Promoter function and structure of the growth factor-inducible immediate early gene *cyr61*

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ABSTRACT

***cyr61* is an immediate early gene that is transcriptionally activated in 3T3 fibroblasts by serum, platelet-derived growth factor, fibroblast growth factor, and the tumor promoter TPA with kinetics similar to the induction of *c-fos*. *cyr61* encodes a secreted protein that is associated with the cell surface and the extracellular matrix, and may play a role in cell-cell communication. We report here the complete nucleotide sequence of the mouse *cyr61* gene, which contains four short introns. The transcription start site was mapped by S1 nuclease and primer extension analyses. A 2 kb 5' flanking DNA fragment functions as a serum-inducible promoter. This DNA fragment contains a poly(CA) sequence that can adopt the Z DNA form. In addition, it contains a sequence that resembles the serum response element (SRE) originally identified in the *c-fos* promoter. We show that deletion of the *cyr61* SRE-like sequence abrogates serum inducibility. Furthermore, this SRE-like sequence is sufficient to confer serum and growth factor inducibility when linked to a basal promoter, and binds the 67 kD serum response factor *in vitro*. We conclude that the *cyr61* SRE functions as a serum response element and may account for the coordinate activation of *cyr61* and *c-fos*.**

INTRODUCTION

The induction of cell growth by serum growth factors is accompanied by the rapid activation of a genetic program (reviewed in 1). The first set of genes expressed following growth factor stimulation, known as immediate early genes, are transcriptionally activated without requiring *de novo* protein synthesis (2–5). Many immediate early genes encode regulatory molecules, including transcription factors and cytokines, that are thought to regulate the subsequent genomic and cellular responses to growth factors (reviewed in 1,6–9). How serum growth factors initiate this genetic program for growth is thus an important problem to solve in understanding growth control.

Among the immediate early genes, the *c-fos* protooncogene has been studied most extensively and serves as a paradigm for growth factor-activated gene expression. Work from a number of laboratories has established that a *cis* acting sequence element in the *c-fos* promoter called the serum response element (SRE) or dyad symmetry element (DSE), is required for the transient transcriptional activation by serum growth factors (reviewed in 10,11). The *c-fos* SRE is a 22 base pair sequence of imperfect dyad symmetry. The SRE is comprised of an inner core known as the CArG box, characterized by the sequence CC(A/T)₆GG, that is also found in the serum response elements of other immediate early genes including *zif268/krox24/NGFI-A/egr-1* (12–15), *krox20/Egr-2* (16,17), and β -actin (18). The *c-fos* SRE binds to a complex of nuclear proteins, one of which is a 67 kD polypeptide known as serum response factor (SRF, 19). Mutational and biochemical studies indicate that binding of SRF to SRE is required for transcriptional activation of the *c-fos* promoter (10,11). For *c-fos*, the SRE not only mediates transcriptional activation by serum growth factors, it is also required for the subsequent transcriptional repression (20–24).

cyr61 is an immediate early gene identified in BALB/c 3T3 fibroblasts that is rapidly induced by serum, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and elevated levels of cAMP (2,3,25). The *cyr61* mRNA is rapidly induced in regenerating mouse liver following partial hepatectomy, suggesting that its encoded protein may play a role during the G₀/G₁ transition in the living animal (26). Its chicken homolog, *CEF-10*, is inducible by the viral oncogene *v-src* (27). *cyr61* encodes a 41 kD secreted protein with heparin binding properties that is associated with the cell surface and the extracellular matrix, and may play a role in cell-cell communication (28).

The induction of *cyr61* in cultured fibroblasts by serum growth factors occurs with kinetics similar to the induction of *c-fos*. However, unlike *c-fos*, which becomes transcriptionally repressed within one hour of growth factor stimulation (29), *cyr61* continues to be transcribed through mid-G₁ (25). To understand how *cyr61* is regulated and how immediate early genes are controlled by growth factors more generally, we have isolated and sequenced the *cyr61* gene, and characterized its promoter. We show that

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q-2062 GAATTCCAACGAATTTCTGCTCTGGGAACAGAAAGTTAAGTCTATATTTAAACAACATTGGA
 -2001 AGCTGTCAAGAATGCCTTGTGGTTGGATAACAGAGGGAGAAAAATGTTAAAAATCGCAGGCTACTGCT **ATACCCAAATATGGAAATAT** TGT **TACGTCT** GGTGTCTGAT
 -1893 TCACCATCTGGAAACTACTTACAACCATGAAGTCAAAATAGAAAAGACGCCACAACAGAGCCAGGAACCTCCTCTCCATTCTGTTTCTCCAGCGCCTCCTTCTACCCGTGTC
 -1781 CTTGCCCTTCTCCACCCTCACCCCTACCCCTCCCGAGCTTCCCTAAGATGGAACAACTGAAGTGTGCGCAGGAGGAACCTGTTCTCTAATAAATCGGCCAGCCGAGGGGACCAG
 -1669 GCCTAGGCTTTGCTTTTCATT **ATTTCAT** AACACTTGACAACCCATTGTCTTTAAAGTCCCAACCCAGAAAGCCTTGGAGAACCTACCGCCTCTAGCAAATTCCTTCC
 -1559 AGGACTTACGAAAGGGCGCTTTTGAATGAATGAACCTTAACTCTTCCAGTAACAGCTGTCTTCACTGGAGTGTGCGCAACAGCCTCTCCTTCTCACTGGAGTCATTATAGA
 -1447 ACAGGCAACGCAGGAGGCTCTCTTAAGCAAGATCGCAAGCTGCAAAAGCAGAGAGGCCCTAAATATTAATAAAGTGAAGAAAGCTATCCAGTTTATGAATGGCATTATATA
 -1335 CCCCATAAGGGTCTGTGTGGGAGGGGTGTCTTCAGTTGTATGTTTAATAAGATCTTAAACCCAACTTCTCTATTACAGTGTGCGCTTCTAAATCCCTCATGTCTCGGA
 -1223 ACTTGGAGGGAGCCACTGACCTACCCCTTGAAGAAGAGCTTCTAAGTGATCCAAAACAAAGAGTTAAACACATGGACGGGCCAGAG **CACACACACACACACACACAC**
 -1113 **CACACACACACACACACTCACACACAC** GGGGGGGGGGCAACAAATACACACAGCGAGACATAGGGGGCGGTGGGGGAGGGTGTCTGCAAAACCCCCGAGTCTTT
 -1003 TTTCCAGTATCCACAGGCAATAGTCTGTTAGAACTGTGTTTGTCCCGGTAGCTTTTGCACCTGGTGATTTCCAGGACAAGTCACTCCAGGGTGGTCTCTCCGCTTCT
 -891 GCTTTGGCTGCTCAGTTTCCACCGCCGCTCAGGAGAGAGGCCAGGAGGGTTGACCCGGCTCCGGCATCCGTGAGGAAGTG **CACGTCA** GGCTCCGGGAGAACTCT
 -781 GCACTCGTTTCTCTTGGATTTCATGCAAGGAGTGGCTGGAACATAAACTGGGAACCTCCAAAAACAAACAGTACAACATACCAAAAAAGGGGAGGGATGAGGGAA
 -668 GACCTCTGGCTGGAATATGCCAGCGAAGAGGGAAGCTTCCCTCCCCCCCCATTCAATAATGCCACTCCGGGTATTAATTTGCAATACACTTCTCTTGGCTAATAACATC
 -555 ATGCCAAAGCTTTGGACTTGATCCGAGCATGCTCTTTGAAGTCCACAAATAGATCCCTGGCTTAGAAACCAACTCCCCCTCCCCTTTGGACTCTCAGTAGATAACTTTACT
 -442 CTCACCTTGGTTGTAAACAAGCAACAGCTCGCTGCCCTTTCCCGGTAGGCATCACCAACAAACCACTTTTGTTCCTCTGTCTC **CCGCCC** TCCCATGTCTAGGCAAGA
 -332 TCTGAAGGGTTACACCAACACATACACCAGCCCCCCCCCATATACCTCTTCAATCGAAATCATCACCTCGCGCCCCAAGCATC **CCGCCC** TCCCAAGGAAGTT
 -222 GTTATTAAAGTGAAGAAAAACGAACCTTTTTTCTCTCTGATGATCTGAGAAGAGGGGGGAAAGGTGCAACGGAGCCAGGGGAGGTGCTTGGCAGCAGCCCGCCCAACC
 -109 AACATTCCTGAGATGTTTGAAGATTCTAGAACGCCCGCAGACAGC **TACGTCA** CTGCAACACCGCGCGCTAGGCAGGCATTATAAA **GGGCGG** GCTCCCGCGCTGGGGC
 +1 AGACCGTGAGGAGAGCGCCAGAGAAGGCCTGCAATCTCGCGCTCTCGCCAGCACCTCGAGAGAAGGACACCCCGCGCTCGGCCCTCGCCTCACCGCACTCCGGG
 +114 CGCATTGTATCCCGCTGCTCGCGCTTGTGTTCTGTGTCGCGCGCTCGCCCCGTTCTCTCGCGCCACAATGAGCTCCAGCACCTTCAGGACGCTCGGTGTGCGCG
 MetSerSerSerThrPheArgThrLeuAlaValAlaV
 +227 TCACCTTCTCCACTTGACGAGCTG GTGAGTTGAGTTTTTTTTTTTAAACACTGCTTCCCGTCTGCCCTCATCGATTCTTTCCCGGGCCATGATCTTACCGC
 alThrLeuLeuHisLeuThrArgLeuIntron I
 +337 AGGGAAAGTTAGACTAAAAGTTCCGAGGTAGCTGTTGCTTTATGCCAGGGGGCTCTTCTGGGACGCAGCCGCTGGGGTGGTCAGGCTGCCGGGACGGATCGAGACACT
 +449 TCTGGTGGACGGAGCCACAGCCAGACCTAGGCTCAGGGCAGTTTACGTGCTCCCGAGCAATGCAAGCAATCTCACGCGCTTCTCCCTCTTGAG CGCCTCTCCAC
AlaLeuSerTh
 +559 CTGCCCGCGCTGCCACTGCCCTCTGGAGGCACCAAGTGCAGCCCGGGAGTGGGGTGGTCCGGGACGGCTGGCGTGTGTAAGGTCTGCGCTAAACAACTCAACGA
 rCysProAlaAlaCysHisCysProLeuGluAlaProLysCysAlaProGlyValGlyLeuValArgAspGlyCysGlyCysCysLysValCysAlaLysGlnLeuAsnGl
 +670 GGACTGCAGCAAACTCAGCCCTGCGACACACCAAGGGTGAATGCAATTTCCGGCGCCAGCTCCACCGCTCTGAAAGGATCTGCAGAG GTAAGAGGCTTGTGTTT
 uAspCysSerLysThrGlnProCysAspHisThrLysGlyLeuGluCysAsnPheGlyAlaSerSerThrAlaLeuLysGlyIleCysArgAIntron II..
 +780 GGGCCCTTTAAAAAATAATAGTCCCATAGTCCAGAAGTTAGTAATTTTGAGACCATGTATGGTGGACGCTTTGTTGATGGTAGCTTGGCAGAAAGGCACATGATTTC
 API
 +892 GCAGTCTGGAATGCAATTACAGAGTGTCTGGGCCAACGAAAGGCATACGGAAGAGTGATTTC **TGACTAA** CTTATTGTTACAGCTCTGGAGTCTCGGGGGAAGCTGAGG

Figure 1. (A) Physical map of the *cyr6l* gene. Two mouse genomic clones, 5A and 3B, encompass six *Eco*RI (E) fragments (1–6) covering approximately 17 kb. The region that is fully sequenced is enlarged, showing the TATA element and the polyadenylation site of *cyr6l*. Exons are shown as heavy black bars, and introns as open bars. Underneath the genomic clone diagram is a representation of the cDNA, with the coding region as a heavy bar. Translation start and stop codons are shown. The sequence element that confers serum inducibility is denoted as SRE. (B) Complete nucleotide sequence of the *cyr6l* gene. Highlighted sequences include the consensus sequences for the octamer binding site (Oct), Sp1 binding sites, cAMP responsive elements (CRE), AP-1 sites, the CARG element, and the FAP site. The poly(CA) sequence that can adopt the Z DNA form is shown in bold. Numbers at left refer to the first nucleotide of the lines, with the transcription start site as +1. Intron sequences are as shown. The first copy of the 49 bp direct repeat in the 3' non-coding region is in bold, the second copy is underlined. The polyadenylation signal is in lower case letters and underlined.

an SRE-like sequence containing a CARG box is located 2 kb upstream of the transcription initiation site and is sufficient to confer serum inducibility on a reporter gene in a transient transfection assay. Furthermore, the *cyr6l* SRE can compete with the *c-fos* SRE for binding to SRF. These results suggest that the coordinate activation of *c-fos* and *cyr6l* can be explained by signals transduced through the SRE.

MATERIALS AND METHODS

Cell culture and transfections

NIH 3T3 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Gibco). Transfections were carried out with 10 μ g of test plasmid and

10 μ g of the reference plasmid pPGK β -Gal bpA encoding β -galactosidase (30, a gift of P. Soriano) per 100 mm dish by the calcium phosphate precipitation procedure (31). Cells were washed in PBS the following day and incubated in media containing 0.5% serum for 48 hours. To control for transfection efficiency, transfected cell extracts containing equal amounts of β -galactosidase activity (31) resulting from the reference plasmid were used for CAT assays (32). Where indicated cells were stimulated by the addition of serum to 20% or PDGF to 50 ng/ml for 4 hours. BALB/c 3T3 clone A31 cells were grown and treated as described (25).

Isolation of genomic clones and sequence analysis

The *cyr6l* gene was isolated from a lambda charon 4 AJ mouse genomic DNA library, obtained from Dr. F. Blattner's laboratory (Madison, Wisconsin). Approximately 2×10^5 recombinant

phages were screened with ^{32}P -labeled *cyr61* cDNA as probe. Sequence analysis was carried out by the dideoxynucleotide chain termination procedure (33) using Sequenase (United States Biochemicals). DNA was sequenced using nested 5' and 3' deletions generated by Bal31 nuclease or by exonuclease III and S1 nuclease. Where appropriate deletions were not available, synthetic oligonucleotides were used as primers for sequencing.

Primer extension and S1 nuclease analysis

An oligonucleotide complementary to nucleotides (nt) +11 to +30 of the *cyr61* mRNA was 5' end-labeled with ^{32}P and hybridized to 30 μg of total cellular RNA isolated from either quiescent cells or cells stimulated with serum and cycloheximide for 3 h as described (25). Following hybridization and subsequent reverse transcription (31), one-twentieth of the reaction products was analyzed on 8% polyacrylamide gels containing 8 M urea. S1 nuclease protection assays were carried out using a single-stranded, 5' end-labeled DNA fragment derived from an *EcoRI/XhoI* fragment of the genomic sequence which spanned the *cyr61* gene from nucleotide -84 to +67. Equivalent amounts of probe were annealed to 50 μg of total RNA from each sample in 50% formamide, 40 mM PIPES pH 6.4, 400 mM NaCl and 1 mM EDTA at 30°C for 16 h. The samples were digested with S1 nuclease (2.5 U/ μl ; BRL) in 0.56 M NaCl, 0.1 M sodium acetate pH 5.2, 9 mM ZnSO_4 , and 16 ng/ μl single-stranded calf thymus DNA at 30°C for 1 h; the products were analyzed on an 8% polyacrylamide gel with 8 M urea.

Promoter-reporter fusion constructs

An *EcoRI-XhoI* fragment from the *cyr61* promoter region containing nt -2062 to +65 was cloned into the *SalI* site of pCAT-Basic (Promega), placing the promoter region DNA upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene. This construct was used to derive other deletions using restriction sites. Insertion of the *cyr61* CARG box oligonucleotides (see below) into the *HindIII* site in the pCAT-Basic vector of construct [-529] in either one or two copies in the wild-type orientation created CARG [-529] and CARG2 [-529], respectively.

Gel mobility shift assay

A plasmid containing the SRF cDNA (a gift of R. Treisman) cloned downstream of the phage T7 promoter (34) was used as template for *in vitro* transcription using T7 RNA polymerase in the presence of a cap analog (New England Biolabs). The resulting SRF mRNA was translated in a rabbit reticulocyte lysate (Promega) for use in DNA binding reactions. Each binding reaction included 2.5 μl of *in vitro* translation products, 80 $\mu\text{g}/\text{ml}$ of poly(dI-dC), 12 mM HEPES pH 7.9, 60 mM KCl, 1 mM MgCl_2 , 5 mM spermidine, 0.5 mM DTT, 12% of glycerol and 3×10^4 cpm of ^{32}P -labeled oligonucleotides. The *c-fos* SRE probe was prepared by end-labeling and annealing the oligonucleotides 5'-AATTACACAGGATGTCCATATTAGG-ACATCTGCGTC-3' and 5'-AATTGACGCAGATGTCTTAATATGGACATCCTGTGT-3'. The *cyr61* CARG box probe corresponding to nucleotides -1912 to -1933 of the *cyr61* sequence was formed by end-labeling and annealing the oligonucleotides 5'-AGCTTCTTGATACCCAAATATGGAAATATTGG-3' and 5'-TCGACCAATATTTCCATATTTGGG-TATCAAGA-3'. Competitions for binding were carried out using a 50 or 200 fold molar excess of unlabeled competitor DNA.

Binding reactions were incubated for 15 min at room temperature and electrophoresed on 4% polyacrylamide (80:1 acrylamide:bis) gels, which were dried and autoradiographed.

RESULTS

Complete nucleotide sequence of the *cyr61* gene

To isolate the *cyr61* gene, the full-length *cyr61* cDNA (25) was used as a probe to screen a mouse genomic DNA library. Two representative clones that cover 17 kb of genomic sequences were further analyzed (Fig. 1A). After restriction mapping and hybridization analysis, the sequence of a 5 kb DNA fragment that contains the entire coding region and 2 kb of 5' flanking region was determined (Fig. 1B). The mRNA cap site was established by S1 nuclease protection assay and by primer extension analysis (Fig. 2). Both procedures show that transcription initiates predominantly at the adenine residue designated +1, located 10 bp upstream of the 5' end of the cDNA (25).

The *cyr61* gene contains four introns of 294, 344, 121 and 230 bp, respectively. The intron-exon junctions show conservation with consensus splice junctions (35). The exon sequences match base for base the *cyr61* cDNA sequence reported previously (25). An unusual 49 bp direct repeat in the 3'-untranslated region of the cDNA (25) is encoded by the fifth exon; thus it is not an artifact of cDNA cloning. Whether this repeat sequence plays a regulatory role is not known.

The 5' flanking region of *cyr61* contains a sequence that resembles the *c-fos* SRE (-1912 to -1933), raising the possibility that this sequence might function as a serum response element. A number of other potential regulatory elements were

Figure 2. Mapping of the transcription start site. (A) Primer extension reaction using total RNA prepared from either quiescent BALB/c 3T3 cells (lane 1) or from cells serum-stimulated for 3 h in the presence of cycloheximide (lane 2). An oligonucleotide complementary to nt +11 to +30 of the *cyr61* sequence was used as primer. Right four lanes show ^{35}S -labeled sequencing reaction products as size markers. (B) S1 nuclease analysis. The labeled antisense strand (shown in lane 1 as undigested probe) was isolated and hybridized to the following RNA samples before being subjected to S1 nuclease digestion: lane 2, tRNA; lane 3, quiescent cell RNA; lane 4, RNA from cells stimulated with serum for 1 h; lane 5, RNA from cells stimulated with serum for 6 h.

also found, notably three Sp1 sites (36), two cAMP responsive elements (CRE, 37) and one octamer binding motif (38, Fig. 1B). An Sp1 site is also found in the 3' untranslated region, and an AP-1 site is present in the second intron (Fig. 1B). A sequence 3' of the CArG box resembles the 'FAP' element (AP1/CRE like element, 39) which is also found 3' of the SREs of the *c-fos*, *zif268/krox24*, and *krox20/Egr-2* promoters. Starting at -1125 there is a poly(CA) minisatellite sequence that can adopt the Z DNA form *in vitro* (40).

5' flanking region confers serum responsiveness to a reporter gene

To test whether the 5' flanking region of *cyr61* functions as a serum-inducible promoter, we fused various segments of the *cyr61* 5' region to the bacterial chloramphenicol acetyltransferase

(CAT) gene in the vector pCAT-Basic (Fig. 3A). The ability of these constructs to express CAT activity in a serum-dependent manner was tested in a transient transfection assay in NIH 3T3 cells. The construct containing nt -2062 to +65 [construct -2062] confers an 11 fold induction of CAT activity upon serum stimulation. When this fragment was cloned in the reverse orientation [+65/-2062], little CAT activity was detectable in either quiescent or stimulated cells (Fig. 3B). It should be noted that we observed a low basal level of CAT activity in cells transfected with the parental vector (pCAT-Basic) that was serum inducible by about 2 fold (data not shown). Therefore, we do not interpret any induction in the neighborhood of 2 fold in our experiments as being significant.

These findings suggested that the *cyr61* serum-inducible promoter is contained within 2 kb of 5' flanking sequence. To

Figure 3. Serum and PDGF induction of the *cyr61* promoter. (A) Schematic diagram showing various segments of the *cyr61* promoter fused to the CAT gene used in transfection assays. The *cyr61* SRE, which contains the CArG box, is shown as a filled square. Extracts from NIH 3T3 cells transfected with these constructs were normalized for transfection efficiency and analyzed for CAT activity. Q, quiescent cells; S, cells serum stimulated for 4 hours. The percent conversion of chloramphenicol to acetylated forms are calculated from 3–5 independent sets of transfection experiments. (B) A representative experiment described in (A) is shown; CAT activity was assayed by thin layer chromatography. The sample labeled CAT represents a control reaction performed with purified CAT (Sigma). (C) Activation by PDGF. Various promoter-CAT constructs were transfected into NIH 3T3 cells. After transfection cells were brought to quiescence; where indicated cells were then re-stimulated with PDGF for 4 hours. Cells extracts normalized for transfection efficiency were assayed for CAT activity by thin layer chromatography. The % conversion of chloramphenicol to the acetylated forms is shown.

further define the sequence requirements for serum inducibility, we tested a series of constructs that deleted various segments of the *cyr61* 5' flanking region (Fig. 3). Whereas construct -2062 conferred serum inducibility, construct -1763 containing nt -1763 to +65 which lacks the SRE-like sequence did not. An internal deletion of construct -2062, $\Delta BgIII$, which removes a *BgIII* fragment (-1285 to -335) but contains the CArG box was serum inducible by 35-fold (Fig. 3). The high level of serum induction in this mutant suggests the possibility that an inhibiting sequence was removed. Constructs -529 (-529 to +65) and -335 (-335 to +65) showed higher levels of constitutive expression, but were not serum inducible. Taken together, these results indicate that a sequence between -1763 and -2062, most likely the SRE-like sequence or the CArG box, is required for serum inducibility of the *cyr61* promoter.

cyr61 SRE functions as a serum response element

To test whether the *cyr61* SRE-like sequence can confer serum inducible expression, we placed either one or two copies of the *cyr61* SRE (-1912 to -1933), which contains the CArG box, upstream of the -529 promoter (Fig. 3). Either one or two copies of the *cyr61* SRE restored serum inducibility to the -529 promoter (Fig. 3), showing that the *cyr61* SRE is a functional serum response element. Since the SREs of *c-fos* and *zif268* also mediate promoter induction by purified growth factors (10–12), we tested whether the inducibility of *cyr61* by PDGF is mediated by the SRE as well (Fig. 3C). In the transfection assay described above, constructs -2062 and $\Delta BgIII$ were able to confer PDGF inducible CAT activity, whereas constructs -529 and -335 were not. Insertion of the *cyr61* SRE upstream of the -529 construct restored PDGF responsiveness (Fig. 3C). These results indicate that the *cyr61* SRE is not only capable of mediating serum induction, but also PDGF inducibility of the *cyr61* promoter.

Interaction of the *cyr61* promoter with the 67 kD Serum Response Factor

Since serum induction of several immediate early genes requires sequences that interact with the serum response factor SRF (10), we tested whether the *cyr61* SRE also interacts with SRF. First, we tested the ability of oligonucleotides containing the *cyr61* SRE (-1912 to -1933) to compete with the *c-fos* SRE for binding to SRF synthesized *in vitro* in a gel mobility shift assay (Fig. 4A). The *cyr61* SRE competed effectively for SRF binding to the *c-fos* SRE, indicating that it can bind to SRF. Moreover, SRF binds to labeled *cyr61* SRE oligonucleotides directly (Fig. 4B). Similar results were observed when nuclear extracts from either quiescent or serum stimulated BALB c/3T3 cells were used in the gel mobility shift assay instead of *in vitro* translated SRF (data not shown). Thus the *c-fos* SRE and the *cyr61* SRE appear to bind some of the same nuclear protein factors.

To determine if there are other CArG box sequences or SRE-like elements in the *cyr61* gene that might bind SRF, we used various genomic fragments to compete with SRE for SRF binding in the gel mobility shift assay. As expected, genomic DNA containing nt -2062 to +65 competed with labeled SRE oligonucleotides for SRF binding, whereas DNA containing nt -1763 to +65 did not (Fig. 4A). Genomic DNA fragments E3 and E5 (Fig. 1A), which contain DNA further 5' of the *cyr61* SRE and 3' of the coding sequence, respectively, failed to compete with SRE for SRF binding (data not shown). These results indicate that the CArG box at -1920 to -1929 is the only SRF binding site in the *cyr61* gene.

Figure 4. Gel mobility shift assays. (A) 5'-labeled *c-fos* SRE oligonucleotides were incubated with SRF translated *in vitro*. First lane on the left contains the reticulocyte lysate incubated without the SRF mRNA. The gel mobility shift due to SRF is competed with a 50 fold and a 200 fold excess of unlabeled DNA: DSE, *c-fos* SRE oligonucleotide; -2062, *cyr61* promoter fragment containing nt -2062 to +65; -1763, *cyr61* promoter fragment containing nt -1763 to +65; CArG, *cyr61* SRE oligonucleotides containing the CArG box. (B) The *cyr61* SRE oligonucleotides were kinase labeled and incubated with SRF translated *in vitro*. Gel mobility shifts were competed by a 50 fold and a 200 fold excess of unlabeled DNA: CArG, *cyr61* SRE oligonucleotides containing the CArG box; DSE, *c-fos* SRE oligonucleotides.

DISCUSSION

We have presented the complete nucleotide sequence of *cyr61*, a mouse immediate early gene that is transcriptionally activated by serum growth factors. *cyr61* is simply organized, with four introns that total about 1 kb in addition to the 2 kb of exon sequences.

We have shown that a single SRE-like sequence located about 2 kb upstream of the transcription start site functions as a serum response element and appears to mediate transcriptional activation by PDGF. Deletion of sequences containing this SRE results in a promoter unresponsive to serum. Furthermore, we have shown that the *cyr61* SRE can bind SRF *in vitro*, and can efficiently compete with the *c-fos* SRE for binding to nuclear factors. These results suggest that *cyr61* and *c-fos* are transcriptionally activated by a similar mechanism mediated through the SRE. Thus a common mechanism of activation may explain the coordinate activation of *c-fos* and *cyr61* during the G₀/G₁ transition (3).

SRE-like sequences have been shown to be required for serum inducibility of a number of other serum responsive genes. For example, several immediate early genes encoding zinc finger proteins including *zif268* (also known as *Egr-1*, *NGFI-A*, *krox-24*) (12) and *Egr-2* (also known as *krox-20*) (17), as well as β -actin (18) are regulated by serum through SREs. Each of these SREs contains a CArG box and binds SRF. Thus these SREs appear to mediate a general mechanism that activates at least a subset of immediate early genes. However, there are other immediate early genes activated with similar kinetics that do not have CArG box sequences in their promoters, including *c-jun*, *junB*, and *nur77* (41–43). These immediate early genes are likely to be activated via a different mechanism. The location of the *cyr61* SRE is further upstream (2 kb) from the transcription start site than those identified thus far, which are generally within 400 bp of the mRNA cap site. This result suggests that SREs can function far upstream of the transcription start site.

The transient nature of the expression of many immediate early genes is due to both transcriptional repression soon after activation and rapid degradation of the mRNA (11). For *c-fos*, the SRE has been shown to mediate both transcriptional activation and subsequent repression (10,11,21). In contrast to *c-fos*, transcription of *cyr61* is maintained at a moderate level for at least 8 hours after stimulation (25), thus transcription is not efficiently repressed after induction. Therefore, even though transcriptional activation of *cyr61* appears to be mediated through the SRE, the mechanism of repression appears to be different from that of *c-fos*. Experiments designed to dissect the sequence requirements for transcriptional repression of *cyr61* are now underway.

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