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Interaction of the nuclear protein CBF1 with the κB site of the IL-6 gene promoter

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ABSTRACT

The nuclear protein CBF1 has been shown to function as an intermediate to target transcription factors, such as the activated Notch receptor, to specific DNA sites. In this paper, we show that CBF1 from cell lines of different origin is able to bind to the κB site of the IL-6 promoter. By transfection analyses performed in HeLa cells, we demonstrate that overexpressed CBF1 acts as a negative regulator of IL-6 gene transcription and is unable to elicit Notch-dependent activation of this gene. Analyses of protein–DNA interactions indicate that the topology of the complex formed by CBF1 and the target DNA is subtly affected by sequences surrounding the recognition site. Furthermore, we show that CBF1 induces DNA bending. This finding suggests that CBF1 may influence IL-6 gene transcription by determining a specific conformation of the promoter region.

INTRODUCTION

Interleukin-6 (IL-6) plays a central role in host defense mechanisms and in haematopoiesis (1,2). Results obtained from both clinical investigations and transgenic animals indicate that deregulation of IL-6 expression is involved in the pathogenesis of various immunologically related diseases (1–3). Numerous studies have shown that IL-6 synthesis is essentially regulated at the transcription level. Among the characterized *cis*-acting elements, the κB site located at positions –73 to –64 plays a central role in the transcriptional activation of the IL-6 gene. Data from several groups have shown that the nuclear factor NF-κB is required for induction of the IL-6 gene by diverse stimuli, including viruses, dsRNA, TNFα and IL-1 (4). Transcription factors of the C/EBP family (NFIL6-C/EBPβ and NFIL6β-C/EBPδ) are also involved in the induction of IL-6 by cytokines such as IL-1 and TNFα (5) and have been shown to bind the sequences located at positions –158 to –145 and –87 to –76. An additional putative regulatory element, a G/C-rich region located between the NFIL6 and the NF-κB sites of the human IL-6 promoter, has also been identified. The homologous sequence of the mouse IL-6 promoter has been shown to bind the constitutively expressed Sp1 transcription factor, playing an important role in both basal and inducible expression of this cytokine (6). In addition, the transcription factor IRF-1 binds to the *cis*-acting regulatory element at positions –267 to –254 and is required for IL-6 induction following stimulation by IFN-γ (7).

Thus, although much is known about the transcription activation of the IL-6 gene, little information is available concerning its repression. Down-regulation by glucocorticoids, estrogens and the tumor suppressor gene products p53 and pRB has been reported (8–11). Specific repressor factors, however, may also play a crucial role in silencing the IL-6 gene. We have previously identified a complex formed by a nuclear protein, not related to the Rel family of transcription factors, which is able to bind the IL-6 κB site. This protein was tentatively identified as CBF1 (12). The identity of this protein was subsequently confirmed by other groups (13,14), as well as by the results presented in this paper. CBF1 is an evolutionarily conserved factor which belongs to the CSL [CBF1, Su(H), Lag-1] (15–20) family of nuclear proteins. CSL members have been shown to be nuclear effectors of the membrane receptor Notch, which regulates lateral inhibition in embryo morphogenesis and tissue differentiation. Notch signaling occurs through the proteolytic release of its cytoplasm domain which translocates to the nucleus and activates responsive promoters by interaction with a CSL protein (21,22). A novel form of CBF1, which binds to a distinct DNA sequence, has been recently identified in the thymus (23) in which Notch-induced signaling seems to be required for the positive selection of CD8 T cells (24) and for αβ/γδ T-cell lineage selection (25). The occurrence of a CSL binding site, however, is not sufficient to ensure Notch responsiveness. Indeed, it has been demonstrated that only one of the two CBF1 target sites within the regulatory region of the *Hairy enhancer of split* (HES-1) gene is able to mediate Notch-dependent activation (26). The EBNA2 transcription factor encoded by the Epstein–Barr virus also interacts with CBF1, thereby activating responsive genes (17,27,28). On the other hand, CBF1 has also been reported to function as a transcription repressor *per se* by competing with activators of the Rel family at overlapping binding sites (13,29) and/or by interacting with corepressor molecules (30) or with components of the transcription preinitiation complex (31). Interaction with

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viral (32) or cellular (33) proteins, which prevent DNA binding, abolishes CBF1 transcription regulation.

We report herein the analysis of various human cell lines for the presence of CBF1 binding activity on the IL-6 promoter. We show that CBF1 down-regulates transcriptional activation of the IL-6 promoter by Rel and C/EBP family members and we report that CBF1 binding to the IL-6 promoter is not sufficient to mediate Notch-dependent activation. In addition, analyses of protein–DNA interactions indicate that CBF1 is a DNA bending protein and that the topology of the complex is subtly affected by sequences surrounding its binding site.

MATERIALS AND METHODS

Cell lines

Breast carcinoma MCF-7, MDA-MB-231 cells were cultured in DMEM; hepatoma Hep G2 and osteosarcoma MG-63 cells were cultured in Eagle's MEM; myeloma U266B1, histiocytic lymphoma U-937, B lymphoma Daudi, acute T cell leukemia Jurkat, myeloma RPMI 8226 and HeLa S3 cells were cultured in RPMI 1640. Culture media (Gibco BRL) were supplemented with L-glutamine, 10% fetal calf serum and gentamycin (50 μg/ml) in 5% CO_2.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to Osborn *et al.* (34), with minor modifications. Protein concentration was measured by the Bradford method (Bio-Rad). EMSAs were performed as described (7). Radiolabeled probe (~0.01 pmol; specific activity $1–2 \times 10^6$ c.p.m./pmol) was added last to each reaction mixture and samples were incubated at room temperature for 20 min. In competition assays, a 100-fold molar excess of unlabeled double-stranded oligonucleotides was added to the reaction mixture. The supershift experiments were performed with anti-CBF1 monoclonal antibody (K0043) (35), kindly provided by Dr Honjo, Kyoto University, Japan. The specificity of the antibody interaction was established by control reactions carried out with the specific antibody in the absence of nuclear proteins, or with an irrelevant antibody. Samples were loaded on a 5% native polyacrylamide gel in 0.5× TBE, run at 250 V, dried and exposed to Kodak films.

Oligonucleotides used are shown in Figure 2. The sequence of oligonucleotide Ino/IL-6 κB, in which the two AT pairs of the CBF1 site of IL-6 promoter were substituted by IC pairs, is the following: ATGTGGGATTIICCCCTG. The sequence of the oligonucleotide binding OCT-1 transcription factor is the following: 5'-GATCTTCACCTTATTTGCATAAGC-3'. All oligonucleotides (Genset, Paris, France) were synthesized so that the double-stranded forms presented three 5' protruding nucleotides on one or both ends for labeling purposes. Hybridized oligonucleotides were labeled using Sequenase (USB), [α-^{32}P]dATP and [α-^{32}P]dCTP. Unincorporated nucleotides were removed by Sephadex G-50 column chromatography.

Transient transfection and reporter gene assay

HeLa S3 cells were permanently transfected with the expression vector pCDNA3 carrying the sequence encoding C/EBPδ. Permanently transfected cells (C/EBPδ/HL) were cultivated as monolayer to subconfluency in 12-well plates. 3×10^5 cells were transfected with 1.5 μg of plasmids with DAC-30 reagent (Eurogentec). The construction of the reporter plasmid pIL-6{-1200}LUC has already been described (12). Reporter plasmids containing point mutations on the IL-6 promoter were obtained by amplifying pIL6{-1200}LUC, according to a standard procedure (36), with oligonucleotides containing the appropriate mutation (AF5 oligonucleotide, see Figure 2, for plasmid mCBF1pIL6{-1200}LUC and the oligonucleotide: GCTAAAGGACGTCACAGTGAACAATCT, in which the mutated bases are underlined, for plasmid mC/EBPpIL6{-1200}LUC). The reporter constructs 4×κBIL6LUC and 4×κBIgLUC were obtained by cloning synthetic four tandem repeats of the κB site of the IL-6 promoter or of the κ light chain immunoglobulin enhancer, respectively, into the pGL3-Promoter vector (Promega). The plasmid HES-1 LUC, described by Jarriault *et al.* (26), was a gift of Dr A. Israel (Institut Pasteur, Paris, France). Eukaryotic expression vectors carrying the coding regions of CBF1, of Notch receptor in which the region coding for the extracellular domain has been deleted (ΔE Notch), and of p65 under control of the CMV promoter were kindly provided by Dr T. Henkel (The Johns Hopkins University, Baltimore, MD, USA), Dr R. Kopan (Washington University, St Louis, MO, USA) and Dr A. Israel, respectively. Expression plasmid encoding the EBNA2 protein under the control of the SV40 promoter was a kind gift of Dr D. Hayward (The Johns Hopkins University, Baltimore, MD, USA). Cells were incubated with plasmids for 8 h. Cell lysates containing an equal amount of protein were assayed for luciferase activity using the Luciferase Assay System (Promega), according to the manufacturer's instructions.

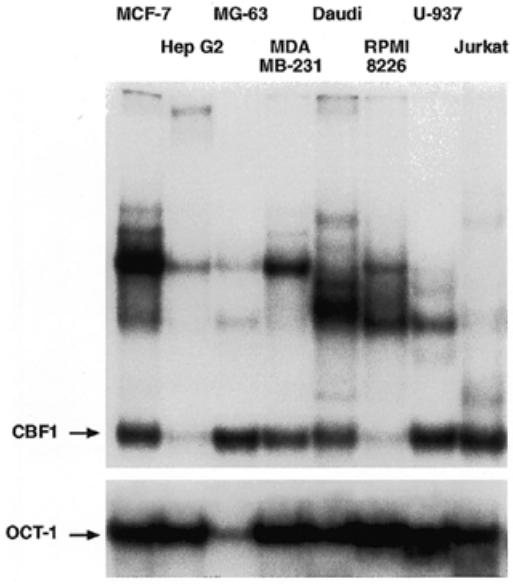

Figure 1. Upper panel: EMSA performed with nuclear extracts from human cell lines MCF-7, MDA-MB-231 (breast carcinoma), Hep G2 (hepatoma), MG-63 (osteosarcoma), Daudi (B lymphoma), RPMI 8226 (myeloma), U-937 (histiocytic lymphoma) and Jurkat (acute T cell leukemia) with the IL-6 κB probe. Lower panel: EMSA performed with the same amount of nuclear extract as in the upper panel using a probe carrying the binding site for the ubiquitous factor OCT-1. In each panel only retarded bands are presented, migration of the free probe is not shown. The relevant complexes are indicated with arrows. In the upper panel complexes which migrate slower than CBF1/IL-6 κB complex correspond to protein endowed with Rel-specific binding activity (data not shown).

	23/24 GTTCCCACGGTTCCCACGC	+
	HES-1 GTGTGAAACTTCCCAACGA	+
	IL-6 κB ATGTGGGATT**TTCCCA**TG	+
	IFNβ κB ATGTAGGAATTTCCCATG	+
	Mad3 CTGGGGTTTTCCCATG	+
	KBF1 AGCTTGGGAATTCCCCAC	+
*	AF4 ATGTAATCTTTTCCCATG	+
*	AF1 ATGTAATATCTTCCCATG	+
*	AF2 ATGTAATATTCTCCCATG	+
*	AF5 ATGAAATGTGGGATTTCCCCATGAGTCTC	
*	AF3 ATGTAATATTTTCCCCTG	
	$H\text{-}2K^b$ GATCTGGGGATTCCCCAT	
	IL-2 κB GATCAAGAGGGATTTCACCCTAT	
	Ig κB GATCCAGAGGGGACTTTCCGAGT	
*	Mut-1 κB ATGTAATATTTTCCCATG	+
*	Mut-2 κB ATGTGGGATTTTAGAATG	
*	6g-1 TTTATCAAATGTGGGACTTTCCCATGAGTCTCAA	+
*	6g-2 TTTATCAAATGTGGGACTTTCCGATGAGTCTCAA	
*	6g-3 TTTATCAAATGTGGGATTTTCCGATGAGTCTCAA	

Figure 2. Sequences of oligonucleotides used as probes and/or competitors in EMSA. 23/24 contains a tandem repeat of the CBF1 binding site, HES-1 corresponds to a CBF1 binding site of the promoter region of the murine *Hairy enhancer of split* gene. IL-6 κB, IFNβ κB, Mad3, KBF1, $H\text{-}2K^b$, IL-2 κB represent binding sites for the nuclear factor NFκB located in the promoter region of genes encoding IL-6, IFNβ, IκBα, p50 subunit of nuclear factor NF-κB, murine MHC class I H-2 gene and IL-2, respectively. Ig κB contains the κB enhancer of the immunoglobulin κ light chain gene. For the 23/24, HES-1, IFNβ κB and KBF1 oligonucleotides, the lower strand is shown. Oligonucleotides containing mutations within the IL-6 κB site or adjacent positions are marked by asterisks; substituted bases are underlined. Nucleotides required for CBF1 binding in IL-6 κB oligonucleotide are shown as emboldened characters. Each oligonucleotide was used as a probe or competitor in EMSAs. Oligonucleotides which are able to form the complex are marked +.

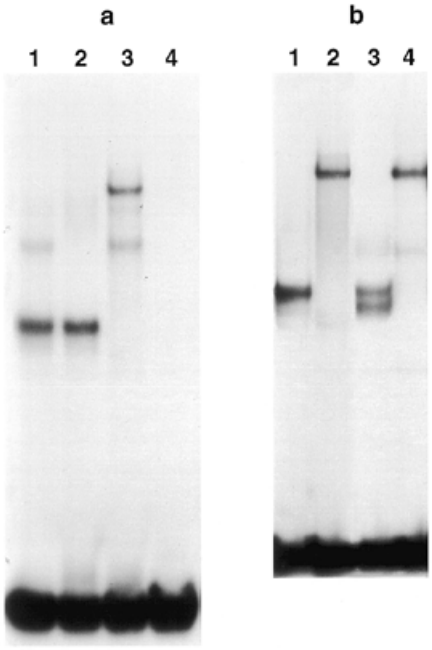

Figure 3. EMSA and antibody supershift analyses performed with crude nuclear extracts or purified CBF1 and the IL-6 κB probe. (**a**) 1, crude nuclear extract from MG-63 cells; 2, as in 1 with the addition of rat non-immune serum; 3, as in 1 with the addition of anti-CBF1 antibody; 4, as in 3 in the absence of nuclear extract. (**b**) 1, as in (a), 1; 2, as in (a), 3; 3, CBF1 purified from MG 63 cells; 4, as in 3 with the addition of anti-CBF1 antibody.

The data presented are representative of at least three independent experiments.

Purification of CBF1

Nuclear extract from MG-63 cells was prepared according to the protocol of Osborn *et al.* (34), with minor modifications. Nuclei were resuspended in buffer A (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM PMSF, 50 μg/ml 4-APMSF, 5 μg/ml each of leupeptin, pepstatin, aprotinin and antipain) and stored frozen. The nuclear suspension was thawed on ice and homogenized in a Dounce potter, after adding, with slow mixing, 5 M NaCl to a final concentration of 0.42 M. The suspension was then stirred for 30 min at 4°C and centrifuged for 30 min at 140 000 *g*. The supernatant was diluted to 0.1 M NaCl with buffer B (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.05% NP-40, 15 μg/ml poly(dI-dC)·poly(dI-dC), 1 mM PMSF, 50 μg/ml 4-APMSF, 5 μg/ml each of leupeptin, pepstatin, aprotinin and antipain) and treated with streptavidin-agarose (Pierce) coupled to the biotinylated IL-6 κB oligonucleotide equilibrated in buffer B with 0.1 M NaCl. Bound proteins were eluted in buffer B with 0.5 M NaCl, then dialyzed against 20 mM MES buffer pH 6, 0.1 M NaCl. Precipitated material was removed by centrifugation and the supernatant applied to HPLC Mono S cation exchange column equilibrated with dialysis buffer. Bound material was eluted with a linear 0.1–1 M NaCl gradient. Fractions containing the CBF1 binding activity were subjected to an additional round of affinity chromatography on streptavidin-agarose $H\text{-}2K^b$ oligonucleotide. Active fractions were eluted with 0.3 M NaCl binding buffer and subjected to affinity chromatography on streptavidin-agarose coupled with the MUT-1 κB (Fig. 2) biotinylated oligonucleotide. Active fractions were eluted with binding buffer containing 0.6 M NaCl. The purified material was analyzed by 8% SDS–PAGE and visualized by silver staining. EMSAs with purified CBF1 were performed using 0.1 μg of poly(dI-dC)·poly(dI-dC) per 20 μl reaction mixture, unless otherwise specified.

Protein–DNA cross linking

Nuclear extracts (~30 μg) were incubated with the appropriate probe, then analyzed by EMSA. Gels were irradiated with a UVG 54 mineral light lamp (Ultraviolet-Prod. Inc. S. Gabriel, CA, USA) at a distance of 10 cm for 20 min at 254 nm on ice and exposed to Kodak film overnight at 4°C. Shifted bands were localized by autoradiography, excised and soaked at 70°C for 10 min in an equal volume of 2× SDS–PAGE loading buffer. The excised bands were deposited into the sample wells of an 8% SDS–PAGE gel and analyzed by electrophoresis. The gels were then fixed, dried and exposed for autoradiography with intensifying screens.

Circular permutation analysis

A 17mer oligonucleotide spanning the IL-6 promoter from –77 to –61 and containing the κB site was cloned into the *Xba*I/*Sal*I sites of the pBend2 plasmid (37). Probes generated by restriction were labeled with [α-^{32}P]dCTP, by filling in 3' recessive ends with Sequenase. Blunt ends probes obtained by *Rsa*I restriction were dephosphorylated and labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. EMSAs using purified CBF1 were carried out at 4°C, under conditions described above. The experiment was repeated three times with different probe preparations. The bending angle was evaluated according to Thompson and Landy (38).

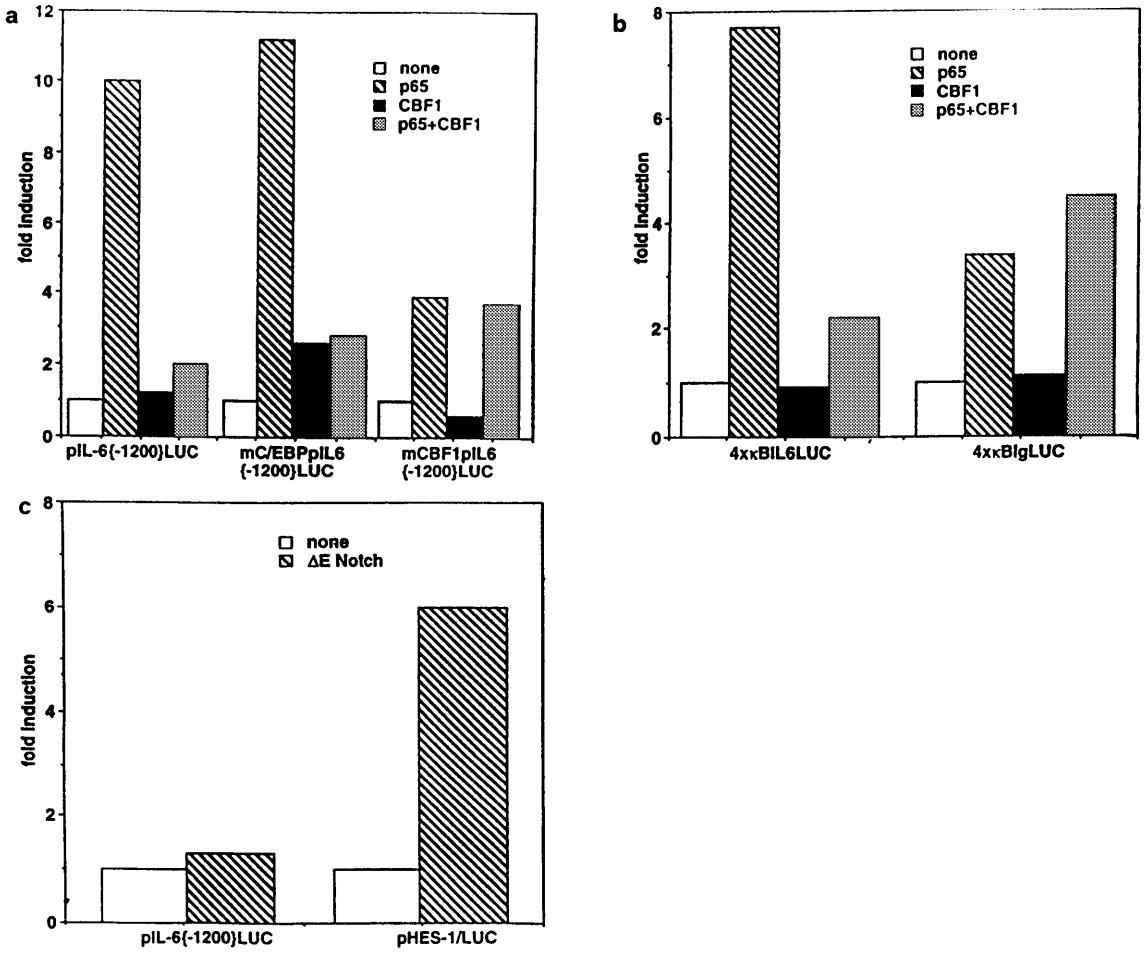

Figure 4. CBF1 activity on the IL-6 promoter. (**a**) C/EBPδ/HL cells were transfected with the luciferase reporter gene under the control of the IL-6 promoter (pIL6{-1200}LUC) or mutants of the IL-6 promoter lacking the C/EBP(–158/–145) binding site (mC/EBPpIL6{-1200}LUC) or lacking the CBF1 binding site (mCBF1pIL6{-1200}LUC). Cells were cotransfected with p65 and/or CBF1 expression vectors. (**b**) CBF1 activity on the κB site of the IL-6 promoter or of the Ig light chain enhancer. C/EBPδ/HL cells were transfected with the luciferase reporter gene under the control of 4×κB site of the IL-6 promoter or 4×κB site of the κ Ig light chain enhancer and cotransfected with p65 and/or CBF1 expression vectors. (**c**) Notch activity on the IL-6 promoter. C/EBPδ/HL cells were transfected with the luciferase reporter gene under the control of the IL-6 promoter (pIL6{-1200}LUC) or the HES-1 promoter pHES-1LUC and cotransfected with the ΔE Notch expression vector.

RESULTS

CBF1 binding activity in human cell lines

We investigated the occurrence and binding specificity of CBF1 in human cell lines of different origin. The oligonucleotide IL-6 κB, spanning the κB site of the IL-6 promoter, formed a fast migrating constitutive complex with all the nuclear extracts tested (Fig. 1). The binding specificity of this complex was the same in all the cell lines examined, as demonstrated by EMSAs (data not shown) using as probes or competitors the κB sites from the promoters of various genes or mutated κB sequences (Fig. 2). The sequence requirements of the target site were very similar to those previously determined by Tun *et al*. (39) for DNA binding of the nuclear protein CBF1. Moreover, a monoclonal antibody raised against CBF1 (35) was able to supershift the complex formed with crude nuclear extracts or with the purified protein from MG-63 cells (Fig. 3). Identical results were obtained with nuclear extracts from the other cell lines (data not shown). These results suggest that the non-Rel protein interacting with the IL-6 κB site, present in human cell lines of different origin, is indeed CBF1.

Shirakata *et al.* (23) have recently demonstrated the existence of a thymic form of CBF1, with a DNA target site different from the canonical sequence previously determined. The thymus-specific protein interacts with a site overlapping the κB site of the MHC class Ia gene. In our experiments this site (H-2K^b oligonucleotide) failed to produce the complex, indicating that the thymic form was not present in nuclear extracts of the cell lines examined, including the T-cell derived lymphoma Jurkat.

Regulation of the IL-6 gene promoter by CBF1

To clarify the role of CBF1 in the regulation of the IL-6 gene expression, we tested the activation of various IL-6 promoter constructs linked to the luciferase reporter gene in HeLa S3 cells permanently transfected with the C/EBPδ cDNA (C/EBPδ/HL). We have previously reported that cotransfection of NF-κB p65 subunit and C/EBP family members leads to maximal activation of the IL-6 promoter (12). As shown in

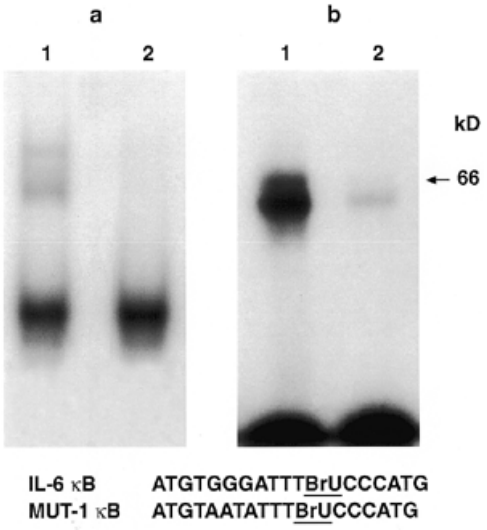

Figure 5. CBF1/DNA cross linking. (**a**) EMSA performed with MG-63 nuclear extracts and probes containing a bromodeoxyuridine residue. Lane 1, IL-6 κB probe; lane 2, MUT-1 κB probe. (**b**) 8% SDS–PAGE of cross-linked complexes obtained with IL-6 κB (lane 1) or with MUT-1 κB probes (lane 2).

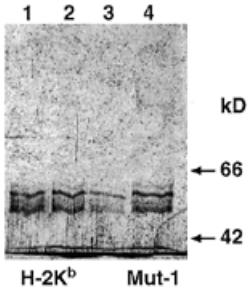

Figure 6. Silver staining of purified CBF1 electrophoresed on an 8% SDS–PAGE. Proteins eluted with 0.3 M NaCl from DNA/H-2K^b affinity chromatography: lanes 1 (5 µl) and 2 (10 µl); proteins eluted with 0.6 M NaCl from DNA/MUT-1 κB affinity chromatography: lanes 3 (5 µl) and 4 (10 µl).

Figure 4a, the wild type pIL6{-1200}LUC as well as the mutant promoter mCBF1pIL6{-1200}LUC, which is unable to bind CBF1, were activated by p65 overexpression. Co-transfection of HeLa cells with an expression vector for CBF1 strongly decreased LUC activity driven by the wild type promoter indicating that CBF1 plays an inhibitory role in the regulation of IL-6 gene transcription. A promoter mutated at the C/EBP binding site from –158 to –145 (mC/EBPpIL6{-1200}LUC) exhibited a level of activation comparable to that of the wild type promoter in the presence of overexpressed p65 and remained sensitive to repression by CBF1. p65-induced activation of the mutant promoter mCBF1pIL6{-1200}LUC was not affected by CBF1 overexpression. As shown in Figure 4b, CBF1 was also able to inhibit the p65/C/EBPδ-induced expression of the LUC reporter gene under the control of four tandem repeats of the IL-6 κB enhancer. CBF1 was without effect, however, on the same construct of the Ig κB enhancer, which is unable to bind the protein. CBF1 is known to act as a DNA targeting intermediate for transcription factors, such as the intracellular domain of the Notch receptor and the EBNA2 protein. In order to determine whether the IL-6 promoter could be activated by such a mechanism, we performed transient transfection experiments using expression vectors encoding the intracellular domain of the murine Notch receptor (ΔE Notch; 26). Overexpression of this protein was unable to promote transcription from the pIL6{-1200}LUC construct, even though it activated the HES-1 promoter directing luciferase expression (Fig. 4c). The EBNA2 transcription activator also failed to up regulate the IL-6 promoter in the same experimental system (data not shown).

Conformational effects of CBF1 on the IL-6 promoter

It is well established that protein/nucleic acid recognition determines sequence-specific DNA deformation and protein conformational changes. To test the hypothesis that the topology of the CBF1–DNA complex could be affected by surrounding sequences, we evaluated the efficiency of two different oligonucleotides to produce UV-induced protein–DNA cross linking. We performed EMSA using ^{32}P-labeled probes containing the CBF1 binding site of the IL-6 promoter or a mutated sequence (MUT-1 κB; Fig. 2) which is unable to bind NF-κB. In both probes a single bromouracil substitution for a thymine residue within the binding site, produced a covalent adduct with the protein forming the complex. As shown in Figure 5a, the amount of CBF1 specific complex formed by the wild type or the MUT-1 κB oligonucleotides was very similar, while the yield of the covalent adduct was greatly reduced with the latter probe (Fig. 5b). Identical results (data not shown) were observed using the protein purified according to the procedure described below. These data suggest that the sequences surrounding the CBF1 target site influence the topology of the protein–DNA interaction. We then addressed the question of whether CBF1 was able to modify DNA conformation at the target site performing a circular permutation analysis with purified CBF1. Figure 6 shows the SDS–PAGE analysis of proteins eluted from the last two steps of CBF1 purification (Materials and Methods). Two major bands corresponding to proteins of 55 and 60 kDa were revealed by silver staining. Consistent with this result, two complexes containing CBF1 were obtained in the EMSA performed with the purified protein (Fig. 3b). Circular permutation analysis was performed with probes generated with the indicated restriction enzymes from the pBend2 plasmid bearing the IL-6 κB site (Fig. 7, left upper side). A position-dependent mobility shift was observed (Fig. 7, left lower side and right side), indicating that CBF1 induced bending of the probes. The amplitude of the observed bending corresponds to a value of 40–42°.

It is known that NF-κB transcription factor interacts with the DNA major groove at its target site (40). The CBF1 recognition element (39) at positions –67 to –62 of the IL-6 promoter partially overlaps the κB site (–73 to –64); therefore, simultaneous binding of the two factors would only be possible if the complex formed by CBF1 would not require DNA major groove occupancy. To clarify this point, we compared the ability of IL-6 κB site and of an oligonucleotide Ino/IL-6 κB (containing IC pairs replacing AT pairs within the CBF1 recognition site) to form a complex with CBF1. As shown in Figure 8, the oligonucleotide Ino/IL-6 κB, which exhibits the same structural features as the wild type IL-6 κB at the minor groove but

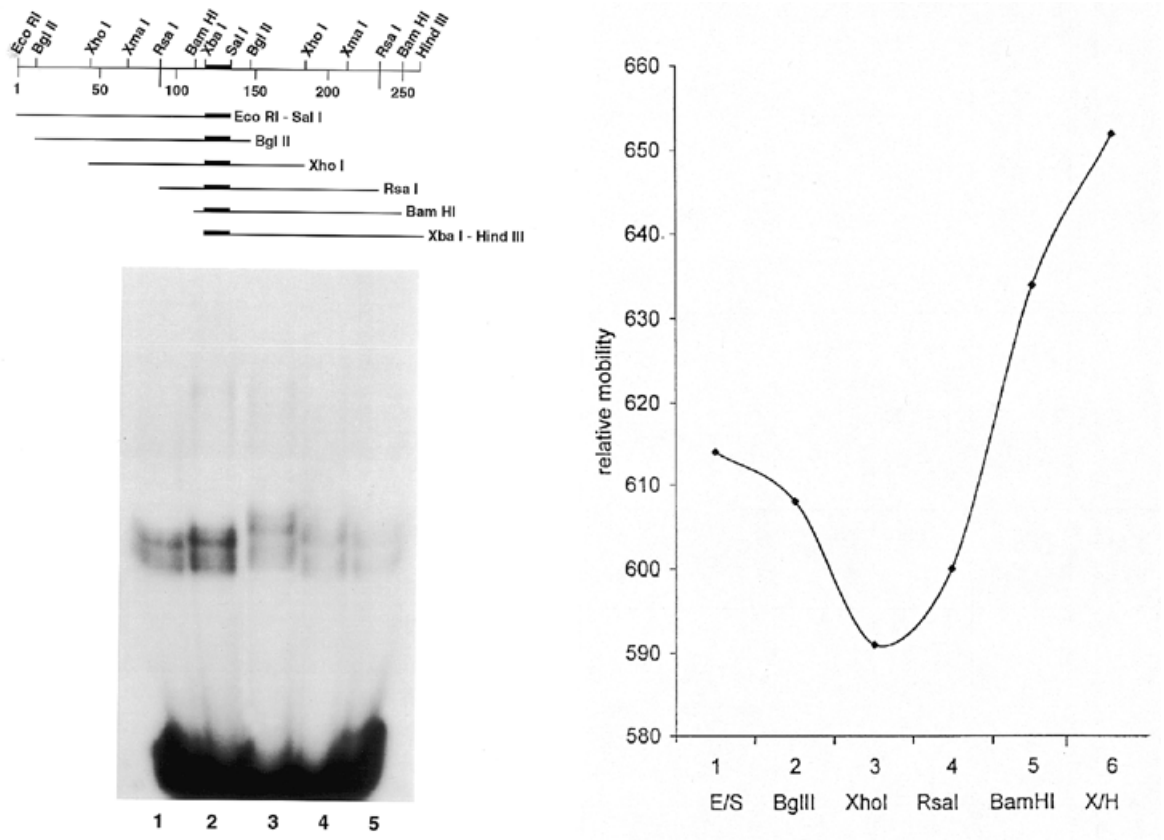

Figure 7. Circular permutation analysis of CBF1 interacting with the IL-6 κB site. Left, upper side: probes used in circular permutation analysis generated by restriction of the pBend2 plasmid; the position of the IL-6 promoter κB site is indicated by a black box. Left, lower side: EMSA of purified CBF1 with circularly permutated probes: 1, *Eco*RI–*Sal*I; 2, *Bgl*II; 3, *Xho*I; 4, *Rsa*I; 5, *Xba*I–*Hin*dIII. Mobility shift with the *Bam*HI probe is not shown. Right side: plot of relative mobility as a function of κB site position within the probe.

differs from it at the major groove, is much less efficient at forming the complex and competes very poorly with the wild type probe. These results suggest that the recognition of the major groove is required for CBF1–DNA complex formation.

DISCUSSION

We and others have recently shown that CBF1 specifically interacts with a DNA region overlapping the κB site of the IL-6 promoter (12–14). We show herein that CBF1 is a negative regulator of IL-6 gene transcription and is able to induce a bend on its DNA binding site.

Plaisance *et al.* (13) have recently demonstrated that the human IL-6 promoter is negatively regulated by CBF1 in murine L929sA fibroblasts. They have proposed that CBF1 competes with NF-κB for the binding to the IL-6 promoter to which the Rel factor displays higher affinity. Kannabiran *et al.* (14) have also reported a negative role of CBF1 in IL-6 gene expression in murine F9 embryo carcinoma cells. They show that this effect is mediated by the κB site and the C/EBP site located immediately upstream (–87/–76), suggesting that it can only be exerted in NF-κB/C/EBPβ coactivation of the IL-6 promoter containing the κB element and the proximal C/EBP site. The discrepancy between the results obtained by the two groups has been ascribed to differences in transfection assays and/or cell phenotypes. Our results, obtained in human HeLa cells, demonstrate that neither the C/EBP site located between positions –87 to –76 nor the site at position –158 to –145 is necessary for repression of IL-6 by CBF1, as indicated by the strong negative activity exerted by this protein on the four tandem repeats of the IL-6 κB site and on the mC/EBPpIL6{-1200}LUC construct.

CBF1 mediates activation of viral and cellular genes by targeting to responsive promoter transcription factors such as the intracellular domain of the Notch receptor or the EBNA2 transcription activator encoded by the Epstein–Barr virus (26–28). The experiments presented herein rule out the existence of a direct effect of these transactivators on IL-6 promoter induction. Thus, our results are in agreement with those of Jarriault *et al.* (26), who showed that CBF1 binding is not sufficient to ensure Notch signaling responsiveness.

To study CBF1/DNA interactions, we have purified the protein from MG-63 cells. Two polypeptide chains of 55 and 60 kDa copurify with CBF1 binding activity. Consistently, EMSAs performed with purified material show two complexes of different mobilities, which are both recognized by a specific monoclonal antibody. Only the lower mobility complex, however, is revealed in EMSAs using crude nuclear extracts. We consider it unlikely that the band of higher mobility represents a proteolytic fragment which retains binding activity, as the ratio of the two complexes remains unchanged during the purification procedure. In addition, we have observed the

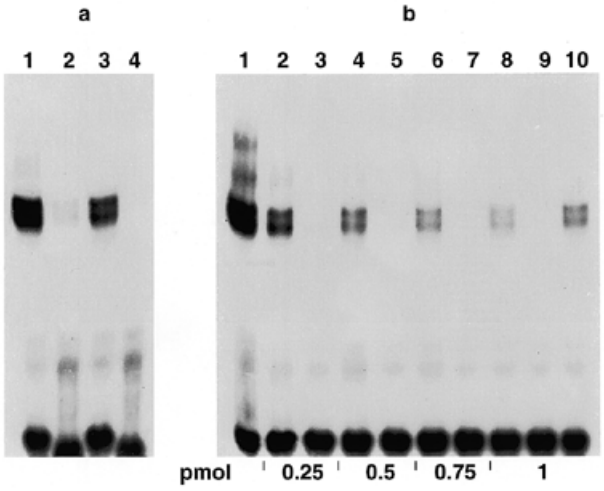

Figure 8. (**a**) EMSA performed with purified CBF1 and the IL-6 κB probe (lanes 1 and 3) or the Ino/IL-6 κB probe (lanes 2 and 4) in the presence of 2 μg/μl (lanes 1 and 2) or 10 μg/μl (lanes 3 and 4) of poly(dI-dC)·poly(dI-dC) as non-specific competitor. (**b**) EMSA performed with purified CBF1 and the IL-6 κB probe (lane 1) and increasing amounts of the Ino/IL-6 κB (lanes 2, 4, 6 and 8) or the oligonucleotide IL-6 κB (lanes 3, 5, 7 and 9) or an unrelated oligonucleotide (lane 10) as competitors. The experiment was performed in the absence of poly(dI-dC)·poly(dI-dC).

same two complexes using a different scheme for CBF1 purification from Jurkat cells (data not shown). In agreement with our observations, Lam and Bresnick (41) have recently reported the purification of two CBF1 forms of 56 and 61 kDa from bovine fetal thymus, which give rise to two different complexes in mobility shift assays. We advance the hypothesis that the polypeptide chain associated with the lower migrating band in EMSAs may represent a CBF1 form which is latent in crude nuclear extracts. The existence of cellular proteins which inhibit CBF1/DNA binding has been recently demonstrated (33) and may account for these observations.

The results of CBF1/DNA cross linking experiments indicate that sequences surrounding the target site affect the contact between DNA bases and the polypeptide chain, in the absence of other proteins. We suggest that these sequences may induce different conformations of the protein–DNA complex, which may play a role in the mechanisms determining the selection of the biological activity displayed by CBF1 at a specific site. In addition, CBF1 binding modifies conformation of the target site causing a bend on the DNA, as shown by circular permutation analysis. HMG-I(Y) proteins have been shown to induce DNA bending, thereby regulating the co-operative assembly of the IFNβ enhanceosome (42). HMG-I(Y) proteins bind to multiple sites of the IFNβ promoter including the κB core sequence, where they contact the DNA minor groove allowing simultaneous binding of NF-κB to the DNA major groove. In contrast, as shown by the results of our experiments, CBF1 recognizes DNA major groove and it is likely to compete with NF-κB for binding at the overlapping sites. Experiments are in progress to further characterize the bending activity of CBF1 in order to fully understand its role in gene regulation.

REFERENCES

1. Barton,B.E. (1996) *Med. Res. Rev.*, **16**, 87–109.
2. Akira,T., Taga,T. and Kishimoto,T. (1993) *Adv. Immunol.*, **54**, 1–78.
3. Peters,M., Schirmacher,P., Goldshmitt,J., Odenthal,M., Peschel,C., Fattori,E., Ciliberto,G., Diens,H.P., Meyer zum Buschenfelde,K.H. and Rose-John,S. (1997) *J. Exp. Med.*, **185**, 755–766.
4. Baldwin,A.S.,Jr (1996) *Annu. Rev. Immunol.*, **1**, 649–683.
5. Akira,S. and Kishimoto,T. (1997) *Adv. Immunol.*, **65**, 1–46.
6. Kang,S.H., Brown,D.A., Kitajima,I., Xu,X., Heidenreich,O., Gryaznov,S. and Neremberg,M. (1996) *J. Biol. Chem.*, **271**, 7330–7335.
7. Faggioli,L., Merola,M., Hiscott,J., Furia,A., Monese,R., Tovey,M. and Palmieri,M. (1997) *Eur. J. Immunol.*, **27**, 3022–3030.
8. Ray,A. and Prefontaine,K.E. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 752–756.
9. Stein,B. and Yang,M.X. (1995) *Mol. Cell. Biol.*, **15**, 4971–4979.
10. Santhanam,U., Ray,A. and Sehgal,P.B. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 7605–7609.
11. Margulies,L. and Sehgal,P.B. (1993) *J. Biol. Chem.*, **268**, 15096–15100.
12. Merola,M., Blanchard,B. and Tovey,M.G. (1996) *J. Interferon Cyt. Res.*, **16**, 783–798.
13. Plaisance,S., Vanden Berghe,W., Boone,E., Fiers,W. and Haegeman,G. (1997) *Mol. Cell. Biol.*, **17**, 3733–3743.
14. Kannabiran,C., Zeng,X. and Vales,L.D. (1997) *Mol. Cell. Biol.*, **17**, 1–9.
15. Hamaguchi,Y., Matsunami,N., Yamamoto,Y. and Honjo,T. (1989) *Nucleic Acids Res.*, **17**, 9015–9026.
16. Israel,A., Yano,O., Logeat,F., Kieran,M. and Kourilsky,P. (1989) *Nucleic Acids Res.*, **17**, 5245–5257.
17. Henkel,T., Ling,P.D., Hayward,S.D. and Peterson,M.G. (1994) *Science*, **265**, 92–95.
18. Schweisguth,F. and Posakony,J.W. (1992) *Cell*, **69**, 1199–1212.
19. Christensen,S., Kodoyianni,V., Bosenberg,M., Friedman,L. and Kimble,J. (1996) *Development*, **122**, 1373–1383.
20. Wettstein,D.A., Turner,D.L. and Kintner,C. (1997) *Development*, **12**, 693–702.
21. Struhl,G. and Adachi,A. (1998) *Cell*, **93**, 649–660.
22. Schroeter,E.H., Kisslinger,J.A. and Kopan,R. (1998) *Nature*, **393**, 382–386.
23. Shirakata,Y., Shuman,J.D. and Coligan,J.E. (1996) *J. Immunol.*, **156**, 4672–4679.
24. Takahama,Y., Tokoro,Y., Sugawara,T., Negishi,I. and Nakauchi,H. (1997) *Eur. J. Immunol.*, **27**, 3318–3331.
25. Robey,E. and Fowlkes,B.J. (1998) *Curr. Opin. Immunol.*, **10**, 181–187.
26. Jarriault,S., Brou,C., Logeat,F., Schroeter,E.H., Kopan,R. and Israel,A. (1995) *Nature*, **377**, 355–358.
27. Ling,P.D., Hsieh,J.J.-D., Ruf,I.K., Rawlins,D.R. and Hayward,S.D. (1994) *J. Virol.*, **68**, 5375–5383.
28. Hsieh,J.J.-D., Henkel,T., Salmon,P., Robey,E., Peterson,M.G. and Hayward,S.D. (1996) *Mol. Cell. Biol.*, **16**, 952–959.
29. Dou,S., Zeng,X., Cortes,P., Erdjument-Bromage,H., Tempst,P., Honjo,T. and Vales,L.D. (1994) *Mol. Cell. Biol.*, **14**, 3310–3319.
30. Kao,H.Y., Ordentlich,P., Koyano-Nakagawa,N., Tang,Z., Downes,M., Kintner,C.R., Evans,R.M. and Kadesch,T. (1998) *Genes Dev.*, **12**, 2269–2277.
31. Olave,I., Reinberg,D. and Vales,L.D. (1998) *Genes Dev.*, **12**, 1621–1637.
32. Cludts,I. and Farrell,P.J. (1998) *J. Virol.*, **72**, 1862–1869.
33. Taniguchi,Y., Furukawa,T., Tun,T., Han,H. and Honjo,T. (1998) *Mol. Cell. Biol.*, **18**, 644–654.
34. Osborn,L., Kunkel,S. and Nabel,G.J. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 2336–2340.
35. Sakai,T., Furukawa,T., Iwanari,H., Oka,C., Nakano,T., Kawaichi,M. and Honjo,T. (1995) *J. Biochem.*, **118**, 621–628.
36. Ho,S.N., Hunt,H.D., Horton,R.M., Pullen,J.K. and Pease,L.R. (1989) *Gene*, **77**, 51–59.
37. Kim,J., Zwieb,C., Wu,C. and Adhya,S. (1989) *Gene*, **85**, 15–23.
38. Thompson,J.F. and Landy,A. (1988) *Nucleic Acids Res.*, **16**, 9687–9705.
39. Tun,T., Hamaguchi,Y., Matsunami,N., Furukawa,T., Honjo,T. and Kawaichi,M. (1994) *Nucleic Acids Res.*, **22**, 965–971.
40. Mueller,C.W., Rey,F.A., Sodeoka,M., Verdine,G.L. and Harrison,S.C. (1995) *Nature*, **373**, 311–317.
41. Lam,L.T. and Bresnick,E.H. (1998) *J. Biol. Chem.*, **273**, 24223–24231.
42. Kim,T.K. and Maniatis,T. (1997) *Mol. Cell*, **1**, 119–129.