Cooperative Influence of Genetic Polymorphisms on Interleukin 6 Transcriptional Regulation*

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Interleukin 6 (IL6) plays key roles in hematopoiesis, immune, and acute phase responses. Dysregulated IL6 expression is implicated in diseases such as atherosclerosis and arthritis. We have examined the functional effect of four polymorphisms in the IL6 promoter (−597G→A, −572G→C, −373A→T, −174G→C) by identifying the naturally occurring haplotypes and comparing their effects on reporter gene expression. The results indicate different transcriptional regulation in the ECV304 cell line compared with the HeLa cell line, suggesting cell type-specific regulation of IL6 expression. The haplotypes showed functional differences in the ECV304 cell line; transcription was higher from the GG8/11G haplotype and lower from the AG8/12G allele. The differences suggest that more than one of the polymorphic sites is functional; the base differences at distinct polymorphic sites do not act independently of one another, and one polymorphism influences the functional effect of variation at other polymorphic sites. These results show that genetic polymorphisms in the promoter influence IL6 transcription not by a simple additive mechanism but rather through complex interactions determined by the haplotype.

IL6,1 a multifunctional cytokine with a central role in host defense (1–3), has diverse functions including stimulation of the hepatic acute phase response to infection and injury (4), differentiation and/or activation of macrophages and T cells, growth and terminal differentiation of B cells, support of multipotent colony formation by hematopoietic stem cells, and neural differentiation. IL6 is not constitutively expressed but is highly inducible and is produced in response to a number of inflammatory stimuli such as IL1, platelet-derived growth factor, tumor necrosis factor α (TNFα), bacterial products such as endotoxin, and viral infection. Glucocorticoids produced as part of the inflammatory response to enhance some IL6 effects, such as acute phase protein synthesis, but down-regulate IL6 expression, providing a negative feedback pathway on the inflammatory response in vivo. Many cell types produce IL6 in response to noxious stimuli, including monocytes/macrophages (5), fibroblasts (6), endothelial cells (7), adipocytes (8), T cells (9), and mast cells (10).

IL6 is an essential mediator of the acute phase response. In IL6 knockout mice, the T cell-dependent antibody response is dramatically compromised in response to localized infection and tissue damage, with impairment of the response to certain viral infections; macrophage stimulation was also deficient (11–13). IL6-deficient mice also show 20–40% reduced numbers of thymocytes and peripheral T cells, suggesting the involvement of IL6 in T cell proliferation.

Dysregulated IL6 production is implicated in the pathology of several disease processes. Constitutively high levels of IL6 in transgenic murine B cell lineages results in fatal plasmacytosis (14) and has been implicated in human multiple myeloma (15–17) and Kaposi’s sarcoma (18). Increased IL6 levels are also a feature of diseases such as systemic onset juvenile chronic arthritis (19), rheumatoid arthritis (20), osteoporosis (21, 22), and psoriasis (23). The symptoms of cardiac myxoma (24) and Castleman’s disease (25) are the consequence of systemic overexpression of IL6, which induces polyclonal B cell activation and leads to hypergammaglobulinemia and autoantibody production. IL6 is the key regulator of the acute phase protein, fibrinogen, which is an important risk factor for atherothrombotic disease and is implicated in thrombus and plaque formation. IL6 mRNA is present in atherosclerotic arteries at a 10–40-fold higher level than in non-atherosclerotic vessels (26). These results suggest the involvement of IL6 in the development of human atherosclerosis. Many of the pleiotropic effects of IL6, such as its ability to stimulate differentiation of monocytes to macrophages (27), may be relevant to the growth of the atherosclerotic plaque.

Circulating levels of IL6 are largely regulated at the level of expression, due to the rapid plasma clearance of this cytokine (28). The transcription of this molecule is tightly regulated by the transcription factors NFIL6, NFκB, FosJun, CRBP, and the glucocorticoid receptor. Experiments in HeLa cells showed the region −180 to −123 in the promoter to be crucial for transcription induction with viruses, second messengers, and cytokines such as IL1, TNFα, platelet-derived growth factor, and epidermal growth factor (29, 30). Activation of the IL6 promoter involves synergism between the transcription factor NFIL6 (−158 to −145), and the transcription NFκB (−73 to −64) (31, 32) (Fig. 1). Potent repression of IL6 expression by steroid hormones such as glucocorticoids and estrogen does not appear to involve high affinity binding of the estrogen receptor or the glucocorticoid receptor to IL6 DNA (33–35). Rather, the estrogen receptor and glucocorticoid receptor ligand complexes interact directly with the transcription factors NFIL6 and NFκB, inhibiting DNA binding and thus repressing transcription (36). Thus, IL6 transcription is regulated by co-ordination among factors binding at distinct sites in the promoter.

Polymorphisms in the promoter region of the IL6 gene may result in inter-individual variation in transcription and expres-
sion. Genetic variants could therefore influence an individual’s susceptibility to a diverse range of diseases. There are precedents for this; for example, the TNFα −308TNF2 allele is associated with elevated risk of cerebral malaria (37). It has been shown that a polymorphism in the 5′ flanking region of the IL6 gene at position −174 (G→C) appears to affect IL6 transcription, and the presence of the C allele may be associated with systemic onset juvenile chronic arthritis (19). Understanding the influence of genetic variation on the control of IL6 expression may provide insight into inter-individual variation in disease risk and underlying pathogenesis.

Polymorphisms do not exist in isolation, and it may be the combination of base changes at several sites, i.e. the haplotype, that influences function. We describe here two point substitutions in the 5′ region of the IL6 promoter at position −572 (G→C), and position −597 (G→A) and study the effect of the previously described −373 AT run polymorphism and the −174 (G→C) point substitution. The position of these polymorphic sites relative to transcription factor binding sites is illustrated in Fig. 1. We aimed to identify the common promoter haplotypes present in a population, and assess the functional consequences of these haplotypes on transcriptional control, in response to activating and inhibitory stimuli in different cell types.

**EXPERIMENTAL PROCEDURES**

**Direct Haplotyping—**Double-ended allele-specific PCR primers were used in combination to allow direct haplotype determination in a group of 182 unrelated individuals from a family study on hypertension (38). These allele PCR products were sequenced to haplotype for the A₄Tₕ run in a small group of 39 healthy controls. PCR primers for detection of the single nucleotide polymorphisms are listed in Table I.

**Haplotyping PCR Conditions—**Haplotype-specific PCRs were carried out as described (39, 40) at a final volume of 13 μl in 96-well plates. Control primers were used in each reaction to avoid false negative interpretation. A total of 5 μl of primer mix with 3 ng/μl control primers 63 and 64 and 10 ng/μl allele-specific primers was overlaid with 10 μl of mineral oil. 8 μl of PCR mix was added. The final concentration of reaction components were as follows: 200 μM each dNTP, 2 mM MgCl₂, 67 mM Tris base (pH 8.8), 16.6 mM ammonium sulfate, 0.01% Tween 20, with 40 ng of genomic DNA and 0.2 units of Taq polymerase (Bioline, London, United Kingdom (UK)). The PCR conditions were 95 °C for 1 min; 5 cycles of 95 °C for 35 s, 70 °C for 45 s, and 72 °C for 35 s; 21 cycles of 95 °C for 25 s, 65 °C for 50 s, and 72 °C for 40 s; followed by 4 cycles of 95 °C for 35 s, 55 °C for 60 s and 72 °C for 90 s. The entire PCR reaction with 10 μl of loading dye was run on a 1% agarose gel in 0.5× TBE at 200 V for 20 min. The various combinations of allele-specific primers and the haplotypes they amplify are shown in Table II.

Automated out of the allele-specific PCR products was carried out by the Department of Biochemistry (University of Oxford, Oxford, UK), using the primer 5′-GCTCCGATGGATCGAG-3′.

**Screening for Coding Region Polymorphisms—**PCR and sequencing of the five exons and intron 1 of the IL6 gene in 20 individuals were carried out using the primer pairs shown in Table III. The PCR reagents for the 40-μl reaction volume were: 67 mM Tris base (pH 8.8), 16.6 mM ammonium sulfate, 0.01% Tween 20, 200 μM dNTPs (Advanced Biotechnologies, Epsom, UK), 5% W1 (Life Technologies, Inc., Paisley, London, United Kingdom (UK)). The PCR conditions were 95 °C for 1 min; 5 cycles of 95 °C for 35 s, 70 °C for 45 s, and 72 °C for 35 s; 21 cycles of 95 °C for 25 s, 65 °C for 50 s, and 72 °C for 40 s; followed by 4 cycles of 95 °C for 35 s, 55 °C for 60 s and 72 °C for 90 s. The entire PCR reaction with 10 μl of loading dye was run on a 1% agarose gel in 0.5× TBE at 200 V for 20 min. The various combinations of allele-specific primers and the haplotypes they amplify are shown in Table II.

### Table I

| Primer name | Primer sequence |
|-------------|-----------------|
| IL6promLH597G AS1 | 5′-AAGTATGCGAAGGTTGAGG-3′ |
| IL6promLH597A AS2 | 5′-AAGTATGCGAAGGTTGAGG-3′ |
| IL6promLH572G AS3 | 5′-GCCGAGGGCAGTTCTAACAGGG-3′ |
| IL6promLH572C AS4 | 5′-GCCGAGGGCAGTTCTAACAGGG-3′ |
| IL6promRH572e AS5 | 5′-TGCTGTCGTCCTTCCTGAGACG-3′ |
| IL6promRH572c AS6 | 5′-TGCTGTCGTCCTTCCTGAGACG-3′ |
| IL6promRH174e AS7 | 5′-TGCAATGACACTTCGCATGACG-3′ |
| IL6promRH174g AS8 | 5′-TGCAATGACACTTCGCATGACG-3′ |
| Control63 | 5′-GCCAATTGCGCTTTGAGGAG-3′ |
| Control64 | 5′-GCCAATTGCGCTTTGAGGAG-3′ |

### Table II

| Reaction no./primer mixes | −597 genotype | −572 genotype | −174 genotype | Size of amplicon (bp) |
|--------------------------|---------------|---------------|---------------|---------------------|
| 1 AS1-AS8 | G | C | C | 471 |
| 2 AS1-AS7 | G | G | C | 471 |
| 3 AS2-AS8 | A | C | C | 471 |
| 4 AS2-AS7 | A | G | C | 471 |
| 5 AS3-AS8 | G | C | G | 438 |
| 6 AS3-AS7 | G | G | G | 438 |
| 7 AS4-AS8 | C | C | C | 438 |
| 8 AS4-AS7 | C | G | G | 438 |
| 9 AS1-AS6 | G | C | C | 73 |
| 10 AS1-AS5 | G | G | C | 73 |
| 11 AS2-AS6 | A | C | C | 73 |
| 12 AS2-AS5 | A | G | G | 73 |

### Table III

| IL6 gene primers | Primer sequence | Region amplified | Product length (bp) |
|------------------|-----------------|-----------------|-------------------|
| Exon1LH | 5′-CCCCGACAAATAATAGGCTGCG-3′ | −35 to +139 | 174 |
| RH1 | 5′-TCCTCTACAGGCGGTCGAC-3′ | −373 to +139 | 472 |
| Intron 1 exon2LH | 5′-GAGCGGAATGATGACGCTT-3′ | +1 to +547 | 496 |
| Intron 3 exon2RH | 5′-GCCCTGCAAATCTAGGTGTC-3′ | +2208 to +2496 | 288 |
| Exon4LH | 5′-AGGTGCGGAATCTGGCAG-3′ | +4108 to +4429 | 321 |
| Exon5LH | 5′-CAGAGCAGATCCACTCTGAG-3′ | −641 to +139 +16 bp | 796 |
| Exon5RH | 5′-CATAGTCTGGCGCTGCGGCGA-3′ | −333 to +139 | 796 |
| L1H1 vector | 5′-TTGCCGCGGCTGCGGCTGCGGCGA-3′ | 472 |
containing a MluI site were used to PCR genomic DNA from individuals with different haplotypes of the IL6 promoter. The resultant MluI-XhoI fragments were cloned into the MluI-XhoI sites of the pGL3-basic luciferase vector. Sequencing of all clones for the multiple cloning sites and the entire cloned IL6 promoter region was carried out to ensure that the only variation between clones was at the desired polymorphic sites.

**Cell Lines and Cell Culture**—The human derived endothelial-like cell line HeLa (ATCC, CCL-2) was chosen as much of the previous analysis of the IL6 promoter had been performed in this cell line. The ECV304 cell line was chosen as it was reported to show some properties of endothelial cells; more recently, it has been suggested that it is epithelial in origin. The rationale for choosing an endothelial-like cell line was because such cells are known to produce IL6 in the atherosclerotic plaque, which may be important in the development of the disease. Haplotype-specific control of IL6 expression was compared in these two cell lines of different origin to investigate potential cell type-specific control of gene expression.

Cell lines were cultured in Eagle’s minimal essential medium (Life Technologies, Inc.) for HeLa cells or M199 (Sigma, Poole, UK), for ECV304.7 cells, supplemented with 10% fetal calf serum (PAA, Teddington, UK), 2 mM l-glutamine (PAA), 45 μg/ml penicillin, 45 μg/ml streptomycin, 90 μg/ml kanamycin. All cells were cultured at 37°C in 5% CO₂.

**Transient Transfections and Reporter Gene Assays**—Plasmid DNA was prepared using a Qiagen endotoxin-free Maxi Prep-500 kit. Two separate preparations of each clone were used, and, for two out of seven clones studied, two separately cloned constructs were used. HeLa and ECV304 cells were transfected during the log phase of their growth using a calcium phosphate co-precipitation method. 1 × 10⁶ HeLa cells were seeded into 9-cm Petri dishes, or 1.9 × 10⁶ ECV304 cells were seeded into six-well plates and incubated for 24 h. HeLa cells were transfected using 125 mM CaCl₂, 140 mM NaCl, 25 mM Hepes free acid, 0.74 mM disodium hydrogen phosphate (pH 7.06) with 10% of the 50% transfected using 125 mM CaCl₂, 140 mM NaCl, 25 mM Hepes free acid, seeded into six-well plates and incubated for 24 h. HeLa cells were washed with phosphate-buffered saline, fresh medium applied and incubated for 16 h after the addition of calcium phosphate co-precipitate, the plates were washed with phosphate-buffered saline, fresh medium applied and incubated for 24 h not treated, treated with interleukin-1 (2.5 ng/ml) only, or treated with IL1 and dexamethasone (3.92 μg/ml). Cell lysates of the transfected cells were prepared and assayed for β-galactosidase using the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (Sigma). CAT was assayed using a standard enzyme-linked immunosorbent assay in microtiter plates (Roche Molecular Biochemicals, Lewes, UK). Quenching of the firefly luciferase and concomitant activation of Renilla luciferase was accomplished by adding Stop & Glo® reagent (Promega). Luminescence was measured in a luminometer (Lucy, Anthos, UK). IL1 was titrated from 0.005 to 10 ng/ml, and a stimulation time course was carried out from 1 to 24 h for both HeLa and ECV304 cell lines. The conditions used above gave a maximal response in these experiments. Dexamethasone concentration was based upon published data (33). All experiments were performed at least twice, with each transfection in duplicate, using two separate DNA preparations. CAT expression was normalized against β-galactosidase activity and firefly luciferase was normalized against Renilla luciferase activity to account for variation in transfection efficiency.

**Statistical Analysis**—The t test (allowing for unequal variance) was used to compare experimental ratios between individual clones and between different treatments. Owing to the large number of comparisons, significance was considered to be at the 0.005 level. Results are shown as mean ± 95% confidence interval.

**RESULTS**

**Haplotype Frequency for Point Substitutions**

\((-174G→C, -572G→C, -597G→A)\)

The haplotypes for the three point substitutions in the promoter region of the IL6 gene, for 182 unrelated individuals, are shown in Table IV.

**Complete Haplotype Frequencies**

We assessed the complete haplotypes for all four promoter polymorphisms in 39 healthy controls (Table V). The 8A/12T allele was shown to be associated with the −597A allele and the −174C allele in all but one case. The −597G and −174G alleles showed more diversity in the associated A₁T₁ alleles, with 9A/11T, 10A/10T, 10A/11T, and an allele with a deletion of the G residue adjacent to the upstream end of the A₁T₁ run identified. The rare −572C allele (0.052) was always associated with the −597G, −174G and 10A/10T alleles. The four most common haplotypes were: −597A −572G 8A/12T −174C; −597G −572C 10A/11T −174G; −597G −572G 9A/11T −174G; −597G −572G 10A/10T −174G.

**Screening of Intron 1 and the 5 Exons**

Screening of intron 1 and the 5 exons of the IL6 gene in 20 unrelated individuals revealed no genetic variation, although we consistently observed variations from the published sequence (GenBank® accession no. Y00081): an additional T residue at position −584, two additional G residues at position +117, AAGG insert at +154, C substituted for an A at +158, CG instead of GC at +165 and +166, an additional C residue at +204, T instead of an A at positions +431 and +506, a C inserted at +465, a C deleted from position +471, a TGC insert at position +478 and a +CC insert at position +490.
Effect of IL6 Promoter Haplotype on IL6 Transcription

TABLE IV

Haplotype frequencies for 3-point substitutions of the IL6 promoter in 182 control individuals

| Haplotype | AGC | GGG | GGC | GGC | AGG | Total |
|-----------|-----|-----|-----|-----|-----|-------|
| No.       | 147 | 195 | 19  | 2   | 1   | 364   |
| Haplotype frequency | 0.40 | 0.54 | 0.05 | 0.006 | 0.003 | 1.00 |

TABLE V

Frequency for complete IL6 haplotypes in 39 control individuals

| Haplotypes | AG8/12C | GG10/11G | GG8/11G | GG10/11G | GC10/10G | AG8/12G | GG10/11G | GG8/11C |
|------------|---------|----------|---------|----------|----------|---------|----------|---------|
| No.        | 28      | 19       | 14      | 9        | 5        | 1       | 1        | 1       |
| Frequency  | 0.36    | 0.24     | 0.18    | 0.12     | 0.06     | 0.01    | 0.01     | 0.01    |

Fig. 2. Effect of the −211 to +13 region of the IL6 promoter on CAT expression in HeLa cells (panel A) and in ECV304 cells (panel B) for the two alleles at the −174 position. CAT activity was corrected for transfection efficiency by co-transfection with a CMV β-galactosidase construct. The values are expressed as mean ± standard deviation of the -fold increase compared with the untreated −174G clone. Cells were incubated for 24 h in the absence of IL1, in the presence of IL1, or in the presence of IL1 and dexamethasone.

Functional Studies

−211+13 Constructs (Figs. 2 and 3)

Transfection of the −221+13 construct into HeLa cells resulted in both alleles showing a significant induction in the presence of IL1 (p < 0.0000002); −174G showed a 9.2 (±0.61)-fold induction, and the −174C allele showed a 12.2 (±1.79)-fold induction after 24 h (Fig. 2). There was no significant difference between the two alleles at any of the time points studied (Fig. 3). Both alleles showed a significant reduction in expression when treated with IL1 and dexamethasone compared with IL1 alone (Fig. 2). Dexamethasone did not reduce IL1 induction of the IL6 promoter to untreated levels (−174G allele = 3.47 ± 0.57, −174C allele = 5.03 ± 1.15). In contrast to HeLa cells, ECV304 cells showed no IL1 induction of transcription with these shorter constructs (Fig. 2).

−641+13 Constructs (Fig. 4 and Table VI)

HeLa Cell Line—All haplotypes tested in the −641+13 constructs showed a significant (p < 0.0003) approximate 6-fold increase (5.4 ± 0.64 to 7.0 ± 1.09) in transcription on IL1 stimulation in HeLa cells. Dexamethasone did not reduce expression for the AG8/12G haplotype, whereas all other haplotypes resulted in a significant reduction in expression (p < 0.005). The haplotypes GG9/11C and GG10/10G showed no difference between transcription levels in the untreated state compared with the IL1- and dexamethasone-treated state, whereas haplotypes GC9/11G, AG8/12C and AG8/12G did not show complete damping down of IL1 induction (p < 0.005) at the dexamethasone concentration used.

There was no significant difference among the individual +641+13 clones in the untreated state, when treated with IL1 alone or in the presence of IL1 and dexamethasone, in the HeLa cell line.

ECV304 Cell Line—In the ECV304 cell line, all −641+13 constructs showed a significant stimulation of expression with IL1. Dexamethasone significantly reduced IL1-stimulated expression for all haplotypes. Dexamethasone reduced expression to a level not significantly different from unstimulated expression levels, with the exception of the AG8/12C construct, which showed significantly higher expression in the untreated state compared with the dexamethasone-inhibited state (p < 0.005) but the absolute difference was small (0.52- versus 1.18-fold).

In the ECV304 cells, there were significant differences between certain long clones. In the untreated state, the haplotype AG8/12C showed significantly higher expression than all other clones (p < 0.005), but, again, the absolute difference was small (1.51 versus 1.23 to 0.83). When stimulated with IL1, the GG9/11G clone showed significantly higher expression (5.02 ± 0.60-fold induction) than all other clones (1.9 ± 0.23 to 3.0 ± 0.52) (p < 0.005). The AG8/12G haplotype showed significantly lower IL1-stimulated expression than the clone differing only at the −174 site, AG8/12C (p < 0.0005), as well as the GG9/11G clone (p < 0.0005), the GG10/11G clone (p < 0.0006), and the GG10/10G clone (p < 0.00005), but was not significantly different from the GC10/10G clone.

In the presence of dexamethasone, there were also significant differences between haplotypes, with the GG10/11G haplotype (0.77 ± 0.02) showing significantly lower expression (greater repression) than the GG9/11G (1.27 ± 0.09) (p < 0.0001), GC10/10G (1.01 ± 0.24) (p < 0.0009) and AG8/12C haplotypes being less effective in downregulating expression.
(1.18 ± 0.03) (p < 0.0002) haplotypes, but absolute differences were small.

**DISCUSSION**

Our findings indicate the essential role of upstream elements for IL1 induction in the ECV304 cell line compared with the region required for IL1-induced transcription in the HeLa cell line. Our results suggest that regulation of IL6 transcription is cell type-specific, which may be of physiological relevance when considering different disease processes. The other important finding is the functional effect of IL6 promoter haplotype on expression levels. The study of a single polymorphism in isolation will not reveal the overall functional effect of the polymorphism in combination with other functional polymorphisms.

In the HeLa cell line, both −174 allelic constructs showed strong induction upon IL1 treatment (11-fold) in contrast to published data (19) on a longer fragment (−550 to +61), which showed that the −174C allele in combination with 8A/12T resulted in a complete lack of transcriptional induction by IL1. The difference in these observations could be explained by the presence of a −174C allele-specific repressor between +13 and +61 or between −211 and −550. Our studies with clones containing the fragment −641 to +13 region of the IL6 promoter on luciferase expression in HeLa cells (panel A), and in ECV304 cells (panel B) for seven common haplotypes. Firefly luciferase activity was corrected for transfection efficiency by co-transfection with a Renilla luciferase construct. The values are expressed as mean ± standard deviation of the -fold increase compared with the untreated AG8/12G clone. Cells were incubated for 24 h in the absence of IL1, in the presence of IL1, or in the presence of IL1 and dexamethasone.

Our results show that the relationship between IL6 expression and the −174 polymorphism is not as simple as might be expected; the A<sub>n</sub>T<sub>n</sub> allele has an influence on expression when comparing the GG9/11G clone and the GG10/11G clone, the GG9/11G haplotype resulting in significantly higher expression than the GG10/11G haplotype, but the increased expression from this one clone is not the consequence of the 9A/11T allele alone as similarly high expression is not seen from the GG9/11G haplotype in isolation.
11C allele. The higher expression is not the result of the −174G allele, as the other −174G haplotypes do not produce the same effect, and the same follows for the −597G allele and the −572G allele. The difference is the result of the combination of alleles for the different polymorphisms, i.e. the haplotype. This same conclusion is drawn for the low expressing haplotype AG8/12G; the low expression is not the result of the −597A allele or the 8A/12T allele, as the other clone containing these two alleles (AG8/12C) shows significantly higher expression. The −174G allele is not the cause alone either, as the same lower transcription is not observed for the other −174G-containing clones. The conclusion is that the different polymorphisms have an influence on transcription but the polymorphisms are not functioning individually. The effect of one polymorphism synergizes with the effect of another, so the difference caused by one variant is not easy to determine. One explanation is that the differences are the result of specific combinations of the A,T run and the −174 polymorphism, but a complete explanation awaits analysis of potential transcription factor binding and/or interactions.

The variation observed in the ECV304 cell line between the clones in the stimulated state may be important physiologically. Owing to the short half-life of IL6 in vivo, the consequence of the strikingly increased induction with IL1 of the GG9/11G haplotype would be increased levels of IL6 locally, and probably not a prolonged acute phase response under normal regulator conditions because the haplotype responds to the down-regulation of expression (e.g. by dexamethasone) in the same way as the other haplotypes. The AG8/12G haplotype resulting in significantly lower levels of expression with IL1 stimulation could also be of physiological importance.

It is also important to consider the reasons for the differential expression of short and long constructs in the ECV304 cells compared with HeLa cells. It may be that transcription from the IL6 promoter reaches a maximum level in HeLa cells, with transcription factors becoming limiting and masking any potential variation between clones. Expression levels in ECV304 are lower for both long and short constructs. From the study of a positive control, it was clear that production of the reporter gene itself was not the limiting factor. Second, the cell type-specific differences may be the results of differential expression of essential transcription factors in the two cell lines or underlying differences in transcriptional control.

When considering these results in the context of potential population-based studies, the overall effect on level if only the −174G→C polymorphism is considered would be that the C allele would show lower expression than the G allele, because the −174G-containing haplotypes with lowest expression levels only account for approximately 5% of the population. This would explain the findings of Fishman et al. (19) that the C allele was associated with significantly lower levels of plasma IL6 levels in a population of healthy subjects, but if future studies show this low expressing AG8/12G haplotype or, indeed, the high expressing GG9/11G haplotype to affect disease susceptibility, genotyping of the −174G→C polymorphism alone would be inadequate.

The overall conclusion of this study is that differences in IL6 promoter haplotype may have an important role in determining levels of transcription for the IL6 gene. The transcriptional control of this gene is complex, and subtle variations in the promoter influence regulation of this system. The observation that base changes at adjacent polymorphic sites are not independent suggests that there may be interaction between the transcriptional machinery at the separate sites. The function of one variation is determined by the effect of other alleles at distinct polymorphic sites, and the effect of altering the DNA sequence in two separate regions does not have the simple combined effect of the alterations individually. A run of A and T nucleotides will result in variation in helical structure with consequent bending in the DNA; it is possible that variation in such bending as the result of different sequences of A,T run could influence the binding of transcription factors to the region and/or the interaction of transcription factors that may bind at sites flanking the putative region of curvature. The difference in transcription factor binding as the result of this structural change would then be compounded by the effects of other variations. This study underlines the importance of studying the transcriptional control region as a whole when considering the functional effects of natural variants, and shows the importance of understanding the function of genetic variants prior to embarking on population-based association studies.

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**Table VI**

The eight haplotypes studied in transient transfection assays in HeLa and ECV304 cells

| IL6 promoter haplotype | Fold increase in corrected luminescence relative to AG8/12G construct |
|------------------------|-------------------------------------------------------------------|
|                        | HELA                  | ECV304                                           |
|                        | Untreated  | IL1 treated | Untreated | IL6 and dex | Untreated | IL6 and dex |
| A                      | G, G       | 9A 11T     | C         | G, G        | 9A 11T     | C          |
| A                      | 1.36 ± 0.15 | 6.52 ± 0.75 | 2.16 ± 1.65 | 1.12 ± 0.07 | 6.68 ± 0.35 | 0.81 ± 0.24 |
| B                      | 1.19 ± 0.11 | 7.04 ± 1.09 | 3.40 ± 0.40 | 1.23 ± 0.08 | 5.02 ± 0.65 | 1.27 ± 0.09 |
| C                      | 0.85 ± 0.20 | 5.93 ± 0.73 | 3.90 ± 0.83 | 1.03 ± 0.09 | 3.65 ± 0.17 | 1.05 ± 0.18 |
| D                      | 1.60 ± 0.19 | 6.37 ± 1.57 | 3.60 ± 0.93 | 0.89 ± 0.15 | 3.01 ± 0.38 | 0.77 ± 0.08 |
| E                      | 1.09 ± 0.11 | 6.37 ± 1.36 | 3.60 ± 0.57 | 1.4 ± 0.17  | 2.70 ± 0.52 | 1.01 ± 0.03 |
| F                      | 1.04 ± 0.11 | 6.52 ± 1.19 | 3.50 ± 0.50 | 1.01 ± 0.07 | 2.72 ± 0.37 | 1.15 ± 0.02 |
| A                      | 1.00 ± 0.10 | 5.41 ± 0.44 | 4.09 ± 1.10 | 1.00 ± 0.10 | 1.93 ± 0.23 | 1.00 ± 0.13 |

†, F clone significantly different, p < 0.005, from all other haplotypes in ECV304 untreated cells; †, B clone significantly different, p < 0.005, from all other haplotypes in ECV304 IL 1-treated cells; †, G clone significantly different, p < 0.0005, from haplotypes B, C, D and F in ECV304 IL1-treated cells; †, D clone significantly different, p < 0.0009, from haplotypes B, E, and F in ECV304 IL 1- and dexamethasone-treated cells.
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