PERGAMON

Molecular Immunology 37 (2000) 961–973

Molecular Immunology

www.elsevier.com/locate/molimm

Identification and characterization of two CD40-inducible enhancers in the mouse TRAF1 gene locus

Ian F. Dunn, Tatyana Y. Sannikova, Raif S. Geha, Erdyni N. Tsitsikov *

Division of Immunology, Children's Hospital, Department of Pediatrics, Harvard Medical School, 300 Longwood Ave., Boston, MA 02115, USA

Received 29 November 2000; accepted 1 March 2001

Abstract

We have shown that CD40 engagement induces TRAF1 gene expression in B lymphocytes. Here we report that CD40-dependent TRAF1 gene transcription in murine B cells is controlled by two enhancer regions. One region is located approximately 2 kb upstream of the transcription start site and the other lies in the intron between exons 5 and 6. The upstream enhancer contains a single NF-κB site in addition to sites that bind constitutive transcription factors. Mutation of this NF-κB site completely abrogates CD40-driven TRAFl transcription. The intronic enhancer contains two sites that strongly bind the CD40-inducible factors NF-κB and AP-1. Simultaneous mutation of the AP-1 site and of the NF-κB site abolishes transcription driven by this enhancer. When cloned together into reporter constructs, the two TRAF1 enhancers do not synergize, suggesting that each enhancer may separately participate in the induction of TRAF1 transcription in B cells following CD40 activation.

Keywords: B lymphocytes; Gene regulation; Transcription; CD40

1. Introduction

Members of the TNF receptor (TNFR) superfamily play important roles in a wide array of biological processes including the acute phase response, cell growth and apoptosis, inflammation and lymphocyte activation (Baker and Reddy, 1996). Receptors in the TNFR superfamily, which lack a death domain, include TNFR2, LT-R, CD27, CD30, CD40, OX-40, and 4-1BB. Each of these may associate with one or several TNF Receptor Associated Factors (TRAF) (Arch et al., 1998). Originally, TRAF1 and TRAF2 were discovered, because of their ability to bind to TNFR2 (Rothe et al., 1994). Altogether, six TRAF molecules have been identified to date (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Regnier et al., 1995; Sato et al., 1995; Cao et al., 1996; Ishida et al., 1996a,b; Nakano et al., 1996). TRAF molecules are generally characterized by the presence of an N-terminal RING finger, several zinc fingers and a C-terminal TRAF domain, which is important for interactions with receptors and other TRAF proteins.

TRAF1 is unique member in that it contains a single zinc finger and TRAF domain. TRAF1 can be recruited to a variety of receptors including TNFR2, CD30, 4-lBB, OX-40, HVEM/ATAR, and TRANCE-R. Little is known about the function of TRAF1 except that when overexpressed in T cells of transgenic mice, it plays an inhibitory role in the antigen-induced apoptosis of $CD8^+$ T lymphocytes (Speiser et al., 1997). Recently, the genes that encode for TRAF1, TRAF2, and the inhibitor-of-apoptosis proteins (IAPs) were identified as targets of NF-κB-dependent transcriptional activity (Wang et al., 1998). Moreover, TRAFl belongs to a group of NF-κB-dependent gene products that function to suppress TNFα-mediated apoptosis and is one of the key regulators of the cellular stress response.

Expression of TRAF1 is tissue-specific and its mRNA has been detected in spleen, tonsil, lung, and

Abbreviations: TNFR, TNF receptor; TRAF, TNF receptor associated factor; TRANCE-R, TNF-related activation-induced cytokine receptor; LT-R, lymphotoxin receptor; HVEM, herpesvirus entry mediator; ATAR, another TRAF-associated receptor; LMP1, latent infection membrane protein 1; HMG, high mobility group.

* Corresponding author. Tel.: +1-617-7354174; fax: +1-617-7358205.

E-mail address: tsitsikov_e@hub.tch.harvard.edu (E.N. Tsitsikov).

0161-5890/01/$ - see front matter
PII: S0161-5890(01)00015-3

testis (Rothe et al., 1994; Mosialos et al., 1995). While TRAF1 message is absent from resting lymphocytes, macrophages, and dendritic cells, it is expressed after cell activation (Durkop et al., 1999). Recently, we demonstrated that TRAFl mRNA is not expressed in non-stimulated splenocytes but can be induced upon activation by different stimuli, including LPS, *anti*-CD3, *anti*-IgM, or *anti*-CD40 antibodies (Dunn et al., 1999). In B cells, TRAFl, TRAF2, TRAF3, and TRAF6, but not TRAF4 or TRAF5, bind directly to the CD40 cytoplasmic domain (Pullen et al., 1998).

We and others have shown that TRAF1 as well as TRAF2 associate with CD30, which was originally identified as a cell surface antigen on Hodgkin and Reed Sternberg cells (Ansieau et al., 1996; Gedrich et al., 1996; Lee et al., 1996; Duckett et al., 1997; Tsitsikov et al., 1997). While nearly all non-Hodgkin's lymphoma show low or no TRAFl expression, TRAFl is highly expressed in most of Epstein-Barr virus (EBV) induced lymphoproliferations and Hodgkin's disease (Durkop et al., 1999). Indeed, human TRAF1 was originally cloned as an EBV-induced protein from EBV-transformed lymphoblastoid cell line (Mosialos et al., 1995). Interestingly, it was shown that EBV latent infection membrane protein 1 (LMP1), which is essential for the immortalization of human B cells, binds several TRAF proteins, including TRAFl, TRAF2 and TRAF3. The LMP1 TRAF binding domain is critical for primary B lymphocyte growth transformation (Izumi et al., 1997, 1999). Furthermore, LMPl expression in Burkitt B lymphoma cells strongly increases TRAF1 expression (Devergne et al., 1998). In agreement with these results, LMPl co-localizes and precipitates with TRAF-l and TRAF-3 in EBV and LMPl-positive tumors (Liebowitz, 1998). Taken together, these observations suggest that TRAF1 is expressed following EBV transformation of B cells and may be important for LMPl signaling in B cells.

In this study we investigated the induction of the murine TRAFl gene in activated B cells. Specifically, we studied TRAF1 gene transcription following CD40 signaling, which is critical for B cell activation, proliferation, immunoglobulin isotype switching, and affinity maturation. We report that induction of the murine TRAFl gene by CD40 is controlled by two enhancer regions. One of these regions is located 2 kb upstream of the TRAF1 transcription start site, while the other is located in the intron between exons 5 and 6. Electrophoretic Mobility Shift Assay (EMSA) analysis revealed that the upstream enhancer contains a single strongly inducible NF-κB site in addition to sites for a number of constitutive nuclear factors. Mutation of this site completely abrogates CD40-driven transcription. The intronic enhancer contains five sites which bind CD40-inducible factors: two sites bind AP-l proteins and three bind NF-κB proteins. Simultaneous mutation of the AP-1 site and of the most avid NF-κB site in the intronic enhancer abolishes enhancer activity.

2. Materials and methods

2.1. Cells and antibodies

The murine M12 B lymphoma cell line (ATCC) and the murine T cell hybridoma 68–41 (a generous gift of Dr Masato Kubo) were maintained in high glucose DMEM medium supplemented with 10% heat-inactivated fetal calf serum (Hyclone), 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 200 u/ml penicillin and 200 μg/ml of streptomycin. The murine J774 monocytic cell line was maintained in LPS-free medium. *Anti*-CD40 (HM40-3), *anti*-CD3 (145-2C11) and isotype control mAbs were purchased from Pharmingen. LPS was obtained from Sigma. Rabbit *anti*-Ets-1, *anti*-Fos and *anti*-Jun antisera were purchased from Santa-Cruz Biotechnology. These antibodies were raised to peptides that are highly conserved among individual members of the particular family and that crossreact with all known members of that family. Normal rabbit serum was purchased from Sigma. *Anti*-NF-κB antibodies were provided by Dr Nancy Rice (NIH) and described previously (Ernst et al., 1995).

2.2. Reporter vector construction

The DNA fragments of the TRAF1 proximal promoter were amplified by polymerase chain reaction (PCR) using corresponding primers and high fidelity Pfu DNA polymerase (Stratagene). Taq polymerase was used to add a 3′ A-overhang to the PCR products, which were later cloned into a pCR2 vector (Invitrogen) and sequenced. These fragments were subcloned in appropriate orientation upstream of the luciferase gene in the luciferase reporter vector pGL3-Basic (Promega). Truncated enhancer fragments were amplified by PCR using primers with additional 5′ nucleotides containing restriction sites. Final PCR products had an *Xho*I site at the 5′ end and *Sal*I at the 3′ end relative to the TRAF1 gene to enable orientation determination when subcloned upstream or downstream of the luciferase gene in pGL3-Basic (Promega). The N2M5 and the P2R2 fragments were ligated together into pGL3-Minimal vector upstream of the minimal promoter and fragment downstream of the luciferase gene, respectively. Site-directed mutagenesis was performed with the QuickChange kit (Stratagene). Using site directed mutagenesis we replaced the NF-κB binding site (GGGTAC) in the UP2 sequence from the N2M5 region corresponding to the 5′ upstream enhancer with a *Hind*III site. We also replaced the AP-1 (TGAGTC) and NF-κB (GGGCTT) binding sites from the IN1 and

1N6 sequences from the P2R2 region of the intronic enhancer with *Sac*I and *Hind*III sites, respectively.

2.3. Transfections and Luciferase assay

M12 cells (3×10^6) were transfected with 1 μg of reporter plasmid using the CellPhect transfection kit (Amersham Pharmacia), split into two wells of the 24-well plate and cultured in medium containing 1% FCS. Six hours later one well of M12 cells was left unstimulated and another well was stimulated with *anti*-CD40 mAb (1 μg/ml) for 24 h. Transfection of 68–41 cells was performed using the CellPhect transfection kit. After transfection 68–41 cells were cultured in complete medium. Six hours later, each culture of transfected 68–41 cells was divided into two wells of the 24-well plate. One well was pre-coated with isotype control mAb while another was pre-coated with *anti*-CD3 mAb. Wells were precoated overnight at 4°C with 5 μg/ml of mAb in PBS. J774 cells were transfected at 2×10^4 cells/well in the 24-well plates using calcium-phosphate transfection kit (Invitrogen) according to manufacturer recommendations for adherent cells. Six hours after transfection one well of J774 cells was left unstimulated while another was stimulated with LPS (l μg/ml). Firefly luciferase activity was measured 24 h after the stimulation with the Luciferase assay kit (Promega) and Turner Luminometer. Protein concentration was determined by bicinchoninic acid (BCA) protein assay kit (Pierce). In each sample, relative luciferase intensity was determined as the amount of luciferase activity per 1 μg of protein. Data for stimulated or unstimulated cells were normalized to the corresponding results obtained after transfection with the pGL3-Basic vector. In each transfection with a particular plasmid, fold induction was calculated as a ratio of the specific activities for stimulated cells to unstimulated cells. The pGL3-Control vector, which contains the SV40 early promoter and enhancer sequences, was purchased from Promega and used as a luciferase assay control. Efficiency of transfection was verified by co-transfection with the pCAT-Control vector. CAT activity was determined as previously described in (Tsitsikov et al., 1995).

2.4. Nuclear extracts

Two hours before stimulation Ml2 cells were transferred into media supplemented with 1% FCS. Cells at 10^6/ml were either left unstimulated or were stimulated with *anti*-CD40 mAb (1 μg/ml) for 1 h. Nuclear extracts were prepared as previously described in (Tsytsykova et al., 1996). Briefly, 3×10^6 of M12 cells were washed twice with ice-cold PBS, resuspended in cold l0 mM Hepes buffer, pH 7.9, containing 0.1% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and Complete Protease Inhibitor (CPI) cocktail (Roche) and incubated for 10 min on ice. For primary B cells, the same cell lysis solution was used, but without NP-40. Nuclei were pelleted for 2 min at 5000 rpm at 4°C and resuspended in ice-cold 20 mM Hepes buffer pH 7.9, containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and CPI cocktail. After incubation on ice for 20 min, debris were removed by centrifugation at 14 K rpm for 10 min at 4°C. The supernatants were aliquoted and frozen at − 80°C. The protein concentration was estimated by the BCA protein assay kit.

2.5. Electrophoretic mobility shift assay

Single-stranded oligonucleotides were 5′-end-labeled with [γ^{32}P]-ATP using T4-polynucleotide kinase, annealed, and purified on 12% PAGE in 1 × TAE. For each reaction, 20×10^3 cpm (~ 0.1 ng) of radiolabeled oligonucleotide probe was incubated with 1–5 μg of nuclear extract in 20 μl of binding buffer for 30 min on ice or at room temperature. The binding buffer contained l0 mM Tris–HCl pH 7.5, 50 mM NaCl, 5% glycerol, 50 ng/ml poly (dI-dC), 1 mM EDTA, 0.1% NP-40, 1 mM DTT, and protease inhibitors cocktail. Samples were then run on 5% polyacrylamide gel electrophoresis (PAGE) in l × TBE. Unlabeled competitors were added at 100-fold molar excess. When indicated, the nuclear extracts were preincubated with the respective immunoreagent for 30 min before addition of radiolabeled oligonucleotides. Gel densitometry was used to determine the intensity of bands after addition of particular antibodies. Sequences for the short UP2, IN1, and IN6 oligonucleotides are GAGTTTTGGGTACTTTCCGAAATTAAA, TGACTTCTGAGTCATAGGTGAACAGCT and TGACTAAAGGGCTTTCCCTTCTATTTC, respectively. Additional oligonucleotides used in these experiments are as follows: NF-κB site of human immunodeficiency virus (HIV) 1 Long terminal Repeat (LTR), TCGCTGGGGACTTTCCAGGGA (Nabel and Baltimore, 1987); consensus AP-1, CGCTTGATGAGTCAGCCG (Promega); and Ets-1 oligonucleotide from human TCRα promoter (Tα2C), (AGAAGCCACATCCTCTGG).

DNA sequence analysis for potential transcription factor binding sites was performed using Genetyx-SV/RC 10.1 software and the All-in-one sequence analyzer (TFSEARCH) by Dr YutakaAkijima (http://www-personal.umich.edu/ ~ ino/blast.html). Sequence analyzer searches highly correlated sequence fragments within the TRANS FAC database (Wingender et al., 2000) of eukaryotic *cis*-acting regulatory DNA elements and *trans*-acting factors (http://transfac.gbf.de/TRANSFAC/index.html). The scoring system is described by the formula:

Score = 100.0 (weighted sum − min)/(max − min).

3. Results

3.1. Characterization of the murine proximal TRAF1 promoter

We have recently cloned the murine TRAF1 gene, determined its transcription start site, and sequenced its proximal promoter (Dunn et al., 1999). We have also demonstrated that TRAF1 mRNA can be induced in the mouse B cell line, M12, following CD40 engagement. To map the CD40 responsive proximal promoter of the TRAFl gene we generated a series of 5′ truncation fragments that begin at different positions (− 762, − 262, − 162, and 102 bp) relative to the transcription start site and that all end within the first exon at + 38 bp. These fragments were placed upstream of the luciferase gene in the pGL3-Basic reporter vector and introduced by transient transfection into M12 cells. Fig. 1A shows that none of these fragments was able to drive reporter gene expression in the M12 B cell line following stimulation with *anti*-CD40 mAb, while CD40 signaling can strongly induce luciferase activity after transfection with the pGL3-Control vector, which is regulated by the SV40 minimal promoter and enhancer. The SV40 enhancer was shown to contain functional binding sites for NF-κB and AP-1 nuclear factors (Lee et al., 1987; Nakamura et al., 1989), which may ascertain successful CD40-inducible transcription of the pGL3-Control vector in the Ml2 cell line. Fig. 1B shows that the shortest fragment (− 102/ + 38 bp) was able to strongly support the SV40 enhancer dependent transcription in M12 cells following stimulation with *anti*-CD40 mAb. Here, the SV40 enhancer may mimic the original TRAFl enhancers located elsewhere outside the − 762 to + 38 bp region. Taken together, these results suggest that the CD40 responsive element/s of the murine TRAF1 gene lies outside the − 762 to + 38 bp region and DNA fragment between − 102 and + 38, relatively to the transcription start site, corresponds to a short proximal promoter capable of initiating mRNA synthesis. The sequence of the − 102/ + 38 bp fragment is shown in Fig. 1C. This fragment is GC-rich (72%) and includes a transcription start site, three 10 bp long identical repeats, and a GC-stretch, but no TATA-box, CAAT sequence, or initiator sequence. The GC rich content suggests that the TRAF1 minimal promoter may be Spl-dependent. We subsequently refer to this fragment as the TRAFl minimal promoter and to the pGL3-Basic plasmid containing this fragment upstream of the luciferase gene as the TRAF1 minimal promoter vector.

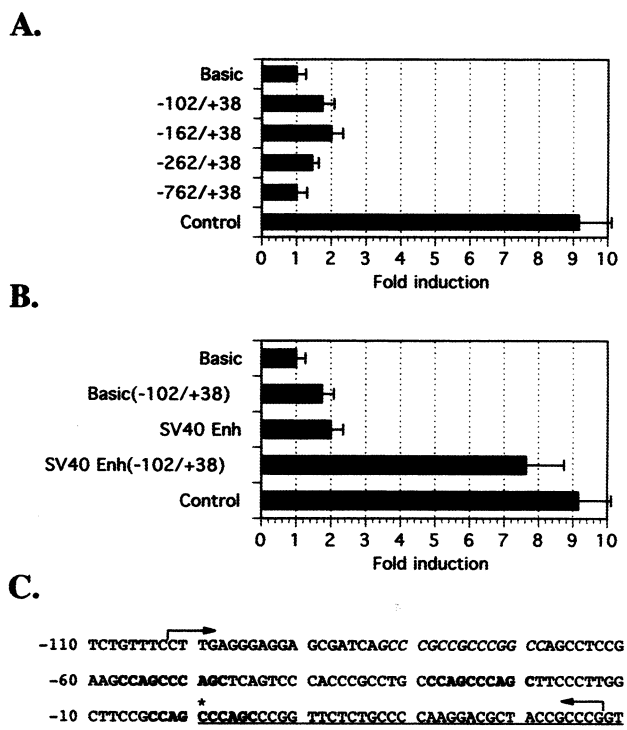

Fig. 1. Characterization of the murine TRAFl gene proximal promoter. (A) Induction of the murine proximal TRAF1 promoter following *anti*-CD40 stimulation. Different 5′ truncated fragments of mTRAFl promoter were cloned into pGL3-Basic vector. (B) Together with SV40 enhancer, the murine TRAFl minimal promoter can drive CD40-inducible transcription. The − 102/ + 38 fragment was ligated into pGL3-Basic (Basic) or pGL3-Enhancer (SV40 Enh) vector. For A and B, the resulting reporter constructs and pGL3-Basic (Basic) were transfected into M12 cells. The pGL3-Control vector (Control), containing the SV40 early promoter and enhancer sequences, was used as a luciferase assay control. After transfection cells were divided into two equal aliquots. One aliquot was left unstimulated, while another was stimulated with *anti*-CD40 antibody. Luciferase activity was determined 24 h later and fold inductions were calculated as described in Section 2. Data represent the mean ratios of three independent experiments with error bars representing the S.E. of the mean. (C) Nucleotide sequence of the TRAF1 gene minimal promoter. PCR primers used to generate the − 102/ + 38 fragment are labeled with arrows. The 10 bp long repeats and GC-stretch sequences are shown in bold and italic, respectively. Transcription start site is indicated by asterisk. Part of the first exon is underlined.

3.2. Localization of the murine TRAF1 enhancers

To map the genomic regions important for CD40-inducible transcription of the murine TRAF1 gene, we subcloned large genomic fragments (Fig. 2A) in sense orientation upstream of the − 102/ + 38 bp fragment into the TRAF1 minimal promoter vector. The resulting constructs were assayed for the ability to drive reporter gene expression following stimulation of Ml2 cells with *anti*-CD40 antibody. These fagments are spread over an 18 kb area of the TRAF1 genomic locus, starting 5 kb upstream of the transcription start site and ending just 5′ of exon 6. This approach allowed us to screen a relatively large area, including the 5′

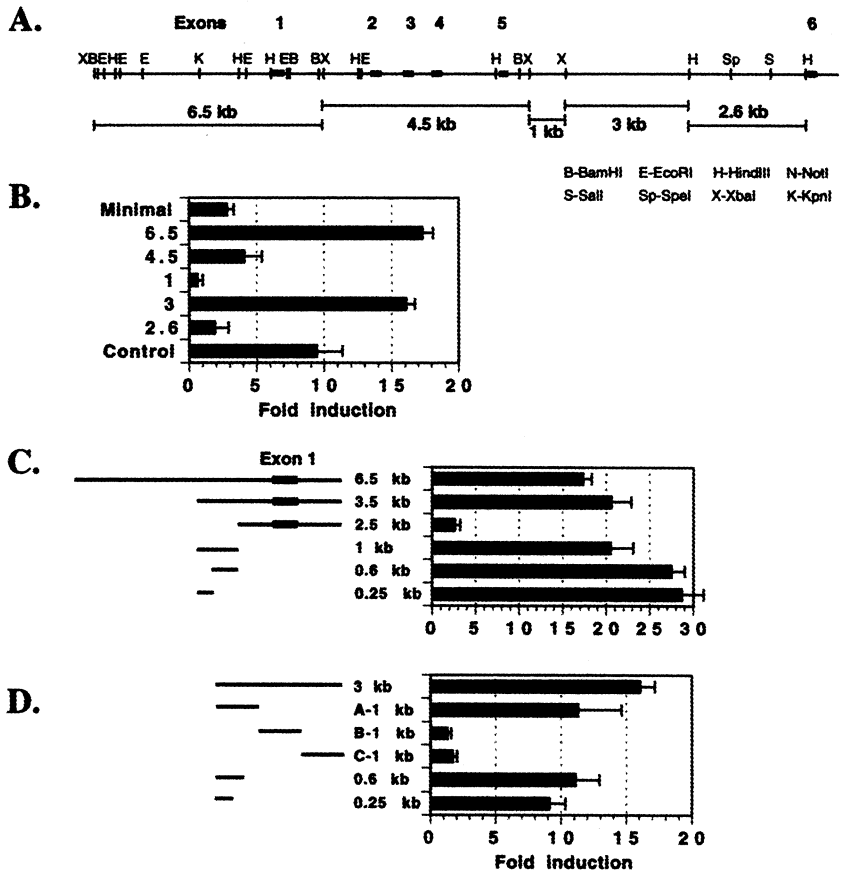

Fig. 2. Localization of the TRAF1 enhancers. (A) Partial restriction map of the murine TRAF1 gene. Different genomic fragments used for transfection study are indicated. (B) The indicated fragments were subcloned upstream of the minimal promoter in the TRAFl minimal promoter vector in sense orientation. Transfection with the TRAF1 minimal promoter vector without any inserts is indicated as minimal. The pGL3-Control vector (Control), containing the SV40 early promoter and enhancer sequences, was used as a luciferase assay control. Localization of the 5′ upstream (C) and intronic (D) enhancers. The indicated fragments were subcloned into the TRAF1 minimal promoter reporter vector. For B, C and D, the resulting reporter constructs were transfected into M12 cells. After transfection cells were divided into two equal aliquots. One aliquot was left unstimulated, while another was stimulated with *anti*-CD40 antibody. Luciferase activity was determined 24 h later and fold inductions were calculated as described in Section 2. Data represent the mean ratios of three independent experiments with error bars representing the S.E. of the mean.

upstream region and the intron between exons 5 and 6, which is not present in the human TRAF1 gene (Siemienski et al., 1997). As shown in Fig. 2B, only two of five fragments tested strongly enhanced CD40-dependent transcription: a 6.5 kb fragment that extends 5 kb upstream of the start site and into the first intron and a 3 kb fragment located in the intron between exons 5 and 6. The enhancing activity of these fragments was independent of position because they enhanced CD40 inducible reporter gene expression to the same degree regardless of whether they were subcloned into the TRAF1 minimal promoter vector upstream or downstream of the luciferase gene (data not shown).

To localize the precise position of the enhancer within the 6.5 kb region, we examined the enhancing ability of smaller fragments in this region. Using in-vector deletions, we narrowed the position of the enhancer activity to a 1 kb KpnI/EcoRI fragment (Fig. 2C). Using a series of nested 5′ and 3′, deletions of this 1 kb fragment, we mapped the enhancer activity to a 0.25 kb region, which is located 2 kb upstream of the murine TRAF1 gene transcription start site.

To finely map the CD40-dependent enhanceris located in the intron between exons 5 and 6, we generated three nested overlapping 1 kb fragments using PCR and localized the intronic enhancer to one of these three fragments (Fig. 2D). Using a series of nested 5′ and 3′, deletions of this fragment, the enhancer activity was mapped to a 0.25 kb region (Fig. 2D) approximately 2 kb downstream of exon 5.

The enhancer activity of the 250 bp regions in both enhancers was found to be position and orientation independent. We cloned each of them in both sense and antisense orientations 5′ and 3′, of luciferase gene in the

reporter vector. Neither position nor orientation change decreased the CD40 responsiveness of each enhancer (data not shown).

3.3. Fine mapping and characterization of the CD40 dependent TRAF1 gene enhancers

To finely map the boundaries of the two enhancer regions, we performed further truncations using PCR. Analysis of the 0.25 kb upstream enhancer revealed a core enhancer 104 bp long, defined by primers N2 and M5 (Fig. 3A). Further deletions (N3 or M6) in this region significantly decreased the enhancer activity. Similar analysis of the 0.25 kb intronic enhancer revealed a core intronic 195 bp long defined by primers P2 and R2 (Fig. 3B). Further deletion in the region using primers P3 or R3 significantly decreased the CD40 inducibility of the intronic enhancer.

Next, transcription activity of the specific N2M5 and P2R2 PCR fragments was ascertained. Each fragment was able to enhance CD40-dependent transcription when placed 5′ upstream of the minimal promoter in the TRAFl minimal promoter vector (Fig. 4A). Similar results were obtained when each enhancer was placed 3′ downstream of luciferase gene

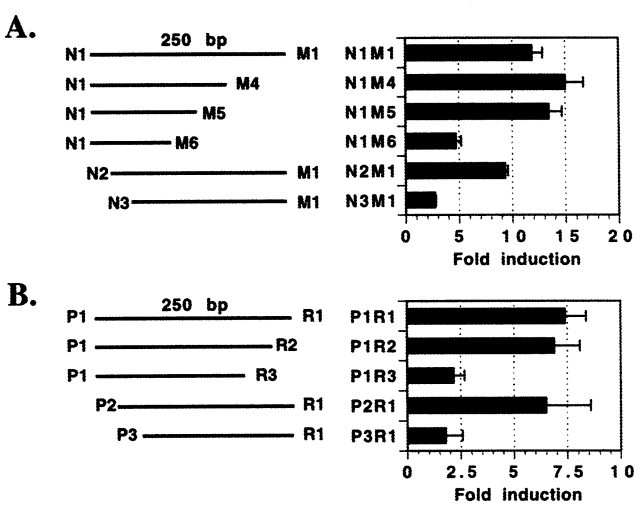

Fig. 3. Fine mapping of the upstream (A) and intronic (B) enhancers. Truncated DNA fragments of the enhancers were generated by PCR using primers shown at the ends. The N1Ml and P1R1 fragments are 250 bp long. Indicated fragments were subcloned into the TRAF1 minimal promoter vector. The resulting reporter constructs were transfected into M12 cells. After transfection cells were divided into two equal aliquots. One aliquot was left unstimulated, while another was stimulated with *anti*-CD40 antibody. Luciferase activity was determined 24 h later and fold inductions were calculated as described in Section 2. Data represent the mean ratios of at least three independent experiments with error bars representing the S.E. of the mean.

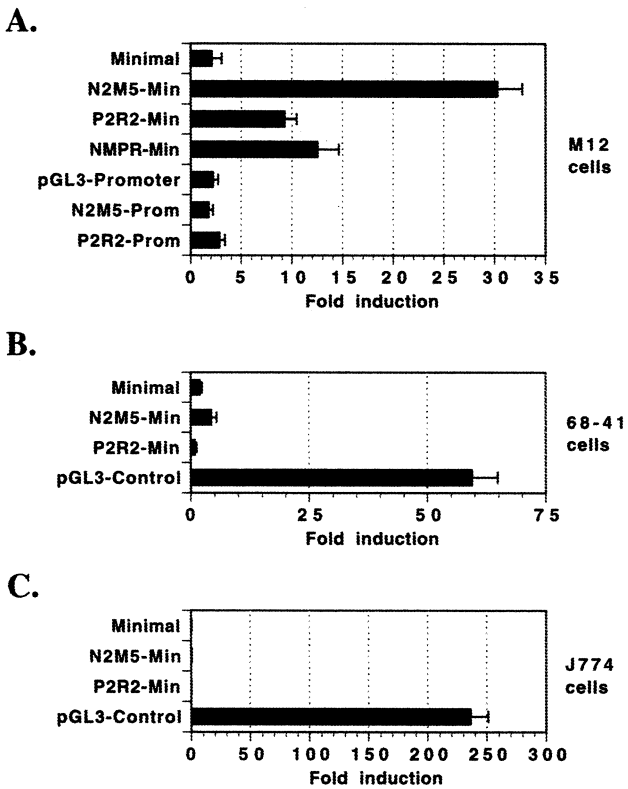

Fig. 4. Characterization of the enhancers. Activity of the enhancers in B cell line Ml2 (A), in T cell line 68–41(B) and monocytic cells line J774 (C). The upstream (N2M5) and intrionic (P2R2) enhancers were subcloned separately or together (NMPR) into the TRAF1 minimal promoter vector (Minimal) or pGL3-Promoter (pGL3-Prom) vector containing the SV40 early minimal promoter. The resulting reporter constructs were transfected into indicated cell lines and transcription activity was determined as described in Fig. 3. Transfection with pGL3-Control (Control) was used as internal control for promoter and enhancer activities.

(data not shown). When placed together into the TRAF1 minimal promoter vector, these two enhancers do not exhibit any synergistic effect, but display activity slightly higher than single P2R2 enhancer and significantly lower than single N2M5 enhancer. These results suggest that these enhancers may function independently of each other. Next, we placed each enhancer into pGL3-Promoter vector containing the SV40 early minimal promoter (Fig. 4A). We found that neither N2M5 nor P2R2 enhancer induced transcription in the context of the TRAFl-unrelated SV40-minimal promoter. To determine tissue specificity of the enhancers, we used generated reporter vectors to transfect T cell line 68–41 and monocytic cell line J774. N2M5 and P2R2 enhancers separately or together did not induce transcription in 68–41 cells stimulated by *anti*-CD3 (Fig. 4B) or J774 cells stimulated with LPS (Fig. 4C), while pGL3-control reporter vector displayed strong activity in either circumstance.

3.4. Dissection of the 5′ upstream enhancer

DNA sequence analysis of the 104 bp long upstream enhancer using the TFSEARCH program and Genetyx-SV/RC 10.1 software revealed the presence of several potential binding sites for transcription factors as shown in Fig. 5A. Binding sites correspond to the following TRANSFAC matrices: AP-l (86.7% homology to M00172), NF-κB (88.3% homology to M00194 and 85.4% homology to M00054), and ets-l (92.2% homology to M00032). We examined the binding of nuclear proteins from M12 cells to the upstream enhancer by EMSA using three double-stranded oligonucleotides (UPl, UP2 and UP3) spanning the entire 104 bp long sequence of the upstream enhancer. These oligonucleotide probes were approximately 50 bp long and shared overlapping 20 bp long regions (underlined in Fig. 5A). Fig. 4B shows that UP1 and UP3 probes interact with nuclear proteins from unstimulated Ml2 B cells (lanes 1 and 5). The intensity of the retarded bands is not increased following *anti*-CD40 stimulation (lanes 2 and 6). Importantly, although UP1 and UP3 oligonucleotides contained potential AP-1 and Ets-l sites, the bands were not competed by consensus AP-1 or Ets-1 oligonucleotides and were not supershifted by antibodies to AP-1 or Ets-1 (data not shown). In contrast, the intensity of the nuclear complex formed by the UP2 probe is significantly increased following CD40 ligation (Fig. 5B, compare lanes 3 and 4). Presence of the NF-κB consensus site within the UP2 sequence and the well known ability of CD40 to activate NF-κB in B cells strongly suggest that NF-κB transcription factor binds to the UP2 oligonucleotide probe. Although the UP1 sequence also contains an NF-κB consensus site, it does not bind NF-κB transcription factor. Probably, a longer flanking region on the 3′ end of NF-κB recognition site in the UP1 probe is required for efficient binding of the NF-κB transcription factor, because the longer exposure of the gel indeed shows a very weak NF-κB binding to the UPl probe (data not shown). Identical results were obtained for UP1, UP2 and UP3 probes with nuclear extracts from spleen B cells stimulated with *anti*-CD40 antibody (data not shown). These data suggest that the UP1 and UP3 oligonucleotides

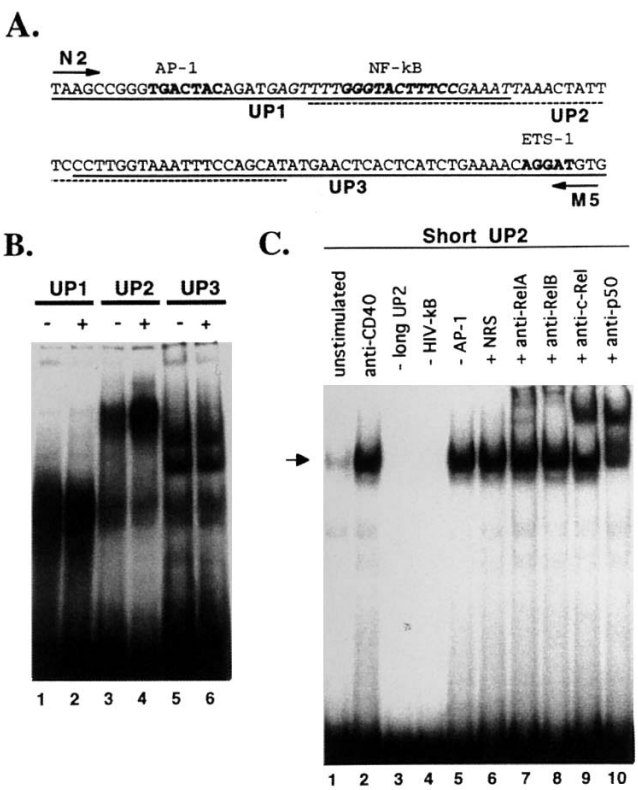

Fig. 5. Dissection of the upstream enhancer. (A) Nucleotide sequence of the upstream enhancer. Nuclear factor binding sites, which identified by using the TFSEARCH and GenetyxSV/RC 10.1 software, are indicated and shown in bold. PCR primers used to generate N2M5 fragment are shown at the ends. (B) Indicated overlapping 50 bp long oligonucleotide probes UP1, UP2 and UP3 (underlined in A) were labeled and used for EMSA with nuclear extracts from unstimulated (−) and *anti*-CD40-treated M12 cells (+). (C) The upstream enhancer binds NF-κB family transcription factors. Short UP2 oligonucleotide probe (shown in italic in A), corresponding to the NF-κB binding site, was labeled and used for EMSA. A 100-fold molar excess of unlabeled competitor oligonucleotides was added as indicated. A consensus AP-1 oligonucleotide was added as an unrelated competitor. Nuclear extracts were pre-incubated with normal rabbit serum (NRS) or antisera to different members of NF-κB family nuclear factors as shown.

bind nuclear factors constitutively expressed in B cells whereas the UP2 sequence binds nuclear factors induced by CD40 stimulation.

Because the UP2 sequence contains a consensus site for NF-κB, we determined whether NF-κB was present in the UP2 nuclear complex. We used a short UP2 probe (27 bp) corresponding to the UP2 sequence that spans the NF-κB putative site. Fig. 5C shows that this probe bound a CD40 inducible nuclear factor from M12 cells (compare lane 1 and 2). This binding was completely inhibited by the long 50 bp UP2 oligonucleotide (lane 3), suggesting that these two probes bind to the same nuclear proteins. Moreover, it was inhibited by an oligonucleotide from the HIV-l LTR known to bind NF-κB (lane 4) (Nabel and Baltimore, 1987), but was not inhibited by an irrelevant control AP-l oligonucleotide (lane 5). The UP2 complex was partially depleted or supershifted by antibodies directed to NF-κB proteins including RelA, RelB, c-Rel, p50, and p52 (lanes 7, 8, 9, 10 and data not shown). Gel densitometry analysis revealed that the UP2-binding NF-κB complex primarily consists of p50 containing heterodimers with other members of NF-κB family members, because *anti*-p50 antibodies strongly decrease the intensity of the major inducible band (lane 10). There is a limited amount of p52-heterodimers, which is represented by the bands not supershifted with *anti*-p50 antibodies. Little or no p50/p50 homodimer was detected in the UP2-binding NF-κB complex, which is consistent with a particular binding pattern of NF-κB subunits in M12 cells following CD40 stimulation (Lin and Stavnezer, 1996). RelB and c-Rel are the major partners of p50 in the UP2-binding NF-κB complex, most likely forming c-Rel/p50 and RelB/p50 heterodimers. There is a RelA/p50 heterodimer present in the complex, but in much lesser amount. These results are in agreement with different NF-κB subunit usage dependent upon the stage of B cell differentiation, where p50 and RelA proteins are mainly expressed in pre-B (and non-B) cells, while p50 and c-Rel proteins predominate in mature B cells, and p52 and RelB are expressed in plasmacytoma cell lines (Liou et al., 1994). Taken together, these results suggest that the upstream TRAF1 enhancer binds NF-κB transcription factor following CD40 stimulation of B cells.

To assess the functional importance of the NF-κB site in the upstream enhancer, we mutated this site within the 104 bp upstream enhancer and compared mutant and native enhancers for their capacity to drive reporter gene expression in M12 B cells. Fig. 6A shows the sequence of the native and mutant 27 bp UP2 oligonucleotides. In contrast to the native UP2 oligonucleotide (lane 3), the mutant oligonucleotide (lane 4) fails to inhibit the formation of a complex formed by the labeled short UP2 oligonucleotide and nuclear extracts from CD40 stimulated cells (Fig. 6B). Fig. 6C shows that in contrast to the native upstream enhancer (N2M5), the enhancer with the NF-κB site mutation (mutUP2) fails to drive reporter gene expression in M12 cells. These results indicate that the activity of the upstream enhancer in response to CD40 is dependent on NF-κB.

3.5. Dissection of the intronic enhancer

DNA sequence analysis of the 195 bp long intronic enhancer revealed the presence of two potential AP-1 binding sites, 3 potential NF-κB sites, and one potential GATA-l site as shown in Fig. 7A. We used six 50 bp long overlapping double stranded oligonucleotides (IN1–IN6) in EMSA assays to examine the binding of transcription factors to the intronic enhancer. The IN1 probe contains a highly conserved AP-1 consensus site, TGAGTCA (98.3% homology to M00199 and 92.9% homology to M00174). IN1 bound a nuclear complex was present in the nuclear extracts from unstimulated M12 cells (Fig. 7B, lane 1). The intensity of this complex increased after activation with *anti*-CD40 mAb (lane 2). Analysis of this complex using a shorter 25 bp IN1 probe, corresponding to the IN1 sequence that spans the AP-1 putative site by oligonucleotide inhibition and by supershift confirmed that it contained the AP-1 proteins Fos and Jun (Fig. 7C). The IN2 probe contained no recognizable binding sites but formed a weak complex (lane 3), the intensity of which increased slightly after CD40 stimulation (lane 4). The IN3 sequence contains potential binding sites for NF-κB and GATA-1, but the IN3 probe formed no complexes (lanes 5 and 6). The IN4 sequence contains a potential AP-1 binding site (90.1% homology to M00174). It formed a weak complex of equivalent intensity in unstimulated and CD40 stimulated cells (lanes 7 and 8). The IN5 sequence contains a potential NF-κB binding site (92.6% homology to M00053) and the IN5 probe bound weakly a CD40 inducible complex (compare lanes 9 and 10). The IN6 oligonucleotide contains an NF-κB (c-Rel) binding site (92.6% homology to M00053) and binds strongly to a CD40 inducible complex (compare lanes 11 and 12). To examine whether NF-κB transcription factors bind to the potential IN6 NF-κB binding site, we performed additional EMSA experiments with a shorter 27 bp long IN6 oligonucleotide probe (Fig. 7D). Analysis revealed that binding to the short IN6 probe was completely inhibited by the long IN6 oligonucleotide (lane 3). These complexes were also completely inhibited by cold NF-κB oligonucleotide from HIV-1 LTR (lane 4), but were not inhibited by an irrelevant control, AP-1 oligonucleotide (lanes 5). Together these data strongly suggest that the 27 bp long and 50 bp long IN6 probes bind to the same nuclear proteins from CD40 stimulated M12 cells and that these nuclear proteins are members of NF-κB

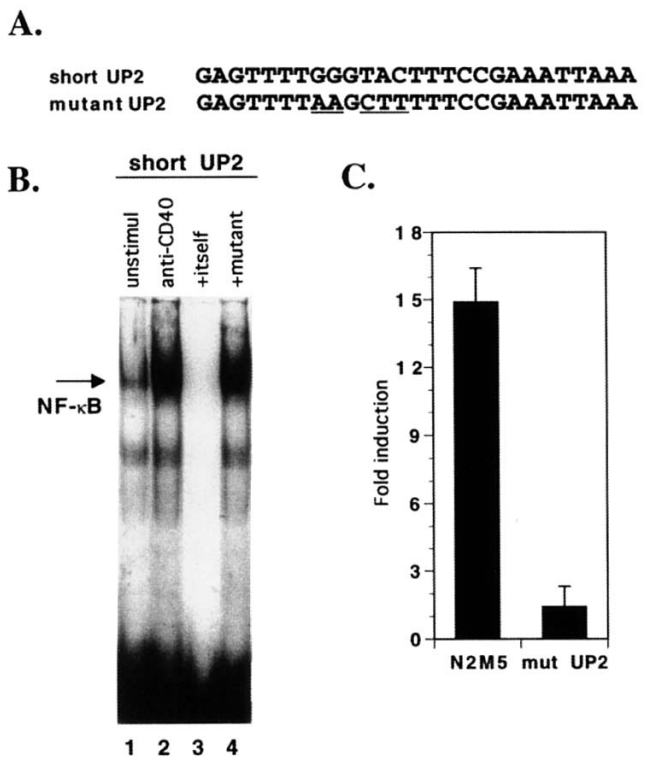

Fig. 6. Mutation of the NF-κB binding site within the upstream enhancer. (A) DNA sequence of the wild type and mutated UP2 oligonucleotides. The mutations of the oligonucleotides are the same as those used for transfection experiments. Mutated bases are underlined. (B) Short UP2 oligonucleotide probe was labeled and used for EMSA with nuclear extracts from unstimulated and *anti*-CD40 stimulated M12 cells. A 100-fold molar excess of normal and mutant competitor oligonucleotides were added as indicated. (C) The upstream (N2M5) enhancer reporter construct and its mutant (mut UP2) were transiently transfected into M12 cells, which were later stimulated with *anti*-CD40 antibody. Specific luciferase activity was determined 24 h later. Fold induction was calculated as a ratio of specific luciferase activity for CD40 stimulated to unstimulated cells. The experiment shown is a representative of four independent experiments.

family. Furthermore, the upper IN6 complex was supershifted by *anti*-c-Rel antibodies (lanes 7 and 9). *Anti*-RelA and *anti*-RelB antibodies had no effect on the IN6 complexes (lane 8). Interestingly, *anti*-p50 antibodies completely supershifted the upper complex as well as the lower complex (lane 10). Thus, these data suggest that the upper complex consists of c-Rel/p50 heterodimers, while the lower IN6 complex consisted of p50 protein.

The same strategy used for the upstream enhancer was used to assess the functional importance of the sites that strongly bind NF-κB and AP-1 in the intronic enhancer. Fig. 8A shows the mutations we introduced within the context of the short INl and IN6 oligonucleotides surrounding the AP-1 site and the NF-κB site, respectively. Fig. 8B shows that in contrast to the native oligonucleotides, the mutant oligonucleotides fail to inhibit the formation of a complex between the corresponding labeled native oligonucleotides and nuclear extracts from CD40-stimulated cells. Fig. 8C shows that introduction of either the AP-1 site mutation or of the NF-κB site mutation in the 104 bp enhancer greatly reduces the ability to drive reporter gene expression in M12 cells. These results indicate that the activity of intronic enhancer in response to CD40 is dependent on both AP-1 and NF-κB.

4. Discussion

The studies were aimed at better understanding of the TRAF1 gene expression in activated murine B cells following CD40 signaling, which is known to induce B cells activation, proliferation, immunoglobulin isotype switching and affinity maturation following somatic hypermutation. Signaling through CD40, besides induction of the transcription of TRAFl and other genes, can induce germline immunoglobulin heavy chain transcription, which is absolutely necessary for isotype switching. We have recently cloned the murine TRAF1 gene and identified the putative transcription start site by 5′-RACE analysis of the TRAF1 transcript using mRNA from activated spleen B cells (Dunn et al., 1999). To study the induction of the murine TRAFl gene by CD40 in B cells, we characterized the minimal promoter sequence required to drive basal transcription

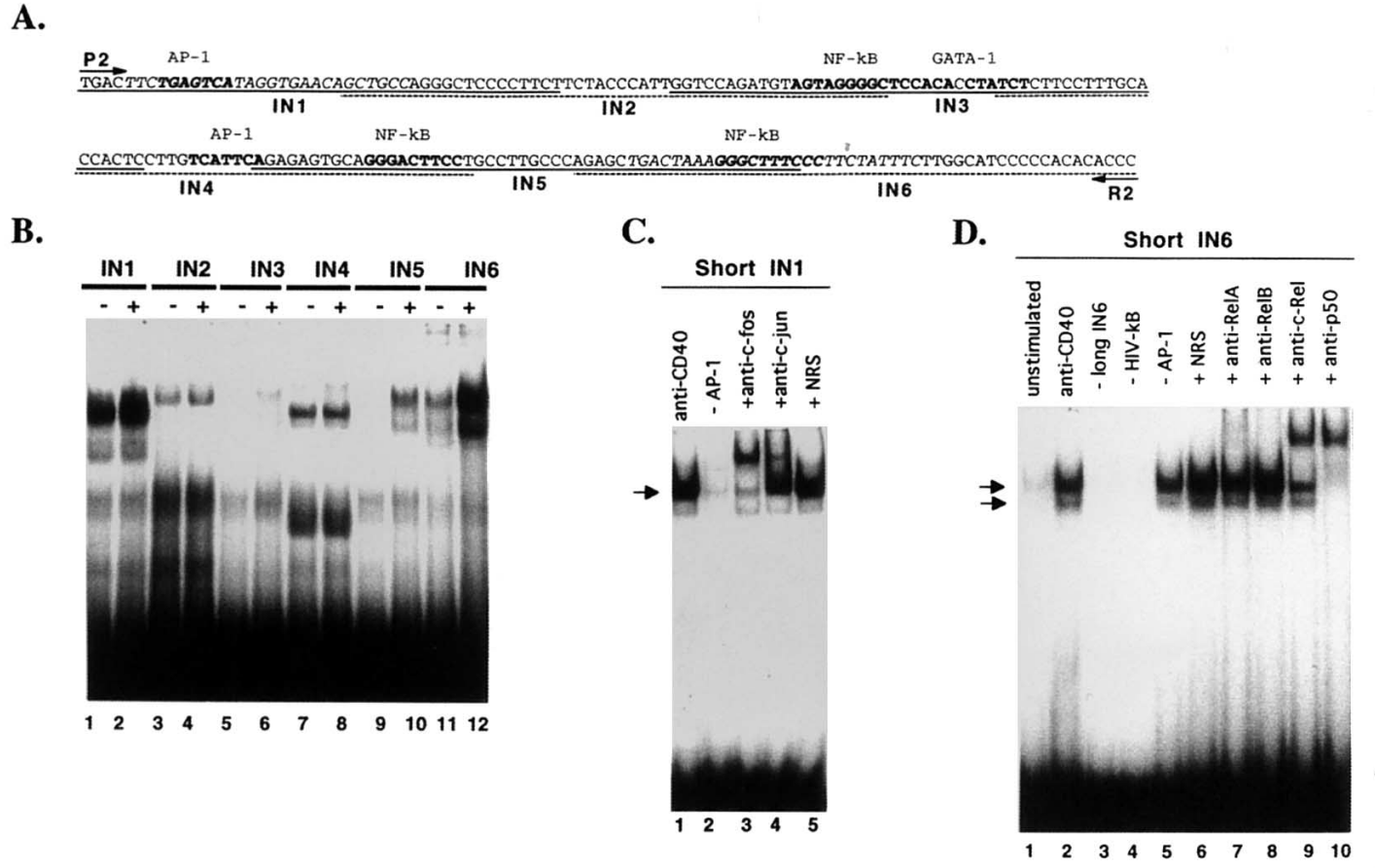

Fig. 7. Dissection of the intronic enhancer. (A) Nucleotide sequence of the intronic enhancer. Nuclear factor binding sites, which identified by using the TFSEARCH and Genetyx-SV/RC 10.1 software, are indicated and shown in bold. PCR primers used to generate P2R2 fragment are shown at the ends. (B) Indicated overlapping 50 bp long oligonucleotide probes IN1–IN6 (underlined in A) were labeled and used for EMSA with nuclear extracts from unstimulated (−) and *anti*-CD40-treated Ml 2 cells (+). (C) The intronic enhancer binds AP-1 family transcription factors. Short IN1 oligonucleotide probe (shown in italic in A), corresponding to the 5′ end AP-l binding site, was labeled and used for EMSA. A 100-fold molar excess of unlabeled consensus AP-1 oligonucleotide was added as indicated. Nuclear extracts were pre-incubated with antisera recognizing c-Fos and c-Jun nuclear factors or normal rabbit serum (NRS). (D) The intronic enhancer binds NF-κB family transcription factors. Short IN6 oligonucleotide probe (shown in italic in A), corresponding to the 3′ end NF-κB binding site, was labeled and used for EMSA. A 100-fold molar excess of unlabeled competitor oligonucleotides were added as indicated. Consensus AP-1 oligonucleotide was added as an unrelated competitor. Nuclear extracts were pre-incubated with normal rabbit serum (NRS) or antisera to different members of NF-κB family nuclear factors.

of a reporter gene in the M12 B cell line, localizing it to within −102 to +38 bp relative to the transcription start site (Fig. 1). The presence of GC-rich sequences within the minimal TRAFl promoter together with the absence of a TATA-box, CAAT sequence, or initiator site suggest that the activity of this promoter might be dependent on Sp-1 to recruit the general transcription machinery to initiate mRNA synthesis (Smale, 1994).

The inability of this TRAF1 minimal promoter to drive CD40-dependent transcription prompted us to look for CD40-dependent transcription regions within the TRAFl gene. We here report that the TRAFl gene contains two enhancer regions, which may mediate CD40-induced transcription. The first of these enhancers is 104 bp long and is located 2 kb upstream of the TRAF1 transcription start site. The other enhancer sequence, 195 bp long and located in the intron between exons 5 and 6. Interestingly, this intron is absent in the human TRAFl gene in which a single exon, exon 3, corresponds to exons 5 and 6 in mouse. When put together into reporter construct, the upstream and intronic enhancers do not display any synergistic effect.

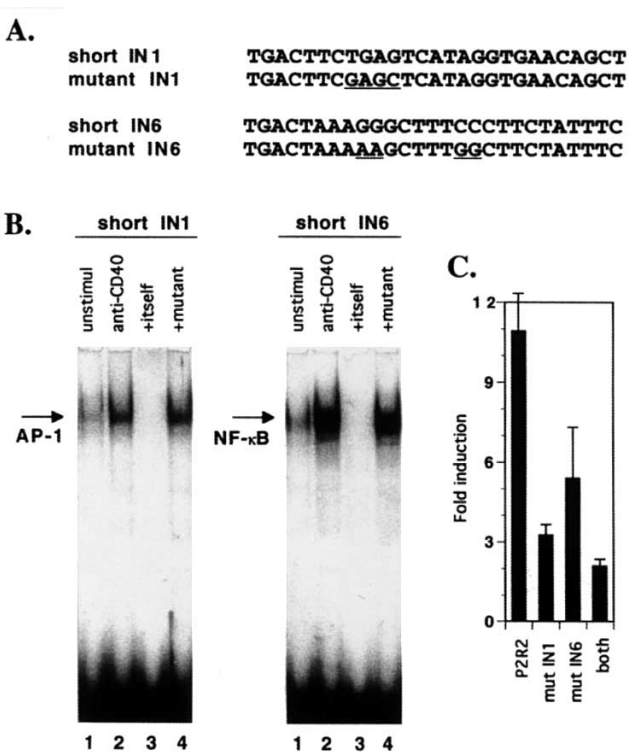

Fig. 8. Mutations of the critical binding sites within the intronic enhancer. (A) DNA sequence of the wild type and mutated IN1 and IN6 oligonucleotides that were tested for the ability to bind nuclear proteins and in EMSA above. The mutations of the oligonucleotides are the same as those used for transfection experiments (see C). Mutated bases are underlined. (B) Indicated oligonucleotide probes were labeled and used for EMSA with nuclear extracts from unstimulated and *anti*-CD40 stimulated M12 cells. A 100-fold molar excess of normal and mutant competitor oligonucleotides were added as indicated. (C) Constructs containing indicated DNA fragments, corresponding to the normal and mutated enhancer were transiently transfected into M12 cells. Cells were stimulated with *anti*-CD40 antibody and specific luciferase activity was determined 24 h later. Fold induction was calculated as a ratio of specific luciferase activity for CD40 stimulated to unstimulated cells. The experiment shown is a representative of four independent experiments. ($^*P < 0.05\%$).

Moreover, together they induce the lower of transcription than the upstream enhancer alone (Fig. 4A). They may compete with each other for binding to components of the general transcription machinery, which participates in the initiation of basal transcription from the TRAF1 minimal promoter. It is possible that under certain circumstances each of these enhancers may separately participate in the induction of TRAFl gene transcription following CD40 signaling in B cells. Further studies are needed to provide a better understanding of the role of each enhancers in CD40-dependent TRAFl gene expression in murine B lymphocytes. Interestingly, neither the upstream enhancer nor the intronic enhancer functions in the context of the SV40 early minimal promoter from pGL3-promoter vector, suggesting that the enhancers require their cognate TRAF1 minimal promoter to achieve full efficiency. This can be explained by the difference of the transcription initiation machinery in the SV40 early minimal promoter and the murine TRAF1 minimal promoter. For example, in contrast to the murine TRAFl minimal promoter the SV40 early minimal promoter is a TATA-box containing promoter (Pauly et al., 1992). All together these data suggest that the murine TRAF1 minimal promoter bind specific transcription factors important for interaction with one of the enhancers.

Each of the two enhancers contains a unique array of multiple sites that bind constitutive, as well as CD40 inducible, nuclear factors. Together, these inducible and constitutive nuclear factors may form multiprotein enhanceosomes. Our analysis of the upstream 104 bp enhancer indicates that it binds to at least two constitutive nuclear factors, UP1 and UP3 probes binding complexes. We also demonstrated that the core of the upstream enhancer, which contains an NF-κB site, binds to NF-κB proteins from nuclear extracts of CD40-stimulated B cells. Mutation of this site completely abrogates CD40-dependent transcription driven by the upstream enhancer (Fig. 7C). These results strongly suggest that NF-κB is critical for activation of the upstream enhancer by CD40. Recently, five putative NF-κB binding sites were found within approximately 1.4 kb long of the transcription start site of the human TRAF1 gene (Schwenzer et al., 1999). Three of these sites bound NF-κB transcription

factors. Their functional importance was confirmed by the demonstration that mutation of each of these sites partially reduced the ability of the 1.4 kb fragment to drive reporter gene expression. Although the genomic organization of the 5′ part of the human and murine TRAF1 genes is different, it appears that transcriptional regulation of both the human and murine TRAFl genes is regulated by upstream NF-κB elements.

We found that the 195 bp TRAF-1 intronic enhancer also binds constitutive nuclear factors as well as CD40 inducible factors. An AP-l consensus site in the 5′ region of this enhancer strongly binds AP-1 transcription factor, whereas an NF-κB consensus site in the 3′, region enhancer strongly binds NF-κB transcription factor. Both sites are functionally important because mutation of either one of them significantly decreased enhancer driven CD40-dependent transcription (Fig. 8C). Simultaneous mutation of both AP-1 and NF-κB binding sites within the intronic enhancer abolishes CD40-dependent induction of transcription driven by this enhancer. These results strongly suggest that both AP-1 and NF-κB are critical for activation of the intronic TRAF-1 enhancer by CD40.

CD40 signaling is known to induce the activation of the AP-1 and NF-κB transcription factors. CD40 stimulates NF-κB through the IkappaB kinase (IKK) (Kosaka et al., 1999), while it triggers AP-1 via the c-Jun N-terminal kinase (JNK) cascade (Sakata et al., 1995). Therefore, it is likely that CD40 activation of the upstream enhancer, which contains a single critical NF-κB site, requires only stimulation of the IKK pathway. On the other hand, CD40 activation of the intronic enhancer containing an NF-κB as well as an AP-1 site, both of which are critical may need IKK as well as JNK. Interestingly, induction of CD40 dependent transcription in the reporter system is consistently higher with the upstream enhancer than with the intronic enhancer (Fig. 4A). That this more avid enhancer is NF-κB-dependent while the intronic enhancer is NF-κB- and AP-1-dependent suggests the presence of a more efficiently assembled enhanceosome at the upstream site. Future studies are necessary to understand the precise molecular mechanisms of the assembly and functioning of each enhanceosome during CD40-dependent transcription of the murine TRAFl gene.

Acknowledgements

We thank Dr Nancy R. Rice for kindly providing of *anti*-NF-κB antibodies. This work was supported by A142031 (ENT) and AR-43985 (RSG) from National Institute of Allergy and Infectious Diseases, USA. IFD was supported by Chrysalis Foundation Research Fellowship

References

Ansieau, S., Scheffrahn, I., Mosialos, G., Brand, H., Duyster, J., Kaye, K., Harada, J., Dougall, B., Hubinger, G., Kieff, E., Herrmann, F., Leutz, A., Gruss, H.J., 1996. Tumor necrosis factor receptor-associated factor (TRAF)-1, TRAF-2, and TRAF-3 interact in vivo with the CD30 cytoplasmic domain; TRAF-2 mediates CD30-induced nuclear factor kappa B activation. Proc. Natl. Acad. Sci. USA 93, 14 053–14 058 retracted by Kieff, E. In: Proc Natl. Acad. Sci. USA 1997 Nov 11; 94(23): 12 732.

Arch, R.H., Gedrich, R.W., Thompson, C.B., 1998. Tumor necrosis factor receptor-associated factors (TRAFs) — a family of adapter proteins that regulates life and death. Genes Dev. 12, 2821–2830.

Baker, S.J., Reddy, E.P., 1996. Transducers of life and death: TNF receptor superfamily and associated proteins. Oncogene 12, 1–9.

Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., Goeddel, D.V., 1996. TRAF6 is a signal transducer for interleukin-1. Nature 383, 443–446.

Cheng, G., Cleary, A.M., Ye, Z.S., Hong, D.I., Lederman, S., Baltimore, D., 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. Science 267, 1494–1498.

Devergne, O., McFarland, E.C., Mosialos, G., Izumi, K.M., Ware, C.F., Kieff, E., 1998. Role of the TRAF binding site and NF-kappaB activation in Epstein-Barr virus latent membrane protein 1-induced cell gene expression. J. Virol. 72, 7900–7908.

Duckett, C.S., Gedrich, R.W., Gilfillan, M.C., Thompson, C.B., 1997. Induction of nuclear factor kappaB by the CD30 receptor is mediated by TRAFl and TRAF2. Mol. Cell. Biol. 17, 1535–1542.

Dunn, I.F., Geha, R.S., Tsitsikov, E.N., 1999. Structure of the murine TRAF1 gene. Mol. Immunol. 36, 611–617.

Durkop, H., Foss, H.D., Demel, G., Klotzbach, H., Hahn, C., Stein, H., 1999. Tumor necrosis factor receptor-associated factor 1 is overexpressed in Reed-Sternberg cells of Hodgkin's disease and Epstein-Barr virus-transformed lymphoid cells. Blood 93, 617–623.

Ernst, M.K., Dunn, L.L., Rice, N.R., 1995. The PEST-like sequence of I kappa B alpha is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or RelA homodimers. Mol. Cell. Biol. 15, 872–882.

Gedrich, R.W., Gilfillan, M.C., Duckett, C.S., Van Dongen, J.L., Thompson, C.B., 1996. CD30 contains two binding sites with different specificities for members of the tumor necrosis factor receptor-associated factor family of signal transducing proteins. J. Biol. Chem. 271, 12 852–12 858.

Hu, H.M., O'Rourke, K., Boguski, M.S., Dixit, V.M., 1994. A novel RING finger protein interacts with the cytoplasmic domain of CD40. J. Biol. Chem. 269, 30 069–30 072.

Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., Yamamoto, T., Inoue, J., 1996a. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an aminoterminal domain of the CD40 cytoplasmic region. J. Biol. Chem. 271, 28 745–28 748.

Ishida, T.K., Tojo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T., Inoue, J., 1996b. TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. Proc. Natl. Acad. Sci. USA 93, 9437–9442.

Izumi, K.M., Kaye, K.M., Kieff, E.D., 1997. The Epstein-Barr virus LMP1 amino acid sequence that engages tumor necrosis factor receptor associated factors is critical for primary B lymphocyte growth transformation. Proc. Natl. Acad. Sci. USA 94, 1447–1452.

Izumi, K.M., McFarland, E.C., Riley, E.A., Rizzo, D., Chen, Y., Kieff, E., 1999. The residues between the two transformation effector sites of Epstein-Barr virus latent membrane protein 1 are not critical for B-Lymphocyte growth transformation. J. Virol. 73, 9908–9916.

Kosaka, Y., Calderhead, D.M., Manning, E.M., Hambor, J.E., Black, A., Geleziunas, R., Marcu, K.B., Noelle, R.J., 1999. Activation and regulation of the IkappaB kinase in human B cells by CD40 signaling. Eur. J. Immunol. 29, 1353–1362.

Lee, S.Y., Kandala, G., Liou, M.L., Liou, H.C., Choi, Y., 1996. CD30/TNF receptor-associated factor interaction: NF-kappa B activation and binding specificity. Proc. Natl. Acad. Sci. USA 93, 9699–9703.

Lee, W., Mitchell, P., Tjian, R., 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49, 741–752.

Liebowitz, D., 1998. Epstein-Barr virus and a cellular signaling pathway in lymphomas from immunosuppressed patients. N. Engl. J. Med. 338, 1413–1421 see comments.

Lin, S.C., Stavnezer, J., 1996. Activation of NF-kappaB/Rel by CD40 engagement induces the mouse germ line immunoglobulin Cgamma1 promoter. Mol. Cell. Biol. 16, 4591–4603.

Liou, H.C., Sha, W.C., Scott, M.L., Baltimore, D., 1994. Sequential induction of NF-κB/Rel family proteins during B-cell terminal differentiation. Mol. Cell. Biol. 14, 5349–5359.

Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., Kieff, E., 1995. The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. Cell 80, 389–399.

Nabel, G., Baltimore, D., 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 326, 711–713.

Nakamura, M., Niki, M., Nagata, K., Ohtani, K., Saito, S., Hinuma, Y., Sugamura, K., 1989. Cell line-dependent response of the enhancer element of simian virus 40 to transactivator p40tax encoded by human T-cell leukemia virus type I. J. Biol. Chem. 264, 20 189–20 192.

Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C.F., Yagita, H., Okumura, K., 1996. TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor. J. Biol. Chem. 271, 14 661–14 664.

Pauly, M., Treger, M., Westhof, E., Chambon, P., 1992. The initiation accuracy of the SV40 early transcription is determined by the functional domains of two TATA elements. Nucleic Acids Res. 20, 975–982.

Pullen, S.S., Miller, H.G., Everdeen, D.S., Dang, T.T.A., Crute, J.J., Kehry, M.R., 1998. CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. Biochemistry 37, 11 836–11 845.

Regnier, C.H., Tomasetto, C., Moog-Lutz, C., Chenard, M.P., Wendling, C., Basset, P., Rio, M.C., 1995. Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. J. Biol. Chem. 270, 25 715–25 721.

Rothe, M., Wong, S.C., Henzel, W.J., Goeddel, D.V., 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell 78, 681–692.

Sakata, N., Patel, H.R., Terada, N., Aruffo, A., Johnson, G.L., Gelfand, E.W., 1995. Selective activation of c-Jun kinase mitogen-activated protein kinase by CD40 on human B cells. J. Biol. Chem. 270, 30 823–30 828.

Sato, T., Irie, S., Reed, J.C., 1995. A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40. FEBS Lett. 358, 113–118.

Schwenzer, R., Siemienski, K., Liptay, S., Schubert, G., Peters, N., Scheurich, P., Schmid, R.M., Wajant, H., 1999. The human tumor necrosis factor (TNF) receptor-associated factor 1 gene (TRAF1) is up-regulated by cytokines of the TNF ligand family and modulates TNF-induced activation of NF-kappaB and c-Jun N-terminal kinase. J. Biol. Chem. 274, 19 368–19 374.

Siemienski, K., Peters, N., Scheurich, P., Wajant, H., 1997. Organization of the human tumour necrosis factor receptor-associated factor 1 (TRAF1) gene and mapping to chromosome 9q33–34. Gene 195, 35–39.

Smale, S.T. (1994). Core promoter architecture for eukaryotic protein-coding genes. In: Carey, M., Smale, S., (ed.), Transcription: Mechanisms and Regulation, p. 63–81. Raven Press, New York.

Speiser, D.E., Lee, S.Y., Wong, B., Arron, J., Santana, A., Kong, Y.Y., Ohashi, P.S., Choi, Y., 1997. A regulatory role for TRAF1 in antigen-induced apoptosis of T cells. J. Exp. Med. 185, 1777–1783.

Tsitsikov, E.N., Fuleihan, R., McIntosh, K., Scholl, P.R., Geha, R.S., 1995. Cross-linking of Fc gamma receptors activates HIV-1 long terminal repeat-driven transcription in human monocytes. Int. Immunol. 7, 1665–1670.

Tsitsikov, E.N., Wright, D.A., Geha, R.S., 1997. CD30 induction of human immunodeficiency virus gene transcription is mediated by TRAF2. Proc. Natl. Acad. Sci. USA 94, 1390–1395.

Tsytsykova, A.V., Tsitsikov, E.N., Geha, R.S., 1996. The CD40L promoter contains nuclear factor of activated T cells-binding motifs which require AP-1 binding for activation of transcription. J. Biol. Chem. 271, 3763–3770.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., Baldwin, A.S. Jr., 1998. NFkappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAPl and c-IAP2 to suppress caspase-8 activation. Science 281, 1680–1683.

Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruss, M., Reuter, I., Schacherer, F., 2000. TRANSFAC: an integrated system for gene expression regulation. Nucleic Acids Res. 28, 316–319.