The immunoglobulin heavy-chain matrix-associating regions are bound by Bright: a B cell-specific *trans*-activator that describes a new DNA-binding protein family

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B lymphocyte-restricted transcription of immunoglobulin heavy-chain (IgH) genes is specified by elements within the variable region (V_H) promoter and the intronic enhancer (E_μ). The gene encoding a protein that binds a V_H promoter proximal site necessary for induced μ -heavy-chain transcription has been cloned. This B-cell specific protein, termed Bright (B cell regulator of IgH transcription), is found in both soluble and matrix insoluble nuclear fractions. Bright binds the minor groove of a restricted ATC sequence that is sufficient for nuclear matrix association. This sequence motif is present in previously described matrix-associating regions (MARs) proximal to the promoter and flanking E_μ . Bright can activate E_μ -driven transcription by binding these sites, but only when they occur in their natural context and in cell lines permissive for E_μ activity. To bind DNA, Bright requires a novel tetramerization domain and a previously undescribed domain that shares identity with several proteins, including SWI1, a component of the SWI/SNF complex.

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Chromatin fibers are normally condensed by histones into nucleosome subunits (for review, see McGhee and Felsenfeld 1980) and then organized into looped domains by attachment to the nuclear matrix (for review, see Gasser and Laemmli 1987). The matrix- or scaffold- associating regions (MARs or SARs) have been defined by in vitro-binding activity to nuclear matrix preparations (Bowen 1981; Mirkovitch et al. 1984; Cockerill and Garrard 1986). MARs may function as boundary elements for transcriptional domains through either physical anchoring or insulating mechanisms (Gasser and Laemmli 1986; Bode and Maass 1988; Phi-Van and Stratling 1988; Kellum and Schedl 1991). However, some MARs can confer position-independent expression of associated genes and create nucleosome-altered environments, suggesting an active role in gene regulation (Grosveld et al.

1987; Stief et al. 1989; Forrester et al. 1990, 1994; Phi-Van et al. 1990; McKnight et al. 1992; Jenuwein et al. 1993).

Tissue-restricted transcription of the immunoglobulin heavy-chain (IgH) gene is controlled by promoter and enhancer elements (Staudt and Lenardo 1991). The heavy-chain enhancer (E_μ) is located in the intron between the J and the constant region gene segments, and functionally consists of two regions; the core and the flanking MARs (Banerji et al. 1983; Gillies et al. 1983; Adams et al. 1985; Cockerill et al. 1987). The core segment has binding sites for numerous transcription factors, and although several of these can *trans*-activate reporter genes, none can activate E_μ -driven IgH transcription efficiently in nonlymphoid cells (Lenardo et al. 1987; Kiledjian et al. 1988; Gerster et al. 1990; Nelsen et al. 1990, 1993; Staudt and Lenardo 1991; Libermann and Baltimore 1993; Rivera et al. 1993). This suggests that one component of tissue restriction may depend on suppression of this locus in non-B cells. The MARs, as well as sites within the core, have been implicated in locus down-regulation (Cockerill et al. 1987; Imler et al. 1987; Wein-

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berger et al. 1988; Scheuermann and Chen 1989; Genetta et al. 1994). In one model, non-B cell factors would bind the MARs and limit core accessibility (Scheuermann 1991; Dickinson et al. 1992).

However, recent work suggests that these MARs facilitate enhancer function in B cells by impacting chromatin structure. In virus-transformed pre-B cells, transgenic $E\mu$ constructs required both flanking MAR sequences for DNase I hypersensitivity and high-level transcription (Jenuwein et al. 1993). A follow-up study in vivo demonstrated that although the core was necessary for locus transcription, IgH transgenes lacking the MARs lost both position-independent high level expression and extended domain DNase I hypersensitivity (Forrester et al. 1994). Thus, the $E\mu$ core in concert with the MARs appears to function as a locus control region (LCR) similar to the LCR of the human β -globin gene (Forrester et al. 1987; Jarman and Higgs 1988; Talbot et al. 1989).

We have developed a system in which a B cell line (BCg3R), transfected with sequences encoding the heavy and light chains of a phosphorylcholine-specific antibody, responds to a combination of antigen plus interleukin (IL) 5 (Webb et al. 1989). The response, an increase in the amount of μ -heavy-chain transcription, resembles that seen when normal B cells are exposed to these stimuli (Alderson et al. 1987; Swain et al. 1988). Subsequent experiments correlated this transcriptional response with induced binding of a B-cell specific complex upstream of the S107 variable region (V_H) promoter,

at -125 to -251 (Tx125) and -424 to -574 (Bf150) from the transcriptional start (Webb et al. 1991a). Sequence analysis revealed both Bf150 and Tx125 to be AT rich and the entire region was shown to function as a MAR (Webb et al. 1991b). Using a Bf150 site concatamer we have cloned a cDNA encoding this B cell-specific protein, termed Bright. Bright has two newly described domains that confer multimerization and DNA binding. We will show that Bright binds the minor groove of a consensus MAR sequence proximal to the S107 V_H promoter and flanking $E\mu$, and that it *trans*-activates gene expression driven by $E\mu$. These observations provide the first evidence for transcriptional regulation by a MAR-binding protein.

Results

The rearranged S107 IgH locus contains three previously defined MARs (Cockerill et al. 1987; Webb et al. 1991b), and several protein-binding sites have been localized in these regions (Fig. 1). A mature B cell complex induced by antigen plus IL-5 binds Bf150 and Tx125 in the promoter MAR (Webb et al. 1991a). NF- μ NR, a complex absent in mature B cells, binds four sites (P1, P2, P3, and P4) in the MARs flanking $E\mu$ (Scheuermann and Chen 1989). SATB1, a T-cell protein, binds sites I–VI with notable overlap at the P2, P3, and P4 sites in regions of high base-unpairing potential (Kohwi-Shigematsu and Kowhi 1990; Dickinson et al. 1992). We have found that Bf150,

Figure 1. Schematic diagram of the murine S107 IgH locus. Rearranged variable, diversity, junctional segments [$V(D)J_1$] and intronic enhancer ($E\mu$) are drawn to scale. MARs and promoter proximal fragments Bf150 and Tx125 are delineated. The 992-bp enhancer fragment is exploded with protein-binding sites marked for the core (open symbols) and MARs (solid symbols). (CAA reactive) High base unpairing potential. The $\Delta E\mu$ and MAR fragments used in subsequent experiments are shown below.

Tx125, and P2 all cross-compete for binding to both the mature B cell factor and NF- μ NR (B. Fishel and C. Das, unpubl.).

Cloning of Bright

To isolate the mature B cell factor we concatamerized a duplexed Bf150 oligonucleotide to seven repeats and screened 10^6 plaques of a λ Zap BCL1 cDNA library by the method of Singh et al. (1988). Three of the clones isolated are shown in Figure 2A. Clone X81 contained a complete 5' end and clone B13 had a complete 3' end with a poly(A) tail. The full-length cDNA of 4843 nucleotides, shown in Figure 2B, was obtained by ligating X81 and B13 at their common AgeI site. The entire sequence consists of a GC-rich leader (261 nucleotides), an open reading frame encoding 601 amino acids, and a long 3'-untranslated region (UTR) (2779 nucleotides) containing

two consensus poly(A) sites. The primary amino acid sequence contains an acidic region (amino acids 128–164), a carboxyl terminus rich in serine and threonine (amino acids 485–601), an alanine-rich stretch with glutamine repeats (amino acids 427–453), but no identifiable DNA-binding motif. We named this protein Bright for B cell regulator of immunoglobulin heavy-chain transcription.

Bright reconstitutes the mature B cell complex

In Figure 3A two protein complexes in nuclear extracts from transformed lymphocyte lines bind the DNA fragment Bf150. Specific binding for the faster migrating B cell complex has been demonstrated previously (Webb et al. 1991a), and this experiment again shows its predominance in mature B cells (lanes 2–5) and induction after antigen plus IL-5 stimulation (lanes 2,3). Equivalence of

Figure 2. Physical characterization of Bright. (A) Maps of full-length cDNA and three representative clones. Bars designate coding (solid) and untranslated (open) regions, with restriction sites indicated. (B) Nucleotide and amino acid sequence of the full-length cDNA. Shown are the acidic region (underlined), the nuclear localization signal (bold/italics; Robbins et al. 1991), the alanine-rich region (bold), and two domains necessary for DNA binding, the conserved domain (single bold box) and the multimerization domain (lighter separated boxes). Two polyadenylation sites in the 3' UTR are boxed.

Figure 3. Bright is identical to the B cell complex described by Webb et al. (1991a). (A) EMSA with transformed lymphocyte nuclear extracts bound to labeled Bf150 DNA. Two protein complexes (indicated by arrows) show a reciprocal binding pattern with induction of the mature B cell complex in 1 μ g of extract from BCg3R cells stimulated with antigen plus interleukin 5 ($\pm$), compared to a 1- μ g extract from unstimulated cells ($\pm$). (B) EMSA with Bright in vitro translate and transformed lymphocyte nuclear extracts bound to labeled Tx125 DNA. Rabbit sera (indicated above lanes) were preincubated at 1:1000 dilution. Anti-Bright serum supershifts the Bright (lane 4) and mature B cell complex (lane 8) but not the NF- μ NR complex in pre-B (lane 11) and T cells (lane 14).

stimulated and control BCg3R extracts was determined by protein concentration and binding of octamer complexes (data not shown). The slower migrating complex in pre-B and T cells has a mobility consistent with NF- μ NR binding (lanes 6–12) (Scheuermann and Chen 1989).

To determine whether Bright was a component of either complex, we produced rabbit antisera against bacterial glutathione *S*-transferase (GST) fusion proteins and added it to the binding reactions (Fig. 3B). In vitro-translated Bright protein and the mature B cell complex bind labeled probe with identical mobility (lanes 1,5). Neither complex is affected by antiserum generated against an irrelevant GST-fusion protein (lanes 2,6) or by preimmune serum (lanes 3,7). However, anti-Bright serum supershifts both the recombinant Bright and mature B cell complexes (lanes 4,8), whereas the slower migrating pre-B and T cell complexes remain unaffected (lanes 11,14). These results demonstrate that Bright alone is sufficient to reconstitute the mature B cell complex and that it is distinct from the NF- μ NR complex seen in pre-B and T cell lines.

Bright expression is lineage and developmental stage restricted

Bright's expression pattern was verified by detection of both mRNA and protein from transformed lymphocyte lines. In Figure 4A, a Northern blot with 32 P-labeled Bright cDNA detects a 5-kb message in poly(A)⁺ RNA (odd-numbered lanes). This message accumulates in mature B cells but not in pre-B or T cell lines. These results are consistent with the full-length cDNA size and the expression pattern predicted from the mobility-shift experiments. Smaller transcripts corresponding to utilization of the upstream poly(A) site have not been detected. A ribonuclease protection assay, shown in Figure 4B, confirmed Bright message accumulation in mature B cells (lanes 8–12) but not in T cells (lanes 16–18) or immature B cells (lanes 13–15). Ribonuclease protection of mouse tissue RNA detected Bright transcripts only in testes (Fig. 4B, lane 7), although more recent nuclease S1 experiments have detected transcripts in lipopolysaccharide-stimulated splenocytes (data not shown).

Western blotting of cell line nuclear extracts, shown in

Figure 4. Bright expression is lymphoid stage and lineage restricted. (A) Northern blot of 10 µg of poly(A)⁺ (odd lanes) or 15 µg of total (even lanes) RNA from transformed lymphocytes with ethidium-stained gel shown below. A Bright cDNA probe detects a 5-kb band in mature B cells. (B) RNase protection of total RNA isolated from mouse tissues, B cell, pre-B cell, and T cell lines. β-Actin controls are shown below. A Bright cDNA fragment (bottom left) was used to synthesize a 223-nucleotide antisense probe that protects a 125-nucleotide species in testis (lane 7) and mature B cells (lanes 8–12). (C) Western blot with anti-Bright serum. Bright protein is detected as a 75-kD band in 1 µg of in vitro translate (lane 1), 10 µg of insoluble nuclear matrix (* lane 6), and 25 µg of soluble nuclear extract from antigen plus IL-5 stimulated (‡, lane 7) or unstimulated (+, lane 8) BCg3R cells and two other mature B cell lines (lanes 9,10). This band is not detected in 25 µg of soluble nuclear extract from pre-B (lanes 11–15) and non-B cell lines (lanes 2–5).

Figure 4C, revealed a 75-kD band consistent with the predicted amino acid sequence size. Similar to mRNA accumulation, Bright protein is detected in mature B cells (lanes 6–10) but not in pre-B (lanes 11–15), T or non-lymphoid lines (lanes 2–5). Bright is also present in the insoluble nuclear matrix fraction isolated from induced BCg3R cells (Fig. 4C, lane 6) and J558 plasma cells (data not shown). The induced binding activity seen after antigen plus IL-5 stimulation (Fig. 3, lanes 2,3) does not appear to be a reflection of increased protein (Fig. 4C, lanes 7,8). There are numerous phosphorylation sites present in Bright, indicating that post-translational mod-

ification may be important for changes in binding activity.

Bright binds in the minor groove and recognizes AT/ATC sequences flanking the promoter and E_μ core

The T cell protein SATB1 binds AT-rich MAR sequences, characterized by all A's, T's, and C's on one strand (ATC sequence), by recognizing a specific sugar-phosphate structure in the minor groove (Dickinson et al. 1992). Knowing that Bright bound MAR sites proximal to the promoter (Bf150, Tx125) and 5' of the en-

Figure 5. Bright binds the minor groove of DNA. EMSA with 10 ng of Bright in vitro translate (lanes 2–7) and 50 ng of a *forkhead* domain GST–fusion protein (lanes 8–13) bound to labeled Tx125 DNA. When added at the indicated dilutions, the minor groove binder distamycin inhibits Bright, but not *forkhead* domain binding.

hancer (NF- μ NR, P2; and SATB1, II), we suspected it might also bind in the minor groove. Distamycin A binds the minor groove of DNA and inhibits several MAR–protein interactions in vitro, including those of SATB1 (Adachi et al. 1989; Kas et al. 1989; Dickinson et al. 1992). In Figure 5, distamycin A completely inhibits Bright binding to a Tx125 probe at ≥ 5 mM (lanes 3–7). The protein QRF-1 contains a *forkhead* domain and binds Tx125 at a different site as both a monomer and dimer, primarily through major groove contacts (Clark et al. 1993; Li and Tucker 1993). Distamycin A, even at 100 mM, has little effect on QRF-1 dimer binding and is unable to block monomer binding (Fig. 5, lanes 9–13). These results show clearly that Bright requires a minor groove interaction to bind its target sequences.

Native binding sites for Bright in the $E\mu$ MARs were identified with mobility-shift competitions. In Figure 6A, the three IgH locus MARs inhibit Bright binding at 30-fold molar excess (lanes 2,3,5). This inhibition is lost when the NF- μ NR sites are deleted from the 5' (Δ P1, P2) and 3' (Δ P3, P4) $E\mu$ MARs (lanes 4,6). We then determined Bright's affinity for these sites. In Figure 6B, Bright binds the $E\mu$ 5' MAR (lane 1), and self-inhibition (lanes 2–4) depends solely on P2, as the Δ P1 fragment inhibits binding completely (lanes 8–10), whereas the Δ P2 fragment does not (lanes 5–7). In Figure 6C, Bright binds the $E\mu$ 3' MAR (lane 1), and self-inhibition (lanes 2–4) depends primarily on P3 (Δ P4; lanes 5–7) and to a lesser extent on P4 (Δ P3; lanes 8–10). In Figure 6D, mobility shift with labeled Δ P fragments demonstrates further Bright's affinity for these sites as P3 (lanes 7–9), $\geq$ P2 (lanes 4–6), $>$ P4 (lanes 10–12), $>>>$ P1 (lanes 1–3).

Oligonucleotide competitions were used to localize binding targets within the promoter fragments Bf150 and Tx125 and the enhancer fragment P2. The oligonucleotide sequences are shown in Figure 6E (BF, TX, and P2). In Figure 6A, concatamers of these oligonucleotides inhibit Bright binding at 10-fold molar excess (lanes 7,8,10), whereas concatamers of a low-affinity oligonucleotide TXD and a mutated P2 oligonucleotide do not (lanes 9,11). Alignment of tested sequences is shown in

Figure 6E. Those with high affinity (P2, P3, BF, TX) contain three features: (1) a core hexamer of AATTAA or AATAAA in an AT/ATC run defined by (2) ≥ 13 bp of ATC sequence that contains (3) a second AT dimer ≤ 6 bp from the hexamer. Of note, the SATB1 footprint falls on top of the AT dimers and extends into the core hexamer at the P2 and P3 sites (Fig. 6E).

Bright binds AT/ATC sequences containing a core hexamer

To further characterize Bright's recognition sequence, we performed a series of cyclic amplification and selection of target (CASTing) experiments (Funk and Wright 1992). Twenty-seven unique sequences were isolated by the CASTing protocol, and their relevant portions are aligned in Figure 7A. In all 27 isolates, the full sequence was $>70\%$ AT rich and contained at least one hexamer consisting of AATTAA, AATAAA, or GATTAA. In 24 isolates, all three features suggested by native site alignment were present. Isolates not fulfilling all three criteria are shown below the consensus in Figure 7A; of these, two had degenerate core hexamers in the AT/ATC run and only one failed to have an AT/ATC run ≥ 13 bp.

To confirm the binding consensus, we determined Bright's affinity for P2 oligonucleotide mutations concatamerized to three repeats in a mobility-shift assay. The mutated oligonucleotides are aligned in Figure 7B. As shown in Figure 7C, a 2- or 3-base change in the core hexamer (mutations 1,3,4) has the greatest impact on binding. A single-base change in the core (mutation 5) has much less effect. When a single-base core change is coupled with loss of the AT dimer (mutation 2) or shortening the AT/ATC run <13 bp (mutation 6), binding activity is affected severely. Shortening the AT/ATC run to 15 bp (mutation 10) has little effect, but shortening it to 12 bp (mutation 8) affects binding dramatically. Mutations 7 and 9 add an additional AT dimer within 6 bases of the core, and as expected, this change augments binding compared to the wild-type concatamer [wt₍₃₎]. These results support the binding consensus suggested

Figure 6. Bright binds specific MAR sites proximal to the promoter and flanking Eμ. (A–D) EMSA with 10 ng Bright in vitro translate, labeled probe, and cold competitor fragments preincubated at the indicated molar excess or titrated at 10-, 20-, and 30-fold excess. MAR fragments are described in Fig. 1. Binding is inhibited by the S107, Eμ5', and Eμ3' MARs and by concatamers of BF, TX, and P2 site oligonucleotides in A; by the Eμ5' and ΔP1 MARs in B; and by the Eμ3', ΔP4, and ΔP3 MARs in C. In D excess poly [d(I–C)] does not inhibit binding to sites P2, P3, and P4 in the ΔP1, ΔP4, and ΔP3 MARs, respectively. (E) Alignment of sites. P1, P2, P3, and P4 are Eμ MAR sites footprinted by NF-μNR. Overhead shaded bars show SATB1 footprints. BF, TX, and TXD are from the Bf150 and Tx125 fragments in the S107 promoter MAR. Summarized features (check mark indicates present, dash indicates absent) include core hexamer (underlined reverse image), ATC sequence ≥13 bp (underlined black), and AT dimer repeats (underlined thick white). Sites with the highest affinity for Bright as determined above contain all three features.

by native site alignment. Thus Bright, like SATB1, requires an ATC sequence for binding; however, Bright also requires a minimum ATC length of 13 bp, a core consensus hexamer, and two closely spaced AT dimers.

The AT/ATC sequence bound by Bright is a matrix attachment site

The ATC sequence bound by SATB1 has been shown to be present in MARs from different species and may represent a consensus recognition site for nuclear matrix association (Dickinson et al. 1992). However, it is unclear whether additional features are required. We took a concatamerized wild-type P2 oligonucleotide [P2-site₍₅₎] and tested its affinity for isolated nuclear matrix as compared to concatamers of two mutated P2 oligonucleotides: one that bound Bright strongly [mutation 10: P2-

mut.10₍₄₎] and one that bound Bright weakly [mutation 2: P2-mut.2₍₈₎] (see Fig. 7B,C). In Figure 8A, mutation 2 (lanes 1,2) can be seen to partition almost entirely in the supernatant fraction (Sn), indicating poor affinity for the nuclear matrix, whereas mutation 10 (lanes 3,4) and P2 wild type (lanes 5,6) partition predominately in the matrix-bound fraction (B). As shown in Figure 7B, all three oligonucleotides have ATC runs ≥13 bp; however, mutation 2 has lost an AT dimer. In Figure 8B, the addition of cold P2 wild type at 100-fold molar excess (lanes 3,4) significantly inhibits matrix association of labeled MAR fragments, whereas cold mutation 2 at 100-fold molar excess (lanes 5,6) has no effect.

A brief inspection of all six SATB1 sites in the Eμ MARs (Dickinson et al. 1992) and the two Bright sites in the S107 5' promoter MAR (see Fig. 6E) reveals the presence of at least two closely spaced AT dimers within an

Figure 7. Bright binds a core hexamer in the context of an AT/ATC sequence. (A) Sequences isolated by CASTing are aligned. Shown are core hexamer (bold), ATC run (underlined in black, length listed at right), AT dimers (underlined in white), and a consensus site matching 24 of the 27 isolates. (B) Sequence alignment of P2 oligonucleotide mutations numbered 1–10. Shown are mutated bases (reverse image) and presence (check mark) or absence (dash) of recognition features. (C) EMSA with 10 ng of Bright in vitro translate bound to concatamerized P2 mutations (three repeats) and wild-type oligonucleotide (five and three repeats). Extensive core mutations (lanes 1,3,4) and mutations affecting the AT dimer (lane 2) or ATC sequence (lanes 6,8) have the greatest impact on binding.

ATC run. This observation, coupled with the above results, suggests that the AT/ATC sequence, in addition to being a requirement for Bright binding, defines a consensus MAR recognition site.

Bright trans-activates only in the context of an active wild-type IgH enhancer

In stable transfections, the promoter proximal site Tx125 was shown to be necessary for inducible heavy-chain transcription (Webb et al. 1991a). However, in a transient system Bright could not *trans*-activate an expression vector containing a multimerized binding site [BFsite₍₇₎-CAT; data not shown]. We then asked if Bright could *trans*-activate through an intact IgH enhancer, reasoning that the Bright sites, located upstream of the promoter and flanking the enhancer core, might be control regions rather than typical promoter or enhancer elements. The 992-bp Eμ fragment (see Fig. 1) was cloned upstream of the ubiquitous promoter [thymidine kinase

(tk)] in a chloramphenicol acetyltransferase (CAT) expression vector. When cotransfected with this construct, Bright increased CAT expression five- to sevenfold over control values in both the plasma cell line J558 and the T cell line EL4 (Table 1). Eμ was barely active in the B cell line M12.4 and accordingly, *trans*-activation by Bright was less apparent, being only twofold over control values (Table 1).

To ensure that *trans*-activation was specific for Bright binding, we tested a CAT vector with the ΔEμ fragment cloned upstream of the tk promoter. All of the Bright and NF-μNR-binding sites are deleted from this fragment (see Fig. 1). Compared to Eμ, ΔEμ was still an efficient enhancer and in control experiments it increased CAT expression 2.7-fold in EL4 and 1.5-fold in J558. However, in the absence of its binding sites Bright could not *trans*-activate this vector (Table 1).

Previous studies demonstrated that Eμ is active in J558, M12.4, and EL4, but not in the T cell line BW5147 (Scheuermann and Chen 1989; Nelsen et al. 1990). As

Figure 8. The AT/ATC sequence is bound by the nuclear matrix. (A) Unbound supernatant (Sn) and matrix-bound (B) fractions are shown side by side from a MAR-binding assay with labeled concatamers of P2 mutation 2 (lanes 1,2), P2 mutation 10 (lanes 3,4), and P2 wild type (lanes 5,6). Mutation 2 has an ATC run ≥ 13 bp but lacks an AT dimer and partitions in the Sn fraction. (B) MAR-binding assay as above with labeled E μ MAR fragments (left) and cold competitor fragments (reverse image above lanes added). P2 wild type inhibits E μ MAR binding (lanes 3,4), whereas mutation 2 does not (lanes 5,6).

anticipated, background levels of E μ -CAT activity that are unaffected by Bright cotransfection were observed in BW5147 cells (Table 1). Thus, Bright-directed *trans*-activation requires the presence of its binding sites in the context of an active enhancer element.

Bright contains a previously undescribed domain necessary for DNA binding

A search of the PIR data base yielded a limited region of homology among Bright and two human proteins, modulator regulatory factors (MRF) 1 and 2A (B. Whitson, T.H. Huang, B.W. Merrills, T. Asai, and K. Itakura, in prep.). These proteins share an identity of 30%–35% (Fig. 9), similar to that seen among helix–loop–helix proteins of different families. Two other proteins, the yeast transcription factor SWI1/Adr6 (Taguchi and Young 1987; Peterson and Herskowitz 1992) and human retinoblastoma-binding protein RBP1 (Fattaey et al. 1993), also share this region of identity (Fig. 9). The most impressive homology is with the *Drosophila* protein, Dead ringer (dri) (S. Gregory, R.D. Kortschak, B. Kalionis, and R. Saint, in prep.). The region of homology between Bright and dri stretches over 132 residues and is 78% identical (Fig. 9). With conservative replacements taken into account, the degree of similarity rises to 87%. This remarkable conservation is comparable to that seen in homeo domains but extends over a larger region (Scott et al. 1989).

MRF1, MRF2A, and dri all bind AT-rich DNA; there-

fore, we tested the importance of the conserved domain for Bright binding. A series of cDNA deletion constructs (Fig. 10A) were translated in vitro and assayed for binding activity in mobility-shift experiments. Figure 10B demonstrates that 5' deletions extended just amino-terminal of the conserved domain in constructs 1 ($\Delta 1$ –178), 2 ($\Delta 1$ –215), and 3 ($\Delta 61$ –224) do not affect binding activity, whereas deletions into the conserved domain do in constructs 4 ($\Delta 61$ –244) and 6 ($\Delta 1$ –247). A small carboxy-terminal deletion in construct 10 ($\Delta 562$ –601) has no effect, although more extensive deletions in constructs 8 ($\Delta 381$ –601) and 9 ($\Delta 489$ –601), despite being well outside the conserved domain, do affect binding (Fig. 10B). Because it seemed unlikely that the DNA-binding domain spanned such a large nonconserved region of the protein, we reasoned that some portion of the carboxyl terminus was required for homotypic interactions.

Bright binds DNA as a tetramer

Several experiments were performed to detect higher order structures in the Bright complex. Nonreducing gels and Sephadex gel filtration of in vitro-translated protein demonstrated high molecular weight complexes corresponding to a Bright homo-tetramer (data not shown). To analyze these complexes, a full-length Bright construct (F.L.) was translated separately or cotranslated with a deletion construct before DNA binding and mobility-shift assay (Fig. 10C). Separate translation and mixing of F.L. and construct 2 ($\Delta 1$ –215) produces two complexes that correspond to those seen with each construct by itself (Fig. 10C). When these two constructs are cotranslated (*), five binding complexes are resolved, corresponding to all combinations of long–short proteins in the tetramer (Fig. 10C). Thus, four copies of the protein exist in the DNA-binding complex. It also appears that formation of the tetramer occurs with assembly of the protein, in the absence of DNA, and is strong enough not to allow “breathing.”

We mapped the tetramer domain with further deletions. Construct 10 ($\Delta 562$ –601) binds DNA with full activity (Fig. 10B). Construct 9 ($\Delta 489$ –601) has no activity either mixed or cotranslated (*) with F.L. (Fig. 10C). Construct 5 ($\Delta 61$ –244/562–601) has a 20-amino-acid amino-terminal deletion in the conserved domain and does not bind DNA when translated separately from F.L. (Fig. 10C). However, when it is cotranslated (*) with F.L. a rescue phenomenon, with the formation of three lower complexes, is observed (Fig. 10C). Apparently, a small deletion in the conserved domain can be complemented by functional protein. Construct 7 ($\Delta 61$ –266) has a larger conserved domain deletion, does not bind DNA on its own, and does not form intermediate complexes when cotranslated (*) with F.L. (Fig. 10C). There is a reproducible loss of intensity from the full-length complex ($\sim 50\%$), suggesting that functional protein is being sequestered with $\Delta 61$ –266 protein into nonfunctional tetramers. This provides further evidence that the conserved domain is a required component for the tetramer to bind DNA.

Table 1. *Bright trans-activates from Eμ in permissive cell lines*

Cell lines	Transfected CAT vectors	Fold increase CAT protein $\pm$ S.D. ^{a,b}		Stimulation index ^b
		{control}	{Bright}	
J558	Eμ-CAT	18.9 $\pm$ 5.4	92.7 $\pm$ 20.6	5.0
	ΔEμ-CAT	28.6 $\pm$ 0.6	18.5 $\pm$ 5.5	0.7
M12.4	Eμ-CAT	2.3 $\pm$ 0.3	4.1 $\pm$ 0.4	1.8
	ΔEμ-CAT	1.4 $\pm$ 0.2	1.6 $\pm$ 0.2	1.1
EL4	Eμ-CAT	5.1 $\pm$ 0.3	33.6 $\pm$ 0.3	6.6
	ΔEμ-CAT	13.8 $\pm$ 0.7	6.7 $\pm$ 0.3	0.5
BW5147	Eμ-CAT	0.9 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0
	ΔEμ-CAT	N.D.	N.D.	N.D.

Tk-CAT vectors containing the intronic enhancer Eμ-CAT or the mutant enhancer ΔEμ-CAT (see Fig. 1) were cotransfected into the indicated cell lines with expression plasmids containing Bright in the antisense (control) or the sense orientation (Bright). Values indicate the fold increase over basal CAT-protein levels. Stimulation index is the ratio of fold increase after Bright cotransfection over fold increase after control cotransfection.

^a(S.D.) Standard deviation.

^b(N.D.) Not determined.

To verify these interactions, we performed immunoprecipitations with hemagglutinin (HA)-tagged full-length Bright. The HA-tagged version was cotranslated with Bright deletion constructs and precipitated with anti-HA monoclonal bound to protein G-agarose. Products that bind DNA, 10 (Δ562–601) and 2 (Δ1–215), were coprecipitated (Fig. 10D). In addition, products 5 (Δ61–244/562–601), 6 (Δ1–247), and 7 (Δ61–266) that have deletions in the DNA-binding domain, but intact carboxy-terminal ends, were also coprecipitated (Fig. 10D). However, products 8 (Δ381–601) and 9 (Δ489–601) that lack the region between amino acids 489 and 561 were not coprecipitated with HA-tagged Bright (Fig. 10D). This region, then, is essential for multimerization and ultimately for DNA binding.

The trans-activation domain lies between the boundaries of the DNA-binding and multimerization domains

Deletion constructs that still maintained DNA-binding

activity were tested for their ability to *trans*-activate the Eμ-CAT construct. Proteins with amino-terminal deletions up to the DNA-binding domain remained efficient *trans*-activators (summarized in Fig. 10A). Thus, the large acidic region, characteristic of several *trans*-activation domains, is not important in this assay system. The carboxy-terminal Δ562–601 deletion also had full *trans*-activation potential, indicating that the *trans*-activation domain lies between the amino terminus of the binding domain and the carboxyl terminus of the tetramer domain.

Discussion

Studies by Webb et al. (1991a) implicated a DNA-binding complex in the up-regulation of IgH expression after stimulation of a responsive B cell line. This report describes the cloning and characterization of this protein, called Bright. We have shown that Bright is restricted to mature stage B cells, binds MAR sequences flanking the

Figure 9. Six-member alignment of a conserved region defines a new DNA-binding domain family. Solid residues are identical to Bright, and shaded boxes indicate conservative substitutions. Numbers indicate amino acid position. Robert Saint (University of Adelaide, Australia) kindly provided the dri sequence. All other sequences were retrieved from the PIR data base or from published data MRF1 (accession nos. S27962); MRF2A (S27963); SWI1/Adr6 (S05728); RBP1 (Fattaey et al. 1993). Both MRF1 and MRF2A were partial cDNAs with incomplete open reading frames.

The IgH MARs are bound by Bright

Figure 10. Bright requires the conserved domain and a second domain to bind DNA as a tetramer. (A) Schematic of the Bright full-length (F.L.) amino acid sequence is shown on top. Deletion constructs (1–10) are shown below, with deleted (Δ) amino acids listed at left and relevant activities summarized at right. (B) Constructs were in vitro translated and bound to labeled Tx125 DNA on EMSA. Those with deletions in the conserved (4 and 6) or tetramer (8 and 9) domains do not bind DNA. (C) EMSA as above with F.L. translated separately or cotranslated (*) with the indicated deletion construct. Construct 2 has both domains intact and forms hybrid tetramers when cotranslated with F.L., whereas Construct 9 lacks the tetramer domain and does not. Construct 5 has a small conserved domain deletion that is rescued into functional F.L. hybrids, whereas a larger deletion in construct 7 is not. (D) An HA-tagged version of Bright F.L. was cotranslated with deletion constructs in the presence of [35 S]methionine. Synthesized products were separated on protein gels before (top) and after (bottom) immunoprecipitation with an anti-HA monoclonal. Products that contain the tetramer domain (10,2,6,5,7) are immunoprecipitated.

$E\mu$ core, and activates transcription driven by $E\mu$. This protein forms a homo-tetramer and binds DNA with a newly described domain that is conserved among several

family members including SWI1. These observations suggest a role for Bright in IgH gene expression.

Bright and NF- μ NR bind similar IgH MAR sequences;

however, their mobility-shift complexes show a reciprocal lymphoid expression pattern. Bright mRNA and protein, although, absent from pre-B and T cell lines, are present in mature B-cell lines. The failure to detect Bright message in resting splenocytes undoubtedly reflects its restriction to activated B cell stages. Activated B cells respond to antigen and T cell signals, and this ability has been modeled in the BCg3R cell line (Webb et al. 1989). The transcriptional response of these cells to antigen plus IL-5 was shown to require a promoter proximal Bright-binding site and to parallel augmented Bright complex formation on mobility-shift assay (Webb et al. 1991a). As suggested by Western blot data, induction of Bright binding may involve some form of post-translational modification. However, Bright's role in relation to steady-state versus stimulated immunoglobulin expression is not known.

Bright binds the minor groove of MAR sequences located upstream of the IgH S107 variable region promoter (Bf150, Tx125) and flanking the intronic enhancer E μ (P2, P3, and P4). Several of these sites also bind NF- μ NR and SATB1, and contain two essential features: (1) an ATC run ≥ 13 bp, and (2) two closely spaced AT dimers. Although Dickinson et al. (1992) first demonstrated the importance of the ATC sequence for matrix association, we have shown that the AT dimer feature is also necessary. This type of ATC sequence (AT/ATC) defines a consensus MAR-binding site and is recognized by Bright, SATB1, and the matrix-associated protein Nucleolin (Dickinson et al. 1992; Dickinson and Kohwi-Shigematsu 1995). Bright binds AT/ATC sequences but only if a core recognition hexamer (AATTAA, AATAAA, or GATTAA) is also present. Bright does not tolerate loss of the AT dimer (mutation 2) or the ATC run (mutation 8), but will tolerate limited degeneracy in the hexamer as evidenced by the P4 site (AATTAT) and mutation 5 (AATTCA). Thus, the core hexamer targets Bright to specific matrix attachment sites.

We demonstrated that Bright *trans*-activates CAT expression vectors in a transient cotransfection system. This *trans*-activation was dependent on enhancer function and correlated with E μ activity in the cell lines tested. It was also specific for DNA binding, as deletion of Bright's binding sites (P2, P3, and P4) from the MARs flanking the E μ core rendered the Δ E μ -CAT vector impervious to activation after Bright cotransfection. However, in control experiments Δ E μ -CAT was more active than E μ -CAT in the T cell line EL-4 and the plasma cell line J558. The deleted sites in Δ E μ -CAT are also bound by putative T cell repressors NF- μ NR and SATB1, giving a rationale for increased expression from this vector in EL-4. The situation for J558 is less clear, but the implication is that these sites also carry a net negative input on enhancer function in this cell line. Whether this results from pure DNA topological constraints or from factor binding in addition to Bright is not known. The deleted sites contain the AT/ATC sequence motif that is recognized by other MAR proteins; therefore, it seems likely that these regions would bind multiple factors. If the MARs act by affecting DNA topology, then proteins

such as histones, binding the MAR, could mediate repression at some level of Bright expression. In this scenario, removal of the MAR sites would relieve repression, as would Bright overexpression by affecting the stoichiometry of factor binding. The same effect is not seen in M12.4 cells. However, the low level of enhancer activity makes differences between E μ and Δ E μ less meaningful. Clearly, Bright does not act alone, but exactly how it activates E μ -driven transcription will require further study.

Bright, like other MAR-binding proteins, exists both free in the nucleus and in association with the nuclear matrix, but remains distinct at the amino acid level from these proteins, including SATB1, which binds a nearly identical sequence (Adachi et al. 1989; Hofmann et al. 1989; Izaurralde et al. 1989; von Kries et al. 1991; Luderus et al. 1992; Romig et al. 1992; Tsutsui et al. 1993; Zhao et al. 1993; Nakagomi et al. 1994; Dickinson and Kohwi-Shigematsu 1995). Bright does share a domain (amino acids 226–360) with the proteins dri, RBP1, SWI1, MRF1, and MRF2A, although the greatest region of homology among all family members is limited to amino acids 249–331. Bright protein with a deletion up to amino acid 246 does not bind DNA but can be complemented by the full domain into a functional binding complex. This suggests that important binding structures reside within the limited domain. Although site-specific DNA binding has not been reported for SWI1 or RBP1, the MRF and dri proteins do bind specific AT-rich sequences (B. Whitson, T.H. Huang, B.W. Merrills, T. Asai, and K. Itakura, in prep.; S. Gregory, R. D. Kortschak, B. Kalionis, and R. Saint, in prep.). We and the groups cloning the MRF and dri genes have named this the ARID domain (AT-rich interaction domain) and a discussion of its secondary structure will be presented elsewhere. By showing that this domain is necessary for binding activity, we have defined a new family of DNA-binding proteins.

We have identified one additional functional domain in Bright, a tetramer domain spanning amino acids 489–561. DNA binding was shown to require four molecules of Bright. However, DNA binding was not necessary for tetramer formation, as deletion constructs devoid of binding activity were able to multimerize if they contained the tetramer domain. There are no obvious structural motifs in this domain, although the amino-terminal half (amino acids 489–527) is rich in polar residues, which often mediate homotypic interactions.

Our studies and experiments by Taguchi and Young (1987), demonstrating regulation of the ADHII yeast gene by SWI1 reveal that proteins containing an ARID domain can mediate transcriptional activation. The SWI/SNF complexes containing SWI1 act as general transcriptional activators in both yeast and humans by altering chromatin structure (Peterson and Herskowitz 1992; Imbalzano et al. 1994; Kwon et al. 1994). Bright is a MAR-binding protein, and it has been suggested that the ability of MARs to regulate transcriptional domains may relate to changes in chromatin structure effected by factors that bind them (Kas et al. 1993; Zhao et al. 1993).

Studies by Forrester et al. (1994) indicate that the IgH locus becomes remodeled or DNase I hypersensitive at the pre-B cell stage and that the E μ MARs are necessary for this event. The β -globin LCR also demonstrates remodeling before active domain transcription (Jimenez et al. 1992). Interestingly, in several genes, transcription factor access to core enhancer elements seems to occur even before these early remodeling events (Jenuwein et al. 1993; Aronow et al. 1995). This suggests that additional topological changes or factors are required to activate transcription. The expression pattern, binding site location, and *trans*-activation potential of Bright put it in a position to fulfill this role. Thus, as B cells differentiate and respond to cytokine and antigen signals, Bright could affect structural chromatin changes through the MARs that activate E μ and drive IgH transcription.

There is no doubt that regulation of immunoglobulin transcription is complex. The growing numbers of factors that bind within the IgH intronic enhancer clearly work together to sustain high level gene expression in B lymphocytes. Although the maintenance of tissue restriction is still an interesting puzzle, the studies described here offer a Bright new path to follow.

Materials and methods

DNA probes and fragments

The S107 5' MAR, Bf150, and Tx125 fragments have been described (Webb et al. 1991a). Scheuermann and Chen (1989) described the 992-bp *Xba*I–*Xba*I murine intronic enhancer (E μ) fragment and the location of the NF- μ NR sites (P1 nucleotides 176–200, P2 nucleotides 285–333, P3 nucleotides 732–753, P4 nucleotides 791–826). The 5' and 3' E μ MARs consisted of a 381-bp *Xba*I–*Pst*I fragment and a 309-bp *Eco*RI–*Xba*I fragment, respectively. The Δ E μ and Δ P1– Δ P4 constructs were made by sequential digestion and religation after insertion of the following restriction sites into the 992-bp E μ fragment with the MutaGene Mutagenesis Kit (Bio-Rad, Richmond, CA): *Spe*I at positions 162 and 203, *Bgl*II at positions 277 and 341, *Xho*I at positions 697 and 759, and *Eag*I at positions 782 and 825. Concatamers were constructed from annealed synthesized oligonucleotides (Applied Biosystems Inc., Foster City, CA) by blunt end or complimentary end ligation, electrophoretic separation of the ligation reaction, gel elution of the appropriate band size, and cloned into Bluescript vectors (Sambrook et al. 1989). When referred to in figures and text *n* = number of concatamerized repeats.

Library screening and clone isolation

An IL-2- and IL-5-stimulated BCL1 λ Zap cDNA library, provided by Mark Davis (Turner et al. 1994), was screened by the method of Singh et al. (1988). Briefly, 5×10^4 phage plaques were grown at 42°C until visible with filter overlays placed at 37°C overnight. Wet filters were blocked for 1 hr in buffer C [50 mM KCl, 20 mM HEPES, 0.5 mM MgCl₂, 0.5% Tween 20 (vol/vol), 0.05% NP-40 (vol/vol) (pH 7.9)] plus 5% (wt/vol) dry milk, washed, and incubated at 4°C overnight in buffer C plus 0.25% dry milk (wt/vol), 10 μ g/ml of sheared salmon sperm DNA, 2 μ g/ml of poly [d(I-C)] and 2×10^6 cpm/ml of ³²P-end-labeled BF site oligonucleotide multimerized to seven repeats. Filters were washed, dried, and exposed to radiographs overnight at –80°C with intensifying screens. Plaques that bound labeled probe

were picked and purified to homogeneity. The library was re-screened by hybridization with labeled cDNA as described by Kaplan et al. (1993). Isolated clones were converted to double-strand plasmids by Stratagene's *in vivo* excision protocol (Stratagene Cloning Systems, LaJolla, CA) and sequenced on both strands with an automated fluorescent-tagged sequencing system (Applied Biosystems).

In vitro translation and transfection vectors

Bright cDNA constructs were cloned into the pBK-cytomegalovirus (CMV) plasmid (Statagene) and used for protein expression in the transfection and mobility-shift experiments. The full-length construct spanned nucleotides 268–2320 and was cloned into the *Xho*I site of pBK–CMV in both sense and antisense orientations. Translation of this construct initiates off of the second in-frame methionine at amino acid 7. Deletion constructs were made through a series of restriction site digests, polymerase chain reaction (PCR), and vector religation of the full-length construct. All constructs were sequenced to confirm proper orientation and in-frame alignment. *In vitro* transcription (T₃ polymerase) and translation was performed with the TNT-coupled reticulocyte lysate system (Promega Corp, Madison, WI). Synthesized products were analyzed by gel electrophoresis to confirm correct expression size.

Electrophoretic mobility-shift assay

Nuclear extract isolation (Dignam et al. 1983) and IL-5 plus antigen induction (Webb et al. 1991a) of the BCg3R-1d cell line have been described. The electrophoretic mobility-shift assay (EMSA) was performed as described by Webb et al. (1991a). Binding reactions consisted of 0.5–2 μ g of nuclear extract or 10 ng of cold *in vitro* cDNA translation reaction (1–3 μ l for single construct and 3 μ l for cotranslated constructs), 1 μ g of poly d(I-C) and DNA probes end labeled (Sambrook et al. 1989) to a sp. act. of 10^5 cpm/ng with [³²P]dATP (ICN, Costa Mesa, CA), with modifications to buffer D [20 mM HEPES (pH 7.9), 20% glycerol (vol/vol), 50 mM KCl, 1% Tween 20 (vol/vol), 0.2 mM EDTA, 5 mM phenylmethylsulfonyl fluoride]. Cold competitor fragments were allowed to bind for 5 min at 37°C before addition of labeled probe.

RNA analysis

Total RNA was isolated from cell lines and frozen mouse tissues with guanidinium and hot phenol (Feramisco et al. 1982). The Northern blot was performed as described in Sambrook et al. (1989). Poly(A)⁺ RNA was selected (Promega PolyATtract mRNA isolation system) from 250 μ g of total RNA and run side by side with 15 μ g of the total sample. Vector templates generating antisense RNA probes for RNase protection were (1) a 750-bp CL8 *Bam*HI fragment cloned into Bluescript KS and linearized at the *Nco*I site (223 nucleotides with 125 nucleotides protected), and (2) pTRI- β -actin–mouse plasmid (Ambion, Austin, TX) linearized at the *Hind*III site (304 nucleotides with 250 nucleotides protected). Labeling, purification, and denaturing gel analysis of riboprobes hybridized at 45°C to 10–20 μ g of total RNA was performed as described previously (Webb et al. 1991a).

Antibody production and Western analysis

One milligram of a bacterial fusion protein, from a pGeX–GST construct (Guan and Dixon 1991) which contained 1 kb of Bright amino-terminal coding sequence up to amino acid 346, was purified over glutathione–agarose beads, then emulsed with

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TiterMax (Sigma Chemical Co., St. Louis, MO) and used to immunize a single rabbit. Serum was collected after 1 month that demonstrated a titer of $>1:10,000$ on ELISA. Antibodies used for Western analysis were purified by acid elution of serum from a agarose-GST-Bright affinity column. Western blots were performed as described in the Western-Light Chemiluminescent Detection Kit (Tropix Inc., Bedford, MA) except that all solutions were 0.6% EIA grade gelatin (Bio-Rad) and 0.06% Tween 20 (Sigma).

Nuclear matrix isolation, MAR-binding assay, and CASTing

BCg3R nuclear matrix was isolated as described by Webb et al. (1991b), and for the MAR-binding assay 6 μ g was washed [50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, 0.25 M sucrose, 0.25 mg BSA/ml] and bound to 1 ng of ³²P-labeled DNA fragment in 50 μ l of buffer D plus 10 μ g of *Escherichia coli* DNA and 5 μ g of poly [d(A-T)] for 1 hr at room temperature. The reactions were microcentrifuged for 60 sec to separate the supernatant and matrix fractions, proteinase K digested for 2 hr, phenol-chloroform extracted, and separated on a nondenaturing gel. The CASTing experiments were performed essentially as described by Funk and Wright (1992). A pool of double-strand 35-mers was created by PCR from a random pool of synthesized 35-base oligonucleotides and allowed to bind to in vitro-translated HA-tagged Bright for 20 min in buffer D. HA-tagged Bright was immunoprecipitated as described below and underwent 18 cycles of PCR. These steps were repeated through seven rounds with the addition of 10 μ g of sonicated salmon sperm DNA to the binding reaction. The final reaction was cloned into pBlue-script, and plasmids containing inserts were isolated and sequenced.

Immunoprecipitations

An HA-tagged version of Bright, containing the 9-amino-acid HA epitope fused in-frame to the amino terminus at amino acid 8, was cotranslated with Bright deletion constructs in the presence of [³⁵S]methionine. Cotranslate (10 μ l) was added to 1 μ g of protein G-agarose-precipitated anti-HA antibody (Boehringer Mannheim, Indianapolis, IN). The reaction was diluted to 50 μ l with buffer D, incubated for 30 min at 4°C, and precipitated. Precipitates were washed twice with both PBS and buffer D (containing 1 mg/ml of BSA and 0.1% NP-40) and separated by protein gel electrophoresis.

Cell culture, transfections, and CAT assays

The CAT expression vectors were constructed by cloning the E μ and Δ E μ fragments (described above) into the 5' polylinker of pBL-CAT2 (Luckow and Schutz 1987). Transfections of cultured cell lines were performed by electroporating 10 μ g of E μ or Δ E μ pBL-CAT2, 15 μ g of sense or antisense pBK-CMV Bright, and 10 μ g of a Rous sarcoma virus (RSV)-luciferase plasmid into 2×10^7 cells with a Bio-Rad Gene Pulser set at 960 μ FD and 280 V for M12.4 and J558 cells, 290 V for BW5147 cells, and 300 V for EL4 cells. Fresh medium was added at 24 hr, and transfected cells were lysed by freeze-thaw at 48 hr in 250 mM Tris-HCl (pH 8.0). Supernatants were tested for CAT protein using CAT-ELISA (Boehringer Mannheim), and values were normalized for protein concentration and luciferase activity (Optocomp II luminometer, MGS Industries).

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