either *mElf3* − 338/ + 703 or *mElf3* − 2236/ + 703$_{\Delta -2142/-339}$ *Elf3* promoter/reporter construct. For the purpose of comparison, the activity of the *mElf3* − 338/ + 703 was set to 1. The values shown are averages ± S.E.M. for triplicate samples from a representative experiment. This experiment was repeated and similar results were obtained.

in this study on the 94 bp sequence between − 2236 and 2143.

3.6. A 36 bp upstream region is required for elevated Elf3 promoter activity

To identify specific DNA sequences within the sequence from − 2236 to − 2143 that are responsible for elevating *Elf3* promoter activity, saturation mutagenesis was employed. Specifically, one 3 bp mutant and fifteen 6 bp mutants were generated to sequentially disrupt the entire 94 bp sequence (Fig. 5A). For this purpose, the wild-type sequence for each of the fifteen 6 bp mutants was replaced with the sequence 5′-CCCGGG-3′. After transient transfection into F9-differentiated cells, the activity of each mutant construct was compared to the activity of *mElf3* − 2236/

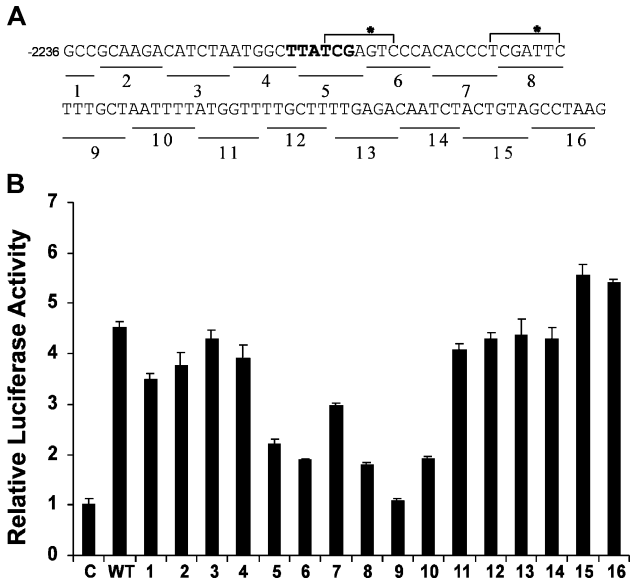

Fig. 5. Mutational analysis of the upstream 94 bp positive regulatory region. (A) The murine 94 bp upstream enhancer region is shown. Sixteen sequences chosen for mutational analysis are sequentially numbered and underlined. A potential GATA site is shown in bold and a repeated sequence is marked with an upper bracket. The single difference in the 7 bp repeat is labeled with an"*" (B) F9-differentiated cells were transfected with the wild-type (*mElf3* − 2236/ + 703$_{\Delta - 2142/ - 339}$) or mutant promoter/reporter constructs containing *Elf3* sequence from − 2236 to − 2143 inserted upstream of the *mElf3* − 338/ + 703 construct. In each numbered construct, the sequence to be mutated was replaced by the sequence 5′-CCCGGG-3′. Cells were assayed for luciferase activity as described in Materials and methods. Values for the mutant constructs were compared to *mElf3* − 338/ + 703, shown as "C", which was set to 1. The values shown are averages ± S.E.M. for triplicate samples from a representative experiment. This experiment was performed four times with similar results.

+ 703$_{\Delta - 2142/ - 339}$. This experiment identified a contiguous core of 36 bp (mutants #5 through #10, − 2215 to − 2180) that influenced *Elf3* promoter activity (Fig. 5B). Each of the six mutant constructs, which encompass this 36 bp region, reduced promoter activity. The largest reduction was observed with mutant #9, which eliminated nearly all the activity of the 94 bp sequence.

Thus far, efforts to identify the factors that bind to this 36 bp region have not been successful. Surprisingly, electrophoretic mobility shift assay (EMSA) failed to demonstrate specific binding to this region by any factors in nuclear extracts from F9-differentiated cells (data not shown). In silico analysis identified a region (− 2216 to − 2211) within the 94 bp sequence that differs by 1 bp from a consensus site for GATA factors (Fig. 5A). This was of interest, because GATA 4 and GATA 6 expression increases when EC cells undergo differentiation (Morrisey et al., 1996; Murakami et al., 1999). However, overexpression of neither GATA 4 nor GATA 6 influenced *Elf3* promoter activity in F9-differentiated cells (data not shown). Interestingly, this region of the *Elf3* gene contains near perfect 7 bp repeats that differ from one another by only 1 bp, and which are separated from each other by 8 bp (Fig. 5A). However, these sequences do not appear to match the consensus sequence for any known eukaryotic transcription factor.

4. Discussion

Previous studies have shown that *Elf3* mRNA increases at least sixfold after EC cells differentiate (Kim et al., 2002). However, changes in Elf3 protein levels in this model system have not been determined due to the lack of an antibody that is specific for mouse Elf3. In this study, we examined the DNA regulatory regions that influence the expression of the *Elf3* gene. Specifically, we mapped the transcription start site of the murine *Elf3* gene in F9-differentiated cells and identified *cis*-regulatory elements located in the proximal promoter region as well as in a region 2 kb upstream of the transcription start site. The upstream regulatory region is localized to a 36 bp sequence, which appears to play an important role in upregulation of *Elf3* when F9 EC cells differentiate.

In this study, 5′-RACE identified a major transcription start site for the murine *Elf3* gene in F9-differentiated cells at position + 32, which is 30 bp downstream from the center of a TATA box at + 2. In our study, 16 of the 17 isolated clones identified a start site at position + 32, while the remaining clone corresponded to a start site at + 33. In contrast to our findings, Tymms et al. (1997) used 5′-RACE and mapped the dominant start site in adult murine lung tissue to + 51. In their study, a total of seven clones were sequenced, and four of the seven clones identified a transcription start site at position + 51. The remaining three clones identified start sites at positions + 42, + 44 and + 68, respectively. An analysis of hundreds of genes by Bucher (1990) found the center-to-center spacing between a TATA box and the cap signal to be 30 ± 2 bp. It is possible that multiple start sites are used for the *Elf3* gene and that different start sites are used in different cell types. However, if functional, the TATA box would direct transcriptional initiation to the site that we identified in F9-differentiated cells rather than to the site found in adult lung. Two studies of the human *ELF3* gene have mapped the transcription start site to − 7 in adult liver cells (Oettgen et al., 1999) and − 8 in a gastric cancer cell line (Park et al., 2001). Oettgen et al. (1999) point out that the difference in start site between the human and murine genes is likely due to the presence of a second TATA box centered at − 39 in the human sequence, which is not present in the murine sequence.

The DNA sequences of the four promoter-proximal *cis*-regulatory elements (ESE, POU, κB and CCAAT box) shown to be functional in F9-differentiated cells (Fig. 2) are 100% conserved between the mouse and human except for one base pair in the ESE site (Neve et al., 1998). This is also true for an overlapping CG box and USF site, but disruption of either site on its own or in

combination failed to affect *Elf3* promoter activity in F9-differentiated cells (data not shown). In contrast to these sites, disruption of the κB site and the ESE site reduced *Elf3* promoter activity 43% and 48%, respectively, (Fig. 2). Earlier studies demonstrated that both sites are functional in the case of the human gene. The κB site was first shown to be functional in the case of the human *ELF3* promoter using a mouse monocytic cell line (Rudders et al., 2001). This study also reported that the κB site of the human *ELF3* gene binds the p50 and p65 subunits of NF-κB in vitro (Rudders et al., 2001). Moreover, recent evidence argues strongly that p50 and p65 activate the human *ELF3* promoter via the κB site in vivo (Grall et al., 2003). Similar to these findings, we determined that c-Rel and the p50 subunit of NF-κB present in nuclear extracts from F9-differentiated cells can bind to the κB site in vitro (data not shown). The ESE site in the human *ELF3* gene was also shown to be functional in an earlier study that used a human gastric tumor cell line (Park et al., 2001). Interestingly, disruption of the ESE site reduced the activity of the human *ELF3* promoter approximately 80% in the gastric tumor cell line. Thus, the overall contribution of the ESE site may differ between the human and the murine gene and/or in different cell types (48% for F9-differentiated cells vs. 80% for gastric tumor cells). Thus far, the nuclear factor shown previously to bind to the ESE site in vitro has not been identified (Park et al., 2001). Given that the ESE site influences *Elf3* promoter activity in at least two cell types, the factor that binds to the ESE site warrants further study. However, it would appear that these studies would be best undertaken using gastric tumor cells.

In this study, we provide the first demonstration that the POU motif and the CCAAT box of this gene influence its promoter activity. Disruption of either site reduced *Elf3* promoter activity more than 65% in F9-differentiated cells. In the case of the CCAAT box, we determined by chromatin immunoprecipitation that NF-Y binds to the promoter of the endogenous *Elf3* gene in F9-differentiated cells, and we determined that NF-Y mediates the positive effect of the CCAAT box with the use of a dominant-negative mutant of NF-YA (Mantovani et al., 1994; data not shown). In other work not described in this report, we determined by electrophoretic mobility shift assay that the transcription factor Oct-1 in nuclear extracts prepared from F9-differentiated cells can bind to the POU motif of the *Elf3* gene in vitro. Given the importance of the POU motif, future studies should determine whether Oct-1 binds to the POU motif in F9-differentiated cells and elevates *Elf3* promoter activity in vivo.

The work reported in this study argues strongly that an upstream regulatory region plays an important role in elevating *Elf3* promoter activity after EC cells undergo differentiation. Earlier studies demonstrated that the steady-state level of *Elf3* mRNA increases after EC cells are induced to differentiate, reaching levels approximately sixfold higher after 3 days and eightfold higher after 5 days (Kim et al., 2002). In the present study, after 3 days of differentiation, the activity of promoter/reporter constructs containing the upstream region is elevated fivefold, while the activity of the proximal promoter appears to increase only twofold (Fig. 3A). We localized this positive regulatory region to a 94 bp sequence between −2236 and −2143 by using a battery of *Elf3* promoter/reporter gene constructs containing the proximal promoter and various portions of the 5′-flanking region of the *Elf3* gene. In F9-differentiated cells, constructs containing this 94 bp region have four- to sixfold higher activities than constructs without this region. This is in contrast to F9 EC cells, where both sets of constructs have similar activities (Fig. 4C). Together, our data argue that differentiation of EC cells significantly increases the activity of the *Elf3* promoter and that full promoter activity is dependent on a distal regulatory region located 2 kb upstream of the transcription start site of *Elf3*. Interestingly, this regulatory region functions in a distance-independent manner, given that it functions even when it is placed 2 kb closer to the *Elf3* promoter (Fig. 4B). We also believe that this region is likely to function in an orientation-independent manner, but this was not tested in this study.

Saturation mutagenesis of the 94 bp regulatory region identified a core of 36 bp from −2215 to −2180, which is required for full *Elf3* promoter activity. The importance of this 36 bp region was demonstrated by six sequential 6 bp mutations, each of which reduced *Elf3* promoter activity. Given that the size of this region is far greater than the size of a typical transcription factor binding site (4–10 bp), and the fact that *cis*-regulatory elements are often clustered in enhancers, we anticipate that more than one transcription factor binds to this regulatory region. Thus far, we have been unable to identify the factors involved. Efforts to identify proteins that bind to this 36 bp region using electrophoretic mobility shift assay have not been successful.

In conclusion, the work reported in this study establishes that the promoter of the murine *Elf3* gene in F9-differentiated cells is controlled by at least four *cis*-regulatory elements located within 250 bp of the transcription start site. Moreover, we have identified a novel 36 bp regulatory region, which is located 2 kb upstream of the transcription start site. Importantly, this regulatory region exerts a strong influence on the *Elf3* promoter activity in F9-differentiated cells and appears to play an important role in the upregulation of *Elf3* gene expression after EC cells undergo differentiation. Given that these cells provide an excellent model for mammalian embryogenesis and that differentiation of EC cells strongly suppresses their tumorigenicity (Adamson and Graham, 1980), it will be of interest to better understand how the *Elf3* gene is regulated in this model system. Such an understanding is likely to provide important insights into the regulation of this gene in both normal and diseased tissues.

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