

B29 Gene Silencing in Pituitary Cells Is Regulated by Its 3' Enhancer

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B cell-specific *B29* (*Igβ*, *CD79b*) genes in rat, mouse, and human are situated between the 5' growth hormone (*GH*) locus control region and the 3' *GH* gene cluster. The entire *GH* genomic region is DNase 1 hypersensitive in *GH*-expressing pituitary cells, which predicts an "open" chromatin configuration, and yet *B29* is not expressed. The *B29* promoter and enhancers exhibit histone deacetylation in pituitary cells, but histone deacetylase inhibition failed to activate *B29* expression. The *B29* promoter and a 3' enhancer showed local dense DNA methylation in both pituitary and non-lymphoid cells consistent with gene silencing. However, DNA methyltransferase inhibition did not activate *B29* expression either. *B29* promoter constructs were minimally activated in transfected pituitary cells. Co-transfection of the B cell-specific octamer transcriptional co-activator Bob1 with the *B29* promoter construct resulted in high level promoter activity in pituitary cells comparable to *B29* promoter activity in transfected B cells. Unexpectedly, inclusion of the *B29* 3' enhancer in *B29* promoter constructs strongly inhibited *B29* transcriptional activity even when pituitary cells were co-transfected with Bob1. Both Oct-1 and Pit-1 bind the *B29* 3' enhancer in *in vitro* electrophoretic mobility shift assay and in *in vivo* chromatin immunoprecipitation analyses. These data indicate that the *GH* locus-embedded, tissue-specific *B29* gene is silenced in *GH*-expressing pituitary cells by epigenetic mechanisms, the lack of a B cell-specific transcription factor, and likely by the *B29* 3' enhancer acting as a powerful silencer in a context and tissue-specific manner.

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Introduction

B29 (*Igβ*, *CD79b*) is an essential component of the B cell antigen receptor, and along with immunoglobulin and mb-1 (*Igα*, *CD79a*), is absolutely required for B cell development and function. *B29* is one of the earliest genes activated in B cell precursors and its expression continues through terminally differ-

entiated antibody-secreting plasma cells.^{1,2} Expression of *B29* is restricted to B cells with the exception of early stages in thymocyte development prior to T cell lineage commitment.³ The rat, mouse, and human *B29* promoters contain highly conserved sequences with a core of essentially identical transcription factor binding motifs.^{4–6} The *B29* promoter in each species has an essential octamer motif whose consensus is competent for Bob1 (OCA-B, OBF-1) co-activator binding.^{7,8} Rat *B29* enhancers, designated DNase 1 hypersensitive sites (DHS) +4.4, +6.0, and +8.7, were identified downstream of *B29* in the intergenic region between the *B29* and the growth hormone (*GH*) genes. These regions are highly conserved among rat, human, and mouse. The *B29* DHS4.4 3' enhancer (*DHS4.4*) shows the highest enhancing activity and contains a consensus

Abbreviations used: *GH*, growth hormone; EMSA, electrophoretic mobility shift assay; DHS, DNase 1 hypersensitive site; TSA, trichostatin-A; 5-aza, 5-aza-2-deoxycytidine; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation.

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octamer-binding site that, when removed, results in a loss of enhancing activity.⁹ This consensus octamer site meets the requirements for Bob1 co-activator binding as well.⁸

The *B29* chromosomal locus in these mammalian species contains several closely linked tissue-specific genes. The *B29* gene is located between the skeletal muscle (SkM)-specific Na-channel α -subunit (*SCN4A*) gene and the pituitary-specific growth hormone (*GH-N*) gene in both human and rat genomes^{10,11} (Figure 1(a)). In mice, *B29* is flanked upstream by the cardiac muscle-specific myosin alkali light chain gene and downstream by the *GH* genes (MGI Genetic map; NCBI). The human *B29* gene is situated between both pituitary and placenta-specific *GH* locus control regions and the downstream genes they control.^{10,11} The pituitary-specific *GH* locus control regions is located only ~1.5 kb upstream from the *B29* gene transcription start site.¹¹ This entire region is enriched in histone H3 and H4 acetylation in pituitary cells suggesting an accessible "open" chromatin configuration.¹² Even so, the human *B29* gene remains silent.¹³ In contrast, the rat genomic *B29* locus is histone H3 and H4 deacetylated while the *GH* region is acetylated in pituitary cells,¹⁴ suggesting that *B29* silencing in rat pituitary cells depends on local histone deacetylation.

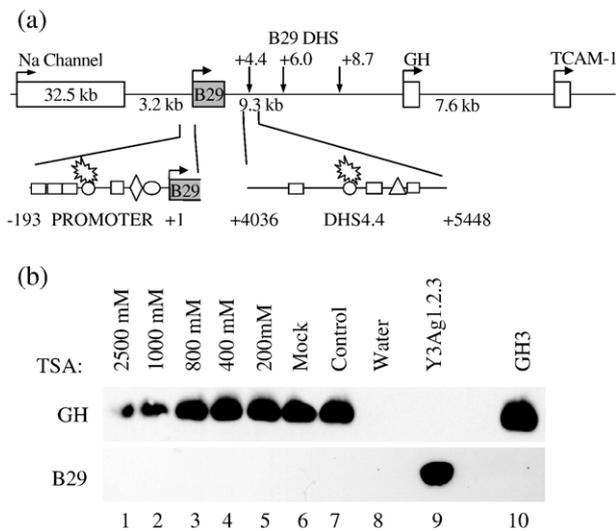


Figure 1. HDAC inhibitor TSA does not reactivate the *B29* gene in pituitary cells. (a) Schematic diagram of the rat *B29* and growth hormone (*GH*) genomic locus. The *B29* promoter, coding sequence and 3'enhancers are located between the muscle-specific sodium channel gene and the pituitary-specific *GH* gene. *B29* promoter and *B29* DHS4.4 3' enhancer transcription factor binding sites are shown. Rectangle, EBF; circle, octamer; spiky oval, Bob1; square, ETS; diamond, Sp1; oval, Ikaros; triangle, NF- κ B. The cartoon is drawn to scale. Numbers indicate distances from the major start of *B29* transcription (+1). (b) Southern blots of *GH* and *B29* RT-PCR gels used to detect gene expression. GH3 pituitary cells were treated with TSA in increasing doses to the maximum tolerated dosage for three days before RNA was prepared, lanes 1–5. Lane 6, mock TSA; lane 7, no treatment; lane 8, water; lane 9, Y3Ag1.2.3 myeloma cell RNA; lane 10, GH3 pituitary cell RNA.

Contrary to expectation, we show that endogenous *B29* is not reactivated when rat GH3 pituitary cells are treated with the histone deacetylase (HDAC) inhibitor trichostatin-A (TSA) or with the DNA methylation inhibitor 5-aza-2-deoxycytidine (5-aza), suggesting that epigenetic modifications alone are insufficient for *B29* silencing in pituitary cells. Transiently transfected *B29* reporter constructs were functional in GH3 pituitary cells, but the addition of the B cell-specific octamer co-activator Bob1 resulted in increased promoter activity that was comparable to the activity seen in B cells. The addition of the *DHS4.4* 3' enhancer increased *B29* promoter activity in rat Y3Ag1.2.3 B cells as expected, but surprisingly inhibited *B29* transcriptional activity in transfected GH3 pituitary cells even when co-transfected with Bob1. Both Oct-1 and Pit-1 transcription factors interact with the *DHS4.4* 3' enhancer, suggesting a role for these factors in *B29* gene regulation. These data indicate that while epigenetic and transcription factor composition have likely roles in controlling *B29* gene expression, it is the paradoxical action of the *DHS4.4* 3' enhancer, acting as a powerful context-specific silencer, that prevents *B29* activity in silent pituitary cells.

Results

HDAC inhibitor TSA did not reactivate *B29* gene expression

The rat *B29* gene promoter and 3' enhancers are acetylated at histone H3 and H4 in *B29*-expressing B cells, while these same regions are deacetylated in *B29*-silent pituitary cells.¹⁴ These data correlate *B29* expression with *cis*-element H3/H4 acetylation and suggest that the acetylation state may control tissue-specific *B29* expression. Treatment of cells with the HDAC inhibitor TSA results in acetylation of chromatin and the reactivation of genes whose expression is regulated by histone H3 and H4 acetylation status.¹⁵ Treatment of GH3 pituitary cells with TSA over three days was used to assess the reactivation of the silent *B29* gene in treated cells. At concentrations ranging from 10 nM to 2500 nM TSA, where the lowest concentration showed no effect on cell growth and the highest concentration showed high cell death, *B29* expression was not detected using sensitive RT-PCR and Southern blotting techniques (Figure 1(b)). These data indicate that the acetylation status of the *B29* promoter and 3' enhancers does not exclusively control *B29* expression.

B29 promoter and DHS4.4 3' enhancer DNA methylation patterns correlate with tissue-specific expression and silencing

Resistance to TSA treatment can be indicative of more advanced gene silencing through dense DNA methylation at CpG dinucleotides.¹⁶ Genomic

bisulfite sequencing was performed to evaluate potential DNA methylation at every cytosine within the *B29* promoter and *DHS4.4* 3' enhancer (Figure 2). DNA methylation patterns were determined for two *B29*-expressing B cell lines (Y3Ag1.2.3, YB2/0), two *B29*-silent pituitary cell lines (GH3 and GC), and one non-B, non-pituitary *B29*-silent kidney cell line (NRK). The promoter region analyzed corresponds to a 488 bp fragment encompassing approximately 200 bp upstream of the *B29* minimal promoter, the entire *B29* minimal promoter, and the start of translation (Figure 2). The *B29* methylation pattern at six CpG dinucleotides shows high or total methylation in the *B29*-silent cell lines while these CpG dinucleotides show minimal to no methylation in the *B29*-expressing B cell lines. The pattern of *DHS4.4* DNA methylation was similar to that determined for the *B29* promoter (Figure 3). Five *DHS4.4* CpG dinucleotides within a 519 bp fragment showed high or total DNA methylation in the *B29*-silent GH3 and GC pituitary and NRK kidney cell

lines, while no DNA methylation was identified in the *B29*-expressing B cell lines Y3Ag1.2.3 and YB2/0 (Figure 3). These data indicate that the lack of *B29* gene expression in pituitary cells is related to DNA hypermethylation.

DNA methylation inhibitor 5-aza did not reactivate *B29* gene expression

Treatment of cells with the DNA methylation inhibitor 5-aza results in the activation of genes whose expression is silenced by CpG methylation.¹⁷ GH3 pituitary cells were treated with 5-aza over three days and reactivation of the silent *B29* gene assessed. At concentrations ranging from 0.1 μM to 10 μM 5-aza, *B29* expression was not detected using sensitive RT-PCR and Southern blotting techniques (Figure 4). These data indicate that the *B29* promoter remains silent in DNA demethylated pituitary cells, possibly due to a lack of appropriate transcription factors.

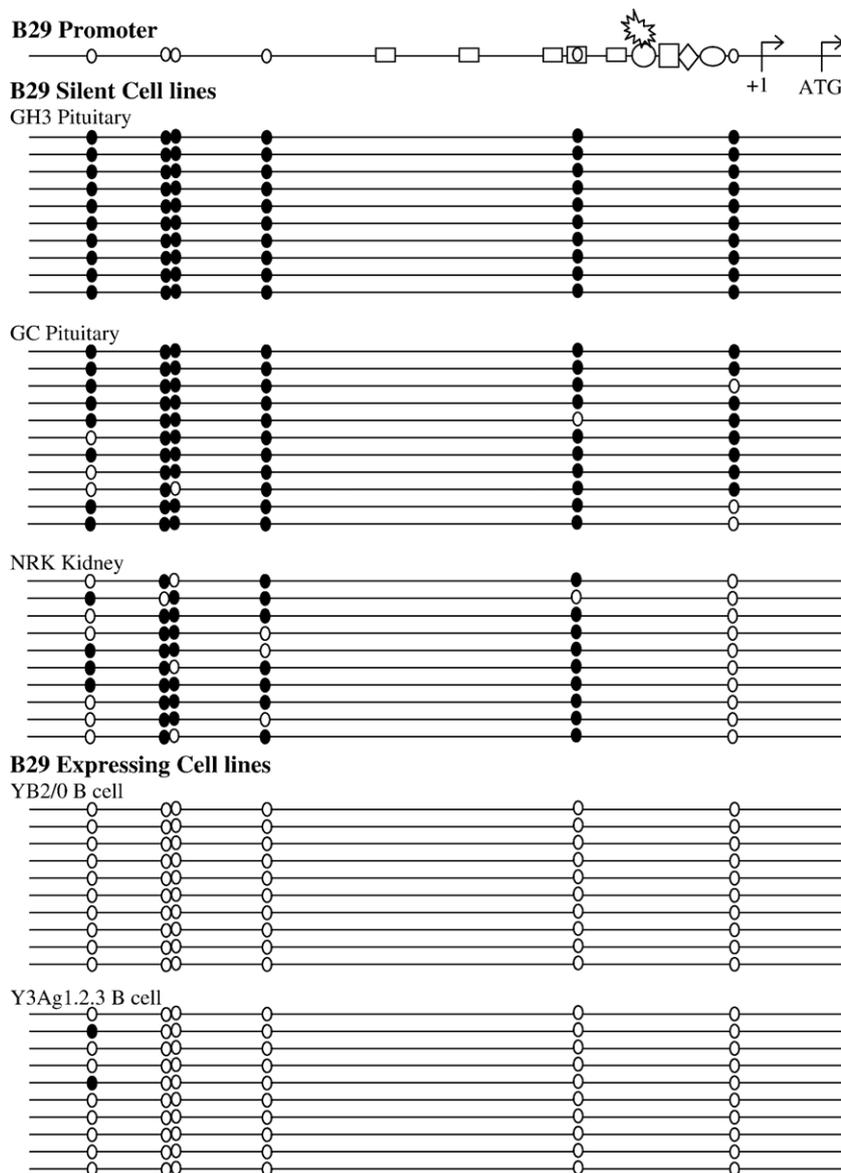


Figure 2. Nearly complete DNA methylation of the *B29* promoter in silent pituitary cells. Genomic DNA from pituitary, kidney and myeloma cell lines was subjected to sodium bisulfite conversion, PCR amplification of a 488 bp fragment, subcloning and cycle sequencing. Unmethylated CpG sites are indicated by small vertical open ovals and methylated CpG sites are indicated by small filled ovals. At least ten clones from each cell line as indicated were bisulfite sequenced to obtain a representative sampling of DNA methylation patterns in each setting. Transcription factor binding sites are as indicated: rectangle, EBF; circle, Octamer; triangle, ETS; diamond, Sp1; oval, Ikaros. +1 indicates the major transcription start site. The cartoon is drawn to scale.

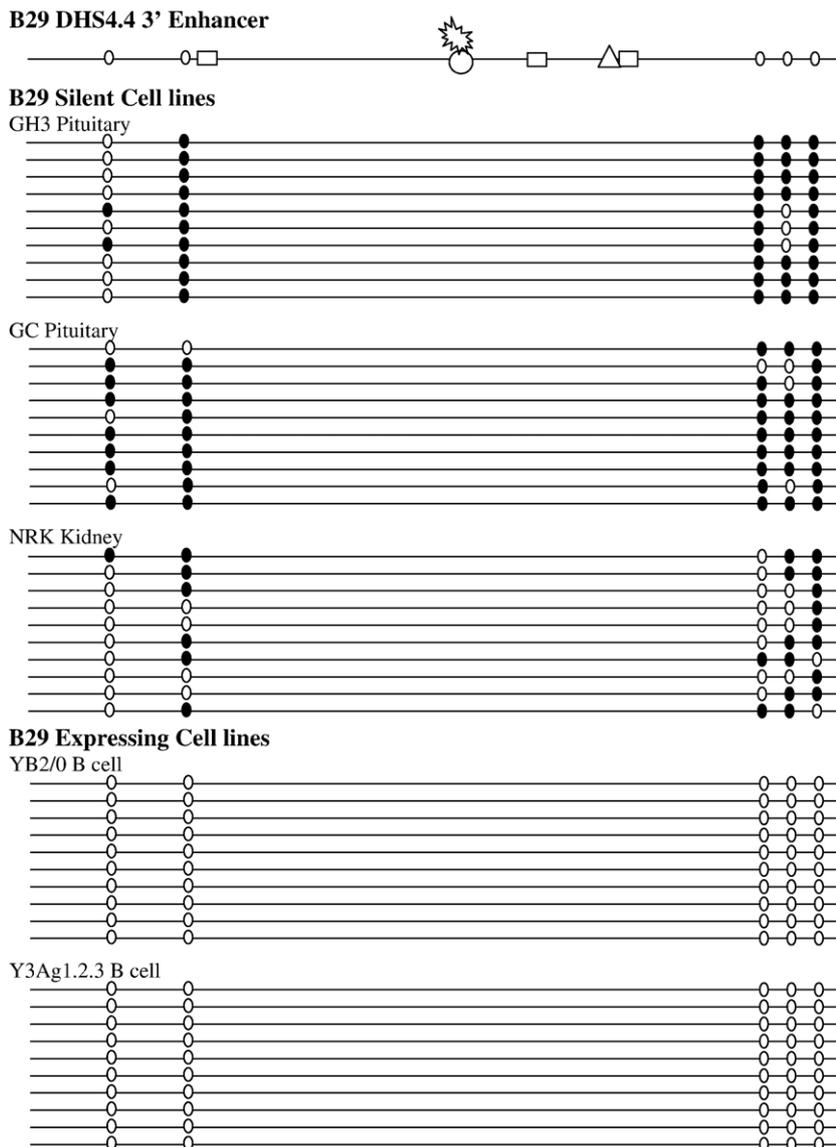


Figure 3. Dense DNA methylation of the *B29* DHS4.4 3' enhancer in pituitary cells. Genomic DNA from pituitary, kidney and myeloma cells was subjected to sodium bisulfite conversion, PCR amplification of a 519 bp fragment, subcloning and cycle sequencing. Unmethylated CpG sites are indicated by small vertical open ovals and methylated CpG sites are indicated by small filled ovals. At least ten clones from each cell line as indicated were bisulfite sequenced to obtain a representative sampling of methylation patterns in each setting. Transcription factor binding sites are by homology only (except Octamer) and are as indicated: rectangle, EBF; circle, Octamer; triangle, NF- κ B. The cartoon is drawn to scale.

B29 promoter activity in B29-silent GH3 cells was enhanced by co-expression of Bob1

The presence and function of essential transcription factors for *B29* promoter expression in GH3 pituitary cells was first analyzed by transient transfection of unmethylated *B29* promoter reporter constructs. *B29* promoter activity was detected that was statistically significant in *B29*-silent GH3 pituitary cells, albeit at lower levels than in *B29*-expressing Y3Ag1.2.3 B cells (Figure 5; B29). The *B29* promoter octamer motif interacts with ubiquitous Oct-1, B cell-specific Oct-2, and the B cell-specific octamer co-activator Bob1 (OCA-B, OBF-1),⁷ a transactivating transcription factor expressed exclusively in B cells.^{18–20} Co-transfection of the Oct-1 co-activator Bob1 elevated *B29* promoter activity in pituitary cells to levels comparable to B cells (Figure 5; B29/Bob). Oct-1 is a ubiquitous transcription factor expressed at high levels in both Y3Ag1.2.3 B cells and GH3 pituitary cells,²¹ suggesting a role for Oct-1 in pituitary cell expression of the *B29*

promoter. The transfected *B29* promoter containing a mutated octamer site was not active (Figure 5; B29mOct), confirming a role for Oct-1 in pituitary cell expression of the transfected *B29* promoter. Co-transfection of a previously tested transactivation-negative but Oct-1-binding competent mutant Bob1 (Bob Δ C) expression construct²² resulted in no significant transactivation of the *B29* promoter construct as expected (Figure 5; B29/Bob Δ C). The co-transfection of Bob1 in Y3Ag1.2.3 B cells showed no increase in activity presumably due to the high levels of endogenous Bob1 in this cell type (Figure 5; B29/Bob and data not shown).⁷ The addition of Bob Δ C resulted in no significant change in *B29* promoter expression in Y3Ag1.2.3 B cells (Figure 5; B29/Bob Δ C). *B29* promoter constructs containing a mutated octamer site were expressed at lower levels in GH3 pituitary cells and Y3Ag1.2.3 B cells, and these levels were unaffected by co-transfection of Bob1 or Bob Δ C expression constructs (Figure 5; B29mOct, B29mOct/Bob, B29mOct/Bob Δ C). These data indicate that the octamer site



Figure 4. Methylation inhibitor 5-aza does not reactivate the *B29* gene in pituitary cells. Southern blots of *GH* and *B29* RT-PCR gels were used to detect gene expression. GH3 pituitary cells were treated with 5-aza in increasing doses to the maximum tolerated dosage for three days before RNA was prepared, lanes 1–6. Lane 7, mock 5-aza; lane 8, no treatment; lane 9, water; lane 10, Y3Ag1.2.3 myeloma cell RNA; lane 11, GH3 pituitary cell RNA.

is largely responsible for *B29* promoter activity in transfected GH3 pituitary cells, and that the lack of the B cell co-activator, Bob1, in these cells dictates, at least in part, the expression level of the *B29* promoter.

DHS4.4 3' enhancer inhibits activity of the B29 promoter in GH3 pituitary cells

In addition to the functional octamer site identified in the *B29* promoter,⁴ the *B29* *DHS4.4* 3' enhancer contains a consensus octamer site predicted by binding sequence to interact with Bob1.²³ Addition of the *B29* *DHS4.4* 3' enhancer to the *B29* promoter reporter construct resulted in the expected enhance-

ment of activity in *B29*-expressing Y3Ag1.2.3 B cells (Figure 6; B29+DHS). Co-transfection of Bob1 with the *B29* promoter + *DHS4.4* construct in Y3Ag1.2.3 B cells showed no increase in activity presumably due to the high levels of endogenous Bob1 in this cell type (Figure 6; B29 + DHS/Bob and data not shown). The addition of transactivation deficient, but Oct-1-binding competent, Bob1 mutant (BobΔC) resulted in no significant change in *B29* promoter + *DHS4.4* expression (Figure 6; B29 + DHS/BobΔC). Surprisingly, transfection of the *B29* promoter + *DHS4.4* construct into GH3 pituitary cells resulted in strong inhibition of promoter activity compared to the transfection of the *B29* promoter alone (Figure 6; B29 + DHS and B29). In fact, addition of the *DHS4.4* inhibited promoter activity to background levels (Figure 6; basic constructs). Further, co-transfection of Bob1 did not overcome the inhibitory effects of the *DHS4.4* in GH3 pituitary cells (Figure 6; B29 + DHS/Bob), but only increased activity to that of the promoter alone. Addition of BobΔC resulted in no significant change in *B29* promoter + *DHS4.4* expression in GH3 cells as expected (Figure 6; B29 + DHS/BobΔC). These data suggest that the *DHS4.4* 3' enhancer has a key, activating role in the activity of the *B29* promoter in B cells and a novel inhibitory effect on *B29* promoter activity in GH-expressing pituitary cells.

B29 DHS4.4 3' enhancer octamer site interacted with both Oct-1 and Pit-1 from GH3 pituitary cells in EMSA

Analysis of the *DHS4.4* octamer site by electrophoretic mobility shift assay (EMSA) showed a specific interaction with Oct-1 protein (Figure 7),

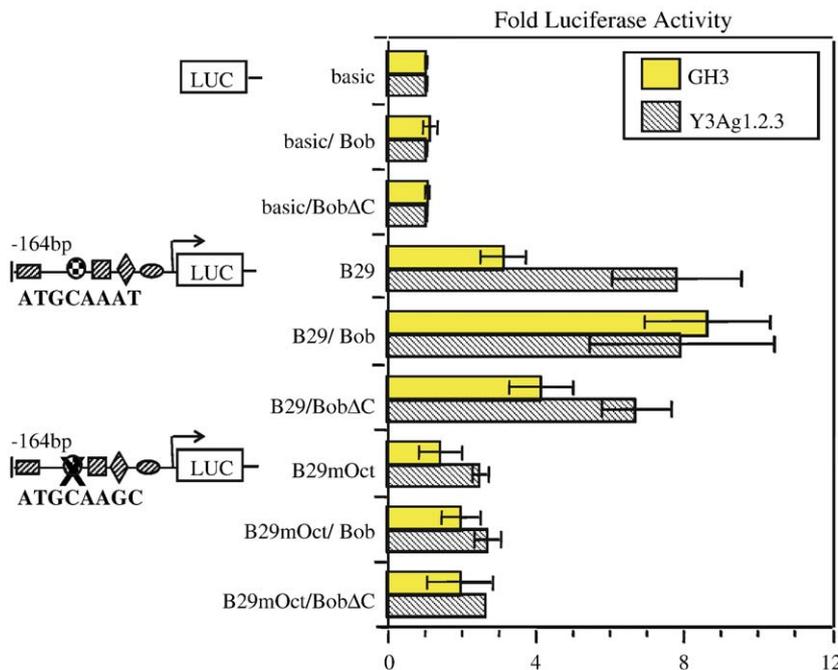
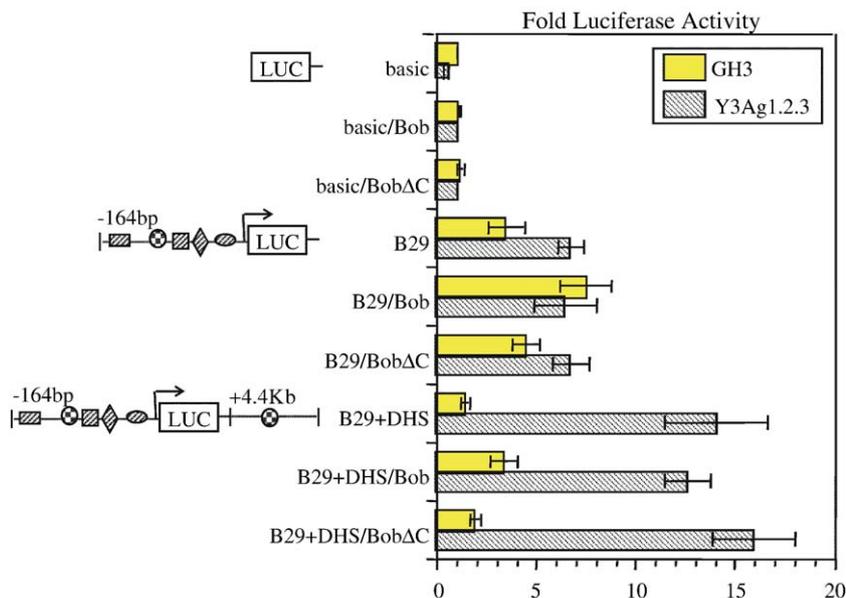


Figure 5. *B29* promoter expression is enhanced by co-expression of Bob1 in pituitary cells. Transient transfections of pGL3 *B29* promoter constructs with and without co-transfection of Bob1 were conducted in *B29*-silent GH3 pituitary cells (shaded bar) and *B29*-expressing Y3Ag1.2.3 myeloma cells (striped bar). The activity of each construct is expressed as the fold activation over the promoterless pGL3 basic firefly luciferase construct. A total of 10 μg of Bob1 expression construct (Bob) or 10 μg of transactivation-negative Bob1 C-terminal deletion mutant construct (BobΔC) was added to transient transfections of the pGL3 basic firefly luciferase construct (basic), the pGL3 *B29* promoter construct (B29), and the pGL3 *B29* promoter with a mutated Octamer motif construct (B29mOct). pGL3 firefly luciferase activities are pRL SV40

renilla luciferase normalized and are the average±SD of at least three independent transfections using at least two preparations of DNA.



pGL3 *B29* promoter and *DHS 4.4* construct (B29+DHS). pGL3 firefly luciferase activities are pRL SV40 renilla luciferase normalized and are the average \pm SD of at least three independent transfections using at least two preparations of DNA.

which was verified by competition with unlabelled octamer site oligonucleotides (lanes 2 and 10) and a lack of competition with unlabelled mutated octamer site oligonucleotides (lanes 3 and 11). Further, the addition of Oct-1 antibody resulted in competition and a supershift of the specific complexes (lanes 6 and 14). Although we assume the Y3Ag1.2.3 myeloma B cell line expresses Oct-2, the Oct-2 antibody did not compete or supershift a complex in Y3Ag1.2.3 under these conditions (Figure 7, lane 7). As expected, the Oct-2 antibody did not compete or supershift a complex in the GH3 pituitary cell line nuclear extracts (Figure 7, lane 15). These results suggest that Oct-1 regulates both the *B29* promoter and *DHS4.4* 3' enhancer in Y3Ag1.2.3 B cells and GH3 pituitary cells.

Pituitary cells express abundant Pit-1 transcription factor, whose consensus binding site is similar to that of the octamer consensus binding site.²¹ Both Pit-1 and Oct-1 interact with A+T-rich *cis*-elements that may deviate from their respective consensus sequences.^{24,25} Figure 7 shows that native Pit-1 specifically interacted with the *B29 DHS4.4* 3' enhancer octamer site, as shown by competition with unlabelled Pit-1 site oligonucleotides (lane 4) and a lack of competition with unlabelled mutated Pit-1 site oligonucleotides (lane 5). Further, the addition of Pit-1 antibody resulted in competition of the Pit-1-specific complex (lane 8). Pit-1 is not expressed in Y3Ag1.2.3 B cells and no Pit-1 complex appears using these extracts (lanes 9–16). *In vitro* transcribed and translated Oct-1 and Pit-1 also interacted with the *DHS4.4* octamer site (data not shown). Native Pit-1 specifically interacted with the *B29* promoter octamer site as well, as shown by competition with unlabelled Pit-1 oligonucleotides, a lack of competition with unlabelled mutated Pit-1 site oligonucleotides, and a competition of the Pit-1

specific complex upon addition of Pit-1 antibody (data not shown). *In vitro* transcribed and translated Pit-1 also interacted with the *B29* promoter octamer site (data not shown). Combined, the data suggest that both Oct-1 and Pit-1 could control the activity of *B29* expression in pituitary cells through exclusive or competitive interactions on functional octamer sites within the *B29* promoter and *DHS4.4*. Augmented activity from co-transfection with Bob1 (Figure 5) also suggests that Oct-1 is bound to at least some of the *B29* promoter reporter constructs even in the presence of Pit-1. Interestingly, the data also strongly suggest a model in which the *B29* promoter octamer site is occupied by Oct-1 at least some of the time, while the *DHS4.4* octamer site is occupied by Pit-1 and/or a powerful Oct-1 antagonist factor(s), since Bob1 transactivation fails in GH3 pituitary cells.

B29 *DHS4.4* 3' enhancer is bound by Oct-1 and Pit-1 *in vivo* in GH3 pituitary cells by CHIP analysis

To corroborate the EMSA data shown above, chromatin immunoprecipitation (ChIP) was performed to determine Oct-1 and Pit-1 binding to the endogenous *DHS4.4* region in the B cell and pituitary cell lines. Figure 8 shows that both Oct-1 and Pit-1 specific antibodies immunoprecipitated the *DHS4.4* region in GH3 pituitary cells (top panel, lanes 3 and 4) whereas the negative control non-specific Ig antibody did not (top panel, lane 2). Oct-1 and Pit-1 specific antibodies were unable to immunoprecipitate the GAPDH promoter that does not contain a consensus octamer binding sequence (top panel, lanes 8 and 9), illustrating the specificity of these ChIP experiments. These data show that both transcription factors are bound to the *DHS4.4* region *in vivo* in GH3 pituitary cells. As expected, only the

Figure 6. Control of *B29* expression by a context-dependent *B29 DHS4.4* 3' enhancer molecular toggle. Transient transfections of pGL3 *B29* promoter and *DHS4.4* 3' enhancer constructs with and without co-transfection of Bob1 in *B29*-silent GH3 pituitary cells (shaded bar) and *B29*-expressing Y3Ag1.2.3 myeloma cells (striped bar). The activity of each construct is expressed as the fold activation over the promoterless pGL3 basic firefly luciferase construct. A total of 10 μ g of Bob1 expression construct (Bob) or 10 μ g of transactivation-negative Bob1 C-terminal deletion mutant construct (Bob Δ C) was added to transient transfections of the pGL3 basic firefly luciferase construct (basic), the pGL3 *B29* promoter construct (B29), and the

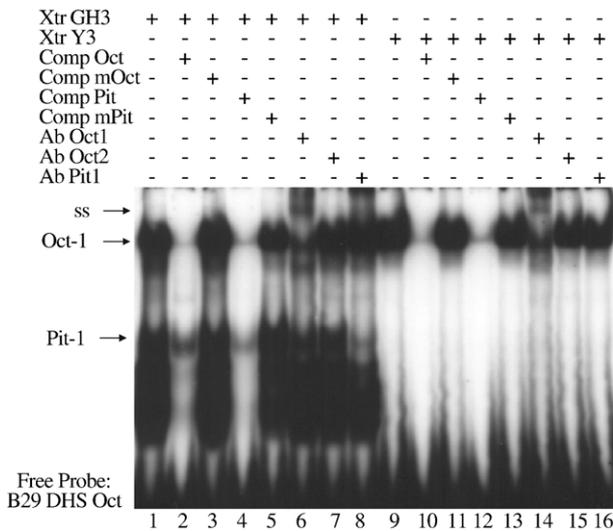


Figure 7. *B29 DHS4.4* 3' enhancer binds Oct-1 and Pit-1 by EMSA. Double-stranded oligonucleotides corresponding to the *B29 DHS4.4* 3' enhancer Octamer site (*B29 DHS Oct*) were end-labelled and used in EMSA. The *B29 DHS Oct* probe was incubated with 20 μ g of GH3 pituitary cell line nuclear extract (lanes 1–8) and 20 μ g of Y3Ag1.2.3 B cell line nuclear extract (lanes 9–16). Reactions were co-incubated in the presence of: 500-fold molar excess of unlabeled *B29 Octamer* motif (Comp Oct, lanes 2 and 10); 500-fold molar excess of unlabeled *B29 mutant Octamer* motif (Comp mOct, lanes 3 and 11); 500-fold molar excess of unlabeled *Pit-1 consensus* motif (Comp Pit, lanes 4 and 12); 500-fold molar excess of unlabeled *Pit-1 mutant consensus* motif (Comp mPit, lanes 5 and 13); 0.5 μ g of anti-Oct-1 antibody (Ab Oct1, lanes 6 and 14); 2 μ g of anti-Oct-2 antibody (Ab Oct2, lanes 7 and 15); and 1 μ g of anti-Pit-1 antibody (Ab Pit1, lanes 8 and 16). Specifically formed complexes are labeled Oct-1 and Pit-1 and denoted by arrows. Antibody supershifted complexes are denoted by SS and an arrow. Free Probe denotes uncomplexed endlabelled *B29 DHS Oct* probe. Results are representative of at least three independent experiments.

Oct-1 antibody immunoprecipitated the *DHS4.4* region in Y3Ag1.2.3 B cells (bottom panel, lanes 7, 8, and 9). This further corroborates the EMSA data shown above and is logical considering that Y3Ag1.2.3 B cells do not express the Pit-1 transcription factor.^{18–20} Taken together, these data suggest that both Oct-1 and Pit-1 bind the *DHS4.4* region *in vivo* in pituitary cells, suggesting a role for these factors in activating and silencing the *B29* promoter in B cells and pituitary cells, respectively.

Discussion

In rat pituitary cells, the *B29* gene, despite being embedded within the DNase I hypersensitive “open” *GH* chromosomal locus, has features of inactive chromatin that include locally-confined H3 and H4 histone deacetylation and a high level of promoter and 3' enhancer CpG DNA methylation reported here.¹⁴ Combined with the lack of a *B29*-

dependent transcription-augmenting factor, the Bob1 co-activator, the expectation was that these epigenetic and genetic constraints were responsible for maintaining *B29* gene silencing. However, the apparent relief of these constraints did not reverse silencing despite promoter studies that indicated essential transcription factors were present and could promote expression and that Bob1 addition augmented this activity, as expected. Instead, a paradoxical role for the *DHS4.4* 3' enhancer region was discovered because *DHS4.4* unexpectedly functioned as a strong silencing element in pituitary cells.

Multiple mechanisms, including histone deacetylation and CpG methylation, likely contribute to the tissue-specific restriction of the rat *B29* gene in *GH*-expressing pituitary cells. DNA methylation patterns of both the *B29* promoter and *DHS4.4* 3' enhancer correlate with the silencing of the *B29* gene in pituitary and non-B cells and the expression of *B29* in B cells. The *B29* promoter and *DHS4.4* are CpG-poor, and are not considered to be part of so-called “CpG islands”. During normal development, the control of expression for several CpG-poor or non-CpG island genes has been determined by DNA methylation analyses.²⁶ It remains unknown how many CpG-poor promoters are actually controlled by CpG methylation, but the *B29* promoter and *DHS4.4* are potential candidates for such regulation. Conversely, CpG-island containing promoters are generally kept free of DNA methylation and not controlled by methylation during normal development.²⁶ There are many examples of CpG-island containing promoters that are controlled by dense CpG methylation in aberrant silencing that occurs during transformation and cancer, but not normal development.²⁶

Epigenetic silencing of genes is a progression of multiple events that includes histone modifications and DNA methylation.²⁷ Genes that are silenced by histone modifications alone are generally reactivated upon treatment with the HDAC inhibitor TSA. Histone modified and densely DNA methylated genes are generally not reactivated by TSA, but can be reactivated by 5-aza which inhibits DNA methyltransferases, with or without TSA.^{16,17,28,29} These chemical treatments have been shown to reactivate many aberrantly or developmentally silenced genes, including the *B29* gene in classical Hodgkin lymphoma cells³⁰ and the *Pax5* gene in terminally differentiated B cell lines,²⁹ respectively. The fact that neither of these treatments reactivated the *B29* gene in the *GH*-expressing and *B29*-silent pituitary cell line was unexpected given the CpG DNA methylation patterns in the *B29*-silent and -expressing cell lines. These data suggested that the existing transcription factor repertoire may be inadequate for expression or responsible for silencing of the *B29* gene in *B29*-silent, *GH*-expressing pituitary cells, even when the chromatin had apparently been “opened” by inhibitors.

The well-characterized mouse *B29* minimal promoter was linked to a luciferase reporter and tested for activity in transfected *GH*-expressing pituitary

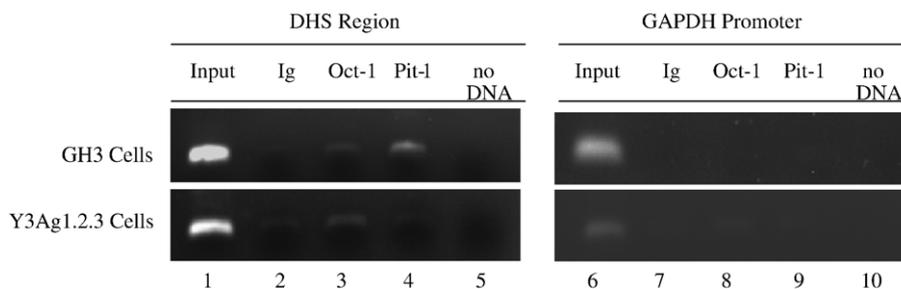


Figure 8. *B29* DHS4.4 3' enhancer binds Oct-1 and Pit-1 by ChIP. Chromatin was purified from GH3 pituitary cells (top panel) and Y3Ag1.2.3 B cells (bottom panel) and used in ChIP analyses. Chromatin was immunoprecipitated using normal rabbit immune sera (Ig, lanes 2 and 7), anti-Oct-1 antibody (Oct-1, lanes 3 and 8), and anti-Pit-1 antibody (Pit-1, lanes 4 and 9). The presence of immunoprecipitated chromatin was determined for the *B29* DHS4.4 3' enhancer region (DHS region, lanes 1–5) and the *GAPDH* promoter negative control (*GAPDH* promoter, lanes 6–10) by PCR using region-specific primers. Lanes 1 and 6 are positive control PCR reactions performed using the purified chromatin before immunoprecipitation (Input) and lanes 5 and 10 are negative control PCR reactions performed without the addition of any chromatin (no DNA).

cells. These transfected constructs remain devoid of both methylation at CpG sites and histone modifications, and should therefore reflect the activity of the *B29* promoter in pituitary cells without these constraints. The minimal *B29* promoter was active, albeit at a low level. This activity (fourfold above a promoterless control) suggests that transcription factors capable of supporting *B29* promoter are present, at least minimally, in *B29*-silent pituitary cells. This *B29* activity is similar to the levels seen in transfections of *B29*-silent T cell and fibroblast cell lines.⁷ *B29* promoter activity comparable to that in B cells was achieved by co-transfection of the B cell-specific co-activator Bob1,^{4,7} which is not expressed in pituitary cells.^{18–20} These data suggest that the low level *B29* promoter construct activity in pituitary cells is at least partly due to a lack of Bob1 co-activator in these cells. They also suggest that lack of Bob1 may have prevented the endogenous *B29* activation with HDAC and methyltransferase inhibitors.

Addition of the *B29* DHS4.4 3' enhancer, which contains a consensus octamer site, was predicted to increase transfected *B29* promoter activity in pituitary cells as in B cells. Genomic bisulfite sequencing analysis revealed the expected pattern of DNA methylation between pituitary cells and B cells. Similar to the *B29* promoter, the DHS4.4 showed high-level methylation at CpG sites in *B29*-silent pituitary and kidney cells and no methylation in *B29*-expressing B cells. From these data, it was expected that addition of DHS 4.4 to the *B29* promoter construct would enhance the activity of the *B29* promoter in both pituitary cells and B cells. Surprisingly, addition of the DHS4.4 strongly repressed *B29* promoter activity in pituitary cells even with co-transfection of Bob1. This paradoxical silencing effect for the *B29* DHS4.4 3' enhancer requires further investigation to resolve its dual roles in controlling tissue-specific expression of the *B29* gene.

The octamer sites in both the *B29* promoter and the DHS4.4 3' enhancer are bound by both Oct-1 and Pit-1 in EMSA and are responsive to Bob1 co-

activation, suggesting that this site plays a pivotal role in *B29* expression in B cells and *B29*-silent pituitary cells. Not all octamer sites that bind Oct-1 are transactivated by Bob1,^{8,23} indicating the importance of this site in *B29* gene regulation. The mechanism of action of the octamer sites in the *B29* promoter and DHS4.4 may involve differential binding of Oct-1/Bob1 and Pit-1 between B cells and pituitary cells, respectively. Pit-1 has been shown to be a potent silencer element as well as its more recognized role as an activator of transcription.³¹ In situations that have yet to be fully delineated, Pit-1 protein dimer bound to DNA recruits the co-repressor N-CoR and inhibits transcription. In the absence of N-CoR co-repressor, Pit-1 dimer bound to DNA is an activator of transcription. The differential activity of Pit-1 dimer is partially based on the Pit-1 binding sequence, but other mechanisms must be involved, since the same GH enhancer Pit-1 site recruits N-CoR in GH-silent pituitary cells but not in GH-expressing pituitary cells.^{21,31} Since the consensus octamer site found in both the *B29* promoter and DHS4.4 varies distinctly from the Pit-1 consensus sequences evaluated in differential N-CoR recruitment studies, it is impossible to predict whether Pit-1 and N-CoR are involved in *B29* gene silencing in pituitary cells.³² Interestingly, the highly methylated DHS4.4 region interacts with both the Oct-1 and Pit-1 transcription factors *in vivo* by ChIP analyses, suggesting that this region is not as "closed" as previously assumed. It is possible that co-transfection of Bob1 did not increase activity of the *B29* promoter and the DHS4.4 3' enhancer construct to greater than promoter alone levels in GH3 pituitary cells due to the presence of Pit-1 binding to the octamer sites in the DHS4.4 and possibly the promoter octamer site as well.

The context-specific inhibitory action of the DHS4.4 may function in concert with *B29* promoter and DHS4.4 methylation to control *B29* gene silencing. It may be possible that the methylation status of the *B29* promoter and DHS4.4 is irrelevant for silencing based on the ChIP results and that the

lack of *B29* gene expression in *GH*-expressing but *B29*-silent pituitary cells is controlled by transcription factor interactions (Pit-1 versus Oct-1/Bob1) and recruitment of co-repressors (N-CoR) at either or both the *B29* promoter and *DHS4.4* octamer-binding sites. The fact that the Oct-1 and Pit-1 transcription factors bind the heavily methylated *DHS4.4* region in pituitary cells adds a complexity beyond simple DNA methylation-induced gene silencing. The striking finding that the *B29 DHS4.4* 3' enhancer functions as a context-dependent molecular toggle switch to activate or repress *B29* opens up the possibility for dissecting the complex combination of genetic and epigenetic factors that control differential expression of the *B29* gene between *B29*-expressing B cells and *B29*-silent pituitary cells.

Materials and Methods

Cell culture, TSA, and 5-aza treatments

Cell lines were maintained at 37 °C in a humidified environment with 5% (v/v) CO₂. The rat GH3 cell line (ATCC) was grown in Ham's F12K (GibcoBRL, Rockville, MD) supplemented with 15% (v/v) donor Horse Serum (Omega, Tarzana, CA), 2.5% (v/v) fetal bovine serum (FBS) (Omega), 2 mM L-glutamine, 10 µg/ml of penicillin, and 10 units/ml of streptomycin. The Y3-Ag1.2.3 cell line (ATCC) was grown in 1.5 g/l sodium bicarbonate DMEM supplemented with 10% fetal bovine serum (Omega), 2 mM L-glutamine, 10 µg/ml of penicillin, and 10 units/ml of streptomycin. The rat NRK kidney cell line (ATCC) was grown in DMEM supplemented with 10% (v/v) calf serum (Omega), 2 mM L-glutamine, 10 µg/ml of penicillin, and 10 units/ml of streptomycin. TSA and 5-aza treatments were essentially as described with the exception of drug concentration optimized for each cell line.³⁰ The GH3 cell line was grown in media supplemented with increasing concentrations of TSA; 0 nM, 200 nM, 400 nM, 800 nM, 1000 nM, 2500 nM or 5-aza; 0 µM, 0.1 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM, 10 µM for three days, exchanging media and drug each day. The Y3-Ag1.2.3 cell line was grown in media supplemented with increasing concentrations of TSA; 0 nM, 10 nM, 15 nM, 25 nM, 50 nM, 100 nM, 200 nM or 5-aza; 0 µM, 0.1 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM, 10 µM for three days, exchanging media and drug each day.

RNA preparation and RT-PCR

Total RNA was prepared from cell lines growing in log phase, TSA treated cell lines, and 5-aza treated cell lines using the RNeasy Miniprep kit with the Qiashredder (Qiagen, Valencia, CA). The primers used for RT-PCR are 5' to 3' as follows: GH forward GCCTGCTGCC-TGC, GH reverse GACTGGATGAGCAGCAG; B29 forward AGAAAAGTTGCAGCCCGTGC, B29 reverse TTGATGGTCCAACCTCAGATGC; GAPDH forward GATGACATCAAGAAGGTGGTG, GAPDH reverse GTCATACCAGGAAATGAGCTTG. Total RNA (200 ng) was subjected to the following RT-PCR conditions using the SuperScript One-Step RT-PCR System with PLATINUM Taq polymerase (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 for 7 min 30 s. B29: 50 °C for 30 min, 94 °C for 2 min; 35 cycles of 94 °C for 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 1% (w/v) agarose gels and subjected to Southern blotting by standard procedures using Spotlight random primer labelled probes and chemiluminescent detection system (BD Biosciences, Palo Alto, CA).

Genomic bisulfite sequencing

Genomic bisulfite sequencing was performed as described.³⁰ Primers used for generating bisulfite treated clones are 5' to 3' as follows:

B29 promoter 5', GTAGTAATATTATAGTTATGAAAGTAG-TAAT,
 B29 promoter 5' nested, GGGGAGGGGTTTTTATAGGAT-TATTAGGAAT,
 B29 promoter 3', CCAATAACAAAACACAAAAACAA-CACCAA,
 B29 promoter 3' nested, CATAATCACTACTCTATCTC-TAAACCCAAA,
 B29 DHS 5', GTATGTGGGAAAGATTGAGATTATAGATGTT,
 B29 DHS 5' nested, GTTGTGGATTAGGTAGGTGAATTT-TAGAT,
 B29 DHS 3', CCCTCTTAAAACAAAACACACCCCAAA,
 B29 DHS 3' nested, CTCCTCCATCAAAAACAAAATAAT-TACTACCC.

4 µl sodium bisulfite-treated DNA was subjected to the following PCR conditions using Taq DNA polymerase (Fisher Scientific): Round 1 PCR, 95 °C for 3 min; 50 cycles of 94 °C for 30 s, 50 °C for 30 s, 70 °C for 2 min 30 s; 72 °C for 10 min. Nested PCR, 95 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 70 °C for 2 min 30 s; 72 °C for 10 min. Resulting PCR products were resolved on 1% BioGel (Q-BIOGene, Carlsbad, CA) 1XTAE gels, excised, purified using Gene Clean (Q-BIOGene), and ligated and transformed using TOPO-TA Cloning (Invitrogen). Plasmids were purified by Wizard minipreps (Promega, Madison, WI) and sequenced using M13 reverse sequencing primers, G-50 auto Seq purified (Amersham) and sequenced (Laragen, Los Angeles, CA).

Plasmid constructs, transient transfections, and luciferase assays

B29 promoter constructs used in transient transfection of Y3Ag1.2.3 B cells were pGL3 basic vectors (Promega) with the promoter sequences inserted HindIII to SacI, pGL3B29 and pGL3B29mOct.⁷ *B29* promoter constructs used in transient transfection of GH3 pituitary cells were pRL null vectors (Promega) with the promoter sequences inserted HindIII to SacI, pRLB29 and pRLB29mOct. *B29* promoter and *DHS4.4* 3' enhancer used in transient transfection of Y3Ag1.2.3 B cells were pGL3B29 with the *DHS4.4* sequences inserted into BamHI, pGL3B29 + DHS and normalized to the simian virus 40 (SV40) promoter and enhancer construct pRL SV40 (Promega). *B29* promoter and *DHS4.4* 3' enhancer used in transient transfection of GH3 pituitary cells were pRLB29 with the *DHS4.4* sequences inserted into BamHI, pRLB29 + DHS and normalized to the SV40 promoter and enhancer construct pGL3 control (Promega). Bob1 and Bob1ΔC

expression constructs were as described.³³ Y3Ag1.2.3 B cells and GH3 pituitary cells were transfected using the Effectene transfection method as described (Stratagene) using 600 ng of pGL3 vectors and 400 ng of pRL vectors. Bob1 and Bob Δ C constructs were used at 1 μ g and total amounts of DNA between samples were normalized by the addition of 1 μ g of pBluescript where Bob1 and Bob Δ C constructs were not used. Transfections were harvested 40–48 h post-transfection and dual luciferase assays were performed as described in the Dual Luciferase Reporter Assay System (Promega). All values are \pm SD of at least three transfections using at least two preparations of DNA.

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

Crude nuclear extracts were prepared as described³⁴ Protein concentration was determined by the Bradford protein assay (BioRad Laboratories, Hercules, CA). Oct-1, Oct-2 and Pit-1 *in vitro* translated protein (IVT) was prepared with TNT T7 coupled Transcription-Translation System (Promega).

Electrophoretic mobility shift assays (EMSA)

EMSA as described⁴ was performed with the exception of 5.5% of 50:1 polyacrylamide/bis-acrylamide; 0.5 \times TBE gels were run at 125 V for 2.5 h at room temperature. The 0.5 to 2 μ l Oct-1, Oct-2, and Pit-1 antibodies (Santa Cruz Biotech, Santa Cruz, CA) used in EMSA were incubated in the binding reaction at 4 $^{\circ}$ C overnight. EMSA probes were double-stranded oligonucleotides 5' end-labelled with [γ -³²P]ATP. EMSA probes were purified by G25 Sephadex spin column chromatography (Amersham). EMSA complementary double-stranded oligonucleotide probes 5'–3' were as follows:

B29 Oct GTCTCAATTTGCATGGCAGG;
 B29 mOct TCCCCTGGGTCTCACTCTAGAGGGCAG-
 GAAGGGGCCT;
 Octamer TGTCGAATGCAAATCACTAGAA (Santa Cruz Biotech);
 mOctamer TGTCGAATGCAAGCCACTAGAA (Santa Cruz Biotech);
 Pit-1 TGTCTTCTGAATATGAATAAGAAATA (Santa Cruz Biotech);
 mPit-1 TGTCTTCTTCTTCTTCTTCTAAGAAATA (Santa Cruz Biotech);
 B29 DHS Oct TTCCTGCTATTTGCATACTCAGGC.

Chromatin immunoprecipitation

A total of 200 \times 10⁶ GH3 or Y3Ag1.2.3 cells were fixed at room temperature for 10 min by adding formaldehyde directly to the culture medium to a final concentration of 1%. The reaction was quenched by adding glycine at a final concentration of 0.125 M for 5 min at room temperature. After two ice-cold PBS washes, the cells were collected and lysed for 10 min on ice in cell lysis buffer (5 mM Pipes (pH 8.0), 85 mM KCl, 0.5% (v/v) NP-40, protease inhibitors). The nuclei were resuspended in nucleus lysis buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% (w/v) SDS, protease inhibitors) and incubated on ice for 10 min. Chromatin was sheared into 500 to 1000 bp fragments by sonication and was then pre-cleared with protein A-Agarose beads. The purified chromatin

was diluted with chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, protease inhibitors) and immunoprecipitated overnight at 4 $^{\circ}$ C using the anti-Pit-1 (BABC0), the anti-Oct-1 (Santa Cruz) or normal rabbit immune sera. Immune complexes were collected with protein A-Agarose beads and were then washed and eluted. After protein-DNA cross-linking was reversed and the DNA was purified, the presence of selected DNA sequences was assessed by PCR. The primer pairs were as follows: DHS region 5'-CTGCCAGAGG-GAACCAGCTT-3' and 5'-CTGGCTGCCTGGTCCC-TAGT-3'; and GAPDH promoter 5'-CCTGGTTCCTGC-AGCTCTCA-3' and 5'-TGGAGTGTGCACCAAGGACA-3'. The PCR products were resolved on a 2% agarose gel.

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