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The chicken immunoglobulin λ light chain gene is transcriptionally controlled by a modularly organized enhancer and an octamer-dependent silencer

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ABSTRACT

Characterization of the regulatory elements involved in V(D)J recombination is crucial for understanding development of the B and T cell immune repertoire. Previously we have shown that the chicken immunoglobulin λ light chain gene (CLLCG) undergoes lymphoid-specific rearrangement in transgenic mice. The whole gene is only 10 kb in length and contains all phylogenetically conserved target sites for recombinational and transcriptional regulation. In this study we have localized an enhancer element in a region 4 kb downstream of the constant (C) region. The 467 bp element can be subdivided into three subfragments. The previously detected silencer element on the V–J intervening sequence is shown to be localized on a 500 bp fragment. Partial silencer activity is retained on a 250 bp fragment, which includes an octamer motif. By mutational analysis this octamer is shown to be essential for B cell- but not for T cell-specific silencer function. The silencer represses transcription directed by heterologous elements like the SV 40 promoter or the Ig κ 3′ enhancer. We propose that transcription of the unrearranged and rearranged Ig genes is regulated by complex interactions between different modules from the promoter, enhancer and silencer, which is eliminated by recombination during B cell development.

INTRODUCTION

The generation of immunglobulin (Ig) and T cell receptor (TCR) diversity in mammals is mediated mainly by somatic DNA rearrangements; a process known as V(D)J recombination, so called for the variable (V), diversity (D) and joining (J) gene segments used (1). The development of an Ig-producing B cell is characterized by three successive gene rearrangements: the first step is linkage of a D and a J segment of the heavy chain, which is followed by recombination to a V gene segment; expression of a functionally rearranged heavy chain then allows V to J rearrangement of either the λ or κ light chain genes. Ig gene rearrangement is a site-specific recombination process that is mediated by an enzyme complex, called V(D)J recombinase, which includes the products of the RAG-1 and RAG-2 genes (2–4).

The chicken Ig λ light chain gene (CLLCG) is particularly well-suited to serve as a recombination substrate for V(D)J recombination studies (5–7). The genomic locus contains only one functional V gene, one J and one C gene; a configuration where V(D)J recombination can only play a minor role in diversification. This function is taken over by another recombination process called gene conversion, which substitutes parts of the rearranged V gene with homologous DNA segments taken from a pool of 25 pseudogenes. These pseudogenes are located in a 19 kb cluster upstream of the functional V gene and do not provide intact heptamer–nonamer signals for the V(D)J recombinase (7). The small size and the unique germline organization of the CLLCG have facilitated studies of the molecular mechanism and timing of V–J joining, not only in the chicken (8), but also as a model system for mammalian B cell development (5).

Transgenic mice carrying the CLLCG locus in the germline configuration have been obtained (5,9). In B cells the transgene is rearranged and expressed, while in T cells there is a lower rate of rearrangement, but no expression. Previously we have shown that the promoter region and two additional gene segments are important for rearrangement: (i) a 1.7 kb fragment 3′ of the C region positively regulates recombination and contains a transcriptional enhancer; (ii) the region between the V and J segments negatively regulates recombination and carries a transcriptional silencer (5).

Identification of these regulatory elements is critical not only for understanding the transcriptional regulation of rearranged genes, but also in order to probe the 'accessibility' hypothesis (10). This hypothesis postulates that Ig germline transcription, resulting from or causing accessibility to a locus, establishes the tissue- and stage-specific targets for the recombinase complex (1,11).

MATERIALS AND METHODS

DNA amplifications

All fragments that were used for plasmid constructions were amplified using the polymerase chain reaction (PCR). The annealing conditions were slightly modified in each reaction according to the melting points of the primers used. The standard reaction was as follows: 94°C, 1 min 20 s; 60°C, 1 min 20 s; 72°C, 2 min; 34 cycles. The *Taq* polymerase was purchased from Perkin Elmer Cetus.

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Table 1. Plasmid constructions used for transient transfection

Plasmid	Vector	Cloning site	Oligonucleotides	Orientation	Digest
pC 41	pCAT	*BglI/Hin*dIII	CLLCG promoter		
pC 42	pC 41	*SalI*	5 kb enhancer		
pC 49	pc 41	*Xba*I	1 + 2	s/as	Ba/Sm
pC 50	pC 41	*Xba*I	1 + 3	s/as	Ba/Sm
pC 52	pC 41	*Xba*I	1 + 4	s/as	Ba/Sm
pC 61	pC 41	*Xba*I	1 + 6	s/as	Ba/Sm
pC 64	pC 41	*Xba*I	1 + 5	s/as	Ba/Sm
pC 97	pC 41	*Sph*I	7 + 8	as	Hi/Pv
pC 102	pC 97	*Xba*I	23 + 24	as	Ba/Ns
pC 114	pC 97	*Xba*I	23 + 24	as	Ba/Ns/Sp
pC 170	pC 41	*Sph*I	7 + 11	s/as	Ba/Bg
pC 201	pGl-2	*BglI/Hin*dIII	CLLCG promoter		
pC 203	pC 201	*Sph*I	7 + 11	s/as	Ba/Bg
pC 205	pC 201	*Sph*I	7 + 9	s/as	Hi/Pv
pC 207	pC 201	*Sph*I	12 + 8	s/as	Ba/Bs
pC 209	pC 201	*Sph*I	10 + 11	s/as	Ba/Bg
pC 211	pC 201	*Sph*I	7 + 8	s/as	Hi/Pv
pC 213	pC 201	*Sph*I	12 + 11	s/as	Ba/Bg
pC 215	pC 201	*Sph*I	27 + 28	s	Ba/EI
pC 216	pC 201	*Xba*I	23 + 24	s	Ba/Ns
pC 217	pC 201	*Xba*I	23 + 24	s	Ba/Ns/Sp
pC 218	pC 215	*Xba*I	23 + 24	s	Ba/Ns
pC 219	pC 203	*Xba*I	16 + 25	s	Hi/Sp
pC 220	pC 203	*Xba*I	16 + 17	s/as	Hi/Sp
pC 222	pC 203	*Xba*I	16 + 18	s/as	Hi/Sp
pC 224	pC 203	*Xba*I	16 + 19	s/as	Hi/Sp
pC 226	pC 203	*Xba*I	16 + 20	s/as	Hi/Sp
pC 228	pC 203	*Xba*I	21 + 18	s/as	BaI/Ns
pC 230	pC 203	*Xba*I	21 + 19	s/as	Ba/Ns
pC 232	pC 203	*Xba*I	21 + 26	s/as	Ba/Ns
pC 234	pC 203	*Xba*I	21 + 22	s/as	Ba/Ns
pC 236	pC 203	*Xba*I	23 + 24	s/as	Ba/Ns
pC 238	pC 203	*Xba*I	23 + 24	s/as	Ba/Ns/Sp
pC 244	pC 201	*BglI/Hin*dIII	SV 40 promoter		
pC 245	pC 244	*Sph*I	27 + 28	s	Ba/EI
pC 246	pC 245	*Xba*I	23 + 24	s/as	Ba/Ns
pC 247	pC 205	*Xba*I	23 + 24	s	Ba/Ns
pC 248	pC 205	*Xba*I	23 + 24	s	Ba/Ns/Sp
pC 249	pC 207	*Xba*I	23 + 24	s	Ba/ Ns
pC 250	pC 207	*Xba*I	23 + 24	s	Ba/Ns/Sp
pC 251	pC 209	*Xba*I	23 + 24	s	Ba/Ns
pC 252	pC 209	*Xba*I	23 + 24	s	Ba/Ns/Sp
pC 260	pC 201	*Xba*I	29 + 18	s	Ba/EV
pC 261	pC 215	*Xba*I	29 + 18	s	Ba/EV
pC 262	pC 245	*Xba*I	29 + 18	s	Ba/EV
pC 263	pC 203	*Xba*I	29 + 18	s	Ba/EV

The enzymes listed in the last column were used to analyse the insert orientations.
Abbreviations: Ba, *Bam*HI; Bg, *BglI*; Bs, *Bss*HII; EI, *Eco*RI; EV, *Eco*RV; Hi, *Hin*dIII; Ns, *NsiI*; Pv, *Pvu*II; Sm, *Sma*I; Sp, *Sph*I.

Plasmid constructions

All CAT expression plasmids described in the paper were derived from PCR fragments cloned into the plasmids pC 41 and pC 42 (5). Luciferase expression plasmids were derived from corresponding CAT expression constructs by substitution of the CAT gene by the luciferase gene from the vector pGl 2 (Promega). A *BglI–Bam*HI fragment, including the CLLCG promoter, was used for subcloning. For better illustration the primers used for PCR are given with continuous numbers. The plasmid constructions are summarized in Table 1. Each plasmid carries a single copy number insert which was determined by *Hin*dIII/*Bam*HI digestion in all cases. Ligation, transformation of *Escherichia coli* Sure cells (Stratagene) and plasmid mini preparations were performed according to standard techniques (12). Restriction endonucleases and T4 ligase were purchased from Boehringer (Mannheim, Germany) and TIB Mol Biol (Berlin, Germany). For transfection experiments plasmids were prepared and purified with Quiagen 100 Midicolumn Kits (Quiagen, Hilden, Germany).

For fragment preparations from agarose gels the Jet-Sorb Kit from Genomed (Bad Oenhausen, Germany) was used.

1 GATCTCTAGAGCTTTGCTAATG
2 GATCTCTAGAACCCCAGACCAG
3 GATCTCTAGAAAGCTCAGGGTC
4 GATCTCTAGAGCGTGGTGGGAG
5 GATCTCTAGACACCCCGAGCAGCAG
6 GATCTCTAGAGACGCGCTGTGCCTTC
7 GATCGCATGCGAAGGCACAGCGCTG
8 GATCGCATGCACCCCGAGCAGCAGC
9 GATCGCATGCTGGAGACTGTTTCG
10 GATCGCATGCCCACGCGCTGGGCAC
11 GATCGCATGCGTGGTGGGAGCGGGC
12 GATCGCATGCCCACGCGCGCCAA
13 GATCGCATGCGCGGCGCGTCTGC
14 GACTCGCATGCTGTGGTTTGTGCA
15 GACTCGCATGCGCATATTTGTGAG
16 GAAACCTCCTTCTAGAGCAAGGAGCAGCTG
17 GCTCTAGATATACCAAGATTTCGAAGAACCAGAAG
18 GCTCTAGATATACCACTGAGCCCTGCTGCCTGCTG
19 GCTCTAGATATACCACAGAGCCAAGGCACTGGCTT
20 GCTCTAGATATACCAGCTCTTTTCCTCAGAGG
21 TGGGATCTAGAAGCACCCTTAGTGTACTCAC
22 TGCAAGTCTAGATAGAAAGAGGCAGAG
23 GATCTCTAGAGCACCTAAGCCTTCC
24 GATCTCTAGACATCCTCCTCAAGAG
25 GGGTTGTCCCGGCTCTAGATATACCA
26 GATCTCTAGAATATGCAAATGCTGTGG
27 GACTGCATGCATGGTGACTGGCCTG
28 GACTGCATGCTGGATACAGCCTTG
29 GATCTCTAGAGCCAGTGCCTTGGCTC

Site-directed mutagenesis

Mutagenesis of the octamer motif in plasmid pC 238 was performed with overlapping PCR amplifications according to Ho *et al.* (13). Primers 14 and 15 were used to introduce the desired mutation, as well as a diagnostic *Sph*I site.

DNA sequencing

All fragments to be sequenced were cloned into pUC 18/19 vectors and primary sequences were determined using a double-strand sequencing kit from Pharmacia (Freiburg, Germany). Alternatively, digoxigenin-labelled primers and the DIG detection system (Boehringer, Mannheim, Germany) were used.

DNA transfection and CAT assay

Transfections were achieved by use of DEAE–dextran, as previously described (14), except that 5 μg plasmid DNA was used to transfect 3×10^6 cells and the incubations were performed for 1 h at room temperature. The CAT assay was also performed according to Martensson and Leandersson (14). For densitometric measurements the autoradiographs were photographed and scanned with a CS-1 Image Documentation System (Cybertech, Berlin, Germany).

DNA transfection and luciferase assay

All luciferase expression constructs were transfected by electroporation at 250 V/900 Ω with a BioRad Gene Pulser. Crude extract preparation and luciferase assay were performed 24 h later according to the manual of the supplier (Promega). Enzyme activity was measured with an Autolumat LB 953 (Berthold). The luciferase expression (5 μg) plasmid was co-transfected with 0.5 μg pCMV β-GAL control plasmid (Promega). β-Galactosidase activity was measured by ELISA according to the suppliers protocol. The values for luciferase activity were then normalized, according to the transfection efficiency determined by ELISA. Transfection variation did not exeed 30% when only Quiagen-purified plasmids were used.

Cell lines and culture conditions are shown in Table 2.

Nuclear extracts and mobility shift assays

Each assay (20 μl) contained 8 μl nuclear extract protein, prepared by the method of Dignam *et al.* (22), and 20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 2 μg poly(dI–dC), as well as 1 ng labelled DNA fragment. The DNA fragments were labelled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase I. Binding reactions were performed at room temperature for 15 min and the mixtures were loaded onto a 4% polyacrylamide gel (acrylamide:bisacrylamide weight ratio 29:1) containing 25 mM Tris, pH 8.0, 25 mM boric acid and 0.5 mM EDTA. The gel was run at 15 V/cm, dried and autoradiographed at –80°C with intensifying screens.

Table 2. Cell lines and cell culture

Cell line	Characteristics	Culture conditions	Source	Reference
230-238	Mouse pre-B cells	RPMI 1640 + 50 mm β-ME	BII	15
3T3	Mouse fibroblast	DMEM	ATCC	16
LG2	Human B cells	DMEM	DRFZ	17
MOLT 4	Human T cells	DMEM	ATCC	18
CH 27	Mouse B cells	RPMI 1640	DRFZ	19
HeLa	Human cervical carcinoma	DMEM	ATCC	20
FF 8	Chicken B cells	IMDM	INP	21

The cell lines used, their characteristics and source, as well as the respective culture conditions, are indicated. All media contained 10% fetal bovine serum, 10 U/ml penicillin, 10 mg/ml streptomycin. Abbreviations: BII, Basel Institue for Immunology; DRFZ, Deutsches RheumaForschungsZentrum; ATCC, American Type Culture Collection; INP, Institut Necker Paris.

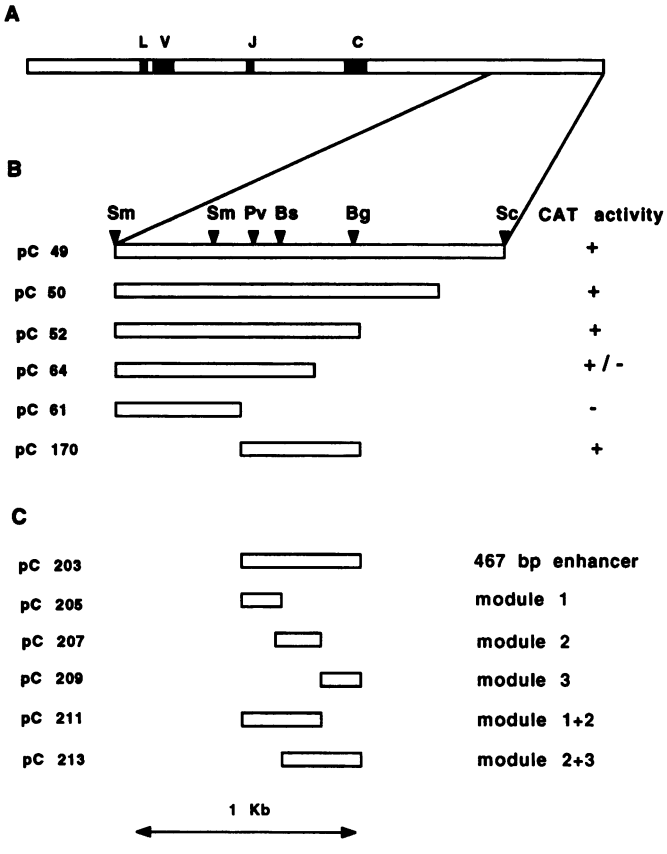

Figure 1. Location of the chicken immunoglobulin λ 3′ enhancer. (**A**) Illustration of the complete chicken immunoglobulin λ light chain gene. (**B**) Plasmid pC 49 carries a 1.7 kb fragment which is localized 4 kb downstream of the C region and which has been shown to include the 3′ transcriptional enhancer (5). All subfragments shown in this part of the figure were amplified by PCR and cloned into CAT expression plasmid pC 41 (see Materials and Methods). Background activity coming from the plasmid promoter is indicated as –; enhancer activity by +. As measured by densitometry, full enhancer function could be localized on a 476 bp fragment (pC 170). The indicated restriction sites were used for orientation determination. Abbreviations: Bg, *BglI*; Bs, *Bss*HII; Pv, *Pvu*II; Sc, *Sac*I; Sm, *Sma*I. (**C**) Dissection of the 476 enhancer fragment. The indicated subfragments were amplified by PCR and cloned into the luciferase expression vector pC 201 (see Materials and Methods). The luciferase activities obtained 24 h after transfection into various cell lines are given in Table 3.

Nucleotide sequence accession number

The 3′ region of the chicken Ig λ light chain gene has been determined with a 25 bp overlap with accession no. M24403 and submitted to the GenBank/EMBL database under accession no. L26587. This fragment (3.2 kb) includes the enhancer sequence shown in Figure 1.

RESULTS

A 467 bp fragment contains the 3′ transcriptional enhancer

In transgenic mice and in transient transfection studies the 3′ transcriptional enhancer element was localized on a 1.7 kb *Sma*I–*Sac*I fragment 4 kb downstream of the C region (5; Fig. 1A). Due to the lack of appropriate restriction sites for subcloning, the whole piece of DNA was sequenced and subfragments were amplified by PCR. The plasmid constructs which were derived after ligation of the fragments into the *Sph*I site of a CAT expression vector (pC 41; see Materials and Methods) are shown in Figure 1B. Each fragment was obtained in both orientations relative to the CLLCG promoter. Only plasmids with single copy insertions were selected and CAT activity was measured 48 h after transfection into the mouse pre-B cell line 230-238. Deletion of 0.7 kb at the 3′-terminus of the *Sma*I–*Sac*I fragment did not affect enhancer function (pC 52 and pC 53). Further deletion of 200 bp, however, produced constructs where only partial transcriptional enhancement could be observed (pC 63/pC 64). No enhancement could be detected on a fragment which was limited to a size of 600 bp (pC 61/pC 62). This set of experiments suggests that the complete orientation-independent enhancer is localized in the overlapping 467 bp (pC 169/pC 170).

The enhancer is lymphoid-specific and can be subdivided into three modules

In order to analyse the molecular organization of the enhancer described above, the 467 bp fragment was further subdivided. Transient transfections were performed with a plasmid vector, where the CAT gene from pC 41 has been substituted by the firefly luciferase gene (pC 203). Among the plasmids that were constructed in this set of experiments, only those defining different modules are shown in Figure 1C. The rate of transcriptional enhancement of the various concstructs in different cell lines is shown in Table 3.

Table 3. Rates of transcriptional enhancement in different cell lines

Construct	FF 8	CH 27	Molt 4	HeLa
201	1	1	1	1
203	5.2	6.6	2.2	1.1
205	2.1	5.6	1.5	1.1
207	1.1	1.6	1.0	1.0
209	1.3	1.9	2.3	1.1
211	3.1	7.3	1.8	1.0
213	2.3	7.6	5.2	1.2
215	2.0	8.8	4.4	1.4

Luciferase activity of plasmid pC 201 (CLLCG promoter) was measured 24 h after transfection of the chicken B cell line FF 8, the mouse B cell line CH 27, the human T cell line Molt 4 and the human carcinoma cell line HeLa. The table gives the factor of enhancement which was observed with the complete enhancer (pC 203) and the different subfragments, which are indicated in Figure 1. Plasmid pC 215 carries the mouse κ 3′ enhancer as a positive control. The numbers represent mean values of two independent experiments, normalized in relation to a co-transfected β-GAL expression plasmid.

Enhancer-module 1 (pC 204/pC 205) spans 157 bp and has the properties of an orientation-independent enhancer. In the mouse B cell line CH 27 and in the chicken B cell line FF8 this element enhances transcription comparable with the complete 467 bp enhancer. Module 2 (pC 206/pC 207) has a 40 bp overlap with module 1 and a total size of 154 bp. It is followed by 190 bp, representing module 3 (pC 208/pC 209). Both elements by themselves enhance the rate of transcription only slightly.

Enhancer function is clearly increased, however, when they are analysed in combination (pC 210/211 and pC 212/213). In plasmid pC 215 the chicken Ig λ 3′ enhancer (pC 203) is substituted by the well-characterized mouse Ig κ 3′ enhancer. The activity of this element is slightly higher in the mouse cell line and slightly weaker in the chicken cell line. The fact that transcription in this vector system cannot be further increased even by the strong κ enhancer is due to the high activity of the CLLCG promoter (pC 201). The luciferase activity of that construct is already 40-fold higher in 3T3 mouse fibroblasts and 100-fold higher in FF 8 chicken B cells compared with the negative controls. Enhancer activity is also clearly detectable in the T cell line Molt 4, but absent in HeLa cells.

Definition of a V–J silencer core element

Transient transfection assays were carried out to localize the silencer element described in the V–J intervening sequence (5; Fig. 2A). Subfragments of the 1.8 kb region were amplified by PCR and cloned into the *Xba*I site of luciferase expression vector pC 203 (Fig. 2B). This vector carries the CLLCG promoter and the complete enhancer element described above. The cloning position of the silencer fragments between promoter and enhancer was selected to reflect the physiological germline configuration. All plasmids were transfected into the mouse B cell line C H 27 and the chicken B cell line FF 8. The luciferase assays were performed 24 h later. As shown in Figure 2B, full silencer activity was found with the constructs pC 219, pC 220 and pC 222, irrespective of fragment orientation. Further reductions of fragment sizes, however, lead to a stepwise loss of silencer activity. The smallest fragment (550 bp) with unimpaired function was identified on plasmid pC 228 (corresponding to positions 1455–2005 of GenBank/EMBL accession no. M24403). A 4-fold reduced, but still significant, silencing activity can be observed with a 254 bp fragment from the central part of the 550 bp silencer (pC 236). It therefore defines a core region of the silencer which, for full activity, needs flanking sequences on both sides. The 5′ flank is represented by plasmid pC 234 and does not show any silencing activity, whereas the 3′ flank (pC 263) reduces transcription comparable with the core region. Outside the V–J intervening sequence no further silencer element was detected (not shown).

Mutagenesis proves that silencer activity depends on the octamer element

The V–J silencer sequence contains an octamer motif ATTTG-CAT which is characteristic for Ig gene promoters (23) and which is also found in some enhancer elements (24–26). To assess the functional importance of this octamer motif for silencer activity, a 254 bp fragment with the octamer in the centre was subjected to mutagenesis and the effects of the mutation on silencing activity were examined by transient transfection assays in various cell lines. The mutation consists of 2 bp substitutions (ATTTG-CAT → ATGCGCAT) with the introduction of a diagnostic *Sph*I restriction site. The constructs which contained wild-type (pC 236) or mutated (pC 238) octamer motifs were sequenced to confirm successful mutagenesis. Both constructs are driven by the CLLCG promoter and carry the 467 bp enhancer fragment (pC 203). In order to analyse whether the different modules of the enhancer (see Fig. 1) are differentially affected by the wild-type

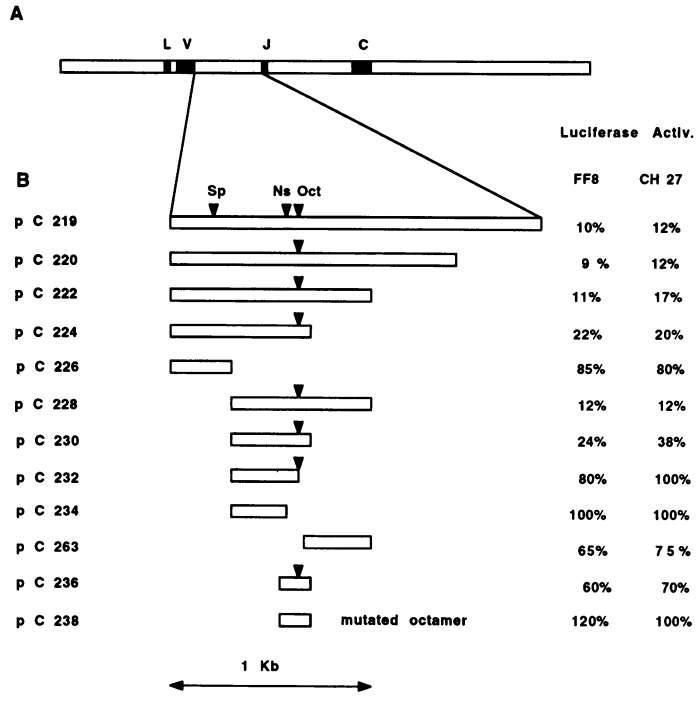

Figure 2. Location of the chicken immunoglobulin λ V–J silencer. (**A**) Illustration of the complete chicken λ light chain gene. (**B**) Fragments from the V–J intervening sequence were PCR-amplified and tested for transcriptional silencer activity by cloning them into luciferase expression plasmid pC 203 (see Materials and Methods). Enzymatic activity was measured 24 h after transfection into chicken B cell line FF 8 and mouse B cell line CH 27. The mean values of two independent transfections were normalized in correlation with the co-transfected β-GAL expression plasmid. The luciferase expression of cloning vector pC 203 was standardized as 100%. Abbreviations: Ns, *Nsi*I; Sp, *Sph*I; Oct, octamer.

and the mutated silencer fragments, various combinations were constructed.

In the chicken B cell line FF 8 the silencer core element reduces transcription irrespective of the enhancer context. As shown in Figure 3, the same rate of reduction is also observed when transcription is driven by the promoter alone (pC 201/pC 216). In the mouse B cell line the same effects can be observed, but they are clearly less pronounced. Mutation of the octamer abolishes the silencer function completely in both cell lines. In combination with enhancer module 3 (pC 250/251) the mutated fragment reproducibly increased the activity of the vector and therefore converted into an enhancer.

In order to analyse whether the loss of silencer function correlates with a loss of protein binding capacity, a mobility shift assay was performed. Figure 4 shows that a specific protein–DNA complex is detectable with the wild-type fragment, but not with the mutated octamer. Analogous mutations have been shown to inhibit the binding of Oct-1 and Oct-2 to the murine heavy chain enhancer octamer (27), suggesting that the inability of these two proteins to bind to the mutated silencer octamer is responsible for the loss of function. Mobility of the complex (C. Scheidereit, personal communication), as well as the observation that Oct-2 is poorly expressed in pre-B cells (28), strongly suggests that Oct-1 is responsible for the detected band shift.

We further addressed the question of whether the octamer by itself in the specific promoter–enhancer context could be sufficient for silencer function. One other octamer motif is located

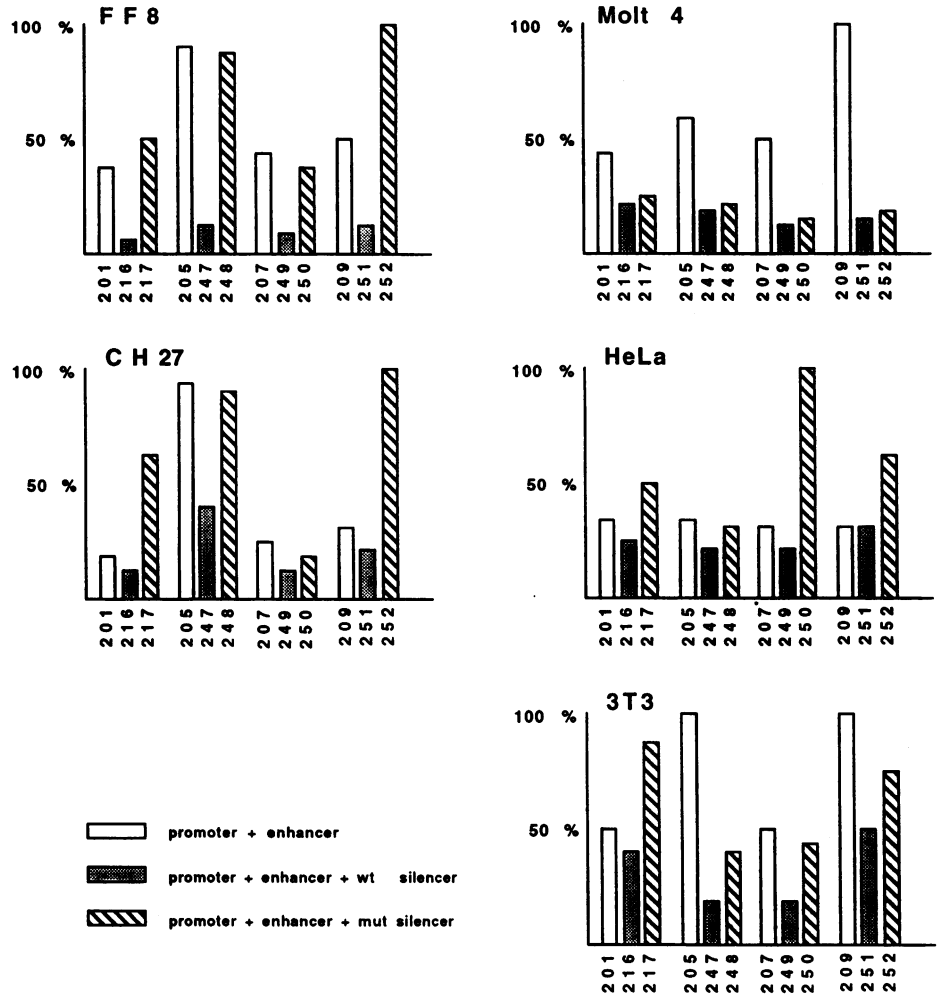

Figure 3. Interactions between the wild-type and mutated silencer core elements and various enhancer elements in lymphoid and non-lymphoid cell lines. A 254 bp silencer core element (wt) was cloned into a luciferase expression vector driven by the CLLCG promoter (pC 201) and into plasmids which in addition carry enhancer module 1 (pC 205), module 2 (pC 207) or module 3 (pC 209). In the plasmids indicated by shaded bars the octamer motif was mutated. All constructs were transfected into the five different cell lines and luciferase activity was measured 24 h later. The figure shows the activities relative to the highest value of each transfection series (100%). The mean value of two independent experiments was normalized in correlation with the co-transfected β-GAL expression plasmid.

300 bp downstream of the enhancer element. A 250 bp fragment with that octamer in its centre was amplified and cloned to replace the silencer core element. Upon transfection into different cell lines, however, the construct did not show any silencer activity (not shown).

The V–J silencer core element has octamer-independent function in T cells

Considering the role of the chicken V–J silencer in recombinational and transcriptional control of transgenic mice (5), we reasoned that its activity would be lymphoid-specific. To test this hypothesis, we assayed the transcriptional activity in two non-lymphoid cell lines (epithelial, HeLa cells; mesenchymal, 3T3 cells), as well as in the human T cell line Molt 4 (Fig. 3). In contrast to the two B cell lines, where silencer activity is completely abolished by the octamer mutation, wild-type and mutant fragments are equally active in the T cell line Molt 4. This result holds true for all silencer–enhancer combinations tested. In HeLa cells a very weak reduction in transcriptional activity with the silencer core element could be observed. Introduction of the mutated fragment, however, increases transcription in each enhancer combination. This phenomenon is also visible in the fibroblast cell line, where the silencer fragment is fully active in connection with all three enhancer modules from the chicken gene.

The silencer acts on heterologous promoters and enhancers

As outlined in Figure 3, the wild-type and mutated silencer elements act quite differently, depending on the silencer/enhancer fragment combination and the cell line used for transient transfections. In order to specify these interactions, the silencer core element and its 3′ flanking element were cloned into plasmids which instead of the CLLCG enhancer carry the mouse Ig κ 3′ enhancer. In the case of plasmid pC 245, the heterologous

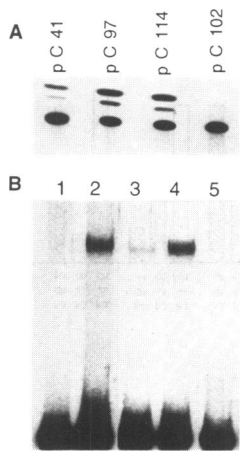

Figure 4. Functional analysis of the mutated V–J silencer octamer motif. (**A**) Plasmid pC 41 carries the chicken light chain promoter pC 97 in addition to the 3′ transcriptional enhancer cloned into a CAT expression plasmid. A wild-type silencer fragment is inserted in the case of plasmid pC 102, a silencer with the mutated octamer in the case of plasmid pC 114. Plasmids were transfected into mouse pre-B cell line 230-238 using the DEAE–dextran method. Cell extracts were assayed for their ability to convert [^{14}C]chloramphenicol to the acetylated form during a 1 h incubation period. (**B**) Mobility shift assay of the chicken λ V–J silencer. The labelled 254 bp fragment carrying the wild-type (lanes 2–4) or the mutated octamer motif (lane 5) was incubated with 8 μg nuclear extracts from mouse pre-B cells (230-238) and analysed by electrophoresis in a 4% polyacrylamide gel. The control lane (lane 1) contained probe alone. In lanes 3 and 4 extracts were pre-incubated with a 20-fold molar excess of specific and non-specific (270 bp mouse κ 3′ enhancer) competitor fragments.

enhancer is combined with the heterologous SV 40 promoter. As shown in Figure 5, transcriptional activity again depends not only on the cell line used for transfection, but also on the combinaton of regulatory elements. In most cases the core element turns out to be more effective than the flanking region. In the construct without enhancer (pC 201), however, this ratio is reversed in the Molt 4 and CH 27 cell lines. In the constructs where transcription is controlled by the mouse κ 3′ enhancer, reduction is comparable with that obtained with the chicken enhancer constructs in the three lymphoid cell lines. No activity, however, could be detected in HeLa and 3T3 cells. The same observations were made when the CLLCG promoter of pC 215 was substituted by the SV 40 promoter (pC 245).

Transcriptional analysis of the shark Ig light chain V–J intervening sequence

The presence of a transcriptional silencer within the CLLCG V–J intervening sequence and its function in mouse pre-B and B cell lines suggests a phylogenetic conservation of Ig gene regulation. To test this hypothesis, we cloned the complete V–J region of the Ig light chain gene of the horned shark (29; kindly provided by J. Rast) between the promoter and enhancer of the luciferase expression vector pC 203. Contrary to our expectation, these constructs (pC 239/240) produced a further 3–5-fold increase in luciferase activity in the chicken and mouse B cell lines, to the highest levels observed in any of our transfection experiments (not included in the figures). In both cell lines the shark sequence therefore behaves as a strong transcriptional enhancer.

DISCUSSION

The interplay of transcription and rearrangement in Ig and TCR genes has been analysed in numerous reports over the last decade (11). Yet it has remained unclear whether it is the transcript itself or the binding of transcription factors at specific target sites which allows access of the recombinase. In the latter case, transcription factors could even be regarded as components of the recombinational machinery itself. One experimental approach capable of distinguishing between these possibilities is a detailed analysis of the DNA elements playing a role in both transcription and recombination. As outlined in the Introduction, the CLLCG can also be regarded as an ideal model for analysing these questions in mammals. It was transgenic mouse technology which first enabled these enhancer and silencer elements to be detected (5).

Transcriptional enhancer elements of immunoglobulin genes have been localized between the J and C regions as internal enhancers and downstream of the C region as 3′ enhancers (24,30). In the case of the mouse heavy chain gene, one internal and two 3′ enhancers have been identified, which complicates the study of their individual roles during B cell development. The 3′ transcriptional enhancer of the mouse κ light chain gene spans only 50 bp and therefore provides only a limited number of protein target sites (31). Despite the small size, however, two subunits (modules) could be identified, which as multimers independently enhance transcription (32). The mouse heavy chain internal enhancer and the rat heavy chain 3′ enhancer have sizes of 600 and 400 bp respectively and represent elements with a more complex organization. Both of them could be subdivided into functional modules which are believed to be activated or repressed at different time points in different tissues (33,34). In the case of the CLLCG, module 1 behaves as an orientation-independent enhancer by itself (Table 3). For stable expression of the rearranged gene, this element alone appears to be sufficient, so that we can only speculate that elements 2 and 3 may be important for the V(D)J recombination process. Besides, a number of proteins which seem to be specific for individual enhancer elements (25,32,33,35), the family of E box binding proteins, has been found to stimulate transcription in various enhancers (36–38). The 467 bp enhancer exhibits five potential E boxes, but no binding sites for NFκB, μB, Oct, PU.1 or Id.

The silencer element adds a new level of complexity to recombinational and transcriptional control of Ig genes. Repression of transcription of these genes in non-B cells has been reported as a function of promoter and enhancer elements (26,38–42). In contrast, transcriptional silencers are defined as elements which exclusively reduce transcription levels (43). They have been found in a variety of eukaryotic genes, such as the the mouse T cell receptor α gene (44), the *Drosophila zen* gene (45,46), the chicken vimentin gene (47), the human interferon β gene (39), human Alu repeats (48), the synapsin I gene (49) and the rabbit angiotensin-converting enzyme gene (50). Little is known, however, of how silencing is achieved at the molecular level.

The chicken light chain gene silencer obviously represses transcription of the unrearranged gene and its deletion during rearrangement allows the recombined gene to be expressed at high levels (5). In mouse and human this function is believed to result from the closer proximity of promoter and enhancer elements after rearrangement. The presence of such silencers, however, cannot be excluded. The fact that the chicken element is well recognized in transgenic mice and in mouse and human

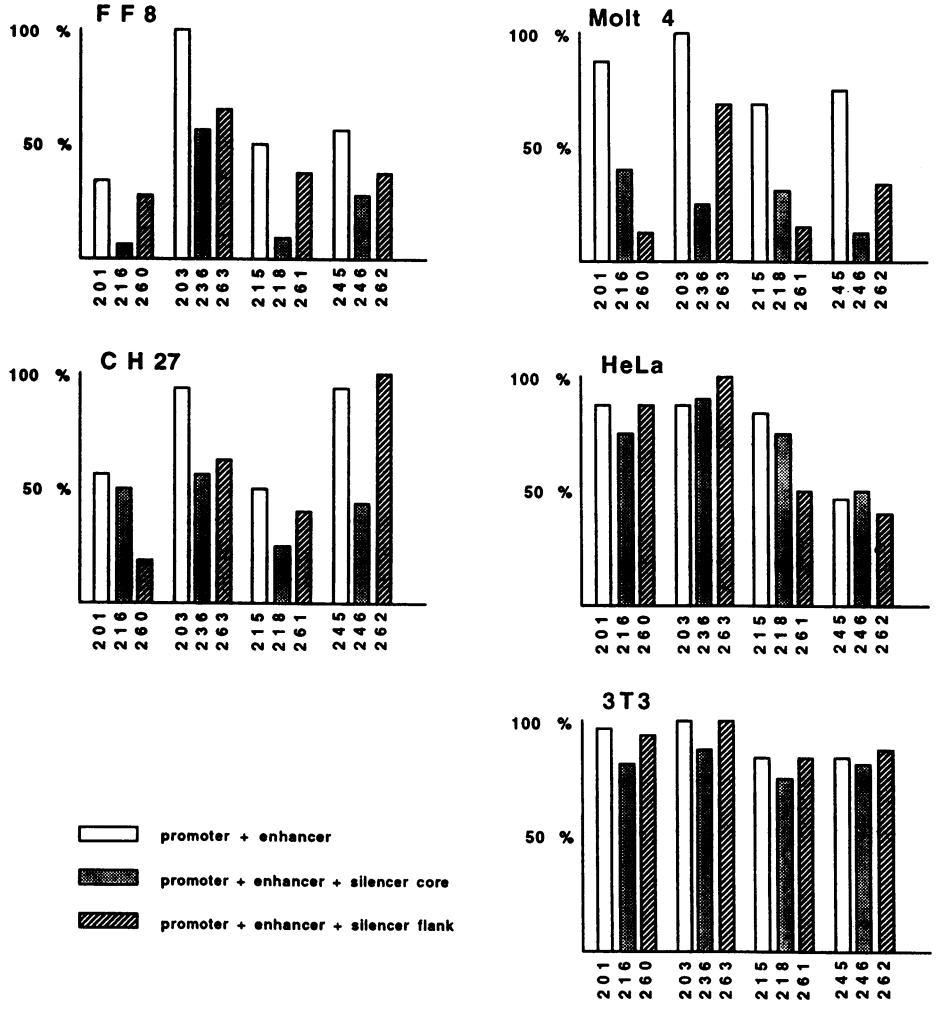

Figure 5. Silencing activity on heterologous promoter and enhancer elements. The silencer core element (dotted bars) and its 3′ flanking region (shaded bars) were cloned into plasmid vectors which in addition to the CLLCG promoter alone (pC 201) carry the complete 467 bp CLLCG 3′ enhancer (pC 203) or the mouse Ig κ 3′ enhancer (pC 215). In construct pC 245 the chicken promoter has been substituted by the SV 40 promoter. All constructs were transfected into the five cell lines indicated and luciferase activity was measured 24 h later. The figure shows the activities relative to the highest value of each transfection series (100%). The mean value of two independent experiments was normalized in correlation with the co-transfected β-GAL expression plasmid.

transfected cell lines clearly shows phylogenetic conservation and suggests the presence of B cell-specific silencers also in these organisms. As a first attempt to prove this hypothesis, the V–J intervening sequence of the horned shark Ig light chain was analysed for silencing activity. In the phylogenetically very distant murine and avian cell lines no transcriptional silencing, but strong transcriptional enhancement, could be observed. This heterologous assay system, however, may not allow the postulation of a transcriptional enhancer element. On the other hand, the result strongly suggests the presence of regulating elements between the V and J elements of the shark Ig light chain gene.

The silencer core element can cleary be shown to depend on the octamer motif. This silencing function is also supported by a recent study, where it was shown that binding of the Oct-2 protein to a heptamer-like sequence in a promoter can be responsible for transcriptional repression (51). Nevertheless, a role of octamer binding proteins as positively acting transcription factors in promoter and enhancer regions and negatively acting factors in silencer elements is difficult to explain. It seems reasonable to assume a control function of Oct proteins, which cooperatively bind other proteins of the transcriptional machinery in order to direct them in a time- and tissue-specific manner to their target sequences. This argument is mainly supported by our observation of octamer-independent silencer function in T cells (Fig. 3). The fact that octamer mutation may convert the silencer element into an enhancer (Fig. 3) clearly demonstrates that enhancer and silencer elements share common binding factors. A similar observation has been made in the case of the *Drosophila zen* silencer (45,46). Sequence comparisons of the 254 bp region with other silencer and repressor elements suggests the existence of an additional protein binding site immediately 5′ of the octamer (not shown). Evidence for this assumption mainly comes from a recent study of myeloma/T cell hybridoma cells, where gene repression is mediated by the octamer/μE4 region of the internal mouse heavy chain enhancer (26).

The complete CLLCG silencer can be subdivided into pieces with partial silencing activity and these elements produce different rates of repression, depending on the promoter and enhancer elements with which they are combined (Fig. 5). This observation strongly suggests the presence of positive and

negative regulating elements even within the silencer. The rate of transcription obviously depends on the composition of promoter, enhancer and silencer binding proteins in each individual construct. Transient transfections as they were carried out in this study can only serve as 'pictures from a movie'. Each cell line will always provide a limited number of partners in the protein–DNA interplay (52). The disruption of the octamer motif in the silencer core region can convert it into an enhancer (see Fig. 3) and demonstrates the proximity of enhancement and silencing. On the basis of this observation it is a reasonable possibility that the V–J intervening sequence of the shark Ig light chain gene functions as a silencer in developing B cells of this species. Phylogenetic loss or acquisition of a single factor could explain its enhancer function in mouse and chicken B cells.

In addition to the plasmids shown in the Results section, several more constructs were tested in order to elucidate silencer function. No effect could be observed when one or two additional copies of the silencer core element were inserted into the expression vector. This element is equally active whether inserted immediately downstream of the enhancer or upstream of the promoter. We could find no dependence of transcriptional activity on spacing between promoter, silencer or enhancer. In summary, the silencer emerges as a negative regulating element which has been separated from the promoter–enhancer complex and come to occupy a position between the V and J elements of the gene during evolution. The transgenic experiments suggested the presence of an anti-silencer element in direct proximity to the V or J region (5,53). This function could not be found in several plasmids that were constructed for this purpose, which again could well be due to the transcription factor expression patterns in the cell lines transfected. A final answer may come from specific disruption of binding sites in a developing organism.

The work presented in this communication opens many new questions. Presently we are in the process of further characterization of the silencer, determining which of the octamer binding proteins is responsible for silencing and what other factors are needed to complete the silencing function.

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