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Enhancer with Cellular Factors in Vivo**

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RESEARCH ARTICLE

B Lineage-Specific Interactions of an Immunoglobulin Enhancer with Cellular Factors in Vivo

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Enhancers are DNA sequences that increase the level of transcription from some promoters when placed within several kilobases of them (1, 2). Mouse immunoglobulin heavy chain genes contain a tissue-specific enhancer, which is located in the intron separating the V region J segment and the constant region (Fig. 1) (3-5). The immunoglobulin heavy chain genes are extensively transcribed in myeloma cells. Cloned copies of these genes are also efficiently transcribed when transfected into cells of the B lineage but not in non-B cells such as L cells, HeLa cells, or lung cells (3, 4).

Analysis of deletions showed that removal of a portion of the intron of the immunoglobulin γ_{2b} gene greatly reduces the level of transcription of the gene upon its transfection into myeloma cells (3). Further experiments revealed that restitution of a 990 base-pair fragment of DNA from the intron to these deletions restores high level transcription in myeloma cells and that this restoration is

independent of both the position and the orientation of the fragment (3). A smaller fragment, 307 base pairs in length (Fig. 1, base pairs 376 to 683) is capable of enhancing transcription in myeloma cells from heterologous promoters and genes such as β -globin and SV40 T antigen (4).

How enhancers work is not known. Although viral transcription enhancers function in most cells regardless of species or tissue of origin, they are most active in cells that are the virus's natural hosts (6-8). The immunoglobulin heavy chain enhancer has a somewhat more restricted tissue specificity and functions most efficiently in cells of the B lineage (3, 4). It seems likely that enhancer activity is related to and dependent on specific cellular factors. In the case of an enhancer showing tissue specificity one might expect to find proteins binding to the enhancer only in cells in which the enhancer is active.

Proteins that interact with specific sequences can prevent or increase the methylation by dimethyl sulfate (DMS)

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of specific guanine (G) residues at the N-7 position in the major groove (9). We have used DMS as a chemical probe to observe the G residues in and around the heavy chain enhancer in the DNA of living cells of diverse origins. The DMS penetrates cells and methylates the accessible G residues. Genomic sequencing (10) can display the state of methylation of the G residues in extracted DNA. We compared the patterns of G reactivity to DMS in several different types of cells, searching for alterations in the intensity of specific G bands that might indicate that sequence-specific proteins were bound. In cells of the B lineage but not in cells of non-B lineage, the DNA of the immunoglobulin heavy chain enhancer exhibited protections and enhancements in its G pattern. The affected G residues were mainly in four clusters, in and around sequences homologous to 5'CAGGTGGC 3' (C, cytosine; A, adenine; T, thymine).

Preliminary considerations. We treated whole cells briefly with DMS, to partially methylate the purines (11). We then extracted the DNA, digested it to completion with a restriction enzyme that cleaves about 100 bp from the beginning of the region being examined, and treated it with piperidine to cause cleavage at all of the methylated G residues. After electrophoretic migration through a denaturing gel and electrophoretic transfer to a nylon membrane, the DNA was cross-linked to the membrane by treatment with ultraviolet light. The membrane was probed with a short, single-stranded, highly radioactive probe made by polymerization across an M13 clone of a 100 to 150 nucleotide DNA fragment that ends at the chosen restriction site. This effectively end-labels the hybridizing DNA and displays a G-specific sequence. Four probes were used. The use of the Eco 5' lower probe (see legend to Fig. 1) and DNA cut with Eco RI is equivalent to end-labeling all hybridizable Eco RI-cleaved genomic fragments at the 5' end of the lower strand. Hybridization with the Hinf 5', Eco 3' upper probe to a Hinf I digest gives the same result as would end-labeling the 5' end of all the upper-strand Hinf I fragments at position 566 or alternatively, in an Eco RI digest, to end-labeling the 3' end of the upper strand fragments at position 683. The four probes enabled us to examine the reactivities to DMS of all the G residues between positions 24 and 997 on the upper strand and, on the lower strand, those between 275 and 574 and 818 and 951. We first describe the upper-strand patterns, then those of the lower strand.

Abstract. The mouse heavy chain immunoglobulin gene contains a tissue-specific enhancer. The enhancer and flanking sequences were studied *in vivo* by carrying out dimethyl sulfate protection experiments on living cells, in combination with genomic sequencing. Relative to reactions on naked DNA, there are changes (protections and enhancements) in the reactivity of guanine residues to dimethyl sulfate within the enhancer sequence in myeloma, B, and early B cells, whereas virtually no alterations appear in cells of non-B lineage. Most of the affected residues are in four clusters, in sequences homologous to the octamer 5'CAGGTGGC 3' (C, cytosine; A, adenine; G, guanine; T, thymine). The alterations in the pattern of G reactivity are consistent with the tissue-specific binding of molecules to the mouse immunoglobulin heavy chain enhancer.

While guanine residues in single-stranded DNA are equally reactive to DMS, in double-stranded DNA they have distinctive reactivities. [An example of this is the systematic underreaction of the middle G in the sequence 5' GGA 3' in double-stranded DNA (12).] Moreover, in living cells, the double-stranded DNA may have proteins bound to it which could affect the reactivity of the G residues. The N-7 position of guanine that is methylated by DMS lies in the major groove; proteins that come close to this N-7 atom can prevent or increase methylation by DMS at that

position (9, 13). To determine whether sequence-specific binding was occurring, we looked for cases in which a G was less reactive than it was in naked DNA (protected), equally reactive (no effect), or more reactive than in naked DNA (enhanced). If a protein is binding, we can expect to find several such protections and enhancements over a short stretch of bases.

Control experiments in which cells were not treated with DMS showed that the patterns observed are not due to the existence of cell-specific endonucleases or depurination; controls without piperi-

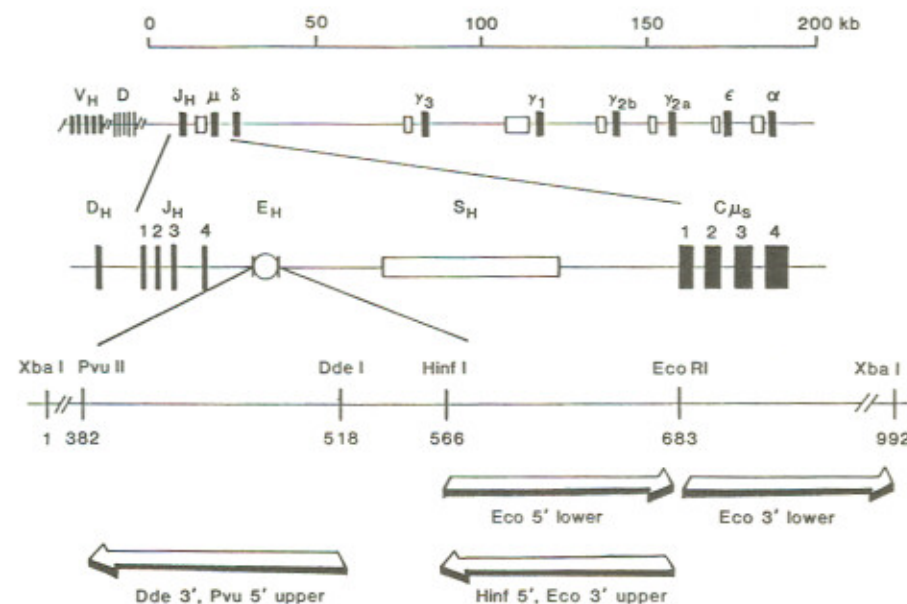


Fig. 1. Scheme of the region examined and of the probes used. The immunoglobulin heavy chain proteins are encoded in several gene segments (black bars). After V-D-J rearrangement (28), a portion common to all productive rearrangements remains, namely, the intron separating J4 and C μ . Within this intron are located the heavy chain enhancer (E_H; white circle) and the μ switch region (S_H; bar). The enhancer is located on a 992-bp Xba I-Xba I fragment whose sequence is shown in Fig. 5. The probes (arrows) have the following nomenclature: name of restriction enzyme with the 5' or 3' end designation and whether it is complementary to the upper or lower strand (for example Hinf 5' upper). Arrows pointing to the left indicate that the probe is specific for upper-strand sequences, and those pointing to the right, for lower-strand sequences. For instance, Eco 5' lower indicates that using this probe to hybridize with DNA cut with Eco RI will be equivalent to end-labeling the 5' end of lower strand genomic DNA fragments at the Eco RI (683) site. The single-stranded DNA probes were synthesized as described (10). Hinf I-Eco RI (566 to 683; Eco 5' lower, Hinf 5' and Eco 3' upper probes) and Eco RI-Xba I (683 to 992; Eco 3' lower) fragments were cloned into the M13mp8 (29) Sma I site as was the Pvu II-Bst NI (382-513; Dde 3' and Pvu 5' upper). The Bst NI site is 5 bp to the 5' of the Dde I site and close enough to use the Pvu II-Bst NI fragment to probe Dde I cut DNA.

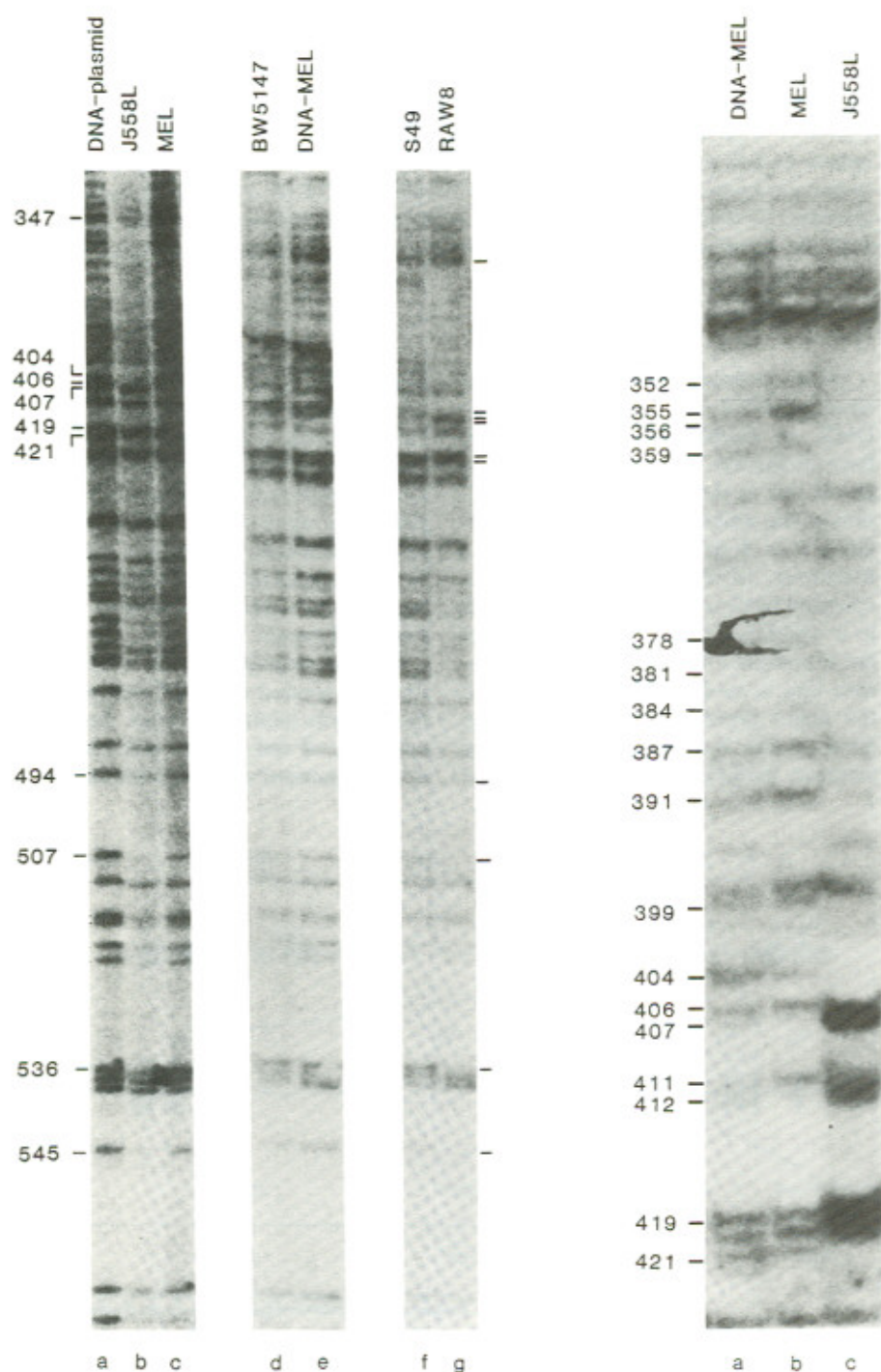


Fig. 2 (left). B lineage-specific protections and enhancements on the upper strand: 560-347. The pattern of reactivity of G residues to DMS in intact cells not of the B lineage (c, d, and f) is identical to that of naked DNA treated with DMS in vitro (e). Relative to this pattern, cells of the B lineage (b and g) show protections at positions 545, 536, 507, 494, 421, 411, 404 and enhancements at 419, 412, and 407. From 404 to 347, numerous protections occur (Fig. 3). Intact cells were treated with DMS in vivo. The DNA was transferred to a membrane which was probed with the Eco 3' upper probe. Plasmid DNA treated with DMS in the presence of formamide (a) has evenly reacted G residues. The systematic underreaction of the middle G in 5'GGA3' sequences in double-stranded DNA is evident when lanes (a) and (c) are compared at positions 419-421; these G residues are part of a stretch of four G residues that look like three residues in all double-stranded non-B lineage DNA's. Close examination shows that this cluster of four G's has, in cells of the B lineage (b), an enhanced G419, a normal G420, and a protected G421. Whether G422 is normal or protected is uncertain since it is weak in all double-stranded DNA's. Just below the G tetramer at 419 to 422 is a 5'GGGA 3' stretch, where cells of the B lineage and non-B cells have the same underreacted 5'GGA 3' middle G. The sequence of this region is shown in Fig. 5. The J558L (30), MPC11 (31), A20-2J (32), RAW8 (33), and MEL (34) cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10 percent fetal calf serum, 10 mM HEPES and $5 \times 10^{-5}M$ 2-mercaptoethanol. BW5147 (35) and S49 (36) were grown in RPMI 1640 (Gibco) with 10 percent fetal calf serum, 10 mM HEPES and $5 \times 10^{-5}M$ 2-mercaptoethanol. The cells were centrifuged, resuspended (10^8 cells per milliliter) in the same medium and kept on ice. Cells were then incubated with or without 0.5 percent DMS for 4 to 5 minutes at 20°C. Reactions were stopped by addition of 10 to 20 volumes of ice-cold PBS. Cells were examined for their ability to exclude trypan blue (37), and in all cases fewer than 1 percent were stained before and after DMS treatment. Cells were washed once with PBS, the nuclei were isolated, and the DNA was extracted according to standard procedures (38). Extracted DNA's were cut with restriction enzymes from New England Biolabs. The G reactions on DNA's that had not been treated with DMS in vivo and strand-scission reactions were as described (11). Reactions on single-stranded DNA were carried out by heating 60 ng of restriction-en-

zyme cleaved plasmid XF' (X18 poly-linker plasmid containing the 683-bp Xba I-Eco RI fragment) with 50 μ g of sonicated salmon DNA, in 80 percent formamide, 10 mM tris-HCl, pH 8.3, 1 mM EDTA at 90°C for 30 seconds, adding DMS to a concentration of 0.5 percent, incubating at 20°C for 1.5 minutes, and precipitating with ethanol. The genomic sequencing protocol (10) was followed, with simplifications. The first two washings after hybridization and the sealing procedure for the last washing were omitted. The damp membranes were placed between taut layers of Saran Wrap for autoradiography. Exposure times were 2 days, with intensifier screens. For exposures without screens, the exposure time was 2 weeks. All DNA's were cut with Eco RI, cleaved with piperidine, and separated by size on a denaturing gel, transferred to nylon, and probed with the Dde 3' upper probe. Naked DNA methylated with DMS in vitro (a) and a non-B lineage cell which was treated with DMS in vivo (b) have very similar patterns of reactivity of G residues with DMS. Their pattern is distinct from the pattern of DNA from a B-lineage cell which was also treated with DMS in vivo (c). Compared with (a) and (b), (c) has protected G residues at 421, 411, 406, 404, 399, 391, 387, 384, 381, 359, 356, 355, and 352; and G residues with enhanced reactivities at 419, 412, and 407. Since these residues are all in G clusters, it was necessary to observe these enhancements on shorter exposures, in order to resolve which band was indeed enhanced. The protection in (c) at G378 was not confirmed in other experiments with J558L nor was G378 protected in other cells of the B lineage. Similarly, G395 (b) was not protected in other experiments with MEL and with other non-B cells. G411 and G406 were slightly enhanced in (b) relative to naked DNA [occasionally in chromatin the first G in 5'GGA 3' and 5'GGC 3' sequences is enhanced compared with naked DNA (39)]. The sequence of this region is shown in Fig. 5.

dine rule out the possibility that the patterns are due to tissue-specific DMS-activated nucleases.

We compared two categories of cells. The first consisted of immortalized cells of the B lineage that express their immunoglobulin genes, namely, two myelomas (J558L and MPC11), a B lymphoma (A20-2J), and an Abelson virus-transformed early B cell (RAW8). The second was composed of non-B cells: a Friend virus mouse erythroleukaemia line, (MEL) and two T lymphomas (S49 and BW5147). All of the B-lineage cells produce some form of immunoglobulin RNA (14-16). Little or no immunoglobulin RNA has been detected in the non-B cells (16, 17).

Changes in G reactivity are B lineage-specific. We examined nearly 1000 bases of DNA for alterations in reactivity of G residues with DMS and found 28 protections and four enhancements. These alterations are specific to cells of the B lineage and all are localized almost precisely to the region defined by transfection experiments as the enhancer (3, 4).

We first examined DNA from cells treated with DMS *in vivo* using the Eco 3' upper probe (Fig. 1). The sequence is most distinct beginning around the Hinf I site and extending 5' from it (Fig. 2). Cells of the B lineage show a G pattern of reactivity distinct from the pattern of non-B cells and of naked DNA. In B cells (Fig. 2, lanes b and g), G's at positions 545, 536, 507, 494, 421, and 411 are protected; G412 and G419 have enhanced reactivities to DMS. In some experiments G460 and G458 were protected. There is a strong enhancement at the 406-407 band, which is followed by a stretch of protected G residues extending to 347. There are no differences in relative G reactivity between DNA's from any cells and naked DNA 5' to 347.

The stretch from 407 to 347 was examined by means of Dde I digested DNA and the Dde 3' upper probe (Fig. 1). This region is protected exclusively in cells of the B lineage. In those cells, the protected region begins with the strong enhancement at 407 (Fig. 3). The G at position 406 is protected as are residues 404, 399, 387, 384, 381, 359, 356, 355, and 352. In some experiments G391 was protected. G388 is a weakly reactive G (the middle G of a 5'GGC 3' sequence) and may, in some experiments, be protected. From G347 to G24, the pattern in B cells is indistinguishable from the pattern in non-B lineage cells or in naked DNA.

The patterns of B-lineage and non-B

cells (with one possible exception) are identical at all positions 3' to 545 (data not shown). DNA digested with Pvu II and probed with the Pvu 5' upper probe reveals the G's between 480 and 750; a Hinf I digest probed with the Hinf 5' upper probe reveals those between 670 and 992 (Fig. 1). Over this entire 500-bp region all the differentiated cells examined had identical patterns of G reactivity to DMS *in vivo*.

The G at 708 is protected in all cells, with one exception. In one experiment, the embryonal carcinoma line PSA-1 (18) was used and showed a pattern indistinguishable from that of naked DNA at all positions including a visible band at 708. The 708 G residue is not an intrinsically weak G residue: it appears as a strong band in all the naked DNA's. In RAW8, A20-2J, J558L, and MPC11 it is completely obliterated, as it is in BW5147 and MEL cells.

The same strategy was used to examine the lower strand in and around the mouse heavy chain enhancer (data not

shown). Figure 4 shows results obtained with the Eco 5' lower probe (Fig. 1): the G residues at 534, 533, 531, and 523 are protected, and that at 530 is enhanced in cells of the B lineage. A second cluster of G's is protected in these cells, at 413, 408, 401, 399, 358, and 357. No other changes appear in the stretch of DNA between 275 and 575.

Outside the enhancer region, we examined some additional 130 bp with the Eco 3' lower probe on Eco RI digested DNA's. No alterations in the G pattern appeared between 818 and 951 (data not shown). In all cases the whole cell patterns were identical and were indistinguishable from naked DNA controls.

Altered G reactivity occurs in conserved sequences within the enhancer. On both strands, more than 30 G residues are protected or enhanced in cells of the B lineage (Fig. 5) within a region of 970 bp on the top strand and 430 bp on the lower. These tissue-specific effects are all localized within a 193-bp stretch of DNA in the middle of the 992-bp region. Most of the protections and enhancements occur in four clusters located at sequences that share homology to the sequence 5'CAGGTGGC 3'. Three of the four clusters match this sequence at seven out of eight positions; the other matches at six out of eight positions (Fig. 6). Three of the clusters have the 5'CAGGTGGC 3' homologous sequence on the top strand; in the fourth cluster, it is found on the bottom strand (Fig. 5).

Within these sequences on the top strand, the G's at positions 3, 4, and 6 are protected; the G at position 7 is either protected, enhanced, or normal. On the lower strand G1 and G8 are protected. We suspect that these sequences may represent recognition sites for enhancer binding proteins. Given the similarity of these sequences, the binding proteins might be identical.

The reason residue G7, in the octamer, is protected in one case, normal in the second, and enhanced in the third and fourth, may be that the positioning of binding molecules is slightly different in each case because of variations in the DNA sequence. An amino acid residue in one position could protect a G from reacting with DMS while a slight shift in its position could expose the G and allow it to react and perhaps even be enhanced because of the proximity of the protein. This happens in the binding of phage lambda repressor to the operator site O_{R1} (19), where the nearly symmetrical site O_{R1} is divided into two half-sites, each of which is recognized by one subunit of the repressor dimer. Since the

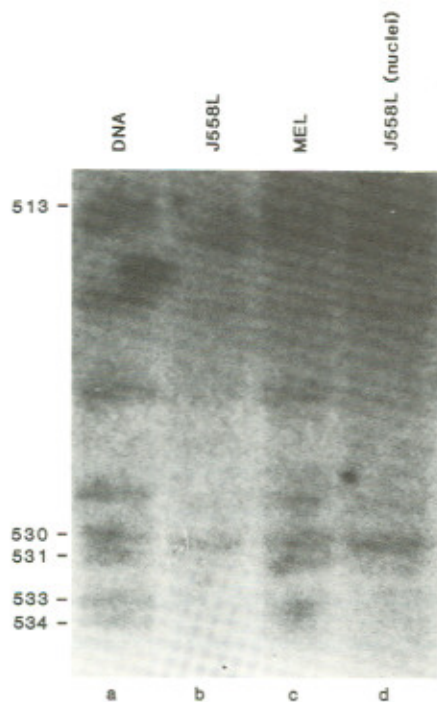


Fig. 4. B lineage-specific protections and enhancements on the lower strand. The membrane shown in Fig. 2 (a, b, and c) was re-probed with the Eco 5' lower probe. G residues at positions 534, 533, 531, and 523 are protected, and G530 is enhanced in myeloma cells and nuclei (b and d) compared with the non-B-cell line (c). Lane a shows denatured plasmid DNA (Fig. 2). At the top of the figure, two closely spaced bands are visible in (a), only one of which is visible in (b, c, and d). These closely spaced bands are part of a 5'GGA 3' stretch (see Fig. 5, 511 to 513, lower strand) in which the middle G is underreacted in double-stranded DNA. The sequence of this region is shown in Fig. 5.

center of symmetry passes through the central base pair, the repressor monomers do not bind with perfect symmetry; a G that is protected in one half-site, is in the other enhanced.

In a companion study, where we examined the heavy chain enhancer in vitro in nuclei (20), we compared B cell to L and Friend cell nuclei. We demonstrated tissue-specific contacts, in the enhancer region, to two sequences inverted with respect to one another and homologous to GCCAGGTGGC (sites 3 and 4). In the study presented here, we show (i) that

the same contacts can be seen in living cells, and (ii) that in these in vivo experiments a further set of protections appears, mostly upstream of sites 3 and 4. The additional contacts to sites 1 and 2 could reflect the binding of the same factor to these more distant homologies or could reflect the binding of other tissue-specific factors to the region, a binding not reflected in vitro.

Some of the protections and enhancements that we see are outside the four octamer sequences. One protection (G545), which maps outside of these

clusters, is located in the sequence TAATTTGCAT. This sequence is homologous to one of the two short sequences found upstream of all immunoglobulin variable region genes and within a region, as defined by transfection experiments, that is essential for correct transcription of those genes (21).

Altered G reactivity correlates with enhancer function. By treating whole cells in vivo with dimethyl sulfate and analyzing their DNA by genomic sequencing, we have shown tissue-specific contact of factors to the mouse immuno-



Fig. 5. Summary of the protections and enhancements. The complete sequence of the Xba I-Xba I fragment which contains the mouse immunoglobulin heavy chain enhancer is shown. Residues whose reactivities to DMS have been examined are indicated by capital letters: G residues 14 through 996 on the top strand and on the lower strand, G residues 275 through 574 and 818 through 951. A fragment spanning bases 376 to 683 has approximately full enhancer activity (4). B lineage-specific protections (○) and enhancements (●) are indicated above upper strand and below lower strand residues. Sequences homologous to those found upstream of all immunoglobulin variable region gene segments (21) are located at 451 to 465 and 539 to 548. The protection at 545 is within one of these sequences. A cross at 708 marks the G which is protected in all differentiated cells examined.

globulin heavy chain enhancer. Almost all of the protections and enhancements we found are specific to cells of the B lineage, which are committed to expressing their immunoglobulin genes. All of the alterations, on both strands, are localized within 200 bp that coincide almost exactly with a fragment defined as sufficient for almost full enhancer activity (3, 4).

Banerji *et al.* (4) transfected into cells the enhancer linked to the SV40 T antigen gene, and assayed T antigen-positive cells. When they deleted sequences 3' to 496, thereby eliminating site 4, activity was reduced. When they deleted sequences 5' to 400, which include sites 1 and 2, the activity fell noticeably, while deleting site 1 alone had no effect. Gillies *et al.* (3), assaying gpt transformants, found that the fragment spanning 387 to 520 (lacking site 4) had half the activity, while Banerji *et al.* (4) showed that the segment from 456 to 627 (lacking 1, 2, and 3) was one-half to one-third as active as the full region, in stimulating β -globin synthesis. These results suggest that these sites can independently provide some enhancer activity.

These distinct patterns, including complete protections, are all in cells that make immunoglobulin messenger RNA (mRNA). There is no striking difference in pattern between immortalized cells believed to represent three distinct stages of B cell development in spite of the fact that these cells make different amounts of immunoglobulin mRNA: myeloma cells express more immunoglobulin mRNA both from their native genes and from transfected copies than do pre-B cells. However, experiments measuring the intrinsic strength of the enhancer in pre-B, B, and myeloma cells have not been reported. The enhancer may be equally active in all cells of the B lineage regardless of their developmental stage; this pattern may reflect general tissue-specific events that make the enhancer active and the gene available for transcription; subsequent changes might then regulate the precise level of immunoglobulin mRNA in cells at different stages of development.

We do not see this pattern of contact in T cells or Friend cells. In fact, the T-cell line S49 makes no detectable immunoglobulin mRNA (16) and BW5147 is reported as making little (three molecules per cell) (17) or no (16) detectable immunoglobulin RNA. Thus the immunoglobulin enhancers in these T cells do not seem to be very active. Although the cell line used in these experiments was not tested, Friend virus erythroleukemia

cells make no detectable immunoglobulin mRNA (16).

In most cells the enhancer exists in two copies which, in the case of B-lineage cells, are often located in different regions of the genome. In J558L, for instance, one copy of the enhancer is appropriately located within the large intron of a productively rearranged α gene. The other copy has been translocated from the immunoglobulin heavy chain locus to the c-myc locus on chromosome 15 (22). Since some of the protections in these cells are complete, it is likely that the reactivities of these G's in both copies of the immunoglobulin gene are being affected by the same factors in the same way.

In double-stranded DNA, as opposed to single-stranded DNA, the sequence 5'GGA 3' consistently yields an underreacted middle G in the reaction with DMS (12). There are five 5'GGA 3' sequences distributed within the enhancer. The reactivities of the middle G's in all 5'GGA 3' sequences in vivo are characteristically weak, like those of double-stranded naked DNA. Thus the immunoglobulin enhancer is for the most part double-stranded.

These patterns are probably not a sim-



Fig. 6. Alignment of clustered protections and enhancements: the octamer CAGGTGGC. The tissue-specific protections and enhancements shown in Fig. 5 are located in clusters within the immunoglobulin heavy chain enhancer. The four clusters have sequence homologies and are similar to the octamer 5'CAGGTGGC 3'. When these G's occur in the sequence, G1, G3, G4, and G6 are protected, and G7 can be either protected, normal, or enhanced. Three clusters (350 to 357, 382 to 389, 401 to 408) are found in one orientation in the enhancer, the other (536 to 529) occurs in the opposite orientation.

ple consequence of transcription since the amount of transcription in the cell lines varies. A more likely interpretation is that these patterns are a consequence of binding of proteins that cause the enhancer activation.

There are other examples of activation of eukaryotic genes by binding proteins. In yeast, elements called upstream activator sequences (UAS) are regulated by *trans*-acting positive regulatory proteins and have some properties similar to those of enhancers (23). The glucocorticoid receptor, which can stimulate the transcription of mouse mammary tumor virus (24, 25), binds in vitro (26) to a fragment of DNA called the glucocorticoid response element (GRE) (27). The GRE, reminiscent of enhancers, has been shown to confer hormone inducibility to heterologous promoters in a distance and orientation-independent fashion.

These experiments demonstrate tissue-specific contacts, most likely made by a binding protein, that correlate with enhancer function. In general, the tissue-specific action of the enhancer could be due to factors positively acting on it in B cells or to negatively acting factors in non-B cells. Our findings support the first model.

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