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Cell type-specificity elements of the immunoglobulin heavy chain gene enhancer

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A strong transcriptional enhancer was created by oligomerization of a short segment from the immunoglobulin heavy chain (IgH) enhancer. This segment was analyzed in parallel for biological activity *in vivo* and factor binding *in vitro*. In transfection experiments the oligomerized segment stimulates transcription in a cell type-specific manner similar to the entire IgH enhancer. Transfections of mutants identified two sequence motifs whose integrity is required for efficient and cell type-specific activity of this enhancer. The first is a sequence suggested previously to be bound by a factor *in vivo*, and the second is a highly conserved decanucleotide which also occurs in Ig variable gene promoters. The ability of these two sequence motifs to bind proteins *in vitro* was tested by band shift assays. Under our *in vitro* conditions we could not detect proteins binding to the *in vivo* footprint region. However, we found protein factors binding to the decanucleotide. A ubiquitous form of this factor is present in every cell line analyzed. Additional variants are detected exclusively in cells where the IgH enhancer and the segment thereof are active. Elimination of the decanucleotide motif is not only a strong down mutation *in vivo* but also abolishes binding of all factor variants *in vitro*. Thus our data suggest that the two enhancer motifs analyzed are involved in positive rather than negative control of transcription.

Key words: cell type-specificity/IgH enhancer/DNA binding proteins/gene regulation/decanucleotide ('octamer') sequence

Introduction

An important goal of developmental biology is the elucidation of the mechanisms responsible for cell type-specific gene expression. Various genes expressed at high levels exclusively in specialized cell types have been studied in detail for several years. As a model system for cell type-specific gene expression we have chosen the genes coding for the antibodies: the immunoglobulins. The immunoglobulins are produced by B lymphocytes and play a crucial role in the humoral part of the immune response (reviewed by Tonegawa, 1983). In the locus encoding the immunoglobulin heavy chain (IgH) genes the first genetic element to confer cell type-specificity to a cellular gene was found. Banerji *et al.* (1983), Gillies *et al.* (1983) and Neuberger (1983) described a transcriptional enhancer sequence located between the joining segments and the constant region of the murine IgH locus. Subsequently, an enhancer was also found in an analogous position within the kappa light chain locus (Picard and Schaffner, 1984; Queen and Stafford, 1984). In transfection experiments these enhancers activated the transcription of linked genes exclusively in cell lines of the B lymphoid lineage (see, for example, Banerji *et al.*, 1983; Gillies *et al.*, 1983; Picard and Schaffner, 1983). Furthermore, in transgenic mice the IgH enhancer is sufficient to trigger the correct developmental program: heterologous genes linked to the IgH enhancer showed an expression pattern similar to the one of an IgH gene (Adams *et al.*, 1985; Gerlinger *et al.*, 1986; for review see Matthias *et al.*, 1987).

Up to now at least three components have been identified that contribute to the cell type-specific expression of immunoglobulin genes. In addition to the enhancer, the promoter in front of the variable region of immunoglobulin genes also shows a cell type preference (Falkner *et al.*, 1984; Foster *et al.*, 1985; Grosschedl and Baltimore, 1985; Mason *et al.*, 1985; Picard and Schaffner, 1985). Furthermore, some intragenic sequences of the Ig gene other than the enhancer are involved in tissue-specificity (Grosschedl and Baltimore, 1985), presumably acting via post-transcriptional mechanisms (Gerster *et al.*, 1986; Kelley and Perry, 1986).

From the discovery of viral enhancers with a host cell preference (de Villiers *et al.*, 1982; Laimins *et al.*, 1982; for review see Picard, 1985), and in particular the finding of the strictly cell type-specific IgH enhancer, the existence of cell type-specific factors that regulate enhancer activity was proposed (de Villiers *et al.*, 1982; Gillies *et al.*, 1983; Mercola *et al.*, 1985; Schöler and Gruss, 1985). A simple model would envisage the presence of positively acting factor(s) in permissive and their absence in non-permissive cells (or in case of negative regulation the absence of repressor(s) in permissive cells). Since it is now generally accepted that enhancers have a modular structure being composed of a multitude of sequence motifs each binding a cellular factor (for reviews see Serfling *et al.*, 1985; Sassone-Corsi and Borelli, 1986), more complex models of regulation are also conceivable.

First attempts to find sequences within the IgH enhancer that are important for cell type-specificity were carried out by Ephrussi *et al.* (1985) and Church *et al.*, (1985). These authors defined by genomic footprinting four regions of lymphoid-specific protection against methylation by dimethylsulphate. The protected sequences resemble each other and it was suggested that they bind a common B cell-specific factor. However, in the meantime it has been found *in vitro* that at least the first and the third copy of these footprint sequences each bind a different factor that is also present in extracts from non-lymphoid cells (Weinberger *et al.*, 1986). It has been reported that a central fragment of the IgH enhancer stimulates transcription also in non-lymphoid cells, at least when brought into the immediate vicinity of the promoter of the test gene (Wasylyk and Wasylyk, 1986; our unpublished data). Adjacent to that central fragment are the fourth copy of the lymphoid-specific footprint regions and a decanucleotide motif (TAATTTGCAT) also found in the variable region promoters of immunoglobulin genes (Falkner and Zachau, 1984; Parslow *et al.*, 1984; Falkner *et al.*, 1986) (some authors refer to this sequence as the 'octamer'). The decanucleotide element is believed to be involved in the cell type-specificity of

immunoglobulin promoters (Bergman *et al.*, 1984; Falkner *et al.*, 1984; Mason *et al.*, 1985; Mizushima-Sugano and Roeder, 1986). The decanucleotide sequence within the IgH enhancer is apparently bound by a factor *in vivo* since a G residue is protected against methylation (Ephrussi *et al.*, 1985). Recently, band-shift and footprinting experiments have shown also *in vitro* binding of a factor present in extracts from lymphoid as well as from non-lymphoid cells (Augereau and Chambon, 1986; Mocikat *et al.*, 1986; Peterson *et al.*, 1986; Schlokat *et al.*, 1986; Singh *et al.*, 1986).

In this paper we compare the biological activity *in vivo* of two sequence motifs of the IgH enhancer in parallel with their potential to bind factors *in vitro*. Transient expression assays with mutants of the decanucleotide sequence show that this sequence element is important for cell type-specific IgH enhancer activity *in vivo*. Using band shift assays to detect binding of factors *in vitro* we find in agreement with recent findings of Staudt *et al.* (1986) and Landolfi *et al.* (1986) that in addition to a factor present in all cell types, there are also lymphoid-specific factors interacting with the decanucleotide motif. Together with our *in vivo* results these binding studies indicate that the lymphoid-specific factors are major determinants of cell type-specific transcription.

Concerning the second sequence motif investigated in this study, namely the fourth lymphoid-specific footprint region located immediately upstream of the decanucleotide, we show that this element is also required for maximal enhancer activity, although we could not detect a factor interacting with this sequence *in vitro*.

Results

The murine immunoglobulin heavy chain gene (IgH) enhancer is known to be located within a 1-kb *Xba*I fragment (Banerji *et al.*, 1983; Gillies *et al.*, 1983; reviewed by Matthias *et al.*, 1987). To characterize potentially interesting sequence motifs within this relatively large piece of DNA we extended the studies of Banerji *et al.* (1983) on the activity of internal subsegments. We found that a central *Hin*fI fragment (nucleotide positions 345–566; numbering according to Ephrussi *et al.*, 1985) was an equally active enhancer as the whole 1-kb *Xba*I fragment (data not shown). A DNA segment of that length might still be too large for a detailed investigation of the interactions between different regulatory factors and enhancer DNA because it represents a potential target for a plethora of transcription factors. Therefore, it is useful to analyze the interaction of a single, or only a few, factor(s) with a defined short sequence element. Since a good enhancer requires a certain length or some multiplicity of factors binding to it, we decided to use for our experiments artificial enhancers composed of multimers of short DNA sequences. Such an approach has been used to identify important sequence motifs in the enhancers of polyoma virus (Veldman *et al.*, 1985), SV40 virus (Schirm *et al.*, 1987) and metallothionein genes (Stuart *et al.*, 1984; G.Westin, M.Petterson and W.Schaffner, unpublished results). As enhancers seem to contain some redundancy of information, mutations in some factor binding sites do not necessarily have drastic effects when assayed in the context of the otherwise complete enhancer (Zenke *et al.*, 1986). We reasoned that the multimerization of short fragments has the advantage that effects of mutations would be potentiated and thus clearly detected.

Multiple copies of a small enhancer fragment act as cell type-specific enhancer

Most of our experiments have been carried out with a *Dde*–*Hin*fI fragment (nucleotides 518–566, the fragment was 51 bp long after filling in the protruding 5′ ends). This region is highly conserved between the IgH enhancers of mouse and man (Rabbitts *et al.*, 1983) and contains the fourth copy (from left to right) of the lymphoid-specific footprint (F) sequences determined by *in vivo* footprinting (Church *et al.*, 1985; Ephrussi *et al.*, 1985) (cf. Figure 3, panel A for a schematic overview). Directly adjacent to it there is a decanucleotide (D) motif that also occurs in the promoters of immunoglobulin variable region genes (Falkner and Zachau, 1984; Parslow *et al.*, 1984; Falkner *et al.*, 1986).

This *Dde*I–*Hin*fI fragment was cloned in one to six copies behind the rabbit β1-globin gene. These DNA constructs were then transfected into X63Ag8 myeloma and epithelial HeLa cells using the DEAE-Dextran procedure. Cytoplasmic RNA was prepared 36–42 h later and analyzed by RNase mapping. The probe for all RNase mapping experiments was an SP6 RNA polymerase-generated transcript complementary to the unspliced precursor RNA of the β-globin gene. Correctly initiated transcripts yield a protected fragment of 145 nucleotides from the initiation site to the end of the first exon (designated β init). Thus, the intensity of this band directly reflects the activity of the enhancer. The band of 210 nucleotides designated β2 + rt represents the major part of the second β-globin exon. This band is mainly generated by correctly initiated transcripts. However, in addition a contribution is also made by read-through transcripts which are initiated far upstream of the promoter (most probably within the plasmid vector) and get spliced to the second exon. As an internal standard a low amount of a truncated β-globin gene (plasmid REFΔ; Picard and Schaffner, 1985) was mixed into all transfections. RNA from this reference gene produces in the RNase mapping assays a cluster of bands ~165 nucleotides in length (designated ref).

Figure 1 shows the result of the transfections of recombinants containing multiple copies of the *Dde*I–*Hin*fI fragment. In X63Ag8 one copy raises the level of β-globin gene transcription only marginally. Increasing the copy number, however, results in a dramatic enhancement of β-globin expression. Interestingly, this effect is cell type-specific: transcription of the β-globin gene is only stimulated in X63 myeloma cells where the complete IgH enhancer is also active. Like the whole 1-kb *Xba*I fragment of the IgH enhancer, the multimers of the *Dde*I–*Hin*fI fragment are also inactive in HeLa cells which are of epithelial origin. The recombinants containing more than two copies of the 51-bp fragment have been constructed by subcloning the fragment first into the polylinker region of plasmid pUC7. For further multimerization they have been reclaimed from pUC7 and ligated in such a way that some polylinker sequences are interspersed between the IgH sequences (cf. Materials and methods). The sharp increase in enhancement betwen two and three copies of the *Dde*I–*Hin*fI fragment might be attributed partially to the presence of these polylinker sequences, possibly because they confer a better spacing for factor binding sites. Whatever the reason for this effect, it is important to note that the polylinker sequences increase enhancer activity only in X63Ag8 cells. In other words, the differential activity of the *Dde*I–*Hin*fI fragment between X63 and HeLa cells seems not to be perturbed by these sequences and the artificial enhancers exhibit in these cells the same specificity as the complete IgH enhancer.

Cell type-specific as well as ubiquitous proteins bind to the DdeI–HinfI fragment of the IgH enhancer in vitro

Having shown that the 51-bp *Dde*I–*Hin*fI fragment was able to act as a cell type-specific enhancer we attempted to detect fac-

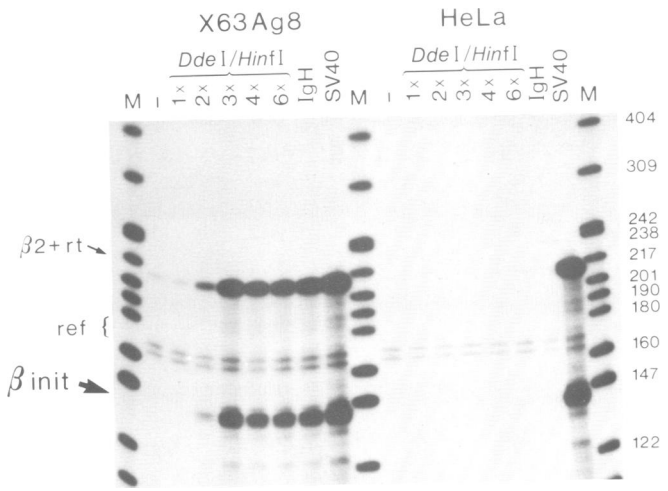

Fig. 1. The *Dde*I−*Hin*fI fragment cloned in multiple copies acts as a cell type-specific enhancer. Multimers of the *Dde*I−*Hin*f fragment were cloned downstream of the rabbit β-globin gene of the GLOTA vector. The following recombinants were transfected by the DEAE-Dextran procedure into X63Ag8 myeloma or into HeLa cells: −: GLOTA without insert; 1×, 2×, 3×, 4×, 6×: GLOTA with a corresponding number of copies of the *Dde*I−*Hin*f fragment; IgH: the 688-bp *Xba*I−*Eco*RI fragment containing the IgH enhancer inserted into GLOTA; SV40: a 196-bp fragment containing the SV40 enhancer inserted into GLOTA. Cytoplasmic RNA was harvested and analyzed by RNase mapping. The significance of the various bands is explained in the text.

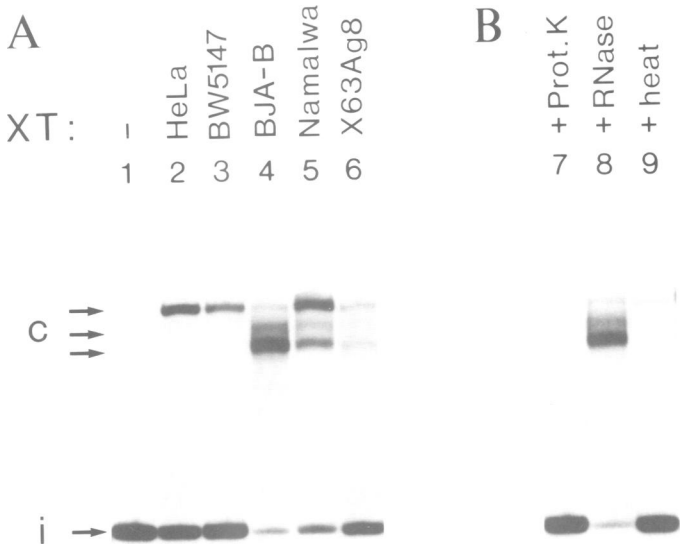

Fig. 2. Cell type-specific and ubiquitous protein factors bind to the *Dde*I−*Hin*fI fragment in nuclear extracts. The end-labelled *Dde*I−*Hin*fI fragment was incubated in nuclear extracts of various cell lines and the resulting complexes 'c' were separated from the unbound input fragment 'i' by electrophoresis on native polyacrylamide gels **(panel A). Lane 1:** no extract added; **lanes 2−6**: nuclear extracts from HeLa (cervix carcinoma), BW5147 (T-cell lymphoma), BJA-B (lymphoblastoid cell line), Namalwa (Burkitt's lymphoma) and X63Ag8 (myeloma), respectively. **Panel B:** binding reactions with BJA-B nuclear extracts were treated with 0.6 mg/ml proteinase K for 10 min **(lane 7)**, 130 μg/ml RNase A and 660 U/ml RNase T1 for 10 min **(lane 8)**, or heated at 65°C for 10 min and then assayed on a band shift gel.

tors binding to that fragment *in vitro*. For this we used the band shift assay technique (Fried and Crothers, 1981; Garner and Revzin, 1981). An end-labelled DNA fragment is incubated with a nuclear extract and is then electrophoresed through a native acrylamide gel. Specific DNA−factor complexes result in a lower mobility of the DNA fragment ('band shift'). In the experiment shown in Figure 2 the end-labelled *Dde*I−*Hin*fI fragment was incubated in nuclear extracts (Dignam *et al.*, 1983) prepared from different cell lines, i.e. HeLa (human cervix carcinoma), BW5147 (mouse T-cell lymphoma), BJA-B (human lymphoblastoid cell line) and Namalwa (human Burkitt's lymphoma), and X63Ag8 (mouse myeloma). Figure 2A shows that all nuclear extracts contain a factor binding to the labelled DNA fragment. Besides that ubiquitous component the extracts from some cell lines contain factors giving rise to two additional, faster migrating bands. The cell lines containing these additional factor(s) are also those in which the IgH enhancer is active (see below). Thus we assume that these additional factors are involved in the tissue specific activity of the IgH enhancer.

The factors binding to the *Dde*I−*Hin*fI fragment appear to be proteins since no band shift is seen when the BJA-B extract is incubated with proteinase K (Figure 2B). RNA components, however, are probably not involved in binding since the DNA−factor complex is RNase-resistant. The ubiquitous and the cell type-specific complexes seem to differ from each other in their physical properties. The cell type-specific bands disappear after short heating (10 min) of the extract to 65°C, whereas the ubiquitous protein is still bound (Figure 2B).

Mutagenesis of potentially important sequence motifs

To analyze specific mutants of the sequences present in the *Dde*I−*Hin*fI fragment in the absence of polylinker sequences, we synthesized a series of deoxyoligonucleotides 47 bp in length (Figure 3). The first oligonucleotide has exactly the same sequence as the corresponding *Dde*I−*Hin*fI fragment of the murine IgH enhancer except that the *Hin*fI site itself is lacking and one base is changed at the 3′ end to create a *Sal*I site for analytical purposes. All oligonucleotides have complementary but not palindromic 5′ ends to allow ligation in a strict head-to-tail fashion only. Besides the wild-type oligonucleotide, which is referred to as FD to indicate intact *F*ootprint and *D*ecanucleotide motifs, two other oligonucleotides were prepared, each containing three transversion mutations in either the lymphoid-specific footprint sequence (fD) or in the decanucleotide motif (Fd). Finally, also a double mutant (fd) was synthesized that combined the mutations of the fD and Fd oligonucleotides.

The decanucleotide is the in vitro factor binding site

By competition studies using the synthetic oligonucleotides we sought to define the sequence element(s) involved in binding to the protein factors detected in the band shift experiments. The end-labelled *Dde*I−*Hin*fI fragment was incubated in nuclear extracts from three different cell lines in the presence of different competitor DNAs (Figure 4). Like the 224-bp fragment containing the whole IgH enhancer, the FD oligonucleotide competes very efficiently, which is not surprising since that oligonucleotide is almost identical to the labelled fragment. Interestingly, the Fd oligonucleotide is not able to compete efficiently. In this DNA fragment the decanucleotide region is mutated. Therefore, we conclude that the decanucleotide is a target for the binding of factor in the extracts. However, a fragment with a mutation (fD) in the region that seems to be bound by a factor *in vivo* (Ephrussi

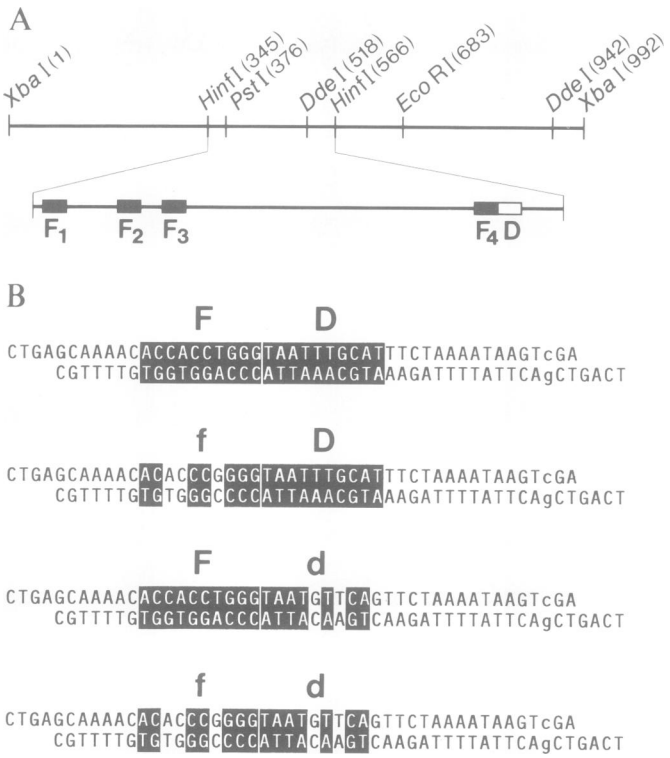

Fig. 3. Synthetic oligonucleotides used in this study. The central *Hin*fI fragment within the 1-kb *Xba*I fragment contains all the IgH enhancer activity. The positions of the four lymphoid-specific *in vivo* 'footprint' sequences described by Ephrussi *et al.* (1985) (F_1, F_2, F_3, F_4) and of the decanucleotide motif (D) are indicated **(panel A). Panel B** shows the sequences of the oligonucleotides synthesized. The sequence of the uppermost oligonucleotide corresponds to nucleotides 518 to 564 of the murine IgH enhancer (numbering according to Ephrussi *et al.*, 1985) except for a base exchange at position 562 (indicated by lower-case letters) to create a *Sal*I restriction site. The *in vivo* footprint region 'F' (Church *et al.*, 1985; Ephrussi *et al.*, 1985) and the decanucleotide 'D' (Falkner and Zachau, 1984; Parslow *et al.*, 1984) are boxed. The lower three oligonucleotides fD, Fd and fd contain mutations in the F and D elements which are indicated by lower-case letters (f or d, respectively).

et al., 1985) is a competitor as efficient as the FD oligonucleotide. This indicates that *in vitro* no stable complex is formed between a factor and the *in vivo* footprint sequence. Nevertheless, we know from transfection experiments that this motif also contributes to enhancer function (see below). As expected, the fd double mutant does not compete for factor binding. An oligonucleotide containing the second and the third of the lymphoid-specific footprints described by Ephrussi *et al.* (1985) does not compete either (F_2F_3 in Figure 4).

Conversely, when labelled oligonucleotides are used for the band shifts it again becomes evident that the decanucleotide is the only DNA element within the *Dde*I−*Hin*fI fragment interacting with a protein under our *in vitro* binding conditions. While the FD and fD oligonucleotides give a band shift pattern like the *Dde*I−*Hin*fI fragment, the Fd and fd oligonucleotides do not produce a retarded band (data not shown).

Although the decanucleotide was originally found by sequence comparisons in immunoglobulin variable segment promoters and the IgH enhancer, highly related sequence motifs also occur in different promoters and enhancers. For example an 8 out of 10 homology is found between the IgH decanucleotides and a sequence adjacent to the *Sph*I site within the 72-bp repeat of the SV40 enhancer (Mattaj *et al.*, 1985; Zenke *et al.*, 1986). A se-

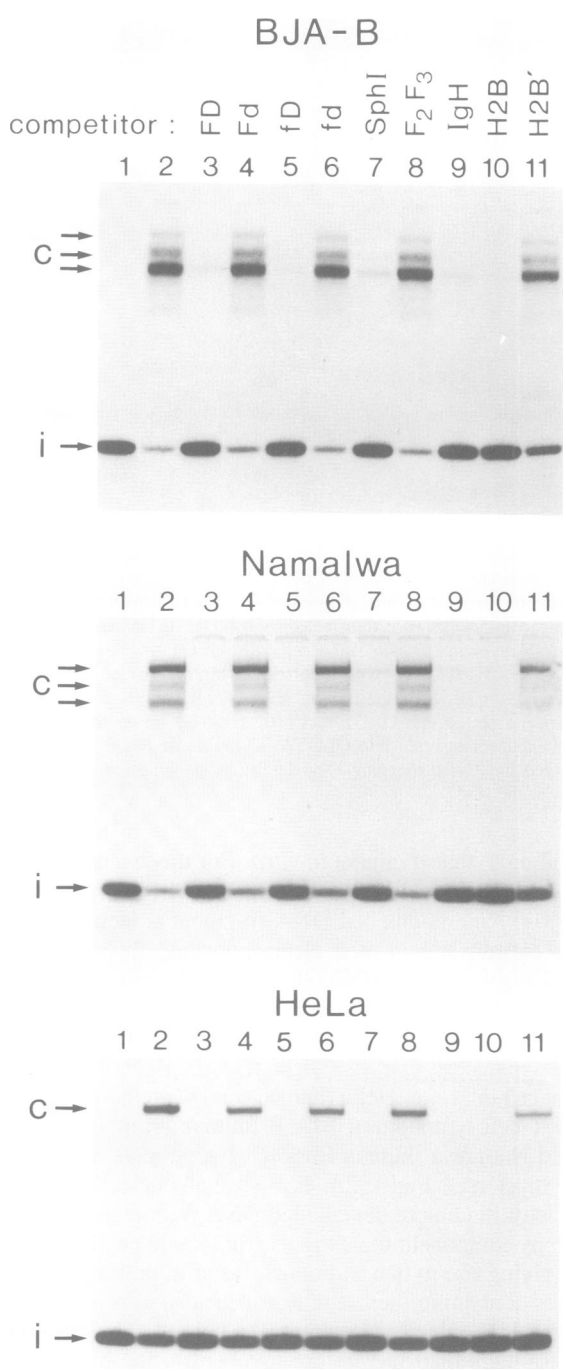

Fig. 4. Competition experiments show that factors bind to the decanucleotide. Competition experiments were done in nuclear extracts of BJA-B, Namalwa and HeLa cells. The endlabelled *Dde*I−*Hin*fI fragment (0.5 ng) was incubated in the indicated nuclear extracts in the presence of a 60-fold molar excess of cold competitor DNA. **Lane 1** shows the free fragment in absence of extract and **lane 2** the *Dde*I−*Hin*fI fragment in presence of extract and absence of competitor DNA. **Lanes 3−11:** competition with the following competitors: oligonucleotides FD, Fd, fD, fd; an oligonucleotide derived from the region homologous to the decanucleotide motif within the SV40 enhancer (Schirm *et al.*, 1987) (*Sph*I); an oligonucleotide spanning the region of the murine IgH enhancer that contains the second and the third region presumed to bind factor(s) *in vivo* (Ephrussi *et al.*, 1985) (F_2F_3); the 224-bp *Hin*fI fragment containing the murine IgH enhancer (IgH); two fragments of a sea urchin sperm histone H2B promoter (A.Barberis and M.Busslinger, personal communication) where once the decanucleotide is intact (H2B) and once truncated (H2B'). For details of the competitor DNAs see Materials and methods.

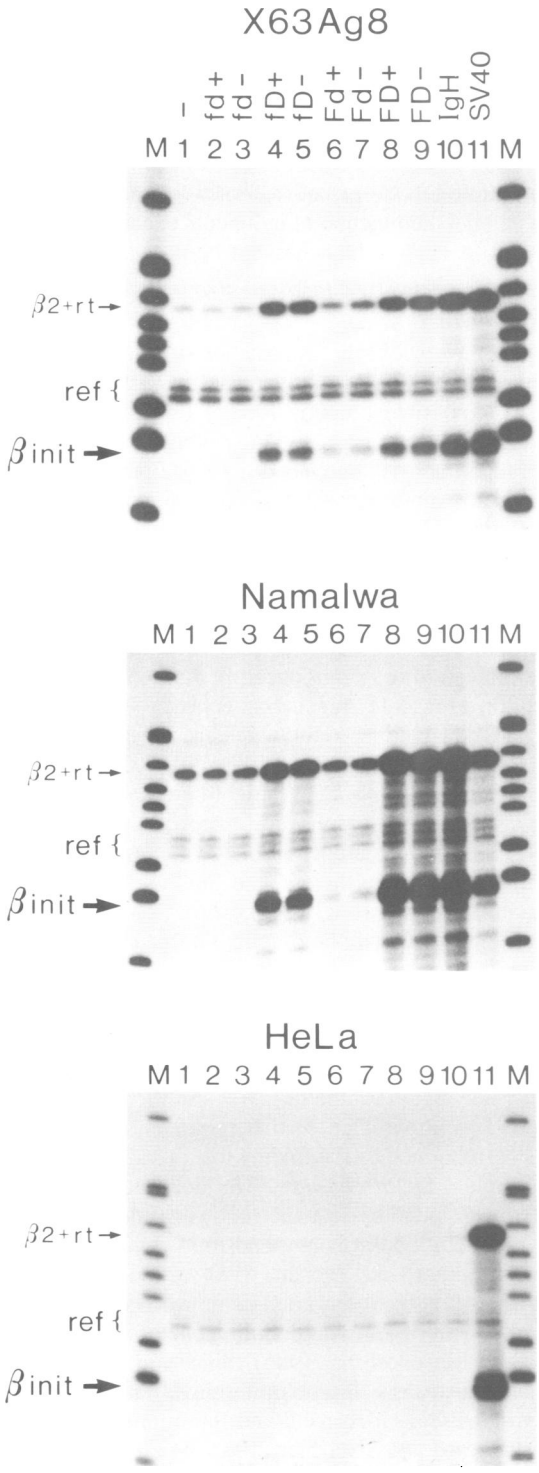

Fig. 5. Both the footprint region and the decanucleotide are important for cell type-specific enhancer activity. Six tandem copies of each oligonucleotide were cloned in both orientations ('+' or '−') downstream of the β-globin gene into vector β1E (cf. Materials and methods) and then transfected into X63Ag8, Namalwa and HeLa cells. Levels of β-globin-specific RNA in the cytoplasm were then determined by RNase mapping. Transfections were done with the following recombinants: **lanes 1**, no insert; **lanes 2** and **3**, fd oligonucleotide; **lanes 4** and **5**, fD oligonucleotide; **lanes 6** and **7**, Fd oligonucleotide; **lanes 8** and **9**, FD oligonucleotide; **lanes 10** and **11**, the 688-bp *Xba*I−*Eco*RI fragment of the IgH enhancer or the 196-bp fragment of the SV40 enhancer inserted into the β1E vector; **lanes M**, size marker (*Hpa*II cleaved pBR322). For details of the constructions see Materials and methods.

quence strongly conserved between all histone H2B gene promoters (Harvey *et al.*, 1982) also shows nearly perfect homology to the IgH decanucleotide (Sive and Roeder, 1986; A.Barberis and M.Busslinger, personal communication). Therefore, we have also carried out competition experiments with oligonucleotides derived from the SV40 enhancer (*Sph*I in Figure 4; Schirm *et al.*, 1987) or restriction fragments from a sea urchin sperm histone H2B promoter (H2B; A.Barberis and M.Busslinger, personal communication). Both DNAs compete efficiently in the band shift assays. A DNA fragment from the H2B promoter with a partially deleted decanucleotide (H2B'), however, does not compete, again demonstrating the involvement of the decanucleotide in factor binding. In all competition experiments there seems to be no major difference between the binding affinity of the ubiquitous or of the cell type-specific forms of the decanucleotide binding proteins. Furthermore, we observe efficient competition for the ubiquitous as well as for the cell type-specific factors when we use competitor fragments derived from an enhancer or a promoter, both of which are active in many different cell types. In agreement with the results described here recent methylation interference experiments suggest that within the *Dde*I−*Hin*fI fragment the only essential contact points for protein binding *in vitro* are located in the decanucleotide region. The binding pattern is indistinguishable between B and non-B cell extracts as well as between the different species observed within the same B cell extract (P.Matthias, unpublished observations).

Functional importance of both the in vivo footprint and the decanucleotide elements

To determine the enhancer acivity of the oligonucleotides *in vivo* we cloned tandem repeats of each oligonucleotide in both orientations downstream of the β-globin gene. These constructs were then tested in transient expression assays in three different cell lines: X63Ag8 (murine myeloma), Namalwa (human Burkitt's lymphoma) and HeLa (human cervix carcinoma) (Figure 5). The data show that six tandem copies of these oligonucleotides act as a bona fide cell type-specific enhancer, i.e. they act in both orientations from a downstream position, and they are active in cells of the B lymphoid lineage but not in HeLa cells. In X63Ag8 a minor, if any, reduction of transcriptional stimulation is seen with the mutation in the footprint sequence (fD). Conversely, the mutation of the decanucleotide region (Fd) results in a clear down mutation. Nevertheless, Fd still has some residual enhancer activity that must be attributed to the intact footprint motif since the double mutant (fd) exhibits no activity at all. This result demonstrates that both the footprint sequence and the decanucleotide motif are of functional importance and that there seems to be a dominance of the mutation in the decanucleotide over that in the footprint sequence.

The results of the transfection experiment with Namalwa cells show a qualitatively similar pattern. The wild-type FD enhancer segment stimulates β-globin transcription very strongly in these cells. The importance of the decanucleotide is again demonstrated by the finding that the Fd mutant shows a drastic reduction of the enhancer effect. Nevertheless, the intact footprint motif prevents the complete loss of activity. On the other hand the fD mutation shows a clear reduction of activity, though less dramatic than that in the case of the Fd mutant. This demonstrates that in addition to the decanucleotide the footprint motif is also of functional importance in these cells. Like in X63Ag8 cells only the combination of both mutations abolishes transcriptional activation completely.

A different pattern emerges in the case of transfections into HeLa cells. In these cells there is no detectable stimulation of

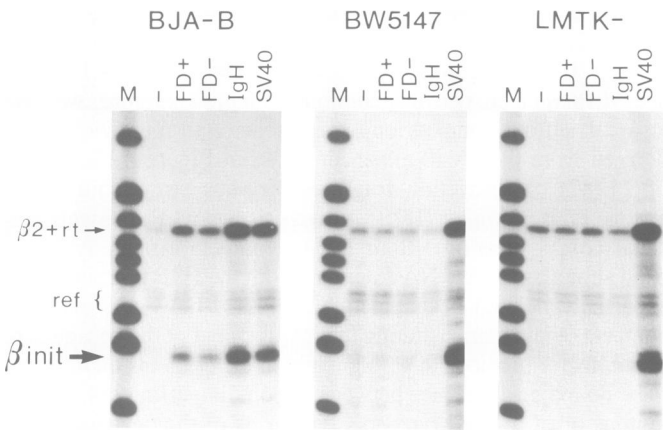

Fig. 6. The FD oligonucleotide is active only in B lymphoid cells. The activity of recombinants β1E, β1E-FD+, β1E-FD−, β1E-IgH and β1E-SV40 was determined by transient expression assays in BJA-B (lymphoblastoid cell line), BW5147 (T cell lymphoma) and LMTK$^-$ (fibroblasts).

transcription by any of the oligonucleotides, nor by the complete IgH enhancer. The internal control shows that the cells in all transfections have taken up similar amounts of DNA. Furthermore, the high level of stimulation of globin expression by the SV40 enhancer demonstrates that these cells are capable of recognizing an enhancer sequence.

In the transfection experiments shown in Figure 5 the six tandem copies of the FD oligonucleotide are active in those cells where the entire IgH enhancer is also active. To corroborate this correlation we tested some other cell lines for activity of the IgH enhancer and the FD hexamer. Figure 6 shows the RNase mappings of transfections into BJA-B (human lymphoblastoid cell line), BW5147 (murine T cell lymphoma) and LMTK$^-$ (mouse fibroblasts). Like the IgH enhancer the FD hexamer is only active in BJA-B cells which are derived from B lymphocytes. In these cells the FD oligonucleotide is not quite as active as the complete IgH enhancer, as was the case in X63Ag8 and Namalwa cells. This fact, which was not further analyzed, could mean that the IgH enhancer is particularly active in BJA-B cells and/or that the six copies of the FD oligonucleotide are relatively less active in these cells. However, in the T-cell line and in the fibroblasts both the oligonucleotide as well as the entire IgH enhancer show no activity at all. Thus, our data clearly demonstrate that both the lymphoid-specific footprint sequence and the decanucleotide are important motifs contributing to the activity and the cell type-specificity of the IgH enhancer.

Discussion

Analysis of isolated subregions of the IgH enhancer

Transcriptional enhancers are composed of different functional domains. These domains can be exchanged or duplicated and still yield functional enhancers (de Villiers *et al.*, 1984; Laimins *et al.*, 1984; Weber *et al.*, 1984; Veldman *et al.*, 1985; Herr and Clarke, 1986; Zenke *et al.*, 1986). Furthermore, some sequences without apparent enhancer activity of their own that flank an enhancer can partially rescue activity when a segment of the adjacent enhancer DNA is deleted (Zenke *et al.*, 1986). Due to this redundancy of information, mutations within an enhancer may have only a weak detrimental effect in the context of the entire enhancer. Our approach of oligomerizing short segments of the IgH enhancer, in conjunction with specific mutagenesis, allowed us to identify small sequence motifs that are involved in IgH enhancer action and in the cell type-specificity of this enhancer. Our data are an important complementation and extension of the studies on larger IgH enhancer segments (Augereau and Chambon, 1986; Peterson *et al.*, 1986; Schlokat *et al.*, 1986; Sen and Baltimore, 1986; Wasylyk and Wasylyk, 1986) since the liberation of enhancer segments from the complicated context of multiple DNA−protein interactions makes it possible to study in parallel the function of individual enhancer modules both *in vivo* and *in vitro*, which has not been done before.

A sequence protected by a factor in vivo is required for maximal enhancer function

In this paper we show the importance of two sequence motifs within the *Dde*I−*Hin*fI segment for the activity of the IgH enhancer. A possible function for the first of these sequences (the F element) was originally suggested from 'genomic footprinting' experiments (Church *et al.*, 1985; Ephrussi *et al.*, 1985). We show that a multimerized oligonucleotide containing this sequence element can enhance transcription in a cell type-specific fashion even in the absence of a functional decanucleotide. However, this motif seems to be less important than the decanucleotide element. It can be mutated with little effect in X63Ag8 cells, as long as the decanucleotide is left intact. In Namalwa cells the effect is more pronounced in that the mutation reduces transcription a few-fold. We and others have not so far been able to detect a factor binding to this sequence *in vitro* (Augereau and Chambon, 1986; Schlokat *et al.*, 1986; Sen and Baltimore, 1986; Weinberger *et al.*, 1986). This might be due to scarcity or low binding affinity of such a factor, inappropriate binding conditions used *in vitro*, instability of the protein, or loss during extract preparation. Nevertheless, the findings of Ephrussi *et al.* (1985) and our data strongly suggest that such a factor exists, is present only in B-lymphoid cells, and is a positive activator of transcription.

A factor binds to the decanucleotide element and is required for cell type-specificity

Our experiments suggest an even greater importance of the decanucleotide region for the cell type-specific activity of the IgH enhancer. The importance of that motif as seen in functional assays is corroborated by the finding that this sequence specifically binds protein factors *in vitro*. The decanucleotide motif has originally been found by sequence comparisons in the promoters of both immunoglobulin heavy and light chain variable region segments (Falkner and Zachau, 1984; Parslow *et al.*, 1984; Falkner *et al.*, 1986). It has been demonstrated that it is a functional component of immunoglobulin gene promoters (Bergman *et al.*, 1984; Mason *et al.*, 1985). Since immunoglobulin promoters show a strong cell type preference (Falkner *et al.*, 1984; Foster *et al.*, 1985; Grosschedl and Baltimore, 1985; Mason *et al.*, 1985; Picard and Schaffner, 1985; Mizushima-Sugano and Roeder, 1986) it was suspected that the decanucleotide is involved in mediating the cell type-specificity of these promoters.

In agreement with these expectations we show that there is indeed a cell type-specificity of the decanucleotide-binding factor. One species of this factor is present in a ubiquitous fashion in B and non-B cells, as already described by Singh *et al.* (1986). However, in addition to this protein species we detect others that bind to the decanucleotide motif exclusively in extracts from cells of B lymphoid origin. Similar results have also been obtained very recently by Landolfi *et al.* (1986) and Staudt *et al.* (1986). This finding is particularly interesting in conjunction with the fact that all the mutants analyzed reduce transcription in lym-

phoid cells, rather than enhancing transcription in non-lymphoid cells. Thus it appears that the two IgH enhancer motifs analyzed are subject to positive rather than negative control of transcription, presumably via availability of activating factors.

Although we find a lymphoid-specific factor(s), it is obvious that both the ubiquitous and the cell type-specific forms bind to the decanucleotide motif of B cell-specific Ig genes. A clue to the role of the ubiquitous factor may come from the fact that sequence motifs related to the decanucleotide occur also in non-B cell-specific promoters and enhancers of the genes encoding histone H2B (Harvey *et al.*, 1982; Sive and Roeder, 1986; A.Barberis and M.Busslinger, personal communication), SV40 T-antigen (Mattaj *et al.*, 1985), or various U snRNAs (Mangin *et al.*, 1986; and references therein). A related sequence element in adenovirus, shown to be bound by a protein factor called NF-III, is thought to be involved in viral DNA replication (Pruijn *et al.*, 1986). In competition experiments we observe that DNA fragments of the IgH enhancer, the SV40 72-bp repeats, and the histone H2B promoter have the same potential to bind to the decanucleotide-binding factors both in extracts of B cells as well as of HeLa cells. Sive and Roeder (1986) and Bohmann *et al.* (1987) have made similar observations. Decanucleotide-binding factors seem to occur also in non-vertebrate cells: Mocikat *et al.* (1986) have reported the presence of such a factor in yeast. Barberis and Busslinger (personal communication) have found a related factor in sea urchin embryos that binds both to a sea urchin histone H2B promoter and the murine IgH enhancer.

Different factors bind to the same regulatory sequences

We thus face the seeming paradox that cell type-specific factors appear to be capable of interacting with genes expressed in a wide variety of different cell types and conversely a ubiquitous protein can interact with DNA motifs of B cell-specific regulatory elements. To explain the preferential activity of the IgH enhancer and immunoglobulin promoters we favor the following model. Only the B cell-specific form(s) of the decanucleotide binding factor are functional by themselves, whereas the ubiquitous form requires an essential co-factor. In the case of the IgH enhancer and the immunoglobulin variable region promoters, the B cell-specific factors bind to the decanucleotide and activate transcription in B cells (by a mechanism yet to be elucidated). In non-B cells, the ubiquitous binding factor interacts with the decanucleotide of the IgH enhancer or the variable region promoters but there are no binding sites for co-factors that must interact with it. Therefore, immunoglobulin genes are silent in non-B cells. However, the promoters of histone H2B genes or the enhancers of U snRNA genes are active in many different cell types. According to our model they would have a different motif arrangement with a specific binding site for another regulatory factor which, in conjunction with the ubiquitous form of the decanucleotide-binding protein, is capable of activating transcription.

Several possibilities can be envisaged as to how the decanucleotide-binding factors could differ from each other. The factors could be single proteins sharing similar or identical DNA-binding domains but differing in some other effector function. Such proteins could be synthesized from closely related genes, or even from the same gene via differential splicing or by post-translational modifications, such as phosphorylation. It will be most interesting to find out how these factor species differ from each other. Therefore we expect that the isolation and closer characterization of the decanucleotide-binding proteins and their interactions with the other components of the transcriptional machinery will further our understanding of cell type-specific gene transcription.

Materials and methods

Construction of plasmids containing multiple copies of short DNA fragments

All enzymatic manipulations were done according to standard procedures (Maniatis *et al.*, 1982). The 51-bp *Dde*I–*Hin*fI fragment of the murine IgH enhancer was reclaimed from clone πUCIgE$^+$ (Gerster *et al.*, 1986), made blunt by filling in and inserted into the *Hin*dII sites of the symmetric polylinker of pUC7 (Vieira and Messing, 1983). Recombinants were obtained which contained either one copy or a dimer of the filled in *Dde*I–*Hin*fI fragment. These monomer or dimer inserts were then excised by *Eco*RI such that on both ends the IgH enhancer-derived DNA was flanked by 15 bp of polylinker DNA. The fragments were ligated at high concentration into the single *Eco*RI site of the plasmid vector GLOTA that contains both the rabbit β1-globin and the SV40 T-antigen genes (E.Schreiber and W.Schaffner, unpublished results). Recombinants were obtained containing a single copy of either the original monomer or of the dimer (designated 1× and 2× respectively in Figure 1). In addition a plasmid was obtained containing two copies of the original dimer (designated 4×). In this case the IgH sequences are separated by 30 bp of polylinker sequences. The constructs having either three copies of the monomer or of the dimer (designated 3× and 6× respectively), have a structure that can be described as follows: Ig sequences–polylinker–Ig sequences–polylinker–Ig sequences. Furthermore, both the 688-bp *Xba*I–*Eco*RI fragment containing the IgH enhancer and a 196-bp fragment (de Villiers *et al.*, 1982) containing the SV40 enhancer were cloned as *Eco*RI fragments into the GLOTA vector as well. To avoid possible influences of T-antigen production on transcription stimulation a plasmid containing only the rabbit β-globin gene was used for further experiments. This recombinant (β1E) is identical to pβG (de Villiers *et al.*, 1982) except that the *Eco*RI site in the third exon is destroyed, leaving a unique *Eco*RI site 3′ of the globin gene. Synthetic oligonucleotides were kinased, ligated, the ends filled in by Klenow polymerase, and the oligonucleotides then purified by polyacrylamide gel electrophoresis. Hexamers of the oligonucleotides were isolated and cloned into the *Eco*RV site of a pUC vector having a synthetic polylinker of the structure *Eco*RI–*Xho*I–*Eco*RV–*Xho*I–*Eco*RI. The six tandem copies were then inserted as an *Eco*RI fragment in both orientations into the *Eco*RI site of β1E. Furthermore, the same IgH and SV40 enhancer fragments cloned into GLOTA were also inserted into β1E.

Cell lines, transfections and RNA analysis

Cells were originally obtained from the following colleagues: HeLa, U.Petterson, University of Uppsala; X63Ag8, S.Y.Chung-Wooley, NIH, Bethesda; BJA-B, H.zur Hausen, DKFZ Heidelberg; Namalwa, I.Gresser, IRSC Villejuif, BW5147, H.Hengartner, University of Zürich; LMTK$^-$, C.Weissmann, University of Zürich. All cell lines were transfected with a DNA mixture [ratio betwen test DNA and REFΔ DNA (Picard and Schaffner, 1985) of 4:1] by the DEAE-Dextran procedure as described (de Villiers and Schaffner, 1983). Modifications of the protocol include a DMSO boost (Lopata *et al.*, 1984) after transfection. On the other hand for all cells except BJA-B and Namalwa the chloroquine treatment was omitted. For these two cell lines a 5-fold increased DNA concentration was used (8.3 μg/ml). RNA isolation and analysis by SP6-polymerase-generated RNA probes was done as described by Picard and Schaffner (1985).

Preparation of nuclear extracts and band shift experiments

HeLa, BJA-B and Namalwa cells were grown in suspension, and BW5147 and X63Ag8 cells were grown on plates. For an extract preparation ~1 l or 40 100-mm dishes were used (about $5 \times 10^8 - 1 \times 10^9$ cells). Nuclear extracts were prepared as described by Dignam *et al.* (1983), with minor modifications. After $(NH_4)_2SO_4$ precipitation, the extract was resuspended in and dialyzed against 20 mM Hepes pH 7.9, 20% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF.

Binding reactions were done by incubating radiolabelled DNA (0.2–0.5 ng, 3000–4000 c.p.m.) with 4–6 μg protein extract and 2 μg non-specific competitor DNA [poly(dI)·(dC)] in a buffer containing: 10 mM Hepes pH 7.9, 110 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM DTT, 4 mM spermidine, 4.2% Ficoll. After 10 min at room temperature, the binding reaction was chilled on ice and then loaded on a 4% polyacrylamide gel which was run at 4°C in Tris–acetate buffer (Singh *et al.*, 1986).

Competitions were done by mixing to the binding reaction the appropriate competitor DNA before adding the extract. One picomole of annealed oligonucleotide (or 200 ng of restriction fragment) was used for each competition reaction. The following competitors were used: FD, Fd, fD, fd, as described in Figure 3. *Sph*I: synthetic deoxyoligonucleotide spanning positions 124–148 and 196–220 respectively of the 72-bp repeats of SV40 5′-GCAGAAGTATGCAAAGCATGCATCT-3′ (Schirm *et al.*, 1987). F_2F_3: oligonucleotide spanning positions

378–421 of the mouse IgH enhancer 5′-GCAGCAGCTGGCAGGAAGCAG-GTCATGTGGCAAGGCTATTTGGG-3′. IgH: the 224-bp *Hin*fI fragment from the mouse IgH enhancer. H2B and H2B′: restriction fragments derived from two *Bal*31-generated deletion mutants of a sea urchin sperm-specific H2B gene (A.Barberis and M.Busslinger, personal communication; a kind gift from M.Busslinger). They extend from a *Sal*I site within the coding sequence (+125) to positions −67 (H2B) or −47 (H2B′), respectively, of the promoter. Thus in the latter case only the 3′ five basepairs of the decanucleotide homology (TCATTTGCAT) are preserved.

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