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A conserved sequence upstream of the *B29* (*Igβ*, *CD79b*) gene interacts with YY1

Lisa Patrone[1], Sarah E. Henson[1], Randolph Wall[1,2] & Cindy Sue Malone[1,*]
[1]*Department of Microbiology, Immunology, & Molecular Genetics and* [2]*The Molecular Biology Institute, University of California, Los Angeles, CA 90095, U.S.A.; *Author for correspondence (Phone: (310) 825-1244; Fax: (310) 825-8088; E-mail: cmalone@ucla.edu)*

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Abstract

The human, murine, and rat *B29* (*Igβ*, *CD79b*) genes are highly conserved in sequence and organization and exhibit strict B cell-specific expression. In the human and rat genomes, the *B29* gene is located between the skeletal muscle-specific Na-channel αsubunit (*SCN4A*) gene and the pituitary-specific growth hormone (*GH-N*) gene. The human pituitary-specific *GH-N* gene is controlled by a tissue-specific locus control region (LCR) located just upstream of the *B29* promoter that mediates tissue-specific enhancement, histone acetylation, and an open chromatin conformation across the *B29* gene in growth hormone (GH)-expressing pituitary cells. Here we show that *B29* mRNA is not detected in a GH-expressing pituitary cell line and that *GH-N* mRNA is not detected in B cells. This differential expression suggests that the *B29* gene is insulated or otherwise protected from the regulatory influences of the closely proximal GH LCR. We searched available sequences upstream of the human, mouse, and rat *B29* genes and found a highly conserved sequence that fulfills the criteria recently established for non-coding DNA elements potentially involved in gene control. This *B29* conserved sequence (BCS) bound ubiquitously expressed nuclear protein complexes. DNase I protection analysis of the BCS revealed a central 'footprinted' core which was confirmed to bind the multifunctional transcription factor, YY1. However, neither the BCS nor the YY1-binding core motif exhibited silencer or enhancer activity in transient transfections or position-independent insulator activity in enhancer-blocking assays. Thus, the BCS may function as a tissue-specific LCR or position-dependent insulator specifically countering the influences of the 5′ GH LCR and controlling *B29* gene expression.

Abbreviations: BCS – *B29* conserved sequence; BTW – between BCS I & II; EMSA – electrophoretic mobility shift assay; GH – growth hormone; *GH-N* – pituitary-specific growth hormone gene; LCR – locus control region; MAR – matrix attachment region; SkM – skeletal muscle; *SCN4A* – SkM-specific Na-channel αsubunit gene.

Introduction

B29 (Igβ, CD79b) is an essential component of the B cell receptor along with immunoglobulin and mb-1 (Igα, CD79a) and is absolutely required for B cell development. The *B29* gene is one of the earliest genes activated in B cell precursors and its expression continues through terminally differentiated antibody-secreting plasma cells [1, 2]. *B29* gene expression is strictly restricted to B cells with the exception of early stages in thymocyte development prior to T cell lineage commitment [3]. We previously characterized murine and human *B29* promoters to identify motifs and factors controlling B cell specificity and general transcriptional activity [4, 5]. Both *B29* promoters exhibited strict B cell specificity and contained highly conserved sequences with a core of essentially identical critical transcription factor motifs [4–6]. The rat *B29* promoter recently has been reported, and also showed high overall sequence homology with the

same core array of transcription factor motifs as the human and murine *B29* promoters [7].

The chromosomal loci of the *B29* gene in three mammalian species contain several closely linked tissue-specific genes. The close proximity of these genes raises questions regarding the differential regulation of their respective patterns of expression. The *B29* gene is located between the skeletal muscle (SkM)-specific Na-channel αsubunit (*SCN4A*) gene and the pituitary-specific growth hormone (*GH-N*) gene in both human and rat genomes [7, 8]. All three genes in the human and rat loci are organized in the same orientation. The murine *B29* gene is flanked upstream by the cardiac muscle-specific myosin alkali light chain gene and followed downstream by the GH genes (according to the MGI Genetic map at NCBI). The different tissue-specific genes in these conserved genomic loci are remarkably close to each other. The human *B29* gene is 8.2 kb downstream of the SkM-specific *SCN4A* gene and 9.7 kb upstream of the pituitary-specific *GH-N* gene, the first gene in the GH gene cluster composed of 5 genes [9]. The rat *B29* gene is similarly situated between these genes and is separated from them by 3.3 and 9.3 kb, respectively [7]. Moreover, the human *B29* gene is situated between both pituitary- and placental-specific GH locus control regions (LCRs) and the downstream genes they control [9, 10]. In the human genome, the pituitary-specific GH LCR is located only ~1.5 kb upstream from the *B29* gene transcription start site (See Figure 1) [9].

The immediate proximity of the human *B29* gene between the pituitary-specific GH LCR and gene prompted us to search for potential 5′ regulatory regions which might function as LCRs or insulators to block the regulatory influences of the 5′ GH LCR on *B29* gene expression. LCRs and insulators are *cis*-acting DNA control elements. LCRs confer tissue specificity and physiological levels of expression on linked genes by stimulating transcription by acting as tissue-specific enhancers [11]. LCRs also establish open chromatin domains and both LCRs and insulators shield these open chromatin domains from both positive and negative regulatory influences of surrounding chromatin. Searching the available human, murine, and rat *B29* genomic sequences by the cross species comparison method [12] yielded a single highly conserved sequence of 160 bp with high homology (63% overall identity) that was located ~500, ~700, and ~ 700 bp, respectively, upstream of the start of transcription. These unique sequences have been designated as *B29* conserved sequences (BCS). The high degree of sequence relatedness and the specific conserved location of the BCS between the human *B29* gene and the nearby 5′ GH LCR suggests a function in maintaining B cell restricted gene expression. The BCS is shown to contain a YY1-binding motif; however, it failed to demonstrate enhancer, silencer, or position-independent enhancerblocking activities. These findings suggest that the BCS may provide tissue-specific restriction of *B29* gene expression in a position-dependent manner.

Materials and methods

Cell culture

Cell lines were maintained at 37 °C in a humidified environment with 5% CO_2. The Ramos human Burkitt lymphoma B cell line was grown in RPMI 1640 with 4 mM HEPES (Omega Scientific, Tarzara, CA), supplemented with 10% FBS (Omega), 4 mM L-glutamine, 0.05 mM β-mercaptoethanol, nonessential amino acids, sodium pyruvate, 10 μg/ml penicillin, and 10 U/ml streptomycin. The rat GH3 cell line was obtained from ATCC and grown in Ham's F12K (GibcoBRL, Rockville, MD) supplemented with 15% donor Horse Serum (Omega), 2.5% FBS (Omega), 2 mM L-glutamine, 10 μg/ml penicillin, and 10 U/ml streptomycin. The rat Y3-Ag1.2.3 cell line was obtained from ATCC and grown in 1.5 g/l sodium bicarbonate DMEM supplemented with 10% fetal bovine serum (Omega), 2 mM L-glutamine, 10 μg/ml penicillin, and 10 U/ml streptomycin.

RNA preparation and RT-PCR analysis

Total RNA was prepared from cell lines growing in log phase using the RNeasy Miniprep kit (Qiagen, Carlsbad, CA) according to manufacturer's instructions. The primers used for RT-PCR are shown in Table 1. GH primers are specific to both human and rat *GH-N*. GAPDH primers are specific to both human and rat GAPDH. 500 ng total RNA was subjected to the following RT-PCR conditions using the SuperScript One-Step RT-PCR system (Invitrogen, Carlsbad, CA): GH: 50 °C for 30 min, 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 70 °C for 1 min; 72 °C 7 min 30 s. Human B29: 50 °C for 30 min, 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 59 °C for 30 s, 70 °C for 1 min; 72 °C 7 min 30 s. Rat B29: 50 °C for 30 min, 94 °C for 2 min; 35 cycles of 94 °C for

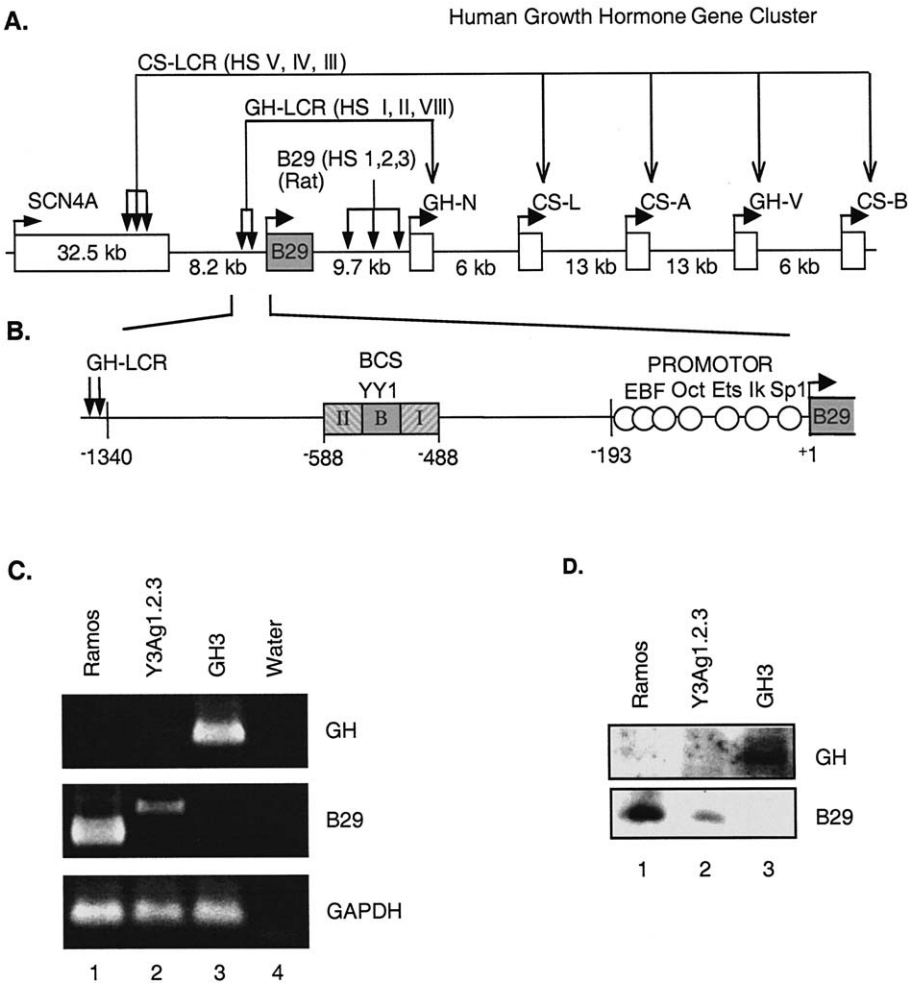

Figure 1. Genomic organization of the human chromosome 17q23 locus and growth hormone expression versus *B29* expression in B cell development and pituitary cells. *a.* The human B29 gene is located between the SCN4A (muscle-specific sodium channel) gene and the growth hormone (GH) cluster composed of the pituitary-specific (GH-N), placenta-specific chorionic somatomammotropin (CS-L, CS-A, CS-B), and placenta-specific growth hormone (GH-V) genes [7]. Two locus control regions, the GH-LCR (composed of HS I and II) and CS-LCR (composed of HSIII, IV, and V), control pituitary and placenta-specific gene expression, respectively [8,10,31]. The rat *B29* gene contains three mapped HS sites (1, 2, and 3) located between the *B29* gene and the *GH-N* gene whose function is not well established [30]. *SCN4A, B29* and *GH-N* are organized in the same orientation in the human and rat loci [7]. *b.* The B29 gene 5′ regulatory region is located between the B29 gene and the GH-LCR. *c.* Growth hormone (*GH-N*) mRNA is not expressed in B cells and *B29* mRNA is not expressed in pituitary cells. Total RNA (500 ng) from the Ramos human B cell line (lane 1), the Y3Ag1.2.3 rat myeloma cell line (lane 2), the GH3 rat pituitary cell line (lane 3) and a negative water control (lane 4) were subjected to RT-PCR analysis using gene-specific primers (Table 1). The *GH-N* product is 290 bp, the human *B29* product is 750 bp, the rat *B29* product is 881 bp, and the *GAPDH* product is 180 bp. For *B29*, the negative water control using rat *B29* primers is shown; the negative water control using human *B29* primers was also negative (data not shown). The human *B29* primers also detected the 450 bp splice variant of *B29* (data not shown). *d.* B29 protein is not expressed in pituitary cells and GH-N protein is not expressed in B cells. Whole cell lysates (10^6 cells/lane) from Ramos human B cell line (lane 1), Y3Ag1.2.3 rat myeloma cell line (lane 2), and GH3 rat pituitary cell line (lane 3) were subjected to Western analysis using anti-human B29 antisera and anti-rat GH antibody.

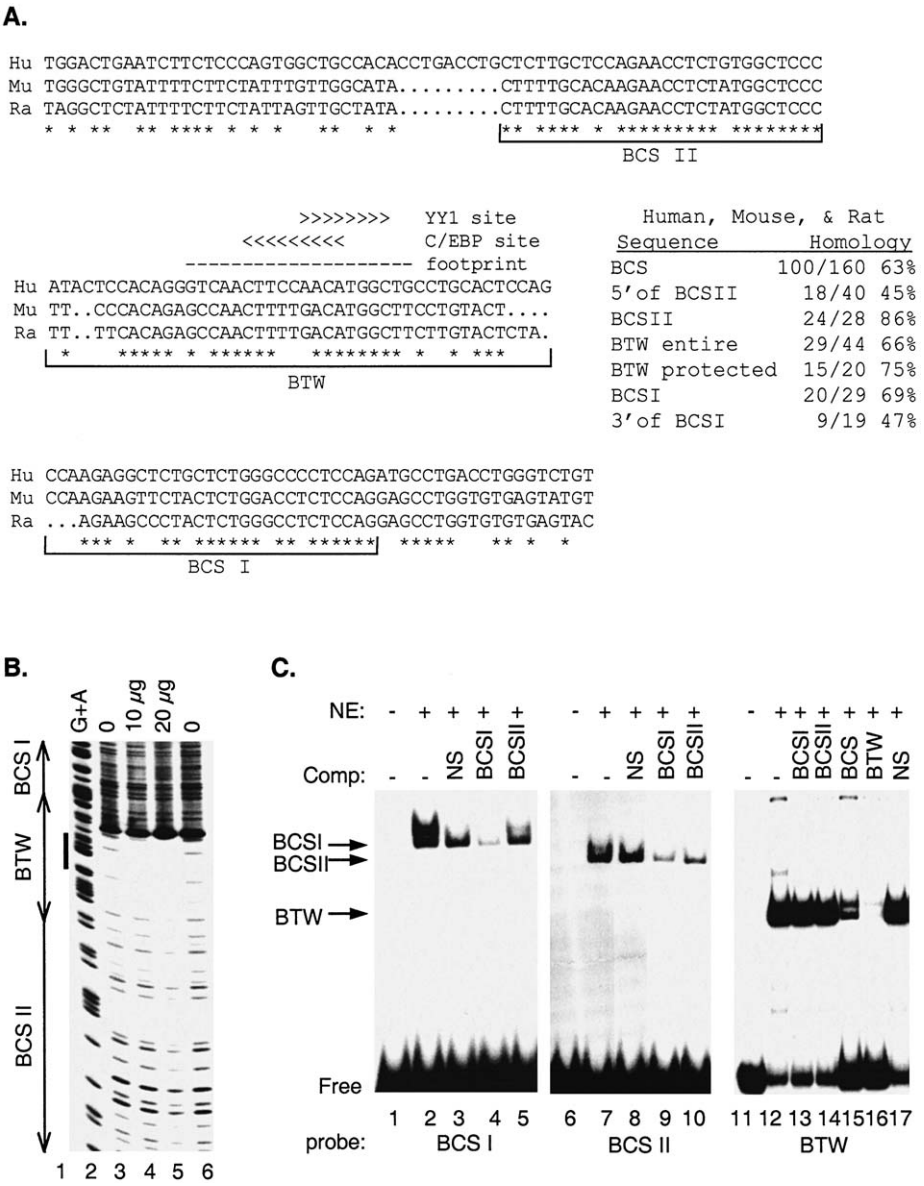

Figure 2. Features of the *B29* conserved sequence (BCS). *a.* Alignment of human, murine, and rat sequences is shown with identity denoted by *; the human BCS is −469 to −628, the murine BCS is −669 to −813, and the rat BCS is −677 to −821 relative to the transcription start sites (putative start site for rat *B29*). The BCS has 3 internal motifs: BCS I, BCS II, and between I & II (BTW). The ∼20 bp of protected sequence within the BTW motif (denoted by − and 'footprint' above the sequence and 'BTW protected' in homology table) and overlapping YY1 (denoted by >> above the sequence) and C/EBP (denoted << above the sequence) transcription factor binding sites were identified. Sequences 5′ of the BCS II are denoted '5' of BCSII' in homology table. Sequences 3′ of the BCS I are denoted '3' of BCSI' in the homology table. *b.* DNase I protection analysis of the BCS revealed a protected region within the BTW motif. DNase I protection using 729 mature B cell nuclear extract (10 μg per reaction, lane 3 and 20 μg per reaction, lane 4) were compared to those without extract (lanes 2 and 5) and reactions were run alongside a G+A sequencing ladder of the probe (lane 1). The BCS I, BTW, and BCS II motifs are shown and the protected region within the BTW motif is denoted by a solid black bar. *c.* BCS I, BCS II, and BTW motifs bind distinct DNA-binding protein complexes by EMSA. End-labeled probes (Table 1) were incubated without nuclear extract (NE-, lanes 1,6,11) and with 20 μg of 729 mature B nuclear extract (NE+, lanes 2,7,12) and with nuclear extract and 1000 fold molar excess of cold oligo competitors (Comp, lanes 3,4,5,8,9,10,13,14,15,16,17, described in Table 1). Binding reactions were run on 5% PAGE (80:1 acrylamide:bis for BCS I and II and 60:1 for BTW) in 0.5× TBE to separate DNA-binding protein complexes. Arrows denote specific DNA-binding protein complexes for BCS I (I), BCS II (II), and BTW (BTW). Free denotes free probe.

30 s, 52 °C for 30 s, 70 °C for 1 min; 72 °C for 7 min 30 s. GAPDH: 50 °C for 30 min, 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 48 °C for 30 s, 70 °C for 1 min; 72 °C for 7 min 30 s. Products were separated by electrophoresis on agarose gels (1% for human and rat B29, 1.5% for GH and GAPDH) and subsequently stained with ethidium bromide.

Whole cell lysate preparation and Western blot analysis

Whole cell lysates were prepared from cell lines growing in log phase with Passive Lysis Buffer (PLB, Promega, Madison, WI) according to manufacturer's instructions. Whole cell lysates (10^6 cells/lane) were separated by electrophoresis on 14% ProSieve 50 gels (BioWhittaker Molecular Applications, Rockland, ME) according to manufacturer's instructions, blotted to nitrocellulose, and blocked overnight with TBST (40 mM Tris HCl, pH 7.4/ 300 mM NaCl/ 0.1% Tween-20) containing 5% nonfat dry milk. Western blot was performed using 1:5000 rabbit anti-human B29 antisera followed by 1:6000 donkey anti-rabbit antibody conjugated to horseradish peroxidase (Jackson Laboratories, West Grove, PA) or 1:500 goat anti-human Growth Hormone (Santa Cruz Biotechnology, declared to be specific for human, mouse, and rat GH) followed by 1:6000 donkey anti-goat antibody conjugated to horseradish peroxidase (Jackson Laboratories). Blotted proteins were detected by Renaissance Enhanced Chemiluminescence (NEN, Boston, MA) and exposed to Kodak XOMATBlue Film.

Nuclear extracts and in vitro *transcribed and translated (IVT) proteins*

Crude nuclear extracts were prepared as previously described [13]. Protein concentration was determined by the Bradford protein assay (BioRad Laboratories, Hurcules, CA). YY1 *in vitro* translated protein (IVT) was prepared with TNT T7 Quick coupled Transcription-Translation System (Promega). pT7-YY1 was kindly provided by Yang Shi (Department of Pathology, Harvard Medical School).

DNase I protection assay

DNase I protection was performed as previously described [4]. The (+) strand probe was generated by 5′ end-labeling with $\{\gamma^{32}\text{P}\}$ATP at the NotI site of pCR-Script (Stratagene) containing a PCR-generated BCS (Table 1) at the SrfI site followed by digestion with PstI. The (−) strand probe was generated by 5′ end-labeling with $\{\gamma^{32}\text{P}\}$ ATP at the BamHI site of the same construct followed by digestion with NotI. Binding reactions were incubated with 30,000 cpm probe, 2 μg of poly dI-dC (Amersham Biosciences, Piscataway, NJ), and 10–20 μg of crude nuclear extract in 4% polyvinyl alcohol for 30 min at room temperature. Binding reactions were treated with DNase I in the presence of 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ and the digestion was stopped with buffer (20 mM EDTA, 1% SDS, 200 mM NaCl, 250 ng/μl tRNA). The G+A ladder was generated by subjecting the $\{\gamma^{32}\text{P}\}$ATP-end labeled probe to the Maxam and Gilbert method of chemical sequencing as described [14]. The 729 B cell nuclear extracts are shown in the DNase I figure for manuscript continuity (Figure 2B), although the Ramos (Burkitt lymphoma) *B29* expressing B cell line, as well as cell lines not expressing *B29* such as the plasmacytoma cell line RPMI.8226, the T cell line Jurkat, the promyelocytic cell line HL60, and the nonlymphoid cell line HeLa (data not shown) also tested positive for protection over the BTW motif.

Electrophoretic mobility shift assays (EMSA)

EMSA was performed as previously described [13]. Briefly, 25 ng of double-stranded DNA oligonucleotide (Table 1) was 5′ end-labeled with $\{\gamma^{32}\text{P}\}$ATP and purified on a G25 Sephadex spin column (Amersham Biosciences). Binding reactions were incubated with 10,000–20,000 cpm of probe, 2 μg of poly dI-dC (Amersham Biosciences), competitor double-stranded DNA (100–1000-fold molar excess), and 20 μg of nuclear extract or 5 μl of *in vitro* translate (IVT) in binding buffer (10 mM Tris-HCl pH 7.4, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) for 30 min at room temperature. For EMSA supershifts, antibody (described in Table 1) in place of competitor double-stranded DNA, was added as per the manufacturer instructions and incubated overnight at 4 °C. 729 B cell nuclear extracts are shown in all EMSA figures for manuscript continuity although the BTW-binding complex was ubiquitously formed in B cell lines representing various stages of development (697, 729, Ramos, HS.Sultan), as well as *B29* non-expressing cell lines RPMI.8226, Jurkat, HL60, and HeLa (data not shown).

Enhancer-blocking assays

Enhancer-blocking assays were performed essentially as described [15–17]. pJC13-1 containing the βglobin

Table 1. RT-PCR primers, PCR primers, oligonucleotides, and antibodies used in this study

Name	Type[a]	Sequence[b]
GH-N	RT-PCR	GCCTGCTCTGCCTGC
		GACTGGATGAGCAGCAG
Human B29	RT-PCR	GGAGCCTCGGACGTTGTCA
		CGACCTGGCTCTCACTCCT
RAT B29	RT-PCR	AGAAAAGTTGCAGCCCGTGC
		TTGATGGTCCAACCTCAGATGC
GAPDH	RT-PCR	GATGACATCAAGAAGGTGGTG
		GTCATACCAGGAAATGAGCTTG
BCS[c]	PCR	(hB29-628) TGGACTGAATCTTCTCCCAGT
		(hB29-469) ACAGACCCAGGTCAGGCAT
BCS I (−516 to −488)	Oligo	CCAAGAGGCTCTGCTCTGGGCCCCTCCAG
BCS II (−588 to −561)	Oligo	CTCTTGCTCCAGAACCTCTGTGGCTCCC
BTW (−560 to −517)	Oligo	ATACTCCACAGGGTCAACTTCCAACATGGCTGCCTGCACTCCAG
m2BTW[d]	Oligo	ATACTCCACAGGGTCAACTTCCAAC**gct**GCTGCCTGCACTCCAG
m3BTW[e]	Oligo	ATACTCCACAGGGTCAACTTCCAACAT**tt**CTGCCTGCACTCCAG
m4BTW[f]	Oligo	ATACTCCACAGGGTCAACT**gCa**AACATGGCTGCCTGCACTCCAG
EBF (−184 to −153)[g]	Oligo	GAGAGAGACTCAAGGGAATTGTGGCCAGCC
YY1c[h]	Oligo	CGCTCCGCGGCCATCTTGGCGGCTGGT
mYY1 c[h]	Oligo	CGCTCCGCG**att**ATCTTGGCGGCTGGT
YY1[h]	Ab	clone H-10 of full length
C/EBPc[h]	Oligo	TGCAGATTGCGCAATCTGCA
mC/EBPc[h]	Oligo	TGCAGA**gactagtc**TCTGCA
C/EBPα[h]	Ab	clone 14AA of internal region
C/EBPβ (NF-IL6)[h]	Ab	clone Δ198 of C-terminus, reacts with C/EBPα, β, γ, ε
C/EBPβ[h]	Ab	clone E19 of lucine zipper

[a]RT-PCR was used to detect human and rat B29, rat GH, and human and rat GAPDH with the primers shown. PCR was used to generate probes from the primers shown. Double stranded oligonucleotides (Oligo) were prepared with the complementary strand. Antibody (Ab) was used in electromobility shift assays (EMSA).
[b]Sequences are shown 5′ to 3′ and the (+) strand is shown for the oligonucleotides. Lower case and bold letters indicate mutations from the wild type sequence.
[c]The location of the oligonucleotide is shown relative to the transcription start site.
[d]Designed to mutate YY1 and C/EBP binding sites.
[e]Designed to mutate YY1 binding site, leaving C/EBP binding site intact.
[f]Designed to mutate C/EBP binding site, leaving YY1 binding site intact.
[g]EBF site from the *mb-1* gene was used as a non-specific (NS) competitor.
[h]Consensus oligonucleotides and antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA). YY1c: YY1 consensus binding site, mYY1c: mutated YY1 consensus binding site, C/EBPc: C/EBP consensus binding site, and mC/EBPc: mutated consensus C/EBP binding site.

HS2 enhancer insulated by a direct repeat of the chicken 1.2 kb βglobin insulator from the γ^{A}-globin promoter-neomycin reporter followed by a direct repeat of the 1.2 kb βglobin insulator was kindly provided by Gary Felsenfeld (NIH, Bethesda, MD). The upstream insulator was excised with KpnI and SacI and closed to create pJC13-0. The upstream insulator was also replaced with a KpnI-SacI fragment containing a direct repeat of BCS or m2BCS (containing a mutated YY1-binding site shown to abolish binding) or 3 consecutive copies of the BTW motif to create pJC-BCS, pJC-m2BCS, and pJC-BTW, respectively. Linearized constructs were purified and quantified by UV absorption. 200 ng of each construct was electroporated (200V, 1180 μF, low Ω) into 10^7 K562 erythroleukemia cells and after 24 h of recovery cells were plated in 0.3% soft agar with 500–1000 μg/ml G418 (GibcoBRL). Colonies were counted after 3–4 weeks of selection and the colony number was normalized to that obtained for pJC13-0.

Results and discussion

A highly homologous DNA sequence is located 5′ of the human, murine, and rat B29 genes

Because of the close proximity of the pituitary-specific *GH* LCR to the *B29* promoter, we first analyzed *B29* and *GH-N* mRNA in GH-expressing pituitary cells (GH3 rat pituitary cell line) and B29-expressing B cells (Ramos human B cell line and Y3-Ag1.2.3 rat myeloma cell line) by RT-PCR. As shown in Figure 1C, *B29* mRNA was not detected in a GH3 rat pituitary cell line that showed abundant *GH* mRNA (lane 3), but was detected in both B cell lines (lanes 1 and 2). *GH-N* mRNA was not detected in either B cell line (Figure 1C, lanes 1 and 2). Western analysis of B29 in Ramos, Y3Ag1.2.3, and GH3 cell lines confirmed our RT-PCR analysis and showed differential expression of B29 protein (B cell lines only, lanes 1 and 2) and GH-N protein (pituitary cell line only, lane 3) (Figure 1D). Human pituitary cell lines are not commonly available and therefore could not be tested. These results suggest that the human *B29* gene may be somehow shielded from inappropriate activation by the active nearby 5′ GH LCR. We searched for potential regulatory sequences between the *B29* gene and the upstream GH LCR using the cross-species comparison criteria [12]. Conserved orthologous DNA sequences that exhibit $\geq$70% identity over at least 100 bp across different species have been shown to exhibit regulatory functions [12]. One DNA segment matching these criteria was found upstream of *B29* gene sequences available in current databases. This region, that we call the *B29* conserved sequence (BCS), was 160 bp long and 69% identical overall between human and murine sequences (Figure 2A). Two aligned sequences in the BCS segment, denoted BCS I and BCS II, were each found to be 86% identical in both species (Figure 2A). No other similarly long, highly homologous DNA sequences were found from alignments of the human and murine genomic sequences between the *GH* LCR and the *B29* promoter. Because of the extreme similarity of the arrangement of the chromosomal loci [7, 8] and similarity of the transcription factor binding site array and overall sequence identity between the human, murine, and rat *B29* promoters [5, 7], we searched the rat *B29* region upstream of the promoter for BSC-like sequences. Figure 2A shows the BCS sequence alignment and homologies among the human, murine, and rat *B29* promoters. These three species have a strikingly similar entire BCS (Figure 2A, 63% identity) and BCS subregions (Figure 2A, up to 86% identity), furthering our suspicions that this region may somehow be involved in *B29* gene regulation.

DNase I protection analysis revealed a region of protection over the BTW motif of the BCS

To identify potential nuclear factor binding site(s) within the human BCS, DNase I protection analysis was performed using multiple *B29* expressing and non-expressing cell lines and 729 mature B cell crude nuclear extracts gave the cleanest protected region and is shown in Figure 2B. A $\sim$20 bp region of protection was identified (Figure 2B) between the BCS I and BCS II segments that we designated as the BTW motif (Between BCS I and BCS II) (Figure 2A). The BTW motif encompasses the 44 bp connecting the BCS I and BCS II sequences (Figure 2A). The $\sim$20 bp 'footprinted' region is designated 'BTW protected' in the homology table and shown as a dashed line above the human, murine, and rat aligned *B29* sequences (Figure 2A). This region within the BTW motif was similarly protected in assays with nuclear extracts from the Ramos (Burkitt lymphoma) *B29* expressing B cell line, as well as cell lines not expressing *B29*, such as the plasmacytoma cell line RPMI.8226, the T cell line Jurkat, the promyelocytic cell line HL60, and the nonlymphoid cell line HeLa (data not shown). These results suggest the BTW motif of the BCS interacts with ubiquitous DNA-binding protein(s).

Although DNase I protection showed a 'footprint' only within the BTW motif of the BCS, DNA-binding complexes were detected by EMSA with the BCS I and II motifs. The BCS I formed a specific DNA-binding complex (Figure 2C), as previously reported [5]. The BCS II exhibited a DNA-binding complex by EMSA that was not competed by non-specific DNA, but was nearly equally competed by both BCS I and II motifs (Figure 2C). The BTW motif formed a DNA-binding complex that was specific to the BTW motif and the entire BCS, and was not competed by non-specific DNA or the BCS I or II motifs (Figure 2C). In addition, the BTW-binding complex was ubiquitously formed in B cell lines representing various stages of development (697, 729, Ramos, HS.Sultan), as well as *B29* non-expressing cell lines RPMI.8226, Jurkat, HL60, and HeLa by EMSA (data not shown). Taken together these results suggest that several distinct DNA-binding complexes interact with the entire BCS.

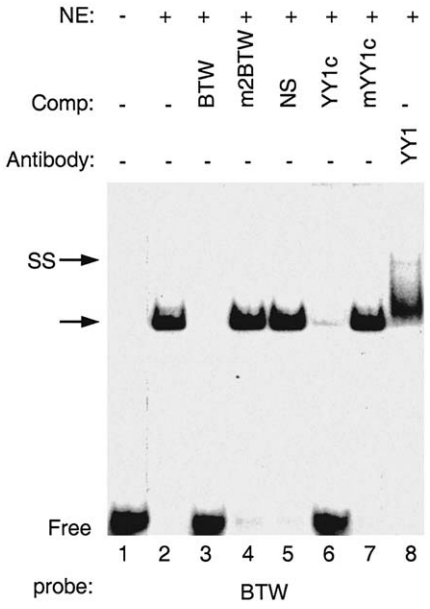

Figure 3. The BTW motif binds the YY1 transcription factor by EMSA. End-labeled BTW motif probe (Table 1) was incubated without nuclear extract (NE-, lane 1) and with 20 μg of 729 mature B cell nuclear extract (NE+, lane 2) and with nuclear extract and 1000 fold molar excess of cold oligonucleotide competitors (Comp, lanes 3–7, described in Table 1) or YY1-specific antibody (lane 8, Table 1). Binding reactions were run on 5% PAGE (60:1 acrylamide:bis) in 0.5× TBE to separate DNA-protein complexes. Arrow denotes specific DNA-binding protein complexes and 'SS' arrow denotes a supershifted DNA-binding protein complex (lane 8). Free denotes free probe.

We next searched for potential nuclear factor binding sites within the BCS. Using available databases, no potential transcription factor binding sites were found in the BCS I and II motifs (data not shown). In contrast, overlapping sequences within the DNase I protected region (Figure 2A, BTW protected) on the (−) strand of the BTW motif matched consensus sequences for C/EBP and YY1 transcription factors (Figure 2A).

The BTW motif interacts with the YY1 transcription factor

The YY1 transcription factor has been shown to interact with the **CCATNTTN**NNW (N=ACTG, W=AT, core shown in bold) consensus sequence [18]. YY1 is a Zn finger, GLI-Kruppel family member containing multiple domains with various functional capacities [19, 20]. Several of these activities are particularly relevant to the location of the YY1-binding motif within the BCS between the *B29* gene and upstream tissue-specific genes and regulatory elements. We tested the effect of YY1 motif competitors (Table 1) in EMSA with the BTW motif probe and mature B cell nuclear extracts. Formation of the BTW-binding complex was blocked by a YY1 consensus binding site oligonucleotide (Figure 3, lane 6, YY1c), suggesting that this DNA-protein interaction was likely mediated by YY1. The BTW-binding complex was not disrupted by a mutated YY1 consensus binding site oligonucleotide (Figure 3, lane 7, mYY1c) or by a mutated BTW motif oligonucleotide (Figure 3, lane 4, m2BTW). This mutant BTW motif also failed to form specific DNA-binding protein complexes when used as a probe in EMSA (data not shown). EMSA with YY1-specific antibody resulted in competition and a weak supershift with the BTW motif (Figure 3, lane 8, YY1 antibody), further confirming a specific YY1 interaction. *In vitro* transcribed and translated (IVT) YY1 formed a complex with the BTW motif probe which was specifically competed with the BTW motif and YY1c, but not mYY1c, and was competed with a YY1-specific antibody (data not shown). These combined data confirm that the BTW motif interacted specifically with the YY1 transcription factor and implicate YY1 as a potential regulator of *B29* gene expression.

The mature B cell expressed C/EBPβ (previously known as NF-IL6) transcription factor interacts with the **TSNNGNAAS** (N=ACTG, S=GT; core shown in bold) consensus sequence, and other known C/EBP family members also interact with this motif and slight variations of this consensus [21]. The human BTW motif contains this C/EBP consensus on the (−) strand: TGTTGGAAG (Figure 2A), and it overlaps the YY1 consensus sequence. Despite this predicted match to the C/EBP consensus sequence, the major BTW-binding complex was not competed by or supershifted by antibodies to the individual family members (data not shown), suggesting that C/EBP family members do not interact with the major BTW-binding complex. A very minor binding complex was visible upon over-exposure of EMSA with the BTW motif probe and mature B cell nuclear extracts. The minor binding complex was specifically competed by the C/EBP consensus site oligonucleotide (C/EBPc), the BTW motif oligonucleotide (BTW), but not the mutated C/EBP consensus site oligonucleotide (mC/EBPc) (data not shown, oligonucleotides described in Table 1). Since the C/EBP and YY1 consensus binding sites overlap in the BTW motif, mutations were engineered to disrupt only a single consensus sequence, and EMSA was performed to identify the DNA-binding proteins in the BTW-binding complex. BTW motif oligonuc-

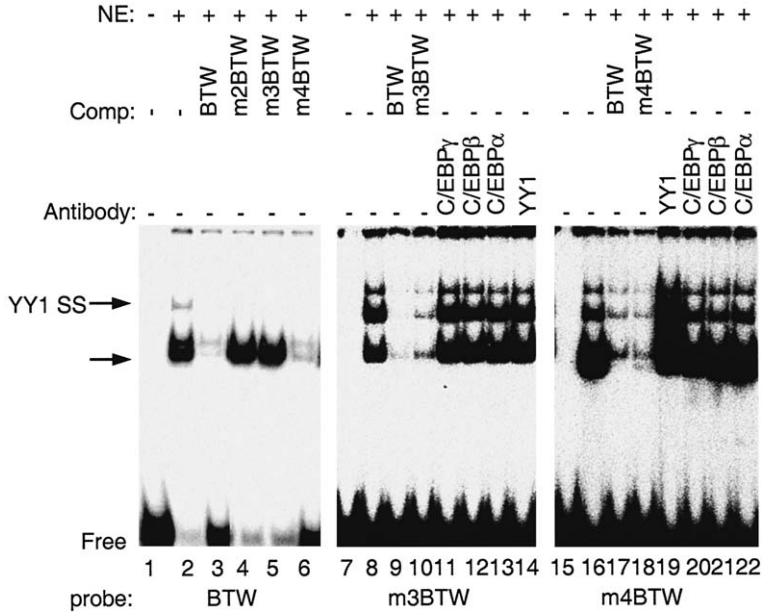

Figure 4. YY1 interaction with the BTW motif is unaltered when the C/EBP binding site is destroyed, while C/EBP does not interact with the BTW motif even in the absence of YY1. End-labeled probes BTW, m3BTW, and m4BTW (Table 1) were incubated without nuclear extract (NE−, lanes 1,7,15), with 20 μg of 729 mature B cell nuclear extract (NE+, lanes 2,8,16), and with nuclear extract and 1000 fold molar excess of cold oligonucleotide competitors (Comp, lanes 3,4,5,6,9,10,17,18, described in Table I) or specific antibodies (Table 1) in EMSAs. Binding reactions were run on 5% PAGE (60:1 acrylamide:bis) in 0.5× TBE to separate DNA-protein complexes. Unlabeled arrow denotes specific DNA-binding protein complexes and 'YY1 SS' arrow denotes supershifted DNA-binding protein complex by YY1-specific antibody (lane 19). Free denotes free probe.

leotides (Table 1) with mutations in the YY1 binding site (m2BTW) and with mutations in the YY1 binding site conserving the C/EBP binding site (m3BTW) failed to compete the wild type BTW-binding complex (Figure 4, lanes 4 and 5). In contrast, the BTW motif oligonucleotides with mutations in the C/EBP binding site while conserving the YY1 binding site (m4BTW) competed the BTW-binding complex in EMSA (Figure 4, lane 6). Further, when the m3BTW oligonucleotide (YY1 consensus binding site mutated with C/EBP consensus binding site intact) was used as a probe, antibodies to individual C/EBP family members and YY1 did not compete or super-shift this complex (Figure 4, m3BTW probe, lanes 11–15). However, when the m4BTW oligonucleotide (C/EBP consensus binding site mutated with YY1 binding site intact) was used as a probe, a complex was supershifted by YY1-specific antibody (Figure 4, m4BTW probe, lane 19), while antibodies to individual C/EBP family members did not compete or super-shift any complex (Figure 4, m4BTW probe, lanes 20-22). C/EBP antibodies to C/EBPγ and C/EBPβ did supershift a complex using our mature B cell nuclear extract and the C/EBP consensus oligonucleotide probe (C/EBPc)(data not shown). C/EBPα is not expressed in B cells and did not super-shift any complex using our mature B cell nuclear extract and the C/EBP consensus oligonucleotide probe (C/EBPc) [21], (data not shown). These results suggest that, even in the absence of YY1 transcription factor binding, C/EBP transcription factor family members did not interact with the BTW motif to any significant degree under the conditions used here in EMSA.

The BCS and individual components (BCS I, BCS II, and BTW) do not function as silencers, enhancers, or insulators

YY1 is known to function as either a silencer or an enhancer, as a MAR, and as a DNA-binding factor that interacts with a variety of chromatin modifying proteins [19, 20, 22]. However, the BCS and individual components (BCS I, BCS II, and BTW) failed to show either silencer or enhancer activities in transient transfections designed to assess the transcriptional regulation of the *B29* promoter by these upstream motifs (data not shown). Additionally, a MAR analysis program found no potential MAR sites in the 1.5 kb region encompassing the BCS, suggesting that neither

the human nor murine BCS functions as a MAR [23, 24]. Tethering of chromatin modifying proteins by YY1 within the BCS also seems unlikely since histone acetylation across the *B29* gene in *GH-N*-expressing cells was reported to be comparable to that over the downstream expressed pituitary-specific *GH-N* gene [25, 26]. Taken together these considerations and findings do not indicate positive or negative effects of the BCS and YY1-binding motif on *B29* gene expression.

The central genomic location of the BCS and YY1-binding motif between the pituitary-specific GH LCR and the *B29* gene prompted us to consider a role in insulation. We utilized the enhancer-blocking stable transformation assay that has identified multiple CTCF transcription factor-containing insulators established for the chicken βglobin insulator and subsequently used with other vertebrate insulators [15–17] to directly assess this activity. Insulation is manifested by a marked reduction in the number of colonies resistant to the selectable marker when the insulator is placed between the enhancer and promoter (i.e., enhancer-blocking). The direct repeat of the upstream chicken βglobin insulator was replaced with two repeats of the BCS and m2BCS (containing a mutated YY1-binding site shown to abolish binding) and three consecutive BTW motifs and stably transformed in the K562 erythroleukemia cell line. The control βglobin-containing insulator produced more than 4-fold insulation in this enhancer-blocking assay; however, no activity was observed with the BCS, m2BCS, or BTW motif (Figure 5).

Although we were unable to detect position-independent enhancer-blocking activity for the BCS and YY1-binding motif, this result does not completely rule out the possibility of insulator function. The RO insulator of *Xenopus* rRNA genes requires its native context, in both position and orientation, for appropriate function [27]. Additionally, the chicken βglobin insulator requires more than the essential CTCF-containing motif to protect from position variegation [15, 28]. In general, insulators are much larger than the 160 bp BCS [29]. Thus, it is conceivable that much larger 5′ *B29* constructs or the complete upstream region containing the BCS in context might be required to demonstrate insulation.

The intergenic region between the *B29* and downstream GH genes has recently been reported to contain three separated DNase I hypersensitive sites in *B29*-expressing rat plasmacytoma cells (HS 1,2,3 Rat in Figure 1A). DNA fragments containing these sequences showed modest enhancer activity using both

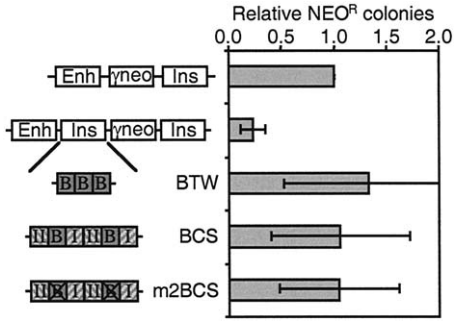

Figure 5. The BCS and YY1-binding motif do not show position independent insulator activity in the enhancer-blocking assay. The insulator construct contains the βglobin enhancer (Enh) 'blocked' from the γ^A-globin promoter-neomycin (γneo) reporter by a direct repeat of the 1.2 kb chicken βglobin insulator (Ins) placed 5′ of the γneo reporter and 3′ of the Enh (row 2). The 5′ Ins was removed and replaced with a multimerized BTW motif (row 3, BTW), a direct repeat of the BCS (row 4, BCS), and a direct repeat of the BCS with the BTW motif mutated to destroy the YY1 and C/EBP binding sites (row 5, m2BCS). Stably transformed K562 erythroleukemia cells were selected in G418 and colony number was normalized to a construct without an inserted test fragment 5′ of the γneo reporter gene (row 1). Each bar represents an average with standard deviation of 6 independent transformations with 2 different preparations of DNA.

the *B29* and *SV40* promoters in transient transfections of rat plasmacytoma cells, and a consensus octamer motif, in one of the three conserved sequences, was shown to be required for this activity. These three DNase I hypersensitive sites are composed of sequences that are >70% identical and >200 bp long between the human and rat sequences [30] and also >70% identical between human and mouse sequences by our own GenBank analyses (data not shown). It remains to be determined how these orthologous conserved 3′ intergenic sequences and the 5′ BCS might function in controlling *B29* gene expression in B lineage cells or in restricting its expression in pituitary *GH-N* expressing cells. Transgenes containing the *B29* gene along with the entire region from the upstream *SCN4A* gene through the downstream *GH-N* gene may be required to establish the activities and interactions of the highly conserved 5′ BCS and 3′ conserved intergenic sequences in determining *B29* gene specificity.

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References

1. Hermanson GG, Briskin M, Sigman D & Wall R (1989) Proc. Natl. Acad. Sci. U.S.A. 86: 7341–7345
2. Benschop RJ & Cambier JC (1999) Curr. Opin. Immunol. 11: 143–151
3. Wang H, Diamond RA & Rothenberg EV (1998) Proc. Natl. Acad. Sci. U.S.A. 95: 6831-6836
4. Omori SA & Wall R (1993) Proc. Natl. Acad. Sci. U.S.A. 90: 11723–11727
5. Thompson AA, Wood WJ, Jr, Gilly MJ, Damore MA, Omori SA & Wall R (1996) Blood 87: 666–673
6. Malone CS, Miner MD, Doerr JR, Jackson JP, Jacobsen SE, Wall R & Teitell M (2001) Proc. Natl. Acad. Sci. U.S.A. 98: 10404–10409
7. Nakazato S, Nomoto K, Kazahari K & Ono M (1998) Genomics 48: 363–368
8. Bennani-Baiti IM, Jones BK, Liebhaber SA & Cooke NE (1995) Genomics 29: 647–652
9. Bennani-Baiti IM, Cooke NE & Liebhaber SA (1998) Genomics 48: 258–264
10. Jones BK, Monks BR, Liebhaber SA & Cooke NE (1995) Mol. Cell Biol. 15: 7010-7021
11. Li X, Liu D & Liang C (2001) Int. J. Biochem. Cell Biol. 33: 914–923
12. Loots GG, Locksley RM, Blankespoor CM, Wang ZE, Miller W, Rubin EM & Frazer KA (2000) Science 288: 136–140
13. Damore MA, Omori SA & Wall R (1996) J. Immunol. 156: 2451-2457
14. Maxam AM & Gilbert W (1977) Proc. Natl. Acad. Sci. U.S.A. 74: 560–564
15. Chung JH, Whiteley M & Felsenfeld G (1993) Cell 74: 505–514
16. Bell AC, West AG & Felsenfeld G (1999) Cell 98: 387–396
17. Filippova GN *et al.* (2001) Nat. Genet. 28: 335–343
18. Shrivastava A & Calame K (1994) Nucl. Acids Res. 22: 5151-5155
19. Shi Y, Lee JS & Galvin KM (1997) Biochim. Biophys. Acta 1332: F49–F66
20. Thomas MJ & Seto E (1999) Gene 236: 197–208
21. Akira S & Kishimoto T (1992) Immunol. Rev. 127: 25–50
22. Eliassen KA, Baldwin A, Sikorski EM & Hurt MM (1998) Mol. Cell Biol. 18: 7106–7118
23. Shewchuk BM, Cooke NE & Liebhaber SA (2001) Nucl. Acids Res. 29: 3356–3361
24. Malone CS, Patrone L, Buchanan KL, Webb CF & Wall R (2000) J. Immunol. 164: 2550–2556
25. Ho Y, Elefant F, Cooke N & Liebhaber S (2002) Mol. Cell 9: 291–302
26. Elefant F, Cooke NE & Liebhaber SA (2000) J. Biol. Chem. 275: 13827–13834
27. Robinett CC, O'Connor A & Dunaway M (1997) Mol. Cell Biol. 17: 2866–2875
28. Litt MD, Simpson M, Recillas-Targa F, Prioleau MN & Felsenfeld G (2001) Embo. J. 20: 2224–2235
29. Bell AC, West AG & Felsenfeld G (2001) Science 291: 447–450
30. Komatsu A, Otsuka A and Ono M (2002) Eur. J. Biochem. 269: 1227–1236
31. Bennani-Baiti IM, Asa SL, Song D, Iratni R, Liebhaber SA & Cooke NE (1998) Proc. Natl. Acad. Sci. U.S.A. 95: 10655–10660