Activation of the immunoglobulin κ 3' enhancer in pre-B cells correlates with the suppression of a nuclear factor binding to a sequence flanking the active core

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ABSTRACT

Both the κ intron and the κ 3' enhancer are required for high levels of immunoglobulin κ gene expression. The activity of both enhancer elements can be induced by LPS in pre-B cells. While the LPS induction of the κ intron enhancer is mediated by NF- κ B, this factor is not responsible for activation of the 3' enhancer. Dissection of the 3' enhancer has shown that in pre-B cells the activity of the κ 3' enhancer is repressed by a region flanking an active core element. We have now scanned this flanking region for nuclear factor binding sites and have identified sites for B-cell specific E47/E12-like proteins and two ubiquitous nuclear proteins. Furthermore, we have identified a nuclear factor in pre-B cells whose binding activity is suppressed in response to LPS. In its tissue-distribution and binding specificity this factor appears to be identical to the lymphoid specific protein LEF-1. The position of the LEF-1 binding site within the 3' enhancer and its response to LPS raise the possibility that LEF-1 may be the target for a second pathway able to mediate LPS induction of immunoglobulin κ gene transcription.

INTRODUCTION

Expression of the immunoglobulin (Ig) κ light chain gene is activated at the developmental switch from pre-B to B cells. Three elements that contribute to the developmental and cell-type specific control of expression have been identified: the lymphoid specific V κ -promoters, the κ intron enhancer (reviewed in [1]) and more recently the κ 3' enhancer [2]. Like the κ intron enhancer, the activity of the κ 3' enhancer can be induced in pre-B cells by lipopolysaccharide (LPS) stimulation [3, 4]. In the case of the κ intron enhancer this induction is mediated by the nuclear factor NF- κ B. In pre-B cells NF- κ B exists as an inactive cytoplasmic precursor, complexed to the inhibitor molecule I- κ B. Upon LPS stimulation, post-translational modifications dissociate the complex and active NF- κ B is translocated into the nucleus (reviewed in [1]). The κ 3' enhancer, on the other hand,

does not require NF- κ B for its function since it is active in a cell line that lacks this nuclear factor [3, 5]. The molecular mechanism by which LPS can induce transcriptional activation via the κ 3' enhancer is thus not understood.

So far only a few nuclear factor binding sites have been identified within the 3' enhancer. In mouse plasma cells, where the enhancer was first defined as a 800bp *SacI*–*XbaI* fragment [2], most of the activity of the 3' enhancer resides in a central 124bp core [3]. Within this core region binding sites for the B-cell specific nuclear complex PU.1/NF-EM5 and E47/E12-like factors have been identified [4, 6]. Functional analysis and *in vivo* footprinting of the core region in the highly homologous human Ig κ 3' enhancer have confirmed the presence of the PU.1 and the E47/E12 sites and, in addition, have identified a third B-cell specific region designated DR [7]. While most of the activity resides in the core, a larger 450bp region of the κ 3' enhancer is remarkably conserved between species, with 79% conservation between mouse and human [8] and 75% conservation between rabbit and mouse [9]. This is likely to reflect functional conservation of these sequences.

Indeed, transient transfection assays have implicated the region flanking the core as important control elements. In pre-B cells the full length enhancer is inactive, but its activity is LPS inducible. However, the 124bp enhancer core is already active in this cell-type [10], suggesting that the flanking regions can silence enhancer activity in pre-B cells and thereby control its developmental pattern of activity. In keeping with this observation a stepwise deletion of sequences downstream of the core led to a stepwise increase in enhancer activity. In particular, sequential 3' deletions from bp 658 to bp 523 led to an 8-fold increase in enhancer activity in the pre-B cell line 3-1 [10]. Within this region Park and Atchinson identified a binding site for NF-E1 (simultaneously cloned as YY-1, δ) [10–12]. This nuclear factor can act either as positive or negative transcriptional regulator depending on the context in which it functions [13]. However NF-E1/YY-1 appears to be an ubiquitously expressed protein and it is as yet unclear how it contributes to the developmental regulation of the κ 3' enhancer.

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We thus decided to scan the region downstream of the core for additional DNA binding proteins that may regulate the activity of the κ 3' enhancer. Interactions were found with several ubiquitously expressed and B-cell specific nuclear proteins. In addition we noted a site that is able to bind several distinct, developmentally regulated, lymphoid-specific proteins, one of which appears to be LEF-1/TCR-1 α [14, 15]. The LEF-1-like complex is inhibited by LPS stimulation, raising the possibility that this factor may be a second target for LPS mediated induction of Ig κ transcription in pre-B cells.

MATERIALS AND METHODS

Cell lines

Pre-B cell lines were maintained in RPMI supplemented with 10 % foetal calf serum, 50 μ M β -mercaptoethanol. The IL-3 dependent pro-B cell lines BaF3 [16] and LyD9 [17] were from R.Palacios and were maintained as the pre-B cell lines, except that the medium was supplemented with 10% supernatant of the IL-3 producing cell line WEHI3D+. All other cell lines were grown in DMEM, 10% foetal calf serum. The cell lines PD31, 70Z/3, 38B9 (pre-B), A20, WEHI231 (B cell lymphoma), S107, J558L and NS0 (plasmacytoma), EL4 and BW5147 (T cell lymphoma) were from our laboratory collection. In addition the cell lines 3-1 and 1-8 (pre-B) were obtained from K.Nelms, ψ 2 (a derivative of 3T3 fibroblasts), MEL cells and K562 cells from S.Wright, WEHI3D+ and WEHI274 were from A.Green and SOAS-2 and 1BR cells from T.Kouzarides (see Table 2).

Nuclear extracts

Large scale nuclear extracts were prepared according to the method of Dignam *et al.* [18] except that the nuclear proteins were extracted with buffer C containing 0.4M NaCl. Small scale nuclear extracts were prepared by NP40 lysis [19] of cells with subsequent elution of DNA binding proteins in 20mM Hepes, pH7.9, 0.4M NaCl, 1mM EDTA, 1mM DTT. Protease inhibitors (1mM phenylmethylsulfonylfluoride, 10 μ g/ml leupeptin and 1 μ g/ml aprotinin) were used in all extraction buffers.

Figure 1. Diagram of the immunoglobulin κ 3' enhancer, showing the enhancer core, the overlapping oligonucleotides A, B, C and D used in this study, and the oligonucleotide NF-E1 used in [10].

Electrophoretic mobility shift assay

EMSA was carried out with 15000cpm radioactively end-labelled oligonucleotide, which was incubated for 20 mins at room temperature with 15 μ g nuclear extract in a final volume of 20 μ l binding buffer (80mM NaCl, 10mM Tris-HCl, pH 7.9, 1mM EDTA, 10% glycerol) including 1 μ g polyd(I-C). Samples were separated on 4.5% (40:1 cross-linked) polyacrylamide gels with 5% glycerol, which was run in 0.5 $\times$ TBE buffer. Quantitation was carried out using a phosphorimager and ImageQuant software.

Oligonucleotides

Oligonucleotide A extends from bp 527 to 556 (also see Table 1), Oligo-B from bp 550 to 570, Oligo-C from bp 568 to 590 and Oligo-D from bp 577 to 606 (numbered as in [2]). These oligonucleotides, as well as m4, are flanked by *Hind*III sites, while A', m1, m2 and LEF-1 (see Table 1) are flanked by *Bam*HI sites. Other oligonucleotides used are the NF- κ B and κ E2 sites from κ intron enhancer [20], the USF site from the IgH 3' enhancer [21], the BSAP site from the H2B.2.2 promoter [22] and the EBF site from the mb-1 promoter [23].

NF- κ B: AGCTTAGAGGGGACTTCCGAGAGGAGCT
 κ E2: AGCTTAGGCACATCTGTTGCTTTCAAGCT
 USF: AGCTTCTGTGGCCACGTGGAGACAGAAGCT
 BSAP: TGTGACGCAGCGTGGGTGACGACTA
 EBF: AGCTGAGACTCAAGGGAATTGTGGCCAG
 LEF-1: GATCCGTAGGGCACCTTTGAAGCTCTCCAGATC

RESULTS

Nuclear proteins binding to Oligo-B, -C and -D

Figure 1 shows the four overlapping oligonucleotides, A, B, C and D, which were analysed. Oligonucleotide B and C both contain CANNTG motifs which form the consensus motif for helix-loop-helix transcription factor binding sites. An

Figure 2. EMSA of oligonucleotides B and C. (a) The probe indicated below the panel was incubated with nuclear extract from 3-1 pre-B cells and 5pmol of competitor oligonucleotides as indicated above each lane. (b) Oligo-B was incubated with extracts from B (A20), pre-B (3-1), T cells (BW5147) and fibroblasts (HeLa).

electrophoretic mobility shift assay (EMSA) reveals that both oligos give rise to a pattern that is indistinguishable from that obtained with a κ E2 binding site [24]. Using either Oligo-B or Oligo-C as probe, self and κ E2 oligonucleotide compete efficiently for the bands formed (Fig. 2a). Oligo-A on the other hand does not interfere with binding. Extensive analysis of the κ E2 site has shown that the major proteins binding to this site are E47/ E12 and ITF-2, which are able to give rise to B-cell specific complexes [25]. We find that Oligo-B gives rise to a B-cell restricted band (Fig. 2b), which is likely to be formed by the same proteins. Results obtained with Oligo-C were identical. Oligo-D, on the other hand, binds a nuclear factor which we have detected in B cells, T cells and fibroblasts. It can be competed with self, but not with the Oligo-A (data not shown).

Identification of a pre-B cell specific complex

Whilst the proteins binding to Oligo-B, -C, and -D are present in pre-B as well as B cells, Oligo-A gave rise to a pre-B cell specific band in an EMSA. Using nuclear extracts from different stages of B cell development two protein-DNA complexes were seen (Fig. 3). The lower band was only present in nuclear extracts from pre-B cell lines (1-8, 70Z/3, 3-1 and 38B9) while a second band is seen in extracts from cells of all stages of B cell development. The latter was also observed in HeLa cells (data not shown). The EMSA was repeated with nuclear extracts from 3-1 cells using Oligo-A' (which lacks 5bp at the 3' end) and a mutant oligo, m4 (shown in Table 1). The A' oligonucleotide formed the same complexes as Oligo-A, whilst the m4 oligonucleotide bound the ubiquitous, but not the tissue-specific nuclear complex, thus indicating that the two proteins bind to different sites within the Oligo-A.

To address the specificity of complex formation, either cold Oligo-A or oligonucleotides with binding sites for the pre-B cell specific nuclear factors EBF [23] or BSAP [22] were used as competitors in the EMSA. Using extracts of the pre-B cell line 3-1 we found that cold Oligo-A competed efficiently for both the ubiquitous band and the tissue-specific band, but binding sites for the pre-B cell specific factors BSAP or EBF did not interfere

with complex formation (Fig. 4). We will subsequently refer to the tissue-specific complex as κ 3'-A1.

Formation of the pre-B cell specific complex is repressed by LPS

As the LPS induction of the κ 3' enhancer is controlled by the flanking region [10] [own observations], the pre-B cell specific κ 3'-A1 nuclear factor is clearly a candidate to mediate this response. After 48 hrs of LPS stimulation of 3-1 cells the pre-B cell specific band is reduced approximately 4 fold (Fig. 5), while the ubiquitous band is not affected and serves as an internal control. The response of the pre-B cell line 70Z/3 is similar.

A particular hallmark of NF- κ B induction by LPS is its extremely rapid activation, which is achieved by post-translational control [1]. We were thus interested to compare this induction to the kinetics of the LPS mediated repression of the κ 3'-A1 complex. To facilitate a time-course analysis, a protocol for small scale preparation of nuclear extracts based on NP40 cell lysis

Table 1.

(a)	
mouse :	CACACCCTTTCA AGTTTCCA AAGCCTCATA
human :	-----GA-----C-----G---C-G-G
rabbit :	--AG-----T-GA-----TTG---T----
(b)	
Oligo-A :	CACACCCTTTCAAGTTTCCAAAGCCTCATA
m1 :	-----A--G--G-----
m2 :	-----G--G--T-----
A' :	-----
m4 :	-----GT-GACG--A-----
(c)	
Oligo-A :	CACACCCTTTCAAGTTTCCAAAGCCTCATA
LEF-1 :	GTAGGGCACCTTTGAAGCTCTCC
κ 3' core :	AAGACCCTTTGAGGAAGTGAAC

(a) Alignment of sequences homologous to Oligo-A from the mouse, human [8] and rabbit κ 3' enhancers [9].

(b) Oligo-A and its mutant derivatives.

(c) Alignment of Oligo-A to the LEF-1 site of the TCR- α enhancer and a homologous sequence from the mouse κ 3' enhancer core (bp 439-461).

Figure 3. EMSA of (a) Oligo-A and (b) its mutant derivatives Oligo-A' and m4 using 15 μ g of nuclear extracts of the cell lines shown above each lane.

Figure 4. Competition experiment of the bandshift obtained with Oligo-A using 15 μ g of nuclear extract from pre-B cell line 3-1. Increasing concentrations of the relevant cold competitor oligonucleotide (none, 5pmol, 25pmol or 50pmol) are indicated above the lanes.

Figure 5. The effect of LPS on nuclear complex formation using Oligo-A and nuclear extracts from 3-1 and 70Z/3 pre-B cells lines before and after 48 hrs of LPS stimulation.

Figure 6. Time course analysis of DNA-protein complex formation in nuclear extracts from LPS stimulated 3-1 cells on (a) Oligo-A, (c) NF- κ B, and (d) USF. (b) Quantitation of complex formation shown in panel a. The intensities are given relative to the strength of complex formation in extracts with no LPS stimulation, which was taken to be 1 unit.

was utilised. When assayed with Oligo-A the resulting extracts contained high levels of the tissue-specific band (χ 3'-A1), whilst the ubiquitous band was now no longer detectable. Extracts were prepared after 0, 0.5, 3, 6 and 24 hrs of LPS stimulation and

Figure 7. EMSA of Oligo-A (upper panel) and USF (lower panel) using extracts from lymphoid and fibroblasts cell lines. Complexes formed by the nuclear factors χ 3'-A1, A2 and A3 are indicated by arrows. All cell lines are derived from mouse except 1BR which is human in origin.

tested for binding of Oligo-A, NF- κ B or USF (Fig. 6, panel a, c and d). In response to LPS, NF- κ B is fully induced within 30 mins, whilst the amount of χ 3'-A1 complex decreases slowly with time. USF was employed as control, because it is not affected by LPS treatment. Quantitation of the Oligo-A gelshift (Fig. 6a) using a phosphorimager shows an exponential decay of band intensity, with binding being reduced to about 50% after 24 hrs (Fig. 6b). Such kinetics would be expected if either no new active protein is produced or if an inhibitor of binding, which accumulates with time, is synthesized.

Oligo-A spans a binding site for LEF-1 and distinct, lymphoid specific nuclear factors

As Oligo-A appears to span a developmentally regulated site, we extended our analysis of complex formation to a wider panel of lymphoid cell lines. Using small scale nuclear extracts several distinct DNA-protein complexes were observed (Fig. 7). The high-mobility form (χ 3'-A1) seen in pre-B cells is also observed in two T cell lines (EL4 and BW5147), but in addition these cells also give rise to a low mobility band (χ 3'-A3). This low mobility form differs from the ubiquitous band seen in Dignam extracts since it is not detectable in plasma cells (NS0, MPC11) or non-lymphoid cell lines (ψ 2, 1BR). A band of low mobility is also present in the pro-B cell extracts from LyD9 and BaF3 cells. The pro-B and T-cell low mobility complexes do not appear to comigrate (Fig. 7). Further biochemical characterisation is

Table 2.

Lymphoid cells			
cell type	cell line	nuclear factor	elec. mobility
pro-B	LyD9	$\kappa 3'$ -A3	L
	BaF3	$\kappa 3'$ -A3	L
pre-B	1-8	$\kappa 3'$ -A1	H
	PD31	$\kappa 3'$ -A1	H
	70Z/3	$\kappa 3'$ -A1	H
	3-1	$\kappa 3'$ -A1	H
	38B9	$\kappa 3'$ -A1	H
B cells	A20	$\kappa 3'$ -A2/3	M/L
	WEHI231	$\kappa 3'$ -A2/3	M/L
	BCL1	$\kappa 3'$ -A2/3	M/L
plasma	S107	-	
	J558	-	
	NS0	-	
	MPC11	-	
T cells	EL4	$\kappa 3'$ -A1/3	L/H
	BW5147	$\kappa 3'$ -A1/3	L/H
Non-lymphoid (mouse or human)			
erythroid	MEL	-	
	K562	-	
monocytic	WEHI3D+	-	
	WEHI274	-	
myeloid	$\psi 2$	-	
	1BR	-	
	HeLa	-	
fibroblast	SOAS-2	-	

Tissue distribution of Oligo-A binding factors from different lymphoid and non-lymphoid cell lines. All lines are derived from mouse, except for the human K562, 1BR, HeLa and SOAS-2 cell lines. Electrophoretic mobility: L: low, M: medium, H: high.

required to establish whether this reflects post-translational modifications or whether the proteins are non-identical. Using B-cell extracts we observed the formation of two weak complexes with low ($\kappa 3'$ -A3) and medium ($\kappa 3'$ -A2) electrophoretic mobility. In extracts prepared by the Dignam method these bands were masked by the presence of the ubiquitous protein-DNA complex (Fig. 3). However in the previous analysis of extracts from the B cell line A20 the upper band does in fact appear as a doublet (Fig. 3). The lower panel of figure 6 shows a control EMSA with the USF oligonucleotide, using the same nuclear extracts as in the upper panel. No Oligo-A binding factors were detected in non-lymphoid or non-haematopoietic cell lines (Table 2). Thus, in conclusion, Oligo-A binding factors are strictly lymphoid specific and are regulated in lymphoid development.

The tissue-distribution of the $\kappa 3'$ -A1 complex corresponds exactly to the expression pattern of the LEF-1/TCF-1 α gene [14, 15]. Furthermore Oligo-A and the binding site for LEF-1 are highly homologous (see Table 1). Indeed the LEF-1 site from the TCR- α enhancer can compete for $\kappa 3'$ -A1 complex formation and the two sites appear to bind the nuclear factor with similar affinity (Fig. 8a). In contrast, the low mobility complexes ($\kappa 3'$ -A3) seen in T cells and pro-B cells were not sensitive to competition with the LEF-1 site from the TCR- α enhancer (Fig. 8b). A 3bp mutation which destroys the LEF-1 site (m1, Table 1) significantly reduced competition for the high mobility band, strengthening the view that LEF-1 and $\kappa 3'$ -A1 may be identical. The m1 oligo still effectively competed for the $\kappa 3'$ -A3 complex, indicating that the formation of the $\kappa 3'$ -A1 and -A3 complexes is independent and is likely to be mediated by different proteins. The m2 oligo with a mutation in the 5' end of Oligo-A competed

Figure 8. Competition analysis of nuclear complexes formed on Oligo-A; (a) complex A1 formed by extracts from 3-1 pre-B cells competed with 1, 5 and 25 pmol of the competitor shown above the lane; and (b) complexes A1 and A3 formed by nuclear extracts from pro-B cells (BaF3) and T cells (EL4) competed with 25pmol of each of the cold oligonucleotides indicated above each lane.

efficiently for all complexes seen. Within the $\kappa 3'$ enhancer a second site with strong homology to the LEF-1 site was identified in the enhancer core (Table 1). However, when this site and Oligo-A were examined in EMSA, no comigrating bands were seen. The homologous site in the enhancer core overlaps the previously described PU.1 site and steric hindrance by PU.1 may prevent LEF-1 binding.

Thus, we have identified three lymphoid specific nuclear complex, $\kappa 3'$ -A1, A2 and A3. In its tissue-distribution and its ability to compete for binding, the $\kappa 3'$ -A1 complex is indistinguishable from the cloned nuclear factor LEF-1. In pre-B cells the $\kappa 3'$ -A1 complex is sensitive to LPS stimulation, which makes it a candidate for mediating the developmental control of $\kappa 3'$ enhancer activity.

DISCUSSION

The work presented here describes a number of nuclear proteins that may be involved in controlling the activity of the Ig κ 3' enhancer. We have identified two binding sites for the

transcription factors E47/E12 (Oligo-B and -C) and binding sites for two ubiquitous nuclear factors (Oligo-D and the 5' end of Oligo-A). However, of particular interest is the identification of a potential binding site for the nuclear factor LEF-1/TCF-1 α , which was cloned as a lymphoid specific factor binding to the TCR- α enhancer [14, 15]. Several lines of evidence suggest that the protein (κ 3'-A1) binding to a site just downstream of the κ 3' enhancer core (Oligo-A, see Fig. 1) is LEF-1. Firstly, the LEF-1 site and Oligo-A show the same affinity for κ 3'-A1 binding, and secondly, the tissues in which complex formation is seen correlate perfectly with the expression pattern of the LEF-1 gene [14, 26]. Strikingly, LPS stimulation, which induces κ gene expression in pre-B cells and inhibits LEF-1 expression [14], also leads to a decrease in the amount of κ 3'-A1 complex formed (Figs. 5 and 6). The LEF-1 site of the κ 3' enhancer lies within a region that had previously been implicated in the negative regulation of κ enhancer activity in pre-B cells [10]. Furthermore, the LEF-1 expression pattern within the lymphoid lineage correlates exactly with the absence of the κ gene rearrangement and expression. We therefore speculate that LEF-1 plays a negative regulatory role in κ gene expression.

Within the context of the TCR- α enhancer, LEF-1 has been shown to function as a transcriptional activator [14, 15, 27] and its activation domain has been mapped [28, 29]. However, a multimerized LEF-1 site is unable to drive expression of a reporter gene construct [14, 30] and, when linked to a heterologous enhancer element, the LEF-1 site can act as a repressor of enhancer function in T cells [31]. In keeping with these observations we find that a multimerized Oligo-A is unable to stimulate transcription from a c-fos promoter in either pre-B or T cells (data not shown). Upon binding to DNA, LEF-1 induces a sharp bend in its target site and it was postulated that LEF-1 may act, in part, by allowing the functional interaction between proteins bound at widely separated sites [32]. In the same way LEF-1 may distort the DNA to prevent the functional contact between bound transcription factors, thereby acting as a repressor. As LEF-1 function is highly context-dependent [27, 28], extensive mutagenesis will have to be carried out to assess the function of the LEF-1 site within the κ 3' enhancer.

In addition to LEF-1 further lymphoid-specific nuclear complexes were formed on Oligo-A. A complex with low electrophoretic mobility (κ 3'-A3) differed in its binding site specificity from the LEF-1-like complex suggesting that it is formed by distinct proteins. In analogy to the TCR- α enhancer function, additional proteins may be required to achieve cell-type specific control via the LEF-1 site [29–31, 33]. This may be particularly important in switching from suppressor to enhancer function [31].

Previous analysis of the κ 3' enhancer region that can silence enhancer activity has identified a binding site for NF-E1/YY-1. By itself, YY-1 appears to lack the ability to either activate or repress transcription but instead mediates transcriptional repression or activation by regulating contact between other proteins through DNA bending [34]. Thus two proteins binding within close proximity in the 3' enhancer can bend DNA and the potential interference or cooperativity between the LEF-1 and YY-1 sites needs to be examined. Evidence for a potential interaction comes from the observation that mutating the NF-E1/YY-1 site alone will only lead to a 30% increase in enhancer activity while the 3' flanking region encompassing NF-E1/YY-1, LEF-1 and possibly additional sites can reduce activity by 71 to 89% [10].

For high levels of transcription of the Ig κ gene both the κ intron enhancer and the κ 3' enhancer are required [3, 35]. While the developmental induction of the κ intron enhancer is mediated by NF- κ B it remains an outstanding question how the κ 3' enhancer is switched on. We present here the identification of the first nuclear factor that binds within the κ 3' enhancer and is responsive to LPS treatment in pre-B cells. This factor, likely to be LEF-1, may thus be involved in controlling the developmental regulation of κ 3' enhancer activity. It is interesting to note that LEF-1 is expressed not only in pre-B cells but also in T cells. An artificial rearrangement construct including unrearranged V κ - and J κ -genes, a κ constant region and the κ intron enhancer will rearrange in B cells but, unlike the endogenous gene, the construct will also recombine in thymus [36] indicating that additional control elements may be required to restrict κ gene expression to the correct tissue. It will be interesting to examine whether the LEF-1 site within the κ 3' enhancer can play such a role.

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