# An Upstream Oct-1- and Oct-2-Binding Silencer Governs *B29* (Ig$\beta$) Gene Expression[1]

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**The B cell-specific *B29* (Ig$\beta$) gene is activated in the earliest B cell precursors and is expressed throughout B cell development. Tissue-specific expression of the murine *B29* gene is controlled by a B cell-specific promoter whose activity is governed by a cassette of upstream transcriptional silencers. This study describes a potent new silencer that is located 5′ of the previously identified *B29* silencer elements, FROG and TOAD. Like these known elements, the new *B29* silencer is not restricted to the *B29* promoter. Nuclear proteins from all cell lines tested interacted with this A+T-rich sequence, which closely resembled a noncanonical octamer binding motif and also conformed to the consensus sequence for nuclear matrix attachment regions. Interaction of Oct-1 and Oct-2 with the *B29* A+T-rich sequence was confirmed using octamer-specific Abs. Oct-1/Oct-2 binding was required for the inhibitory activity of this sequence because mutations that blocked Oct-1/Oct-2 binding also eliminated inhibition of the *B29* promoter. This *B29* A+T-rich sequence specifically interacted with isolated nuclear matrix proteins in vitro, suggesting that it may also function as a matrix attachment region element. Maintenance of the level of *B29* gene expression through the interaction of the minimal promoter and the upstream silencer elements FROG, TOAD, and the A+T-rich Oct-1/Oct-2 binding motif may be essential for normal B cell development and/or function.** ***The Journal of Immunology*, 2000, 164: 2550–2556.**

The product of the *B29* (Ig$\beta$) gene is an essential component of the B cell receptor (BCR)[4] that plays an indispensable role in B cell development. The *B29* gene product is disulfide-linked to the *mb-1* (Ig$\alpha$) gene product, and this heterodimer is associated with Ig to form functional BCR complexes on B cells. *B29-mb-1* heterodimers control $VDJ_H$ recombination, allelic exclusion, surface translocation of Ig, and signal transduction events that occur through the BCR (reviewed in Refs. 1–3).

Due to the critical role the *B29* gene plays in B cell maturation and function, we have characterized the features regulating its transcription and B cell specificity. Previously, we demonstrated that the B cell specificity of *B29* is determined by a minimal promoter that contains multiple transcription factor motifs that collectively determine its tissue specificity and transcriptional activity. These motifs include early B cell factor, Ikaros, ETS, SP1, and octamer (4, 5). The *B29* octamer motif was shown to interact with both Oct-1 and Oct-2 transcription factors (6). This octamer motif is a major determinant of *B29* promoter activity in that mutations that eliminated Oct-1 and Oct-2 binding also abolished *B29* promoter function (5).

Expression of the *B29* minimal promoter is also regulated by upstream transcription control elements with inhibitory activities. We previously identified two silencer elements, FROG and TOAD, upstream of the *B29* minimal promoter that coordinately govern its level of expression (7). To date, the FROG- and TOAD-interacting proteins have not been identified. These *B29* silencer elements are equally active in both orientations, are position-independent, can affect heterologous promoters, and are not tissue restricted (7). These features of the *B29* silencers resemble other functionally defined *cis*-acting negative regulatory DNA sequences that down-regulate gene transcription and belong to the class of silencer elements that restrict levels of gene expression rather than impart tissue specificity. Other examples of silencers reported to govern gene expression like the *B29* silencer are present in the *bcl-2* (8), ETS-1 (9), and $\lambda 5$ (10) genes. It has been postulated that this class of inhibitory transcription control elements functions in maintaining the level of specific gene expression, thereby preventing deleterious consequences of overexpression (7). For example, moderate overexpression of *bcl-2* is linked to the prolonged survival of neoplastic B cells in follicular lymphoma and chronic lymphocytic leukemia (11, 12). *bcl-2* is governed by silencer elements similar in nature to the silencers of the *B29* gene in that they affect *bcl-2* expression levels but do not impart tissue specificity (8). Elevated CD3$\epsilon$ expression in T cells blocked early T cell development (13) and in prothymocytes functioned as an oncogene (14). These consequences of CD3$\epsilon$ gene overexpression are particularly relevant in that CD3$\epsilon$ fulfills the same role in the TCR as *B29* does in the BCR.

Further functional analysis of the region upstream of the *B29* minimal promoter has identified a new region of potent silencer activity 5′ of the previously identified FROG and TOAD silencer elements. Like these previously characterized *B29* silencer elements, this new 5′ *B29* silencer is not *B29* promoter-restricted.

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[4] Abbreviations used in this paper: BCR, B cell receptor; MAR, matrix attachment region; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; Bright, B cell regulator of IgH transcription.

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DNase I protection assays over this region delineated a well-defined protected area with an extended central A+T-rich sequence. This central A+T-rich sequence is homologous to the degenerate A+T-rich motifs recognized by octamer transcription factor family members (15). Both Oct-1 and Oct-2 factors bound the *B29* A+T-rich sequence in EMSA. Site-directed mutations incorporated into this A+T-rich predicted octamer motif eliminated Oct-1 and Oct-2 binding, which in turn abolished the silencer activity of this region.

The *B29* A+T-rich sequence also conforms to all the consensus sequence criteria for nuclear matrix attachment regions (MARs). MARs mediate chromatin association with the nuclear matrix (16, 17), are usually located within or near transcriptional regulatory elements (18–22), and are postulated to function in regulating gene expression by acting as boundary elements for transcription (20), by creating altered nucleosome environments (23, 24), and by affecting enhancer and silencer functions by impacting chromatin structure (25–27). The *B29* A+T-rich sequence selectively interacted with nuclear matrix proteins, suggesting that this segment may also function as a MAR in *B29* gene control.

## Materials and Methods

### *Plasmid construction and mutagenesis*

The chloramphenicol acetyltransferase (CAT) reporter constructs were made using the pCAT Basic vector (Promega, Madison, WI) backbone. *B29* promoter 5′ deletions −565, −354, and −164 were generated by restriction digest and were blunt ligated into the *Sal*I site of pCAT Basic. *B29* 5′ deletion −411 was generated by PCR using the pCR-Script vector (Stratagene, La Jolla, CA) and was subsequently subcloned into the *Hin*dIII site of the pCAT Basic vector (Promega) with a deleted *Sph*I site (GenBank accession number AF002279). In all constructs, the *B29* endogenous ATG was destroyed and the first methionine codon was that of the CAT gene. The 5′ −565/−355 region silencer construct was generated using PCR and the pCR-Script vector (Stratagene), and was subsequently ligated in both orientations immediately upstream of the *B29* (−164) promoter into *Sac*I/*Cla*I sites of pCAT Basic. These sites were carried over from the pSP73 cloning vector from the original subcloning of the −164 and −354 *B29* promoter deletion fragments into the pCAT Basic construct. Mutagenized constructs were created using the Quik-Change mutagenesis kit (Stratagene) and the following complimentary oligonucleotides: 5′-GAAGTAG CAACAAAAgTTAAcTTATGGTTGGGCG-3′ (mutant (m)A+T-rich1).

### *DNA transfections and CAT assays*

The B cell line M12 was transfected by the DEAE-dextran method (28). Cells were cotransfected with 5 μg of CAT reporter plasmid and 1 μg of pRSV-luciferase. Extracts were prepared and assayed as described (5) and were quantitated by Phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). Results were normalized to luciferase activity and are the averages of at least three independent transfections using at least two preparations of DNA.

### *Nuclear extracts and DNA-binding assays*

Preparation of crude nuclear extracts from M12 cells was previously described (29). DNase I footprinting was performed as described (29). The DNase I footprinting probe was generated by 5′ end-labeling with [γ-$^{32}$P]ATP at the *Cla*I site of pCR-Script containing the −565/−355 *B29* upstream promoter PCR fragment and then digestion with *Sac*I. DNase I footprint probes were purified by polyacrylamide gel electrophoresis. EMSA was performed as described (5) with modifications of 2 μg poly(dI·dC) and analysis in 4.5% of 60:1 polyacrylamide:bis-acrylamide using 0.5× TBE gels (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH8) at 125 V for 2.5 h at room temperature. Oct-1 in vitro translate was prepared using the TNT coupled reticulocyte lysate system (Promega). Oct-1, Oct-2, and Bob1 Abs and Oct-1 and Oct-2 blocking peptides (Santa Cruz Biotechnology, Santa Cruz, CA) used in EMSA were incubated in the binding reaction at 4°C overnight. EMSA probes were double-stranded oligonucleotides 5′ end-labeled with [γ-$^{32}$P]ATP. EMSA probes were purified by G25 Sephadex spin column chromatography (Sigma, St. Louis, MO). EMSA oligonucleotide probes were as follows: 5′-GAAGTAGCAA CAAAAATTAATTTATGGTTGGGCG-3′ (A+T-rich motif), 5′-GAAG TAGCAACAAAAGTTAACTTATGGTTGGGCG-3′ (mA+T-rich motif), 5′-TGTCGAATGCAAATCACTAGAA-3′ (octamer; Santa Cruz Biotechnology), 5′-TGTCGAATGCAAGCCACTAGAA-3′ (mOctamer; Santa Cruz Biotechnology), and 5′-TCTTCCAGAGCAAGGCAACCA CAGGAGACC-3′ (nonspecific TOAD motif).

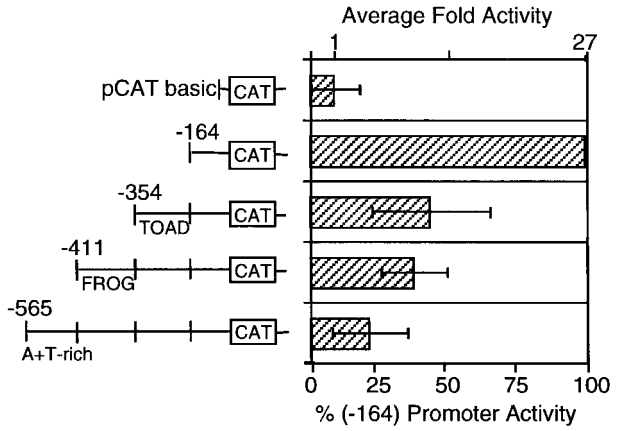


**FIGURE 1.** *B29* A+T-rich motif contributes to the negative regulation of the *B29* minimal (−164) promoter. Transient transfection to detect transcriptional expression of *B29* promoter deletion constructs were conducted in the M12 B cell line. The activity of each construct is expressed as a percentage of the CAT activity obtained with the *B29* (−164) minimal promoter construct (100%). Deletion construct −411 was created to separate the activities of the A+T-rich motif from the FROG motif silencer element to determine transcriptional activity of the A+T-rich motif region. Deletion construct −411 excludes the A+T-rich motif but contains both the FROG and TOAD silencer elements. Deletion constructs −164 (minimal promoter), −354 (TOAD motif), and −565 (TOAD, FROG, and A+T-rich motif) are identified by nucleotide numbers with respect to the major start site of transcription (+1) and are shown for comparison. CAT activities are RSV-luciferase normalized and are the average ± SD of six independent transfections using at least three preparations of DNA. Absolute fold induction for the *B29* (−164) minimal promoter construct ranged from 8- to 60-fold (average, 27-fold) over vector alone (pCAT basic). The *p* values for *B29* constructs are as follows: −164, $p < 0.001$; −354, $p < 0.001$; −411, $p < 0.5$; and −565, $p < 0.02$.

### *Nuclear matrices and in vitro nuclear matrix attachment assays*

Isolation of nuclei and nuclear matrices was performed according to Cockerill and Garrard (30), as previously described (31). Briefly, nuclei were isolated by dounce homogenization and purified by centifugation through a 2-M sucrose cushion. Endogenous DNA was degraded with DNase I for 1–2 h, and histones were extracted by sequential washes with 2 M NaCl. The resulting nuclear matrices were stored for up to 6 mo at −20°C after combination with an equal volume of glycerol. Before use, matrices from ~$1.6 \times 10^7$ cells were washed three times in 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 0.25 M sucrose, and 0.25 mg/ml BSA by centifugation for 30 sec at 10,000 × *g* at 4°C and were resuspended in 10–90 ml of assay solution. The final reaction mixture consisted of 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.25 M sucrose, 0.25 mg/ml BSA, 20 ng/ml [γ-$^{32}$P]ATP end-labeled DNA fragments, and 50–200 mg/ml sonicated *Escherichia coli* DNA unlabeled competitor. The reaction mixture was shaken for 1–3 h at 23°C; washed three times; solubilized in 0.5% SDS and 0.4 mg/ml proteinase K with 10 μg of unlabeled carrier DNA; and incubated overnight at 37°C. Material was phenol-extracted and ethanol-precipitated, and the resulting purified matrix-bound DNA fragments were electrophoretically resolved on 5% polyacrylamide-0.1% SDS gels. The gels were dried and visualized by autoradiography.

## Results

### *A new silencer element 5′ of the* B29 *minimal promoter*

Previous studies defined two *B29* closely linked 5′ silencer elements, FROG (−381) and TOAD (−349), which function cooperatively to govern the expression of the *B29* minimal promoter (7). Further deletion analysis of the region 5′ of the FROG element revealed a new upstream negative control region (−565 to −411)

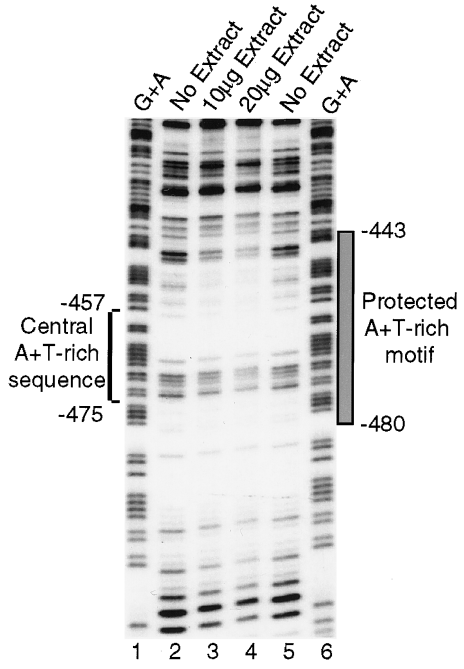


**FIGURE 2.** DNase I footprinting analysis reveals a region of protection corresponding to the A+T-rich motif in the *B29* promoter. The endpoints of the DNase I footprint are identified by nucleotide numbers with respect to the start site of transcription (+1). DNase I footprints using M12 B cell crude nuclear extracts are indicated by either "10 $\mu$g extract" or "20 $\mu$g extract" (*lanes 3* and *4*, respectively). Lanes containing reactions incubated without extract are indicated by "no extract" (*lanes 2* and *5*). Reactions were run alongside a G+A ladder of the probe (*lanes 1* and *6*). Results are representative of three independent experiments.

with greater activity than FROG and TOAD combined. Fig. 1 shows that the −565 *B29* deletion construct exhibited significantly reduced ($p < 0.02$) transcriptional activity compared with the −411 deletion construct that contained both the FROG and TOAD silencer elements. DNase I footprinting was used to detect DNA-protein binding sites in the −565 to −411 *B29* gene segment. A single, strongly protected DNA segment (i.e., −480 to −443) was obtained with nuclear protein extracts from M12 B cells (Fig. 2). This protected segment contained a central A+T-rich DNA sequence (−475 to −457) that closely conformed to noncanonical octamer factor binding motifs and to the consensus MAR motif (16, 17, 19, 25, 32).

### *The* B29 *A+T-rich motif interacts with Oct-1 and Oct-2 transcription factors*

A double-stranded oligonucleotide probe (A+T-rich motif) corresponding to the −480 to −443 DNase I protected *B29* segment (GAAGTAGCAACAAAAATTAATTTATGGTTGGGCG) formed protein complexes with M12 B cell nuclear extracts when analyzed in EMSA (Fig. 3*A*, *lane 2*). These complexes were specifically competed by cold A+T-rich motif oligonucleotides but were not disrupted by cold nonspecific competitor oligonucleotides (Fig. 3*A*, *lanes* 5 and 7). Identical complexes were formed in EMSA with all nuclear extracts tested from pre-B, T, myeloid, and fibroblast cell lines, suggesting that the DNA-binding protein(s) in these complexes may be ubiquitously expressed (data not shown). Octamer family members recognize degenerate A+T-rich sequences like those in this *B29* segment and are expressed in different cell lineages (15). They have also been implicated as silencers in addition to their well known role as stimulatory factors (33–39). Accord-

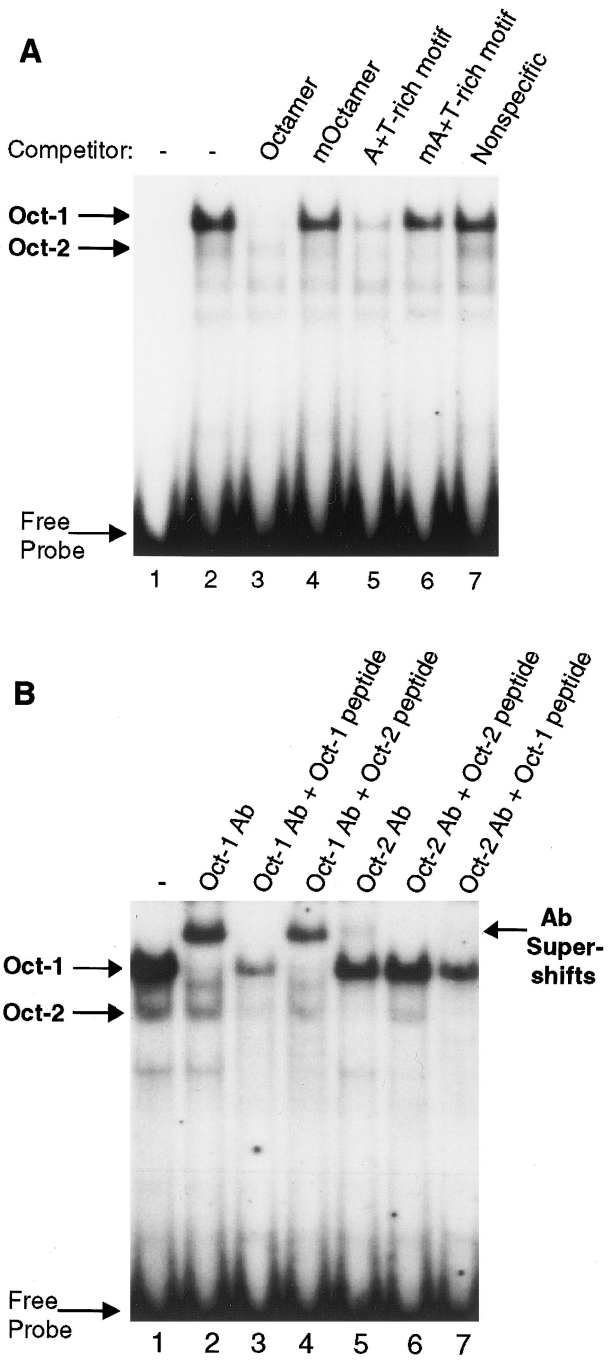


**FIGURE 3.** The *B29* A+T-rich motif specifically interacts with transcription factors Oct-1 and Oct-2. Double-stranded oligonucleotides corresponding to *B29* −482/−448 were end-labeled and used in EMSA. *A*, *Lane 1* contains A+T-rich motif probe alone. The A+T-rich motif probe was incubated with 5 $\mu$g M12 B cell nuclear extract (*lanes 2–7*). Reactions were incubated in the presence of 1000-fold excess of cold octamer consensus competitor (*lane 3*); mutated octamer competitor (*lane 4*); A+T-rich motif competitor (*lane 5*); mutated A+T-rich motif competitor (*lane 6*); or nonspecific *B29* promoter motif competitor (*lane 7*). EMSA in the presence of 100-fold and 500-fold cold competitors showed identical results. *B*, The A+T-rich motif probe was incubated with 5 $\mu$g M12 B cell nuclear extract (*lanes 1–7*). Reactions were incubated in the presence of 2 $\mu$g anti-Oct-1 Ab (*lane 2*); 2 $\mu$g anti-Oct-1 Ab and 2 $\mu$g Oct-1 blocking peptide (*lane 3*); 2 $\mu$g anti-Oct-1 Ab and 2 $\mu$g Oct-2 blocking peptide (*lane 4*); 2 $\mu$g anti-Oct-2 Ab (*lane 5*); 2 $\mu$g anti-Oct-2 Ab and 2 $\mu$g Oct-2 blocking peptide (*lane 6*); or 2 $\mu$g anti-Oct-2 Ab and 2 $\mu$g Oct-1 blocking peptide (*lane 7*). The specifically formed complexes are indicated by arrows. Results are representative of at least three independent experiments.

ingly, we tested the effect of octamer motif competitors in EMSA with the *B29* A+T-rich containing sequence. Formation of the −480 to −443 protein complex was blocked by octamer consensus binding site oligonucleotide cold competitors, suggesting that this DNA-protein interaction was likely to be mediated by an octamer family member (Fig. 3*A*, *lane 3*). The DNA-protein complex was not disrupted by a mutated octamer binding site oligonucleotide competitor (Fig. 3*A*, *lane 4*) or by a mutated A+T-rich sequence (mA+T-rich motif) competitor oligonucleotide (Fig. 3*A*, *lane 6*), further confirming a specific octamer factor interaction. This mutant *B29* A+T-rich motif also failed to form protein complexes when used as a probe in EMSA (data not shown).

Octamer-specific Abs were used in EMSA to identify the octamer factor(s) interacting with this *B29* segment. EMSA including Oct-1 Ab resulted in a super shift with the *B29* A+T-rich motif probe (Fig. 3*B*, *lane 2*). The addition of Oct-1 blocking peptide eliminated the super-shifted complex (Fig. 3*B*, *lane 3*), whereas addition of the Oct-2 blocking peptide had no effect (Fig. 3*B*, *lane 4*). In vitro-translated Oct-1 specifically formed a complex with the *B29* A+T-rich motif probe, whereas the negative control (in vitro-translated luciferase) showed no complex formation (data not shown). Oct-2-specific Ab competed the faster migrating protein complex with the *B29* A+T-rich motif probe, which resulted in a modest super-shifted complex (Fig. 3*B*, *lane 5*). The addition of Oct-2 blocking peptide eliminated the super-shifted complex (Fig. 3*B*, *lane 6*), whereas addition of the Oct-1 blocking peptide had no effect (Fig. 3*B*, *lane 7*). These combined data show that the 5′ *B29* segment containing the A+T-rich motif interacts specifically with both Oct-1 and Oct-2 and suggest that this DNA-protein interaction may be involved in the transcriptional regulatory activity of this segment.

### *Site-directed mutations in the 5′* B29 *A+T-rich motif that disrupt DNA-Oct-1/Oct-2 binding also eliminate silencer activity*

The two engineered point mutations in the *B29* A+T-rich motif that prevented Oct-1 and Oct-2 binding in EMSA were tested for silencer activity in the context of the *B29* promoter construct containing the complete upstream sequence to −565. These introduced mutations in the A+T-rich Oct-1/Oct-2 binding site abolished the transcriptional silencer activity of this extended 5′ segment and restored transcriptional activity to a level slightly greater than that of the −354 *B29* segment (Fig. 4). This result strongly suggests that the Oct-1/Oct-2 binding motif is responsible for the silencing activity of the A+T-rich sequence in the −565 construct. The previously identified TOAD and FROG silencer elements (7) are presumed to account for the residual negative transcriptional activity of the −565 *B29* promoter construct containing the mutated A+T-rich motif (Fig. 4).

The FROG and TOAD silencer elements functioned as silencer elements when associated with heterologous promoters (e.g., mb-1, c-*fos*) in addition to their silencer activity in the *B29* minimal promoter (7). Next we tested whether the wild-type and mutant octamer binding sites in the *B29* A+T-rich sequence exhibited similar broad activity. Isolated 5′ *B29* fragments (−565 to −355) containing either the wild-type or the mutated *B29* A+T-rich octamer motif were placed directly upstream of the minimal *B29* promoter (−164) or the heterologous c-*fos* promoter (Ref. 7 and Fig. 5). The segment containing the wild-type *B29* A+T-rich octamer motif inhibited the c-*fos* promoter comparably to the way it inhibited the *B29* minimal promoter ($p < 0.001$), indicating that this 5′ silencer element is active on heterologous promoters. Silencing activity of the −565 to −355 segment on both the *B29* and c-*fos* promoters was completely eliminated in constructs containing the mutated Oct-1/Oct-2 binding site ($p < 0.001$). This reaffirms that the A+T-rich octamer motif is the primary silencer element in this upstream *B29* gene segment. The −565 to −355 *B29* segment tested in these experiments included the wild-type FROG (−381) motif but not the TOAD (−349) motif. The complete elimination of silencer activity in the −565 to −355 segment with the mutated *B29* A+T-rich octamer motif suggests that the retained wild-type FROG motif without the TOAD motif was unable to exert silencer activity on either *B29* or c-*fos* promoters. When both the FROG and TOAD motifs are present and the A+T-rich octamer motif is mutated (as in Fig. 4), residual silencing activity remains. These data suggest a cooperative and interdependent function for the two closely linked *B29* silencer elements TOAD and FROG that is independently augmented by the Oct-1/Oct-2 binding site in the upstream A+T-rich sequence.

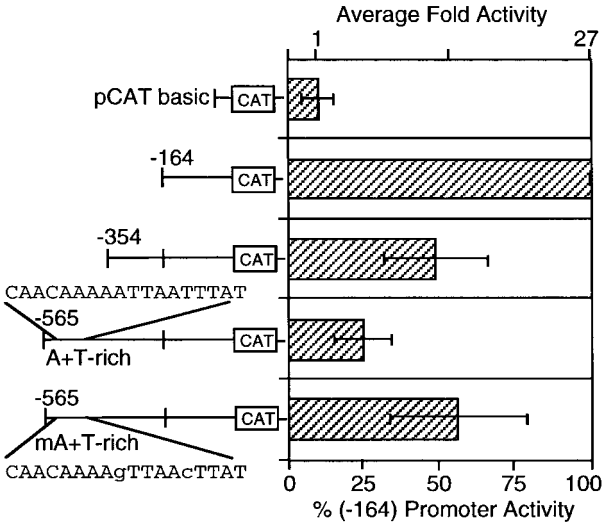


**FIGURE 4.** Site-directed mutagenesis of the *B29* A+T-rich motif alleviates negative regulation of the *B29* promoter. Transient transfections to detect transcriptional expression of *B29* promoter deletion construct −565 with introduced point mutations in the A+T-rich motif were conducted in the M12 B cell line. Mutagenized nucleotides are indicated by lowercase script. Wild-type sequence of the A+T-rich motif is 5′-GAAGTAGCAA CAAAAATTAATTTATGGTTGGGCG-3′ (A+T-rich). Mutated sequence of the A+T-rich motif is 5′-GGAGTAGCAACAAAAgTTAAcT TATGGTTGGGCG-3′ (mA+T-rich). The activity of each construct is expressed as a percentage of the CAT activity obtained with the *B29* (−164) minimal promoter construct (100%). Deletion constructs −164, −354, and −565 are identified by nucleotide numbers with respect to the major start site of transcription (+1) and are shown for comparison. CAT activities are RSV-luciferase normalized and are the average ± SD of seven independent transfections using at least three preparations of DNA. Absolute fold inductions for the *B29* (−164) minimal promoter construct in repeated experiments ranged from 8- to 60-fold (average, 27-fold) over vector alone (pCAT basic). The *p* values for *B29* constructs are as follows: −164, $p < 0.001$; −354, $p < 0.001$; −565, $p < 0.001$; and −565 mA+T-rich, $p < 0.001$.

### *The* B29 *A+T-rich sequence preferentially binds to the nuclear matrix*

The *B29* A+T-rich octamer motif sequence matches the sequence criteria for MAR binding protein interactions. In fact, this sequence exactly conforms to the consensus motif for a known B cell-specific MAR binding transcription activator protein known as B cell regulator of IgH transcription (Bright) (40). The Bright consensus sequence consists of an A+T-rich hexamer core sequence flanked by at least two AT dimer repeats ≤6 bp from the core hexamer that are all contained within an ATC-restricted sequence

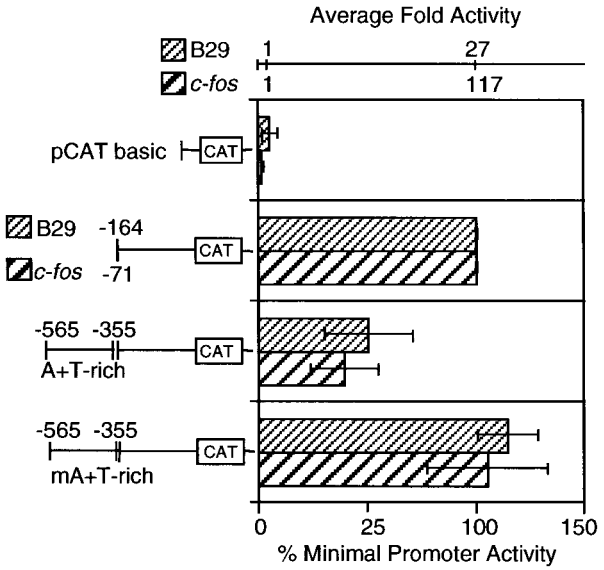


**FIGURE 5.** Targeted disruption of the *B29* A+T-rich motif alleviates its silencing activity on *B29* promoter and c-fos promoter constructs. Transient transfections to detect transcriptional expression of site-directed mutations within *B29* and c-*fos* promoter-silencer constructs were conducted in the M12 B cell line. The A+T-rich fragment position and orientation relative to the promoters are as indicated. The activity of each construct is expressed as a percentage of the CAT activity obtained with each minimal promoter construct (100%). Promoter sequences are identified by nucleotide numbers with respect to their start sites of transcription (+1). CAT activities are RSV-luciferase normalized and are the average ± SD of three independent transfections using two preparations of DNA. Absolute fold inductions for the each promoter construct in repeated experiments ranged from 8- to 60-fold (*B29*) and from 40- to 400-fold (c-*fos*) over vector alone (pCAT basic). Average fold induction for the *B29* (−164) minimal promoter and c-*fos* (−71) promoter are 27-fold and 117-fold, respectively, over pCAT basic. The *p* values for *B29* constructs are as follows: −164, $p < 0.001$; −565/−355 construct, $p < 0.001$; −565/−355 mA+T-rich construct, $p < 0.001$. The *p* values for c-*fos* constructs are as follows: −71, $p < 0.001$; −565/−355 construct, $p < 0.001$; −565/−355 mA+T-rich construct, $p < 0.001$.

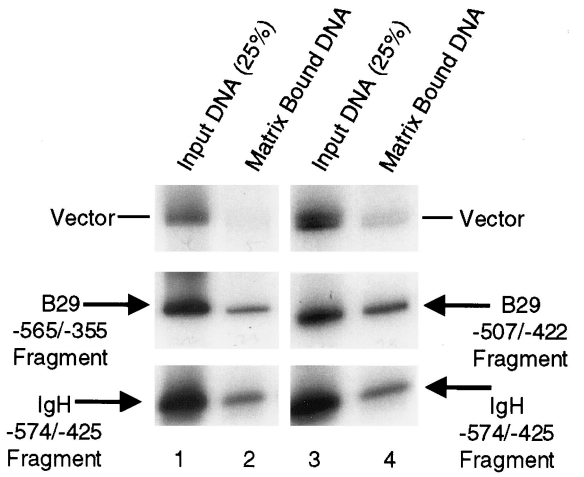


**FIGURE 6.** The 5′ A+T-rich region of the *B29* promoter preferentially binds to the nuclear matrix in vitro. The *B29* A+T-rich region was analyzed for its ability to bind the nuclear matrix. Nuclear matrix preparations were incubated with whole 5′ end-labeled *Pvu*II-digested plasmids containing the −565/−355 *B29* promoter fragment and the −574/−425 (bf150) IgH V1 promoter fragment (*lanes 1* and *2*) and the −507/−422 *B29* promoter fragment and the −574/−425 (bf150) IgH V1 promoter fragment (*lanes 3* and *4*). The matrix-retained fragments obtained after washing away the unbound DNA and digesting away matrix proteins with proteinase K are shown in *lanes 2* and *4*. *Lanes 1 and 3* contain 25% of the free-input DNA originally incubated with the matrix. *B29* and IgH matrix-bound DNA is indicated by arrows.

of at least 13 bp (40). The *B29* A+T-rich octamer motif sequence consists of an AATTAA hexamer core with two AT dimers found 1 and 5 bp away from the hexamer core, and the ATC-restricted sequence stretches over 18 bp (i.e., $^{-475}$CAACAAAAATTAATT TAT$^{-457}$). However, *B29* 5′ DNA segments containing the A+T-rich octamer sequence and Bright consensus sequence (−507 to −422 and −565 to −355) did not interact with Bright protein from BCg3R-1d B cell transfectants, which express Bright protein (40, 41), and did not bind in vitro translated Bright protein (Ref. 41 and data not shown). Anti-Bright Ab also had no effect on the protein complexes seen with the *B29* A+T-rich octamer motif in EMSA (data not shown). Thus, the Bright transcription activator does not appear to bind to the *B29* A+T-rich octamer motif or to be a component of the protein complexes detected in EMSA with this sequence.

Even though the *B29* A+T-rich octamer motif did not interact with the MAR binding protein Bright, this sequence still conforms to all of the sequence criteria of a MAR (16, 17, 19, 30, 32). Next we tested nuclear matrix protein binding of the −565 to −355 *B29* A+T-rich octamer motif segment. Specifically, two overlapping *B29* promoter fragments encompassing the *B29* A+T-rich octamer motif (−475 bp), −565 to −355 and −507 to −422, were used in standard in vitro nuclear matrix protein binding assays (31) along with the positive control IgH V1 promoter (S107) MAR-containing DNA fragment known to interact with the nuclear matrix (31, 41, 42). Restriction-digested plasmids containing these DNA fragments from the *B29* and IgH (S107) genes were labeled and mixed with isolated nuclear matrices. After multiple washes to remove non-matrix-bound DNA, the resulting nuclear matrix-DNA pellets were subjected to proteinase K digestion and were separated by electrophoresis. Fig. 6 shows that both *B29* DNA fragments were bound by nuclear matrix preparations at levels comparable to that of the IgH positive control DNA fragment. Control non-MAR vector DNA fragments were not retained by the nuclear matrix preparations. These data indicate that the *B29* A+T-rich octamer motif segment exhibits specific binding to nuclear matrix proteins.

## Discussion

These studies functionally define a new *B29* silencer (−475) located upstream of the *B29* promoter in a 5′ regulatory region that includes the previously identified silencer elements FROG (−381) and TOAD (−349) (7). The *B29* A+T-rich octamer motif appears to be a more potent silencer than the combined activities of the *B29* FROG and TOAD silencer elements. We previously attributed residual silencer activity seen with the *B29* −565 to −355 DNA fragment to the FROG silencer element present in this fragment (7). However, results from the present study now conclusively indicate that the A+T-rich segment (−475 to −457) is the primary determinant of the silencer activity of this region. Site-directed mutagenesis of the *B29* A+T-rich octamer motif, both in its natural context upstream of the *B29* promoter (Fig. 4) and in truncated test constructs with the *B29* promoter and the c-*fos* promoter, completely eliminated the silencer activity of this upstream segment (Fig. 5).

The binding of Oct-1 and/or Oct-2 with the degenerate *B29* A+T-rich octamer motif is essential for the silencer activity of this 5′ *B29* gene segment. Direct evidence for this negative regulatory

role of Oct-1 and/or Oct-2 in *B29* gene expression was demonstrated by site-directed mutations in the *B29* A+T-rich octamer motif, which destroyed silencer activity on both the *B29* and the heterologous c-*fos* promoter (Fig. 4 and 5) and also eliminated Oct-1 and Oct-2 binding (Fig. 3). Octamer factor binding is now implicated in the activity of silencers in a number of other genes. Oct-1 was recently reported to act as a silencer protein in the *hTSHβ* promoter (33), the CYPA1 gene promoter (34), and the *PIT-1/GHF1* gene promoter (36). Oct-2 functions as a silencer factor in the *IL-2* enhancer (35), the tyrosine hydroxylase promoter (43), and several viral promoters including the herpes simplex virus immediate-early gene 3 promoter (44). Additionally, both Oct-1 and Oct-2 have been shown to repress the HIV long terminal repeat promoter (45). These examples and the results presented here indicate that Oct-1 and Oct-2 function as transcriptional silencers in addition to their better known roles as transcriptional activators (37–39).

It is now evident that *B29* transcriptional control is determined by Oct-1 and/or Oct-2 functioning in opposing fashions. In one context, Oct-1 and/or Oct-2 function as transcriptional inhibitors when bound to the 5′ A+T-rich octamer motif silencer, and in the other they act as activators when bound to the canonical octamer motif in the *B29* minimal promoter (5, 6). These opposing Oct-1- and/or Oct-2-mediated functions in *B29* gene control could be regulated by the differential interaction of bound Oct-1/Oct-2 with the B cell octamer transactivator or cofactor Bob1 (OCA-B, OBF-1) (46–48). Based on the specific octamer binding site sequence requirements for Bob1 binding (46, 49–51) and on the fact that no ternary complexes (reflective of Bob1 interactions) were identified in our EMSA with the *B29* A+T-rich motif (Fig. 3), we predict that Bob1 would not effect *B29* A+T-rich octamer motif silencer activity. In contrast, we have found that Bob1 is a potent transactivator in conjunction with Oct-1/Oct-2 binding to the canonical octamer motif in the minimal *B29* promoter (Malone et al., manuscript in preparation). These findings provide a plausible explanation for the dual and opposing activities of Oct-1 and/or Oct-2 in the regulation of the *B29* promoter.

The *B29* A+T-rich octamer motif not only interacts with the Oct-1 and Oct-2 factors but also binds to nuclear matrix proteins as well (Fig. 6). *B29* promoter fragments encompassing the *B29* A+T-rich octamer motif showed binding to isolated nuclear matrices equivalent to that of the IgH V1 promoter MAR element used as the positive control in these experiments. Preliminary results from in vivo nuclear matrix association assays conducted according to Refs. 52 and 53 indicate that the *B29* A+T-rich octamer motif is preferentially associated with nuclear matrices in isolated chromatin (results not shown). These findings suggest that this region is likely to function as a MAR. However, the present studies are not able to resolve whether the *B29* A+T-rich octamer motif also functions as a MAR because transient transfections do not reflect nuclear matrix interactions (54). These studies do establish that the silencer activity of the *B29* A+T-rich octamer motif can function independent of any MAR involvement. Additional studies using either stably transformed cells and/or transgenic mice will be necessary to determine whether the *B29* A+T-rich Oct-1/Oct-2 motif functions both as a silencer and as a MAR.

Recent results with the hTSHβ promoter (33) provide a compelling case in which an Oct-1 binding motif was shown to have the dual functions of a silencer and a MAR. The hTSHβ promoter contains an A+T-rich Oct-1 binding motif with MAR homology (33), like the *B29* A+T-rich octamer motif. Additionally, the *B29* A+T-rich octamer motif conferred negative regulation on the *B29* minimal promoter as has been shown for the A+T-rich Oct-1 binding motif and the hSTHβ promoter (33). Furthermore, the *hSTHβ* promoter Oct-1 binding site was tethered to the nuclear matrix in vivo (33). Oct-1 recently has been shown to be a component of the insoluble nuclear matrix in addition to its well known role as a soluble nuclear transcription factor (33, 55), along with several other transcription factors including SP-1, AP-1, and C/EBP (55). By analogy to these findings with the *hTSHβ* promoter, the *B29* A+T-rich octamer motif similarly may serve a dual function in *B29* gene regulation by mediating silencer activity on the *B29* promoter and by facilitating *B29* gene association with the nuclear matrix (33, 55). Confirmation of such a duality would open a new dimension in *B29* gene expression in B cell development.

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