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Combinatorial Regulation of Transcription II: Revie the Immunoglobulin μ Heavy Chain Gene

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The regulated transcription of a gene expressed in the immune system depends, as in any cell system, on contributions from multiple regulatory proteins and regulatory events. The existence of complex mechanisms of gene regulation was postulated many years ago (Gierer, 1973; Britten and Davidson, 1969; Georgiev, 1969) to explain how a genome of limited size can provide sufficient information for dictating an enormous number of expression patterns. The first installment of this review (Ernst and Smale, 1995) provided an overview of the multiple types of control regions and controlling events that contribute to the combinatorial regulation of transcription in eukaryotes. This installment focuses in greater detail on studies of the transcriptional regulation of a single gene, the murine immunoglobulin μ (Igμ) heavy chain gene.

Transcriptional regulatory mechanisms have been studied in greater detail for the Igμ gene than for any other gene specific to the immune system (for detailed reviews, see Staudt and Lenardo, 1991; Nelsen and Sen, 1992; Kadesch, 1992; Calame and Ghosh, 1995). These studies have demonstrated that a diverse array of proteins are involved in Igμ transcription. The identities of many of the regulatory proteins remain unknown and the precise regulatory mechanisms have not been clearly established. However, the knowledge generated since Igμ regulatory regions were first reported in 1983 has inspired several recent experiments that provide novel insights into regulatory strategies. Following a brief introduction to the Igμ gene as a model for B cell–specific transcription, specific experiments to illustrate these strategies are described.

The expression of immunoglobulin genes is unique to, and essentially defines, the B cell lineage. The Igμ locus appears to be activated at a very early stage of B cell development, preceding V(D)J recombination and possibly preceding commitment to the B cell lineage. The initial activation of the locus has been studied by analyzing changes in methylation status and DNase I hypersensitivity of Igμ regulatory regions (Mather and Perry, 1983; Storb et al., 1981). Hypomethylation and DNase I hypersensitivity have been observed not only in B lineage cells, but also in multipotential hematopoietic precursors (Ford et al., 1992). Transcripts initiating within the locus are detectable at an early progenitor B cell stage (Yancopoulos and Alt, 1985, 1986). Following gene rearrangement (reviewed by Yancopoulos and Alt, 1986), mature transcripts increase throughout the subsequent preantigen recognition stages of differentiation (Perry and Kelley, 1979). Igμ mRNA is further induced in an antigen- and T cell–dep dent manner (Melchers et al., 1980; Raynal et al., 19 Yuan et al., 1992).

The analysis of Igμ transcription is complicated by gene rearrangements that take place in immature c and by the isotype switching events that take place mature cells. Some control elements may play a dual r of enhancing recombination/switching and transcript initiation. Furthermore, in addition to the Igμ enhancer scribed below, enhancers and locus control regions (LC are present downstream of the entire IgH locus and in vicinity of the coding segment for at least one other isot (see Calame and Ghosh, 1995; Madisen and Groud 1994; and references therein). For simplicity, we will fo our attention on the control regions needed for the B c specific transcription of a fully rearranged Igμ gene.

As shown in Figure 1, rearranged Igμ genes contai core promoter, regulatory promoter, enhancer, and ma attachment regions (MARs) that are typical for a mamma gene transcribed by RNA polymerase II (pol II; Staudt Lenardo, 1991; Calame and Ghosh, 1995). The core pro ers for the numerous V_H gene segments contain an A/T- sequence that appears to function as a TATA box by dir ing transcription initiation from a site located 25–30 downstream. The regulatory promoters are located prim upstream of the TATA box. Although a few common c trol elements are found in the promoters of most V_H g segments, the promoter sequences and control eleme vary. One element, called an octamer, is highly conser among the V_H and V_L promoters and between spec Other conserved elements that influence promoter stre include a heptamer element 2–20 bp upstream of the tamer, as well as C/EBP and μE3 elements further stream (Figure 1). An enhancer of approximately 25(is located in the J–Cμ intron and is flanked by two MA As described below, the intronic enhancer combined the MARs comprises an LCR. Control elements within enhancer include the C/EBP, μE1, μE5, μE2, μA (o μE3, μB, μE4, and octamer elements. Proteins that h been shown to bind to these elements are indicate Figure 1.

The control regions described above appear to be p cipally responsible for the precise expression level expression pattern observed with the endogenous arranged Igμ gene. Rearranged murine Igμ transge which include a V_H promoter, an intronic enhancer, MARs, are expressed at levels comparable to that of endogenous Igμ gene (Grosschedl et al., 1984; Jenuw and Grosschedl, 1991). The murine transgenes also clo mimic the expression pattern of the endogenous g both are expressed primarily in B cells and, in both ca transcripts are detectable at low levels in a similar se other cell types (Jenuwein and Grosschedl, 1991; A weiler et al., 1992). Furthermore, transcription from μ hancer–containing transgenes and from the endoge Igμ gene can be induced in vitro by differentiation

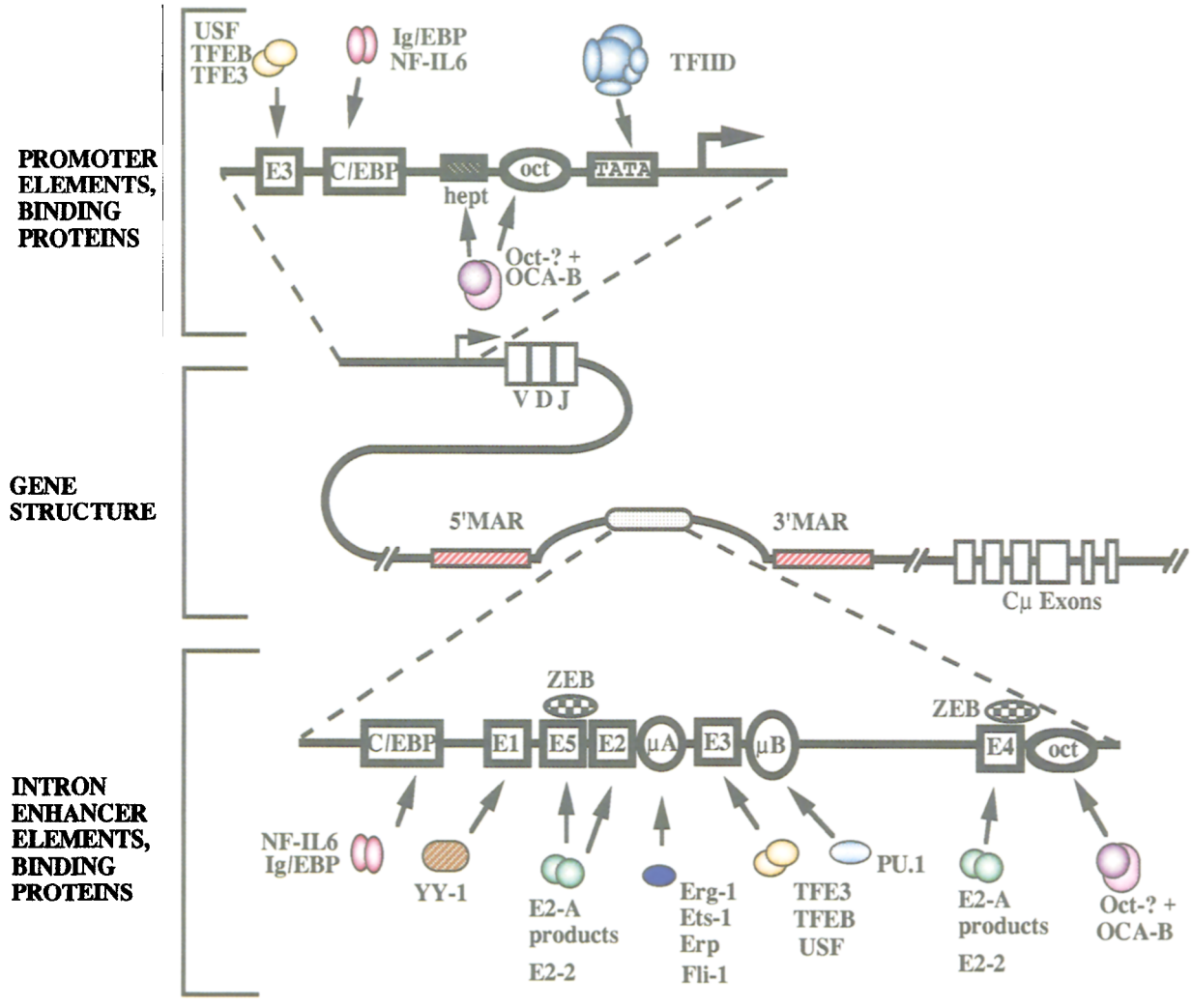

Figure 1. The Murine Immunoglobulin μ Heavy Chain Gene and Control Elements

growth signals, such as interleukin-6 and lipopolysaccharide (Yuan et al., 1992; Raynal et al., 1989; Miller et al., 1992).

In the context of a rearranged gene, all of the control regions (core promoter, regulatory promoter, enhancer, and MARs) appear to be essential for efficient μ transcription in transgenic mice (Jenuwein and Grosschedl, 1991; Annweiler et al., 1992; Forrester et al., 1994). However, in transgenic mice or in transfection experiments, mutations in individual enhancer elements only partially reduce enhancer activity (Jenuwein and Grosschedl, 1991; Annweiler et al., 1992; Lenardo et al., 1987; Kiledjian et al., 1988; Perez-Mutul et al., 1988; Tsao et al., 1988; Libermann et al., 1990; Nelsen et al., 1990, 1993). By using enhancer subfragments, instead of the entire enhancer, a more complete inhibition of activity can be obtained with a mutation in an individual enhancer element.

B cell–specific transcription can be directed by either the promoter or the enhancer, if the promoter is accompanied by a heterologous enhancer or if the μ enhancer is accompanied by a heterologous promoter (Garcia et al., 1986; Grosschedl and Baltimore, 1985). Thus, Igμ transcription is regulated in a manner that appears to be redundant, in that no single control region or control element is solely responsible for B cell specificity. This concept is exemplified by the experiments described below, which reveal that several ubiquitous and cell-specific proteins contribute to the precise expression pattern of the Igμ gene.

A B Cell–Specific Coactivator Regulates Transcriptional Activation by Oct Proteins

The octamer was the first regulatory element identified in the immunoglobulin heavy chain and light chain genes (for review, see Staudt and Lenardo, 1991). This element was conspicuous because of its conservation at a similar position (about 40 bp upstream of the TATA box) in almost all V_H and V_L promoters. In transfection assays and in transgenic mice, mutations in this element strongly reduce the activity of V_H and V_L promoters. Furthermore, insertion of a single octamer element upstream of a simple core promoter generates a promoter that is selectively active

in B cells. A control element matching the octamer motif is also present in the Igμ enhancer; mutation of this octamer element reduces, but does not eliminate, tissue-specific enhancer activity in transgenic mice (Jenuwein and Grosschedl, 1991; Annweiler et al., 1992). These and other results (Staudt and Lenardo, 1991) demonstrate that the octamer element plays a critical role in regulating the B cell specificity of Igμ transcription.

Electrophoretic mobility shift assays led to the identification of multiple proteins that interact with the octamer motif (Singh et al., 1986; Staudt et al., 1986; Landolfi et al., 1986). Isolation of cDNAs encoding these proteins revealed that they are members of a large class of DNA-binding proteins referred to as the POU domain proteins (Herr et al., 1988; Rosenfeld, 1991; Schöler, 1991). The POU domain, which is approximately 150 aa in length, consists of two adjacent regions that are homologous among family members: the POU-specific domain and the POU-homeo domain. Both of these regions contain helix–turn–helix motifs that are required for high affinity DNA binding. Numerous POU domain proteins have been identified, many of which are expressed in a cell type–specific manner (Rosenfeld, 1991; Schöler, 1991). Not surprisingly, numerous genes with widely varying expression patterns contain binding sites for these proteins, with many of the binding sites closely resembling the octamer elements found in the immunoglobulin genes.

Oct-1 and Oct-2 are two POU domain proteins that have been characterized extensively and bind with high affinity to the octamer elements in the immunoglobulin genes (Staudt and Lenardo, 1991). These proteins are encoded by two different genes, but the *Oct-2* transcripts undergo alternative splicing, resulting in the production of several distinct protein isoforms (Wirth et al., 1991). These isoforms contain the same POU domain, but the surrounding sequences differ, leading to variable transcriptional activation properties (Annweiler et al., 1994). Oct-1 and Oct-2 appear to bind equally well to the immunoglobulin octamer motif, but Oct-1 is expressed in most cell types, whereas Oct-2 is expressed primarily in B cells (Staudt and Lenardo, 1991).

The identification of an Oct protein that is expressed primarily in B cells suggests a simple model: only Oct-2 can function through the immunoglobulin octamer elements, thereby leading to the B cell specificity of immunoglobulin transcription. Recently, however, this model has been disproven, as homozygous disruption of the Oct-2 gene did not reduce immunoglobulin expression in mice or in cultured B cells (Corcoran et al., 1993; Feldhaus et al., 1993). Instead, Oct-2 appears to be essential primarily for events that occur during terminal differentiation of B cells (Corcoran et al., 1993; Corcoran and Karvelas, 1994; Radomska et al., 1994). Consistent with these results are several studies that have demonstrated equally efficient transcriptional activation of immunoglobulin promoters by both Oct-1 and Oct-2 (LeBowitz et al., 1988; Pierani et al., 1990; Pfisterer et al., 1994; and references therein). With an in vitro transcription assay, for example, an immunoglobulin promoter was strongly activated by either purified Oct-1 or purified Oct-2 in a B cell nuclear extract depleted of Oct proteins (Pierani et al., 1990). Thus, the ubiquito Oct-1 protein appears to be perfectly capable of activati transcription through a control element that imparts B c specificity to an immunoglobulin promoter.

An attractive explanation for the above results h emerged from studies performed both in vitro and in vi Extending the in vitro results described above, Pierani al. (1990) found that neither Oct-1 nor Oct-2 could e ciently activate transcription from an immunoglobulin p moter in a nonlymphoid (HeLa) cell extract, even thou both proteins strongly activated transcription from a h tone H2B promoter, which contains a similar octamer e ment. Similar results were found in vivo, most recently Pfisterer et al. (1994), who stably expressed various Oc isoforms in fibroblast cell lines at levels comparable to Oct-2 levels in B cells. Despite the presence of signific Oct-2 protein in the transfected fibroblasts, synthetic p moters under the control of multiple octamer eleme were inactive. These observations suggest that a disti B cell–restricted activity may be required for immunogl ulin transcriptional activation by both Oct-1 and Oct-2

A novel activity that appears to be responsible for above results has been defined as a B cell–restricted activator, called OCA-B (Pierani et al., 1990; Luo et 1992; Luo and Roeder, 1995). In general, two biochemi activities have been attributed to OCA-B. When addec a HeLa cell extract, it facilitates transcriptional activat of an immunoglobulin promoter (but not an H2B promo by either Oct-1 or Oct-2. In addition, OCA-B physic associates with both Oct-1 and Oct-2, but not with anot Oct protein, Oct-3. Purified OCA-B preparations cont two protein species with molecular masses of 34 and kDa (Luo and Roeder, 1995). From partial amino acid quences of the purified proteins, a cDNA has been isola that encodes both proteins, with the 35 kDa protein ap ently derived by posttranslational modification (Luo Roeder, 1995). Antibodies directed against the recon nant protein inhibit OCA-B-mediated activation of an munoglobulin promoter, confirming that the isolated cD encodes the OCA-B activity. Interestingly, the *OCA-B* g was independently isolated by two other laboratories fi cDNA expression libraries screened for proteins that rectly bind to Oct-1 or Oct-2 (Gstaiger et al., 1995; Stru et al., 1995).

The properties of the OCA-B coactivator descri above provides an explanation for the B cell specif attributed to the octamer elements of immunoglobulin moters. At the same time, several new questions eme Principally, why is a specific coactivator needed for t scription from immunoglobulin promoters, but appare not for transcription from other promoters that can be vated by Oct-1, like the *H2B* promoter? The specif does not appear to reside in the immunoglobulin pro ers, as synthetic promoters containing octamer elem also exhibit B cell specificity (Pfisterer et al., 1994, references therein). One simple explanation may be the spacing between the octamer and the TATA bc shorter in the vertebrate *H2B* promoters than in the im noglobulin promoters (Luo et al., 1992); the short spa could allow Oct-1 to function in the absence of a coac

tor. An alternative explanation is that, in the *H2B* genes, Oct-1 may act in concert with another protein that binds to the promoter. Utilizing recombinant coactivator and Oct proteins, this and other questions regarding the mechanisms of activation through octamer elements can now be addressed.

Interplay Between Positive and Negative Regulators at the μE Elements

The studies described above demonstrate that ubiquitous Oct proteins contribute to the B cell specificity of Igμ transcription by employing a tissue-restricted coactivator. This section describes studies of another family of transcription factors, the bHLH proteins, which provide further insight into the mechanisms by which ubiquitous proteins may influence Igμ regulation. Two related questions are addressed by these studies: how do ubiquitous bHLH proteins regulate B cell–specific transcription? If bHLH family members with the capability of binding to μ enhancer elements are present in nonlymphoid cells, why is the Igμ gene inactive in these cells?

Proteins of the bHLH family recognize the μE elements within the μ enhancer (Kadesch, 1992; Murre et al., 1994; Calame and Ghosh, 1995). The bHLH domain that is characteristic of this family contains a stretch of basic amino acids followed by two amphipathic helices that are separated by a spacer (i.e., loop). The bHLH proteins bind to DNA as heterodimers with other family members or, in some cases, as homodimers; the basic region mediates the protein–DNA interactions and hydrophobic surfaces of the HLH domain mediate dimerization. A core binding sequence for bHLH dimers is CANNTG, with the two central nucleotides and flanking nucleotides involved in determining the precise binding specificity for a homodimer or heterodimer. Although considerable effort has been applied towards isolating a bHLH protein that is expressed specifically in lymphocytes, none has been found. Instead, the bHLH proteins expressed in lymphocytes appear to be expressed in many cell types.

Two general classes of bHLH proteins bind to the μE elements in lymphocytes. One class includes protein products of the *E2A*, *E2–2*, and *HEB* genes (Murre et al., 1989a; Henthorn et al., 1990; Hu et al., 1992). The proteins in this class are capable of binding to the μE2, μE4, and μE5 elements as homodimers or as heterodimers. The *E2A* gene produces three protein products through alternative splicing: E12, E47, and E2–5. Most studies described below have employed the protein isoforms derived from the *E2A* gene. We will not distinguish between these isoforms for simplicity and because it is not yet clear which products actually function through each control element.

The second class of bHLH proteins that interacts with the μ enhancer contains several members, including TFE3, TFEB, and USF (Calame and Ghosh, 1995; Beckmann et al., 1990; Carr and Sharp, 1990; Gregor et al., 1990; Roman et al., 1992). Members of this class bind as homodimers, or as heterodimers with each other, to the μE3 element in the enhancer and to μE elements found in some IgH promoters (Artandi et al., 1994). A unique feature of this family is the presence of a leucine zipper (Kerppola and Curran, 1991) as a second protein–protein interaction motif, C terminal to the bHLH domain. In this family, which is sometimes referred to as the bHLH–ZIP family, the zipper domain appears to be a primary determinant of the dimerization specificity (Beckmann and Kadesch, 1991; Fisher et al., 1991; Roman et al., 1992). The presence of two protein–protein interaction motifs makes it possible for the bHLH–ZIP proteins to form higher order multimers; in fact, tetramers of the TFE3 and USF proteins have been identified (Ferre-D'Amare et al., 1994; Artandi et al., 1994). This finding suggests that bHLH–ZIP tetramers, by binding simultaneously to μE elements in both the Igμ promoter and Igμ enhancer, may provide a physical bridge between the two control regions. Support for this idea recently has been provided by experiments that demonstrate that TFE3 possesses a fairly unique ability to stimulate transcription from a distant enhancer site in a manner that is absolutely dependent on the presence of TFE3 binding sites in the promoter (Artandi et al., 1994).

Despite the apparent absence of a lymphocyte-specific bHLH protein, considerable evidence suggests that products of this family directly participate in B cell–specific Igμ transcription. The first evidence for this role was provided by in vivo genomic footprinting experiments, which revealed protein–DNA contacts at the μE motifs in B cells but not in nonlymphoid cells (Ephrussi et al., 1985; Church et al., 1985). More recently, overexpression of an E2A protein was found to activate rearrangement and transcription of the endogenous Igμ gene in a progenitor T cell line (Schlissel et al., 1991). Furthermore, mice containing a homozygous disruption of the *E2A* gene specifically lack progenitor and mature B cells (Bain et al., 1994; Zhuang et al., 1994). Together, these results suggest that E2A proteins, and possibly other bHLH proteins, play major roles in regulating the B cell specificity of Igμ transcription.

Electrophoretic mobility shift assays performed with nuclear extracts provided a clue as to the mechanism by which ubiquitous bHLH proteins might direct cell-specific transcription, by revealing the formation of B cell–specific protein–DNA complexes with DNA probes containing μE2, μE4, or μE5 (Murre et al., 1991; Bain et al., 1993; Jacobs et al., 1993). The presence of E2A proteins within these complexes was demonstrated by their reactivity with monoclonal antibodies. Further characterization of the complexes suggested that they include E2A or E2–2 homodimers or heterodimers. The molecular mechanism responsible for the B cell–specific dimerization of ubiquitous proteins has not been established, but four possible mechanisms (which are not mutually exclusive) have been proposed. First, a posttranslational modification of the ubiquitous proteins could regulate the formation of B cell–specific dimers. Second, the formation of an intermolecular disulfide bond, which has been identified in E2A homodimers, may be regulated in a B cell–specific manner (Benezra, 1994). Third, preferential heterodimer formation with cell-specific bHLH proteins could prevent the ubiquitous proteins from forming dimers with each other in non-B cells. In muscle cells, for example, E2A proteins preferentially form heterodimers with muscle-specific bHLH proteins like MyoD (Murre et al., 1989b; Lassar et al., 1991).

A fourth mechanism for preventing the dimerization of ubiquitous bHLH proteins with each other is through sequestration by HLH proteins that are dedicated to this function. Two such proteins are Id and Id2 (Benezra et al., 1990; Sun et al., 1991). Both of these proteins contain HLH domains that are homologous to the HLH domains of E2A proteins, but the Id proteins lack a basic domain. Therefore, although Id and Id2 form heterodimers with the E2A proteins, the heterodimers are not capable of binding to DNA. The Id proteins were first implicated in Igμ transcription and B cell development by the observation that both Id and Id2 are expressed at high levels in progenitor B cell lines that lack detectable Igμ transcription, but are not expressed in more mature B cells (Wilson et al., 1991; Sun et al., 1991). Furthermore, ectopic expression of Id in cultured cells led to repression of the μ enhancer, presumably by forming dimers with E2A proteins (Wilson et al., 1991). Recently, constitutive expression of Id in the B cell lineage of a transgenic mouse was found to block B cell development at an early stage (Sun, 1994). Although these studies primarily confirm that the heterodimer partners of Id are required for B cell development, the results are intriguing and consistent with the idea that Id proteins may play a direct role in regulating Igμ transcription.

Although E2A homodimers appear to form only in B cells, it has been well established that E2A proteins form potent transcriptional activators in other cell types as heterodimers with cell-specific proteins like the muscle-specific MyoD protein (Lassar et al., 1991). In fact, a subset of the μE sequences are identical to the E boxes found in muscle-specific genes, which are strongly activated by MyoD/E2A heterodimers (Lassar et al., 1991; Weintraub et al., 1994). This observation highlights the critical question of why these heterodimers do not activate Igμ transcription in muscle cells. Clearly, the lack of B cell–restricted proteins like OCA-B and PU.1 (see below) in muscle cells helps to prevent efficient Igμ transcription. However, recent experiments have suggested that a more direct strategy has evolved to insure that Igμ transcription is not induced in non-B cells. This strategy involves negative regulation through the μE4 and μE5 elements.

The μE4 element was implicated in negative regulation through the analysis of B × T hybrid cell lines (Shen et al., 1993). In these hybrids, transcription of the endogenous Igμ gene is repressed, presumably because transacting repressors expressed by the T cell partner interfere with activators expressed by the B cell partner. By transfecting the hybrids with reporter genes under the control of various μ enhancer fragments and mutant enhancers, the μE4 element was found to contribute to enhancer repression (Shen et al., 1993). The μE5 element was implicated in negative regulation through analyses of enhancer mutants in nonlymphoid cells. In cultured cell lines and in transgenic mice, mutation of the μE5 element led to enhancer activity in multiple cell types (Kadesch et al., 1986; Wasylyk and Wasylyk, 1986; Weinberger et al., 1988; Ruezinsky et al., 1991; R. Grosschedl, personal communication).

The negative effects observed with the μE4 and μE5 elements suggest that they interact with a repressor that prevents adjacent control elements (e.g., μE2 and μ from functioning in non-B cells. Indeed, a candidate this repressor protein, which binds to both μE4 and μ recently has been isolated (Genetta et al., 1994). This p tein, called ZEB, contains a zinc finger DNA binding main and is expressed ubiquitously. E2A homodimers displace ZEB from the μE5 element, but ZEB overexpr sion cannot repress the μ enhancer in B cells. This re suggests that ZEB and E2A proteins do not carry ou simple competition for the μE5 site.

The above studies suggest that the μE4 and μE5 ments contribute to the B cell specificity of Igμ transc tion at three distinct levels. First, they interact with a pressor that directly inhibits the binding of bHLH din in non-B cells. Second, they serve as binding sites for I homodimers, which displace the repressor and contrib to transcriptional activation in B cells. Third, repres binding to the μE4 and μE5 elements in non-B cells in feres with transcriptional activation through surround enhancer elements. A detailed analysis of this third fu tion for the μE5 element recently was reported by W traub et al. (1994). The goal of the study was to determ why MyoD/E2A heterodimers do not activate the μ hancer in muscle cells, even though the heterodimers b tightly to μE elements in vitro. The initial observation transient assay in fibroblasts was that transfection MyoD expression plasmid led to strong transcriptional vation of a cotransfected reporter gene under the con of E box multimers from a muscle-specific gene, but of a reporter gene under the control of multimers μ enhancer fragment containing the μE5, μE2, and elements. In contrast, transfection of an E2A expres plasmid led to strong activation of both reporters. Anal of mutations in each of the three μE elements revea that μE5 was responsible for the inability of MyoD to vate the reporter containing μE5–μE3–μE2 multimers MyoD strongly activated a reporter gene under the con of μE5 mutant–μE3–μE2 multimers. These results con that μE5 plays a role in negative regulation and dem strate that the putative repressor influences MyoD tr activation, but not E2A transactivation.

The ability of E2A homodimers to displace the puta repressor was confirmed by coexpression of MyoD an E2A mutant containing its bHLH domain but lac its transcriptional activation domains (Weintraub et 1994). In the presence of the coexpressed E2A bHLH main, MyoD strongly transactivated the μE5–μE3–μE porter, presumably because the E2A bHLH homodim displaced the repressor from the μE5 element, allow MyoD-containing dimers to activate through the othe elements. Detailed characterization of the μE5-depen repression revealed that it is specific to bHLH prote as the μE5 element could not repress transcription w placed adjacent to binding sites for other activators. M over, the ability of the μE5 element to repress transcri was dependent on only 4 bp flanking the core E box quence, as a 4 bp mutation converted the μE5 elem from a repression element into a MyoD-responsive ac tion element. Equally subtle characteristics of the E2A teins were involved in repressor displacement, since

placement of two amino acids in the basic domain of an E2A protein with two amino acids from MyoD strongly reduced the ability of E2A to displace the μE5 repressor. In other words, highly specific sequence differences between bHLH proteins and their binding sites appear to be responsible for dramatic functional differences.

To summarize, the μE elements and ubiquitous E2A proteins (and possibly other ubiquitous bHLH proteins) contribute to B cell–specific Igμ transcription through a variety of mechanisms. In nonlymphoid cells, E2A homodimers do not form. In addition, ZEB or ZEB-like repressor proteins bind to μE5 and μE4 and prevent ubiquitous enhancer-binding proteins from stimulating enhancer activity. In lymphoid cells, E2A homodimers bind to the μE4 or μE5 elements, or both, and contribute to enhancer function through their transcriptional activation domains. The E2A proteins also displace the ZEB or ZEB-like repressors from the μE4 and μE5 elements, allowing bHLH proteins to stimulate transcription from the nearby μE2 and μE3 elements, through functional cooperation with the tissue-restricted μA- and μB-binding proteins described in the next section.

Context-Dependent Activation Through the μA, μE3, and μB Elements

The studies of Oct and bHLH proteins have elucidated mechanisms employed by ubiquitously expressed proteins to influence the B cell specificity of Igμ transcription. Moreover, the detailed analysis of the μE5 and μE4 elements has revealed the critical interplay between positive and negative regulation. This section describes studies of a third family of DNA-binding proteins, the Ets family, whose cell type–restricted expression patterns contribute to B cell–specific Igμ transcription. These studies demonstrate that the activity of a transcription factor can depend strongly on the context of its binding site; in other words, a transcription factor may be nonfunctional or may function aberrantly unless bound at a specific location relative to other DNA-binding proteins. In addition, these studies highlight a basic principle of combinatorial gene regulation: that B cell specificity may be generated by two proteins that both are expressed in multiple cell types, if their expression patterns overlap only in the B cell lineage.

Mutational studies of the entire μ enhancer and of enhancer fragments have revealed that the μA (or π) and μB elements, as well as the intervening μE3 element, are needed for optimal enhancer activity in B cells (Nelsen et al., 1990, 1993; Libermann et al., 1990; Libermann and Baltimore, 1993; Rivera et al., 1993). A member of the Ets family, called PU.1, binds tightly to the μB element (Nelsen et al., 1993) and several other Ets family members bind to the μA element (Nelsen et al., 1993; Rivera et al., 1993; Lopez et al., 1994). The prototypic member of the Ets family of DNA-binding proteins is c-Ets-1, which is the cellular homologue of the E26 avian retrovirus v-Ets protein (for review, see Wasylyk et al., 1993). Members of this family range from highly tissue-restricted proteins to ubiquitously expressed proteins. The Ets family members contain a homologous 84 aa domain that interacts as a monomer with DNA sequences containing a GGA or GGAA core. Sequences surrounding this core determine the precise sequence specificities of the many family members, but, in general, multiple Ets family members will bind in vitro to any given GGA-containing control element. For example, at least five different Ets family members are capable of binding to the μA element, presumably with varying affinities (Nelsen et al., 1993; Rivera et al., 1993; Lopez et al., 1994). The structure of the Ets domain has recently been solved by multidimensional nuclear magnetic resonance, revealing a new type of helix–turn–helix motif (Liang et al., 1994).

A common characteristic of many Ets family members is that they appear to activate transcription poorly by themselves, but instead function cooperatively with other proteins (Wasylyk et al., 1993). PU.1 provides one example of this characteristic. When multimers of the μB element were inserted upstream of a simple promoter and reporter gene, only weak promoter activity was observed following transfection into B cell lines that contain high levels of PU.1 (Nelsen et al., 1993). Furthermore, overexpression of PU.1 did not strongly stimulate transcription through the μB element (Nelson et al., 1993). These results contrast with those obtained with some of the Oct and bHLH proteins, which efficiently activated transcription when multiple binding sites for the proteins were inserted into a reporter plasmid and tested in a transient transfection assay. The weak activity detected with μB multimers suggests that PU.1 does not possess a strong transcriptional activation domain that is capable of independently enhancing transcription initiation. In fact, a transcriptional activation domain has been identified at the N terminus of the PU.1 protein (Hagemeier et al., 1993; Shin and Koshland, 1993), but this domain only weakly activates transcription of reporter plasmids containing multiple PU.1 binding sites.

In the μ enhancer, efficient transcriptional activation by PU.1 through the μB element depends on the presence of the adjacent μA and μE3 elements. When a reporter gene under the control of two copies of a fragment containing these three elements was tested by transient transfection in a B cell line, strong enhancer activity was detected (Nelsen et al., 1993). Mutation of any of the three elements abolished activity. Thus, this control region appears to depend on functional interactions between three proteins.

Transient transfection experiments in fibroblasts suggest that the enhancer activity of this tripartite fragment depends on Ets-1 bound to μA, PU.1 bound to μB, and TFE3 bound to μE3. In fibroblasts, cotransfection of either an Ets-1 expression plasmid or a PU.1 expression plasmid with the reporter plasmid resulted in only weak activation (Nelsen et al., 1993). However, cotransfection of both of these expression plasmids with the reporter plasmid led to strong activation (Nelsen et al., 1993). Although the μE3 element was required for efficient activation in these experiments, ectopic expression of a bHLH was not needed, presumably because the functional μE3-binding protein is present in fibroblasts.

Evidence in support of TFE3 as the functional μE3-binding protein has been derived from coexpression ex-

periments, which showed that TFE3 can cooperate with either PU.1 or Ets-1 for transcriptional activation (R. Sen, personal communication). In particular, with a reporter gene under the control of the μA–μE3–μB fragment, overexpression of any one protein, Ets-1, TFE3, or PU.1, failed to activate transcription significantly in fibroblasts. However, simultaneous overexpression of any two proteins, Ets-1 and TFE3, PU.1 and TFE3, or Ets-1 and PU.1, led to strong activation. These results suggest that when TFE3 is overexpressed, it can functionally cooperate with either PU.1 or Ets-1 during transcriptional activation. However, when lower (endogenous) concentrations of TFE3 are present, activity of the μA–μE3–μB fragment depends on both Ets-1 and PU.1, in addition to TFE3. These results suggest that both Ets-1 and PU.1 may be capable of recruiting TFE3 to the enhancer; depending on whether high or normal concentrations of TFE3 are present within the cell, one or both of the Ets family proteins may be needed for efficient recruitment.

The precise mechanism by which Ets-1, TFE3, and PU.1 cooperate with each other during transcriptional activation remains unknown. However, insight into this issue has been provided by studies of the protein domains required for activity. For each of the three proteins, discrete domains have been defined that are needed for transcriptional activation of a reporter gene under the control of multiple copies of the binding site of that protein (Hagemeier et al., 1993; Shin and Koshland, 1993; Schneikert et al., 1992; Beckmann et al., 1990). Most likely, these domains lead to transcriptional activation by communicating directly with the general transcription machinery or with a coactivator. Interestingly, for activity of the μA–μE3–μB fragment, the transcriptional activation domain of Ets-1 is required, but only the DNA-binding domain of PU.1 is needed (R. Sen, personal communication; the TFE3 domains required for activity of the μA–μE3–μB fragment have not been determined). One possible explanation for this result is that the primary role of PU.1 is to help recruit TFE3, Ets-1, or both to the enhancer. The PU.1 DNA binding domain could carry out this role either through direct protein–protein interactions with TFE3 or Ets-1 or through an indirect mechanism (e.g., by preventing the binding of nucleosomal or repressor proteins). The requirement for the transcriptional activation domain of Ets-1 and possibly of TFE3 suggests that these proteins may be responsible for communicating with general transcription factors. The activation domains of Ets-1 and TFE3 may function independently, possibly by interacting with different general factors or coactivators, or may function together by forming a composite activation domain; either of these mechanisms could lead to cooperative, or synergistic, activation. To summarize, the functional cooperativity between Ets-1, TFE3, and PU.1 may result from cooperative DNA binding, cooperative transcriptional activation, or a combination of the two.

The studies described above reveal specific functional interactions between Ets-1, TFE3, and PU.1. These proteins and interactions may be critical for activity of the μ enhancer. However, it should be stressed that these experiments, like most of the experiments presented with the octamer and μE elements, were not performed in t context of the entire enhancer. Moreover, an exhaust analysis of all Ets family and bHLH family members w not carried out, making it possible that other members these families may function similarly with the μA–μE3– fragment or, more importantly, may function preferenti in the context of the endogenous μ gene. In support this possibility, studies of the μA element by itself or w the adjacent μE2 element, have suggested that other family members, including Fli-1, Erg-3, or ERP, may c tribute to enhancer activity (Rivera et al., 1993; Lopez al., 1994). Further experiments will be needed to de mine whether only one Ets family member is capable carrying out the precise protein–protein interactions quired for μA function; whether multiple Ets family me bers have evolved to function through μA, providing a gree of redundancy; or whether the multiple Ets fam members function through μA at distinct stages of dev opment. Similar possibilities exist for the multiple bHL ZIP proteins that bind to the μE3 element, as well as the multiple Oct proteins that bind to the octamer eleme and the multiple bHLH proteins that bind to the μE2, μ and μE5 elements.

The cooperative interactions between Ets-1, TFE3, a PU.1 appear to contribute to the B cell specificity of transcription, based on the finding that the μA–μE3- fragment functions in B cells, but not in other cell ty that have been tested (Nelsen et al., 1993). Reminisc of the Oct and μE5–μE4 examples, none of the functio DNA-binding proteins appear to be expressed exclusiv in B cells. In the previous examples, B cell specificity re on a B cell–specific coactivator or on B cell–specific dim ization. In this case, the B cell specificity results from overlapping expression patterns of DNA-binding prote that function cooperatively. PU.1 is expressed prima in the B cell and myeloid lineages, and possibly in hem poietic progenitors (Wasylyk et al., 1993; Scott et 1994). Ets-1 is expressed in B and T cells and in a var of other cell types (Wasylyk et al., 1993). With the μE3–μB fragment, neither protein functions independe preventing significant activation in T cells by Ets-1, example. The expression patterns of the two proteins o lap primarily in B cells, resulting in considerable B specificity of the enhancer fragment. Most likely, a tional functional cooperativity with other μ promoter enhancer fragments, like the octamer and μE5–μE4 ments, results in the creation of a highly restricted exp sion pattern.

Regulation of Chromatin Structure at the Igμ Loc

The studies described in the preceding sections sug that Igμ transcription is regulated by a complex arra ubiquitous and cell-specific proteins that are capabl binding to promoter and enhancer elements. These teins are likely to interact with each other and, with help of coactivators, to regulate the formation of a II-containing preinitiation complex at the transcription site. Thus far, however, we have described these prot protein and protein–DNA interactions as they might o on a naked DNA template. In fact, the Igμ locus most l

is assembled into an inaccessible chromatin configuration prior to gene activation (Jenuwein et al., 1993; Forrester et al., 1994). Although the structure of the inaccessible chromatin remains undefined, it may be in the form of a 30 nm filament (see Ernst and Smale, 1995).

During B cell development, changes in chromatin structure within the Igμ locus occur prior to or concomitant with the activation of gene rearrangement and transcription initiation (Ford et al., 1992). Two recent studies by Grosschedl and colleagues (Jenuwein et al. 1993; Forrester et al., 1994) have suggested that the intronic enhancer and its flanking MARs (Cockerill et al., 1987) are largely responsible for inducing these chromatin alterations. These studies characterized transcription and chromatin structure in mice containing rearranged Igμ transgenes. The transgenes contained the entire intronic enhancer or enhancer fragments in the presence or absence of the flanking MARs.

In the transgenic mice, efficient, position-independent transcription from a V_H promoter within the transgene was detected when both the enhancer and MARs were included, but not when either of these regions was deleted. Chromatin accessibility throughout the locus, as measured by DNase I sensitivity, was similarly dependent on both the enhancer and MARs. These results suggest that the combination of the intronic enhancer and the MARs is sufficient for inducing locus accessibility and position-independent Igμ transcription, functions that define an LCR.

One limitation of the above experiments is that they do not establish whether the enhancer and MARs are directly responsible for the chromatin alterations, or whether the chromatin alterations are the result of transcription initiation and elongation by pol II. To distinguish between these possibilities, Jenuwein et al. (1993) measured chromatin accessibility with transgenes that link a bacteriophage T7 promoter instead of a pol II promoter to the enhancer/MARs. Pre-B cell lines derived from the mice were then tested for locus accessibility by adding T7 RNA polymerase to isolated nuclei. This elegant strategy uncoupled the enhancer from transcription, thereby ruling out the possibility that the enhancer influences chromatin structure only as a result of transcriptional enhancement. In addition, the measurement of T7 transcripts provided an assay that was more specific and more quantitative than DNase I for measuring chromatin accessibility. The results confirmed that, indeed, the intronic enhancer and flanking MARs could induce accessibility of the DNA to T7 RNA polymerase, regardless of whether the T7 promoter was close to or at a distance of 1 kb from the enhancer.

A similar set of experiments was performed with transgenes containing the intronic enhancer in the absence of the MARs (Jenuwein et al., 1993; Forrester et al., 1994). The enhancer could not stimulate transcription initiation from the V_H promoter, but was capable of inducing chromatin alterations in the local vicinity of the enhancer. In fact, a 95 bp fragment of the enhancer (which lacks the μE4 and octamer elements) was sufficient for inducing local access to T7 polymerase in a position-independent fashion. This fragment, however, did not induce DNase I hypersensitivity within the enhancer and did not induce DNase I hypersensitivity or T7 accessibility at a distance of 1 kb from the enhancer. These results demonstrate that the intronic enhancer is sufficient for inducing specific local chromatin alterations, but both the enhancer and MARs are needed for long-range alterations. In addition, the results suggest that the T7 assay measures a more subtle chromatin change than does the DNase I assay, implying that multiple specific levels of chromatin alterations are needed for complete locus accessibility.

The precise protein–DNA and protein–protein interactions responsible for the above results are not known. However, a model that is consistent with the known facts is as follows: in the inaccessible chromatin, one or more cell type–specific proteins (e.g., PU.1, Ets-1, or an E2A homodimer) may be capable of binding to control elements within the 95 bp enhancer fragment. This interaction may impart a local chromatin alteration, making the enhancer accessible to other cell type–specific and ubiquitous DNA-binding proteins (and accessible to T7 RNA polymerase in the Jenuwein et al. [1993] assay). The formation of a stable protein–DNA complex over the entire 220 bp enhancer may alter chromatin structure more severely (and induce DNase I hypersensitivity). The MARs then appear to be responsible for propagating the chromatin alterations throughout the locus, allowing for efficient transcription initiation from the V_H promoter. The mechanism of action of the MARs remains unknown, but may involve MAR-binding proteins like topoisomerase II, SATB1, or NFμ-NR (Gasser and Laemmli, 1987; Dickinson et al., 1992; Scheuermann and Chen, 1989).

For the most part, the results found in transgenic animals are consistent with results found using stable or transient transfection assays. However, with the transfection assays, the core enhancer imparts strong transcriptional enhancement in the absence of the MARs (e.g., Gilles et al., 1983; Banerji et al., 1983). Thus, introduction of the gene into the germline appears to be critical for establishing the chromatin configuration that requires the enhancer–MAR combination for disruption. Forrester et al. (1994) suggest that DNA methylation may be responsible for the differences between the transfection and germline transformation assays, as the germline Igμ transgenes most likely are methylated prior to transcriptional activation during B cell development (Jahner et al., 1982).

These results highlight the current difficulties in distinguishing between the functions and properties of enhancers, LCRs, MARs, and insulators. Enhancers generally are capable of increasing gene transcription in transient and stable transfection assays. LCRs provide position-independent transcriptional enhancement in transgenic mice by increasing locus accessibility. MARs provide attachment sites to the nuclear matrix and, in some instances, are thought to define chromosomal boundaries by insulating a locus from adjacent loci. The intronic control regions within the Igμ locus do not match these general definitions. The MARs are within the locus and therefore are unlikely to function as a boundary or insulator element. Instead, the MARs appear to function in combination with the enhancer as an LCR. Finally, a 95 bp fragment of the intronic enhancer appears to be

sufficient for inducing a position-independent local chromatin alteration, a novel property for an enhancer. The ambiguities between enhancers, LCRs, MARs, and insulator highlight the need for further experiments to determine whether precise properties can be assigned to each of these regulatory regions or whether they comprise a wide range of overlapping functions, depending on their precise components and proximities to other control regions.

Concluding Remarks

The studies described above demonstrate that numerous proteins, which carry out a complex array of protein–protein and protein–DNA interactions, are required for appropriate Igμ regulation. Despite this complexity, the experiments suggest that either the OCA-B coactivator, the E2A homodimer, or the Ets-1/PU.1 combination is sufficient for imparting considerable B cell specificity to Igμ transcription. This suggestion immediately leads to the question of why three different strategies are needed for a single B cell–specific gene. A partial, if not complete, answer to this question is provided by the combinatorial theory. Perhaps, each of the three mechanisms only leads to partial cell type specificity (e.g., E2A homodimers may actually function in a few non-B cell types, where they may regulate an unrelated set of genes). Instead, all three mechanisms may need to be combined to achieve the precise expression pattern observed with the endogenous Igμ gene. It may be worth reiterating that the endogenous murine Igμ gene and Igμ transgenes, despite their complex regulatory regions, are not perfectly B cell-specific, since weak transcription has been detected in several other tissues (Jenuwein and Grosschedl, 1991). The widespread "leakiness" may result from expression of OCA-B or E2A homodimers in a few non-B cell tissues, from the absence of ZEB or a ZEB-like repressor in other tissues, and from the combined expression of PU.1 and Ets-1 in yet other tissues; possibly, B cells are unique only in that all of these mechanisms function simultaneously to efficiently activate Igμ transcription. An alternative explanation for the leakiness is that inappropriate members of the relevant transcription factor families (the POU, bHLH, and Ets families) may be capable of functionally interacting with the μ regulatory elements with low efficiency. In either case, the important observation is that the desired expression pattern is not absolutely attained, despite the fact that numerous proteins and multiple distinct strategies contribute to Igμ regulation. Given this fact, it might be expected that most genes will depend on a similarly complex set of proteins and regulatory strategies in attempting to achieve an appropriate pattern of expression.

In light of the studies reviewed in this article, it might be valuable to conclude by considering the progress of the eukaryotic transcription field through the past 10 years. The second half of the 1980s witnessed an explosion in the delineation of cis-acting control elements required for appropriate regulation of tissue-specific genes, and in the identification of trans-acting factors that bind to those control elements. At that time, many molecular biologists anticipated that the straightforward approach, of control element to DNA-binding protein to DNA-binding protein gene, would rapidly lead to an understanding of the mechanisı by which numerous genes are regulated. However, more and more control regions and transcription factı were characterized, several results revealed that trı scriptional regulatory mechanisms would be extremely ficult to dissect: Numerous cis-acting control elemeı were often needed for appropriate regulation of a giv gene; transcription factors rarely mimicked the precise pression patterns of their putative target genes; multi members of a transcription factor family typically were pable of binding to a given control element; DNA frı ments containing the promoter and enhancers for a giv gene were insufficient for appropriate regulation in tra genic mice; and transcriptional activators were incapa of stimulating transcription by the general transcript factors in the absence of additional coactivators. Althou many of these findings were predicted by the combina rial principles that were proposed 20 years earlier (Gieı 1973; Britten and Davidson, 1969; Georgiev, 1969), degree of complexity was nearly overwhelming.

The experiments and concepts described here rev that tremendous progress has been made in the first h of the 1990s in furthering our understanding of the mec nisms of transcriptional control. For the most part, t progress can be attributed to the increased use of ge knockout technology, as well as to three advanced proaches to the study of regulated transcription. The f approach has been to study control elements in the c text of a natural promoter or enhancer, rather than indi ually. This strategy, which is exemplified by the stud of repression through the μE5 element and of activat through the μA–μE3–μB region, clearly is leading t more accurate picture of the protein–DNA and prote protein interactions needed for appropriate regulati The second advanced approach has been to emplo vitro transcription assays reconstituted with highly purif or recombinant proteins for the study of transcriptic activation and repression. These studies, which are aic by powerful genetic approaches in Saccharomyces ce visiae, have facilitated the identification of coactivat and have led to rapid advances towards an understanc of the precise mechanisms by which regulatory prote modulate the frequency of transcription initiation by II. The third approach has been to apply more intens efforts towards studying the mechanisms through wh genes are regulated in the context of an intact chro some, by analyzing the roles of chromatin proteins of specialized control elements like insulators, silenc LCRs, and MARs. Continuing efforts in each of these th directions will certainly lead to further advances in second half of the decade. Eventually, these advan may allow us to determine whether the complex regula schemes that have been observed rely on a relatively predictable combination of differentially expressed t scription factors or an intricate network of regulatory tors arranged in a rational hierarchical order.

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