Interleukin-6 repression is associated with a distinctive chromatin structure of the gene

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ABSTRACT

Expression of the interleukin-6 (IL-6) gene is usually tightly controlled and may be induced in specific tissues only after treatment with appropriate stimuli. The molecular mechanisms responsible for IL-6 gene repression in specific tissues or cell lines remain poorly defined. In order to address this question we have studied two human breast carcinoma cell lines, MDA-MB-231, in which the IL-6 gene is expressed, and MCF-7, in which it is not. The promoter region of the IL-6 gene was analysed in both cell lines with reference to two different parameters: (i) DNase I hypersensitivity; (ii) the in vivo pattern of DNA–protein interactions. We show herein that the mechanism responsible for silencing IL-6 gene expression in MCF-7 cells most probably involves a modification of chromatin structure, as suggested by a decreased sensitivity of the IL-6 promoter to DNase I relative to the IL-6-expressing cell line MDA-MB-231. Moreover, we show that a ‘closed’ nucleosomal structure in MCF-7 cells does not inhibit the binding of nuclear proteins to IL-6 gene regulatory sequences in vivo. We suggest, therefore, that, in non-expressing cells, local chromatin remodelling at the proximal promoter is inhibited by negative regulators, as suggested by two specific hallmarks of nuclear factor binding that are not observed in expressing cells: an additional in vivo footprint spanning positions −135/−116 and an additional DNase I hypersensitivity site far upstream, around position −1400. Furthermore, a specific factor binding in vitro to the −140/−116 region of the IL-6 promoter is found in MCF-7 cells.

INTRODUCTION

Interleukin-6 (IL-6) is a multifunctional cytokine the production of which is rapidly and strongly induced by several pathological and inflammatory stimuli. Thus, IL-6 can be induced according to a tissue-specific pathway of expression in response to bacterial endotoxin (LPS), viral infection, phytohaemagglutinin, or a variety of other cytokines such as TNF-α, IL-1β, PDGF and interferons (1). IL-6 has been found to play a central role in the defense mechanism(s), by regulating several aspects of the immune response, haematopoiesis and the acute phase reaction. As would be expected for such a pleiotropic cytokine, expression of the IL-6 gene is tightly controlled, and unregulated IL-6 synthesis is associated with a number of pathological conditions, including certain autoimmune diseases, some types of tumours, and both acute and chronic inflammatory diseases (1,2).

A 1.2 kb fragment of the 5’-flanking region of the IL-6 gene (3–5) contains the cis-acting elements necessary for induction of the gene by all known inducers. The nuclear factors κB (NF-κB) (6) and interleukin-6 (NFIL6 or C/EBPβ) (6) have been reported to play a crucial role in the induction of IL-6 gene expression, even though other nuclear factors have also been shown to modulate IL-6 gene expression under certain circumstances (5). Transfection studies have shown that overexpression of NFIL6 and the p65 subunit of NF-κB synergistically activates an IL-6 promoter–reporter construct in the murine embryonic carcinoma cell line P19 (7), indicating that these two factors are sufficient to sustain activation of the IL-6 gene. Furthermore, we have shown that the κB element alone is sufficient to confer an IL-1β- and TNF-α-induced response in HeLa cells, in the presence of the p65 subunit of NF-κB and the nuclear factors NFIL6 and/or NFIL6β (C/EBPβ) (8), suggesting a direct interaction of NF-κB with non-Rel transcription factors (6). Down-regulation of IL-6 expression by diffusible factors has been reported in the presence of glucocorticoids (9), oestrogens (10) and the tumour suppressor gene products p53 and pRB (11).

Although much is known about inducible expression of the IL-6 gene, almost no information is available concerning the mechanisms which mediate tissue-specific expression/regression of the gene. It has previously been reported that in normal liver (12,13), in polymorphonuclear cells (14) and in the MCF-7 human breast carcinoma cell line (15) activation of NF-κB, NFIL6 and NFIL6β in response to TNF-α and IL-1β fails to induce IL-6 production. Furthermore, our studies on MCF-7 cells have shown that, in a transient expression assay, an IL-6 promoter–reporter construct is fully functional, suggesting that in this cell line the chromatin surrounding the IL-6 gene is in a repressed state (15). All the studies carried out to date which have addressed the mechanism of regulation of IL-6 gene expression have, however, employed in vitro systems or transfection
assays, which may be inadequate to predict protein–DNA interactions in intact cells. Indeed, in the eukaryotic nucleus DNA is organized in a highly complex chromatin structure, and thus protein–DNA interactions within whole cells may be determined not only by the presence of regulatory proteins but also by the accessibility of their target sequence (16). It has been generally assumed that nucleosomes negatively regulate gene expression by restricting access of the transcriptional machinery to the DNA (17). Thus, chromatin structure remodelling appears to be a key step in transcriptional activation by increasing the ability of transcription factors to bind the appropriate regulatory elements. Remodelled chromatin has been detected classically as DNase I hypersensitive sites which can form either before the activation of a gene (persistent or preset DNase I hypersensitive sites) or upon inducible binding of transcription factors (18).

In order to study the mechanisms underlying tissue-specific expression/repression of the IL-6 gene we have chosen two breast carcinoma cell lines, MDA-MB-231, which produce high levels of IL-6 in response to IL-1β and TNF-α, and the MCF-7 cell line, which fails to produce both IL-6 protein and mRNA under the same experimental conditions (15). The promoter region of the IL-6 gene was analysed in both cell lines with reference to two different parameters: (i) DNase I hypersensitivity; (ii) the in vivo pattern of DNA–protein interactions. We show herein that the mechanism responsible for silencing IL-6 gene expression in the human breast carcinoma cell line MCF-7 most probably involves a modification of chromatin structure as suggested by a decreased sensitivity of the IL-6 promoter to DNase I relative to the IL-6-expressing cell line MDA-MB-231. Moreover, we show herein that the nucleosomal structure in MCF-7 cells does not inhibit binding of nuclear factors to IL-6 gene regulatory sequences in vivo, rather it allows the establishment of an additional in vivo footprint. This result suggests the presence in these cells of an additional factor which may correlate with silencing of the IL-6 gene. In agreement with this observation, a cell-specific factor binding to the same region can be detected by in vitro assay in MCF-7 cells.

MATERIALS AND METHODS

Materials

Recombinant human TNF-α (sp. act. 2 × 10^7 U/mg protein) and recombinant human IL-1β (sp. act. 1 × 10^6 U/mg protein) were purchased from Genzyme and were both used at a concentration of 10 ng/ml. Oligonucleotides were synthesised with an Applied Biosystem oligosynthesiser.

Cell lines

Human breast carcinoma cell lines MDA-MB-231 and MCF-7 and the human hepatoma cell line Hep G2 were cultivated in DMEM (Gibco BRL) supplemented with 10% foetal calf serum, antibiotics and L-glutamine, in an atmosphere of 5% CO₂ in air.

DNase I hypersensitivity

The procedure employed was a slightly modified version of the method of Rigaud et al. (19). Genomic DNA was purified by phenol/chloroform extraction, RNase A treated, re-extracted and finally ethanol precipitated. An aliquot of 25 μg of purified DNA was digested with EcoRI (Pharmacia), separated by 1% agarose gel electrophoresis and transferred onto nylon membrane (Hybond N; Amersham). Hybridization was performed using as probe a 257 bp fragment, spanning the sequence between restriction sites SmaI and EcoRI in intron 2 of the IL-6 gene, labelled with 32P (ICN) by random priming (Sigma). The same DNA samples were submitted to the LMPCR procedure (see below). To obtain in vitro DNase I-digested DNA, naked DNA was treated with 20 μg/ml DNase I in 10 mM Tris–HCl, pH 7, 5 mM MgCl₂, 1 mM EDTA for various incubation times at 4°C.

In vivo and in vitro DMS/piperidine cleavage of DNA

Adherent cells were washed with PBS and then treated for 5 min at room temperature with 0.2% dimethyl sulphate (DMS) (Sigma) in complete medium. The DMS reaction was stopped by washing with a large volume of PBS and the genomic DNA was purified by proteinase K digestion and phenol/chloroform extraction. Ten micrograms of in vivo methylated DNA were ethanol precipitated, dried, resuspended in 100 μl of 1 M piperidine (Sigma) and treated for 30 min at 95°C. Piperidine was removed under vacuum and the DNA was washed twice with water and finally ethanol precipitated.

DNA was methylated in vitro by adding 1 μl of DMS to 200 μl of naked DNA (1 μg/ml in 10 mM Tris–HCl, pH 8, 1 mM EDTA) and incubating for 90 s at 20°C. The reaction was stopped with 50 μl of a solution containing 1.5 M sodium acetate, pH 7, and 1 M β-mercaptoethanol. The DNA was then ethanol precipitated twice, resuspended in 200 μl of 1 M piperidine and treated as described for in vivo methylated DNA.

In vivo footprinting with the LMPCR procedure

Aliquots of 1 μg of genomic DNA, digested with DNase I or cleaved with DMS/piperidine, were submitted to the LMPCR procedure as described (20,21). The same DNA sample was used for two-strand analysis. DNA was amplified for 30 cycles as follows: 94°C for 30 s; 68°C (lower strand) or 64°C (upper strand) for 3 min; 73°C for 2 min. For the probing step the samples were amplified for nine cycles as follows: 94°C for 30 s; 72°C (lower strand) or 67°C (upper strand) for 3 min; 73°C for 2 min. One tenth of each sample was loaded on a conventional 8% polyacrylamide–urea sequencing gel. Following electrophoresis the gel was fixed, dried and autoradiographed without an intensifying screen. Genomic DNA chemically cleaved by the modified procedure of Saluz and Jost was used for the A/G ladder (22). The nucleotide sequences of the primers used were as follows. (i) Lower strand: primer 1, GAAATTAAGGAA-GAGTGGTTC; primer 2, TCTTACCCTAGCCCTCAATGAGC- CGAC; primer 3, AGGCTCAATGAGCAGCTAAAGCTGCA- CTTT. (ii) Upper strand: primer 1, CTGTTGGAAGGA-GTTCTAG; primer 2, GTGGCTCGAGGGCAGAATGAGCG- CTG; primer 3, GCCGAAATGACGCTTGACATCATCTCCA- GTC.

Protection and hypersensitivity relative to control DNA were determined by densitometric scanning with a Molecular Dynamics PhosphorImager.

EMSA

Cells were scraped, washed twice with cold PBS, and transferred to a microfuge tube. All the subsequent steps were performed at 4°C. To prepare nuclear extracts the cells were
resuspended in ~3 packed cell pellet vol of hypotonic buffer A [10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, containing 0.1% NP-40, PMSF and protease inhibitors (Complete™; Boeringer Mannheim) with EDTA] and were incubated for 10 min. The cellular lysate was centrifuged for 10 min in a refrigerated table centrifuge at 12 000 r.p.m. The nuclear pellet was gently resuspended in 50% packed nuclei pellet vol of low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.5 mM DTT, PMSF and protease inhibitors) and 50% packed nuclei pellet vol of high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.5 mM DTT, PMSF and protease inhibitors). After 15 min incubation the sample was centrifuged for 30 min in a refrigerated table centrifuge at 12 000 r.p.m. and the supernatant was recovered, frozen in aliquots in liquid nitrogen and stored at –80°C. The final KCl concentration of the nuclear extract was ~375 mM. The protein concentration was measured with the Ready-to-Use Coomassie blue G-250-based reagent from Pierce, using BSA as standard protein, and was usually 8–10 mg/ml. Binding reactions were carried out in a final volume of 20 μl using 10 μg nuclear extract in 10 mM HEPES, pH 7.9, 5 mM Tris–HCl, pH 7.9, 1 mM DTT, 10% glycerol, 50 (MDA-MB-231 cell extract) or 100 mM KCl (MCF-7 cell extract) and 2 μg poly[(dI·dC)(dI·dC)] (Pharmacia). Radiolabelled probe (40 000 c.p.m.) was added last to each reaction mixture and samples were incubated at room temperature for 30 min. In competition assays, a 200-fold molar excess of unlabelled double-stranded oligonucleotide was added to the reaction mixture (only the sequence of the sense strand is indicated) was WT probe, 5′-AAGGTTTTCCAATCAGCCCCACCCCGC-3′ (from positions –140 to –116 of the IL-6 gene promoter). Hybridized oligonucleotides were 5′-32P-end-labelled using T4 polynucleotide kinase (Biolabs).

RESULTS

DNase I hypersensitivity of the IL-6 promoter

We have shown previously (15) that transcription of the IL-6 gene is strongly induced by IL-1β and TNF-α in the human breast carcinoma cell line MDA-MB-231, while MCF-7 cells fail to produce IL-6 mRNA under the same experimental conditions. Analysis of the transcription factors involved in the IL-6 gene expression revealed that the MDA-MB-231 and MCF-7 cell lines possess the same profile of transcription factors. Moreover, a luciferase reporter gene under the control of the IL-6 promoter was found to be efficiently expressed in both cell lines following transient transfection, leading to the hypothesis that chromatin structure may play a role in repression of the IL-6 gene (15). Differences in chromatin arrangement between cell lines (or in the same cell line under different conditions) are generally elucidated by analysing the susceptibility of a target region of the genome to the action of DNase I. Active promoters in vivo are, indeed, almost invariably contained in regions characterized by DNase I hypersensitive sites indicating that ‘normal’ nucleosome structures are temporarily or permanently absent (18).

We have compared the pattern of DNase I hypersensitivity of the IL-6 gene in uninduced and IL-1β- or TNF-α-induced MDA-MB-231 and MCF-7 cells. DNase I digestion of genomic DNA in the whole cell was achieved by treating a cell suspension in a permeabilising buffer containing increasing amounts of DNase I. Southern blotting using EcoRI-digested DNA and a probe which spans a 257 bp region between the EcoRI and Smal sites in intron 2 of the IL-6 gene (see Fig. 1C) revealed five major DNase I hypersensitive sites (Fig. 1A and B). A site located around position +400 (DH 1) relative to the transcription initiation site (+1) of the IL-6 gene in uninduced (left) or TNF-α-induced (90 min) (right) MDA-MB-231 (A) and MCF-7 (B) cells was probed with a Smal–EcoRI restriction fragment included in intron 2 of the IL-6 gene. The right hand side arrows indicate DNA fragments generated by DNase I digestion. The position of DNA markers is indicated on the left hand side of the figure. (C) A schematic representation of the region of the IL-6 gene analysed. EcoRI digestion generated a 3500 bp fragment spanning +1000 to +2500 relative to the transcription initiation site (+1). Positions of the probe used for Southern blot analysis and of exons 1 and 2 are indicated as full bar and open boxes, respectively. Vertical arrows indicate the positions of the hypersensitive sites on the IL-6 gene in MDA-MB-231 and MCF-7 cells.

Figure 1. DNase I hypersensitivity of the IL-6 gene. NP-40 permeabilized cells were treated with 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/ml DNase I for 3 min prior to isolating genomic DNA for Southern blot analysis. EcoRI-digested genomic DNA from uninduced (left) or TNF-α-induced (90 min) (right) MDA-MB-231 (A) and MCF-7 (B) cells was probed with a Smal–EcoRI restriction fragment included in intron 2 of the IL-6 gene. The right hand side arrows indicate DNA fragments generated by DNase I digestion. The position of DNA markers is indicated on the left hand side of the figure. (C) A schematic representation of the region of the IL-6 gene analysed. EcoRI digestion generated a 3500 bp fragment spanning +1000 to +2500 relative to the transcription initiation site (+1). Positions of the probe used for Southern blot analysis and of exons 1 and 2 are indicated as full bar and open boxes, respectively. Vertical arrows indicate the positions of the hypersensitive sites on the IL-6 gene in MDA-MB-231 and MCF-7 cells.
IL-1β (data not shown). A site in the region of position –150 (DH 3), which corresponds to sequences of the IL-6 promoter, is present in MDA-MB-231 cells with an intensity similar to that of the other sites, while it is almost completely absent in MCF-7 cells. A fifth site in the region of position –1400 (DH 5) is present only in MCF-7 cells. The relative intensity of the bands corresponding to each site remains unchanged after treatment with the inducers in both cell lines. Hence, the results obtained by DNase I hypersensitivity assay indicate that in the IL-6-expressing MDA-MB-231 cell line, chromatin appears to be constitutively sensitive to DNase I digestion at various sites and, particularly, at site DH 3 which covers the 5′-flanking region of the IL-6 gene considered crucial for its transcription. Moreover, the data suggest that the regions of the IL-6 gene corresponding to the hypersensitive sites are devoid of nucleosomes or contain an altered nucleosomal structure. In the IL-6 non-expressing MCF-7 cell line the accessibility of chromatin to DNase I is restricted at site DH 3. This result indicates that the IL-6 promoter in MCF-7 cells is organised in a closed nucleosomal structure and suggests that IL-6 expression may involve a remodelling of chromatin structure. A pattern of DNase I hypersensitivity similar to that shown for MCF-7 cells has also been obtained with Hep G2 cells (data not shown), which are unable to produce IL-6 (unpublished data) while they synthesize IL-8 (23,24), a cytokine which requires the same array of transcription factors as IL-6 for its induction by IL-1 or TNF-α (7).

**In vivo** DNase I footprinting of the IL-6 promoter

To determine whether the differential pattern of DNase I hypersensitivity between cells which express IL-6 and cells which do not corresponds to a differential pattern of regulatory factor–DNA interactions, we analysed the interaction of nuclear factors with the IL-6 promoter in vivo in both the MDA-MB-231 and MCF-7 cell lines. Nuclear extracts from both cell lines have been shown by gel mobility shift assays to contain inducible factors of the Rel family and a constitutive non-Rel protein binding to the kB site of the IL-6 promoter (15). The constitutive protein has been identified as RBP-Jκ/CBF-1 and has been demonstrated to play a repressive role in basal and inducible expression of the IL-6 promoter which contains three repeats of the element CCACC and plays an important role in basal and inducible expression of the IL-6 gene (29). Consistent with these results, we have

*Figure 2.* (Opposite) *In vivo* DNase I footprint of the IL-6 promoter. (A) A DNase I footprinting experiment performed on the upper strand of the IL-6 promoter. Cells were treated with 0.8 mg/ml DNase I for 3 min. Naked DNA was digested with 20 μg/ml DNase I for 3 or 6 min. A/G and naked correspond to the deproteinised genomic DNA cleaved in *vitro* at purine-specific locations (30) or with DNase I, respectively. At the top of the figure is indicated the cell line from which the genomic DNA was derived. Where indicated, cells were treated with inducers (IL-1β or TNF-α) for 90 min prior to DNase I digestion. (B) Analysis of the lower strand. Samples were treated as in (A), except that digestion of genomic DNA purified from MDA-MB-231 and MCF-7 cells was achieved using 0.7, 0.8, 0.9 and 1 mg/ml DNase I. The principal protected sites (open symbols, circles and squares) or hypersensitive sites (filled symbols, circles and squares) compared to the control (naked) are indicated on the right hand side of the gels. Only differences which were found to be reproducible in at least two separate experiments are indicated. Circles and squares specify protection or hypersensitivity found for MCF-7 (open and closed circles) or MDA-MB-231 (open and closed squares) cells, respectively. Locations of transcription factor consensus elements are indicated on the left side of the gels. The same pattern as for uninduced cells was obtained with IL-1β- and TNF-α-induced cells (not shown). (C) Patterns of DNase I protection and hypersensitivity of the IL-6 promoter in MDA-MB-231 and MCF-7 cells. Protected and hypersensitive sites are marked with symbols as in (A) and (B). Black lines with one arrow represent lower strand and upper strand primer 3 (see Materials and Methods). Boxed sequences indicate recognition binding elements for the transcription factors given underneath. Position +1 indicates the initiation transcription site.
also observed that the promoter of the human IL-6 gene is able to bind a constitutive factor(s) in both MDA-MB-231 and MCF-7 cells, as demonstrated by the footprints obtained in the regions corresponding to putative Sp1 binding sites.

In vivo DMS footprinting of the IL-6 promoter

Since DNase I footprinting can be carried out only on isolated nuclei or permeabilized cells, by procedures which may cause labilisation of protein–DNA interactions, we also analysed the sensitivity of the IL-6 promoter by treatment of intact cells with DMS. This small molecular weight reagent penetrates cells readily and methylates double-stranded DNA principally at guanines and to a lesser extent at adenines. Piperidine treatment results in cleavage at the modified guanines (30). It has to be pointed out, however, that as DMS can gain access to protein binding sites more readily than DNase I, only a few residues may appear protected or enhanced, and some DNA–protein complexes might not cause altered DMS sensitivity. A representative DMS footprint of the upper strand of the IL-6 promoter is shown in Figure 3A. A difference in the pattern of DMS methylation relative to naked DNA is observed only in MCF-7 cells where protection of a guanosine residue at position –174 is revealed: its relevance has to be further clarified by in vitro functional assays, as this region of the IL-6 promoter has not yet been demonstrated to play a role in the regulation of IL-6 gene expression. The pattern does not change after treatment with the inducers (Fig. 3A for TNF-α; data not shown for IL-1β). Examination of the lower strand of the IL-6 promoter (Fig. 3B) reveals a reduction in the methylation intensity of two guanosine residues (–64 and –65) belonging to the NF-κB binding site. Interestingly, the extent of protection seems to increase with time of inducer treatment (Fig. 3B for TNF-α; data not shown for IL-1β). Also, the NFIL6/NFIL6 and the distal Sp1 binding sites show footprints, relative to naked DNA, characterised by hypomethylated guanosines. Other guanosine residues located outside the previously described canonical binding sites (–128, –132, and –133) appear to be constitutively protected. No differences in the pattern of DMS methylation between MDA-MB-231 and MCF-7 cells were observed. Some hyper-reactivities corresponding to adenosine residues, which are present only in in vivo treated DNA, probably result from the cleavage at N3 methylated adenosines during DNA purification (31).

Figure 3. In vivo DMS footprint of the IL-6 promoter. Analysis of the upper (A) and lower (B) strands of the IL-6 promoter. Whole cells were treated with TNF-α for 15 and 90 min and 6 and 24 h prior to the addition of 0.2% DMS for 5 min. Genomic DNA purification and cleavage of methylated bases are detailed in Materials and Methods. A/G and naked correspond to the deproteinised genomic DNA cleaved in vitro at purine-specific locations (30) or in vitro methylated with DMS, respectively. For symbols, A/G and naked ladders see Figure 2. Only differences which were found to be reproducible in at least two separate experiments are indicated. The footprint obtained was found to be identical when IL-1β was used as inducer (not shown).
To establish whether the in vivo EMSA with the –140/–116 region of the IL-6 promoter lines with IL-1 retarded complexes remain unchanged after induction of both cell –119 and from position –148 to –136. The patterns of the specific this cell line. This result is in agreement with the presence of the Instead, a complex of higher mobility appears with extracts from which is not obtained with MDA-MB-231 nuclear extracts. contain a constitutive factor giving rise to a specific retarded band of specific retarded protein–DNA complexes. A 200-fold excess of unlabelled –140/–116 oligonucleotide (self) or an unrelated oligonucleotide (non-specific) was added to the binding mixture where indicated. Free DNA bands and slot areas were cut off.

**EMSA with the –140/–116 region of the IL-6 promoter**

To establish whether the in vivo DNase I footprint covering positions –135/–119 of the IL-6 promoter observed only in MCF-7 cells correlates with the binding in vitro of a nuclear factor, we performed an electrophoretic mobility shift assay using a probe spanning the region between nucleotides –140 and –116 and nuclear extracts from MCF-7 or MDA-MB-231 cells. As shown in Figure 4, nuclear extracts from MCF-7 cells contain a constitutive factor giving rise to a specific retarded band which is not obtained with MDA-MB-231 nuclear extracts. Instead, a complex of higher mobility appears with extracts from this cell line. This result is in agreement with the presence of the two differential in vivo footprints between MCF-7 and MDA-MB-231 cells which span, respectively, from position –148 to –119 and from position –148 to –136. The patterns of the specific retarded complexes remain unchanged after induction of both cell lines with IL-1β or TNF-α (data not shown).

**DISCUSSION**

The present study was undertaken to investigate the molecular mechanism of tissue-specific expression of the IL-6 gene. In this report we show, using the techniques of DNase I hypersensitivity and in vivo footprinting, that differences do indeed exist in the pattern of sensitivity to DNase I and of protein–DNA interactions between MDA-MB-231, an IL-6-expressing human carcinoma cell line, and MCF-7 cells, a human carcinoma cell line which does not express IL-6.

The region located between positions –2500 and +1000 (EcoRI sites) relative to the transcription initiation site of the IL-6 gene contains five DNase I hypersensitive sites in the region of positions –1400 (DH 5), –600 (DH 4), –150 (DH 3), +200 (DH 2) and +400 (DH 1). Two major differences have been identified in the IL-6-expressing cells (MDA-MB-231) compared to cell lines which do not express IL-6 (MCF-7 and Hep G2). First, sites DH 1 and DH 3 are very strong only in the MDA-MB-231 cell line. Site DH 1, which is located in the transcribed region of the gene, could represent a 3′ enhancer. The differential sensitivity of the DH 3 site, which covers the 5′-flanking region of the IL-6 gene considered crucial for its transcription, suggests that the two types of cells differ in their nucleosomal structure on the IL-6 promoter. Site DH 3, as well as the other DNase I hypersensitive sites, are detected in the absence of any inducer and remain unchanged in MDA-MB-231 chromatin after treatment of cells with IL-1β or TNF-α, the most potent physiological inducers of IL-6 gene expression described to date. These findings indicate that the DNase I hypersensitive sites in the MDA-MB-231 chromatin are not generated as an immediate response to treatment with the inducer and could be regarded as persistent sites. Thus, the occurrence of nucleosomal remodelling on the IL-6 gene in MDA-MB-231 chromatin is not related to induction. The second difference between the IL-6-expressing and non-expressing cell lines concerns site DH 5, located in the region of position –1400 relative to the transcription initiation site of the IL-6 gene, which is present only in MCF-7 and Hep G2 cell lines. To our knowledge, the furthest upstream DNA region of the IL-6 gene analysed to date by transfection assays extends to ~1200 bp upstream from the starting point of IL-6 gene transcription and does not include the hypersensitive site DH 5. Thus, our results suggest that the –1400 region of the IL-6 promoter is involved in certain repressive functions of the chromatin surrounding the IL-6 gene.

The differences in nucleosomal structure of the IL-6 promoter detected in the MDA-MB-231 and MCF-7 cell lines suggest that the pattern of protein–DNA interactions in vivo may also differ in the two types of cells. Indeed, protein–DNA interactions are determined not only by the presence of regulatory proteins but also by the accessibility of target sequences to such proteins, which in turn depends upon the organisation of chromatin. We have shown previously that following induction with IL-1β or TNF-α, the pattern of activation of NF-kB, NFIL6 and NFIL6β, which are considered to be sufficient to sustain transcription of the IL-6 gene, is similar in both MDA-MB-231 and MCF-7 cells (15). We have now shown, using in vivo footprinting analysis of the human IL-6 promoter, that in both cell lines proteins are present constitutively within a region of ~150 bp upstream of the transcription initiation site. These results demonstrate that in MCF-7 cells the binding of putative regulatory proteins to the promoter is compatible with the retention of a nucleosomal structure. This observation is in agreement with numerous reports illustrating that the binding of many different transcription factors to nucleosomal DNA in purified in vitro systems results in the formation of ternary complexes containing transcription factors, DNA and histones (17,32). Moreover, in vivo footprinting studies suggest that numerous factors can bind to the MMTV promoter in the continued presence of the underlying nucleosome (28). The constitutive in vivo footprints observed in MDA-MB-231 and MCF-7 cells within the NF-kB and NFIL6 binding sites as well as within the putative Sp1 site of the IL-6 promoter are in keeping with the constitutive presence of NF-kB and NFIL6 in both nuclear extracts from the two carcinoma cell lines (15) and in nuclear extracts from HeLa cells (33). Furthermore, these results are in agreement with the finding that the same binding sites within the murine IL-6 promoter are constitutively occupied by proteins (29). Evidence has already been obtained...
which suggests that the promoters of other rapidly induced genes such as c-fos (34) and the IFN-α1 gene (35) are also occupied in vivo both before and after induction. On the other hand, induction of other genes, such as human hsp70 (36), ISG54 (37) and guanylate-binding protein genes (37) and rat tyrosine aminotransferase gene (19), resulted in the induced protection of the respective responsive elements in vivo. Thus, it seems likely that genes which can be immediately activated may have proteins bound to their promoters before induction that may or may not exchange with other proteins after induction.

The pattern of protein–DNA interactions in vivo differs between MDA-MB-231 and MCF-7 cell lines at various sites (Fig. 2C). The major difference, which is observed in the region between positions –135 and –119, correlates with the pattern of the mobility shift assay showing specific constitutive binding activities to a fragment spanning the –140/–116 region of the IL-6 promoter. In contrast, the other differences, which concern mostly single base protection or enhancement, may reflect the positioning of the same nuclear factors on a stable nucleosomal structure in MCF-7 cells, or on histone-free DNA in MDA-MB-231 cells. Such sterically different arrangements of chromatin may determine a differential sensitivity of the corresponding regions to footprinting reagents, even when the same factors are bound.

In conclusion, we have demonstrated by DNase I hypersensitivity assays that the IL-6 promoter is organised in a closed nucleosomal structure in the IL-6 non-expressing MCF-7 cells and we suggest that this feature may play a critical role in IL-6 gene repression. The results obtained by in vivo footprinting experiments and mobility shift assays show the presence of a specific nuclear factor(s) in MCF-7 cells, which binds to the –140/–116 region of the IL-6 promoter comprising a closed nucleosomal structure. This factor could function as a DNA target for the recruitment of a repressor complex responsible for chromatin condensation. Repressor complexes have, indeed, already been described (38) which consist of a tissue-specific DNA-binding protein recruiting a complex machinery responsible for histone deacetylation (39) or other unknown chromatin modifications (40). The higher mobility complex which has been found in nuclear extracts of MDA-MB-231 cells correlates with the less extended area of protection determined in the in vivo footprint of this cell line relative to MCF-7 cells. We suggest that the nuclear factor present in the cell may be able to bind the same sequence as the MCF-7 factor but may have the opposite function, i.e. it recruits to the promoter a complex machinery responsible for nucleosomal disruption and gene activation. Further characterisation of the observed binding activities will determine the validity of this speculation. Other silencing factors may also interact with the –1400 hypersensitive site DH 5 that is present only in cells which do not express IL-6. The structure of the IL-6 promoter in vivo in MDA-MB-231 cells would be in agreement with the classical assumption that nucleosomal disruption associated with a promoter is required for gene transcription and is generally detected by the presence of DNase I hypersensitive sites.

To our knowledge, the results presented herein provide the first indication of the involvement of chromatin organisation in the regulation of tissue-specific expression of the human IL-6 gene.

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