Functional analysis of defined mutations in the immunoglobulin heavy-chain enhancer in transgenic mice

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ABSTRACT

We have analyzed the effect of defined mutations in the mouse immunoglobulin heavy-chain enhancer after introduction into the germline of transgenic mice. We have tested a mutation of the enhancer octamer motif, a double mutation of the octamer motif and the μB-site, and a triple mutation in the μE2, μE3 and μE4-sites. All constructs are expressed in the spleen of transgenic mice. Furthermore, expression is exclusively detectable in lymphoid organs and not in several non-lymphoid tissues. Whereas mutations in the μE-sites have a more pronounced effect on transgene activity in thymocytes as compared to bone marrow and spleen cells, the octamer/μB double mutation shows significantly reduced expression levels only in B-cells. Finally, our results demonstrate that the intrinsic heavy-chain enhancer element does not contribute to the increase in steady state levels of heavy-chain mRNA after stimulation of spleen cells with LPS.

INTRODUCTION

The regulated rearrangement and expression of immunoglobulin (Ig) genes in B-lymphocytes represents a paradigm for a cellular differentiation program. The lymphoid-specific expression of the Ig-genes is controlled by several cis-acting elements, promoter elements present upstream of all variable region genes, an intrinsic enhancer element as well as enhancer elements localized 3' of the Ig-loci (reviewed in 1). In addition, less well defined intragenic elements might also contribute to efficient B-cell specific expression of Ig-genes (2). Members of this family of transcription factors are the muscle specific regulators MyoD and Myogenin, drosophila regulatory gene products as well as additional mammalian DNA-binding proteins (10,11). Further ubiquitously active elements in the heavy-chain enhancer, i.e. a binding site for a C/EBP like factor and several copies of the enhancer core motif have also been identified (12,13).

The two best characterized elements conferring B-cell specific activity to the enhancer element are the μB-site and the octamer motif. A 96 kD nuclear protein that binds to the μB element of the human heavy-chain enhancer can activate transcription of this enhancer in a mouse fibroblast cell line (14). The corresponding site in the murine heavy-chain enhancer has been shown to be critical for the B-cell-specific activity of this enhancer element (15,16). The octamer motif is recognized by several transcription factors that are either expressed ubiquitously or in a tissue-restricted fashion (17,18,19). cDNAs encoding the B-cell-specific octamer transcription factor Oct2 have been isolated and their characterization showed that Oct2 belongs to a family of homeodomain proteins termed the POU-domain proteins (20,21,22,23). Members of this family of transcription factors are characterized by a bipartite DNA-binding domain made up of a POU-specific- and a POU-homeo-domain (24,25). In mouse B-cells Oct2 is expressed as a family of alternatively spliced isoforms that all retain the POU-domain but differ in their amino- and carboxy terminal sequences (26). Other members of the POU-domain transcription factor family seem to have critical functions in mammalian pituitary differentiation and early embryogenesis (27,28). Recently a third B-cell-specific enhancer element, the μ-site, has been identified. This site seems to be preferentially active in pre-B cells (1).

Previous experiments had indicated that at least some aspects of the differential Ig-gene activation and expression can be studied using either rearranged immunoglobulin gene constructs or chimeric gene constructs with Ig-regulatory elements in transgenic mice (29,30,31,32). From these analyses it became obvious that the Ig-heavy-chain enhancer could confer lymphoid-specific expression onto a heterologous promoter and reporter gene (31). We have characterized several mutations in the heavy-chain enhancer after introduction into transgenic mice. We show that none of the mutated sites is absolutely essential to achieve transgene activation by the enhancer element. Furthermore, our analyses indicate that a different set of transcription factors interacts with the enhancer element in different lymphoid organs.
MATERIAL AND METHODS

Plasmid constructions

The plasmid containing the minimal promoter element has been described (54x, (33). The 700bp fragments containing wild type or the indicated mutant heavy chain enhancers were isolated as TaqI fragments, the ends were filled in with the Klenow fragment of DNA polymerase and inserted in the correct orientation into the filled in SalI site of plasmid 54x. The various enhancer mutations have been described previously (15,34). For transient transfection assays the AatII-Xhol fragments that exclude the SV40 enhancer present in the parental 54x plasmid were transferred into pUC19 (AatII-Sall).

Generation of transgenic mice

For generation of transgenic mice the AatII to Xhol fragments were gel purified twice and diluted to 1ng/ml. A few picoliters were microinjected into fertilized BDF1 xBDFl (F2) mouse eggs following standard procedures. Transgenic animals were identified by Southern blot analyses of tail DNA form 15 day old mice. On the average, 25% of the mice born carried the transgene and about 70% of them passed the transgene through the germ line.

Cells and tissue culture and transfections

S194 mouse plasmacytoma cells were maintained in Iscove’s modified DMEM supplemented with 5% fetal calf serum, 5% calf serum, 10µM β-mercaptoethanol and antibiotics. Transient transfections by the DEAE-Dextran method were performed as described previously (33). Cytoplasmic RNA for primer extension assays (see below) was prepared 48hrs after transfection. Transfections were controlled by including RSV-lacZ and whole cell free fraction was extracted from 20% of the transfected cells 48hrs after transfection. For LPS stimulations single cell cultures of spleen cells in Iscove’s modified DMEM supplemented with 10% fetal calf serum, 2mM glutamine, 10µM β-mercaptoethanol and antibiotics were incubated with 50µg/ml LPS (Sigma) for the indicated times.

RNA analyses

Total RNA from the various tissues and LPS-stimulated spleen cell cultures by the Guanidinium-CsCl method (35). For primer extension analyses the following primers were used:

β-globin 5' GGGTTGTGAGTCCAGATCGATCTG 3'
GAPDH 5' CCACCTTTGCCAATCGCA 3'

The β-globin primer specifically recognizes the transgenic globin RNA because it overlaps a CcaI linker that was inserted into the HincII site 15 nucleotides downstream of the cap-site (36). The GAPDH primer is homologous to the published mouse GAPDH sequence from +1 to +105 (37). Primers were endlabelled with T4-polynucleotide kinase and 30,000 cpm of each primer were mixed and coprecipitated with 15 µg of RNA. Primer extension reactions were performed as described (26) and analyzed on 8% sequencing gels. For quantitations at least two different autoradiographic exposures were scanned using a Hirschmann densitometer. All transgene expression values were normalized for the respective GAPDH signal.

For Northern analyses 5µg of spleen or LPS induced spleen cell RNA was separated on a 1% formaldehyde agarose gel, transferred to zetaprobe (Biorad) membrane and probed with 32P-labelled (random priming) µ-cDNA or GAPDH fragments.

Southern blot analyses

About 1cm of tail was surgically removed from anesthesized mice, cut into pieces and incubated o/n in 500µl 50mM Tris/HCl pH 7.5, 100mM EDTA, 100mM NaCl, 1% SDS, 0.5mg/ml protease K at 55°C. After 1 hr treatment at 37°C with RNaseA (0.5µg/ml) and a second incubation with proteinase K (1hr, 55°C) DNA was phenol and chloroform extracted, precipitated with isopropanol and washed with 70% ethanol. Liver DNA was prepared accordingly. BamH1/ EcoRl digests were probed with a fragment representing the respective part of the human β-globin gene. For estimations of transgene copy numbers appropriate dilutions of plasmid DNA carrying the transgene were used as reference.

RESULTS

To analyze the effect of the various enhancer mutations we generated transgenic mouse lines that carry the constructs depicted in Figure 1. The enhancer versions were inserted upstream of the β-globin gene in front of a minimal promoter containing a synthetic octamer motif next to the β-globin TATA box (33). The reason for using this promoter/ enhancer construction was the intention to mimic the in vivo situation where the enhancer interacts with the immunoglobulin promoter. At the same time we wanted to exclude the contribution of less well defined promoter elements potentially present in natural immunoglobulin promoters. The different chimeric constructs were initially tested by transient transfection into mouse S194 plasmacytoma cells. As expected, the octamer mutation showed a moderate decrease of enhancer activity to 60–70% of the wild type level. Both the µB/octa double mutation and the µE234 triple mutation further decreased enhancer activity to a residual 30–40% of wild type levels.

Figure 1. Structure of the transgene and of point mutations in the heavy-chain enhancer. On top the 3kb DNA fragment injected into mouse oocytes is outlined. The enhancer element and the minimal promoter are indicated as hatched boxes. Open boxes represent the β-globin exons, the introns and 3' flanking region are shown as line. The short segment (80bp) between the upstream AatII site and the enhancer element is derived from pBR322 vector sequences. A fragment of the enhancer element is shown enlarged below. Some of the characterized transcription factor binding sites are depicted as stippled boxes (binding sites for ubiquitous proteins) and hatched circles (binding sites for B-cell-specific proteins). Wild type and mutated sequences of the relevant sites are shown beneath. Mutations are indicated by lowercase letters.

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Two transgenic lines each were obtained for the wild-type enhancer construct and the octamer mutation in the enhancer. For the μB/octa double mutation as well as the triple mutation of the μE-sites, five transgenic mouse lines were derived and characterized. Transgenic lines carried less than 5 copies of the transgene per haploid genome. Only line E234-9 contained between 10 and 12 copies (data not shown). All transgenic lines transmitted the transgene in a mendelian fashion and analyses were performed on heterozygous F1 animals or further progeny. Transgene expression was assayed by primer extension analysis of total RNA. All reactions were controlled by simultaneous inclusion of a primer that recognizes mRNA for the glyceraldehyde phosphate dehydrogenase (GAPDH) gene.

At first we wanted to determine whether all the enhancer mutants would still allow proper activation of the transgene in the spleen. Transgene-specific RNA was detectable in the spleens of essentially all lines indicating that the various enhancer mutations did not interfere with the activation of the gene (Figure 2). Individual lines bearing the same mutation did not yield identical expression levels, however, but rather showed some heterogeneity in expression levels. These variations were most likely due to influences of the chromatin region flanking the inserted transgene. Two of the mouse lines, μB/octa-17 and E234-8 showed essentially no expression in spleen or in any other organ tested (Figure 2 and data not shown). These two lines were therefore not considered in any of the subsequent experiments, the reasons for this low expression were not pursued further. They could either represent integrations into heterochromatin or alternatively, they might have rearranged the transgene in a subtle way that is not easily detectable by Southern blotting. This latter explanation could apply to the μB/octa-17 line which probably carries only a single copy of the transgene (data not shown).

The transgenic lines carrying the mutant octamer in the enhancer showed no reduction at all in expression levels indicating that this motif is not required for full activity in the presence of the complete enhancer element. In contrast, the μB/octa double mutant and the E234 triple mutant mice showed a clear reduction in expression levels that resembled the results obtained in the transient transfection experiments. On the average, expression in the E234 triple mutants and the μB/octa double mutants was reduced 2—5 fold compared to the wild type levels. The μB/octa-3 mice behaved exceptional as they did not show a reduction when compared to the wild type enhancer bearing mice but rather overexpressed the transgene. Again this result could most likely be accounted for by integration site heterogeneity. The observed variations in expression level indicate that whereas all the information for proper gene activation was retained in the enhancer element, additional regulatory elements important for integration site independence, such as dominant control regions (38), were absent from the constructs.

The octamer element and the μB site interact with B-cell specific transcription factors. To determine whether these mutations altered the tissue-specificity we compared transgene expression in brain, kidney, liver, thymus and spleen. The results for a representative line for each transgenic construct are shown in Figure 3. Transgene expression was restricted to lymphoid tissues and not detectable in any of the other organs. This lymphoid specificity was maintained even with the enhancer construct where both binding sites for B-cell specific transcription factors were mutated (Figure 3D). Even on longer exposures no specific signal could be seen with brain, kidney or liver RNA from the various mouse lines (data not shown). Considering the detection limits of the assay we conclude that transgene expression in brain, liver and kidney was at least 100—200 fold lower than in spleen and thymus. Identical results were obtained for the other mouse lines analyzed (data not shown).

On comparison of the spleen and thymus signals in Figure 3 it became obvious that expression in these two organs differed within the same transgenic line. To test whether this difference was subline-specific or a characteristic of the respective transgenic construct we compared transgene expression in the thymuses of the individual mouse lines (Figure 4A). The octamer mutation alone did again not affect expression levels. Expression levels in the μB/octa double mutant mice were reduced but to a much lesser extent than in the spleen. In contrast, the E234 triple mutation showed a significantly more pronounced effect on transgene expression levels in thymus compared to spleen (compare lanes 6—9 in Figure 2 with lanes 5—8 in Figure 4A). The only exception was the E234-9 line. This transgenic line has the highest transgene copy number and this might compensate for the mutations to some extent. Despite some variations in

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Figure 2: Detection of transgene specific RNA in the spleen. Primer extension analysis on total spleen RNA with a transgene specific (β-globin) and a GAPDH primer. The position of the correct cDNA extension products are indicated at the right side. Endlabelled Mspl digested pBR322 DNA was used as size marker in the left lane (M). The identity of the transgenic mouse lines and respective mutations is given on top. In non-transgenic litters no signal is observed with the β-globin primer (data not shown).
Figure 3: Tissue-specificity of transgene expression. Total RNA from the indicated organs was analyzed by primer extension as in Figure 2. Only the relevant regions of the autoradiographs are shown. The organs are indicated on top of each panel, the upper part shows the transgene signal (β-globin), the lower one the GAPDH control. Transgenic mice carrying either the wild type enhancer (panel A), the enhancer with a mutated octamer motif (panel B), the triple mutation in the E-box motifs (panel C) and the μB/octa double mutation (panel D) are shown. The identical tissue-specificity was observed for the other transgenic lines tested (data not shown).

Figure 4: Analysis of transgene expression in the thymus and bone marrow. A) Primer extension analysis on total thymus RNA. B) Total bone marrow RNA was prepared from femurs and tibias from mice of the indicated transgenic lines and analyzed by primer extension. Only the relevant regions of the autoradiograph are shown.

Expression levels between individual transgenic lines these results demonstrate that the transacting factors interacting with the E234 motifs are more important for thymic activity of the heavy-chain enhancer than μB and octamer transcription factors.

The thymus is the primary site for T-cell maturation. The corresponding site for B-cell maturation in mice is the bone marrow. When we analysed expression in the bone marrow we again failed to detect an effect of the octamer mutation alone (Figure 4B). The E234 triple mutation showed only a slight, at most 2 fold reduction, whereas the transgene expression in mice bearing the μB/octa double mutation was reduced about 3–5 fold. Consistent with the previous findings this result indicated that
We have shown here that a wild type immunoglobulin heavy-chain enhancer in the B lymphoid lineage.

It had been reported that stimulation of splenic B lymphocytes with the B-cell mitogen lipopolysaccharide (LPS) led to a specific increase of \( \mu \) heavy-chain transcription by 8–10 fold in addition to an increase in \( \mu \)-RNA stability (39). We were therefore interested to determine whether the intronic heavy-chain enhancer is involved in this transcriptional stimulation and more specifically whether the presumed role might be influenced by any of the introduced mutations. Total spleen cells from the transgenic lines containing the wild type enhancer construct were stimulated with LPS and RNA was prepared 72 or 96 hours later. As expected, the LPS treatment resulted in a dramatic increase in steady state levels of endogenous \( \mu \)-mRNA, as judged from Northern blot analyses using a cDNA probe specific for \( \mu \)-mRNA (data not shown). When the steady state levels of transgene RNA from unstimulated and LPS-stimulated spleen cells were compared, it was evident that mitogenic stimulation led to a dramatic increase in transgene RNA levels (Figure 5). This stimulation was more obvious for the wild type 16 mouse line. Consistent with previous findings (39), LPS stimulation also results in a roughly 10 fold overall stimulation of transcription, evident in the GAPDH control panel. When activation of transgene transcription was corrected for this general transcriptional induction no specific stimulation of the transgene remained, however. This result suggests that the heavy-chain enhancer did not contribute to the LPS induced transcriptional stimulation of \( \mu \)-mRNA. When LPS induction was analyzed in mice bearing the various mutations no significant differences became obvious (data not shown).

### DISCUSSION

We have shown here that a wild type immunoglobulin heavy-chain enhancer as well as several mutant enhancer versions are all capable of activating a heterologous gene in a tissue-specific fashion. We demonstrate that mutations in binding sites for B-cell-specific and ubiquitous transcription factors behave differently in the different lymphoid tissues analyzed. The \( \mu B / \text{octamer} \) double mutation leads to a significant reduction of enhancer activity in bone marrow and spleen, whereas the E234 triple mutant reduces activity in spleen and even stronger in the thymus. Furthermore, our data show that the heavy-chain enhancer and the octamer motif in the promoter position do not contribute to the specific stimulation of \( \mu \)-gene transcription after LPS stimulation of spleen cells.

For all the constructs tested, transgene expression was exclusively detectable in lymphoid tissues. This result is consistent with previous transfection experiments that had shown that chimeric constructs driven by the heavy-chain enhancer are transcriptionally active in B-cell lines but not in fibroblasts and a number of other cell lines tested (2,34,40,41,42). Furthermore the minimal promoter element consisting of only an octamer motif upstream of the TATA box had also been shown to function preferentially in cell lines of B-cell origin (33). In a previous report a \( \beta \)-globin transgene driven by the heavy-chain enhancer upstream of the conalbumin promoter was only active in B-cells and not in thymocytes or splenic T-cells (31). The reasons for this difference in the expression pattern are not known. Gerlinger and colleagues used a different promoter region and a larger enhancer fragment for their transgenic mice (a 1kb XbaI fragment rather than the 700bp XbaI to EcoRI fragment). A potential explanation for the observed differences could be the presence of negatively acting elements identified in the region that was missing in our enhancer fragment (43). However, transgenic mice containing a complete rearranged immunoglobulin heavy-chain transgene showed significant expression in the thymus similar to the expression pattern observed with our transgenic constructs (29,32,44).

Interestingly, the tissue-specificity was retained even when two binding sites for B-cell-specific transcription factors were mutated. Several explanations could account for this observation. There could be additional binding sites for B-cell-specific transcription factors present in the heavy-chain enhancer. In fact, a motif localized next to the \( \mu E3 \) site (the \( \pi \)-site) has been shown to interact with a nuclear protein from pre-B-cells specifically (1). Alternatively, the presence of negative regulators could inhibit the activation of the enhancer in all the non-lymphoid cells. At least two regions have been characterized in the 700bp fragment of the heavy-chain enhancer that reduce activity of the enhancer in non-B-cells (45,46,47). However, in a recent analysis of transgenic mice carrying a rearranged immunoglobulin heavy-chain transgene under the control of various mutant enhancer elements, reproducible low transgene expression was detected in brain and kidney RNA. This low expression was dependent on the presence of the heavy-chain enhancer element but independent of the octamer motif in the promoter.

In transient transfection experiments the contribution of the octamer motif to full enhancer activity was only evident when short fragments of the enhancer were analyzed (15,34). It was therefore not completely unexpected that the octamer mutation had no effect in the transgenic mice as we used the 700bp enhancer fragment. There were reasons to invoke the enhancer octamer in a more general role for locus activation. The octamer element in the enhancer had been shown to be important for initiation of \( \mu E \) transcripts (48). The occurrence of these transcripts is one of the earliest molecular events characterizing heavy-chain locus activation. The transcriptional activation had been implicated in the establishment of permissive chromatin (49).
We do not think that the nearby promoter octamer motif could compensate for the mutation in the enhancer because; i) it would require that the activating complex of which the protein interacting with the octamer motif (most likely Oct2) is a component displays a significant amount of flexibility, because the octamer element is displaced by about 200bp as compared to its normal position; ii) Jenuwein and Grosschedl who have investigated the effect of the enhancer octamer mutation in the context of a rearranged immunoglobulin heavy-chain gene in transgenic mice also failed to detect a phenotype for the octamer mutation (44). Furthermore, when they combined the enhancer octamer mutant with an octamer mutation in the promoter, the enhancer mutation did not lead to a further decrease of the residual activity of the promoter mutant.

The µB/octa double mutation reduced activity to an average of 20—30% of the wild type enhancer activity in bone marrow and spleen in agreement with results from transfection experiments were this mutant showed 15—25% of wild type activity (15). The µB/octa double mutation had only a marginal effect on transgene expression in the thymus, however. The µB and Oct2 proteins have been identified in a number of T-cell lymphoma cell lines and Oct2 specific RNA had been detected in primary thymocytes (16,18,26). It is unclear whether low amounts of the respective proteins in primary thymocytes are the reason for the reduced effect of the double mutation in these cells. From the intensities of the obtained signals we estimate that overall expression levels for the transgenes containing the wild type enhancer or the octamer mutation are comparable in spleen and thymus. The observation that the µB/octa double mutation is more significantly affected in spleen cells could indicate that thymocytes utilize a distinct set of transcription factors from spleen cells to obtain the high level of enhancer activity. This interpretation finds support in the behaviour of the E234 triple mutations. Transgenic mice bearing this mutation are barely affected in transgene expression in bone marrow and they show an intermediate effect in spleen. In the thymus the E box motifs seem to contribute strongly to full enhancer activity, however.

The E box motifs interact with ubiquitously expressed transcription factors like the E12/E47 E2A gene products or TFE-3 proteins (6,7). These transcription factors are members of a family of proteins characterized by a helix-loop-helix dimerization motif and it had been demonstrated that different members of this family have the capability to form heterodimers (50) and that these heterodimers can synergistically activate transcription (51). It is conceivable that the ubiquitous E-box-binding proteins interact with distinct cell-type-specific transcription factors in bone marrow, spleen cells and thymocytes and thus contribute differentially to overall enhancer activity.

Splenic B-cells can be induced to proliferate and differentiate into antibody-secreting plasma cells by the polyclonal mitogen LPS. In the course of this differentiation the amount of µ-RNA increases 800 fold (52) while overall polyA+ RNA content increases 10 fold. From nuclear run-on determinations it was estimated that the µ-RNA synthesis rate was specifically induced 10 fold under these circumstances (39). As the intronic heavy-chain enhancer is a major determinant of µ-gene transcriptional regulation, we expected it to be responsible for this increased transcriptional activity. Although we did observe an increase in transgene expression after LPS stimulation, the correction for general transcriptional induction via the GAPDH control did not leave any specific transgene induction. We therefore conclude that neither the intronic enhancer element, nor the octamer motif in the promoter position are targets of the LPS induced transcriptional stimulation in the context of our construct. Steady state levels of RNA for a human γ1 heavy-chain transgene could be induced 50 fold by treatment of spleen cells with LPS for 72 hours (53). From those experiments it was unclear whether the increase was due to transcriptional induction or changes in RNA turnover.

Our finding is surprising in light of the fact that LPS stimulation of spleen cells leads to an increase in the amount of the Oct2 transcription factors (54). It is, however, conceivable that the existing amounts of Oct2 protein were sufficient to maximally stimulate the promoter and enhancer elements. The increased amounts of Oct2 might alternatively be important for the mitogenic stimulation as it has been shown that Oct2 like Oct1 can efficiently stimulate adenovirus replication in an in vitro system (55). Our results resemble those of transfection experiments into B-cell lines representing different stages of B-cell development (2,56). These studies had shown that the heavy-chain enhancer functions equally well in a variety of B-cell lines, regardless of whether they represented the pre B-cell stage, mature B-cells or plasma cells. Recently, several binding sites for a LPS inducible factor (LR1) have been described in the heavy-chain locus (57). Most of the binding sites occurred in the immunoglobulin switch regions, a weak binding site was localized in the enhancer fragment, however. It is at present unclear whether LR1 is involved in the transcriptional stimulation of the heavy chain gene. The single weak binding site for LR1 in the enhancer region is obviously not sufficient to mediate the stimulatory effect.

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