

Genes Activated in the Presence of an Immunoglobulin Enhancer or Promoter Are Negatively Regulated by a T-Lymphoma Cell Line

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The tissue-specific expression of immunoglobulin genes can be partially explained by a requirement for activating factors found only in B lymphocytes and their derivatives. However, loss of immunoglobulin expression upon fusion of an immunoglobulin-producing myeloma cell with a T lymphoma cell (BW5147) or fibroblast (L cell) suggests that negatively acting factors also play a role in the tissue specificity of immunoglobulin genes. Expression of a cloned immunoglobulin heavy-chain gene introduced into myeloma cells was suppressed after fusion of the myeloma transformants with BW5147. The presence of either the immunoglobulin heavy-chain enhancer or promoter conferred suppression, under similar conditions, upon a heterologous gene that is normally expressed in both B and T lymphocytes. These immunoglobulin heavy-chain gene control regions, or gene modifications induced by them, are subject to negative control by T-lymphocyte-derived factors.

Immunoglobulin heavy-chain (IgH) gene expression is under tissue-specific control. Transfected IgH genes are expressed at high levels in cells of the B-lymphocyte lineage, but are only expressed at low levels when placed into inappropriate cell types such as T lymphocytes or fibroblasts (17, 20, 24, 34). This is due, in part, to an intragenic tissue-specific enhancer element found within productively rearranged IgH genes (5, 17, 38). In addition to the enhancer, promoter regions and other intragenic sequences have also been implicated in the tissue-specific control of these genes (20, 34).

Several lines of evidence indicate that B-lymphocyte-derived cell lines contain regulatory factors that are required for activation of the IgH enhancer (12, 33, 35, 44). The observed tissue specificity of this enhancer might be explained if non-B cells lacked such factors. However, positive regulation does not completely account for the tissue-specific control of IgH gene expression. Several regions of the IgH enhancer when fused to the promoter or enhancer region of a heterologous gene will prevent activation of that gene when it is introduced into a non-B cell (e.g., fibroblast [23]). It has been suggested that the lack of IgH enhancer activity in non-B cells, then, is at least partially due to negative regulators that prevent enhancer activation, perhaps by inhibiting some of the stimulatory, *trans*-acting factors that normally participate in that activation.

Another kind of evidence for the negative regulation of immunoglobulin genes in non-B cells is the finding that IgH production generally ceases when B-derived cells are fused to either T cells or fibroblasts (9, 19, 25, 40). This observation suggests the existence of a *trans*-acting negative regulator in non-B cells that can extinguish immunoglobulin gene expression even after the gene has been activated. The sequence elements which interact with this negative regulator have not yet been defined.

Extinction of tissue-specific functions is common in fusions involving dissimilar cell types. Extinguished traits may be reexpressed in some hybrid subclones after the segrega-

tion of chromosomes which presumably carry genes encoding negative regulators (28). The reexpression of specific traits has been correlated with the loss of specific chromosomes (28). Thus, negative regulatory factors responsible for extinction may be specific for particular genes.

The studies reported here were undertaken to determine whether the IgH enhancer is involved in the extinction of IgH gene expression. Myeloma transformants containing genes under the control of the IgH enhancer were fused to T-lymphoma cells. Expression of the transfected genes was evaluated both before and after cell fusion. Our results indicated that even a heterologous gene when activated by the IgH enhancer is subject to T-cell-mediated negative control. Further, we found that the presence of the IgH promoter similarly affected a gene that normally shows no tissue specificity.

MATERIALS AND METHODS

Cell lines. Cell lines were maintained in Dulbecco modified Eagle medium containing 15% horse serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. 45.6.2.4 is a $\gamma 2b/\kappa$ -producing BALB/c mouse tumor, MPC-11 (32). In the present study, we refer to this cell line as MPC-11. J558L is a variant myeloma line which produces λ light chains but no immunoglobulin heavy chains (39). BW5147.G.1.4.OUAR.1 was obtained from the American Type Culture Collection (Rockville, Md.) and is a variant subline of the AKR/J mouse thymoma-derived cell line BW5147. The BW5147.G.1.4.OUAR.1 variant is resistant to 10^{-4} M 6-thioguanine and to 10^{-3} M ouabain (22).

Plasmid constructions. The constructions of p97 $\gamma 2b$ and p97 Δ Enh $\gamma 2b$ have been described previously (53). To construct pESgpt, a 1.0 kilobase (kb) *Xba*I restriction fragment containing the IgH enhancer was isolated from a low-melting-point agarose gel and 5' overhanging sequences were filled in with Klenow polymerase. This fragment was then inserted via *Sal*I linkers into a *Sal*I-digested, calf intestinal phosphatase-treated derivative of pSV2gpt lacking simian virus 40 (SV40) enhancer sequences ($\Delta 232$ [15]). The resultant plasmid containing the *xgpt* gene under the control of an SV40 promoter and an IgH enhancer was designated pESgpt (see map, Fig. 4B).

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To construct pE_{sv}P_{ig}pt (see Fig. 4B), we deleted a *Pvu*II-*Bgl*II fragment containing the SV40 promoter, enhancer, and origin of replication from pSV2gpt. Inserted in its place were an SV40 enhancer-containing fragment of 263 base pairs (positions 37 to 294 with respect to the origin in the SV40 genome) and a 162-base-pair fragment consisting of nucleotides -153 (an *Eco*RI site) to +6 (*Bcl*I) with respect to the cap site of the MPC-11 heavy-chain gene. The SV40 enhancer fragment is in the opposite orientation with respect to *xgpt* transcription.

The E_{sv}P_{sv}IgH gene construction consists of a 356-base-pair SV40 fragment mapping from nucleotide 5171 through the origin to position 294 (this fragment includes both the SV40 promoter and enhancer), and a γ 2b gene identical to that in p97X γ 2b except that the gene is fused at position +6 (*Bcl*I site) with respect to its cap site to the 356-base-pair SV40 fragment described above. The result is a γ 2b gene in which the IgH enhancer and promoter have been substituted by the SV40 enhancer and promoter, both of which lie 5' of the V region encoding sequences. Finally, E_{sv}P_{sv}IgH contains *Bam*HI-*Eco*RI-digested pSV2gpt. This vector is ligated to the newly formed γ 2b transcription unit to allow for selection of transformants in mycophenolic acid-hypoxanthine-xanthine (MHX) medium.

Cell fusion. A 6-thioguanine-resistant ouabain-resistant subclone of BW5147 was fused with equal numbers of cells from various cell lines by using 50% polyethylene glycol. Cells were then plated at 5×10^4 total cells per well in a 96-well plate. Hybrids were selected in complete Dulbecco modified Eagle medium supplemented with 1×10^{-4} M hypoxanthine, 4×10^{-6} M aminopterin, 1.6×10^{-4} M thymidine, and 1×10^{-3} M ouabain.

ELISAs. IgG-specific enzyme-linked immunosorbent assays (ELISAs) were performed in 96-well Immunlon 2 plates (Dynatech Laboratories, Inc., Alexandria, Va.) coated overnight at 4°C with 50 μ l of 0.1-mg/ml affinity-purified goat anti-mouse IgG, Fc fragment specific (Cappel Worthington). The coated wells were then incubated for 1 h at room temperature with 50 μ l of serial dilutions from 0.5% Nonidet P-40 lysates of 10^6 cells. IgG was detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Cappel Worthington) as the second-step antibody, using *p*-nitrophenolphosphate as the enzyme substrate. The A₄₀₅ was measured in an ELISA plate reader (Biotek).

DNA and RNA blot analyses. DNA and RNA blot analyses were performed as previously described (52).

Transcription rate assays. Transcription rates were measured in an in vitro nuclear run-on assay (8). Cells (5×10^7) were gently lysed in a 0.2% Nonidet P-40 solution, and transcription was allowed to continue for 30 min at 30°C in the presence of 0.25 Ci of [³²P]UTP. Labeled run-on transcripts were hybridized with an excess of plasmid DNA which had been size fractionated by agarose gel electrophoresis and blotted onto a nitrocellulose filter.

RESULTS

Extinction of endogenous IgH gene expression. MPC-11 is a cell line derived from a γ 2b/ κ -producing mouse myeloma (32). BW5147 is a cell line derived from a mouse thymic lymphoma (22). MPC-11 was fused with a 6-thioguanine-resistant ouabain-resistant subclone of BW5147, and individual hybrids were selected in hypoxanthine-aminopterin-thymidine medium supplemented with ouabain. Such mouse-mouse hybrid cells have been shown to occasionally lose one or a few chromosomes in the first few generations after

fusion. After this, the chromosomal makeup of the hybrids becomes stable (11). Of 16 independent hybrid cell lines examined, 10 had retained the functional IgH gene of MPC-11 (Fig. 1A). However, none of these hybrid cells contained any γ heavy-chain protein as measured by ELISA (Fig. 2A).

Immunoprecipitation of cell lysates with an anti- κ antiserum followed by polyacrylamide gel electrophoresis demonstrated that light-chain as well as heavy-chain production is extinguished in these hybrid cells (data not shown). Assays which measured relative transcription rates demonstrated that the observed extinction of heavy-chain production was at the level of RNA transcription (Fig. 2B).

Interestingly, the shutdown of IgH transcription was accompanied by de novo methylation at an *Msp*I-*Hpa*II restriction enzyme site located 5' of the IgH enhancer (between J_{H4} and the enhancer, see maps in Fig. 3). Blackman and Koshland (6) have shown that this site becomes undermethylated with the onset of IgH gene expression during B-lymphocyte differentiation. In the present analysis, DNA was isolated from BW5147, MPC-11, and a hybrid cell line, digested once with *Bam*HI, and then digested again with either *Msp*I or *Hpa*II. *Msp*I and *Hpa*II recognize the same DNA sequence (CCGG), but *Hpa*II cannot cleave at this sequence if the inner cytosine is methylated. Digested DNA was size fractionated, transferred to nitrocellulose, and then hybridized with the pJ11 probe. As shown in the maps and autoradiograph in Fig. 3, pJ11 will hybridize to two fragments in *Bam*HI-*Msp*I-digested DNA from MPC-11 (0.9 and 3.5 kb). Similarly digested and hybridized DNA from BW5147 yields two fragments of 0.9 and 4.9 kb. The two cell lines differ because of the class-switch rearrangement that has taken place in MPC-11. MPC-11 DNA digested with *Bam*HI-*Hpa*II yielded the same pattern seen with *Bam*HI-*Msp*I-digested DNA; the *Msp*I site between J₄ and the enhancer is not methylated and remains susceptible to *Hpa*II cleavage. In contrast, this site is methylated in BW5147 so that *Hpa*II-*Bam*HI-digested DNA hybridized with pJ11 yielded a single fragment of 5.8 kb (the sum of the 4.9- and 0.9-kb fragments). In neither cell line is the more 3' (with respect to immunoglobulin gene transcription) *Msp*I site methylated.

As expected, DNA from the hybrid between MPC-11 and BW5147 contained the sum of the *Bam*HI-*Msp*I bands seen in the two parents (0.9, 3.5, and 4.9 kb). However, this was not true of the *Bam*HI-*Hpa*II pattern. *Bam*HI-*Hpa*II-digested hybrid DNA was missing the MPC-11-derived bands and contained instead a new band of 4.4 kb. This is the size expected if the *Msp*I site between J₄ and the enhancer was methylated (the sum of the 3.5- and 0.9-kb fragments). In the hybrid then, the MPC-11-derived IgH gene has been both extinguished and newly methylated.

Extinction of transfected IgH gene expression. J558L is a myeloma-derived cell line which produces a λ light chain but no endogenous heavy chain (39). We have previously described stable transformants of J558L whose genomes contain the cloned MPC-11 heavy-chain gene either with or without a 1.0-kb region that encompasses the IgH enhancer (Fig. 4A) (53). Those transformants which had received the cloned MPC-11 IgH gene with an intact enhancer produced about 85 times the amount of IgH produced by enhancer-deficient transformants (53). An example of each transformant was fused with BW5147 to determine whether or not the transfected gene could be extinguished. For each transformant, four independent hybrid cell lines that retained the transfected IgH gene were isolated (Fig. 1B). Cytoplasmic IgH in transformants (e⁺) that carried an intact gene was still

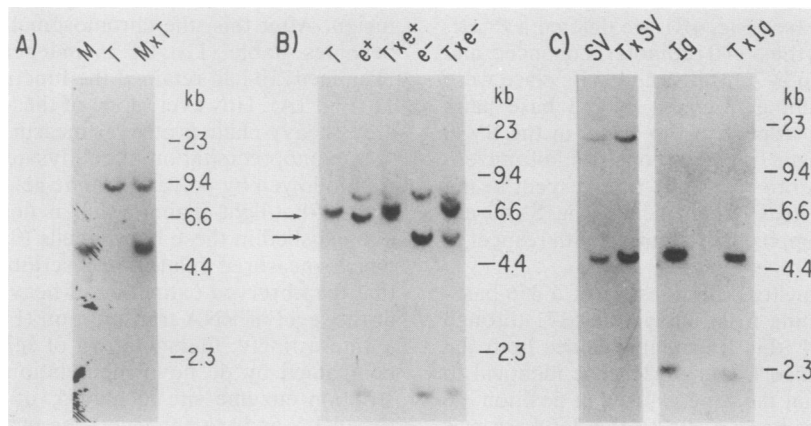


FIG. 1. Restriction analysis of genomic DNA from hybrid cells. (A) Genomic DNA was isolated from MPC-11 (M), BW5147 (T), and a somatic cell hybrid between MPC11 and BW5147 (M \times T). DNA was digested with *Bam*HI, electrophoresed on a 0.8% agarose gel, blotted to nitrocellulose, and then hybridized with 32 P-labeled J11 DNA. The J11 probe (indicated by the black bar in Fig. 3A) is derived from sequences within the expressed MPC-11 IgH gene (10). It does not detect sequences on the unexpressed IgH chromosome of MPC-11. (B) Genomic DNA was isolated from BW5147 (T), J558L transformants containing cloned heavy-chain genes with (e^+) or without (e^-) the enhancer, and somatic cell hybrids between BW5147 and either J558L transformant (T \times e^+ , T \times e^-). DNA was digested with *Eco*RI and then treated as described in panel A. The arrows indicate the size of the restriction fragments corresponding to the transfected heavy-chain genes (6.0 or 5.0 kb). The two additional bands seen in J558L transformant DNA (7.5 and 1.9 kb) are due to sequences endogenous to J558L which are recognized by the J11 probe. (C) Genomic DNA was isolated from J558L transformants containing the pSV2gpt (SV) or pESgpt (Ig) plasmid and from hybrids between BW5147 and either transformant (T \times SV, T \times Ig). DNA was digested with *Eco*RI and then hybridized with the pSV2gpt plasmid. Since the entire transfected gene was used as a probe in these blots and two bands were detected, presumably a single copy of the gene had integrated into the genome of each transformant (hybridizing bands correspond to restriction fragments containing the junction between plasmid and genomic sequences). The second pESgpt transformant used in these experiments also appeared to be a single integrant by the same criteria.

detectable after fusion with BW5147, although the IgH levels were dramatically reduced (Fig. 5). In fact, the level of IgH in these enhancer-containing hybrids closely corresponded to that found in J558L transformants with enhancer-deficient IgH genes (e^-). In the latter transformants, no change in heavy-chain protein levels resulted from fusion with BW5147 (Fig. 5). Similar results were obtained when BW5147 was fused with other independent J558L transformants, in each of which the cloned IgH gene has presumably been integrated into a different genomic location (data not shown).

In hybrids between J558L transformants and BW5147, the transfected IgH gene was expressed at low levels (Fig. 5). This was in contrast to the complete extinction seen with the endogenous IgH gene in MPC-11. In the IgA-secreting myeloma J558 from which J558L was derived, heavy-chain production is also completely extinguished after fusion with BW5147 (data not shown). This makes it unlikely that the residual IgH expression seen in the $e^+ \times$ BW5147 hybrids represents variations in intracellular environment among myeloma cell lines. It is possible that the low level of cytoplasmic IgH detected in these hybrids is due to the influence of pSV2gpt plasmid sequences present in the transfected gene, as low levels of IgH production were observed when the cloned MPC-11 IgH gene, with or without the enhancer, was transfected directly into BW5147 (data not shown). However, it is also possible that complete extinction requires DNA sequences that lie outside of the cloned gene used in these experiments or requires a particular sequence modification or chromatin structure associated with the endogenous, but not the transfected, IgH gene. The endogenous gene may differ from the transfected gene as a result of its distinct chromosomal location or developmental history or both.

Extinction of heterologous gene under control of the IgH enhancer. The previous experiments demonstrated that a

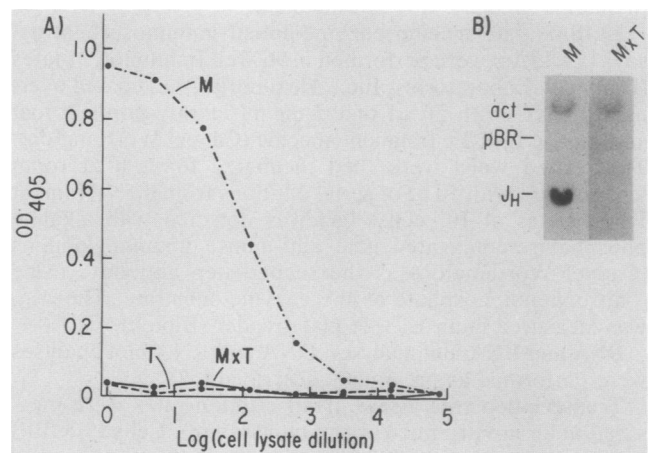


FIG. 2. (A) ELISA comparing heavy-chain protein levels of MPC-11 (M), BW5147 (T), and a somatic cell hybrid between MPC11 and BW5147 (M \times T). Cells (10^6) from each cell line were lysed with 100 μ l of a 0.5% Nonidet P-40 solution. Serial dilutions of cell lysates were assayed for γ heavy chain by an ELISA (see Materials and Methods). A representative curve for the M \times T hybrids is shown. An alkaline phosphatase-conjugated goat antibody to mouse immunoglobulins was used as a second-step reagent in the assay. The reaction of this reagent with *p*-nitrophenolphosphate substrate yields a colored product with an absorbance peak at 405 nm. OD, Optical density. (B) Comparison of IgH transcription rate in MPC-11 (M) and a somatic cell hybrid between MPC-11 and BW5147 (M \times T). Labeled run-on transcripts (see Materials and Methods) were hybridized with an excess of DNA which had been size fractionated by agarose gel electrophoresis and blotted onto a nitrocellulose filter. DNA bound to the filter included an actin cDNA (act), pBR322 vector (pBR), and J11 (J_H). A bar above the MPC-11 IgH gene map in Fig. 3 signifies the region of the γ 2b transcript complementary to J11 DNA.

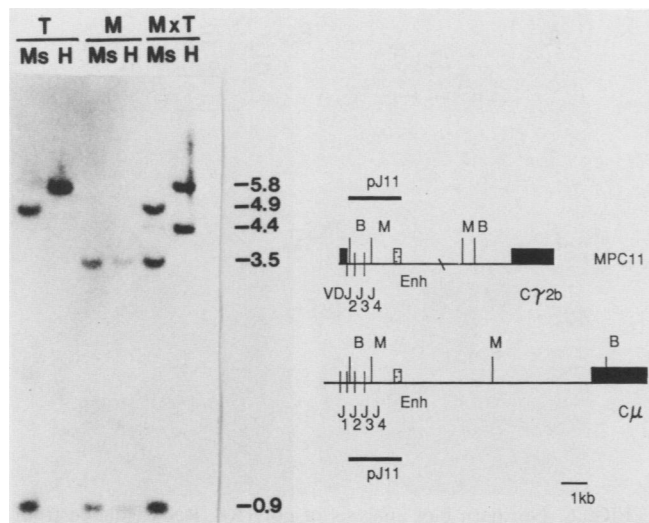


FIG. 3. De novo methylation of an extinguished IgH gene. Genomic DNA was isolated from BW5147 (T) and MPC-11 (M) and a somatic cell hybrid between these two parental lines (M × T). DNA was digested with both *Bam*HI and either *Msp*I (Ms in autoradiograph; M in gene maps) or *Hpa*II (H). The DNA was then size fractionated on a 0.8% agarose gel, blotted to nitrocellulose, and hybridized with ³²P-labeled pJ11 DNA (area of homology with MPC-11 and BW5147 genes is indicated in maps). Sizes of the restriction fragments seen in the autoradiograph are indicated in kilobases. In the maps shown, exons are designated by solid boxes, the enhancer by an open box, and the site of class-switch rearrangement in the MPC-11 gene by a diagonal line between the variable (VDJ₂)- and constant (Cγ2b)-region coding sequences. Joining gene segments are designated J₁ to J₄.

transfected IgH gene, like an endogenous IgH gene, was subject to negative control in myeloma × BW5147 hybrid cells. To assess more directly the role of the IgH enhancer element in this gene extinction, we transfected J558L cells with the bacterial xanthine-guanine phosphoribosyl transferase (*xgpt*) gene under the control of an SV40 promoter and either an SV40 enhancer (pSV2gpt [37]) or an IgH enhancer (pESgpt) (Fig. 4B). Both kinds of transformants could grow in medium containing mycophenolic acid, hypoxanthine, and xanthine (MHX), which is a selective medium requiring expression of the *xgpt* gene. Fusion of these transformants with BW5147 was used to determine whether a nonimmunoglobulin gene activated by the IgH enhancer is subject to extinction. pSV2gpt served as a control in these experiments since the SV40 enhancer, unlike the IgH enhancer, functions efficiently in both B and T lymphocytes. pSV2gpt and pESgpt transformants were each fused to BW5147, and hybrids of both types were selected in medium supplemented with hypoxanthine-aminopterin-thymidine and ouabain. DNA was isolated from surviving cell lines, digested with *Eco*RI, and analyzed on DNA blots with a pSV2gpt probe to identify hybrids that had retained the transfected genes (Fig. 1C). Independent hybrids were then tested for survival in medium containing MHX to determine whether the *xgpt* gene remained active.

Sixteen hybrids between BW5147 and a pSV2gpt transformant were tested. More than 70% of these hybrids, as analyzed on DNA blots, retained the transfected pSV2gpt gene. All these hybrids grew in MHX medium. A total of 32 independent hybrids were generated from the fusion of BW5147 with two independent pESgpt transformants. Only one of these survived in MHX medium, although more than

60% of the hybrids analyzed had retained the transfected pESgpt gene. Therefore, in all but one of the hybrids between BW5147 and pESgpt transformants, expression of the *xgpt* gene was extinguished. A likely explanation for the single hybrid that survived in MHX medium is that the T-cell chromosome(s) carrying a negative regulatory factor(s) had been lost. Consistent with this interpretation, the endogenous λ light chain of J558L, normally extinguished in such hybrids (Fig. 5B), continued to be expressed in this exceptional hybrid. The pESgpt gene in this hybrid was not altered with respect to the original transformant or any of the other transfected gene-containing hybrids, eliminating the idea that the *xgpt* gene had escaped the negative influence of the IgH enhancer by gross gene rearrangement.

The lack of survival in MHX medium after cell fusion provided evidence for *xgpt* gene extinction in the pESgpt

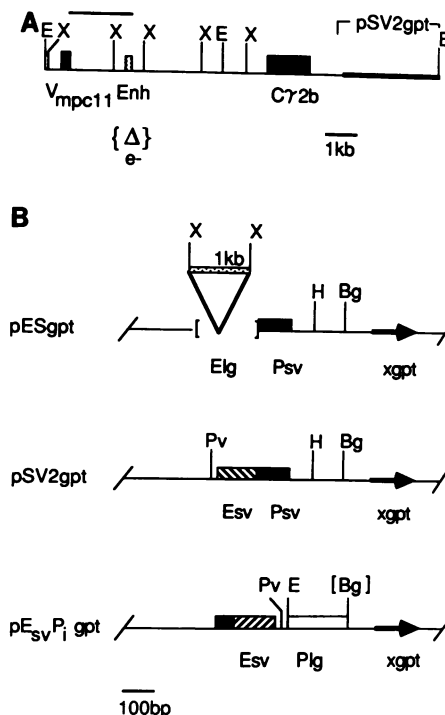


FIG. 4. (A) Restriction map of γ2b constructions used to transform J558L. Exons are designated by solid boxes, and the IgH enhancer is represented by a stippled box (Enh). The deletion of a 1.0-kb *Xba*I fragment to remove the enhancer is indicated {Δ}. A bar above the map indicates the region of hybridization with J11. (B) Map of *xgpt* constructions indicating the positions of the SV40 enhancer (Esv; box with diagonal stripes), the IgH enhancer (EIg; stippled box), the IgH promoter (PIg; open box), and the SV40 promoter (Psv; solid box). A large arrow marks the translational start site of the *xgpt* gene as well as its direction of transcription. pSV2gpt is as constructed by Mulligan and Berg (37). Only the modified regions of the pSV2gpt plasmid map are shown. In pE_{sv}-P_igpt, a small portion of the SV40 promoter region remains (the 21-base-pair repeats at nucleotide positions 62 to 103 in the SV40 genome; solid box), but this and the SV40 enhancer are inverted in relation to *xgpt* transcription and with respect to their position in the SV40 genome and in pSV2gpt. To construct pESgpt, we inserted a 1.0-kb *Xba*I fragment encompassing the IgH enhancer, via *Sal*I linkers, into a derivative of pSV2gpt lacking SV40 enhancer sequences (Δ232 [15]). The *Xba*I fragment insertion is at the site of the SV40 enhancer deletion. More complete descriptions of this and the pE_{sv}-P_igpt construction are in Materials and Methods. Restriction endonuclease recognition sites: E, *Eco*RI; X, *Xba*I; H, *Hind*III; Pv, *Pvu*II; Bg, *Bgl*II; [Bg], *Bgl*II site destroyed in cloning strategy.

transformants. To confirm this, we isolated poly(A)⁺ mRNA from a pSV2gpt transformant, a pESgpt transformant, and representative hybrids between those transformants and BW5147. RNA was size fractionated on a denaturing agarose gel, blotted to nitrocellulose, and hybridized with an *xgpt* probe. Similar levels of *xgpt* mRNA were detected in the two transformants (Fig. 6A). The level of *xgpt* mRNA remained unchanged in hybrids containing the plasmid with the SV40 enhancer (pSV2gpt), whereas hybrids containing the plasmid with the IgH enhancer (pESgpt) did not contain any detectable *xgpt* mRNA (four hybrids were examined; a representative hybrid of each type is shown in Fig. 6A). The blot was erased and rehybridized with an actin cDNA probe to confirm that approximately equal amounts of RNA were loaded in each line (data not shown). The lack of *xgpt* mRNA in hybrids containing pESgpt implies that association with the IgH enhancer can place even a heterologous gene that usually shows no tissue specificity under tissue-specific negative control.

Extinction of heterologous gene under control of the IgH promoter. Since the IgH enhancer can mediate extinction of an associated gene when a cell carrying such a construction is fused with a T lymphoma cell, we wondered whether loss of the enhancer by an endogenous immunoglobulin gene would make that gene no longer susceptible to extinction. We and others (1, 10, 29, 30, 48, 53) have described cell lines whose endogenous IgH genes lack an IgH enhancer and yet continue to produce high levels of immunoglobulin. When one of these cell lines, 9.9.2.1, was fused to BW5147, IgH production was turned off (data not shown). While this result is consistent with a model for negative regulation in which the regulator recognizes an enhancer-induced modification(s) of the active gene, not the enhancer sequences

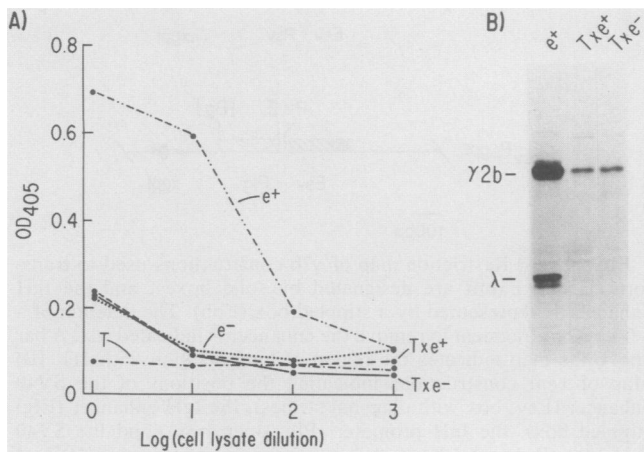


FIG. 5. Comparison of heavy-chain protein levels of BW5147 (T), J558L transformants containing cloned heavy-chain genes with (e⁺) or without (e⁻) the enhancer, and somatic cell hybrids between BW5147 and either J558L transformant (T × e⁺, T × e⁻). (A) ELISA (methods were as described in the legend to Fig. 2). OD₄₀₅. Optical density at 405 nm. (B) Autoradiogram of a sodium dodecyl sulfate-10% polyacrylamide gel showing [³⁵S]methionine biosynthetically labeled immunoglobulin. Immunoglobulin from lysates of 5 × 10⁶ cells was precipitated with an excess of heat-killed *Staphylococcus aureus* Cowan strain I. Positions of the γ2b heavy chain (produced by the transfected γ2b gene) and the λ light chain (produced by the endogenous gene of J558L) are indicated. Note that λ light-chain expression, like κ light-chain expression in the MPC-11 × BW5147 hybrids (see text) is extinguished upon fusion with BW5147.

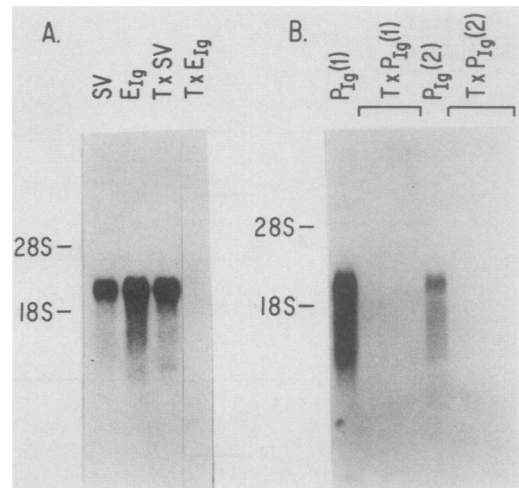


FIG. 6. Northern blot analysis of poly(A)⁺ RNA isolated from J558L cells transformed with pSV2gpt (SV), pESgpt (E_{Ig}), pE_{sv}P_{Ig}gpt (P_{Ig}), and representative somatic cell hybrids between BW5147 and these J558L transformants (T × SV, T × E_{Ig}, T × P_{Ig}). (A) Two T × SV and two T × E_{Ig} hybrids were analyzed as representative of the many collected and tested in MHX medium; RNA data from one of each type of hybrid are shown. The blot was hybridized with ³²P-labeled *xgpt* DNA. The same blot was also hybridized with ³²P-labeled actin cDNA to verify that approximately equal amounts of intact poly(A)⁺ RNA were loaded in each lane (data not shown). (B) P_{Ig}(1) and P_{Ig}(2) are two independent transformants of J558L carrying the pE_{sv}P_{Ig}gpt construction. Both were fused to BW5147, and RNA data for two representative hybrids from each of these fusions are shown. The RNA blot shown was hybridized with ³²P-labeled *xgpt* DNA. The difference in the intensity of signal seen for P_{Ig}(1) and P_{Ig}(2) corresponds to a difference in the quantity of RNA in these two gel tracks; an actin cDNA probe showed the same difference in signal. By the same criteria, poly(A)⁺ RNA from the hybrids was present in as great or greater quantity than that from the parent transformants (data not shown).

themselves (see Discussion), we thought it also possible that T-cell-negative regulators were capable of acting on more than just the enhancer (or enhancer-induced changes) in an IgH gene.

Another likely candidate for tissue-specific negative control in this system is the IgH promoter, since it, like the enhancer, acts more efficiently in B cells than in non-B cells (20, 21). We constructed a third version of the pSV2gpt plasmid (pE_{sv}P_{Ig}gpt) in which an IgH promoter was substituted for the SV40 promoter normally used to drive *xgpt* expression (see Materials and Methods and map in Fig. 4B). The SV40 enhancer remained in this construction so that *xgpt* expression in transfected J558L cells was at the same high levels as seen with pSV2gpt (Fig. 6B). Two independently derived pE_{sv}P_{Ig}gpt transformants were fused to BW5147. Of eight hybrids shown by Southern analysis to have retained the pE_{sv}P_{Ig}gpt construction, all failed to survive in MHX medium. Consistent with this, when poly(A)⁺ RNA preparations from four representative hybrid lines were analyzed, none contained detectable *xgpt* mRNA (Fig. 6B). A non-tissue-specific gene, then, when under the influence of either the IgH promoter or the IgH enhancer will cease expression when placed into the cellular environment of a T × B cell hybrid.

IgH enhancer and promoter are the only IgH gene sequences involved in IgH gene extinction. To determine whether other IgH gene sequences were involved in the IgH gene extinction seen in B × T cell hybrids, we constructed an IgH gene

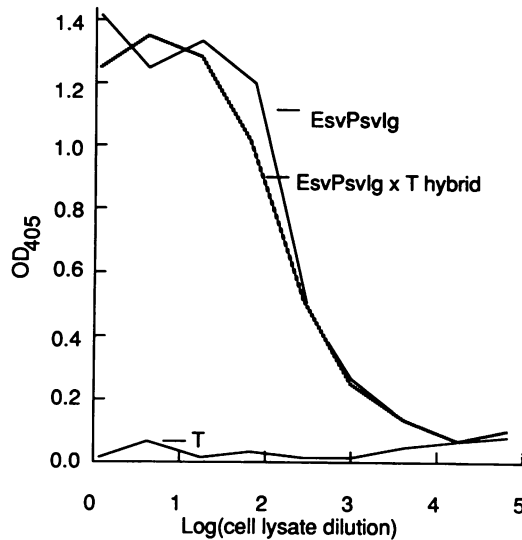


FIG. 7. ELISA comparing heavy-chain protein levels in J558L transformants containing the $E_{sv}P_{sv}Ig$ gene construction (Esv PsvIg), in BW5147 cells (T), and in hybrids between these transformants and BW5147 (EsvPsvIg \times T hybrid). Methods were as described in the legend to Fig. 2. OD_{405} , Optical density at 405 nm.

that lacked the IgH enhancer and promoter but retained all other regions of the IgH transcription unit. To promote and enhance the expression of this gene in the J558L myeloma, we provided it with an SV40 enhancer and promoter, both of which (as shown with the pSV2gpt construction) function in BW5147 and are unaffected in B \times T cell hybrids. The construction of this IgH gene is described in Materials and Methods. When transfected into J558L, it was expressed at high levels, and upon fusion with BW5147, the hybrids continued to produce IgH chain at the same high levels (but in the absence of light chain) (Fig. 7). As in all our cell fusion experiments, genomic DNA analysis of the hybrids was done to confirm their hybrid nature (retention of DNA fragments derived from both the T- and the B-cell parental lines [data not shown]).

DISCUSSION

The results of these studies demonstrate that genes activated in a myeloma cell in the presence of either the IgH enhancer or promoter are subsequently inactivated when the myeloma is fused to a T lymphoma cell. It is unlikely that this gene inactivation is simply due to fusion-induced loss of chromosomes encoding factors necessary for IgH enhancer or promoter activity. Although chromosome loss does occur in intraspecific hybrids, it is an infrequent event, and we examined many independent hybrids in each fusion experiment. As noted above (Results), among the ~ 19 hybrids between pESgpt transformants and BW5147 in which the transfected gene was retained, only 1 continued to express *xgpt*. If that hybrid were explained as the loss of a pair of homologous chromosomes encoding a negative regulator, the frequency of such exceptional hybrids would correspond to a segregation frequency of ~ 20 to 25% per chromosome (assuming random chromosomal segregation). This is not far from the frequency of loss we saw for the productively rearranged IgH chromosome of MPC-11 as followed directly in MPC-11 \times BW5147 hybrids (6 of 16, $\sim 37\%$, see Results). On the other hand, for 18 of 19 hybrids not to express *xgpt*

because each is missing a chromosome essential for IgH enhancer activity requires either that individual chromosomes segregate at a frequency of $>94\%$, with a diploid dose of the activator gene being essential for enhancer activation, or that enhancer activity depend on a diploid dose of all of several genes, each mapping to a different chromosome and each expressed only in B lymphocytes. The products of these genes must be not only tissue specific but also IgH enhancer specific since, in the fusions involving pSV2gpt transformants, the SV40 enhancer remained active. Both the SV40 enhancer and the IgH enhancer have been shown to bind multiple factors, some in common (7, 16, 31, 35, 41, 44-46, 51). However, as yet, only one of the IgH enhancer-binding factors has proven to be B-cell specific (16, 31, 46). The same reasoning applies to the extinction seen with IgH promoter-containing gene constructions.

A more probable explanation for the loss of *xgpt* expression seen in the myeloma cell \times T-lymphoma cell hybrids is that, in BW5147, there is a negative regulator(s) that acts specifically on genes carrying either the IgH enhancer or promoter. Evidence has been presented to suggest that viral gene products (adenovirus E1a proteins) can similarly repress IgH enhancer-activated genes and that this interaction plays a role in the shutdown of IgH production after adenovirus infection (21). Unlike the cellular factor(s) implicated in the present study, E1a products have a similar effect on the SV40 enhancer.

How might the T-cell-derived negative regulator operate? Since the enhancer is located in the middle of the heavy-chain gene, the binding of a negative regulator to this enhancer could block transcription through the IgH gene. However, *xgpt* gene expression in hybrids containing pESgpt was extinguished even though the IgH enhancer was inserted 5' of the *xgpt* gene. In addition, *in vivo* (12) and *in vitro* (41, 44) binding assays have, as yet, failed to detect T-cell-specific factors that bind to the IgH enhancer.

The negative regulator could interfere in some way with the positive factors that interact with the enhancer or promoter or both. This might be the mode of action of a negative regulator(s) that prevents IgH enhancer-mediated activation in non-B cells. Such negative regulators were postulated when it was found that deletion of some regions of the 1-kb *Xba*I fragment resulted in an increase in activity of the IgH enhancer in non-B cells (23, 26, 50). However, the results of several studies suggest that continuous interaction of positive factors with the enhancer (4, 49), and, in fact, the enhancer region itself (29, 53) are not required to maintain high levels of gene expression. Consequently, a negative regulator which blocked this interaction would be expected to have little or no effect on a gene that had already been enhancer activated. The negative regulator responsible for gene extinction in B \times T hybrids, then, is unlikely to operate in this way. Such a regulator may act, instead, by altering gene modifications that have been introduced as a result of IgH enhancer-mediated activation. For example, enhancer activation might involve the formation of a transcription complex that, once formed, remains stable even in the absence of the enhancer. The negative regulator might mediate its effect through disruption of this complex either by binding to one or more of the proteins in the complex or by interfering in a more indirect way with the production or assembly of these proteins. It might have a role in the *de novo* methylation of the IgH gene we see after cell fusion, although it is not clear whether this methylation is a cause or simply an effect of the silencing of this gene.

We found that the presence of the IgH promoter, like the

IgH enhancer, can render a non-tissue-specific gene susceptible to T-cell-mediated extinction. The simultaneous activity of the IgH enhancer-driven *xgpt* gene and the endogenous λ light-chain gene in the one exceptional hybrid we recovered suggests a common negative regulator for these two elements (an enhancer has not yet been identified in the λ gene). The IgH promoter and enhancer share a consensus sequence, generally referred to as the octamer, which has previously been shown to play a role in the tissue specificity of IgH gene expression (see, e.g., references 13, 14, 16, 18, 20, 34, 36, 42). Experiments are under way to determine whether the same or different regulators are involved in the extinction of the IgH promoter-*xgpt* and IgH enhancer-*xgpt* gene constructions and, more specifically, whether the octamer plays a role in this extinction.

Other types of evidence for a negative regulators in non-B cells include studies of IgH gene expression in mouse L cells (a fibroblastic cell line). Mouse L cells transfected with an IgH gene expressed the gene in an enhancer-dependent manner after treatment with cycloheximide (24, 33). Cycloheximide treatment may have eliminated a labile negative regulator. Scholer and Gruss (43) have suggested, using an *in vitro* transcription system, that nuclear extracts from a human T-cell line, MOLT4, contain a repressor factor that binds to a portion of the IgH enhancer. Surprisingly, the factor identified in these assays appeared to bind (and mediate a negative effect on) both the B-cell-specific IgH enhancer and on an enhancer (lymphotropic papovavirus) normally functional in both T and B lymphocytes.

It has been postulated that transcription may stimulate immunoglobulin gene rearrangements (2, 47). A negative factor that specifically represses immunoglobulin gene transcription might serve to prevent these rearrangements in T lymphocytes while still allowing rearrangements at other loci (e.g., T-cell-receptor genes). Transcription does occur at the immunoglobulin locus in some T-cell lymphomas (not BW5147) (3, 27). It will be of interest to see whether such T-cell lines are lacking the negative regulators required to extinguish IgH enhancer- or promoter-regulated gene expression.

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