Mutational analysis of the contribution of sequence motifs within the IgH enhancer to tissue specific transcriptional activation

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ABSTRACT

We have investigated the role of sequence motifs in the immunoglobulin heavy chain (IgH) enhancer on its activity in myeloma and fibroblasts cell-lines. In transient transfection assays the transcription stimulatory activity of the enhancer is decreased in myeloma cells by mutating the E motifs 1, 2 and 3, the core motifs C1, C2, C3 and the octamer motif (OC) and in fibroblasts by mutating E2, E3, and C2. Our results suggest that transcription factors binding to E1, C1, C3 and OC contribute in a positive manner to the tissue specificity of the IgH enhancer.

INTRODUCTION

Enhancers are promoter elements that can activate transcription over large distances, although less efficiently than over short distances. They stimulate transcription in cis and in an orientation-independent manner from both homologous and heterologous promoter elements. Specific trans-acting factors interact with defined sequence motifs in enhancers. An important goal is to understand how the combined activity of these trans-acting factors results in the modulation of enhancer activity during differentiation and in response to various signals in the cell (for reviews see 1-3).

One of the best characterized cellular enhancers is the immunoglobulin heavy chain (IgH) enhancer, which is preferentially active in lymphoid cells (4,5). However a central fragment of the IgH enhancer can efficiently activate transcription in non-lymphoid cells (6-8). The IgH enhancer has been proposed to contain eight motifs (see Fig.1) : E1-4 ("Ephrussi" sequences related to 5'-CAGGTGGC-3' (9,10)), C1-3 ("core" sequences related to 5'-GTGG^{TTT}_{AAA}G-3' (4,5)) and OC ("octanucleotide" sequences related to 5'-ATTTCAT-3', (11,12)). Studies using various cell-extracts have identified proteins which interact with some of these motifs (Fig. 1 and 13-27). However from in-vitro studies it is not clear whether these proteins are transcription factors, and if they are important for determine the speci-

ficity of the IgH enhancer. One approach to this problem is to determining the effects of point mutating their binding sites on the activity of the enhancer in vivo. We report here the effect of mutating the eight proposed IgH enhancer motifs on its ability to activate transcription in both myeloma and fibroblast cell-lines.

MATERIALS AND METHODS

All recombinants were constructed by standard techniques (28). The wild type and mutated enhancer fragments A,B,C and D (Fig.1) were assembled from synthetic oligonucleotides by the shot-gun ligation technique (29). The wild type and mutant fragments were sequenced to assure that the only differences between them were the intended mutations. These fragments were transferred into the reporter recombinants pG4 and pG1 (see Fig. 1) using appropriate unique restriction sites which were introduced on the ends of the fragments during synthesis. Naturally occurring restriction sites were used to assemble the ABC fragment (see Fig. 1). The reporter recombinants were transfected into MPC11BU4 and LMTK⁻ fibroblasts, and 48h later total RNA was extracted and analysed by quantitative S1 nuclease mapping (6,30,31). Suitably exposed autoradiograms were analyzed by scanning densitometry. The average standard deviation was $\pm 10\%$, and the largest standard deviation observed was $\pm 15\%$ (see error bars on Figs).

Fig. 1 - Structure of recombinants (A) and sequence of wild type and mutant IgH enhancer DNA fragments (B). A : the reporter recombinants pG4 and pG1 contain rabbit β globin gene sequences including 109 bp of upstream promoter sequences (from +1 to -109). The DNA fragments A,B,C,D and ABC (part B) were introduced into polylinker sequences at the indicated positions. B : the enhancer motifs E1-4, C1-3 and OC are boxed. The sequences above these motifs show the mutations which were introduced in the enhancer. Naturally occurring unique restriction sites at the extremities of the A, B and C fragment are indicated. The filled and open circles on the sequence indicate G residues which are either more or less reactive, respectively, to modification in in-vivo footprinting experiments (9,10). The arrows below the sequence indicate the specific binding sites for proteins identified in cell-free extracts [NF- μ E1, NF- μ E2, NF- μ E3:(17,20); LN1+2, LN4, LC1, LC2 (13); IgPE-1, IgPE-2:(18); NFA:(19,22) B, C1-3, 8:(16)].

Fig. 2 - Quantitative S1 nuclease analysis of RNA from transfections in MPC11BU4 myeloma cells with pG4, pG4MIX and pG4-ABC. 1 μ g reporter recombinant and 1 μ g of the internal control p8CMX were transfected into myeloma cells, and after 48h total RNA was analysed for transcripts initiated from the β globin promoter (GLOB) of the reporter recombinant and the conalbumin promoter (CON) of the internal control. Lanes 2 and 3 contain 1/35 and 1/61 of the amount of RNA used for lanes 4 and 5 respectively, to permit accurate quantitation of autoradiographic signals of very different intensities (ie between pG4 and pG4-ABC or pG4MIX).

RESULTS

Mutation of the OC, C1, C3, E1 and E2 motifs decreases IgH enhancer activity in myeloma cells.

We studied the effect of mutating individual motifs of the mouse IgH enhancer on its activity *in vivo*. The mutations were introduced into DNA fragment ABC extending from the HinfI (345) to AvaII (590) sites of the 1kb IgH gene XbaI fragment which contains the IgH enhancer (4,5 and Fig. 1). Enhancer activity was measured as the ability to stimulate, from a distant location, specific transcription from the promoter of the rabbit β globin reporter gene (see recombinants pG4, pG4MIX, pG4-ABC, Fig. 1). The reporter

Fig. 3 - Effect of mutating IgH enhancer motifs on enhancer activity in myeloma cells. 1 μ g reporter recombinants containing mutations in one of the enhancer motifs and 1 μ g internal control (p β CMIX) were transfected into mouse myeloma cells. Total RNA was analysed by quantitative S1 nuclease mapping. The activity of the wild-type enhancer fragment ABC was taken as 100 %. The value of standard deviation is indicated by the error bars. The position of the motifs in the ABC fragment is indicated below the graph.

recombinants were co-transfected into murine myeloma cells (MPC11BU4 line) with an internal control recombinant (p β CMIX or p β CBx2, see 30) to correct for variations in transfection efficiency. After 48h total RNA was extracted and analysed by quantitative S1 nuclease mapping in order to measure the amount of specific RNA initiated from the reporter and control recombinants (see bands labelled REPORTER (GLOB) and CONTROL (CON) respectively in Fig. 2). The transfections were repeated at least 3 times, with at least two different DNA preparations. Suitably exposed autoradiograms were analysed by scanning densitometry. The standard deviation was on average ± 10 %. We found that the IgH enhancer fragment ABC stimulated β globin transcription about 30 fold (compare lanes 1, 2, 4, Fig. 2), in contrast to the XbaI fragment which stimulated transcription approximately 60 fold (compare lanes 1, 3, 5, Fig. 2). These results show that the ABC DNA fragment is an efficient enhancer in myeloma cells, and is almost as efficient as the 1kb XbaI fragment.

Fig. 4 - Stimulation of β globin transcription by IgH enhancer fragments A,B,C and D in myeloma and fibroblast cell-lines. The pG1 based reporter recombinants (1 μ g in myeloma cells, 3.5 μ g in fibroblasts) and the internal control recombinants (1 μ g pBCMIX in MPC11BU4, 3.5 μ g pBCBX2 in fibroblasts) were transfected into myeloma (M) and fibroblast (F) cell-lines, and total RNA was analysed by quantitative S1 nuclease mapping. The results are presented as the amount of specific RNA transcribed from the reporter recombinants containing the WT fragments A,B,C and D divided by the amount transcribed from the recombinant pG1 which lacks IgH enhancer sequences.

Point mutations were created in the different motifs of the ABC DNA fragment (see Fig. 1 and Materials and Methods), and their effects on enhancer activity in myeloma cells was determined. Mutation of the OC, C1, C3, E1 and E2 motifs reproducibly decreased enhancer activity (Fig. 3). Mutating the OC motif has the largest effect suggesting that this motif is particularly important for IgH enhancer activity in myeloma cells.

E3 and C2 are also functional motifs in myeloma cells

Mutations in E3, C2 and E4 had only small inhibitory effects, which were not very significant due to the experimental errors (see error bars which give the value of one standard deviation). The effects of mutating the latter motifs might not be very large because the other motifs compensate for loss of enhancer activity. Consequently, we subdivided the enhancer into

Fig. 5 - Effect of mutating motifs in fragments B or C on their ability to stimulate β globin transcription in myeloma and fibroblast cell-lines. The pG1 based reporter recombinants, containing either wild type or mutated fragments B or C, (1 μ g in myeloma cells, 3.5 μ g in fibroblasts) were transfected with a control recombinant (1 μ g p β CMIX in myeloma cells, 3.5 μ g in fibroblasts) in myeloma (M) or fibroblast (F) cell-lines, and total RNA was analysed by quantitative S1 nuclease mapping. The results are presented as a percentage of the amount transcribed from recombinants containing the WT fragments B or C. The value of one standard deviations is indicated by the error bars. The position of the motifs in fragments B or C is indicated below the graph.

smaller fragments, thereby decreasing the number of motifs which are present in any particular fragment. To compensate for loss of transcription stimulatory activity, the fragments were introduced in the reporter recombinant pG1, directly adjacent to the β globin promoter (see pG1-A, pG1-B, pG1-C, pG1-D, Fig. 1), since enhancers are most efficient when they are close to the TATA box region of the promoter (32,33). The DNA fragments B, C and D stimulated globin transcription in myeloma cells (Fig. 4, bars labelled M). In contrast fragment A was inactive (Fig. 4), despite the presence of the E1 motif which is functional in the context of the ABC enhancer fragment (see Fig. 3). Mutations in the B fragment of the E3 and C2 motifs, as well as the E2, C1 and C3 motifs, decreased its transcription stimulatory activity (Fig. 5). The latter motifs (E2, C1 and C3) had already been shown to be functional in the context of the ABC enhancer fragment (Fig.3). Mutation in the C fragment of the OC motif abolished the stimulatory activity of the fragment, whereas mutation of

the E4 motif had no effect (Fig. 5). These results show that the E3 and C2 motifs can function in myeloma cells, even though their mutation does not lead to a large decrease in the activity of the ABC enhancer fragment. In contrast, mutation of E4 apparently had no effect using either type of reporter recombinant (Figs. 3, 5). However, we cannot exclude that the mutation of the motif could have had an effect in another context. This is strikingly demonstrated by the behaviour of the E1 motif. This motif was functional in the ABC fragment of the pG4 reporter, but not in the A fragment in the pG1 reporter (Fig. 4 and results not shown). The D fragment contains the E1-3 motifs, and activates transcription in myeloma cells (Fig. 4). Mutating the E1 motif did not decrease the activity of this fragment, whereas mutating E2 and E3 decreased its activity (results not shown). This suggests that the E1 motif may only be active when motifs other than E2 and E3 are present (see Discussion).

The E3, E2 and C2 motifs are functional in fibroblasts

We and others have shown that a central fragment of the IgH enhancer can stimulate transcription in non-lymphoid cells, such as mouse fibroblasts (6-8). In agreement with these results we found that fragment B in pG1-B stimulated transcription from the β globin promoter in LMTK⁻ fibroblasts, whereas fragments A, C and D were inactive (Fig. 4). Mutations in the E3, C2 and E2 motifs decreased the transcription stimulatory activity of the B fragments, whilst mutations in C1 and C3 had no effect (Fig. 5). These results show that the E3, E2, C2 IgH enhancer motifs are functional in fibroblasts, whereas the OC, C1, C3 and E1 motifs are only functional in myeloma cells. It is surprising that the D fragment is inactive in fibroblasts despite the presence of two functional motifs.

DISCUSSION

We have shown that mutating the E1-3, C1-3 and OC motifs of the IgH enhancer decreases its ability to stimulate transcription in myeloma cells, whereas mutating the E4 motif has no effect. The results for the E and OC motifs agree with those of several recent reports. Lenardo et al. (34) found that the E1-4 and OC motifs were functional in myeloma cells. Kiledjian et al. (35) found that the E2-4 and OC motifs were functional. Gerster et al. (22) only studied the E4 and OC motifs, and found that both were functional transcription elements. The overall conclusion is that all four E motifs, and the OC motif are active. However in each study the effects of certain mutations could only be detected in some recombinants and not in others. Two

phenomena appear to account for these results. Firstly, the effects of mutations in certain motifs are apparently masked by compensation from other sites (see Results and 34). Secondly, there could be preferred interactions between motifs, so that the effects of mutations in a particular motif are only seen in the context of some other motifs. Such synergism between transcription elements has already been described for the IgH enhancer and promoter (36). It is striking that we only observed an effect of mutating E1 in the context of the whole enhancer fragment ABC, and not in fragments A or D (see results). Kiledjian et al. (35) could not detect any effect due to mutation of E1, in an enhancer fragment (domain) which contained the E1-3 and C1-3 motifs. In the case of Lenardo et al. (34) the effect of mutating E1 was observed in the context of a complete enhancer fragment with mutated E3 and E4 motif. In each of these studies, the effects of mutating E1 are only observed when the DNA fragment contains the OC motif, suggesting that the presence of this motif is required for E1 activity. Experiments with combinations of mutations in the OC and E1 motifs will be required to test this hypothesis. Similar considerations may account for our inability to detect a decrease in enhancer activity due to mutation of E4. The effects of point-mutating the C motifs have not been reported previously, and our results clearly show that all three C motifs are functional transcription elements in myeloma cells.

A central fragment of the IgH enhancer has been shown to have significant activity in fibroblasts (6-8). We found that the E2, E3 and C2 motifs are required for this activity, whereas the other motifs (E1, E4, C1, C3 and OC) appear to be inactive in these cells (see results and data not shown). In agreement with our results, Kiledjian et al. (35) reported an effect of E2 and E3 mutations and deletion of the region containing the three C motifs on enhancer activity in fibroblasts, and Lenardo et al. (34) demonstrated that mutation of E3 had an effect in these cells. Furthermore, we have shown that mutating C1 and C3 only affects the activity of the B fragment in myeloma cells, whilst mutating C2 affects the activity of this fragment in both cell types. These results suggest that the C motifs interact with at least two different factors, one of which is ubiquitous, the other B cell specific. However, we cannot exclude that there is a fibroblast specific motif, that substitutes for the effects of C1 and C3 in these cells. The SV40 enhancer has two C-like motifs, which interact with two different factors, neither of which is present in MPC11BU4 cells (37-39). These results suggest that the factors binding to the SV40 enhancer motifs are different from those which

interact with the IgH enhancer, and that there are at least 4 transcription factors which interact with the C motifs in different enhancers. It remains to be seen if they are related and if they belong to the same gene family.

The effects of the mutations in either B or non-B cells can be correlated with the presence or absence in cell extracts of proteins that bind specifically to these regions. Factors interacting with the E1,E2,E3 and OC motifs have been detected in B cells (13-27). In contrast no factor has been detected which binds to the E4 motif, even though there is evidence for the presence of this factor, from both in-vivo footprinting (9,10) and transfection experiments (22,34,35). Our results suggest that B cells contain factors which interact with the three C motifs. Two studies with in-vitro extracts suggest that these cells contain proteins which bind to the C1 motif (see ref. 18 and IgPE-1, Fig. 1, and ref. 13 and LN1+2, LC1, Fig. 1) whereas proteins binding to the C2 and C3 motifs have not been reported. In addition, the in-vivo footprinting data did not suggest that proteins are present on the C1-C3 motifs (Fig. 1B, 9, 10). Conversely, specific enhancer binding proteins have been described whose activity has not been studied in vivo in transfection assays (eg. see ref. 13 and LN4, LC2 Fig.1 and ref. 18 and IgPE-2, Fig. 1). In addition, factors have been detected which bind to sequences adjacent to the ABC fragment (24) and we found that sequences outside this fragment and in the larger XbaI fragment contribute to enhancer activity, suggesting that these proteins may be transcription factors. Non-B cell extracts contain factors binding to E1, E2, E3 and OC (13, 16, 17, 19-22, 24, 25) but factors binding to C2 have not been reported although this motif is active in vivo. Clearly further work is required to establish why the E1 and OC factors, which are present in these extracts, apparently do not functionally interact with the IgH enhancer in vivo, and to search for a factor which binds to the C2 motif.

The combined activity of multiple factors accounts for the overall activity of enhancers (see reviews 1-3). The emerging mechanism which accounts for the specificity of the IgH enhancer in vivo is that both cell-specific and ubiquitous positively acting transcription factors interact with and account for the activity of the enhancer in B cells. The inactivity of the enhancer in non-B cells results both from the absence of some of positive transcription factors which can functionally interact with the enhancer, and from the presence of negative elements which influence its cell-type specificity (6-7,31,40).

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