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A dominant transcriptional silencer located 5′ to the human T-cell receptor Vβ2.2 gene segment which is activated in cell lines of thymic phenotype

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ABSTRACT

We have identified a transcriptional regulatory sequence located 5′ to the human T-cell receptor Vβ2.2 promoter. The upstream part of this sequence acts as a transcriptional activator in the three cell lines, Jurkat, MOLT4 and HSB2, which all have a thymic phenotype. The downstream part of the sequence exerts a dominant silencing activity in the Jurkat and MOLT4 cell lines, but not in the immature HSB2 cell line. The silencing sequence binds nuclear factor(s). Mutations of nucleotides in a short stretch of sequence, demonstrating methylation interference, abolish both the factor binding and the silencing effect. Replacement of the silencing element by a homologous sequence found 5′ to the human Vβ8.1 segment, leads to a protein binding pattern which shows some DNA–protein specific complexes identical to those observed with the Vβ2.2 sequence. Interestingly, binding of nuclear factors to the Vβ2.2 silencing sequence is also observed using thymic extracts, but not using extracts from samples enriched for CD34+ cells, PBL, EBV cell lines or the HeLa cell line.

INTRODUCTION

The specificity of antigen recognition by Ig and TCR is due to somatic rearrangements which involve V, J and in some cases D gene segments and which occur during the early stages of lymphoid differentiation. Regulation of V(D)J recombinations occurs at least at two levels (1–3). The first process ensures that V(D)J rearrangements only occur in immature lymphoid cells. The restriction of the simultaneous expression of recombination activating genes (RAG 1 and RAG 2) to immature lymphoid cells is probably the most important factor in this regulation (4). The second regulatory level targets gene segments for recombination. This regulation is important for understanding the lineage specificity of Ig/TCR rearrangements and also for the understanding of allelic exclusion which is observed at a genomic level for certain antigen receptor genes. Since RAGs are expressed in immature cells of the B and T lineage and since transfection of these genes into fibroblasts does not allow endogenous Ig/TCR genes to rearrange, factors other than RAG are clearly involved. Methylation of CG and germline transcription of unrearranged segments are considered to be important factors regulating gene segment accessibility to the recombinase machinery.

The TCRβ gene appears to be a good model to analyze this regulatory process. TCRβ rearrangements occur in two steps. During the first one, which occurs early during T-cell differentiation, one Dβ segment recombines to one Jβ segment. This step is probably not completely restricted to T-cells. The second step occurs in more mature thymocytes and involves a secondary Vβ to DβJβ rearrangement. As shown by transgenic studies this last recombination event is highly restricted to the T-cell lineage (5). If this rearrangement encodes a functional protein, the Vβ gene rearrangement process is switched off, defining a near complete allelic exclusion (6). In the context of the transcriptional regulation of the V(D)J recombination, these data suggest the existence of regulatory sequences which may switch on/off the Vβ segment germline transcription.

Transcriptional regulation of mouse and human rearranged TCRβ genes (7,8) involves an enhancer element located 3′ of the Cβ2 region and a promoter located 5′ of each Vβ segment. The 3′ enhancer is T-cell-specific and is active in αβ and γδ cells. As shown by transient transfection assays, Vβ promoters display a weak activity which is not restricted to T-cells. These promoters can be activated by the TCRβ enhancer in T-cells and by heterologous enhancers in a variety of cells including T- and B-cells. A binding site for CREB/ATF factors is located upstream to the Vβ transcriptional start (in most cases within 100 bp) and may be important for Vβ transcription, at least in mice. In addition to this well defined region, the presence of a T-cell-specific enhancer located 570 bp 5′ to the human Vβ8.1 segment transcriptional start has been previously suggested (9).

In this study, we have examined the transcriptional regulatory activity of a 974 bp non-coding region located 5′ to the human Vβ2.2 translational start site. Using gene reporter based constructions, we have characterized two adjacent regulatory sequences. The more 5′ sequence is inactive in EBV transformed or epithelial cell lines, but exerts enhancing activity on a thymidine kinase (tk) promoter in three cell lines of thymic phenotype, which include one immature, HSB2, and two more mature, MOLT4 and Jurkat

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cell lines. The more 3′ sequence acts as a dominant transcriptional silencer which represses the enhancing activity of the 5′ sequence in MOLT4 and Jurkat, but not in HSB2. This silencer specifically binds nuclear factors from MOLT4, Jurkat and thymocytes. As shown by mutation analysis, binding of these factors is essential for the silencing effect.

MATERIALS AND METHODS

Cells and cell lines

The human cell lines Jurkat, MOLT4, HSB2, Reh and HeLa were obtained from the American Type Culture Collection (ATCC), corresponding to ATCC number 152, 1582, 120.1, 8286 and 2, respectively. The EBV 1341 B-cell line was a gift of J. Dausset (C.E.P.H, Paris, France). Human Ficoll purified peripheral blood leukocytes (PBL) and purified $CD34^+$ cells were obtained from normal donors. The percentage of $CD34^+$ cells was 70%. Unfractionated human thymocytes were prepared from a 24 week normal fetus. Phenotypic and genotypic characteristics of the four T-cell and pre-B-cell lines are indicated in Table 1.

DNA fragments, PCR products, oligonucleotides and CAT expression vectors

The 5′ flanking region of a previously described Vβ2.2–Jβ2.6 rearrangement from a pre-B-cell leukemia (10) was cloned and sequenced according to standard procedures (11). An *Rsa*I–*Xba* fragment (corresponding to positions –974 to +160 from the Vβ2.2 translational start site) was blunt ended and ligated to the blunt ended *Xba*-digested pBL CAT2 vector upstream to the tk promoter which controls the chloramphenicol acetyl transferase (CAT) gene (12). Different DNA fragments were similarly subcloned in the pBL CAT2 vector. The fragments (Fig. 1A) and cloning procedures were as follows: XR: the *Xba*–*Rsa*I fragment (corresponding to positions +160 to –974) was blunt ended and ligated to the blunt ended *Xba* digested pBL CAT2 vector; RS: the *Rsa*I–*Ssp*I fragment (corresponding to positions –974 to –717) was ligated to the blunt ended *Xba* digested pBL CAT2 vector; AS: the *Rsa*I–*Ssp*I insert from RS construction was digested by *Alu*I and eluted. Then, the 3′ *Alu*I–*Ssp*I fragment (corresponding to positions –891 to –717) was ligated to the blunt ended *Hin*dIII and *Xba* digested pBL CAT2 vector; RA: the *Rsa*I–*Ssp*I insert from RS construction was digested by *Alu*I and eluted. Then, the 5′ *Rsa*I–*Alu*I fragment (corresponding to positions –974 to –892) was ligated to the *Xba*-blunt ended and *Bam*HI digested pBL CAT2 vector.

Several polymerase chain reaction (PCR) products of the corresponding region on RS template were also used in the present study: af (positions –904 to –717), using the following synthetic primers: 5′-GAAACATACAAAGCTAAATTGAGA-3′ and 5′-ATTGATAAAGCACAGGCCAG-3′; ae (positions –904 to –764), using the following synthetic primers: 5′-GAAACATACAAAGCTAAATTGAGA-3′ and 5′-CATAATCATGTTGAAGCCACTTCATCA-3′; ef (positions –790 to –717), using the following synthetic primers: 5′-TGATGAAGTGGCTTCAACATGATTATG-3′ and 5′-ATTGATAAAGCACAGGCCAG-3′; ad (positions –904 to –822), using the following synthetic primers: 5′-GAAACATACAAAGCTAAATTGAGA-3′ and 5′-ATCT CATCACAATTTCCCTC-3′; de (positions –845 to –764), using the following synthetic primers: 5′-GAGGGAAATTGTGATGAGAT-3′ and 5′-CATAATCATGTTGAAGCCACTTCATCA-3′.

Synthetic oligonucleotides were also used as double-strand probes after annealing: bc (positions –884 to –845: 5′-GAGAAATATTATTAGAATGTTTAGAATTAAGTCCTTTATA-3′ and 5′-TATAAAGGACTTAATTCTAAACATTCTAATAATATTTCTC-3′); bc/m (derived from bc by introducing 13 mutations: 5′-GAGAAATATTATTACTTTCCCTAGAACCAAGTAACCCATA-3′ and 5′-TATGGGTTACTTACTTGGTTCTAGGGAAAGTAATAATATTTCTC-3′); bc/mA (derived from bc by introducing eight mutations: 5′-GAGAAATATTATTACTTTCCCTAGAACCAAGTCCTTTATA-3′ and 5′-TATAAAGGACTTACTTGGTTCTAGGGAAAGTAATAATATTTCTC-3′); bc/mB (derived from bc by introducing five mutations: 5′-GAGAAATATTATTAGAATGTTTAGAATTAAGTAACCCATA-3′ and 5′-TATGGGTTACTTAATTCTAAACATTCTAATA ATATTTCTC-3′); bc/Dia (derived from bc by introducing three mutations and two deletions: 5′-GAGAAATATTATTCGAAGTTTATATTAGGTCCTTTATA-3′ and 5′-TATAAAGGACCTAATATAAACTTCGAATAATATTTCTC-3′).

Some of these fragments were subcloned within the RA construction previously digested by *Bam*HI and blunt ended, leading to the following pBL CAT2 constructions: RA-ad, RA-bc and RA-bc/m (Fig. 1A).

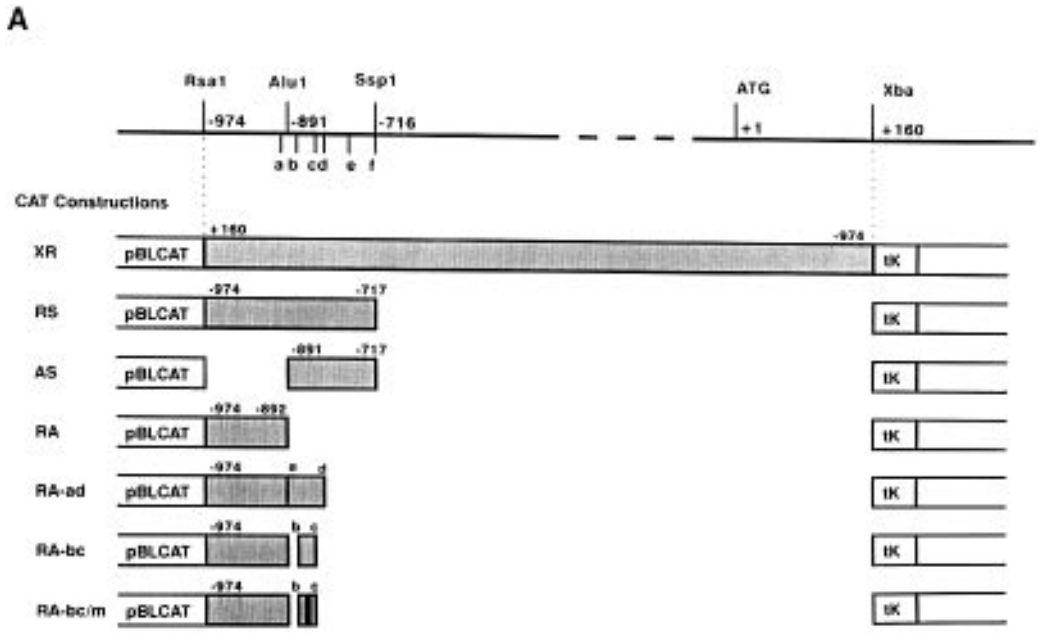
The promoterless pBL CAT3 vector (12) and a A11TF vector containing a strong enhancer cloned upstream of the tk promoter (13) were used as controls.

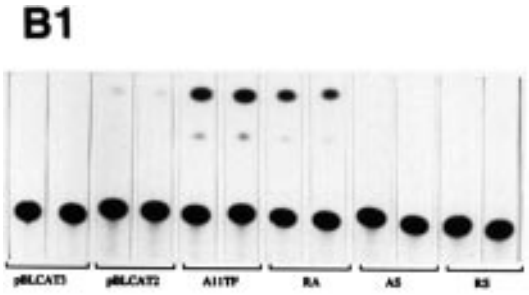
Cell cultures, transfection and CAT assays

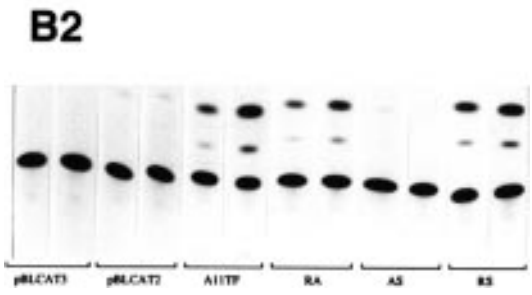
T-cell lines, EBV-transformed B-cell lines, HeLa and Reh cell lines were maintained at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM sodium pyruvate, 2 mM glutamine. Prior to transfection, cells were washed in serum-free RPMI medium and resuspended at $5–10 \times 10^6$ cells/250 µl RPMI together with 10 µg plasmid and electroporated. Electroporation parameters were set at 960 µF and 250 V. Transfected cells were incubated at 37°C and 5% CO_2 for 20–24 h. CAT activity was determined as described (14), and quantified by liquid scintillation counting of spots cut from chromatography plates. A ratio was calculated between non-acetylated and acetylated forms and expressed in percent of acetylation. Each CAT assay was performed in duplicates for a minimum of two independent series.

Nuclear extracts and mobility shift DNA-binding assay

Nuclear extracts of cells were prepared as described previously (15). Oligonucleotides were 5′-end-labeled using T4 polynucleotide kinase in the presence of γ-^{32}P. Free nucleotides were eliminated using a spun column and the labeled strand was annealed with its unlabelled complementary strand in 100% excess. The probe was incubated in 20 µl of different binding reaction buffers containing 2 µg poly(dI–dC) and 2 µg nuclear extract in binding buffer. Only one Zn^{++} buffer (20 mM Tris–HCl pH 7.4, 70 mM KCl, 2 mM $MgCl_2$, 20 µM $ZnCl_2$, 0.2 mM DTT, 6% glycerol, 0.1% NP-40) was retained for experiments. The resulting DNA–protein complexes were separated by electrophoresis on a 4% acrylamide gel containing 0.5× TBE. The qualitative and quantitative comparability of these nuclear extracts was controlled using target sequences for ubiquitous binding factors (AP1 and octamer) as probes.







C

Plasmid	Cell lines					
	HSB2	MOLT4	Jurkat	Reh	EBV 1341	Hela
pBL CAT3	0.1	0.8	0.2	0.05	0.02	0.05
pBL CAT2	1	1	1	1	1	1
A11TF	12	8.5	13.5	7.9	5.8	3.8
XR	3.1	0.8	0.5	7.2	0.1	0.1
RS	7.6	0.8	0.5	7	0.9	0.5
RA	6	7	8	6.7	0.2	0.8
AS	0.9	0.8	0.5	3.2	0.7	0.5

Figure 1. Localisation of transcriptional enhancing and dominant silencing activities in the 5′ flanking region of the human Vβ2.2 gene segment. Functional analysis by CAT assays. (**A**) Restriction sites used for subcloning in pBL CAT vector are located. All DNA fragments used later in gel shift experiments are indicated (af, ae, ad, bc...). Precise descriptions are given in the Materials and Methods section. All pBL CAT constructs are shown, except pBL CAT3, pBL CAT2 and A11TF. Plain area indicates that mutations have been done within the bc fragment (see Materials and Methods section, as well as Fig. 4B). (**B**) Representative experiments in the mature Jurkat (B1) and immature HSB2 (B2) T-cell lines. (**C**) Quantification of the results by scintillation counting. Each plasmid (10 mg) was transfected into cell lines by electroporation. Cell extracts were tested for CAT activity after 24 h of culture. Results are expressed as fold increase of CAT activity compared with the pBL CAT2 plasmid for which activity was set at 1. Experiments were performed in duplicate and repeated at least twice. The mean values are indicated.

Methylation interference analysis

Duplex oligonucleotides labeled on one strand were prepared as described in the previous section. The binding reaction (50 µl) contained 10^6 c.p.m. of end-labeled methylated probe and 40 µg of nuclear extract diluted in the same buffer used for electrophoretic mobility shift assays. Nucleoprotein complexes were resolved through a 4% native polyacrylamide gel. After 3 h photographic exposure of the gel, the bands corresponding to the protein–DNA complex and to the free DNA probe were excised from the gel and eluted overnight in 20 mM Tris–HCl pH 7.0 and 0.1% SDS. After phenol–chloroform extraction the DNA was precipitated with

2.5 vol ethanol in the presence of 300 mM sodium acetate. The pellet was washed with 80% ethanol, processed through NaOH 1 N as described (16). Equal amounts of radioactivity from bound and free materials were run on 8% sequencing gels.

RESULTS

A dominant transcriptional silencing sequence is located 5′ to the human Vβ2.2 promoter and differentially activated in T-cell lines of thymic phenotype

We have previously cloned a Vβ2.2–Jβ2.6 rearrangement from a pre-B-cell leukemia (10). An *Xba–Rsa*I (**XR**) fragment which included 974 nt upstream of the Vβ translational start site was subcloned, sequenced (submitted to EMBL/GenBank, accession number Z49234) and various restriction fragments were cloned in a reporter vector in which the transcription of the CAT gene is under the control of the tk promoter (pBL CAT2) (Fig. 1A). CAT activity was assessed after transfection in various cell lines.

As shown in Figure 1, the *Rsa*I–*Alu*I (**RA**) restriction fragment (corresponding to positions –974 to –892, 83 bp long) displayed an enhancing transcriptional activity in the three T-cell lines studied (HSB2, MOLT4 and Jurkat). No significant enhancing activity was detected in an epithelial cell line (HeLa) and in one EBV transformed B-cell line (EBV 1341). Interestingly, an enhancing activity was found in the pre-B Reh leukemic cell line which displays TCRβ gene rearrangements (Table 1).

To verify whether **RA** function is conserved in a larger context, CAT activity was assessed after transfection of the entire **XR** construction. The enhancing activity was only found in the immature T-cell line HSB2 and in the pre-B Reh cell line, but not in the two more mature MOLT4 and Jurkat T-cell lines. These data suggested that a dominant silencing sequence was present in this fragment and that this sequence was activated in Jurkat and MOLT4. Analysis of CAT activity from various constructions allowed the mapping of this sequence into a 175 bp *Alu*I–*Ssp*I (**AS**) fragment adjacent (downstream) to the **RA** fragment. This **AS** sequence alone did not display any significant transcriptional activity in T-cell lines and, as expected, the *Rsa*I–*Ssp*I (**RS**) fragment (which included the activating **RA** fragment and the silencing **AS** fragment) displayed an activity which was similar to that of the entire **XR** fragment. A representative experiment is shown in Figure 1B.

These results (summarized in Fig. 1C) revealed an activating/silencing sequence located 5′ to the Vβ2.2 segment. The overall effect of this sequence was to exert an activating activity on the tk promoter in the immature HSB2 T-cell line, but not in the more mature MOLT4 and Jurkat T-cell lines due to the dominant effect of the silencing sequence.

A nuclear protein or protein complex binds the silencing sequence in MOLT4 and Jurkat T-cell lines, but not in the HSB2 immature T-cell line

An **af** DNA fragment (188 bp), which included the 175 bp **AS** sequence, which supported the dominant silencing activity, and 13 adjacent nucleotides from the **RA** fragment, was split into two fragments: **ae** (141 bp and 5′ located) and **ef** (47 bp and 3′ located) (Fig. 1A). These two fragments were used as probes for nuclear protein binding in gel shift assays. No specific complex was observed using the ef fragment. One specific complex C was detected using the ae fragment and nuclear extracts from Jurkat and MOLT4 T-cell lines. This complex was only barely detectable using HSB2 immature T-cell line nuclear extracts and not detectable using Reh pre-B cell line nuclear extracts (Fig. 2A). This pattern correlated perfectly with the cell specificity dominant functional silencing activity of AS.

Interestingly, in extended mobility shift assay experiments, specific binding to the **ae** fragment was also found using extracts from unpurified human thymocytes showing that the presence of the corresponding DNA binding factors is not restricted to established leukemic cell lines (Fig. 2B). In contrast, no binding could be demonstrated with extracts from enriched preparations of $CD34^+$ cells, Ficoll purified PBL (Fig. 2B), EBV cell lines and with extracts from HeLa (data not shown).

To more precisely define the sequence involved in nuclear protein binding, the ae fragment was split into two fragments: **ad** (83 bp and 5′ located) and **de** (58 bp and 3′ located) (Fig. 1A). Binding of the specific complex C was found using the ad fragment as a probe, with the same cell line specificity as that observed using the larger **ae** fragment (data not shown). Interactions between proteins included in the complex C and DNA were then studied by methylation interference analysis. This analysis was performed using the coding and the non-coding strands of the **ad** fragment and Jurkat nuclear extracts. As shown in Figure 3, interferences were detected on both strands of a small DNA region of the **ad** fragment. Relevant residues were distributed among two close short stretches of sequence: six residues were located between the –870 and –864 positions; five consecutive residues were located between the –852 and –848 positions. It was of interest to note that a repeated TTAGAAT motif was present between the –873 and –858 positions.

Table 1. Phenotypic, cytogenetic and genotypic characteristics of the Jurkat, MOLT4, HSB2 and Reh cell lines

Cell line	Phenotype	TCRβ configuration	
		Cβ	Vβ
Jurkat	$CD3^+$, $WT31^+$, $CD4^+$, $CD8^-$	R $Cβ_2$/GL	Vβ8
MOLT4	$CD3^-$, $WT31^-$, $CD4^+$, $CD8^+$, $CD3_c^+$, $β_c^+$	R $Cβ_2$/GL	Vβ2
HSB2	$CD3^-$, $WT31^-$, $CD4^-$, $CD8^-$, $CD7^+$, $CD2^-$, $CD3_c^+$, $β_c^+$	R Cβ1/GL	Vβ5
Reh	$CD10^+$, $CD19^-$, $CD20^-$, $CD3^-$, $CD7^-$, $CD2^-$, $β_c^-$	R Cβ2/R Cβ2	Vβ3/?

WT31 monoclonal antibody (Becton Dickson) recognizes the cell surface TCRαβ complex. $CD3_c$ and $β_c$ denotes intracytoplasmic CD3 and TCβb chain, respectively. $β_c$ was detected using βF1 murine monoclonal antibody (Bioadvance). TCRβ status was studied with Southern blot analysis using a specific Cβ probe and genomic PCR analysis using specific Vβ and Jβ primers, as previously described (10). R and GL denote rearranged and germline, respectively.

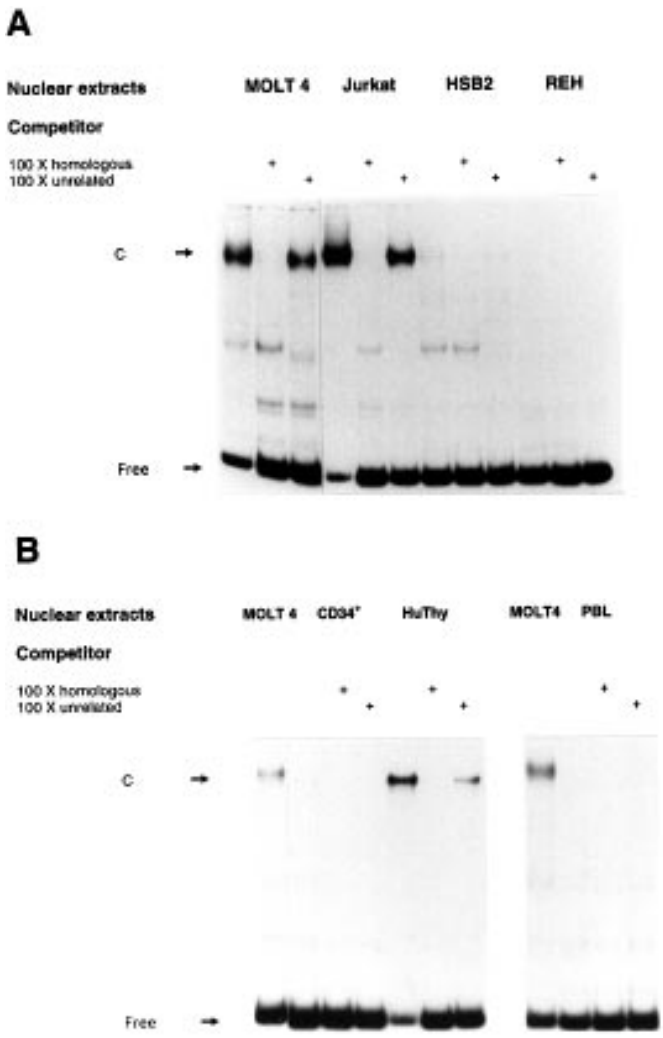


Figure 2. Electrophoretic mobility shift assay of the silencing 'ae' fragment using lymphoid cell lines and primary cell nuclear extracts. The location of the 'ae' fragment used as probe is indicated in Figure 1 and described in the Materials and Methods section. A specific complex C is indicated by the arrow. (**A**) Cell lines (see Table 1). (**B**) Primary cells. $CD34^+$, nuclear extracts of enriched preparations of CD34 positive cells obtained from normal donor bone marrow (70% enrichment); HuThy, nuclear extract of unpurified human neonatal thymocytes; PBL, nuclear extracts of Ficoll purified peripheral blood lymphocytes.

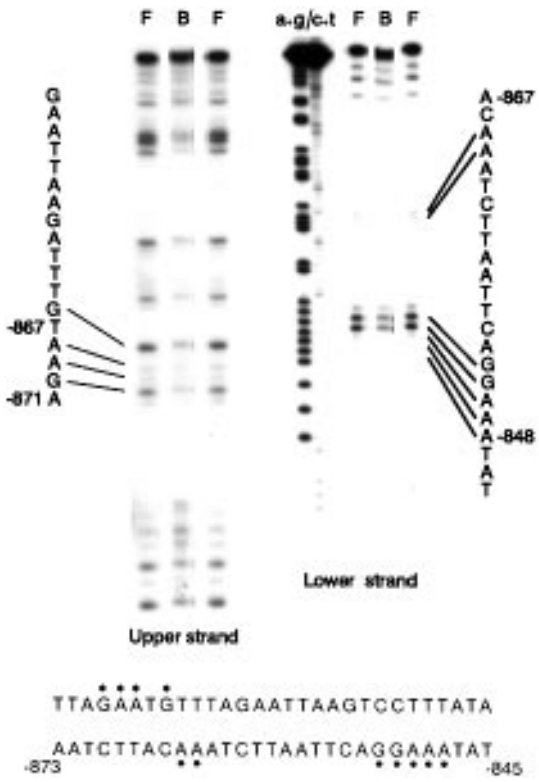


Figure 3. Methylation interference analysis of the 'ad' fragment. Protein bound (B) and unbound (F) complexes were isolated by electrophoretic mobility shift assay using Jurkat nuclear extracts, cleaved, and analysed on sequencing gel after treatment by NaOH. Eleven G and A residues interacting with complex formation when methylated were detected either on the coding or the non-coding strand. These residues, indicated by filled circles, appeared as two splits within the interacting stretch of sequence (–870/–864 and –852/–848, respectively). Most of these interactions were partial and sometimes barely detectable (positions –848, –849, –850 on the lower strand, for example).

Further characterization of the silencing sequence in Jurkat cell line

To further characterize the binding properties of the silencing sequence, competitive gel shift assays were performed on resolutive gels using the **ad** fragment and shorter fragments, derived from the **ad** fragment, as probes. These fragments are shown in Figure 4A and described in the Material and Methods section. All these assays were performed using Jurkat nuclear extracts.

Using the **ad** fragment as a probe, it appeared that the binding involved at least four specific C1, C2, C3 and C4 complexes, as shown in Figure 5. The high intensity C1 complex might correspond to the C complex, previously observed when using the larger **ae** fragment. Only one of these four complexes corresponding to C2 was observed using the –873/–840 or –878/–853 fragments as probes (data not shown). The simple presence of all residues detected by methylation interference analysis was thus insufficient to allow the binding of the four specific complexes.

Conversely, the four specific complexes were still observed using the a/–853 and –878/d fragments as probes (data not shown). Moreover, the shorter 40 bp bc fragment described in Figure 4A and including all interacting nucleotides was also sufficient to bind the four specific complexes, although with a different intensity pattern (Fig. 6 compared with Fig. 5). Adjacent sequences located either upstream or downstream of the interacting region were thus required to allow the formation of these four complexes.

The involvement of interacting residues detected by methylation interference analysis in complex formation was studied by nucleotide mutation analysis. Three mutated fragments (bc/m, bc/mA, and bc/mB) are shown in Figure 4B and described in the Materials and Methods section. Mutations of all interacting nucleotides inhibited the formation of the four specific complexes (Fig. 6 with bc/m fragment as probe). Mutations of all the five consecutive –852/–848 interacting nucleotides had no apparent effect on C1, C2, C3 and C4 formation (Fig. 6 with bc/mB fragment as probe). On the contrary, the mutations of all the six remaining interacting nucleotides were sufficient to inhibit the formation of the four specific complexes (Fig. 6 with bc/mA fragment as probe). These observations argued for the predominant role of these six residues in the formation of the complexes, when tested in a larger DNA context including either 5′ and/or 3′ adjacent sequences.

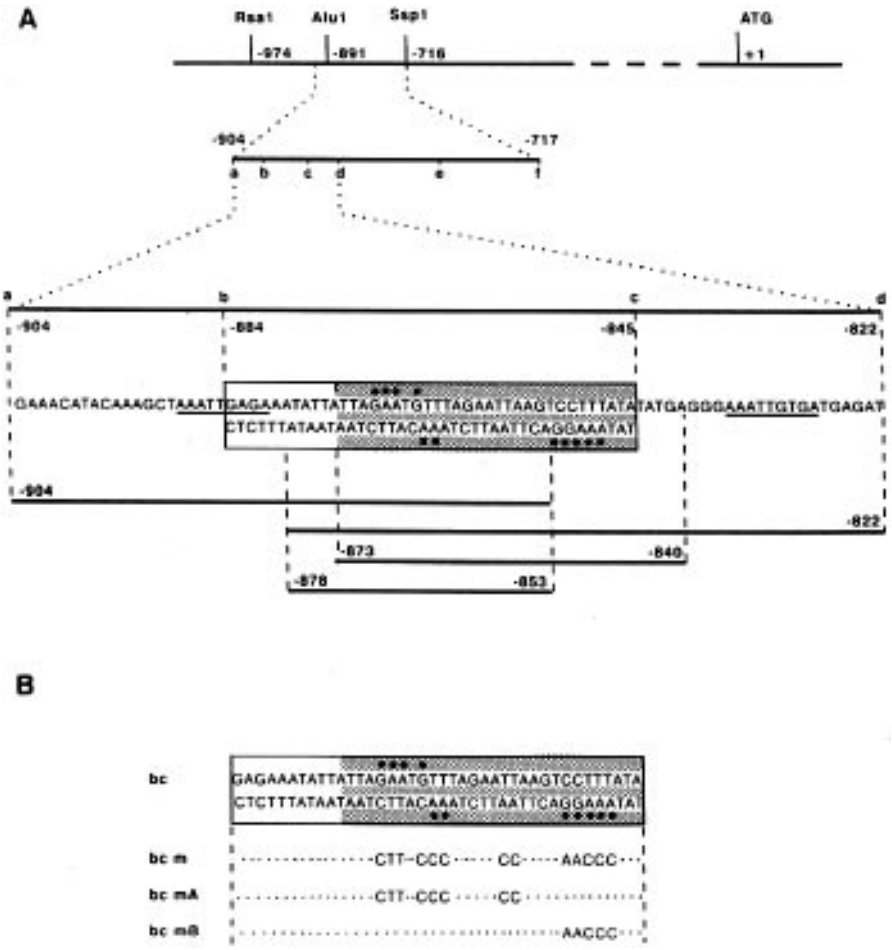


Figure 4. Fragments used as probes in gel shift assays. All these fragments are described in the Materials and Methods section. (**A**) The ad fragment (–904/–822), corresponding to the binding region of the larger ae fragment that supported the dominant silencing activity is shown and its nucleotide sequence is given. Shorter fragments derived from this ad fragment are indicated: the –878/–853 fragment; the –873/–840 fragment; the a/–853 fragment; the –878/d fragment; and the bc fragment. Interacting residues are indicated by filled circles. Two 8/9 homologous sequences flanking the interacting region are underlined. (**B**) Three mutated synthetic oligonucleotides were derived from the bc fragment sequence: the bc/m fragment which includes the mutation of all interacting residues; the bc/mA fragment which includes the mutation of all interacting residues located within the –870/–864 region; and the bc/mB fragment in which all interacting residues located within the –852/–848 region were mutated. Only mutated residues are indicated by letters under the complete unmutated bc sequence.

To correlate the consequence of site mutations on binding properties with the functional activity, unmutated and mutated fragments were included together with the RA enhancing sequence in pBL CAT2 constructs. First, ad and bc fragments which were sufficient to observe DNA–protein complex formation, were also sufficient to support the dominant silencing activity on the RA enhancing sequence (Fig. 7 with the RA-ad and RA-bc constructs). Secondly, the bc/m fragment, which does not allow the formation of specific complexes in gel shift assays, also abolished the dominant silencing effect observed in the Jurkat cell line (Fig. 7 with the RA-bc/m construct).

Replacement of the Vβ2.2 interacting region by a homologous sequence from the human Vβ8.1 promoter leads to a similar pattern of specific binding in the Jurkat cell line

Interestingly, the interacting DNA region had 16 homologous residues with a 19 bp fragment located upstream from the human Vβ8.1 gene segment previously described by Diammond *et al.* (9). This homology is shown in Figure 8A and no additional homology was found within the 5′ and 3′ flanking sequences. Seven of the 11 Vβ2.2 residues detected by methylation interference analysis were shared with this Vβ8.1 homologous sequence (all the six interacting residues from the –870/–864 region and only one of the five consecutive interacting residues from the –852/–848 region).

We tested the binding properties of the 19 bp sequence from the Vβ8.1 corresponding region when introduced within the bc fragment to replace the Vβ2.2 homologous sequence (bc/Dia oligonucleotide). Using bc/Dia as a probe and Jurkat nuclear extracts, the formation of C1 and C2 specific complexes was still observed, while C3 and C4 specific complexes became undetectable (Fig. 8B). Competition experiments using bc as the probe and bc/Dia as a competitor were consistent with these observations.

DISCUSSION

In this paper, we have shown that a dominant transcriptional silencing sequence is present 845–884 bp upstream of the Vβ2.2 ATG. This sequence specifically binds nuclear proteins in the Jurkat and MOLT4 T-cell lines but not in the HSB2 immature thymic cell line. The presence of this sequence in reporter constructs inhibits the enhancing effect of an upstream adjacent

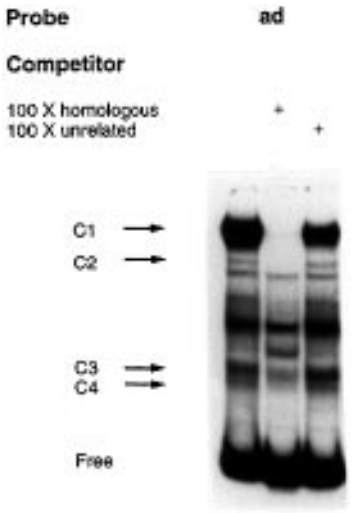


Figure 5. Binding properties of the silencing 'ad' sequence. Gel shift assay using Jurkat nuclear extracts. Four specific complexes C1, C2, C3 and C4 are observed with the ad probe using high resolution conditions.

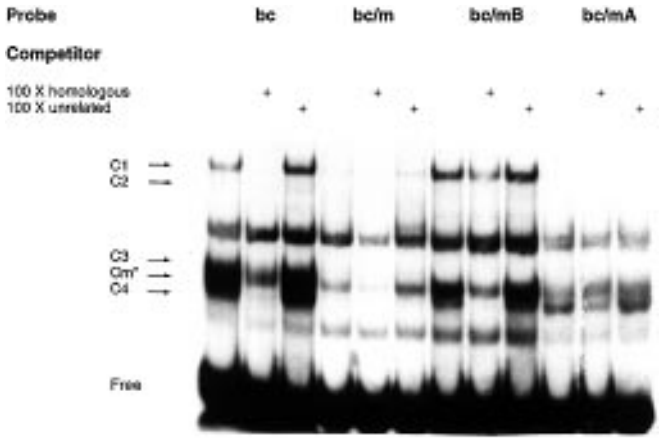


Figure 6. Binding properties of unmutated and mutated fragments. Gel shift assay using Jurkat nuclear extracts. All fragments used as probes (bc, bc/m, bc/mA and bc/mB) were described in the Materials and Methods section and shown in Figure 4B. Despite a difference in complex intensities (compared with Fig. 5), heterologous competitions between the ad and bc fragments were consistent with a common specific C1-C2-C3-C4 binding (data not shown). Mutations of all interacting residues (bc/m fragment) or only of those located within the –870/–864 interacting region (bc/mA fragment) did not allow the formation of the four C1, C2, C3 and C4 specific complexes. *Cm indicates a specific complex that appears using bc/m as probe; this fifth complex is also detectable using bc/mB as probe, although comigrating with the C3 complex; furthermore, this complex Cm is not shifted by bc or bc/mA as heterologous competitors (data not shown). The appearance of Cm could thus be linked to nucleotides introduced within the –852/–848 interacting region in replacement of wild-type residues.

sequence which is apparently activated in many different stages of thymic differentiation.

Repression of transcription in eukaryotic cells involves multiple mechanisms including regulation of chromatin condensation, nucleosome positioning and repression by inhibitory factors (17). Among these factors, certain passively repress gene transcription by competition with activating factors or by titration. In other cases, repression is active and due to the binding of inhibiting factors to specific DNA sequences leading to inactivation of the transcriptional process. Results shown in this paper for the region located 845–974 bp 5′ to the human Vβ2.2 gene segment ATG are consistent with this model. Indeed, repression is detected in cells in which the 5′ located enhancing sequence is active. It could be hypothesized that factors that interact with the silencing sequence inhibit activation of the promoter by activating factors.

Gel-shift experiments testing interactions between nuclear factors and the silencing DNA sequence that are reported here are consistent with a silencing mechanism that requires several components for protein–DNA interaction and could involve protein–protein interactions. First, the presence of sequences adjacent to the interacting region is required to observe the entire pattern of protein–DNA interactions. Secondly, the variation of intensity observed in the specific complexes when using different DNA fragments as probes, suggests that either the DNA binding affinity and/or the protein–protein interactions within complexes may vary, depending on the presence or absence of adjacent DNA sequences. For example, the stabilization of a high affinity C1 complex would be linked to the presence of 5′ and/or 3′ sequences adjacent to the bc fragment. The presence of two AAATTGA/TGA homologous sequences flanking the bc fragment might be of relevance in this stabilization.

Vβ promoters have been mostly studied in mice showing the presence of a conserved decamer (18,19) upstream of the initiation start site. This sequence, which is also found in the human Vβ8.1 (9) and Vβ2.2 (this study, EMBL/GenBank accession number Z49234) promoters, binds several ATF/CREB cAMP induced factors (19). Interestingly, this sequence binds thymus specific complexes, one of them appearing at day 16 of gestation which coincides with the initial activation of the Vβ locus (20). In a previous study, Diammond *et al.* (9) identified, by CAT assays, an enhancing T-cell-specific sequence which was active in a murine T-cell line EL4 and in the human Jurkat T-cell line, but not in cell lines of the B-cell lineage. Binding of this sequence by nuclear factors has not been studied (9). The presence of a well conserved 14 bp sequence in the DNA fragment described by Diammond *et al.* and in the RA segment we have isolated upstream of the Vβ2.2 promoter, suggests that these fragments are homologous. Moreover, analysis of CAT assays previously performed on the Vβ8.1 promoter suggest that one DNA fragment may also contain a transcriptional silencer (possibly between positions –510 and –445 from the putative Vβ8.1 transcriptional start site) (9). It is therefore possible that sequences homologous, at least functionally, to those described in this paper are also present in the 5′ non-coding region of other Vβ segments and may be the target of shared *trans*-acting factors.

It is improbable that the nuclear factor binding sequences we have described here significantly contribute to the transcriptional regulation of TCRβ rearranged genes as suggested by the strong expression of complete Vβ2(Dβ)Jβ-Cβ RNA in the MOLT4 cell line (data not shown), in which the silencing effect is detected by transient transfection of reporter constructs. In this configuration, the Vβ promoter is located in the vicinity of the 3′ Cβ enhancer and all the effects mediated by the sequences described here are relatively weak. More probably, these sequences may have a biological role in regulating transcription of Vβ segments in germline configuration as previously hypothesized for the decamer sequence (20).

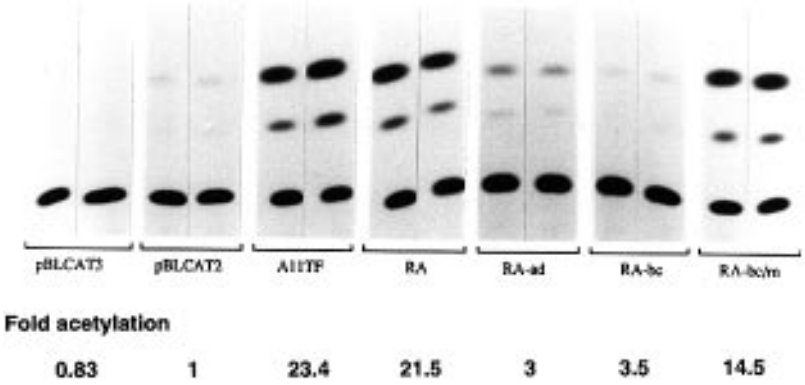


Figure 7. Functional consequences of 'bc' mutations. Transcriptional activity in Jurkat cell line of the ad, bc and bc/mfragments when cloned between the RA enhancing fragment and the tk promoter in the pBL CAT2 vector. The description of each plasmid is shown in Figure 1A and described in the Materials and Methods section. Quantification of the results by scintillation counting are expressed as fold increase of CAT activity compared with the pBL CAT2 plasmid for which activity was set at 1.

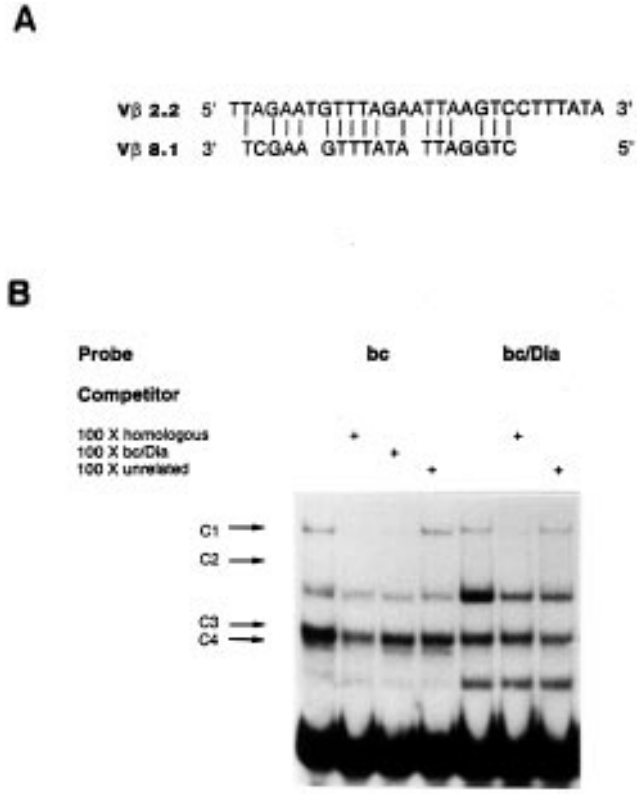


Figure 8. Replacement of the Vβ2.2 interacting region by a homologous sequence from the human Vβ8.1 promoter. (**A**) Comparison of the Vβ2.2 sequence (positions –873 to –845) with the –505 to –487 Vβ8.1 sequence (9) shows 16 homologous residues indicated by vertical lines. (**B**) Gel shift assay using Jurkat nuclear extracts with bc and bc/Dia fragments as probe. Description of the mutated synthetic bc/Dia oligonucleotide, which derives from the bc fragment by introducing the Vβ8.1 sequence to replace the corresponding homologous Vβ2.2 sequence, is given in the Materials and Methods section. Results of competition are consistent with a specific C1 and C2 binding to the bc/Dia oligonucleotide, without C3 and C4 formation. The C2 complex is only barely detectable on this 4 h exposure autoradiograph.

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REFERENCES

1 Alt, F.W., Oltz, E.M., Young, F., Gorman, J., Taccioli, G. and Chen, J. (1992) *Immunol. Today*, **13**, 306–314.
2 Schatz, D.G., Oettinger, M.A. and Schlissel, M.S. (1992) *Annu. Rev. Immunol.*, **10**, 359–383.
3 Okada, A., Alt, F.W. (1994) *Sem. Immunol.*, **6**, 185–196.
4 McBlane, J.F., Van Gent, D.C., Ramsden, D.A., Romeo, C., Cuomo, C.A., Gellert, M. and Oettinger, M.A. (1995) *Cell*, **83**, 387–395.
5 Ferrier, P., Krippl, B., Blackwell, T.K., Furley, A.J.W., Suh, H., Winoto, A., Cook, W.D., Hood, L., Constantini, F. and Alt, F.W. (1990) *EMBO J.*, **9**, 117–125.
6 Malissen, M., Trucy, J., Jouvin-Marche, E., Cazenave, P.A., Scollay, R. and Malissen, B. (1992) *Immunol. Today*, **13**, 315–322.
7 Leiden, J.M. (1993) *Annu. Rev. Immunol.*, **11**, 539–570.
8 Leiden, J.M. and Thompson, C.B. (1994) *Curr. Opin. Immunol.*, **6**, 231–237.
9 Diammond, D.J., Nelson, F.B. and Reinherz, E. (1989) *J. Exp. Med.*, **169**, 1213–1231.
10 Dombret, H., Loiseau, P., Bories, J.C. and Sigaux, F. (1992) *Blood*, **80**, 2614–2621.
11 Manniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor University Press, Cold Spring Harbor, NY.
12 Luckow, B. and Schütz, G. (1987) *Nucleic Acids Res.*, **15**, 5490.
13 Haddad, P., Wargnier, A., Bourge, J.F., Sasporte, M. and Paul, P. (1993) *Eur. J. Immunol.*, **23**, 625–629.
14 Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
15 Blanchet, O., Bourge, J.F., Zinszner, H., Israel, A., Kourilski, P., Dausset, J., Degos, L. and Paul, P. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3488–3492.
16 Maxam, A. and Gilbert, W. (1980) *Methods Enzymol.*, **65**, 499–550.
17 Herschbach, B.M. and Johnson, A.D. (1993) *Annu. Rev. Cell. Biol.*, **9**, 479–509.
18 Anderson, S.J., Chou, H.S. and Loh, D.Y. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3551–3554.
19 Anderson, S.J., Miyake, S. and Loh, D.Y. (1989) *Mol. Cell. Biol.*, **9**, 4835–4845.
20 Lanier, E., Brown, R. and Kraig, E. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8131–8135.