

The transcriptional regulation of human arachidonate 12-lipoxygenase gene by NF κ B/Rel

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Abstract As examined by the luciferase assay, a negative regulatory region including the NF κ B motif was found in the 5'-flanking region of the 12-lipoxygenase gene in human erythroleukemia cells. The negative control was abolished by a site-specific mutation of the NF κ B motif. Probes including the NF κ B region gave positive bands upon a gel-shift assay. The bands were super-shifted by antibodies for NF κ B p50, NF κ B p65 and c-Rel, and were lost by a NF κ B competitor DNA. Furthermore, the NF κ B sequence was protected in DNase I footprinting. Thus, two kinds of heterodimer (p50 and p65; p50 and c-Rel) seemed to control the over-expression of the human 12-lipoxygenase gene.

Key words: 12-Lipoxygenase; Transcription factor; NF κ B; Rel

1. Introduction

Lipoxygenase enzymes are dioxygenases which incorporate one molecule of oxygen at specific positions of unsaturated fatty acids. 12-Lipoxygenase oxygenates the 12-position of arachidonic acid, and produces regio- and stereo-specifically 12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid. There are two isoforms of 12-lipoxygenase which are distinguishable in terms of substrate chain length specificity, reactivity with antibody, homology of their amino acid sequences and exon-intron structures of their genes [1]. When the genomic DNAs of 12-lipoxygenases of porcine leukocytes [2], human erythroleukemia (HEL) cells [3,4], murine platelets [5], and murine leukocytes [5] were cloned and sequenced, neither typical TATA box nor CCAAT box was found in their promoter regions, suggesting that the 12-lipoxygenase is a product of a housekeeping gene. SP1 consensus sequences and also other regulatory elements such as AP2 and NF κ B sites were present in the human 12-lipoxygenase gene [3,4].

NF κ B was originally found as a binding protein to immunoglobulin κ light-chain enhancer [6]. Motifs for NF κ B have been found in many genes encoding immunoreceptors (immunoglobulin κ light chain and major histocompatibility complex class I), cytokines (interleukins-2 and -6 and tumor necrosis factor- α) and viral proteins (human immunodeficiency virus-1, cytomegalovirus and simian virus 40) [7–11]. The NF κ B is now considered as a ubiquitous transcription factor. It is constitu-

tively present in the nuclei in B cells, certain T cell lines and monocytes, and has the structure of either homodimer or heterodimer [8–11]. The heterodimer of p50 and p65 subunits has higher affinity to the NF κ B site than the homodimer of either p50 or p65 [12]. Moreover, Rel proteins were found as oncogene products of reticuloendotheliosis virus strain T in turkey [13], and also has been shown to function as a transcription factor. The NF κ B and Rel proteins are a family with high homologies in their amino acid sequences.

In this study, we found that NF κ B p50 and p65 or c-Rel heterodimers suppressed the over-expression of 12-lipoxygenase gene in HEL cells. This is the first demonstration of the NF κ B/Rel protein as a transcription factor regulating the gene expression of mammalian lipoxygenases.

2. Materials and methods

2.1. Cell culture of HEL cells

The HEL cell line was kindly provided by Dr. Y. Eto of Ajinomoto Central Research Laboratories and Dr. S. Narumiya of Kyoto University. The cells were cultured in RPMI 1640 medium (Gibco-BRL) containing 10% fetal calf serum (FCS) (Biological Industries) and streptomycin sulfate (100 mg/l) adjusted to pH 7.4 by the addition of sodium bicarbonate [14]. The cells were cultured at an initial density of 1×10^5 cells/ml in 100-mm plastic dishes at 37°C in a humidified atmosphere of 7% CO₂ in air, and grown for 3–4 days to a density of $10\text{--}12 \times 10^5$ cells/ml.

2.2. Construction of the luciferase reporter vector

DNA fragments of various lengths containing human 12-lipoxygenase promoter regions were prepared either by restriction enzyme digestion of the genomic clone for pXLO-01, 1, 3 and 4-1 or by the polymerase chain reaction (PCR) amplification method for pXLO-4-2, 4-4, 4-3, 5-1, 5-2, 5-3, 6 and 7. The PCR amplification was performed for 25 cycles (94°C for 1 min, 55°C for 2 min, and 74°C for 2 min) using Pfu polymerase (Stratagene). The DNA fragments were ligated into a luciferase plasmid pXP-1 [15] as described by Sambrook et al. [16]. A mutant at the site of NF κ B (pXLO-NFM1) was constructed by the site-specific mutagenesis method as described by Higuchi et al. [17]. All the plasmids for transfection were purified by the use of Qiagen-tip 100 (Qiagen) or by equilibrium centrifugation in CsCl/ethidium bromide gradients.

2.3. Transfection of plasmids to HEL cells

The lipofection method was performed using Lipofectamin (Gibco-BRL) according to the manufacturer's instructions with a slight modification. HEL cells (1×10^5 cells/ml) were cultured up to a density of 1×10^6 cells/ml, harvested by pipetting, washed with RPMI 1640 medium without FCS and streptomycin sulfate, and resuspended in Opti-MEM medium (Gibco-BRL) at a density of 1.5×10^6 cells/ml. The suspension (0.8 ml) was seeded in 35-mm plastic dishes. Lipofectamin (15 μ l) was incubated with 3 μ g of pXLO luciferase plasmid and 1 μ g of pCMV β -galactosidase plasmid (Clontech) in 200 μ l of Opti-MEM medium in a CO₂ incubator for 30 min at 37°C. The mixture was added to the above-treated cells, followed by incubation for 20 h at 37°C in a humidified atmosphere of 7% CO₂. RPMI 1640 medium with 10% FCS (4 ml) was added to the dishes, and the cells were incubated for additional 18 h.

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Abbreviations: HEL, human erythroleukemia; EMSA, electrophoretic mobility-shift assay; PMSF, phenylmethylsulfonyl fluoride; bp, base pair(s); FCS, fetal calf serum; PCR, polymerase chain reaction.

2.4. Luciferase and β -galactosidase assays

The luciferase activity was measured using the PicaGene luciferase assay system (Toyo Ink Corporation). The 1.2×10^6 transfected cells were harvested, washed with phosphate-buffered saline by centrifugation, and resuspended in 40 μ l of PicaGene lysis buffer. After a 15-min incubation at room temperature, the suspension was centrifuged at 12,000 rpm for 15 s, and the supernatant solution was used as the cell lysate. PicaGene luciferase assay substrates in 100 μ l were mixed with 20 μ l of the cell lysate, and then the luciferase activity was measured by a Berthold Lumat LB 9501 luminometer. For β -galactosidase assay, 15 μ l of the cell lysate were mixed with 285 μ l of 0.1 M sodium phosphate buffer at pH 7.5 containing 10 mM KCl, 1 mM $MgCl_2$, 0.1% (v/v) Triton X-100 and 5 mM β -mercaptoethanol, and the mixture was kept at 37°C for 10 min. The reaction was started by the addition of 50 μ l of *o*-nitrophenyl- β -D-galactopyranoside solution (4 mg/ml in 0.1 M sodium phosphate buffer at pH 7.5), and continued for 2 h at 37°C. The reaction was stopped by the addition of 150 μ l of 1 M Na_2CO_3 , and absorbance at 420 nm was measured by a Beckman DU 640 spectrophotometer. The luciferase activities were normalized to the β -galactosidase activities.

2.5. Preparations of nuclear extracts from HEL, U937 and HeLa cells

Nuclear extracts were prepared as described by Dignam et al. [18] with a slight modification. HEL cells were grown to a density of 1.5×10^7 cells/10 ml in 100-mm plastic dishes. The cells from 20 dishes were collected (3×10^8 cells), suspended in 5 ml of 10 mM HEPES at pH 7.9 containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), incubated on ice for 10 min, and homogenized by a Dounce homogenizer B pestle. The cell homogenate was centrifuged at $1,000 \times g$ for 10 min at 4°C, and the pellet was suspended in 2 ml of 20 mM HEPES at pH 7.9 containing 25% (v/v) glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.5 mg/ml pepstatin A (Peptide Institute), 0.5 mg/ml leupeptin (Peptide Institute), and 1.3 μ g/ml spermidine (Sigma). The suspension was stirred at 30 rpm for 1 h at 4°C, and centrifuged at $100,000 \times g$ for 30 min at 4°C. The resulting supernatant was dialyzed twice each for 12 h against 1.5 liter of 20 mM HEPES at pH 7.9 containing 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM PMSF. The dialysate was centrifuged at $30,000 \times g$ for 30 min at 4°C. The supernatant was stored at $-80^\circ C$ until use. U937 cells were grown to a density of 1.0×10^7 cells/10 ml with RPMI 1640 medium and 10% FCS in 100-mm plastic dishes. HeLa cells were grown to a density of 5×10^6 cells/10 ml with DMEM medium and 10% FCS in 100-mm plastic dishes, and treated with 2.5 μ M phorbol 12-myristate 13-acetate for 30 h. The nuclear extracts from U937 and HeLa cells were prepared as described above.

2.6. DNase I footprinting analysis

Two probes were prepared for the DNase I footprinting reaction. The probes were amplified by PCR method as described above for the luciferase reporter gene construction. We used two primers: upstream primer (–618 to –595), 5'-TCGTTGACGGAAGCACAGCGTTC-3'; downstream primer (–435 to –416), 5'-ATGAGTCCATCTTCCAAACG-3'. Either of the two primers was labeled with [γ - ^{32}P]ATP (DuPont-NEN) using T4 polynucleotide kinase (New England Biolab). Amplified DNA fragments were purified electrophoretically on a 5% polyacrylamide gel, and used as sense and antisense probes. The DNase I footprinting reaction was performed as described by Roesler et al. [19]. DNA sequencing with the sense strand was carried out using Sequenase sequence system (U.S. Biochemical Corporation). G + A reaction was performed as described by Sambrook et al. [16]. These sequence ladders were used as reference. The reaction mixtures were denatured at 95°C for 5 min, and loaded onto an 8% polyacrylamide sequencing gel containing 8 M urea. The gel was dried and exposed to Kodak XAR film with an intensifying screen for three days at $-80^\circ C$.

2.7. Electrophoretic mobility-shift assay (EMSA)

Two pairs of oligonucleotide targeting NF κ B site from –520 to –544 bp were synthesized: *oligo 1*, 5'-GCCGCGGGACATCCCCAGACC-CAG-3' and *oligo 2*, 5'-CTGGGGTCTGGGGATGTCCCGCGGC-3'; *oligo 3*, 5'-GCCGCGGGACATCCCCAGACC-CAG-3' and *oligo 4*, 5'-CTGGGGTCTGGGGATGTCCCGCGGC-3'. *Oligo 3* and *oligo 4* were site-specific mutants for NF κ B motif (the underlined bases were changed). Each pair of oligonucleotide (*oligo 1* and *oligo 2*, or *oligo 3*

and *oligo 4*) was annealed as described by Berger and Kimmel [20]. Annealed oligonucleotides were isolated electrophoretically on a 15% polyacrylamide gel, end-labeled with [γ - ^{32}P]ATP, and used as probes (g6 or g7, respectively). For competition analysis, an annealed oligonucleotides (κ 3 sequence of immunoglobulin enhancer as NF κ B competitor, CAGAGGGGACTTCCGAGAGG [6]) was prepared by the same method described above. The binding reaction was performed in a 15- μ l mixture containing 1 μ g of poly(dI-dC)·poly(dI-dC) (Pharmacia), 20 mM HEPES at pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 2% (w/v) polyvinyl alcohol, 2.5 μ g of the nuclear extracts of HEL or HeLa or U937 cells, and the probe (1×10^4 cpm, about 25 fmol). Each antibody (1 μ g) against NF κ B p50 (Santa Cruz Biotechnology), NF κ B p65 (Serotec), or c-Rel (Serotec) was added to the mixture for the supershift assay. The mixtures were incubated at room temperature for 30 min, and loaded on a 4% polyacrylamide gel. Electrophoresis was carried out at a constant voltage of 150 V for 2 h. The gel was dried, and analyzed by a Fujix Bio-image analyzer BAS 2000.

3. Results and discussion

Various response elements are found in about 750 base pairs (bp) of the 5'-flanking region of human 12-lipoxygenase gene

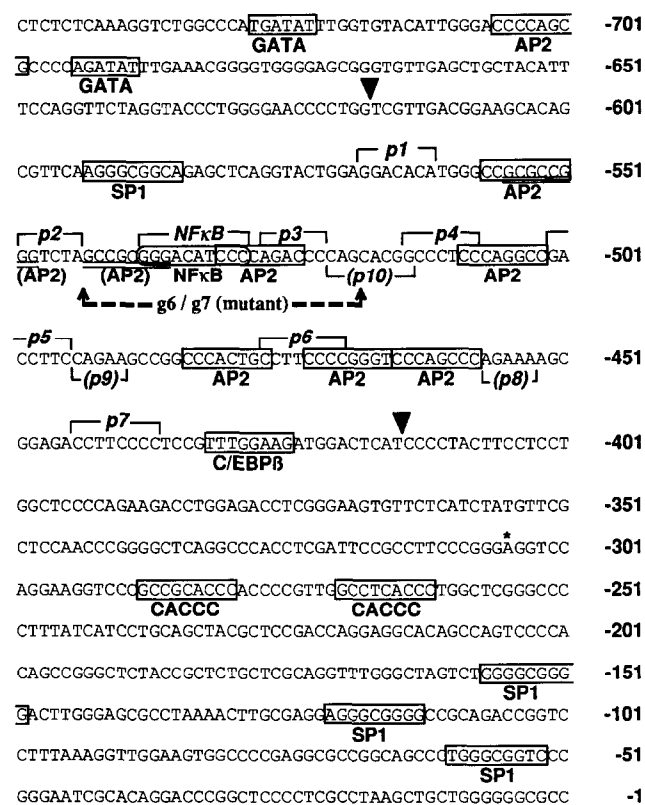


Fig. 1. Regulatory elements of human 12-lipoxygenase gene promoter. Each putative consensus sequence in the 5'-upstream region of human 12-lipoxygenase gene [3] is enclosed with square. Double underline shows consensus sequence on the antisense strand. Abbreviations used are: SP1, SP1 binding site; CACCC, CACCC box found in β -globin gene promoters; AP2, binding site of AP2; NF κ B, binding site of NF κ B; C/EBP β , binding site for CCAAT/enhancer-binding protein; and GATA, GATA box. An asterisk denotes the transcription start site. p1–p7 and NF κ B indicate the protected regions on the sense strand upon DNase I footprinting analyses, and p8–p10 are those on the antisense strand (See Fig. 4). Footprinting probes were made according to the sequence between two arrow heads (–618 to –416 bp). g6 and g7 are the EMSA probes the positions of which are indicated by broken line and arrows.

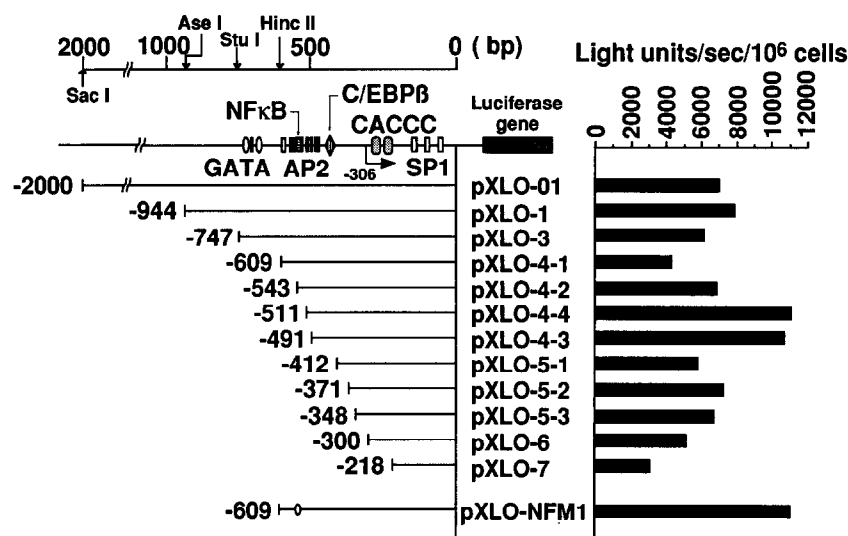


Fig. 2. Construction of luciferase expression vectors and transfection analysis of human 12-lipoxygenase gene promoter. Truncated promoter fragment was ligated into a luciferase plasmid. Numbers indicate distance in base pairs from the start of translation. pXLO-NFM1 contains a mutant NFκB motif at -539 to -530 bp (GGGACATCCC changed to GGCCCATCCC). Restriction enzyme cleavage sites and putative regulatory elements are also shown at the top of the figure. A mixture of each plasmid (3 μg) and β-galactosidase plasmid (1 μg) was transfected into HEL cells (1.2×10^6 cells) by the lipofection method. The luciferase activity was normalized with the β-galactosidase activity, and expressed as units/s/10⁶ cells.

(Fig. 1). There are consensus elements for AP2 (-558 to -459 bp and -707 to -700 bp counted from the initiation codon), NFκB (-539 to -530 bp), SP1 (-158 to -53 bp and -594 to -586 bp) and C/EBPβ (-433 to -426 bp). Two GATA boxes (-729 to -690 bp) and two CACCC boxes (-289 to -263 bp) are also found.

For deletion analysis of the promoter region, we constructed luciferase vectors of various lengths covering the region from about -2000 to -218 bp (Fig. 2). Each vector was transfected to HEL cells by the lipofection method, and the luciferase activity was measured with the cell lysate. The luciferase activity decreased when three positive regions (-944 to -609 bp, -491 to -412 bp, -348 to -218 bp) were deleted. In contrast, the luciferase activity increased when the negative region from -609 to -511 bp was absent. There were several response elements such as SP1, AP2 and C/EBPβ in the positive regions mentioned above, and there were AP2 and NFκB sites in the negative region (Fig. 2).

The experimental results presented below suggest that NFκB is constitutively expressed in the nucleus of HEL cells, and binds to the corresponding site on the human 12-lipoxygenase gene promoter. First, NFκB motif is present at -539 to -525 bp as illustrated in Fig. 1. Secondly, as shown in Fig. 3, two shifted bands (complexes c and d in lane A1) were found in EMSA using a probe targeting NFκB site and the HEL cell nuclear extracts, and the bands were lost by the addition of competitor DNA κ3 (lanes A3–A5). Thirdly, further addition of antibodies against three subunits (NFκB p50, p65 and c-Rel) brought about new bands; bands a and b by anti-p50 (lanes A2 and A8), band g by anti-p65 (lane A9), and band f by anti-c-Rel (lane A10). Complex c became faint by the addition of anti-c-Rel antibody (lane A10). NFκB p50 homodimer presented as complex e in lane B2 was different from complexes c and d. For further characterization of these complexes binding to NFκB motif in HEL cells, nuclear extracts were also prepared from

U937 and HeLa cells, and used in EMSA. It is known that NFκB and Rel proteins are expressed in the nuclei of HeLa and U937 cells [21,22]. When these nuclear extracts were added, the complex d appeared (lanes C2 and C3). When the amounts of nuclear extracts were doubled to 5 μg, complex h appeared (lanes C5–C7). Furthermore, c-Rel protein is 75 kDa which is a little bigger than p65 (65 kDa). Thus, complex h is presumably a heterodimer of c-Rel and p50. Since NFκB motif was identified first as an enhancer of immunoglobulin κ light-chain gene, we end-labeled κ3 double-stranded oligonucleotides and used them as probes in EMSA for our 12-lipoxygenase gene. As shown in Fig. 3D, complex β was found using the three nuclear extracts, and complex α appeared using HEL and U937 cell nuclear extracts (lanes D1–D3). Complexes α and β were different from complex γ which was an NFκB p50 homodimer (lane D4). Complex α may be a P50 and c-Rel heterodimer, and complex β is presumably a P50 and p65 heterodimer. Thus, the complex d is a heterodimer of NFκB p50 and p65, and complex h is a NFκB p50 and c-Rel heterodimer. It is known that c-Rel protein interacts with many factors [23–27]. Therefore, the complex c may be composed of NFκB p50, c-Rel protein and other proteins interacting with c-Rel protein. In addition, mutation of the NFκB motif in the human 12-lipoxygenase gene increased the luciferase activity (pXLO-NFM1 in Fig. 2). Using a site-specific mutant probe of NFκB site (g7), complexes c and d were not detected (lane B4), and a much less dense band of complex e was observed indicating the decrease in its affinity (lane B5). These findings suggest a negative regulation of 12-lipoxygenase gene expression by complexes of proteins binding to NFκB motif in HEL cells.

We also demonstrated three positive regulatory regions (-944 to -609 bp, -491 to -412 bp, -348 to -218 bp) by the luciferase assay (Fig. 2), but these regions have no known response elements. We prepared sense and antisense probes from -618 to -416 bp (Fig. 1), and used them in the foot-

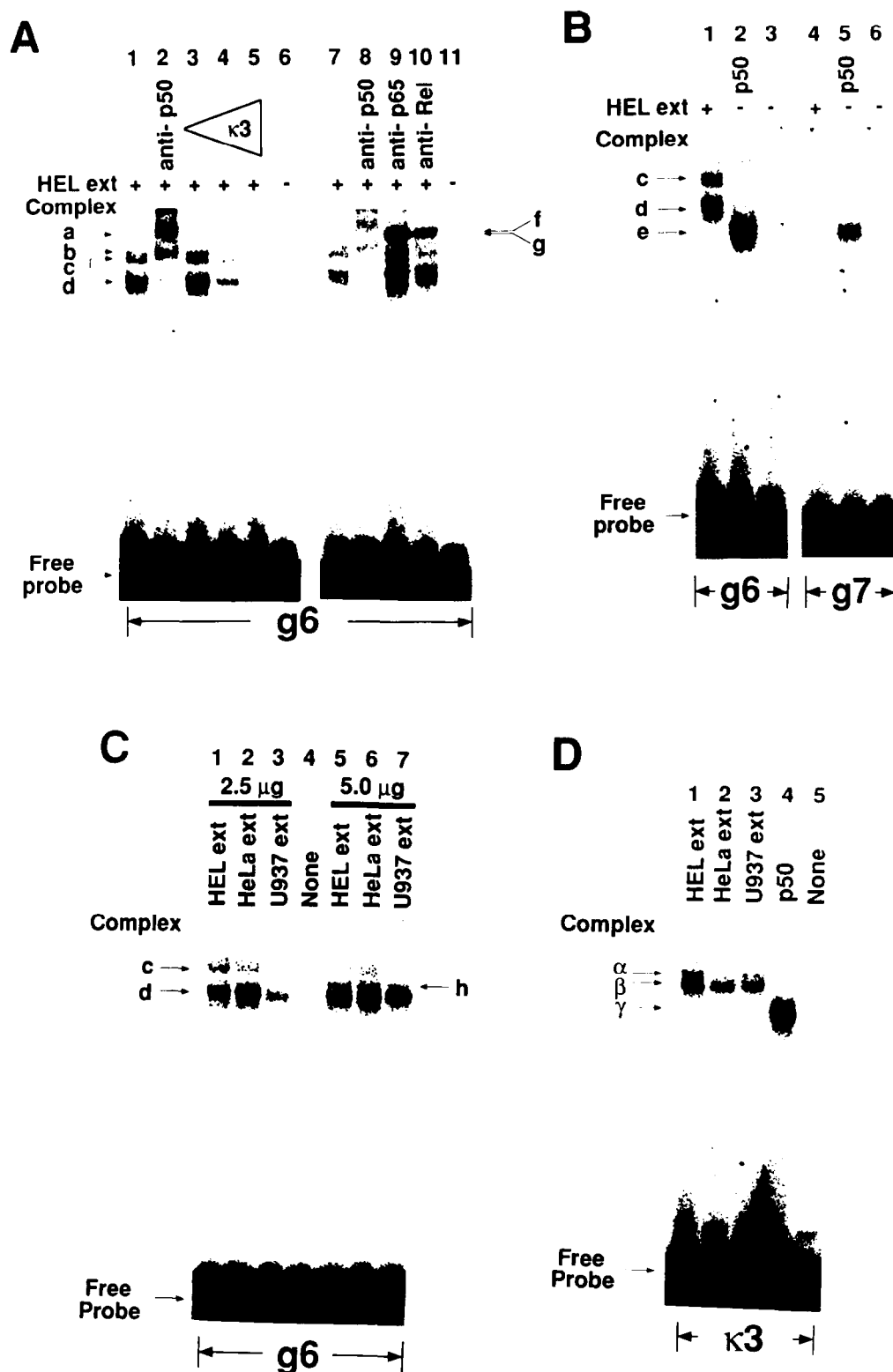


Fig. 3. Electrophoretic mobility-shift assay. Shifted bands are indicated by arrows a–g and α – γ . The nuclear extracts (2.5 μ g protein) were incubated with each probe. (A) The HEL cell nuclear extracts and probe g6 targeting NF κ B site were used. Lanes 1 and 7 were for the HEL cells nuclear extracts alone. κ 3 competitor DNA of NF κ B site was added; 50 fmol (2-fold molar excess against g6 probe, lane 3), 1 pmol (40-fold, lane 4), 50 pmol (2000-fold, lane 5). Anti-NF κ B p50 (lanes 2 and 8), anti-NF κ B p65 (lane 9) or anti-c-Rel (lane 10) antibodies were incubated in addition to the nuclear extracts. Lanes 6 and 11 were for incubation with no protein. (B) Probe g6 and probe g7 which contained a mutation for NF κ B site were used. Lanes 1 and 4 were for the HEL cells nuclear extracts alone. For controls, 50 ng of purified NF κ B p50 protein (lanes 2 and 5) were incubated. Lanes 3 and 6 were for incubation without proteins. (C) g6 probe was used with HEL (lanes 1 and 5), HeLa (lanes 2 and 6) and U937 (lanes 3 and 7) cell nuclear extracts. Nuclear extracts were incubated in an amount of 2.5 μ g (lanes 1–3), 5.0 μ g (lanes 5–7) and none (lane 4). (D) κ 3 probe was incubated with HEL (lane 1), HeLa (lane 2) and U937 (lane 3) cell nuclear extracts; NF κ B p50 protein (lane 4) or none (lane 5).

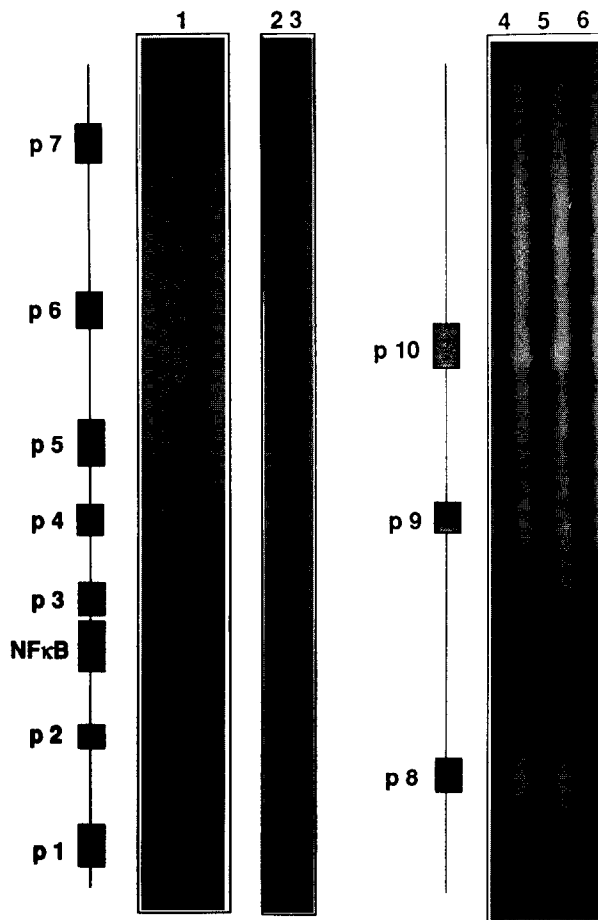


Fig. 4. DNase I footprinting analysis. Two probes (2×10^4 cpm) were prepared and allowed to react with the HEL cell nuclear extracts (27 μ g). Sense strand probe was used in lanes 2 and 3, while antisense strand probe was used in lanes 4, 5 and 6. Lane 1, sequencer ladders for sense-strand; lanes 2 and 5, with the nuclear extracts; lanes 3 and 6, without the nuclear extracts; lane 4, G + A reaction. The protected regions p1–p10 and NF κ B sequence are shown by bars on the left side.

printing analysis with the nuclear extracts of HEL cells (Fig. 4). The sense probe showed DNase I protection in the regions of p1–p7 and NF κ B consensus sequence (lane 2), and the regions of p8–p10 in the antisense probe were also protected (lane 5). As shown in Fig. 1, p6, p7 and p8 are found in one of the positive regions (–491 to –412 bp) demonstrated by luciferase assay. However, their binding proteins are still unidentified. It should be noted that p6 and p7 had the same sequence CCTTCCCC. A certain transcription factor binding to this CCTTCCCC motif may work as a positive transcriptional regulator for human 12-lipoxygenase.

Little is known about the transcriptional regulation of mammalian lipoxygenases. Human 5-lipoxygenase gene has many GC-rich regions (–212 to –88 bp from the initiation codon), and these regions have positive regulatory elements as examined by chloramphenicol acetyltransferase assay. Transcription factor SP1 was bound to these regions judging from EMSA using HeLa cell nuclear extracts and an SP1 competitor oligonucleotide [28]. The SP1 may be involved in lipoxygenase

gene expression since SP1 consensus sequences are present in all mammalian lipoxygenase gene promoters so far studied [2–5, 28–30]. In addition to human 12-lipoxygenase, NF κ B consensus motifs were found in several mammalian lipoxygenase genes: two types of murine 12-lipoxygenase (–383 to –374 bp in platelet-type; –485 to –476 bp and –808 to –799 bp in leukocyte-type) [5], and human (–445 to –436 bp) [28] and guinea pig (–327 to –318 bp) [29] 5-lipoxygenases. In contrast, the gene promoters of porcine 12-lipoxygenase and rabbit 15-lipoxygenase have no NF κ B motif within the sequences already determined [2,30]. When these NF κ B sites and their vicinity are compared between human and murine platelet-type 12-lipoxygenase genes, we should note that there are three common sequences: NF κ B, GGGACATCCC (human) and GGGCATCCCC (murine); AP2, CCCCAGAC (human) and CCCCAGGC (murine); p4 (Fig. 1), GCCCTCC (human and murine) [3–5].

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