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In Vivo Protein-DNA Interactions at the Kinin B_1 Receptor Gene Promoter: No Modification on Interleukin-1 Beta or Lipopolysaccharide Induction

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Abstract The kinin B_1 receptor (B_1R) gene is strongly upregulated following tissue injury and inflammation. In an attempt to define the regulatory elements that account for the control of B_1R gene expression, we have conducted in vivo footprinting analysis of the B_1R gene promoter region in three human cell types: embryonic lung fibroblast cells (IMR-90), embryonic kidney cells (HEK-293), and primary cultures of vascular umbilical smooth muscle cells. Initial in vitro delineation of the B_1R gene promoter by transient transfection experiments with a reporter gene indicated that a 1.4-kb region, located just upstream of the transcription initiation site, bears all the characteristics of a core promoter with a functional TATA box and additional positive and negative control elements, as some of them could be tissue-specific. In vivo ultraviolet and dimethylsulfate footprinting analyses of the 1.4-kb region revealed no difference between the footprint patterns in the three cell types studied. We found that even in the noninduced state, the B_1R gene promoter is possibly bound by several sequence-specific DNA binding proteins (GATA-1, PEA3, AP-1, CAAT, Sp1, Pit-1a, Oct-1, CREB). Some other footprints were detected on sequences that do not correspond to any known transcription factor binding site. No additional changes in protein-DNA complexes were observed upon treatment with interleukin-1 beta (IL-1β) or bacterial lipopolysaccharide, shown previously to induce B_1R gene expression. These results indicate that complex protein-DNA interactions exist at the B_1R gene promoter prior to induction by external stimuli even in cells (HEK-293) that do not express a functional B_1R. J. Cell. Biochem. 78:278–296, 2000.

Key words: G protein-coupled receptor; transient transfection; chloramphenicol acetyl transferase reporter gene; transcription factor binding motif; in vivo genomic footprinting; ligation-mediated PCR; primary cultures of human smooth muscle cells

Kinins are blood-derived peptides that influence circulation and renal function, and act as inflammatory mediators via two subtypes of kinin receptors, B_1 and B_2, which have been characterized on a pharmacological basis [Regoli and Barabe, 1980]. Whereas the B_2 receptor (B_2R) is responsive to the intact kinins, bradykinin (BK) and Lys-BK (kallidin), the B_1 receptor (B_1R) has higher affinity for the carboxypeptidase metabolites of kinins, des-Arg^9-BK and des-Arg^{10}-kallidin [Marceau et al., 1998]. Expression cloning of B_1R and B_2R cDNAs have confirmed the existence of these two subtypes and revealed that they both belong to the superfamily of G protein coupled receptors [reviewed in Marceau et al., 1998]. Although the B_2R is constitutively expressed in a variety of tissues and cultured cell lines and mediates most of the in vivo effects usually assigned to kinins, the B_1R is not present to any significant extent in normal tissues. However, functional responses to the B_1R are rapidly up-regulated from a null basal level in biologic systems following some types of tissue injuries and inflammation, notably the injection of bacterial

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materials to rabbits, rats, or pigs, with effects pertaining to hemodynamics, smooth muscle contractility, pain perception, and leukocyte recruitment [Marceau et al., 1998]. For example, B_1R gene transcription has been observed in hearts from rabbits pretreated with bacterial lipopolysaccharide (LPS), but not in organs from control animals [Marceau et al., 1997]. Some of these observations are based on tissues of human origin [e.g., Menke et al., 1994; Sardi et al., 1998]. Thus, a fast and specific genetic program recruits the expression of the B_1R gene in smooth muscle cells (SMCs), endothelial cells, fibroblasts, and a few other cell types. The cytokine network and some mitogen activated protein kinase pathways have been linked to B_1R gene expression in functional experiments [Larrivée et al., 1998]; the gene for this receptor is known to be strongly upregulated by interleukin-1 beta (IL-1β), a proinflammatory cytokine [Marceau et al., 1998]. However, the molecular mechanisms involved in the regulation of the B_1R gene expression have not yet been clearly defined.

We and others have studied the genomic structure of the human B_1R gene [Bachvarov, 1996; Yang and Polgar, 1996]. Convergent results indicate that this receptor gene contains three exons separated by two introns. The first and the second exons are noncoding, whereas the coding region and the 3′-flanking region are located entirely at the third exon. Recently, several studies based on transient transfection experiments of promoter fragments fused to a reporter gene have indicated the presence of positive and negative control regions in the human B_1R gene promoter region [Yang et al., 1998] and the putative implication of the nuclear factor-kappa B (NF-κB) in the IL-1β upregulation of the B_1R gene expression [Ni et al., 1998; Schanstra et al., 1998; Zhou et al, 1998]. As potential features of a strongly regulated gene were found, we have conducted a high resolution in vivo footprinting analysis of the human B_1R gene promoter region in three human cell types: IMR-90 cells and primary cultures of human umbilical artery SMCs (both of which represent a relevant model for the regulated expression of the human B_1R gene in functional studies [Marceau et al., 1998; Menke et al., 1994; Abbas et al., 1998]) and in the human embryonic kidney (HEK-293) cell line (which does not exhibit endogenous B_1R binding [Yang et al., 1998] and was used for comparison).

MATERIALS AND METHODS

Materials

Radiolabelled nucleotides [α-^{32}P] dCTP (3000 Ci/mmol) and [α-^{35}S] dATP (1,250 Ci/mmol) were obtained from New England Nuclear (Mississauga, ON, Canada). Oligodeoxynucleotide primers were purchased from Life Technologies, Inc. (Burlington, ON, Canada). Restriction and modification enzymes were obtained from New England Biolabs, Inc. (Mississauga, ON, Canada) and from Pharmacia (Baie d'Urfé, QC, Canada). Dideoxy-sequencing kits were from Pharmacia.

Cell Types and Cultures

The human lung fibroblast cell line IMR-90 (CCL 186, American Type Culture Collection, Rockville, MD) was grown in minimal essential medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin (Life Technologies, Inc. Burlington, ON). The human embryonic kidney cell line HEK-293 (CRL-1573, American Type Culture Collection, Rockville, MD) was grown in Eagle's minimum essential medium with 2 mM L-glutamine and Earle's supplements adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% FBS. Primary cultures of SMCs were obtained in a pure form from the human umbilical artery and propagated as described previously [Marceau et al., 1990]. For both transfection analysis and genomic footprinting, the cells were used at passage four or five.

Analyses of B_1R mRNA Expression

Primary SMCs were used for the isolation of total RNA. Some cells were exposed before RNA extraction to 500 pg/ml IL-1β (R & D Systems, Minneapolis, MN) for 1 h, or to 10 μg/ml LPS for 24 h. In some cases, 25 ng/ml interleukin receptor antagonist (IRA; R & D Systems) was added prior to IL-1β or LPS treatment. For B_1R gene expression analysis, total RNA was prepared from treated and control cells as shown previously [Chomczynski and Sacchi, 1987]. The B_1R gene expression was determined by semi-quantitative duplex reverse transcriptase polymerase chain reaction (RT-PCR) as described [Dukas et al., 1993]. Briefly, 20 μg of total RNA were treated for 30 min at 37°C in 20 mM Tris · HCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl, 60 U RNA guard

(Pharmacia) with 2 U of DNase I (amplification grade; Life Technologies, Inc.) to remove traces of genomic DNA. The mixes were then extracted with phenol/chloroform and the RNA was ethanol-precipitated and resuspended in 20 μl of sterile water. Two μg of DNase I-treated RNA were denatured at 94°C for 3 min in 50 mM Tris · Cl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 250 ng of oligo-dT_{15}. Then, 50 U of RNA guard, 1 mM of each dNTPs, and 200 U of Moloney Murine leukemia virus (MMLV) reverse transcriptase (Life Technologies, Inc.) were added and the mix was incubated for 10 min at room temperature followed by 75 min at 37°C. One tenth of the RT reactions was further used for PCR amplification. Each 50 μl PCR reaction contained 2 μl RT mix, 10 mM Tris · HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 2% dimethyl sulfoxide, 60 μM of each deoxynucleotide triphosphates (dNTPs), 250 ng of each of the B_1R primers, 25 ng of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) primers (needed for the amplification of an internal standard), and 1.5 u of *Taq* polymerase (Life Technologies, Inc.).

The following oligonucleotides were used as PCR primers: 5′-TGTGCATGGCATCATCCTGGC-3′ and 5′-GGCAACCACGAGCGTGAGGAT-3′ were used as sense and anti-sense primers, respectively, for the amplification of a specific human B_1R fragment. The primer sequences were selected from the published human cDNA sequence [Menke et al., 1994]. 5′-CACCATCTTCCAGGAGCGAGATCC-3′ and 5′-GTCTTCTGGGTGGCAGTGAT-GGC-3′ were used as sense and anti-sense primers, respectively, for the amplification of a specific human GAPDH fragment (primer sequences selected from Applequist et al. [1995]). The samples were denatured initially for 3 min at 94°C and then submitted to 24 cycles of PCR (45 s at 94°C, 45 s at 64°C, 75 s at 72°C) followed by a 10-min final elongation step at 72°C. The number of cycles was chosen to keep the PCR-amplified DNA in the exponential phase of amplification. One tenth of the PCR reactions was run on a 1% agarose gel in 1 × Tris-acetate (TAE) buffer and then transferred to BrightStar-Plus nylon membrane (Ambion, Inc., Austin, TX). The membranes were prehybridized for 1 h at 65°C in a buffer containing 6× SSC, 5× Denhardt's, 0.5% sodium dodecyl sulphate (SDS), 100 μg/ml salmon sperm DNA; then 10^6 cpm/ml of human B_1R gene random-primed- ^{32}P-labeled probe were added and hybridization was carried out for 8–16 h at 65°C. The membranes were repeatedly washed (final wash 0.1× SSC /0.1% SDS at 65°C) and exposed to Kodak Biomax MS (InterSciences Inc., Markham ON, Canada) autoradiographic films. The membranes were then stripped in boiling 0.1% SDS and rehybridized with a rat GAPDH probe used as an internal standard. The resulting autoradiograms were scanned with a ScanJet 6 (Hewlett-Packard) and analyzed with 1D-main densitometry software (AAB Software).

Plasmid Constructions

Different segments from the 5′-noncoding region of the human B_1R gene were generated by PCR technology and then subcloned (some of them in both orientations) into the unique *Hin*dIII blunted site of the promoterless vector pCAT-Basic (Promega Corp., Madison, WI) in front of the reporter gene chloramphenicol acetyltransferase (CAT). Previously constructed plasmids [Bachvarov et al., 1996], which contain cloned human B_1R gene fragments, were used as templates for the PCR amplifications. Table 1 presents the list of the PCR primers used together with the positions (relative to the transcription initiation site as indicated in Bachvarov et al. [1996]) of the amplified promoter fragments and the designations of the CAT constructs. The orientation of the cloned fragments in each construct was checked by both restriction mapping and sequencing.

Transient Transfections and Determination of Reporter Activities in Cell Types

Plasmid DNAs used for the transfection experiments were double-purified on CsCl gradients. The constructs were transiently transfected in IMR-90 and HEK-293 cells, by the Ca phosphate coprecipitation procedure [Sambrook et al., 1989]. Transient transfection in primary SMCs was performed using the cationic polymer ExGen 500 (MBI Fermentas Inc., Flamborough, ON, Canada), as described by the manufacturer. All transfections were performed in duplicate, and each construct was tested in at least two independent experiments using different batches of plasmid preparations. The pCAT-Basic vector was transfected as a negative control and the values of the basal CAT activity were subtracted from those obtained with the promoter constructs. The

TABLE I. PCR Primers Used for the Isolation of Human B_1R Gene Promoter Fragments[a]

Sense primer (5′—3′)	Anti-sense primer (5′—3′)	PCR fragment position[b]	Plasmid
TGCAAAGTGAAATGAGAGTGGATTTTTTC	TCAGGCAGAAAATGAAGGCGT	−139/+86	p-139-CAT
CCCATGCAGACATAATTTGAG	TCAGGCAGAAAATGAAGGCGT	−566/+86	p-566-CAT
GAATTCTGGCATGAATGAGTTC	TCAGGCAGAAAATGAAGGCGT	−734/+86	p-734-CAT
CCTAAGAACAAACGCTTCTCAG	TCAGGCAGAAAATGAAGGCGT	−1247/+86	p-1247-CAT
AGCAGAGGATTACACCAGGAC	TCAGGCAGAAAATGAAGGCGT	−4203/+86	p-4203-CAT
CCCATGCAGACATAATTTGAG	GTGATGTCATTGTGGGATTG	−566/−45	p-566/-45-CAT
CCTAAGAACAAACGCTTCTCAG	CCTGTCAACTTACAAATTAAG	−1247/−662	p-1247/-662-CAT
AGCAGAGGATTACACCAGGAC	AGGCTCAGTAGCGGATGTACA	−4203/−949	p-4203/-949-CAT
AGCAGAGGATTACACCAGGAC	CCTGTCAACTTACAAATTAAG	−4203/−662	p-4203/-662-CAT

[a]Primers were cloned in the pCAT-Basic vector in front of the CAT reporter gene.
[b]Positions of the PCR fragments are given relative to the transcription initiation site [Bachvarov et al., 1996].
PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase.

pcDNA3-CAT vector (Invitrogen, Carlsbad, CA) was used as a positive control. The cells were harvested 72 h after transfection and the CAT activity was measured by the phase-extraction assay, as described [Kingston et al., 1992]. The results were normalized for protein content and for β-galactosidase activity by co-transfection with a second reporter gene (pβGAL; Promega Corp., Madison, WI). In some cases the transfected cells were treated for 24 h before recuperation with IL-1β (R & D Systems Inc.) (20 ng/ml culture medium), or immediately after transfection with anti-IL-1α antibodies (R&D Systems Inc.) (1 μg/ml culture medium).

Genomic Footprinting of the B_1R Gene Promoter Region

Drug incubations.

IMR-90, HEK-293, and primary SMCs (80–90% confluent) were treated with IL-1β (0.5 ng/ml culture medium) for 0, 7.5, 15, 30, or 60 min at 37°C. In some cases, SMCs were treated with LPS (10 μg/ml culture medium) for 24 h. After the incubation, the culture medium was removed from the Petri dishes and the cells were washed with unsupplemented Dulbecco's minimum essential medium (DMEM).

Dimethylsulfate treatment and chemical cleavage of N7-methylguanines.

Living cells and purified DNA from each cell type (referred to in this article as in vivo and in vitro, respectively) were treated by dimethylsulfate (DMS). The cells were incubated with unsupplemented DMEM containing 0.2% DMS (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for 6 min at room temperature [Drouin et al., 1997]. After cell detachment by trypsinization, nuclei were isolated and methylated-DNA purified as described [Pfeifer and Riggs, 1993; Drouin et al., 1996a]. Purified DNA was treated by DMS as usual [Maxam and Gilbert, 1980]. Methylated guanines were chemically converted to single-strand DNA breaks by hot piperidine (Sigma-Aldrich Canada Ltd.) [Pfeifer and Riggs, 1993]. The single-strand break frequencies were estimated using alkaline gel electrophoresis [Drouin et al., 1996b].

Ultraviolet irradiation and enzymatic cleavage at cyclobutane pyrimidine dimers.

Purified DNA and cells were irradiated on ice with two 254-nm germicidal lamps [Drouin and Therrien, 1997]. The ultraviolet C dose

and the irradiation time were 1 500 J m^{-2} and 32 s, respectively. Cell lysis, nucleus sedimentation, and DNA purification were performed as described [Drouin et al., 1996a; Tornaletti and Pfeifer, 1996]. The enzymatic conversion of cyclobutane dimers to single-strand DNA breaks was carried out as published [Pfeifer et al., 1992; Tornaletti and Pfeifer, 1996]. The single-strand break frequencies were also estimated by alkaline gel electrophoresis [Drouin et al., 1996b].

Ligation-mediated polymerase chain reaction (LMPCR).

Details of the LMPCR protocols used for this work have already been published [Drouin et al., 1996a; Tornaletti and Pfeifer, 1996]. Approximately 1.4 kb of the human B_1R gene promoter region (GenBank accession no. U48230: from nt −1356 to +90 upstream and downstream of the transcription initiation site; numbers as in Bachvarov et al. [1996]) were analyzed on both strands using the primer sets presented in Table 2. DNA analysis by chemical cleavage reaction was carried out as previously reported [Pfeifer and Riggs, 1993; Iverson and Dervan, 1987]. The chemically cleaved G, A, T+C, and C samples done on purified genomic DNA from human peripheral blood lymphocytes were included along with the other samples in the LMPCR assays as sequence markers. Only clear band intensity differences (more than 50% lower intensity or more than twofold higher intensity) between in vivo and in vitro samples were considered to be footprints. Experiments were repeated at least twice to ensure reproducibility.

Sequence analysis.

To identify potential transcription regulatory sites in the human B_1R gene promoter, we referred to our published data [Bachvarov et al., 1996] and also used TESS (Transcription Element Search Software) as implemented in Computational Biology & Informatics Laboratory (URL: http://www.cbil.upenn.edu/tess/index.html). This software accesses the TRANSFAC MATRIX database 3.3, which contains the consensus binding sites for a variety of transcription factors.

RESULTS

Preliminary in Vitro Analysis (Delineation) of the Human B_1R Gene Promoter

Initially, we have analyzed the putative promoter region of the human B_1R gene by cloning different PCR-generated fragments in front of the CAT reporter gene. The region we have studied comprises a 4.2-kb fragment upstream from intron I, and including the consensus TATA box, located by us previously [Bachvarov et al., 1996] (see Fig. 1). All the constructs shown on Fig. 1 were transiently transfected into three different cell types: human lung fibroblast cells (IMR-90), primary cultures of human SMCs, and human embryonic kidney 293 cells (HEK-293). The first two cell types are known to endogenously express functional B_1Rs [Menke et al., 1994; Abbas et al., 1998]. Due to variations in transfection efficiency, it is difficult to make an accurate comparison of the basal activities of the same construct in different cells. We therefore compared the basal activities of a series of deletion constructs within each cell type; as the base of comparison was the promoter activity of the smallest (p-139-CAT) construct.

Our initial results have demonstrated the functional significance of the TATA box: all constructs, containing 5′-flanking region fragments in which the TATA box has been truncated (nt −566 to −45, nt −1247 to −662, nt −4203 to −662 and nt −4203 to −951), showed no promoter activity in the three cell types studied (data not shown). Two other domains from the region analyzed have been indicated to be important in the regulation of human B_1R gene expression. A 169-bp region (nt −734 to −566) upstream from the transcription initiation site contains element(s) functioning as a positive regulator(s) of the receptor gene expression (Fig. 1). This positive regulatory region is capable of increasing substantially the promoter activity in IMR-90 cells as the p-734-CAT construct displays a 14-fold higher promoter activity than the p-139-CAT construct, and is only 40% weaker than that of the strong cytomegalovirus promoter (the construct pcDNA3-CAT) used as a positive control in our experiments (data not shown). However, the nt −734 to −566 region has quite a moderate effect in HEK-293 and, surprisingly, also in primary SMCs (about 1.5-fold increase of promoter activity compared with the basal p-139-CAT construct), indicating that it holds cell-type specific features.

Immediately upstream of this domain, a strong negative control element was located at position nt −1280 to −734. This element completely inhibited the function of the 169 bp positive regulatory region in IMR-90 cells and

TABLE II. Synthetic Oligonucleotide Primers for LMPCR Analysis of the Human B_1R Gene Promoter

Primer	Sequence (5′—3′)	Position[a]	T_m (°C)[b]
Non-transcribed (upper) strand			
57.1	CACAGACTGTCTTCCAGC	−1356 to −1339	57.2
57.2	CAGCAAGAGGCAAAGCAGAGATGAGACCTG	−1342 to −1313	67.5
57.3	CAGAGCTGGCCTTATACGCACTCCTG	−1296 to −1271	66.7
55.1	CAGGAAAGGCTAAGATTTGG	−1143 to −1124	55.7
55.2	GAGCCTTCCTCCGGTTCAGATCTATCC	−1124 to −1098	66.5
55.3	GGGAGTAATGGAAGATGCTTGCCTGTTGAC	−1096 to −1067	66.1
51.1	AGAATGCTATTTGTTCCACC	−949 to −930	53.7
51.2	AGGTCTTCAGCAACAGGAGAAGACAGCAGG	−913 to −884	67.5
51.3	AGGAAAACTTGAAGTGCAGTTGCGTCCCGC	−886 to −857	67.5
53.1	AGGGAACACAAGCAAAAGGC	−834 to −815	57.7
53.2	TCTAAAGACAGAGGTGACACCTTCTGCTCC	−809 to −780	66.2
53.3	GACACCTTCTGCTCCAAACATTTGG	−794 to −770	61.9
49.1	TCTCCTACGTGTAGGGG	−714 to −698	57.0
49.2	GTAAGTTGACAGGTAAGGGGCCTTCAGTTGG	−674 to −644	67.3
49.3	ATCTTGTCACCTGATTCTCAAAGCCAAA	−640 to −613	60.5
47.1	TCTGTTTGTGTGATGGGTCC	−463 to −444	57.7
47.2	GATGGGTCCACTCTGATGGATTCTTGGCGA	−452 to −423	67.5
47.3	TCTTTCATGACCAAAATTCAAAAGAGGCCA	−418 to −389	60.6
45.1	AACATACAGGTCTTGGGCA	−234 to −216	55.4
45.2	TCTGCAGATGGTACCAAAGTAAAGGGGGAG	−203 to −174	66.1
45.3	GATGGTAGCTGAATAATCTCTTATCATCCC	−174 to −145	62.0
Transcribed (bottom) strand			
56.1	CAAGCATCTTCCATTACTCC	−1076 to −1095	55.7
56.2	GGATAGATCTGAACCGGAGGAAGGCTC	−1098 to −1124	66.5
56.3	CTCCAAATCTTAGCCTTTCCTGTTACTTAG	−1122 to −1151	62.0
54.1	TGACATCTCAGCGGGAC	−847 to −863	57.0
54.2	AAGTTTTCCTGCTGTCTTCTCCTGTTGCTG	−877 to −906	64.7
54.3	TTGCTGAAGACCTTAAAACTGTGGAAGTAGG	−901 to −931	63.3
50.1	ATTCTCCAACTGAAGGCC	−639 to −656	55.0
50.2	TACAAATTAAGATCTTTCCAGGCCACCCC	−672 to −701	64.7
50.3	CCTACACGTAGGAGAACTCATTCATGCCAG	−700 to −729	66.1
52.1	CAGAGAGCTAGGAAAACG	−460 to −477	55.0
52.2	GACTCATGCAATGTTCAGAGTTGAGTAAGACCAGA	−481 to −515	65.5
52.3	AGATACATTTTTTCCTAATCCTCCTTGGCG	−513 to −542	62.6
48.1	TCATGAAAGATACATCGCCA	−409 to −428	53.7
48.2	TCGCCAAGAATCCATCAGAGTGGACCCATC	−423 to −452	67.5
48.3	ATCACACAAACAGAGAGCTAGGAAAACG	−450 to −477	62.0
46.1	TTATTCAGCTACCATCTCCC	−159 to −178	55.7
46.2	CTTTACTTTGGTACCATCTGCAGAGGTCTG	−180 to −209	64.7
46.3	GCAATCCTGCCCAAGACCTGTATGTTGGCA	−209 to −238	67.5
44.1	TCAGGCAGAAAATGAAGGCGT	+88 to +68	58.2
44.2	GAAGGCGTTCACAACTGTAGTTGGGTTTTC	+75 to +46	64.3
44.3	GTTGGGTTTTCTGATAGTGAGAGCTGC	+56 to +30	63.5

[a]Primer positions are given relative to the transcription initiation site [Bachvarov et al., 1996].
[b]T_m determined by the GeneJockey software program.
LMPCR, ligation-mediated polymerase chain reaction.

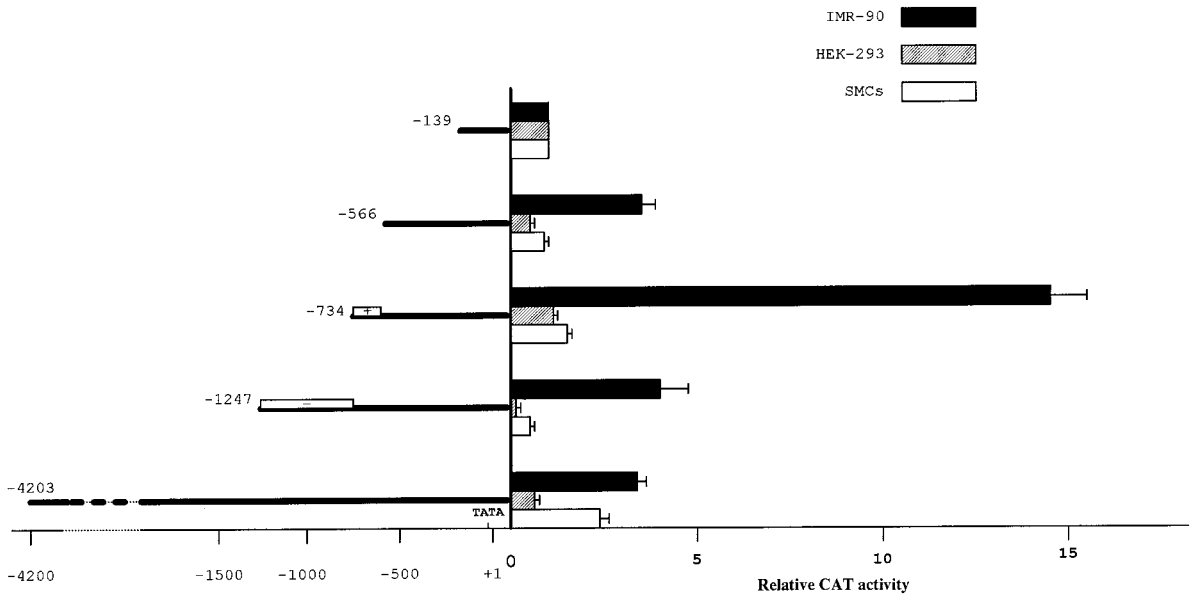

Fig. 1. Promoter activity of different fragments of the 5′-flanking promoter region of the human B_1R gene in IMR-90, HEK-293, and primary smooth muscle cells (SMCs). The promoter region is schematically represented at the lower left of the figure and the TATA box is indicated. The positive (+) and the negative (−) control regions are displayed with boxes. Deletion constructs were co-transfected into the three cell types as described in Materials and Methods. The cells were harvested for both chloramphenicol acetyltransferase (CAT) and β-galactosidase assays. Transfections were normalized to the β-galactosidase control activity, and the activity of the CAT reporter gene is expressed relative to that generated by the p-139-CAT construct in the three cell types studied. The data are presented as means ± standard errors of triplicate determinations.

strongly diminished the promoter activity in primary SMCs and HEK-293 cells (Fig. 1). The transfection experiments performed in HEK-293 cells suggest the existence of an additional negative control element in the nt −566 to −139 region, which probably represents a tissue-specific control domain. Based on absolute values, the promoter activity of all constructs transfected in HEK-293 cells was considerably weaker (at least four to five fold) than those displayed in IMR-90 and SMCs.

Induction of the B_1R Gene Expression

The observation of local production of IL-1β during inflammation accompanied by induction of the B_1R gene expression in several tissues has resulted in the hypothesis that this cytokine is directly involved in the upregulation of this receptor gene [Marceau et al., 1998]. Furthermore, binding studies have shown a sevenfold increase in B_1R binding sites after treatment of IMR-90 cells with IL-1β [Marceau et al., 1998]; and Northern experiments have confirmed the strong upregulation of B_1R mRNA with this cytokine in different cell types [Menke et al., 1994; Bachvarov et al., 1996; Yang, et al., 1998] (see also Fig. 2). In search of IL-1β–responsive elements in the control region studied, we have transfected the constructs shown on Fig. 1 in IMR-90 and primary SMCs that were treated or not with IL-1β post-transfection. The increase in promoter activity observed with some of the promoter constructs varied between 10% and 20% (data not shown), which is not significant and does not correlate to the rate of B_1R gene upregulation in IMR-90 and SMCs upon IL-1β treatment, as measured by Northern experiments [Bachvarov et al., 1996] and functional studies [Menke et al., 1994; Sardi et al., 1998]. Literature data indicate that different types of human cultured cells are capable of expressing both intracellular and secreted forms of IL-1β, especially in stressful conditions (see for example Potvin et al. [1997]), and the serum in the culture media can upregulate gene expression. We made an additional effort to decrease the influence of the autocrine IL-1β and the serum by adding immediately after transfection anti-IL-1a antibodies in the culture medium containing low serum (0.4%), but these additional treatments did not lead to any significant

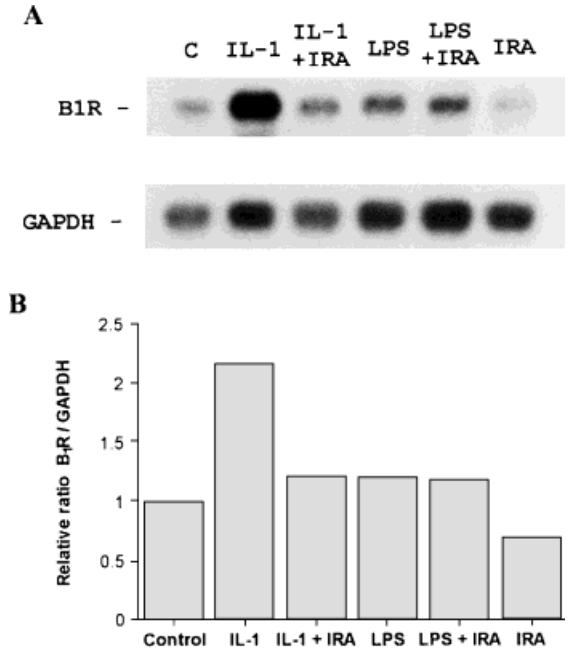

Fig. 2. Duplex reverse transcription-polymerase chain reaction (RT-PCR) analysis of the B_1R mRNA expression in human smooth muscle cell (SMC) cultures. Total RNA was isolated from different SMC cultures [control, or treated with IL-1β, IL-1β + interleukin receptor antagonist (IRA), lipopolysaccharide (LPS), LPS + IRA, and IRA] and used for the synthesis of cDNA by an RT and an oligo-dT primer. This cDNA was used as a template for the simultaneous amplification of two PCR fragments: one corresponding to the B_1R and the other corresponding to a glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) fragment, serving as an internal standard. One tenth of each PCR reaction was loaded on agarose gel and transferred on nylon filters. The transferred DNA was probed with a B_1R-specific labeled fragment and a labeled GAPDH fragment (see Materials and Methods for details). The positions of the B_1R and the GAPDH PCR fragments are indicated. The treatments of the SMCs before cell harvesting and RNA isolation are displayed above each lane. **A:** Representative RT-PCR Southern Blot analysis of control and treated cells showing B_1R versus GAPDH expression. **B:** Quantification of the B_1R gene expression after analyzing the autoradiographic signals. Data are shown upon densitometry and after normalization to GAPDH mRNA levels.

changes of promoter activity upon consecutive induction with IL-1β in all promoter constructs studied (data not shown).

In Vivo Genomic Footprinting of the B_1R Gene Promoter

To localize in living cells DNA-protein interactions within the promoter region of the human B_1R gene and to investigate possible changes after IL-1β and LPS treatments, we conducted a detailed in vivo footprint analysis of the B_1R gene promoter applying the LMPCR approach. The LPS treatment was additionally included in our footprinting experiments because we found experimental evidence that LPS may induce, although modestly, the B_1R gene expression through a different, IL-independent mechanism. The RT-PCR analysis of B_1R expression that we have performed in primary SMCs indicates that pretreatment with IRA can abolish the up regulation of B_1R expression by IL-1β, but IRA has no effect on the LPS-stimulated expression of this receptor gene (Fig. 2). Two independent approaches were used in our LMPCR experiments: footprinting with DMS and footprinting with ultraviolet (UV).

Principles of in vivo DMS and UV footprinting by LMPCR.

Dimethylsulfate is a very reactive molecule that easily diffuses through the cell outer membrane and into the nucleus. It preferentially methylates guanine residues at the N-7 position through the major groove and adenine residues at the N-3 position through the minor groove, but with much less efficiency. Theoretically, each guanine residue of purified DNA displays the same probability of being methylated by DMS. Because DNA inside living cells forms chromatin and is often found associated with a number of proteins, it is expected that its reactivity towards DMS will differ from that of purified DNA. Indeed, the association between DNA and protein will either increase (hyperreactivity) the accessibility of specific guanines to DMS or decrease it (protection) [Pfeifer et al., 1990]. Although protection of specific guanines (negative footprint) nearly always indicates protein binding, hyperreactivity (positive footprint) can either show the edge of a DNA-protein interraction or a special in vivo DNA structure [Cartwright and Kelly, 1991]. Hot piperidine is able to cleave the glycosylic bond of methylated guanines and adenines, leaving a 5′-phosphate [Maxam and Gilbert, 1980]. Single-stranded breaks can be quantitatively detected and amplified by LMPCR, a powerful genomic sequencing technique having a nucleotide resolution level. First, a gene-specific primer 1 is extended to produce a blunt end on one side of each single-strand break generated previously. The ligation of the linker provides a common nucleotide sequence to all 5′ blunt-end DNA molecules regardless of their size. A nested primer 2 in conjunction with a linker primer will amplify all fragments of the

genomic ladder sequence and preserve the quantitative representation of each DMS-induced strand break. These DNA fragments are size-fractionated on a sequencing gel and transferred to a nylon membrane by electroblotting.

In vivo DNA analysis studies using DMS alone can miss sequences with protein-DNA interactions. First, AT-rich DNA sequences cannot be mapped efficiently for DNA-protein interactions because DMS methylates mainly guanine residues. Second, some proteins may not modify the accessibility to DMS. Third, it might also be possible that certain loose DNA-protein interactions will be missed by DMS because of its high reactivity causing the release of these proteins from their DNA sequences. Due to these DMS-related limitations, it is important to apply an alternative in vivo footprinting approach. Ultraviolet light (UVC: 200–280 nm; UVB: 280–320 nm) can also be used for in vivo footprinting [Tornaletti and Pfeifer, 1995]. As for DMS, UV photons are able to reach DNA in the nucleus of living cells without any cell conditionning (plasma membrane permeabilization) that could affect the transcriptional factor binding state. Cyclobutane pyrimidine dimers are the major adducts produced by UV radiation and are formed between the 5,6 bonds of two adjacent pyrimidines [Cadet et al., 1992]. Thus, UV footprinting analyses are limited to sequences with at least two adjacent pyrimidines. The distribution of UV-induced lesions along genomic DNA inside living cells is also affected by chromatin structure and the binding of proteins. These UV lesions can specifically be converted into ligatable DNA single-strand breaks for LMPCR by cleaving them with T4 endonuclease V and photolyase. In addition to DNA-protein interactions and special in vivo DNA structures, UV footprinting can also reveal positioned nucleosomes [Pfeifer et al., 1992; Tornaletti and Pfeifer, 1995]. In summary, it is the difference in reactivity between purified DNA (in vitro) and genomic DNA inside living cells (in vivo) to a damaging agent combined with the specificity and reproducibility of LMPCR that makes in vivo footprinting possible and informative.

The UV and DMS footprinting approaches were applied to three cell types (IMR-90, HEK-293, and primary SMCs) to study the in vivo protein-DNA interactions on a 1.4-kb DNA sequence spanning between −1356 upstream and +90 downstream the transcription initiation site of the human B_1R gene promoter region. This genomic DNA region was analyzed on both strands (sense and anti-sense) and several DMS and UV footprints were identified in the three cell types studied (Figs. 4–8). Every band was carefully evaluated and in vivo/in vitro ratios were estimated. To establish these ratios, the intensity of the band obtained after in vivo treatment was divided by the intensity of the corresponding band obtained upon treatment in vitro. A *hyperreactive* footprint (ratio $>$ 1) was identified if the intensity of the in vivo band was higher than that of the in vitro band. Alternatively, a *protected* footprint (ratio $<$ 1) was identified if the intensity of the in vivo band was weaker than that of the in vitro band. In general, there was no significant difference in the footprinting patterns displayed in the three cell types upon both (DMS and UV) treatments. However, in vivo/in vitro band intensity ratios were higher (the ratios were often either <0.5 or >2) on the LMPCR autoradiograms featuring primary SMCs. Indeed, with the exception of one guanine residue, the in vivo/in vitro ratios from IMR-90 and HEK-293 cell lines were either >0.5 or <2. Moreover, induction experiments using various treatments with IL-1β and LPS failed to detect any additional or missing footprinted bands in this promoter region, by using both the UV and the DMS approaches.

Initially, we analyzed by LMPCR the −740 to −537 promoter region (both strands) that comprises the 169 bp positive control domain (nt −734 to −566) as determined by our in vitro B_1R gene promoter studies, but we were not able to discover any reproducible in vivo footprints in the region studied (Fig. 3). Upon further LMPCR analysis, we have detected several regions in the B_1R gene promoter that displayed reproducible in vivo footprints. In particular, three regions from the human B_1R gene promoter displayed in vivo footprints in the three cell lines studied: region A, spanning from nt −533 to −484 (Figs. 4 and 5); region B, spanning from nt −336 to −274 (Fig. 6); and region C, spanning from nt −89 to −26 (Figs. 7 and 8; all numbers are relative to the transcription initiation site). In parallel, the TESS (Transcription Element Search Software) program was used to look for putative transcription factor binding sites within the B_1R gene

promoter sequence (Fig. 9A). The analysis of this tissue-specific gene promoter revealed a large diversity of putative protein binding sequences. Among others, several common AP-1, Sp1, and NF-1 binding sites were identified. A sample of other potential binding sites included the sequences for GATA, Pit-1a, PEA3, and a CAAT box. In region A, DMS protections were mapped at nt −533 to −532 on the upper strand and at nt −505 to −503 on the bottom strand, and nt −509 on the bottom strand proved to be DMS hyperreactive. On the upper strand, nt −530 to −529, nt −508 to −507, and nt −504 to −503 displayed UV protection and nt −514 to −513, nt −505 to −504, and nt −500 to −499 were hyperreactive to the same UV. On the bottom strand, nt −524 to −520 and nt −485 to −484 were protected and nt −528 to −527 were hyperreactive. The region B displayed mostly a UV-hyperreactive bottom strand footprinting activity (Fig. 6) localized at nt −336 to −335, nt −328 to −325, nt −287 to −286, and nt −275 to −274. A unique DMS-hyperractive guanine was mapped at nt −285 on the upper strand. Two distinct domains in the region C have shown footprinted nucleotides: nt −89 and nt −80 motif displayed DMS-protected (−84) and DMS-hyperreactive (−80) footprinted guanines on the upper strand and UV protection (nt −89 to −88 and −83 to −81) on the bottom strand; whereas the nt −52 to −26 motif, nt −52, nt −50, nt −47, and nt −45 exhibited DMS-protected sites, and nt −48 to −47, nt −36 to −35 and nt −27 to −26 displayed UV protection on both strands, as nt −38 to −37 were hyperreactive to UV on the bottom strand. Outside regions A, B, and C, UV protections were seen between nt −188 to −186, and nt −183 to −181 (bottom strand) and nt −1686 to −1685 (upper strand).

Many of the in vivo identified footprinted sequences correspond to known transcription

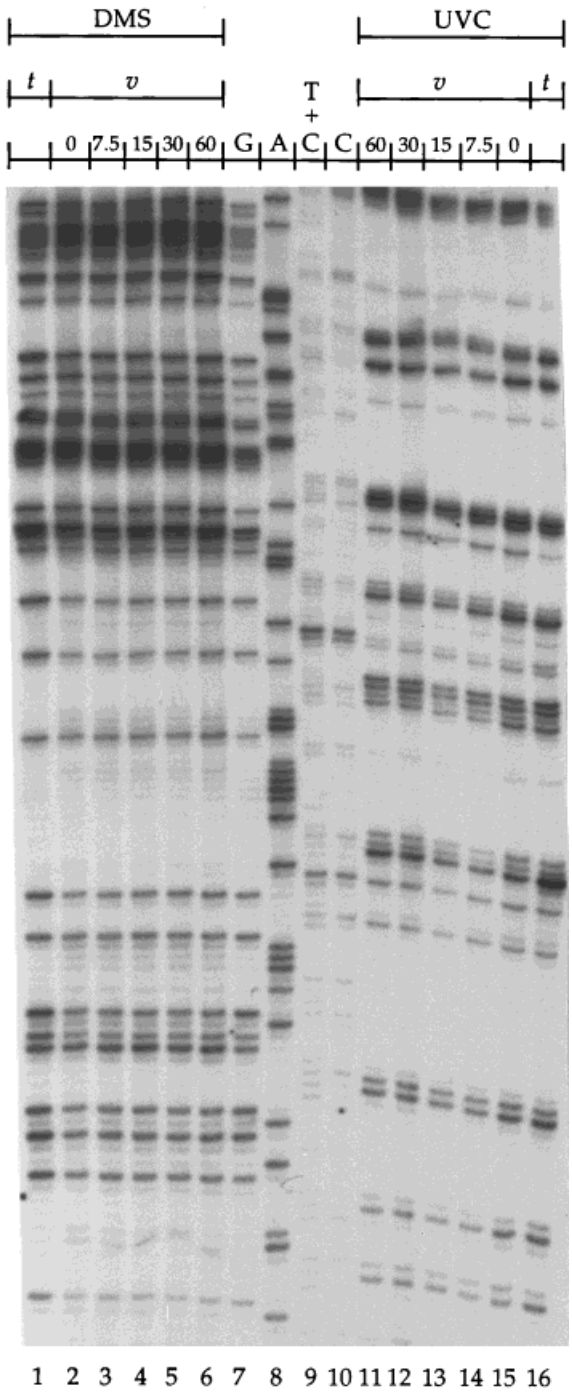

Fig. 3. Genomic footprinting of the human B_1R gene promoter. The region shown was analyzed with primer set 52 (primers 52.1, 52.2, 52.3, Table 2) to reveal upper strand sequences from nt −710 to −537 relative to the major transcription initiation site. Lane 1: ligation-mediated polymerase chain reaction (LMPCR) of naked DNA, purified from primary smooth muscle cells (SMCs), that was treated in vitro (*t*) with dimethylsulfate (DMS). Lanes 2–6: LMPCR of DNA purified from primary SMCs that were treated with IL-1β for 0 min (lane 2), 7.5 min (lane 3), 15 min (lane 4), 30 min (lane 5), or 60 min (lane 6) and then treated in vivo (*v*) with DMS prior to DNA purification. Lanes 7–10: LMPCR of DNA treated with standard Maxam-Gilbert cleavage reaction. Lanes 11–15: LMPCR of DNA isolated from primary SMCs that were treated with IL-1β for 60 min (lane 11), 30 min (lane 12), 15 min (lane 13), 7.5 min (lane 14), or 0 min (lane 15) and then irradiated in vivo (*v*) with ultraviolet C (UVC) prior to DNA purification. Lane 16: LMPCR of naked DNA, purified from primary SMCs irradiated in vitro (*t*) with UVC. No footprints were reproducibly observed on this autoradiogram.

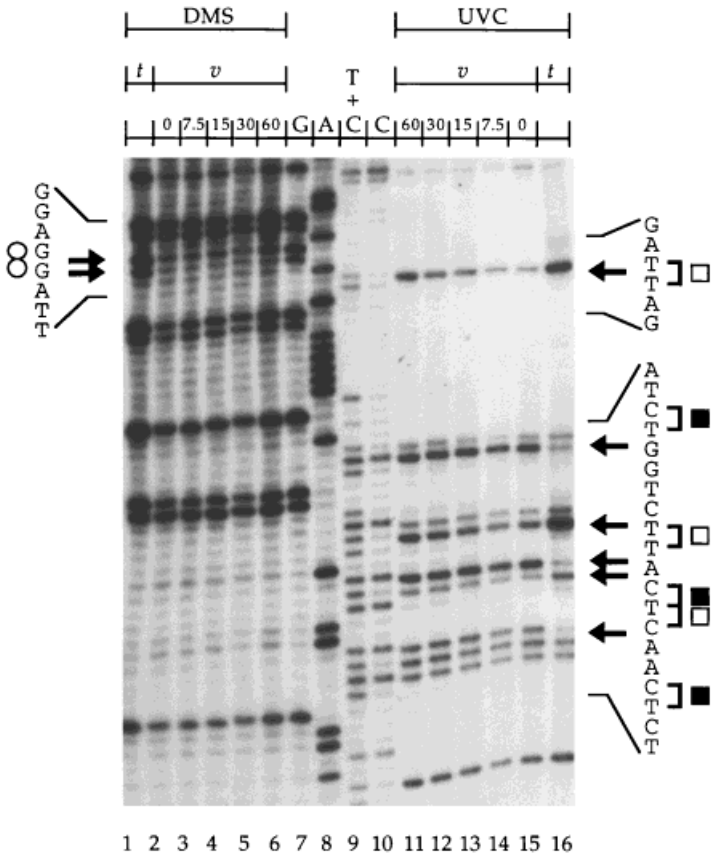

Fig. 4. Genomic footprinting of the human B_1R gene promoter. The region shown was analyzed with primer set 48 (primers 48.1, 48.2, 48.3, Table 2) to reveal bottom strand sequences from nt −541 to −491 relative to the major transcription initiation site. Lanes 1–16 are as described in the legend of Fig. 3. Dimethylsulphate (DMS) protection is indicated by open circles (displayed on the left); Ultraviolet C protection is indicated by open squares and UVC hyperreactivity is marked with closed squares (displayed on the right). As reference, a small portion of the Maxam-Gilbert-derived sequence is shown on both sides of the autoradiogram. Arrows indicate the footprinted bands.

factor binding sites (summarized in Table 3; only sites displaying 15% mismatch or less with the TESS program were considered). The consensus binding sites for the CAAT box (−338 CCAATAG -−332) and the TATA box (−32 TATATAAT −25) were UV-footprinted (Figs. 6 and 8). In region A, the footprinted sequence (−534 AGGATTAGGAAAAAATG-TATCT −513) contains a consensus binding site for PEA3 (AGGAAA) and possesses homology with GATA-1 (AaGATTAG) and Pit-1a (AtGAAtAAAT) (Fig. 9B). Another footprinted sequence (−510 TCTTACTCAA −501) is equivalent to the consensus binding sites for C/EBP (TCTTACTC) and GCN4 (AaGAGTCAA) or AP-1 (TTACTCA). Eighteen bp downstream from this sequence, a protected photofootprint was found within a consensus AP-1 sequence. In section B, the hyperreactive photofootprinted sequence between nt −328 and −322 corresponds to an Sp1 (GGGGCC) consensus binding site. This is unusual because Sp1-footprinted sequences are generally DMS protected. A UV-protected sequence (−188 AAAGTAAAG −180) is similar to an Oct-1 (AtGTAAAG) binding site. Finally, one of the two footprinted sequences found in region C is similar to a CREB (TGACATCA) consensus binding site. The protected photofootprint seen at nt −1686 to −1685, the hyperreactive photofootprint seen at nt −287 to −286, as well as the two DMS footprinted guanine residues combined with the three photofootprinted residues (−89 GTCCCGAAAG −80) did not show any homology with known transcription factor binding site. The two photofootprints just upstream of the TATA box displayed no match with any known transcription factor binding site. However, in the DNA segment spanning nt −287 to nt −274 (GAGCCTTGGGGCAA), a putative myogenin (TTaGGGCA) transcription factor binding site is present; although no footprint was found within the consensus sequence, it is bordered by both UV and DMS hyperreactive footprints.

Figure 9 schematically summarizes all the putative consensus transcriptional factor binding sites identified with the TESS program and the in vivo genomic footprints identified by LMPCR (using both UV and DMS treatments)

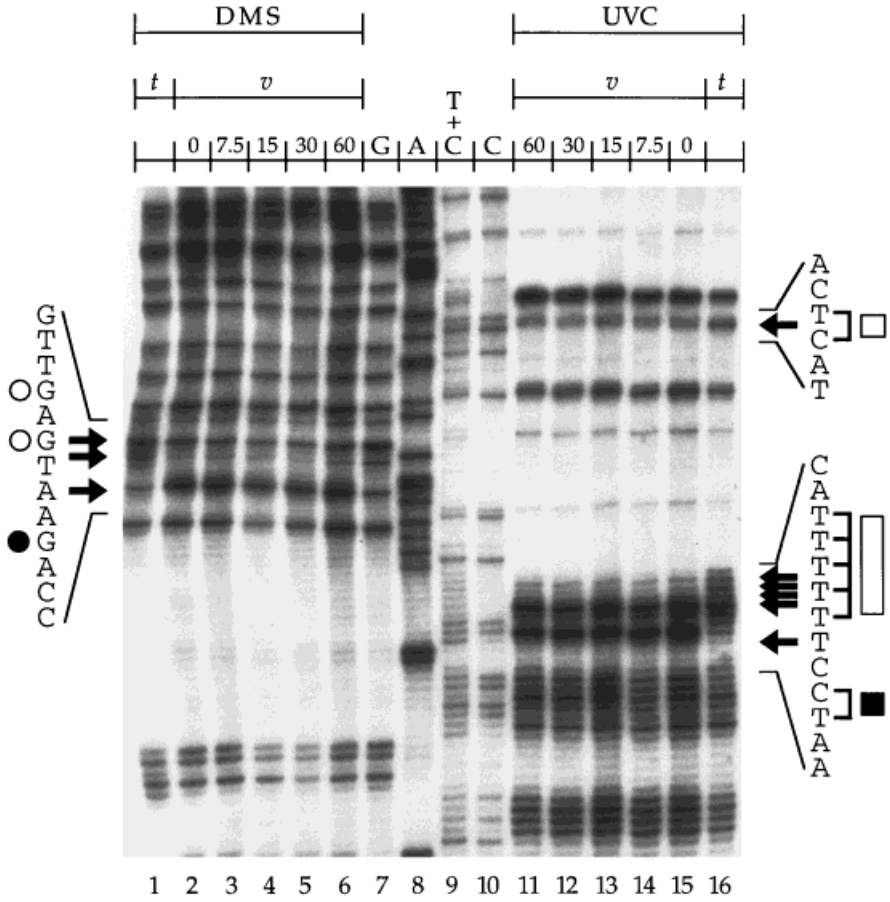

Fig. 5. Genomic footprinting of the human B_1R gene promoter. The region shown was analyzed with primer set 49 (primers 49.1, 49.2, 49.3, Table 2) to reveal bottom strand sequences from nt −459 to −549 relative to the major transcription initiation site. Lanes 1–16 are as described in the legend of Fig. 3. Dimethylsulfate (DMS) protection is indicated by open circles and DMS hyperreactivity is marked with a closed circle (displayed on the left). Ultraviolet (UVC) protection is indicated by open squares and UVC hyperreactivity is marked with a closed square (displayed on the right). As reference, a small portion of the Maxam-Gilbert-derived sequence is shown on both sides of the autoradiogram. Arrows indicate the footprinted bands.

in the human B_1R gene promoter region. The TESS program identified 86 putative transcription binding consensus sites (Fig. 9A) that will likely display gel-shifts in vitro when mixed with nuclear extracts from the cell types studied. However, it appears that only few of these putative consensus sequences effectively show in vivo protein-DNA interactions in the cell types studied (Fig. 9B).

DISCUSSION

The genomic organization of the human B_1R gene was studied previously in our laboratory [Bachvarov et al., 1996]. The sequencing analysis of the 5′-flanking region showed the presence of a consensus TATA box and of numerous candidate transcription factor binding motifs (see above). These results, along with other studies [Yang and Polgar, 1996] strongly indicate that this region contains the core promoter of the human B_1R gene. In the present study, we have applied an in vivo DNA analysis technique (LMPCR) to functionally characterize this control region by searching for protein-DNA interactions and special DNA structures. Initially, we have performed preliminary in vitro delineation of the putative promoter region as we analyzed a 4.2-kb fragment, situated immediately upstream of the first intron of the receptor gene, by transient transfection of different promoter fragments in front of the CAT reporter gene. Our results demonstrate that this region has all the characteristics to represent the core promoter of the human B_1R gene with a functional TATA box and additional control elements, up- and down-regulating the expression of the receptor gene, as some of these elements might be tissue specific. Quite a recent study, published in the course of our experiments [Yang et al., 1998] has also revealed the presence of negative and positive control elements in the B_1R gene promoter region applying similar transient DNA transfection analyses in IMR-90 cells. Two positive regions at nucleotide positions −733 to −685 and −607 to −451 have been identified,

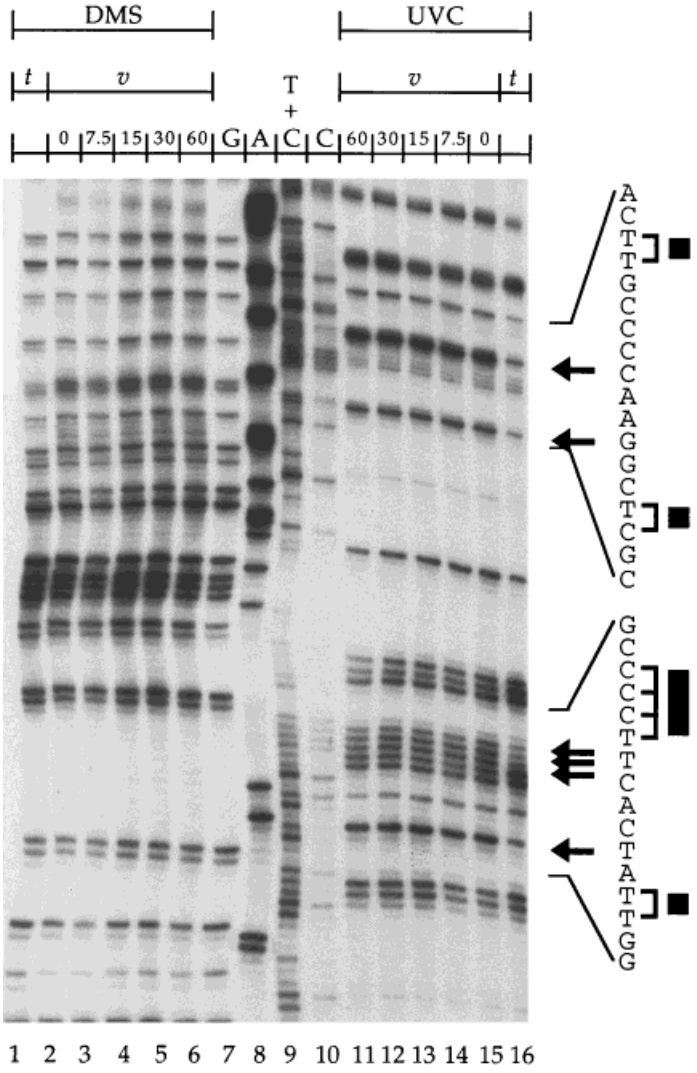

Fig. 6. Genomic footprinting of the human B_1R gene promoter. The region shown was analyzed with primer set 47 (primers 47.1, 47.2, 47.3, Table 2) to reveal bottom strand sequences from nt −244 to −352 relative to the major transcription initiation site. Lanes 1–16 are as described in the legend of Fig. 3. Ultraviolet C (UVC) hyperreactivity is marked with closed squares (displayed on the right). As reference, a small portion of the Maxam-Gilbert-derived sequence is shown on the right of the autoradiogram. Arrows indicate the footprinted bands.

separated by a strong negative regulatory region (nt −685 to −607) as additional negative regulatory regions were also identified (nt −1748 to −733, and nt −451 to −387). A powerful enhancer was located at nt −551 to −451 bp that is tissue-specific for IMR-90 cells, similar to our positive control region. There is a significant overlap between the positive and negative regulatory domains in this study and our positive and negative control regions, although some differences exist in the localization of these domains due to the different promoter constructs used in both studies.

As the expression of the human B_1R gene is strongly regulated by the cytokine network and especially modulated by IL-1β (see Introduction) we also tried to identify the presence of any IL-1β responsive element(s) in the 5′-control region of the receptor gene. Our transfection experiments in three cell types failed to identify IL-1β-responsive elements in the promoter region studied. Similar studies by others [Yang et al., 1998] have not found any domains in the human B_1R gene promoter region involved in the induction of expression by LPS, TNF-α, and PMA in IMR-90 cells. However, two other groups [Ni et al., 1996; Schanstra et al., 1998] have recently reported that upregulation of the human B_1R gene by IL-1β is controlled at the transcriptional level and is strongly correlated to the activation of transcription factor NF-κB, although there is a contradiction concerning the localization of the putative NF-κB-binding domain. Although one of the studies locates this motif at positions −1172 to

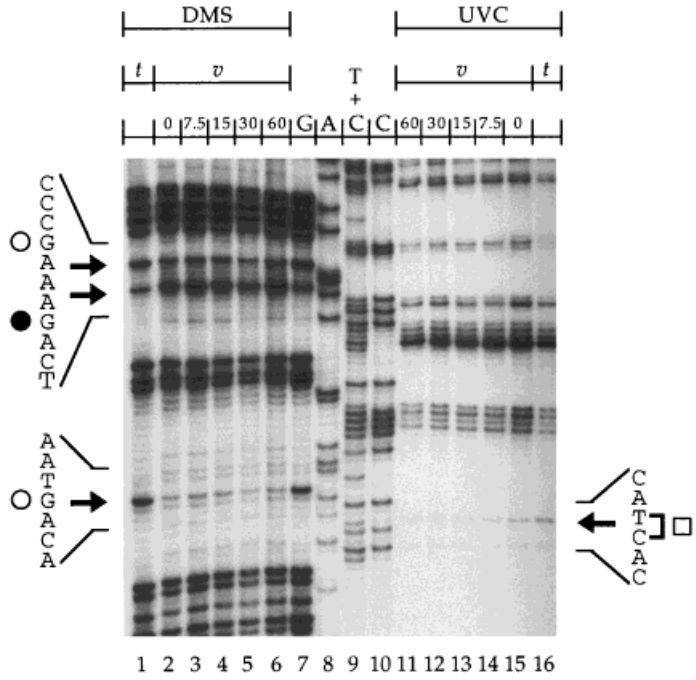

Fig. 7. Genomic footprinting of the human B_1R gene promoter. The region shown was analyzed with primer set 44 (primers 44.1, 44.2, 44.3, Table 2) to reveal upper strand sequences from nt −103 to −38 relative to the major transcription initiation site. Lanes 1–16 are as described in the legend of Fig. 3. Dimethylsulfate (DMS) protection is indicated by open circles and DMS hyperreactivity is marked with a closed circle (displayed on the left). Ultraviolet C (UVC) protection is indicated by an open square (displayed on the right). As reference, a small portion of the Maxam-Gilbert-derived sequence is shown on both sides of the autoradiogram. Arrows indicate the footprinted bands.

−1162 by transient transfections in IMR-90 cells [Schanstra et al., 1998], the other study, using a similar approach in rat SMCs, claims the functional NF-κB-binding domain to be located at positions −67 to −57 [Ni et al., 1998].

Because the differences in signal transduction between the native promoter, with the target gene in vivo, and the chimeric promoter, transiently transfected and linked to a reporter gene, may be critical for protein-DNA contacts and response to stimuli (IL-1β or LPS), we performed a high-resolution in vivo DNA analysis of a 1.4 kb human B_1R gene promoter region in three human cell types (IMR-90, primary SMCs, and HEK-293). We have used UV and DMS treatments in our footprinting experiments because both approaches are complementary and thus cover more completely the spectrum of DNA-protein contacts (see Results). The footprints we have obtained were very reproducible but, surprisingly, there were no differences between the footprint patterns in the three cell types. However, the primary SMCs displayed greater in vivo/in vitro band intensity ratios than those obtained with the transformed cell types and for this reason we are presenting only the SMCs footprinting data. In principle the LMPCR technique has the property to preserve the original selection of single-strand break samples through the different steps of the procedure. Thus, a weak difference in the in vivo/in vitro ratio (>0.5 or <2) at a particular sequence might indicate that only a fraction of the treated cells had protein-DNA interactions at the time of the treatment. In the present case, the greater ratios in SMCs could be attributed to a higher fraction of these primary cells displaying protein binding activity in the B_1R gene promoter region in comparison with IMR-90 and HEK-293 cells. Alternatively, a stronger, more efficient protein binding may exist at the B_1R gene promoter region in primary cultured cells than in the transformed cell types.

In particular, three domains from the human B_1R gene promoter have displayed reproducible footprints: nt −533 to −484 (region A), nt −336 to −274 (region B) and nt −89 to −26 (region C). From these, region A overlaps with the 5′-end half of the previously identified enhancer region [Yang et al., 1998], and region C is located just upstream from the TATA box. No significant footprinting activity was detected in the negative control region, characterized in vitro by us and others [Yang et al., 1998]. The footprinting analysis shows no indication of tissue specificity of the positive control region and the enhancer region in IMR-90 cells, as postulated by us and others [Yang et al., 1998]. Both TATA and CAAT boxes were UV footprinted, revealing an active promoter.

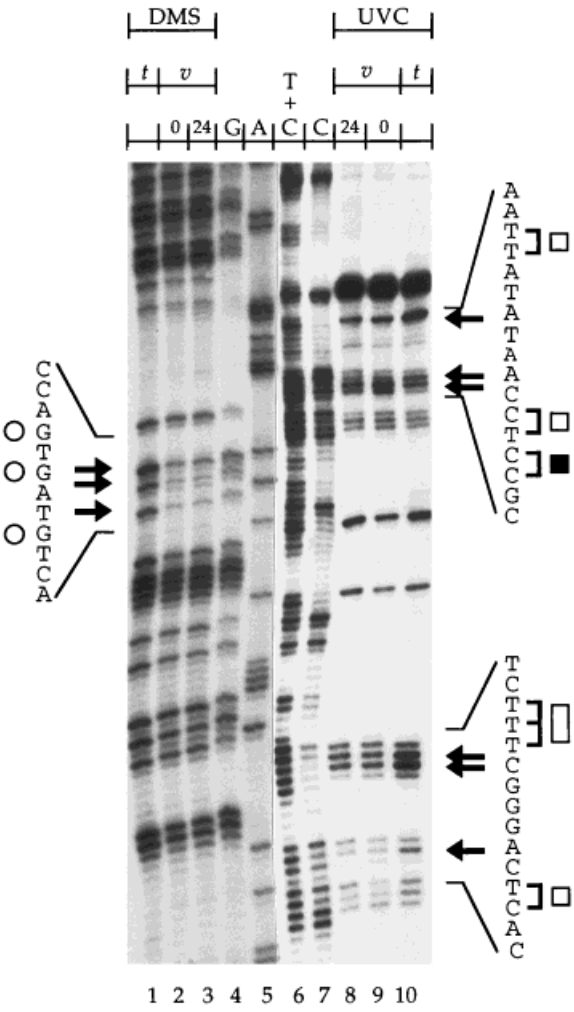

Fig. 8. Genomic footprinting of the human B_1R gene promoter. The region shown was analyzed with primer set 45 (primers 45.1, 45.2, 45.3, Table 2) to reveal bottom strand sequences from nt +3 to −98 relative to the major transcription initiation site. Lane 1: Ligation-mediated polymerase chain reaction (LMPCR) of naked DNA, purified from primary smooth muscle cells (SMCs), that was treated in vitro (*t*) with dimethylsulfate (DMS). Lanes 2 and 3: LMPCR of DNA purified from primary SMCs that were treated with lipopolysaccharide (LPS) for 0 (lane 2) or 24 h (lane 3) and then treated in vivo (*v*) with DMS prior to DNA purification. Lanes 4–7: LMPCR of DNA treated with standard Maxam-Gilbert cleavage reaction. Lanes 8 and 9: LMPCR of DNA isolated from primary SMCs that were treated with LPS for 24 (lane 8) or 0 h (lane 9) and then irradiated in vivo (*v*) with ultraviolet C (UVC) prior to DNA purification. Lane 10: LMPCR of naked DNA purified from primary SMCs irradiated in vitro (*t*) with UVC. DMS protection is indicated by open circles (displayed on the left). UVC protection is indicated by open squares and UVC hyperreactivity is marked with a closed square (dispalyed on the right). As reference, a small portion of the Maxam-Gilbert-derived sequence is shown on both sides of the autoradiogram. Arrows indicate the footprinted bands.

We found that even in the noninduced state, the B_1R gene promoter is possibly bound by several sequence-specific DNA binding proteins (presented in Table 3). Some other footprints were detected on sequences that do not correspond to any known transcription factor binding site, as accessed by the TESS data bank.

Surprisingly, no additional changes in protein-DNA complexes were detected upon stimulation of B_1R gene expression with IL-1β or LPS. In particular, no footprints were identified (for both untreated or IL-1β- and LPS-treated cells) at the two NF-κB binding sites, postulated to be responsible for the IL-1β-determined transcriptional activation of the B_1R gene [Ni et al., 1998; Schanstra et al., 1998]. Because UV and DMS treatments have different sequence preference, are sensitive to different types of protein-DNA contacts, and respond differently to structural perturbations in the DNA double helix thus complementing each other, we are confident that any significant rearrangements of protein-DNA interactions upon IL-1β or LPS treatment, would have been detected in the promoter region studied. Given our data, it is likely that complex protein-DNA interactions exist at the B_1R gene promoter prior to induction by external stimuli even in cells (HEK-293) that do not express a functional B_1R; thus the signal transduction process following IL-1β or LPS treatment targets transcription factors at the B_1R gene promoter that are already bound to DNA before induction that might be sufficient for basal transcription activity. Related results were obtained upon in vivo footprinting analysis of the c-jun gene promoter because it was shown that a modification in the transactivating domain of

Fig. 9. Summary of in vivo dimethylsulfate (DMS) and ultraviolet C (UVC) footprinting data and the consensus sequences for transcription factors of the human B_1R gene promoter. **A:** The consensus sequences of different transcription factor binding sites are shown, as identified by the TESS program. The arrows are oriented in the direction of the consensus DNA sequences. **B: S**ummary of the results of our in vivo DMS and UV footprinting experiments. DMS protection is indicated by open circles and DMS hyperreactivity is marked with closed circles. UV protection is indicated by open squares and UV hyperreactivity is marked with closed squares. The symbols above the sequence indicate footprinted nucleotide(s) on the upper strand; the symbols below the sequence indicate footprinted nucleotide(s) on the bottom strand.

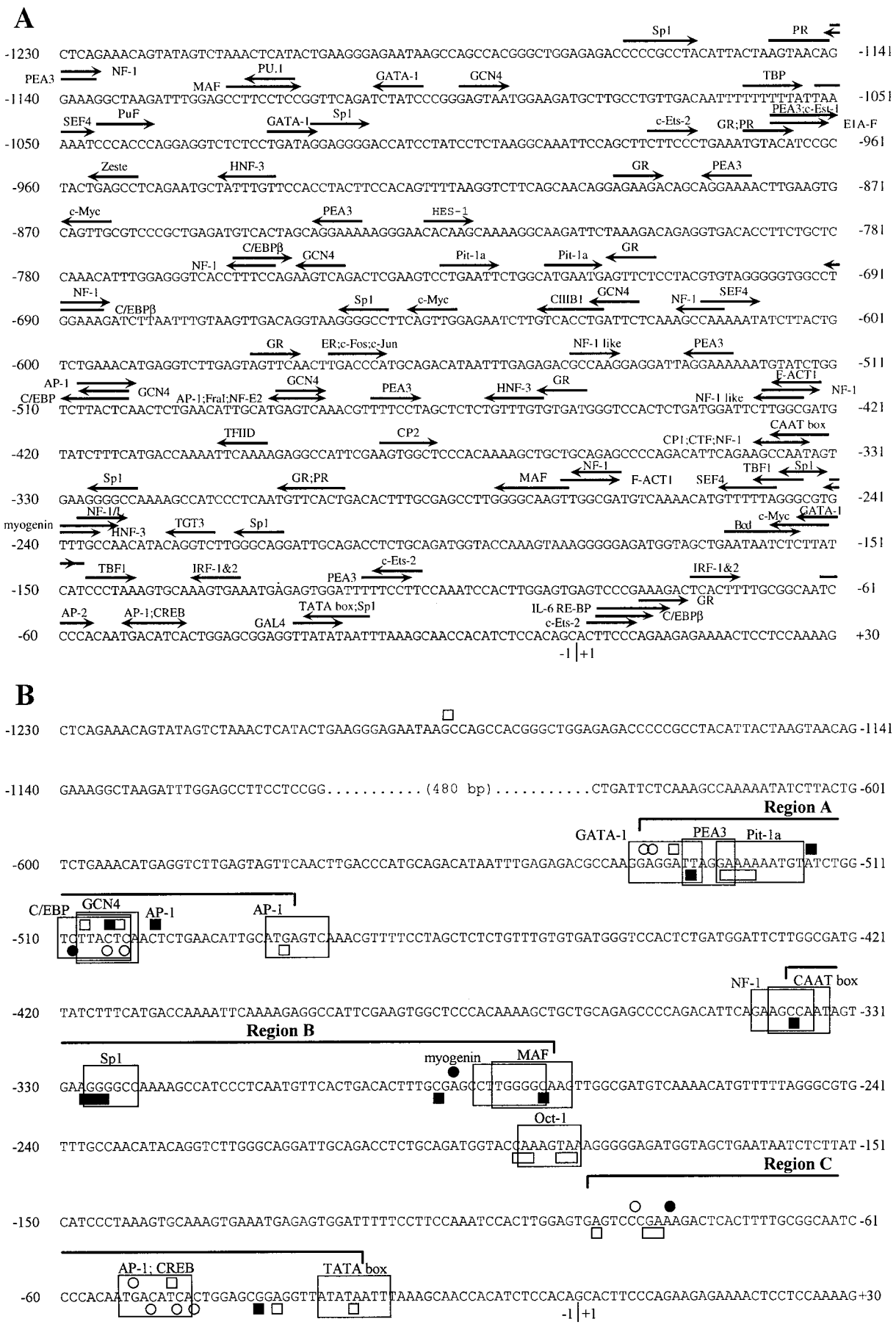
A
-1230 CTCAGAAACAGTATAGTCTAAACTCATACTGAAGGGAGAATAAGCCAGCCACGGGCTGGAGAGACCCCCGCCTACATTACTAAGTAACAG -1141
-1140 GAAAGGCTAAGATTTGGAGCCTTCCTCCGGTTCAGATCTATCCCGGGAGTAATGGAAGATGCTTGCCTGTTGACAATTTTTTTTTATTAA -1051
-1050 AAATCCCACCCAGGAGGTCTCTCCTGATAGGAGGGGACCATCCTATCCTCTAAGGCAAATTCCAGCTTCTTCCCTGAAATGTACATCCGC -961
-960 TACTGAGCCTCAGAATGCTATTTGTTCCACCTACTTCCACAGTTTTAAGGTCTTCAGCAACAGGAGAAGACAGCAGGAAAACTTGAAGTG -871
-870 CAGTTGCGTCCCGCTGAGATGTCACTAGCAGGAAAAAGGGAACACAAGCAAAAGGCAAGATTCTAAAGACAGAGGTGACACCTTCTGCTC -781
-780 CAAACATTTGGAGGGTCACCTTTCCAGAAGTCAGACTCGAAGTCCTGAATTCTGGCATGAATGAGTTCTCCTACGTGTAGGGGGTGGCCT -691
-690 GGAAAGATCTTAATTTGTAAGTTGACAGGTAAGGGGCCTTCAGTTGGAGAATCTTGTCACCTGATTCTCAAAGCCAAAAATATCTTACTG -601
-600 TCTGAAACATGAGGTCTTGAGTAGTTCAACTTGACCCATGCAGACATAATTTGAGAGACGCCAAGGAGGATTAGGAAAAAATGTATCTGG -511
-510 TCTTACTCAACTCTGAACATTGCATGAGTCAAACGTTTTCCTAGCTCTCTGTTTGTGTGATGGGTCCACTCTGATGGATTCTTGGCGATG -421
-420 TATCTTTCATGACCAAAATTCAAAAGAGGCCATTCGAAGTGGCTCCCACAAAAGCTGCTGCAGAGCCCCAGACATTCAGAAGCCAATAGT -331
-330 GAAGGGGCCAAAAGCCATCCCTCAATGTTCACTGACACTTTGCGAGCCTTGGGGCAAGTTGGCGATGTCAAAACATGTTTTTAGGGCGTG -241
-240 TTTGCCAACATACAGGTCTTGGGCAGGATTGCAGACCTCTGCAGATGGTACCAAAGTAAAGGGGGAGATGGTAGCTGAATAATCTCTTAT -151
-150 CATCCCTAAAGTGCAAAGTGAAATGAGAGTGGATTTTTCCTTCCAAATCCACTTGGAGTGAGTCCCGAAAGACTCACTTTTGCGGCAATC -61
-60 CCCACAATGACATCACTGGAGCGGAGGTTATATAATTTAAAGCAACCACATCTCCACAGCACTTCCCAGAAGAGAAAACTCCTCCAAAAG +30
-1 +1
B
-1230 CTCAGAAACAGTATAGTCTAAACTCATACTGAAGGGAGAATAAGCCAGCCACGGGCTGGAGAGACCCCCGCCTACATTACTAAGTAACAG -1141
-1140 GAAAGGCTAAGATTTGGAGCCTTCCTCCGG.....(480 bp).....CTGATTCTCAAAGCCAAAAATATCTTACTG -601
Region A
GATA-1
PEA3
Pit-1a
-600 TCTGAAACATGAGGTCTTGAGTAGTTCAACTTGACCCATGCAGACATAATTTGAGAGACGCCAAGGAGGATTAGGAAAAAATGTATCTGG -511
C/EBP
GCN4
AP-1
AP-1
-510 TCTTACTCAACTCTGAACATTGCATGAGTCAAACGTTTTCCTAGCTCTCTGTTTGTGTGATGGGTCCACTCTGATGGATTCTTGGCGATG -421
NF-1
CAAT box
-420 TATCTTTCATGACCAAAATTCAAAAGAGGCCATTCGAAGTGGCTCCCACAAAAGCTGCTGCAGAGCCCCAGACATTCAGAAGCCAATAGT -331
Region B
Sp1
myogenin
MAF
Oct-1
-330 GAAGGGGCCAAAAGCCATCCCTCAATGTTCACTGACACTTTGCGAGCCTTGGGGCAAGTTGGCGATGTCAAAACATGTTTTTAGGGCGTG -241
-240 TTTGCCAACATACAGGTCTTGGGCAGGATTGCAGACCTCTGCAGATGGTACCAAAGTAAAGGGGGAGATGGTAGCTGAATAATCTCTTAT -151
Region C
-150 CATCCCTAAAGTGCAAAGTGAAATGAGAGTGGATTTTTCCTTCCAAATCCACTTGGAGTGAGTCCCGAAAGACTCACTTTTGCGGCAATC -61
AP-1; CREB
TATA box
-60 CCCACAATGACATCACTGGAGCGGAGGTTATATAATTTAAAGCAACCACATCTCCACAGCACTTCCCAGAAGAGAAAACTCCTCCAAAAG +30
-1 +1

Figure 9.

TABLE III. Putative Transcription Factors Binding at the Human B_1R Gene Promoter, as Found by In Vivo DMS and UV Genomic Footprinting

Region	Transcription factor	Consensus sequence[a]	Nucleotide position[b]
A	GATA-1	AaGATTAG [Simon, 1993]	−534 to −527
	PEA3	AGGAAA [Xin et al., 1992]	−528 to −523
	Pit-1a	AAAAATGcAT [Ohta et al., 1992]	−524 to −515
	C/EBP	TCTTACTC [Agre et al., 1989]	−510 to −503
	GCN4	TTACTC [Hope and Struhl, 1986]	−508 to −503
	AP-1	TTACTCA [Bohmann et al., 1988]	−508 to −502
	AP-1	TGAGTCA	−486 to −480
B	NF-1	AGCCAAT [Rossi et al., 1988]	−340 to −334
	CAAT box	CCAATAG [Bakker and Parker, 1991]	−338 to −332
	Sp1	GGGGCC [Pugh and Tjian, 1990]	−327 to −322
	myogenin	TTaGGGCA [Buchberger et al., 1994]	−282 to −275
	MAF	GGGGCAAGT [Blank and Andrews, 1997]	−280 to −272
	Oct-1	AtGTAAAG [Strom et al., 1996]	−187 to −181
C	CREB	TGACATCA [Andrisani, 1999]	−53 to −46
	AP-1	TGACATCA	−53 to −46
	TATA box	TATAATTT [Maniatis et al., 1987]	−30 to −23

[a]Small letters in the consensus sequences indicate mismatches with corresponding sequences in the human B_1R promoter gene (see Fig. 9B).
[b]Corresponding positions are given relative to the transcription initiation site [Bachvarov et al., 1996].
DMS, dimethylsulfate; UV, ultraviolet.

pre-existing DNA-bound factor, and not a newly bound activator, is responsible for the rapid induction of the c-jun gene by UV light [Rozek and Pfeifer, 1993]. Since the NF-κB factor is involved in the IL-1β-induced B_1R gene upregulation that can be blocked with putative NF-κB inhibitors [Zhou et al., 1998] we could presume that NF-κB may interact with the AP-1 or CREB binding proteins as previously postulated [Stein et al., 1993; Cogswell et al., 1994]. Other mechanisms of IL-1β regulation may also exist because it was shown recently that post-transcriptional mRNA stabilization, together with transcriptional activation, may be involved in the IL-1β-based upregulation of B_1R gene expression [Zhou et al., 1998].

We did not investigate the influence of the serum in the culture media in the in vivo footprinting analyses because, as indicated in the Results section, our in vitro transfection experiments with all promoter constructs studied showed no significant changes in promoter activity upon IL-1β induction of transfected cells, kept in low serum (0.4%), and pretreated with anti IL-1a antibodies. Nevertheless, we are considering to address this question in our future LMPCR experiments, in which we intend to use an alternative in vivo footprinting approach, upon treatment with DNase I. We also cannot completely exclude the possibility that an IL-1β- and/or an LPS-responsive element(s) could exist outside the B_1R gene promoter region studied, although our in vivo footprinting analyses cover a large (1.4 kb) region from the B_1R gene promoter that includes all putative functional regulatory domains, determined by in vitro transient transfection experiments. Two introns (7 kb and 1 kb) were identified in the 5′ noncoding region of the human B_1R gene downstream from the promoter region [Bachvarov et al., 1996] and until now there were no serious attempts of identifying any control domains in these genomic DNA regions.

Regulation of gene expression is a complex mechanism controlled by many different factors. Among them transcription factors, as well as special chromatin and DNA structures, play dominant roles. Identification of peculiar DNA structures and sequences showing DNA-protein interactions at the promoter of an active gene is one of the important works to forgo in order to better understand the mechanisms involved in gene regulation. While it is commonly accepted that recombinant or native

proteins are able to bind purified DNA fragments at specific consensus DNA sequences, it frequently appears that these same sites are not occupied by any proteins in transcriptionally active genes inside a living cell. Special chromatin and DNA structures are also lost in cloned or purified DNA. Thus, based on the results of this and previous studies [Drouin et al., 1997], we suggest that initial in vivo DNA analysis approaches are necessary to effectively map promoter sequences showing DNA-protein interactions or special DNA structures. However, further in vitro studies should be then performed to confirm the DNA-protein interactions and hopefully identify the proteins responsible for the in vivo footprints. In this way, we should rapidly and efficiently come to a better understanding of the mechanisms regulating the expression of a specific gene in living cells.

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