Lymphoid-specific transcriptional activation by components of the IgH enhancer: studies on the E2/E3 and octanucleotide elements

Graham P. Cook and Michael S. Neuberger
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

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ABSTRACT

The IgH enhancer is a strong lymphoid-specific activator and is composed of multiple factor-binding motifs. One of these, the octamer, is common to enhancer and promoter, binds ubiquitous and lymphoid-specific factors and is able to act as a lymphoid-specific transcriptional activator. However, it is also found as an essential component of promoters active in non-lymphoid cells. From analysis of the activities of synthetic promoters, we suggest that recruitment of the lymphoid-specific octamer-binding protein next to the TATA is sufficient to create a functional lymphoid-specific promoter whereas the ubiquitous octamer binding protein is not active in single copy but can act in concert with other promoter binding factors. However, the activity of the IgH enhancer is not dependent on the octamer and we identify the E2/E3 elements as also being sufficient to confer lymphoid-specificity on a linked gene. Activity of the E2/E3 region results from the synergistic activity of the two motifs, E2 alone being able to confer a low level of activity which is dramatically increased by the adjacent E3. Thus, in the case of both the E2/E3 and the octamer motifs, interactions between adjacent elements can play a critical role in determining the tissue specificity of activity.

INTRODUCTION

Enhancer elements function through the binding of specific transcription factors. Studies on the SV40 enhancer have established that the broad cell-type specificity of this enhancer results from the presence of multiple elements, each with different cell-type specificities (1,2). Certain elements from the SV40 enhancer can act alone whilst others need to interact functionally with other, distinct elements in order to generate transcriptional activity (3,4). In contrast to the SV40 enhancer, the immunoglobulin heavy chain (IgH) enhancer displays rigid cell-type specificity (5,6). Genomic footprinting of the IgH enhancer has revealed a number of lymphoid-specific protections (7) termed E1, E2, E3 and E4. Nuclear factors designated NF-\(\mu\)E1, NF-\(\mu\)E2 and NF-\(\mu\)E3 have subsequently been identified which bind in vitro to elements E1, E2 and E3 respectively (8–10).

The octanucleotide element, ATTTGCAT or its invert, is found in both the IgH enhancer and in V gene promoters (11,12) and is bound by factors both in vivo and in vitro. Two factors have been shown to bind to this motif in vitro: Oct-1, which has been found in all cell types examined and Oct-2, which is largely confined to lymphoid cells (13–17). The octanucleotide motif can itself be sufficient to confer lymphoid specificity on a linked gene (18–20) consistent with the tissue distribution of the Oct-2 protein. However, the octanucleotide is also found as a functional component of promoters whose activity is not restricted to the lymphoid lineage, such as those of the histone H2B and snRNA genes (see 17 for references). This ability of the octanucleotide to function both as a lymphoid-specific and ubiquitous transcription element has been the subject of much discussion (21,22).

Whilst being necessary and sufficient for V gene promoter function (23,24), the octanucleotide is dispensable for the lymphoid-specific activity of the enhancer (9,25,26). This implies the existence of other elements within the IgH enhancer that are capable of conferring lymphoid-specific transcriptional activation. Here, we have used mutational and sufficiency analyses to search for such elements and have found that cell-type specific transcriptional activity is conferred by the functional interplay of two adjacent E elements, E2 and E3. This requirement for synergistic activity of two individual E elements to make a transcription activator suggested, by analogy, a possible solution to the octanucleotide paradox. Whereas the octanucleotide on its own (presumably through binding of Oct-2) is sufficient to activate transcription in lymphoid cells, its activity in non-lymphoid cells (where only Oct-1 is available) may depend on an additional activation function in the form of an adjacent linked factor-binding motif. We present data which strongly support this model and which therefore emphasize the importance of combinations of transcription elements in regulating tissue specificity.

MATERIALS AND METHODS

Plasmid construction

Point mutants were constructed using oligonucleotide directed mutagenesis (27) and checked by sequencing using the chain termination method (28). Enhancers containing point mutations were cloned into the XbaI site of plasmid p\(\beta\)Genh\(^+\) [p\(\beta\)G800; (29)]; deletion mutants were inserted into the vector using the
XbaI site and a second site created by insertion of BglII or XhoI linkers into the SacI site. For assay of individual elements cloned upstream of the β-globin ATA, a derivative of plasmid pG128SV3 (29) was used in which the SV40 enhancer is located at the 3' end of a β-globin gene which is itself deleted of sequences upstream of position -35 relative to the cap site (23). Complementary oligonucleotides were hydrogenised together and ligated into either the SmaI site or the XbaI and SacI sites. Orientation and sequence of all inserts was checked by sequencing of the double-stranded plasmid DNA (30). Construction of the e2-E3 and E2-e3 mutants was achieved by hybridising the mutant oligonucleotide to the wild type opposite strand oligonucleotide and ligating the mismatched duplexes into the vector. Mutants were then identified by DNA sequencing.

Cell lines and DNA transfection
MPC11 cells were a gift from B. Wasylyk, HeLa cells (clone S3) were from our own collection. Cell lines were maintained in DMEM/10% FCS with antibiotics. Cells were transfected by the calcium phosphate procedure (31,32) using 20μg of test plasmid and 5μg of the α-globin reference plasmid [αSVHPC3; (29)].

RNA extraction and ribonuclease protection analysis
Total cytoplasmic RNA was prepared as previously described (23) and a sample (10μg) analysed by ribonuclease protection using probes made from the plasmids pSP66132 and pSP66350 (29) with SP6 RNA polymerase.

RESULTS
Identification of a second lymphoid-specific element in the IgH enhancer
Mutagenesis of the IgH enhancer was carried out in order locate other elements, apart from the octanucleotide, which are sufficient to confer lymphoid-specificity; candidate elements were then subsequently tested for their ability to function outside the context of the full enhancer. The mutated enhancers were assayed by transient transfection assays in which they were linked to a β-globin gene which is poorly transcribed after introduction into a host cell unless linked to an enhancer element. Thus, following transfection into the mouse myeloma line MPC11, little β-globin mRNA is produced from pβGenh- whereas derivatives that include the SV40 or IgH enhancers produced greatly elevated levels of β-globin mRNA (Figure 1C). Mutated enhancers were
cloned into pβGenh− 800 bp upstream of the β-globin transcription start-site and were transfected together with an α2-globin internal reference plasmid into MPC11 cells. The results confirm that mutation of the octanucleotide does not significantly alter the activity of the enhancer implying the importance of other motifs to activity. Furthermore, whilst the mutagenesis of the E motifs suggests that the E3 element is most important to the activity of the enhancer, no single one of these motifs is essential owing to a functional redundancy. In order to overcome this redundancy, a deletion analysis was used in an attempt to separate the lymphoid specificity of the enhancer from the octanucleotide. Deletions were made using the restriction sites that had been introduced by the specific mutagenesis described above; the deleted enhancers were cloned into pβGenh− with the variable endpoints most 5′ so as to avoid artefacts that might arise by varying the distance between promoter and enhancer (Figure 1B). Ribonuclease protection assays obtained with the most informative endpoints are shown in Figure 1C with quantitation of the activity in MPC11 cells of all the deletion endpoints and point mutants tested being summarised in Figure 1B. The results demonstrate that the enhancer can be divided into at least two domains that are independently capable of transcriptional activation; the left and right-hand domains are designated domain A and B respectively. The various deletion mutants were also tested for activity in non-lymphoid cells; deletions that remained active in MPC11 cells retained a high degree of cell-type specificity (data not shown). Whereas analysis of the deletion endpoints reveals that the octanucleotide is an essential component of domain B, the activity of domain A requires some element that is located between E2 and E4 (Figure 1C) implying the existence of a lymphoid-specific element within this region. This finding coupled with the reduced activity of the E3 mutated enhancer prompted us to analyse the cell-type specificity conferred by individual motifs from this region.

A number of enhancer elements have been shown to function within the context of a promoter, including both the octanucleotide (18,20) and the NF-κB motif (33). Indeed in this type of assay the octamer is sufficient to confer lymphoid-specificity to a promoter (18,20). These and other experiments suggest that promoter and enhancer elements are functionally interchangeable and the mechanisms by which the two types of elements act are fundamentally similar. We therefore used promoter assays in order to test the activity conferred by individual elements of domain A. Single copies of individual motifs were tested for their ability to confer cell-type specific activity on a β-globin gene that has been deleted of its own upstream-promoter region. Individual elements were cloned in single copy into a Smal site placed 30 bp 5′ of the β-globin ATA box (Figure 2A); oligonucleotide sequences were chosen on the basis of the dimethylsulphate protections observed in genomic footprinting experiments (7). An SV40 enhancer located at the 3′-end of the gene drives transcription from these promoters. Strong lymphoid-specific transcriptional activation was achieved with a promoter consisting of the octanucleotide and weaker activation was obtained with the 21 bp oligonucleotide containing the E2 motif (Figure 2B). Little if any stimulation was obtained with the E3 element on its own (Figure 3A) nor was any detected with either E1 or E4 (not shown).

The E2 element is able to confer some degree of tissue specificity since whereas weak transcriptional activation was

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**Figure 2.** Activity of the E2 element. (A) Structure of the plasmids used to assay activity of individual elements. The β-globin sequences are in bold and the positions of the RNA start site (+ 1) and the ATA box are marked. Oligonucleotides were inserted into the Smal site. The sequence given for the E2 motif corresponds to the E2a orientation, the plasmid containing the E2 oligonucleotide in the opposite orientation is designated E2b. The mutated E2 element is designated e2 with the mutant nucleotides being shown in lower case. (B) Activity of the E2 element in lymphoid and non-lymphoid cells. The β-globin exons plus ATA containing no additional upstream elements (pβGF), the octanucleotide (Oct), E2 (E2a etc.) or the β-globin upstream elements (pβGF) are shown. Positions of correctly initiated transcripts are indicated. Quantitation of the activity of these elements is given in Figure 4C.
obtained with MPC11 myeloma cells, no such effect was obtained with HeLa cells (Figure 2B). We were concerned to show that this activity was indeed due to the E2 element itself and therefore, by implication, to the factor NF-κB rather than to some factor that recognises flanking vector sequences or a combination of the vector with the E2 oligonucleotide. We therefore assayed the E2 element in either orientation [E2a and E2b in Figure 2B] and also tested a mutant of E2 [oligonucleotide e2] that is no longer able to bind NF-κB (9). The results (Figure 2B) strongly support the proposal that the weak cell-specific transcriptional activation conferred here is indeed due to NF-κB.

Lymphoid specificity conferred by E2 is increased by the adjacent E3
One of the limitations of assaying individual motifs as upstream promoter elements is that the approach will only detect activity if a single motif provides a sufficient upstream promoter activity.

We wondered whether the activity of the E2 element in this assay
could be increased if it were allowed to act in concert with a second DNA binding motif. We therefore tested a 42 bp oligonucleotide that includes both the E2 and E3 elements. This oligonucleotide conferred strong activation of transcription when assayed in either orientation (Figure 3) and is six to twelve times more active in lymphoid cells than non-lymphoid cells. This contrasts with the weak activation given by E2 alone. Quantitation of the activity conferred by these various elements in lymphoid and non-lymphoid cells is shown, corrected for variations in the α-globin control (Figure 4C). To study whether this activation was indeed dependent on the combination of E2 and E3 binding factors, we made mutants of the 42 bp oligonucleotide which are known from previous work (9) to abolish the binding of NF-μE2 and NF-μE3. The results (Figure 4) show that strong transcriptional activation requires intact binding sites for both NF-μE2 and NF-μE3. These results are consistent with the NF-μE2 and NF-μE3 factors acting in a synergistic manner to create a lymphoid-specific activator when assayed as promoter upstream elements.

The IgH enhancer thus contains at least two elements which are sufficient to confer lymphoid-specificity to a linked gene, the previously identified octamer and the combination of E2 and E3 motifs identified here. An enhancer in which the octamer, E2 and E3 have all been mutated shows a markedly reduced activity as compared to the 1 kb wild-type enhancer fragment (Figure 3A; contrast IgH with ‘230M”), thus confirming the importance of these elements to enhancer activity as a whole.

**Activation by the octanucleotide element in lymphoid and non-lymphoid cells**

The octanucleotide element is sufficient to confer lymphoid specificity on a linked gene but is nevertheless found as an essential component of several promoters active in non-lymphoid cells. The molecular basis of the differential involvement of this element in cell-type specific gene expression has not been elucidated. By analogy to our findings with the E2 and E3 elements, it is attractive to speculate that the octanucleotide binding protein in non-lymphoid cells (Oct-1) may need to act in concert with another DNA binding protein in order to make an effective promoter. In contrast, the lymphoid-specific octanucleotide binding protein (Oct-2) may act without additional factors binding to the upstream promoter region. The differential requirements of the two octanucleotide factors could therefore be the basis of the fact that the octanucleotide motif is sufficient to direct lymphoid-specific gene expression, whereas it is found in promoters of more widespread activity together with additional motifs. Thus for example, the histone H2B promoter could function in a wide variety of cell-types owing to the presence of an octanucleotide element and adjacent motifs, which include a CCAAT box.

We therefore asked whether provision of a CCAAT element upstream of an octanucleotide/TATA promoter would alter the cell-type specificity of this promoter using the assay system described above. Without additional motifs, the promoterless β-globin gene (pβG~) is inactive in MPC11 and HeLa cells (Figure 5C). Insertion of a 17 bp oligonucleotide containing the octanucleotide motif from the IgH enhancer results in the creation of a strong, lymphoid specific promoter (+Oct). The lack of activity obtained using a mutant octanucleotide motif (+OctM) suggests that the activity is dependent on the octanucleotide binding protein. However, the provision of a CCAAT box upstream of these promoters (+Oct+CCAAT) results in a promoter whose activity is no longer confined to the myeloma cells; activity is now observed in both of the cell lines tested. It is notable that the ability of the CCAAT+Oct promoter to function in both lymphoid and non-lymphoid cells is dependent on binding of an octanucleotide factor, since the promoter containing CCAAT+OctM motifs is inactive. These results demonstrate that, whereas a single copy of the octanucleotide can act as a sufficient upstream element in lymphoid cells, it cannot in non-lymphoid cells. Provision of additional motifs to these promoters allows them to function in non-lymphoid cells in an octamer dependent manner; this presumably reflects important differences in the activation functions of the Oct-1 and Oct-2 proteins.

**DISCUSSION**

The IgH enhancer is composed of at least two independently active domains, one centred on the E2/E3 motifs (domain A) and the other on the octanucleotide (domain B). The E2 and E3 motifs have previously been demonstrated to be important for enhancer function (9,25,26). We have now extended these findings to demonstrate that the E2 and E3 motifs are in fact sufficient to direct lymphoid-specific transcriptional activity on a linked gene. The mechanism of their synergistic activity remains unclear. However, E3 placed very close to the TATA box (34) or multimerised at a distance (our own unpublished data) appear to be active in both lymphoid and non-lymphoid cells, consistent with previous mutational analyses (9,25,26). It would therefore seem that E2 plays a dominant role in determining the tissue specificity of the E2/E3 element whilst E3 might determine the actual level of activity. Experiments investigating NF-μE2 binding *in vitro* suggest that it binds relatively weakly to its cognate motif. An attractive possibility therefore is that NF-μE2 is a transcriptional activator whose binding to DNA is enhanced by interaction with an adjacent NF-μE3. Interestingly, comparison of the mouse and human IgH enhancers (35) demonstrates that the E2 element is highly conserved between species but that no homology exists in the human enhancer to the mouse E3 element. Thus, in the case of the human enhancer another element might fulfill the role of E3 whilst retaining the lymphoid-specific E2. Elements that are very similar or identical to E2 and E3 have been implicated in playing a role in the activity of the insulin gene enhancer (36) and cDNA clones have been isolated that encode proteins which bind to sequences similar to E2 (37,38). An understanding of the molecular basis of the cell-type specificity of the E2/E3 region will obviously require a detailed characterisation of the factors which recognise it. It will also be interesting to discover whether the E2/E3 combination shows activity in specific non-lymphoid cell types.

In contrast to the E2/E3 elements, the octanucleotide when placed adjacent to the TATA, can be sufficient to direct lymphoid-specific transcription (18,20,39,40). However, a single copy of the octanucleotide is not sufficient to activate transcription when placed at a distance from the promoter (19,20). Thus, when domain B of the IgH enhancer is assayed at some distance from the promoter, it is likely that the octanucleotide binding protein functions by interaction with some as yet unidentified element within the domain.

The ability of the octanucleotide to confer lymphoid specificity when placed next to the TATA suggests that Oct-2, but not Oct-1, is a sufficient upstream-promoter factor. However, when a CCAAT box is placed next to the octanucleotide, we show that
the result is an octanucleotide-dependent promoter that is active in both non-lymphoid cells and myeloma. This means that the combination of these two factors presumably generates a protein complex that is capable of recruiting the general transcription factors to the promoter. Possibly neither Oct-1 nor the CCAAT-binding protein on their own in single copy contain a sufficient activation domain to recruit RNA polymerase II. The combination of two factors with weak activation domains could create a complex with sufficient activity to enable the transcription machinery to function. These findings emphasise the importance of multiple motifs in promoters dependent on Oct-1, whereas Oct-2 dependent promoters are able to function in the absence of additional motifs. The U2 snRNA enhancer contains adjacent octanucleotide and Sp1 binding sites and its activity in non-lymphoid cells is dependent on both sites being present (41). The requirement for an additional activation function for Oct-1 is also suggested by the finding that the Herpes virus VP16 protein is capable of activating octamer containing promoters in non-lymphoid cells (42).

The model presented above implies that Oct-1 and Oct-2 differ in their transcriptional activation domains. Comparison of the sequences of the two proteins reveals that they are highly related within a region that includes the DNA binding domain but they are less homologous elsewhere (17,43,44). Recent experiments demonstrate that Oct-1 and Oct-2 do indeed have different activation functions; Oct-2 contains two activation domains whereas the Oct-1 protein appears to contain a single domain (45), this further supports the requirement for additional motifs in promoters active outside of the lymphoid lineage.

Thus the synergy of the E2/E3 elements to form a lymphoid-
specific transcriptional activator and of the octanucleotide and CCAAT elements to form a promoter region active in fibroblasts draws attention to the importance of combinational arrangements of transcription elements as a means towards achieving cell-type specificity of transcription.

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