Identification of phylogenetic footprints in primate tumor necrosis factor-α promoters

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The human tumor necrosis factor-α (TNF-α) gene encodes a pleiotropic cytokine that plays a critical role in basic immunologic processes. To investigate the TNF-α regulatory region in the primate lineage, we isolated TNF-α promoters from representative great apes, Old World monkeys, and New World monkeys. We demonstrate that there is a nonuniform distribution of fixed human differences in the TNF-α promoter. We define a ''fixed human difference'' as a site that is not polymorphic in humans, but which differs in at least one of the seven primate sequences examined. Furthermore, we identify two human TNF-α promoter single nucleotide polymorphisms that are putative ancestral polymorphisms, because each of the human polymorphic nucleotides was found at the identical site in at least one of the other primate sequences. Strikingly, the largest conserved region among the primate species, a 69-nt ''phylogenetic footprint,'' corresponds to a region of the human TNF-α promoter that forms the transcriptionally active nucleoprotein–DNA complex, essential for gene regulation. By contrast, other regions of the TNF-α promoter, which exhibit a high density of variable sites, are nonessential for gene expression, indicating that distinct TNF-α promoter regions have been subjected to different evolutionary constraints depending on their function. TNF-α is the first case in which a promoter region dissected by functional analyses can be correlated with nucleotide polymorphism and variability in primate lineages. The results suggest that patterns of polymorphism and divergence are likely to be useful in identifying candidate regions important for gene regulation in other immune-response genes.

Of the genes involved in innate immunity, the cytokine tumor necrosis factor-α (TNF-α) plays a particularly important role in the host response to infectious diseases (1). Stimulation of multiple cell types, primarily cells of the immune system, either through their immunoreceptors or with a variety of infectious agents such as viruses, bacteria, and parasites, results in activation of TNF-α gene expression (1). The biologic actions of TNF-α are initiated by its binding to two cognate receptors, which initiates signaling cascades that result in the activation of multiple transcription factors and the induction of multiple TNF-α-dependent genes involved in the host immune response (2).

The expression of the TNF-α gene itself is tightly regulated at the level of transcription. On cellular stimulation, TNF-α is transcribed in a cell type-specific and stimulus-specific manner (3–7). Functional studies have established that the minimal region of the TNF-α promoter required for induction of the gene by a variety of stimuli in multiple cell types is localized within 200 bp upstream from the start site of transcription (4–14). Recently, we have characterized the activator binding sites in the TNF-α promoter, as well as their spatial arrangement; these sites facilitate a unique pattern of protein-protein and protein-DNA interactions that is required for TNF-α gene regulation. This analysis demonstrated that different activators are recruited to the same set of activator binding sites in the proximal TNF-α promoter, depending on the cell type and the stimulus, resulting in the assembly of distinct nucleoprotein complexes or enhancer complexes (5–7).

The human TNF-α gene is located in the HLA region of human chromosome 6, the most polymorphic region of the human genome (15). Previous studies have identified human single nucleotide polymorphisms (SNPs) located at nucleotides -862, -856, -375, -307, -243, and -237 as well as a single-nucleotide addition at nucleotide $+70$ relative to the TNF-α transcription start site (16–21). None of the SNPs identified to date appear to have an effect on the transcriptional regulation of the TNF-α gene (21–24). However, several of the human TNF-α SNPs (-862, -856, -307, -237, and -243) are in linkage disequilibrium with HLA genes and/or with extended HLA haplotypes (17, 19–21). Therefore, it has been speculated that TNF-α SNPs may serve as markers for linked HLA genes and/or other genes that encode proteins that may influence disease pathogenesis (25).

To determine whether the activator binding sites critical for the regulation of the human TNF-α gene were conserved in the primate lineage, and to investigate whether TNF-α SNPs were ancestral polymorphisms, we isolated TNF-α promoters from representative great apes, Old World monkeys, and New World monkeys, and compared these with the consensus human TNF-α promoter sequence (21). We found 162 nucleotide sites that are not polymorphic in the human sample but that differ in at least one of the primate sequences. We call these ''fixed human differences'' because they are fixed in humans but differ in at least one primate sequence. The fixed human differences are nonuniformly distributed along the promoter. Because our primary objective was to analyze the spatial distribution of fixed human differences in the TNF-α promoter, we made no attempt to determine whether any of the fixed human differences are polymorphic in any of the primate species.

By using a 5-nt sliding window, which was moved along the human TNF-α promoter sequence, the total number of pairwise differences at each nucleotide position was quantified. We identify a striking phylogenetic footprint that contains sequences critical for the regulation of the TNF-α gene and for enhancer complex formation. By contrast, other regions of the TNF-α promoter that are nonessential for gene expression exhibit a high density of variable sites between the primate sequences and are adjacent to, or coincident with, areas of the human TNF-α promoter that contain SNPs. Thus, different regions of the TNF-α promoter have evolved under heterogeneous constraints,

Abbreviations: TNF-α, tumor necrosis factor-α; SNP, single nucleotide polymorphism; NFAT, nuclear factor of activated T cells.

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and sequences required for the binding of activators to the promoter have been conserved.

The importance of TNF-α gene expression in the immune response to infectious disease has made the TNF-α promoter a likely target for natural selection. Here we present the first example of a correlation between variable sites in primate TNF-α promoter sequences, human TNF-α promoter variation, and sequences involved in the regulation of the gene. Our analysis thus provides insight into the origin and evolutionary history of both TNF-α gene regulation and the human genome.

Materials and Methods

Amplification and Sequencing of Genomic DNA. Genomic DNA was isolated from the following cell lines obtained from the American Type Culture Collection: CRL-1847 TANK (*Pan troglodytes*), CRL-1854 ROK (*Gorilla gorilla*), CRL-1850 PUTI (*Pongo pygmaeus*), TIB-201 MLA-144 (*Hylobates lar*), CRL-1495 26CB-1 (*Papio hamadryas*), CRL-1805 LCL 8664 (*Macaca mulatta*), and CRL-1556 OMK (*Aotus trivirgatus*) (26). One individual from each representative nonhuman primate species was studied; hence, there were a total of seven primate sequences to compare with a consensus human TNF-α promoter sequence based on 207 human TNF-α promoter sequences from ethnically diverse groups (21) (J.Y.L. and A.E.G., unpublished data).

TNF-α promoters were amplified by PCR as described previously (21), by using the following primers: -978 (5′-TATGGCCACATGTAGCGGCT-3′) and $+93$ (5′-CGTCTGAGGGTTGTTTTCAG-3′). To amplify the *Macaca* and *Aotus* TNF-α promoters, additional PCRs were performed by using the following primers: *Macaca* -892 (5′-GGGCTATGGGGGTTGAGTAT-3′), *Macaca* -3 (5′-GTGTGCAAACAACTGCGTTTATA-3′), *Aotus* -918 (5′-CATGGCAAAGGGTAGGAGAA-3′), and *Aotus* $+14$ (5′-AGCTTCTGCTGGCTGAGTGT-3′). Each primate TNF-α promoter was amplified in at least two independent PCRs. DNA was then purified and sequenced as described previously (21).

Sequence Analysis. By using the multiple-sequence-alignment program CLUSTAL W (27), nonhuman TNF-α sequences were aligned with the consensus human TNF-α promoter sequence.

Distribution of Fixed Human Differences and Sliding Window Analysis. The Broken Stick Model (28) was applied to the TNF-α promoter sequence to test the statistical significance of the clusters of fixed human differences and the other regions of complete conservation. The Broken Stick Model describes a stick (DNA sequence), along which n number of events (fixed human differences) are scattered, breaking the stick into $n + 1$ intervals, which are then ordered by increasing length. Departure from uniform density of events and the resulting expected distribution of interval lengths would make the longest intervals longer than expected and the shortest intervals shorter.

For our data, the variance of interval lengths and the longest interval were calculated. We subsequently ranked the intervals by increasing length and grouped them into successive sets of three. The sum of the interval lengths in each of these sets defines the length of each third-order interval. Third-order intervals were chosen for analysis because Goss and Lewontin have shown that this test detects clusters of fixed differences with high power (29). A computer program, generously provided by Peter Goss, uses a Monte Carlo simulation to produce critical values under the null hypothesis of uniform distribution of events (29), which we used to analyze our data.

A sliding 5-nt window was moved along the full-length human TNF-α promoter consensus sequence to determine regions with a high density of pairwise differences between any two species. The total number of pairwise differences within each window was assigned to the third position of each window, and these values were graphed as a function of nucleotide position.

Results

Characterization of TNF-α Promoters from the Primate Lineage. We used PCR to amplify TNF-α promoter fragments from a single representative individual from seven nonhuman primate species spanning nucleotides -958 to $+70$ relative to the human TNF-α transcription start site. The species analyzed represent independent genera from the four great apes, two Old World monkeys, and one New World monkey. The TNF-α nonhuman primate promoter sequences were then aligned to the consensus human TNF-α promoter sequence. As shown in Fig. 1, the region of the human TNF-α promoter spanning nucleotides -958 to $+70$ is 84% identical among the representative great apes, Old and New World monkeys, and the human sequence; it is 91% identical when the most divergent species, *Aotus*, is excluded. Furthermore, we found 162 nucleotide sites, called fixed human differences, where at least one of the nonhuman primate sequences differed from the human sequence (Fig. 1).

Interestingly, two previously identified human TNF-α promoter SNPs occurring at nucleotides -375 and -307 (16, 20) are present in nonhuman primate species (Fig. 1). The variant "A" nucleotide at position -375 occurs at the same position in the Old World (*Papio*, *Macaca*) and New World (*Aotus*) representatives. Furthermore, the "A" variant at position -307 in humans also occurs in *Papio*, consistent with a previous study (14), and occurs in *Macaca* (Fig. 1). Thus, the -375 and -307 human TNF-α SNPs are likely to be ancestral in the primate lineage. Moreover, there are 53 additional sites where two or more nonhuman primates share a common variant nucleotide relative to the human sequence but do not coincide with a known human SNP (Fig. 1). Thus, the monomorphic human nucleotides at these sites may represent ancestral nonhuman primate polymorphisms, but ones that became fixed in the human lineage.

Distribution of Fixed Human Differences Relative to Nonhuman Primate TNF-α Promoters Is Nonuniform. Inspection of the sequence alignment of the eight species revealed regions of complete conservation between all of the species and regions with clusters of fixed human differences. Because a single individual of each nonhuman primate species was analyzed, we were unable to determine whether a variable nucleotide at a specific site in a nonhuman TNF-α promoter represented a fixed difference in a particular species. However, we note that of the 162 fixed human differences, all but one also represented sites that differed between the nonhuman primate sequences themselves.

To test the statistical significance of the clusters of fixed human differences, we applied the Broken Stick Model (28) to our data; this model divides the TNF-α promoter sequence into consecutive intervals defined by each fixed human difference. As shown in Table 1, the variance of first- and third-order interval lengths is greater than the critical values generated by Monte Carlo simulation using the specified sequence length of 1,028 nt and the 162 fixed human differences in the sequence (29). Thus, the proportions of small and large interval lengths are higher than would be expected, and these tests reject the null hypothesis of uniform distribution of fixed human differences ($P < 0.001$, Table 1). Furthermore, the longest interval, which corresponds to the conserved 69-nt region spanning nucleotides -131 to -63, is significantly longer than would be expected if fixed human differences were uniformly distributed ($P < 0.001$, Table 1). Thus, there is a nonuniform distribution of fixed human differences and lengths of conserved regions in the primate TNF-α promoters analyzed.

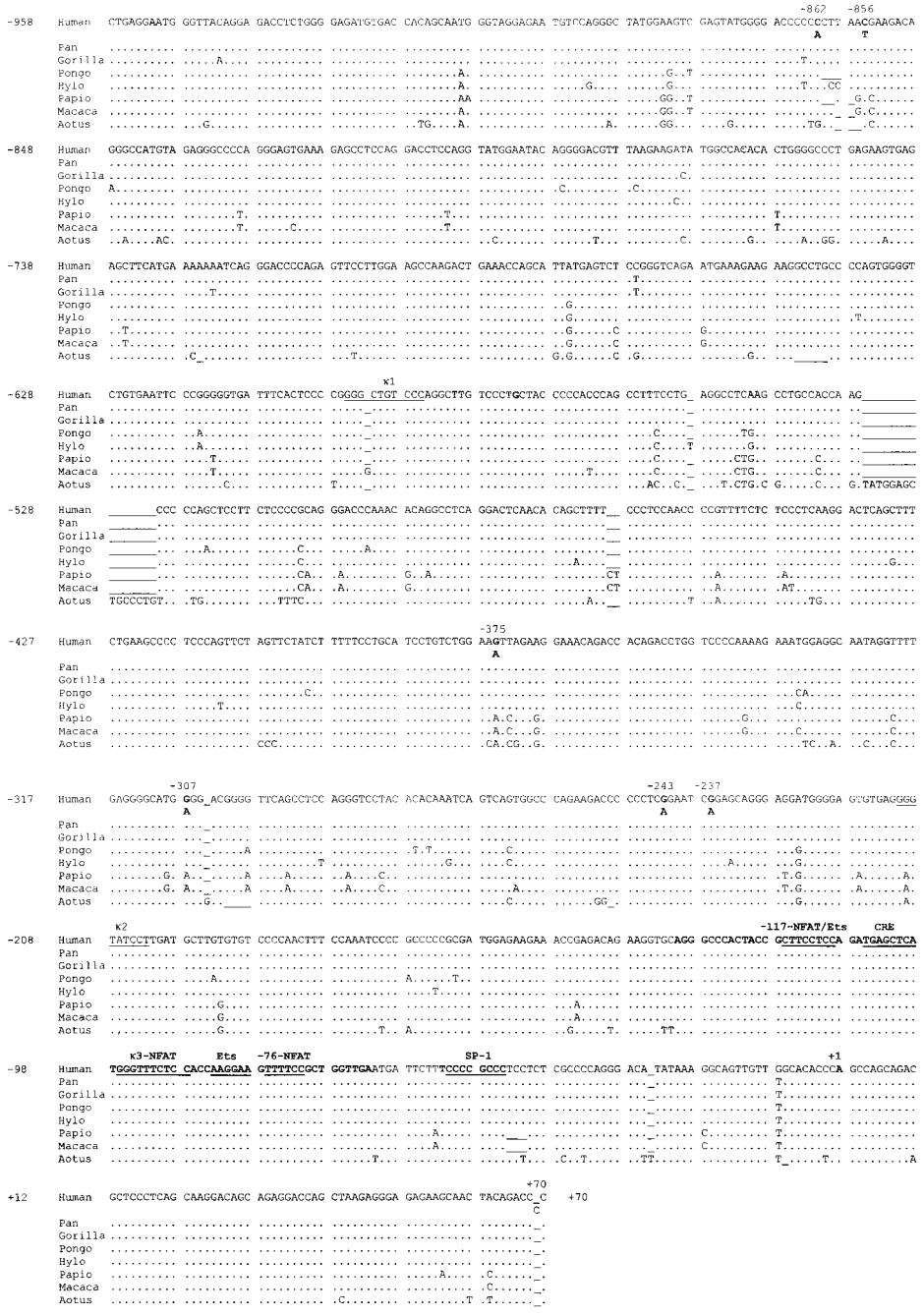

Fig. 1. Isolation and characterization of primate TNF-α promoters. Alignment of the consensus human TNF-α gene promoter and TNF-α promoters from seven nonhuman primate species: *Pan* (chimpanzee), *Gorilla*, *Pongo* (orangutan), *Hylo* (gibbon), *Papio* (baboon), *Macaca* (macaque), and *Aotus* (owl monkey), from -958 to $+70$ relative to the human TNF-α transcription start site. The largest continuous conserved region among all eight species is in boldface. Nucleotide positions marked by ''_'' represent insertion/deletion differences. We observed heterozygosity in the *Macaca* sequence at -445 (C/T) and at -334 (C/T), and in the *Papio* sequence at -294 (A/C). Sequences in GenBank report some differences from our results. A *Papio* sequence derived from the peripheral blood of a *P. hamadryas ursinus* individual contains a C at -294 and a T and -161 (GenBank accession no. AF027198) (14). A sequence in GenBank for *Pongo pygmaeus* assigns a G at -264 (accession no. U42764), a sequence for *Pan troglodytes* assigns a G at -9 (accession no. U42626), and a sequence for *G. gorilla* assigns a G at -9 (accession number U42763). Previously identified human SNPs are indicated (-862, -856, -375, -307, -243, -237, and $+70$). We note that other human SNPs have been reported (-574, -418, -162, and -48), but have been observed only in one individual (16, 21, 36). DNA-binding domains for the transcription factors nuclear factor of activated T cells (NFAT) (117-NFAT/Ets, κ3-NFAT, and -76-NFAT), ATF-2/Jun (cyclic AMP response element) Ets (-117-NFAT/Ets, -84-Ets), and Sp1 (SP-1), which are critical for TNF-α gene regulation (4–7, 12), are noted. Two NF-κB sites (κ1 and κ2) that bind NF-κB proteins in *in vitro* assays (9) that occur in upstream regions not essential for gene regulation are also noted.

Identification of TNF-α Promoter Regions of High Density of Variable Sites and Regions of Conservation. To characterize the spatial pattern of these regions, we subsequently calculated the number of pairwise differences in the eight species within a 5-nt sliding window spanning the TNF-α promoter to determine the density profile of variable sites. As shown in Fig. 2, a plot of these pairwise differences

Table 1. Nonuniform distribution of fixed human differences in the TNF-α promoter among primate species

Test statistic	Observed value	Critical values		P
		5%	1%	
Variance	0.0004881	0.0000478	0.000054	<0.001
Third-order variance	0.0004881	0.000455	0.000474	<0.001
Longest interval	0.06803	0.0574	0.0662	<0.001

Three statistical tests to assess variance of intervals, variance of third-order intervals, and the longest interval between events (fixed human differences) were performed (29). Critical values generated by using a Monte Carlo simulation that correspond to 5% and 1% statistical significance are indicated. For all three tests, the null hypothesis of uniform distribution of fixed human differences was rejected with a P value of less than 0.001.

reveals TNF-α promoter regions with high densities of variable sites. Notably, the region with the highest number of pairwise differences is coincident with the previously identified human TNF-α SNPs at -862 and -856 (19, 21) (Fig. 2). Moreover, the -375 (16) and -307 (20) human TNF-α SNPs are also located within regions of high density of variable sites (Fig. 2).

Strikingly, the 69-nt region between -131 and -63 nt is completely conserved among the eight species (Fig. 2). This region, together with an adjacent conserved region between -53 and -45, contains regulatory elements known to form the TNF-α enhancer complexes (4–8, 12) (Fig. 2). Thus, the largest conserved TNF-α promoter region among the eight species corresponds to the known location of critical functional nucleoprotein binding sites in the human TNF-α promoter.

Discussion

A critical question in the genetic origin and history of humans is what types of nuclear DNA sequence variation have accumulated and the evolutionary forces at work in specific cases. Here we present evidence for a mechanism by which the human TNF-α regulatory region has evolved. TNF-α promoter sequences containing functional activator binding sites have been conserved, whereas regions that are not essential for regulation display a high density of variable sites in the primate lineage. These phylogenetic footprints and nonconserved regions are consistent with TNF-α promoter cold and hot spots with respect to the accumulation of spontaneous mutations.

The TNF-α gene encodes a protein that plays a critical role in lymphocyte biology and in the immune response of the host to a variety of pathogens (1). TNF-α-mediated signaling pathways have also been exploited by a variety of infectious agents that are responsive to TNF-α. For example, when cells latently infected with HIV-1 are stimulated with TNF-α, the transcription factor NF-κB is recruited to the HIV-1 long terminal repeat and HIV-1 replication is induced (30, 31). Thus, patterns of TNF-α gene expression influence both host and pathogen and have likely been targets of natural selection.

Transcriptional activation of eukaryotic genes in response to extracellular signals usually requires the presence of complex regulatory DNA elements called enhancers in their 5′ noncoding regions. Most gene enhancers contain multiple activator binding sites or short cis-regulatory motifs that are recognized by transcription factors. The recruitment of these DNA-binding proteins results in the assembly of the transcriptional machinery and in RNA synthesis (32, 33). Here we have shown that TNF-α promoter sequences containing activator binding sites that are critical in the cell type-specific and stimulus-specific regulation of TNF-α gene expression (5–7) form a phylogenetic footprint in the TNF-α promoter in the primate lineage.

The TNF-α gene lies in the class III HLA region of human chromosome 6, where it is located between the HLA-DR and

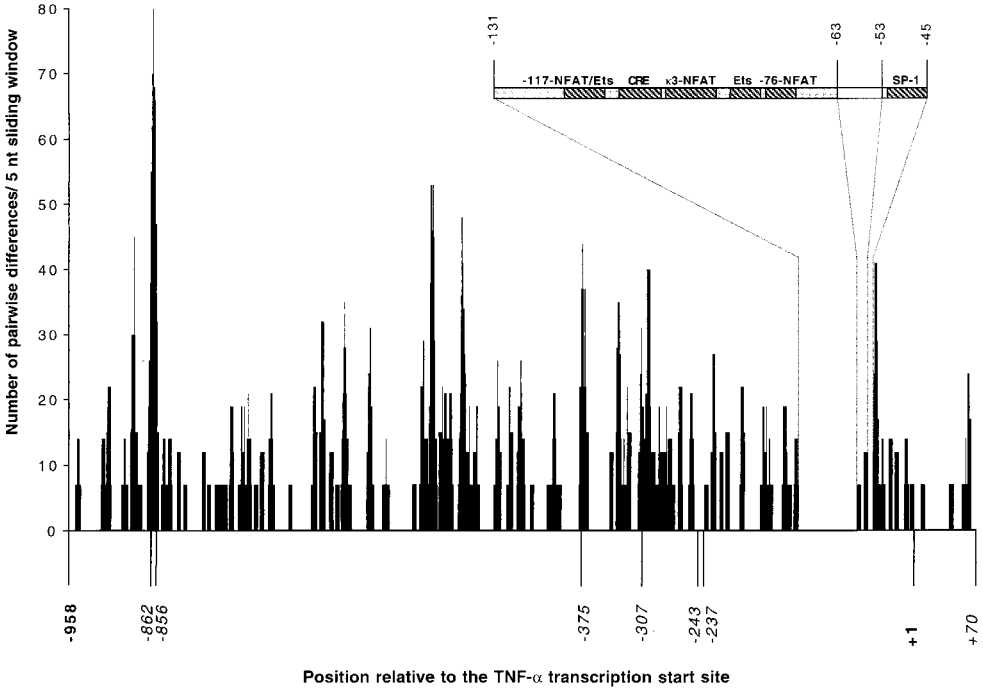

Fig. 2. Identification of regions of high density of variable sites and regions of conservation in primate TNF-α promoters. A density profile of pairwise differences in the TNF-α promoter among eight primate species is displayed. Density is expressed as the number of pairwise differences among the species per 5-nt window of the human promoter and is assigned to the third position of each window. One pairwise difference is defined as a single nucleotide substitution or a single or multiple nucleotide addition or deletion. Positions are relative to the human TNF-α promoter transcription site (+1), and human SNPs are indicated in italics. A diagram of the largest continuous conserved (nucleotides -131 to -63) region and the adjacent conserved (nucleotides -53 to -45) region is also shown. Evolutionarily conserved regions are shaded, and DNA-binding domains for the transcription factors NFAT (117-NFAT/Ets, κ3-NFAT, and -76-NFAT), ATF-2/Jun (cyclic AMP response element) Ets (-117-NFAT/Ets, -84-Ets), and Sp1 (SP-1), which are critical for TNF-α gene regulation (4–8, 12), are hatched.

EVOLUTION

HLA-B genes (1). Interestingly, previously identified human TNF-α SNPs, which are in linkage disequilibrium with nearby HLA genes, are either within or adjacent to areas of the promoter that have a high density of variable sites in the primate lineage. For example, the -307 TNF-α SNP, which is associated with the extended HLA haplotype defined by HLA-DR3 and HLA-B8 alleles in Caucasians (20) and with HLA-DR3 in Asians (34), occurs in a hot-spot region of promoter variability. It is thus interesting to speculate that polymorphisms in the TNF-α promoter, which may be maintained because of linkage disequilibrium with flanking HLA genes, or other yet to be identified genes, are tolerated because they have little effect on the transcription of the gene.

Interestingly, the chimpanzee (*Pan*) TNF-α promoter differs from the human promoter at only two positions, nucleotides -667 and -9. Thus, the chimpanzee TNF-α promoter is no more divergent from the consensus human TNF-α promoter than is a typical human sequence, because two human TNF-α SNPs have been reported to occur on the same chromosome (21, 22). We note that the -9 difference is also found in the gorilla. Neither the -667 nor the -9 polymorphisms have been identified in humans by large-scale sequencing of full-length TNF-α promoters from ethnically distinct human populations from Asia, Africa, or North America (ref. 21; J.V.L. and A.E.G., unpublished observations). Given our finding that the -307 and -375 human SNPs are probably ancestral polymorphisms, we anticipate that the -667 or -9 changes as well as other differences found in nonhuman primate promoters may eventually be identified as SNPs in humans.

Previous studies have suggested that a phylogenetic approach to promoter analysis might reveal promoter regions that are potentially critical for gene regulation (34, 35). By using this approach, phylogenetic footprints, or regions of complete conservation between species in the regulatory regions of candidate genes, have been identified (35, 36). The study presented here provides an example of a correlation between a primate phylogenetic promoter footprint and a well-characterized human promoter region shown to be functionally critical in enhancer formation and in gene regulation.

Although increasing amounts of information are available on the evolution of protein-coding sequences, the mechanisms involved in the evolution of regulatory sequences of eukaryotic genes remain poorly defined. Here we have shown that the accumulation of mutations in distinct TNF-α promoter regions has been nonrandom since the time of the common ancestor of the primate species examined and has been driven by the conservation of TNF-α activator binding sites. We have also identified nonconserved regions with a high density of variable sites, which coincide with human TNF-α promoter regions that are not essential for gene expression and that contain SNPs. It is thus intriguing to speculate that certain promoter regions may experience stronger constraints on length and location than on specific nucleotide sequence, as if they functioned primarily as "spacers." We anticipate that studies similar to ours, which correlate nucleotide sequence variation in primates with human promoter function and polymorphism, will prove useful in the characterization of the regulation of other genes.

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