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# Cloning and Characterization of the 5' Upstream Region of the Human Primary Response Gene *EGR-3*

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**Abstract**

***Egr-3* is an immediate-early primary response gene encoding a zinc finger transcription factor. We cloned the human *Egr-3* genomic locus including greater than 1100 bp of the 5' flanking region and analyzed this region for putative cis-acting elements. The GC-rich promoter forms part of a representative CpG island that extends into the genomic locus. The *Egr-3* promoter contains a region of TATA homology located 25bp upstream from a major transcriptional start site. One serum response element and two variant *Egr* consensus sequences were identified. Features that distinguished *Egr-3* from other human *Egr* gene promoters included the presence of at least five E-box motifs and a retinoblastoma response element. In addition, an overlapping tandem repeat of 16 GC-rich nonamers was identified in the flanking region that may represent a novel regulatory region for this primary response gene. Reporter constructs coupled with *Egr-3* flanking sequences in sense and antisense orientation were tested in transient transfection assays. The functional activity of the *Egr-3* regulatory region was position-specific. Deletional analysis in serum stimulated embryonic lung fibroblasts identified that the major elements responsible for growth-induced *Egr-3* expression are located within the first 378 bp upstream of the major transcription start site. Analysis of the human *Egr-3* genomic locus revealed a complex regulatory organization with significant differences from other *Egr* genes. These findings may provide insights into the expression of *Egr-3* in normal and neoplastic tissues.**

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**Introduction**

The primary response genes encode a spectrum of structural and regulatory proteins including several families of nuclear transcription factors that presumably regulate a select group of target genes involved in tissue- and signal-specific responses (Herschman, 1991). Among them, the *Egr* (early growth response) gene family is a structurally related group consisting, to date, of four zinc finger transcription factors, *Egr-1*, *Egr-2*, *Egr-3*, and pAT 13 (Sukhatme *et al.,* 1988; Joseph *et al.,* 1988; Muller *et al.,* 1991). The *Egr* genes encode proteins with three tandem zinc finger motifs of the Cys2- His2 subclass that are highly homologous and mediate sequence-specific DNA binding. The prototypic member of this group, *Egr-1*, has been most extensively studied in this regard. A GC-rich nonameric consensus sequence (GCGGGGGCG) was initially identified as an *Egr-1* binding site Christy and Nathans, 1989; Cao *et al.,* 1993), and the interaction of murine *Egr-1* with the GCGTGGGCG motif was characterized by X-ray crystallography studies (Pavletich and Pabo, 1991). Binding to the nonameric consensus sequence has been demonstrated for all the *Egr* family members, which complex to this domain with different levels of affinity. Other putative *Egr* response elements include a homopurine/homopyrimidine domain (TCCTCCTCCTCCTCTCC) (Wang and Deuel, 1992) and variations of the consensus sequence (Swirnoff and Milbrandt, 1995; Nakagama et al., 1995). Some of the *Egr*-binding sequences are present in the promoter region of various genes involved in cell proliferation, thus linking this family of regulatory proteins to transcriptional control of cellular growth processes.

A potential relationship of some *Egr* genes and related zinc finger proteins to the development of the malignant phenotype has also been suggested. *Egr-1* is located on chromosome 5q31, a region commonly deleted in therapy-related myeloid leukemias (Nucifora *et al.,* 1993). Dys-regulated expression of *Egr-1* and *Egr-2* by human retrovirus-transformed cells (Wright *et al.,* 1990) and soft-tissue sarcomas has been identified. Putative tumor suppressor activity of *Egr-1* has been described based on studies showing inhibition of v-sis transformation in murine fibroblasts co-transfected with an expression vector containing *Egr* or *Egr* gene fragments (Huang *et al.,* 1994). Finally, the *WT1* gene, the loss of which results in the development of Wilms tumors, encodes a transcription factor with zinc finger regions that share a high level of sequence homology to the corresponding region of *Egr* proteins and binds the *Egr* consensus sequence as well (Nakagama *et al.,* 1995).

The human and murine *Egr-3* genes were isolated from a serum-activated cDNA library by low-stringency hybridization with an *Egr-1* probe containing the zinc finger (Patwardhan *et al.,* 1991). The putative *Egr-3* protein featured three tandem zinc finger motifs that were 90%

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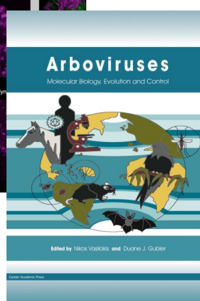

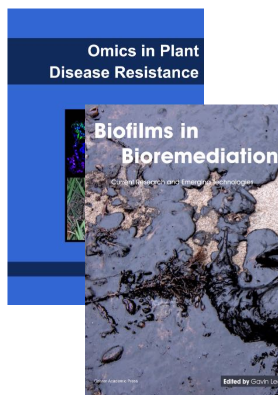

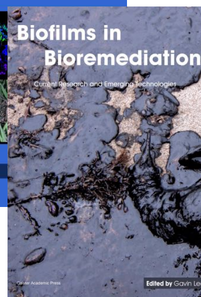

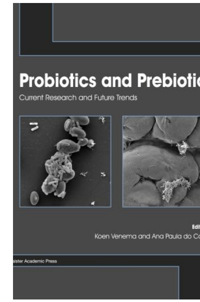

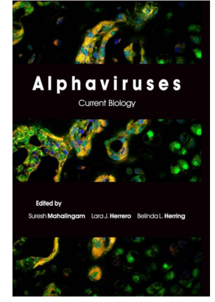

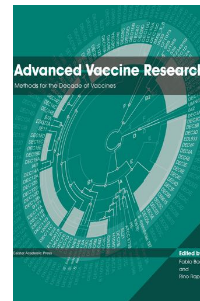

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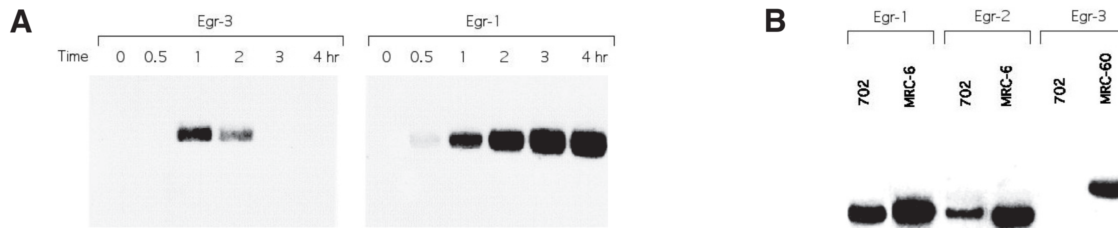


Figure 1. Northern blot analysis of discordant patterns of mRNA expression by related *Egr* genes. A. Kinetics of *Egr-1* and *Egr-3* expression following serum stimulation. Left panel: *Egr-3* expression and right panel: *Egr-1* expression. MRC-9 cells were synchronized in G0 by serum deprivation, and total RNAs were extracted at 0, 0.5, 1, 2, 3, and 4 hr following 20% serum stimulation. Ten $\mu$g of total RNA was electrophoresed and hybridized with 32P-labeled *Egr-3* cDNA probe. B. Comparison of *Egr-1*, *Egr-2* and *Egr-3* expression by human HTLV-1 transformed. T-lymphocyte cell line 702 and fetal lung cell line MRC-9. Cells were harvested at 60 min following serum stimulation of quiescent cells. Ten $\mu$g of total RNA was electrophoresed per lane and hybridized with the respective 32P-labeled cDNA probes.

homologous to the *Egr-1* zinc finger domains as well as a significant degree (~35%) of similarity in the N-terminus to the corresponding region of *Egr-1* and *Egr-2*. Analysis of an *Egr-3* genomic clone identified a gene structure similar to *Egr-1* and *Egr-2* with one intron approximately 1.3 kb in size located between two exonic regions beginning at nt 435 of the known *Egr-3* cDNA sequence. The *Egr-3* genomic locus mapped to human chromosome 8p21-23. Although *Egr-3* RNA expression in response to serum- and phytohemagglutinin-induction resembled the activation pattern of *Egr-1* and *Egr-2*, several features of *Egr-3* regulation apparently are in contrast to these other *Egr* family members. *Egr-3* expression was not identified in any adult rat tissues despite high levels of *Egr-1* expression in rat brain, lung, and heart. In addition, although *Egr-1* is highly expressed in rat PCJ 2 cells following nerve growth factor stimulation, *Egr-3* is not activated under similar conditions.

In this report, we provide the results of cloning and characterization of the human *Egr-3* promoter as the first step in identifying mechanisms responsible for *Egr-3* regulation. Our findings suggest significant differences in *Egr-3* regulation that may be mediated at the transcriptional level.

Table 1. Potential regulatory elements present in the promoter region of *Egr-3*.

| REGULATORY MOTIF | | SEQUENCE | LOCATION |
|---|---|---|---|
| TATA | | TAAATA | -25 to -30 |
| CCAAT | | | -345 to -349 |
| | | | -623 to -627 |
| SP-1 | | GGGCGG | -665 to -670 |
| Serum Response Element | Consensus | GATSYCCWWATWWGGRSATC | |
| | | TCTGTCCATATATGGCAGC | -60 to -79 |
| cAMP Response Element | Consensus | TGACGTCA | |
| | | TGACGTCG | -101 to -108 |
| EGR-1 Canonical Binding Site | Consensus | GCGGGGGCG | |
| | | GCAGGGGCG | -486 to -494 |
| | | GCAGGGGCG | -666 to -674 |
| ETS-Binding Site | Consensus | SMGGAWGY | |
| | | AGGGAAGC | -549 to -556 |
| | | GTGGAAGC | -1047 to -1054 |
| | | TAGGAAGC | +26 to +33 |
| E-Box | Consensus | CANNTG | |
| | | CACTTG | -382 to -387 |
| | | CAGATG | -426 to -431 |
| | | CAACTG | -747 to -752 |
| | | CATATG | -776 to -781 |
| | | CACGTG | - 844 to -849 |
| PAX-2 Binding Site | | GTTCC | -129 to -133 |
| Rb Control Element | | CCACCC | -367 to -372 |
| Insulin Response Sequence | Consensus | TRTTTTG | |
| | | TGTTTTC | -422 to -428 |
| IGF-2 Site | Consensus | CTTTCTCTTTT | |
| | | CTTTCTCAACT | -405 to -414 |
| | | CTTTCTCTTTA | -534 to -543 |
| GATA Response Element | Consensus | WGATAR | |
| | | AGATAA | -28 to -33 |
| | | GGATAG | -523 to -528 |
| GCF Binding Site | Consensus | SCGSSSC | |
| | | CCGGGCC | -38 to -42 |
| | | GCGCCCC | -95 to -101 |

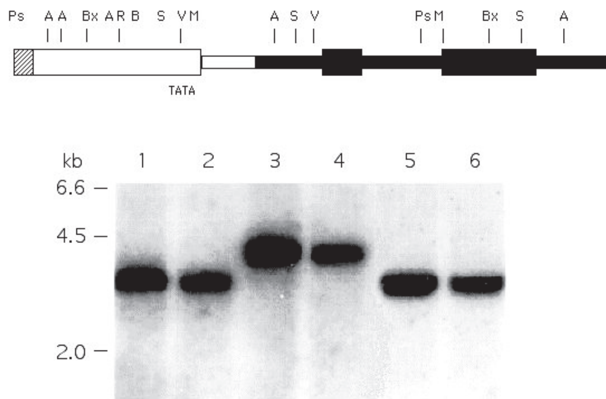


Figure 2. Isolation of *Egr-3* genomic locus. Top: Schematic representation of *Egr-3* gene with 5' and 3' flanking regions including the 3 kb *Pst* I fragment subcloned from a human placenta library. Restriction endonuclease sites identified are *Pst* I (Ps), *Ava* II (A), *BstX* I (Bx), *EcoR* I (R), *BamH* I (B), *Sph* I (S), *Pvu* II (V), and *Bsm* I (M) Filled boxes represent *Egr-3* intron (small diameter) and exonic (large diameter) regions described by Patwardhan et al. Open boxes represent putative 5' cDNA terminus (small diameter) and 5' flanking regulatory region (large diameter). A cross-hatched region upstream of the promoter was not fully sequenced. Bottom: Southern blot analysis of genomic DNA from human breast cancer cell line MCF-7 (lanes 1, 3, 5) and a human placenta genomic clone (lanes 2, 4, 6).

## Results

### Discordant Expression Patterns by Related Egr Genes

A rapid and coordinate up-regulation of primary response genes *Egr-1* and *Egr-3* was observed following serum stimulation of quiescent MRC-9 fetal lung fibroblast cells, synchronized in G0 by serum deprivation. *Egr-3* gene expression was detectable after 30 minutes following serum stimulation and peaked at approximately 1 hr post-serum exposure. In fact, the induction was so transient that *Egr-3* mRNA level was undetectable by 3 hr. (Figure 1A, left panel). Activation of *Egr-1* was also time-dependent but significantly stronger and of longer duration. The *Egr-3* induction following serum induction indicated that a common regulatory motif, specifically one or multiple serum response elements, might be present in the *Egr-3* promoter region.

In contrast to the co-regulated induction of *Egr-1*, *Egr-2,* and *Egr-3* in serum-activated cells, high levels of *Egr-1* were expressed in human fetal and adult organs such as lung, mammary gland but fetal brain and liver as well as placenta had very low or undetectable levels. Under similar assay conditions, *Egr-3* expression was present in all fetal and adult primary tissues (data not shown). In addition to this discrepancy in tissue patterns of expression, alternative regulation of these genes was evident in T lymphocytes transformed by the human retrovirus HTLV-1. The HTLV-I transformed cell line 702 constitutively expresses high levels of *Egr-1* and *Egr-2* but *Egr-3* expression is undetectable (Figure 1B). Since HTLV-I activation of the *Egr-1* and *Egr-2* promoter has been attributed to the HTLV-I Tax protein, corresponding promoter motifs regulated by Tax may be inaccessible or not present in the *Egr-3* promoter.

### Molecular Cloning of the 5' Promoter Region of Egr-3

The *Egr-3* genomic locus was subcloned from a human placenta genomic library by screening 1 x 106 recombinant phage clones under high stringency conditions with two PCR-generated probes from the *Egr-3* 5' coding region. One clone was studied that contained a 12 kb *Not* I fragment spanning the entire length of the *Egr-3* gene including 5' and 3' flanking sequences. Following analysis by restriction endonuclease digestion and Southern blotting, an approximately 3.0 kb *Pst* I fragment was identified that encompassed about 1.5 kb of the 5' regulatory region of *Egr-3*. This fragment was subcloned into Bluescript SK (-) plasmid and M13 vectors and over 1.2 kb of the upstream region was sequenced on both strands. Southern blot analysis of human genomic DNA revealed restriction fragments identical to those in the clone, suggesting that gene rearrangement did not occur during subcloning (Figure 2).

### Structural Features of the 5' Flanking Region

The sequence of 1368 bp of a previously unreported genomic segment flanking the *Egr-3* gene is shown in Figure 3.

The nucleotide composition of the region was GC-rich (61.3% GC in composition) and contained expected numbers of CpG and TpG dinucleotides. A 200 bp segment at the downstream end was exceptionally GC rich (75% GC). Analysis of the region with the Xgrail computer program identified this segment of the promoter as part of a CpG island that extended into the coding region of the *Egr-3* gene (Figure 4).

The published *Egr-3* cDNA sequence includes 282 bp of 5'-untranslated region (Patwardhan *et al.,* 1991). Sequence analysis of the 5'-flanking region identified a canonical TATA box approximately 200 bp upstream from the published 5' end. We performed anchored PCR cloning of cDNAs from the human breast carcinoma cell line MCF-7 as part of a more comprehensive approach to identify the *Egr-3* transcription start site. More than 20% of these 5' RACE' clones began 25 bp downstream from the TATA box indicating that it serves as a major site of TATA directed transcription initiation (data not shown). The potential regulatory elements present in the promoter region are listed in Table 1.

The conserved palindromic CRE (cyclic AMP response element) sequence has been identified in all other *Egr* promoter regions consistent with the demonstrated regulation of these genes by the cyclic AMP signal transduction pathway (Irving *et al.,* 1989). Recently, it was shown that the CRE of the *Egr-1* promoter is essential for the transcriptional response to the cytokines *GM-CSF* and IL3 (Sakamoto *et al.,* 1994). The CRE site may be a focal regulatory point for integration of additional signals as well. The CRE of the *Egr-1* promoter is involved in the regulation of this gene by *TAX-1* and *TAX-2*, the transactivating proteins of human retroviruses HTLV-1 and HTLV-2 respectively (Sakamoto *et al.,* 1992).

The similar kinetics of *Egr-1*, *Egr-2*, and *Egr-3* expression in response to serum activation suggested that either one or multiple serum response elements (SRE) or CArG boxes, the core of an SRE, were likely to be present in the *Egr-3* promoter since these motifs mediate serum

```
-1368  TGCCTCGCAGCAGGACCCTTAAGGAGCACAGCAGCCGNCCCCGGGATCTCTGCCGCATCC  -1309
-1308  TGGAGGTGAAAGTGCTCCGGGTCGGCACACAGGCCGCCCTTGACTGCGGAGCGCCGGCAG  -1249
-1248  GCAGCAATGGGGACAGCCCTGTGGAAGGGGAGGGGGAGGGNAGAGACTAGGGGAGGNCCC  -1189
-1188  CAGTGGCTCAGAGACTGTGGGTTCGGCGTCCTCCTTCTGGAACTCTGGCGGGCCCTTCGC  -1129
-1128  AGGCTGCAGTAGACAGCGACTGCGCCCGGGCCTGCTGGGCCCTCCTGCCATCCTGTGGCC  -1069
-1068  ACAGAGGCTGCTTGGAACCGTTAGGGAATTTACTGGGGATTCAGTGCACGTGTTTATCTA  -1009
-1008  TCCCTTCAACTTCATTCTTTCCTCTTCACCACGAAAACCCGCACATCCTCGACCCCATAT  -949
 -948  GGATGGGGCTCTTGTCTTTGGGGCAACTGCTGGGGTGCTCCGAATCTCCTCGCCACTGCG  -889
 -888  CTCAGCCTAGAGTCAGCGCCGGGTCAGGGGCCCAGCGAGGGGCAGGGGCGGCGAATCAGA  -829
 -828  GTGAAGTTGGCACAAAAGTTCTTGGGATATTGGACCAAGGAATTCTGACAAGTTGAAACA  -769
 -768  CCCCCCCAACCCTGCGCGCACCCCTCCTTCCCCCCACAAAGGGAAGCACTCCCTTTCTCT  -709
 -708  TAATCTCGGATAGGATCCCGAACGCTGGAGCCGCTGAGGTAGCAGGGGCGCAACGCACGC  -649
 -648  CTTCCGGGTTCCCCAGCAGTTCCCGGGCTGCAAAGGGATTTCGCCAGATGTTTTCAACTC  -589
 -588  TCTTTCTAACTGCTGGGGTGTTGGAGACCACTTGCAAACAACCCCACCCCACCGCCCACC  -529
 -528  TCTCCCCAATGCGCTCTCCTAACGCAAACCTCAAATAAACACACACAATGAGTTACTGGT  -469
 -468  GTTTCGACTCCACGCTACGCTTTGCGCTTTGCATGCAGCTGTATCCTTCTTTCCTCTCTC  -409
 -408  TTCTCGCATTCTTCTTGTTTCTTCTCCCCTTCTCTTTTTTTCAAGAGCGCGAGCGTGCGN  -349
 -348  GCNCCCGACGTTGGCTGCGGCCCGCTTCCTGCTTTCTAATGTTCCATTGTGAGGAGCTTC  -289
 -288  CATTGTGACGTCGCGCCCCTTCGGCTGGGCTTTGTCTGTCCATATATGGGCAGCTACGTC  -229
 -228  ACGGAGCTTTCCCGGGGCTCAGATAAATAGGCTGGTGGAGTTCCCTGGCTGGGAGCTTTT  -169
 -168  TGGCAGCAGTGAGCTTGCTAGGAAGCGGCGGGGCTGGTGGTGGTGGTAGCAGCGGCGGCA  -109
 -108  TCGGCGGCAGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGTGGCGGTGGCGGTGGCG  -49
  -48  TTGGCAGATCGGGGGGCGGGGGGGCGGCGGGCGCGTGTCTGTTTGTGA  -1
```

Figure 3. Sequence of *Egr-3* 5' flanking region. Nucleotides are numbered from the previously published 5' end (nt-1) (19). CAAT and TATA sites are boxed. CArG box is underlined. Filled arrowhead indicates the major transcription start site. For a detailed listing of the putative regulatory motifs, please refer to Table 1. The sequence was assigned the genebank accession number AY026865

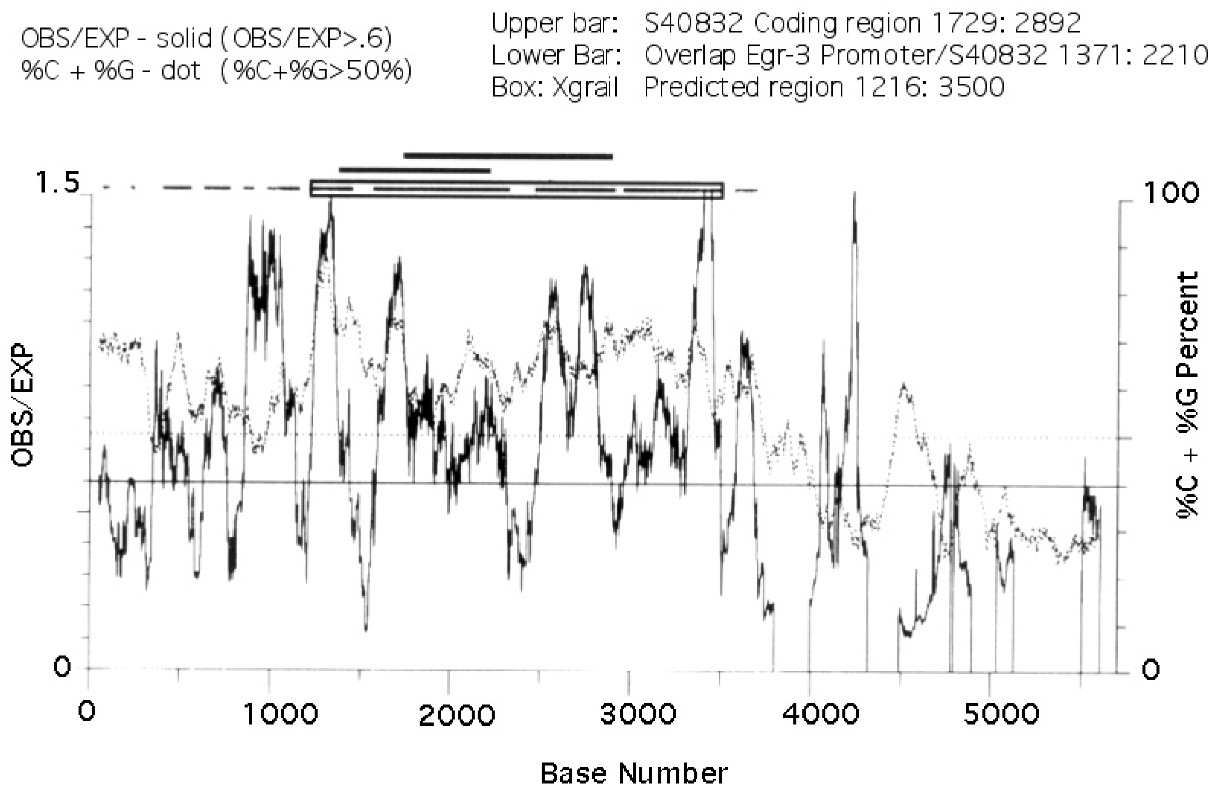


Figure 4. Sequence of *Egr-3* 5' flanking region. The lower bar represents overlap *Egr-3* promoter/S40832 1371:2210 and the upper bar represents S40832 Coding region 1729:2892.

```
GCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGTGGCGGTGGCGGTGGCGGTGGCA
   GCGGCGGCG
      GCGGCGGCG
         GCGGCGGCG
            GCGGCGGCG
               GCGGCGGCG
                  GCGGCGGCG
                     GCGGCGGCG
                        GCGGCGGTG
                           GCGGTGGCG
                              GTGGCGGTG
                                 GCGGTGGCG
                                    GTGGCGGTG
                                       GCGGTGGCG
                                          GTGGCGGTG
                                             GCGGTGGCA
```

Figure 5. Unusual overlapping tandem array of GC-rich nonameric segments in the 5' untranslated region of *Egr-3* downstream of the TATA box. The nucleotides that are different from those of the consensus *Egr* binding site are underlined.

A

B

Figure 6. Transient expression analysis of *Egr-3* 5' flanking region. Three constructs were made by subcloning into the pLG-2 reporter the promoter region of *Egr-3* from the same position from 3' end (nt+33) to &emdash;345, -545, and &emdash;965, respectively. A. Promoter activity of the *Egr-3* 5' flanking region in different cell lines. Hatched bars represent the 998 bp 5' flanking fragment of *Egr-3* in the report vector in sense orientation, while the dotted bars represent the antisense construct and clear bars show the basic activity of control pGL-2 vector. The luciferase measurements were scaled up by 100-fold for Saos-2 and 10-fold for MCF10A over their actual readings. Error bars represent standard deviations calculated from a duplicate set of experiments. B. Identification of the serum responsive region of the *Egr-3* promoter. Clear bars show the 0 time point value while black bars show the activity that tested at 7 hr point after serum stimulation.

induction of the *Egr-1* and *Egr-2* genes (Christy and Nathans, 1989; Rangnekar *et al.,* 1990). In support of this hypothesis, one serum response element was identified that was present in the *Egr-3* proximal promoter region located within 35 bp 5' of the TATA box. The core consensus sequence (GGAAG) of an *ETS* motif, the recognition site for the *ETS* family of transcription factors, is frequently located in the 5' flanking arm of SRE in the promoter region of primary response genes such as c-fos, *Egr-1* and *Egr-2* (Treisman, 1990). Formation of a ternary binding complex at the SRE by serum response factor (SRF) and *ETS* proteins is essential for transcriptional activation through the SRE by serum growth factors. An *ETS*-binding site was not identified adjacent to the *Egr-3* SRE, suggesting an alternative mechanism of SRF-mediated induction at this response element. The *Egr-3* promoter contained two *ETS* motifs and a third *ETS*-binding site was present approximately 50 bp 3' of the TATA box. None of these segments, however, matched the CGGAAG sequence characteristic of the subset of high affinity *ETS* sites involved in SRE-*ETS* protein interactions.

Five F box elements (CANNTG) were scattered throughout the *Egr-3* promoter region. These motifs are potential binding sites for helix-loop-helix transcription factors with leucine zippers (bHLH-LZ) that regulate growth and differentiation, such as c-*myc* and myoD, the later being considered a "master" regulator of myogenic dif ferentiation (Desprez *et al.,* 1995; Kim *et al.,* 1995). The E box sequence CACGTG, located at nt-844 is a high-affinity binding site for *myc*, max, and several other bHLH-LZ transcription factors (Miltenberger *et al.,* 1995).

In addition to these cis-acting sequences, there are two other putative binding sites in the *Egr-3* promoter region for various cellular proteins associated with malignant transformation. One such protein includes the retinoblastoma (Rb) tumor suppressor protein that recognizes the CCACCC sequence located in the flanking region. Of interest, this sequence is also a frequent erythroid gene promoter motif (Pan and McEver, 1993). Moreover, two variant *IGF-2* motifs, which serves as response elements for several interferon-induced regulatory factors including*IRF-1* were also present (Tanaka *et al.,* 1993).

*Egr*-binding sites, including the nonameric consensus sequence (GCGG/TGGGCG), the TC-rich tract (TCCTCCTCCTCCTCTCC) or the iterative PCR-selected site (TGCGTG/AGGCGGT) were not present in the *Egr-3* promoter region. However, two identical one nucleotide variants of the *Egr* nonameric consensus sequence (GCAGGGGCG) were located in this regulatory region at nt-486 and nt-666. These variant motifs are altered at the same nucleotide as an *Egr*-binding segment in the human neurofilament light promoter and may therefore bind *Egr* (Pospelov *et al.,* 1994). A *SP-1* site overlaps the variant *Egr*-binding segment in the distal *Egr-3* promoter. This same structural relationship has been noted in the promoter region of several other genes (Patwardhan *et al.,* 1991; Ackerman *et al.,* 1991; Harrington *et al.,* 1993; Shingu and Bornstein, 1994) and is believed to have important consequences for transcriptional regulation by these *Egr* and *SP-1* binding sites.

An unusual structural feature of the *Egr-3* flanking

region, not present in any of the other *Egr* genomic loci, was an overlapping tandem array of GC-rich nonameric segments located 106 to 159 bp downstream of the TATA box. In a region that spans 45 bp, a total of 16 overlapping nonameric segments can be defined that include eight identical nonamers and eleven nonameric units that vary from the *Egr* consensus sequence at an identical single nucleotide (Figure 5). The remaining five nonamers in the region vary from the canonical *Egr* binding site by two or three base pairs.

Another repetitive DNA segment was noted in the *Egr-3* genomic clone isolated during this study. A tetranucleotide tandem repeat consisting of eleven ATGG sequences was present in the 5' region of the intron. In addition to representing a microsatellite repeat sequence that potentially may be useful as a marker of genetic heterogeneity, the tetranucleotide repeat unit is a binding site for transcription factor YY1 (Shi *et al.,* 1991) which regulates several immediate early genes such as c-fos and c-*myc*. Therefore, this intronic repeat sequence may identify a region involved in transcriptional regulation of *Egr-3* expression.

**Transient Expression Analysis of the 5' Flanking Region of Egr-3**

We wished to determine whether *Egr-3* genomic flanking sequences could act as a functional promoter and whether this region was serum-inducible. To test for this activity in transient cell transfection assays, heterologous constructs were generated using a promoterless plasmid with a luciferase reporter gene by inserting *Egr-3* flanking segments beginning at nt+33bp downstream of TATA in the sense and antisense orientation. Luciferase expression by reporter constructs with 998 bp of *Egr-3* promoter sequence in either orientation, was measured following transfection of three cell lines, lung cancer cell line H-23, bladder carcinoma cell line SAOS-2, and an immortalized but non-malignant mammary epithelial cell line, MCF10A (Figure 6A). The *Egr-3* promoter sense constructs exhibited varying levels of total luciferase activity that differed by approximately 10- to 100-fold with the highest levels of luciferase expression in the H-23 cell line. These variances were independent of transfection efficiency indicating significant differences in basal *Egr-3* activation in the individual cell lines. In all cases, however, activation of the sense construct was substantially higher (15- to 100-fold) than following transfection with antisense constructs or background levels with the pGL-2 Basic vector alone. The orientation-dependent increase in luciferase gene activation indicated that sequences supporting promoter activity were present in these regions of *Egr-3* flanking sequence.

Serum responsiveness of the *Egr-3* promoter was tested next using reporter constructs with serially deleted *Egr-3* promoter fragments spanning 378, 568, and 998 bp. The MRC-9 cell line was used since our previous studies showed *Egr-3* induction in these cells by serum (Figure 1A). Luciferase expression by serum-deprived MRC-9 cells (0 time) transfected with each of the three promoter fragments was uniformly low and equivalent to background expression levels in pGL-2 Basic transfectants. Following serum activation, a 50- to 100-fold increase in promoter activity above 0 time was measured in multiple assays confirming that the *Egr-3* promoter was indeed serum-inducible. However, there was not a significant difference in normalized luciferase expression levels between the three constructs with serially truncated fragments, i.e., the extent of activation was not correlated to the length of the promoter region. Luciferase measurement in serum-activated cells transfected with the 378 was equivalent to the 568 and the 998 bp construct (Figure 6B). These results, reproducible in multiple assays, also were independent of transfection efficiency and thus suggested that the elements that are responsible for the induced *Egr-3* expression is located within the first 378 bp region of the *Egr-3* promoter.

**Discussion**

*Egr-3* is an immediate early gene product with transcriptional regulatory activity that is expressed during extracellular ligand-activated transition from G0 to G1 stages of the cell cycle, and presumably plays a critical intermediary role in regulating the cascade of genetic events leading ultimately to cell division. Activation of *Egr-3* and other *Egr* genes occurs in response to a number of common mitogenic stimuli. But like many structurally related families of transcription factors, there is evidence that individual family members are differentially expressed in similar biological processes, suggesting alternative regulatory influences that may be controlled at the level of transcription (Morgenbesser *et al.,* 1995). Thus, activation of *Egr-3* by nerve growth factor or basal *Egr-3* expression in rat primary tissues is undetectable despite induction of *Egr-1* under similar conditions (Patwardhan *et al.,* 1991). We also have identified differential expression of *Egr-1* and *Egr-3* at the RNA level in several human fetal primary tissues. In addition, we have shown that *Egr-3* is not constitutively expressed by a human HTLV-1 transformed cell line in contrast to *Egr-1* and *Egr-2* that are upregulated in these cells.

The cloning and transfection analysis of *Egr-3* upstream flanking sequences has identified a number of response motifs that may regulate *Egr-3* transcription and govern the differential co-expression of *Egr-3* and other *Egr* family members. TATA and CCAAT motifs that normally promote accurate initiation of transcription were evident in the *Egr-3* promoter. Preliminary studies indicate that a major transcriptional initiation site exists approximately 25 bp downstream of the TATA box. The *Egr-3* promoter contains several regulatory motifs in common with previously characterized flanking segments of *Egr-1*, *Egr-2* and *NGFI-C*, the rat homologue of the fourth *Egr* family member (Crosby *et al.,* 1991) including binding sites for cyclic AMP responsive element binding protein (CREB) and transcription factors *Sp-1* and *NF-k B*. One serum response element (SRE) was identified in the *Egr-3* promoter. In contrast, five SRE are located in the *Egr-1* promoter (Sakamoto *et al.,* 1991) while 2 CArG elements, the core sequence of an SRE, are identified in the *Egr-2* upstream regulatory region (Rangnekar *et al.,* 1990). This may partially explain why the *Egr-3* induction level was lower than that of *Egr-1*. The promoter region of *NGFI-C* contains no SRE or CArG elements, and activation of this gene by serum is markedly reduced compared to other *Egr* genes. An *ETS* motif was not located adjacent to the *Egr-3* SRE

as described for SRE in c-fos, *Egr-1* and *Egr-2*. *Egr-3* thus falls into a class of primary response genes, including cyrGi, SRF, and Xenopus laevis type 5 actin (Williams and Lau, 1993) with promoters containing CArG boxes without associated *ETS* motifs. Regulation of these genes may involve recruiting a more distal *ETS* motif to the SRE or activation through an entirely different pathway.

The presence of multiple E-box motifs, the binding site for bHLH-LZ proteins may signal a role for *Egr-3* in myogenesis. A total of five E-box core elements were present in the *Egr-3* promoter, while not one E-box is identified in greater than 690bp of *Egr-1* promoter and only one E-box is present in the *Egr-2* promoter. Tissue-specific class B bHLH15-LZ proteins have important regulatory functions in several differentiation pathways including skeletal myogenesis, hematopoiesis, and neurogenesis. The E-box at nt-844 of the *Egr-3* promoter is a common E-box sequence (CACGTG) that in various systems is a high-affinity binding site for bHLH-LZ transcription factors including Myc, max, *USF*, *TFE3*, *CBF-1* and *TFEB*. The prominent representation of E-box binding motifs in the *Egr-3* promoter may indicate that the gene is a target for lineage-specific or stage-restricted regulation by bHLH-LZ transcription factors during differentiation

The sequence CCACCC may serve as a retinoblastoma control element (RCE) for mediating p105Rb (Rb) protein regulation of gene expression (Kim *et al.,* 1991). An RCE is located at nt-367 of the *Egr-3* promoter, the only known human *Egr* gene promoter with this response element. The transcriptional regulatory potential of Rb extends to a number of other primary response genes such as c-fos and c-jun. Rb-mediated regulation may, in some instances, occur through cell-lineage specific interactions with other transcription factors that center around the RCE or other response elements (e.g. *Sp-1*, *ATF2*) and may include positive or repressive effect (Bremner *et al.,* 1995). The CCACC sequence has also been identified in many erythroid-specific gene promoters including ß-globin and the porphobilinogen carboxylase genes and has been implicated in a number of cell-specific signaling pathways presumably by serving as a binding site for several regulatory proteins (Frampton *et al.,* 1990).

*Egr-3* is not constitutively activated in the HTLV-1 transformed cell line 702 in contrast to *Egr-1* and *Egr-2*. Expression of these and other primary response genes by HTLV-transformed cells results from transactivation by the HTLV regulatory gene TAX. Although TAX does not bind directly to DNA, TAX-responsive regulatory domains have been identified in the HTLV long terminal repeat (LTR) and in the promoter regions of TAX target genes suggesting that TAX-mediated stimulation requires other specific cellular factors. The TAX gene product of HTLV-I and HTLV-II transformed cells may activate *Egr-1* expression through different response elements based on work by Sakamoto who identified the *Egr* canonical response element and the cyclic AMP response element.

Transfection of the approximately 1 kb of 5'-flanking region of *Egr-3* linked to a luciferase reporter vector indicated that the essential elements for *Egr-3* promoter activity is present in the region between nt-965 and nt+33, which support high-level gene expression in H-23 but only basal expression in MCF-10A cells. These significant differences in basal *Egr-3* activation in individual cells suggest that the 998 base pair flanking sequence contains sufficient information for promoter-specific expression in cultured cell lines. Moreover, the fact that activation of the sense construct was substantially higher than that observed with transfection of antisense construct indicated that sequences that support promoter activity are also orientation-dependent. Serum stimulation experiments using serially truncated segments of *Egr-3* promoter revealed that the positive regulatory region responsible for serum stimulated activation of *Egr-3* expression exists within the first 378 bp of the 5' flanking sequence. In this region, there is a SRE element that has been shown to mediate the expression of mitogen inducible genes such as *c-fos* (Treisman, 1987), *EGR-1* (Christy *et al.,* 1988) and *Egr-2* (Rangnekar *et al.,* 1990). Mutagenesis of this CArG box in *Egr-3* promoter is needed to determine whether this motif is solely essential for serum reponsiveness.

In conclusion, cloning and analysis of the human *Egr-3* 5'-untranslated region revealed a unique complex regulatory organization. The biological activity of the *Egr-3* promoter has been demonstrated by means of differential transcription activation and serum-induced transactivation. Considering the large number of putative regulatory elements present in this region, it is conceivable that *Egr-3* may play a significant role in transcriptional control of cellular proliferation, differentiation, or other growth processes.

### Experimental Procedures

#### Isolation and Sequence Analysis of the Egr-3 Promoter

Recombinant phage clones containing the *Egr-3* gene were isolated by plaque hybridization from a *Mbo I*-generated human placental genomic DNA library in the Lambda Fix II vector (Stratagene, La Jolla, Calif.). The phage library was screened with an equal mixture of two *Egr-3* probes generated by PCR amplification based on the previously isolated *Egr-3* cDNA and were random prime-labeled with [$\alpha$-$^{32}$P] dCTP (Amersham 3000Ci/mmol). The first probe was a 242 bp fragment of exon 1 extending from nt 253 to 495 and the second probe was 662 bp in length, spanning nt 511 to 1173 of *Egr-3* exon 2. Approximately $10^6$ clones were screened from the library. Filter membranes were hybridized at 420C in Rapid Hyb Buffer (Amersham) and washed four times at 65°C for 15 minutes. A 3 kb genomic phage isolate positive on quaternary screening was subcloned into plasmid vector Bluescript SK (-) (Stratagene). DNA restriction fragments from this clone were analyzed by restriction endonuclease mapping and Southern blotting using standard techniques.

Dideoxy sequencing of double-stranded templates with vector- and sequence-specific oligonucleotide primers was performed using Sequenase II (U.S. Biochemical Corp) or "hot tub" sequencing kit (Amersham) as described by the manufacturers. Computer-based sequence analyses were performed with the Xgrail program (Uberbacher and Mural, 1991; Guigo et al., 1992).

#### Construction of Luciferase Reporter Vectors

Segments of the *Egr-3* promoter region were amplified by PCR using sequence-specific oligonucleotide primers designed with a terminal *Bgl* II enzyme site. The 3' end of all *Egr-3* promoter fragments began at nt +33. *Egr-3* promoter fragments of 378 (nt -345), 568 (nt -535) and 998 (nt -965) base pairs in length were subcloned in the sense and antisense orientations into the PGL2 Genelight vector (Promega; Madison, WI) which contains the luciferase gene without a promoter. Full sequence analysis of the constructs was performed to verify orientation and fidelity with the genomic template sequence. The reporter plasmids were purified by CsCI density gradient centrifugation.

#### Transfection and Luciferase Assay

Transient transfections of the *Egr-3* promoter constructs were performed at 75%-80% confluence in 6 well plates. The cell lines used in these studies

were MRC-9 human fetal lung cells, lung cancer cell line H-23, bladder carcinoma cell line SAOS-2, and immortalized mammary epithelial cell line MCF10A. The cell culture conditions were modified according to the individual cell lines. In general, equal volumes of test plasmid (2-3 $\mu$g/well) and 15-20 $\mu$l of Lipofectin (Bethesda Research Lab.) diluted in 0.1 ml of RPMI 1640 medium (Bethesda Research Lab) with 0-5% serum were mixed and incubated for 15 minutes at room temperature before addition to cells. The pSV-ß-gal plasmid (1$\mu$g/well) was co-transfected to normalize for transfection efficiency. After overnight incubation at 37°C, the transfection media was replaced by media with 10% serum and incubated for another 24-48 hr before being harvested for luciferase and ß-Gal activity assays. To test for the effects of serum stimulation, medium with 0.25% serum was added after overnight transfection and incubated for an additional 48 hr. Cells were then serum-stimulated by addition of medium containing 20% FCS. At 1-7 hr after serum addition, cells were harvested for luciferase and ß-galactosidase assays. In brief, following lysis with Tropix's Galacto Lysis Buffer and removal of cell debris by centrifugation, 40 $\mu$l of cellular extract was used to determine luciferase and ß-gal activity. Reactions were initiated with injection of 100 $\mu$l of 1 mM luciferin (Promega) and Galacton Substrate (Tropix), respectively, and enzyme activity was then determined using a luminometer (Berthold L8955).

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