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Mutational Analysis of TNF-α Gene Reveals a Regulatory Role for the 3'-Untranslated Region in the Genetic Predisposition to Lupus-Like Autoimmune Disease

Chaim O. Jacob, Sun Kyung Lee,* and Gideon Strassmann

Lupus-prone mice show reduced production of TNF-α and, upon long-term treatment with recombinant TNF-α, significant protection from disease development. Mutational analysis of the 5'-untranslated region (UTR) and 3'-UTR of the mouse TNF-α gene reveals a marked degree of polymorphism. Transient expression experiments in the RAW 264.7 macrophage-like cell line using the luciferase reporter system suggest an important role for the mutations in the 3'-UTR in the biosynthesis of TNF-α, and provide a molecular explanation for the reduced TNF-α production in lupus-prone NZW mice. The Journal of Immunology, 1996, 156: 3043-3050.

In the search for mechanisms responsible for immune dysregulation that may cause tolerance breakdown in autoimmune diseases, cytokines have received much increased attention. One particular cytokine, TNF-α, plays under appropriate conditions an important protective role in systemic lupus erythematosus (SLE) (1–3). TNF genes, which include TNF-α, LT-α (TNF-β), and LT-β, are unique among all cytokines and growth factors because they are encoded by genes located within the central portion of the MHC (4–6). The location of TNF, as well as its potential biologic activities are related to immunoregulation and inflammation, suggesting the possibility that an "abnormal" TNF gene could be involved in autoimmune diseases. In experimental tests, we have shown (1, 3) that activated peritoneal macrophages from NZW mice produce 5- to 10-fold lower levels of TNF-α than macrophages derived from non-autoimmune strains. Furthermore, this low TNF-α production is associated with a unique polymorphism in a simple tandem repeat sequence identified within the promoter region of the TNF-α gene (3, 7). More importantly, the treatment of NZB × NZW mice with rTNF-α causes a significant reduction in the incidence and severity of nephritis (1, 3). Although one would predict that TNF-α would cause deleterious effects in autoimmune disease based on the in vitro activities of TNF, additional investigations (2) repeating these experiments independently have established the protective effect of TNF-α in the lupus nephritis.

A basic question arises as to whether the results obtained in mouse models are relevant to autoimmune disease in humans. As in the mouse, the human TNF-α gene is located within the HLA (5), and TNF-α production in humans is variable. Differences of up to 20-fold in the levels of TNF-α release are found in normal healthy individuals (8). In addition, the production of TNF-α in humans is associated with the MHC class II genotype; individuals possessing the DR2/DQw1 have low levels of TNF-α production, while DR3- and DR4-positive subjects have significantly higher levels (8–10). We (8) and others (11) also have shown that the subset of SLE patients who carry HLA-DR2/DQw1 alleles have low TNF-α production levels, and a 3- to 5-fold higher incidence of nephritis. On the other hand, DR3-positive SLE patients have normal TNF-α production and no increased risk of nephritis. The DR4 haplotype in SLE patients is associated with high-level TNF-α production, and has a negative correlation with lupus (8, 12).

A fundamental question involves the molecular mechanism that leads to low TNF-α production in the development of lupus-like autoimmune disease. We show here that the mutations in the 3'-UTR of the TNF-α gene play an important role in the down-regulation of TNF-α biosynthesis in autoimmune-prone NZW mouse.

Materials and Methods

Mice

Most of the mouse strains used were purchased from The Jackson Laboratory (Bar Harbor, ME). The Mus spretus mice were a gift from Dr. V. Chapman, Roswell Park Cancer Institute (Buffalo, NY). The NOD, NON, B10.NZW, and B10.KPA44 were from Dr. H. McDevitt's colony at Stanford University School of Medicine, Stanford, CA.

Macrophage cultures and bioassays

Thio-glycerolate broth-elicited peritoneal macrophage cultures were prepared as reported (3). Peritoneal lavage was performed using 10 ml cold HBSS containing 10 U/ml heparin. Exudate cells obtained by lavage were washed and resuspended in complete medium (RPMI 1640 containing 10% heat-inactivated FCS, penicillin, and streptomycin), then plated in 12-well tissue-culture plates at a cell density of 1 × 10⁶/well. After an incubation for 90 min, the cells were washed three times with complete medium. The cell monolayers were routinely found to contain approximately 95% mononuclear phagocytes. The cultures were activated using the indicated stimulator for 18 h. Conditioned medium was removed and cellfree supernatants were stored at −20°C until analysis. The monolayers were washed three times with PBS, solubilized, and cellular protein was quantified by the biocinchonic acid method (Pierce Chemical, Rockford, IL). Variation between the samples tested did not exceed 10%. The presence of TNF-α in serum dilutions...
of the conditioned medium was quantified by the standard L929 based bioassay (3). The addition of neutralizing rat anti-mouse TNF-α (20 μg/ml), but not of isotype control Ab (IgG1), reduced the cytotoxicity of the diluted supernatants by about 96%. The presence of total IL-1 activity was quantified by the standard co-mitogenic stimulation of CHI/HeJ thymocytes in the presence of Con A (13).

Cloning and sequencing the mouse TNF-α upstream and 3'-UTR regions

For the promoter and 5'-UTR amplification, two sets of oligonucleotide primers were synthesized with HindIII restriction site placed at the 5' end for cloning purposes. The primer sequences were: M1: 5'-CTCAAGCTTATCCCATGCCCCAGGGCAAA; M2: 5'-GAGGAATGGGTGTTCATCCATCCATGG. Amplification of various mouse genomic DNA were performed using M1 and M2 or alternatively M3 and M4 primers and 30 cycles of amplification: M1: 5'-CTCAAGCTTAGTGAAAAGGC; M2: 5'-CTCAAGCTTAGTGAAAAGGC; M3: 5'-CTCAAGCTTAGTGAAAAGGC; M4: 5'-CTCAAGCTTAGTGAAAAGGC. An amplification of various mouse genomic DNA were performed using M1 and M2 or alternatively M3 and M4 primers and 30 cycles of amplification: 94°C (0.5 min), 55°C (0.5 min), and 70°C (1.5 min). The amplified DNA was purified on 1% agarose gel, cut with HindIII and cloned into pBlueScript II SK+ (Stratagene, San Diego, CA). For TNF-α 3'-UTR amplification and sequencing we tested the primer pairs flanking the AU-rich region: 5'-AGGCTCTCTTCTGAGCCGGGAGG; and 5'-GCCAGACGTTCTGAGAGGG. Amplification of mouse genomic DNA was performed using primer and mouse mammary leukemia virus reverse transcriptase.

Oligonucleotide synthesis

The oligonucleotides were synthesized on an Applied Biosystem 391 DNA synthesizer (Applied Biosystems, Foster City, CA) and then desalted by Centricon-10 (Millipore, Bedford, MA). The purified fragments were ligated with the dephosphorylated arms from -700 bp to +75 bp containing the promoter and 5'-UTR of TNF-α. The purified DNA was amplified with 20 ng of each primer, 2.5 mM MgCl₂, 100 μM of each dNTP, and 2.5 U of Taq polymerase (Promega, Madison, WI). The PCR products were analyzed by agarose gel electrophoresis. The amplified PCR product was purified from agarose gel, cut with HindIII and cloned into pBlueScript II SK+. The primers designed for the promoter and 3'-UTR regions were used for the amplification of the mouse TNF-α promoter and 3'-UTR regions.

Luciferase reporter constructs

PT3/T7LUC was partially digested with HindIII and, linear DNA was isolated on a gel after dephosphorylation. Fragments of approximately 800 bp (from ~700 bp to ~750 bp) containing the promoter and 5'-UTR of TNF-α from the NZW, NZB, NOD, and SWR mouse strains cloned into pBlueScript KS+ for sequencing purposes were digested with HindIII and isolated on agarose gel. The purified fragments were ligated with the dephosphorylated arms of PT3/T7LUC to produce four different plasmids, which were NZW luciferase, SWR luciferase, NZW- luciferase, and SWR-luc-NZW DNA sequencing was performed to identify the correct orientation of each insert by using the Sequenase kit.

Transfections

NEW 264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were subcultured on the day before transfection. RAW 264.7 cells were transfected with each plasmid by electroporation as described by Economou et al. (14). Cells were suspended in DMEM with 20% FCS at 10^6 cells to 10 μg plasmid DNA were added. Electroporation was performed with single pulse from a Gene Pulser apparatus (Bio-Rad) with a capacitance extender unit (250 V, 960 μF). Before and after the electroporation, cells were incubated on ice for 10 min. As a control for transfection efficiency, cells were co-transfected with 3 μg pSv2PAP, which encodes human placental alkaline phosphatase gene controlled by SV40 promoter. The promoterless pT3/T7LUC plasmid was used as a negative control, and the positive control was pSv2LUC encoding firefly luciferase gene controlled by SV40 promoter.

Enzyme assays

Transfected cells were incubated in 10% FCS supplemented DMEM for 24 h followed by activation with either LPS (1 μg/ml) or combination of LPS with IFN-γ (100 U/ml) for 6 h. Cells were washed and resuspended in 1 ml of TBS. Two-tenths milliliter of the cell suspension was used for placental alkaline phosphatase assay as described (15) and 0.8 ml of cell suspension was used for luciferase assay using the Promega Luciferase system (Promega, Madison, WI) according to the procedure suggested by the manufacturer. Biolumat LB950A luminometer (Berthold) was used for the measurement of luciferase activity. The luciferase activity values were normalized to the values of the human placental alkaline phosphatase.

Isolation of RNA and cDNA synthesis

Cells were lysed with 4 M guanidium isothiocyanate and RNA was isolated according to standard protocol. Complementary DNA was synthesized from RNA by priming approximately 2 μg of total RNA at 42°C in a final volume of 20 μl containing 2.5 μM of oligo dT(18) primer, 1 mM of each dNTP, and mouse mammary leukemia virus reverse transcriptase at 25 U/μl RNA (Perkin Elmer, Norwalk, CT).

Normalization of sample cDNA content by competitive PCR

To accurately compare cytokine (TNF-α) or luciferase mRNA expression in different samples, it is critical to use equivalent amounts of starting cDNA. We normalized all samples for GAPDH cDNA content. To accomplish this we use competitive PCR, during which one set of primers is used to amplify both the GAPDH cDNA and a second DNA construct (MIMIC) which generates a PCR product of a size different from that of the target DNA (Clontech Laboratories, Palo Alto, CA). Or cloned into a plasmid containing a unique restriction site placed at the 5' end for cloning purposes. The primer sequences for competitive PCR are shown in Table I. For each sample, a mixture containing 0.6 attomol of MIMIC cDNA was used as substrate and amplified by PCR with primers specific for the TNF-α or luciferase cDNAs, respectively. The PCRs were carried out in a total volume of 25 μl: 0.5 μM of primers, 0.2 μM of each dNTP, 2.5 mM MgCl₂, and 0.25 U of AmpliTaq DNA polymerase (Perkin Elmer). A total of 35 cycles of amplification were performed on a 9600 Perkin Elmer Cetus
which high producers, such as SWR and C3H.

2) These variabilities are also found (1 S), we analyzed the possibility further (19). In comparing regulatory sequences, we cloned and sequenced the regulatory sequences. We cloned and sequenced the human TNF-a gene were in fact found (1S), we analyzed the possibility further (19). In comparing regulatory sequences, we cloned and sequenced the human TNF-a gene were in fact found (1S), we analyzed the possibility further (19).

DNA thermocycler with denaturation at 94°C (45 s), annealing at 62°C (45 s), and elongation at 72°C (1.5 min). Serial dilutions of MIMIC cDNA (100 attomol to 10-6 attomol/μl) were added to each reaction. PCR products were subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. The density of samples and MIMIC PCR products were subjected to densitometry with IPLab Gel program software (BT Scientific Technologies, Carlsbad, CA). Upon PCR amplification of serial dilutions of sample cDNAs, a concomitant decrease in sample PCR product and increase in MIMIC PCR product were observed, indicating that our PCR conditions were not within the plateau phase of amplification. When the sample and the respective MIMIC PCR product are found to be equal, the amount of substrate sample cDNA is equal to that of substrate MIMIC cDNA. Therefore, by plotting the ratio of sample band density to MIMIC PCR product against the known amount of MIMIC substrate cDNA, the amount of target cDNA in each sample was calculated.

Results

Genetic basis of reduced TNF-a production in NZW mice

We examined the possibility that reduced TNF-a production has a genetic basis, or perhaps merely represents an epigenetic phenomenon. To evaluate these two distinct possibilities, we compared the levels of TNF-a production in various mouse strains, including congenic mice. The results, represented in Table II, may be summarized as follows. 1) Significant variability exists in the production of TNF-a between the different mouse strains. When, under conditions that induce optimal levels of TNF-a (carried out in preliminary experiments not shown), identical numbers of macrophages are activated with IFN-γ and LPS, the different strains can be subdivided into three categories: low producers, such as NZW and NOD, intermediate producers, such as NZB and C57BL/6, and high producers, such as SWR and C3H. 2) These variabilities are selective to TNF-a production, because another cytokine, IL-1, which is produced by macrophages under similar conditions, does not show the same variations. 3) The variability in TNF-a production is not random, but rather appears to be MHC associated. For example, all of the H-2d mouse strains tested (SWR, DBA/1, and B10.Q) produce similar levels of the cytokine. All of the H-2d mice studied (BALB/c, NZB, and DBA/2) show similar levels of TNF production. Studies of H-2 recombinant mouse strains (B10 congenic strains) suggest a linkage between TNF-a production and H-2. Thus, B10.NZW do not produce the same levels of TNF-a as does the parental B10, with which this strain shares most of its genome. Rather, the B10.NZW shows levels similar to the NZW strain, with which it shares the H-2 locus. The B10.KPA44 strain, which has a wild-type H-2 but shares the z haplotype in the region of TNF-a and class I, produces levels of TNF-a similar to the NZW and B10.NZW mouse strains.

Molecular basis of reduced TNF production in the NZW mouse

The experiments shown above suggest that a major component responsible for the low TNF production resides within the MHC. The observed response of NZW macrophages to LPS and IFN-γ stimulation, which eventually leads to the production and secretion of TNF, probably involves many genes. Included among those that are known, some of which fall within the MHC, are CD14, LPS, TNF and IFN-γ receptors, NF-kB, and IkB. Furthermore, recent analysis of NZB × NZW mice suggests a strong linkage between the TNF-a gene and autoimmune disease development (17).

Initially we believed that the regulation of the TNF-a gene itself (located in the MHC) might be responsible for the observed phenotype, since, due to structural constraints, it would be uncommon to find mutations within the coding sequences of the TNF-a gene. Because some mutations in the human TNF-a gene were in fact found (18), we analyzed the possibility further (19). In comparing the TNF-a coding sequences from the NZW mouse with the C57BL/6 mouse, however, we found no mutations in the coding sequences. This in turn led us to analyze the TNF-a noncoding regulatory sequences. We cloned and sequenced the 5' regulatory

Table II. Comparison of TNF-a and IL-1α production by peritoneal macrophages from inbred and congenic mouse strains in vitro

<table>
<thead>
<tr>
<th>Strain</th>
<th>H-2 Loci</th>
<th>Alleles at the H-2 Loci</th>
<th>Cytokine Production</th>
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<td></td>
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<tr>
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<td>k</td>
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<tr>
<td>M.spretus</td>
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*Thioglycollate elicited peritoneal macrophages, 5 x 103 cells were activated for 18 h, and TNF-a and IL-1α in the supernatants were assayed using standard bioassays. Mean values ± SD representing intermouse variability within that strain are given. The numbers in parentheses indicate the number of mice tested separately from each strain. Designation of alleles of H-2 complex at K, A, E, S, and D are from Klein et al. (16). TNF-α and Hsp68 alleles are from Jacob and Hwang (7).
MOLECULAR BASIS OF TNF-α PRODUCTION

A  A G CAG
A  A G CAG
A  A G CAG

SJL 11  (CA1, A  A  (CA),, A  G G TAG
NOD 8  (CA1, A  A  (CAI, A  G G TTG
MRL 8  (CAI, A  G  (CA),, A  G G CAG
BXSB 8  (CA), A  A (CA),, A G G CAG
PiJ 7  (CA), A  A  (CA),,  A  A  G CAG

FIGURE 1. Mutational analysis of the upstream regulatory region and 5'-UTR of TNF-α genes in various mouse strains. Sequence comparison was done against the sequence in EMBL/GenBank accession number Y00467. The position of several consensus elements are shown. Only the position and the nature of the change in sequence are shown. For the C stretch and the AC repeat, the number of copies in the different strains are given.

regions from high- and low-TNF-α producer mice. The results, presented in Figure 1, show various mutational events identified among the different mouse strains. None of the point mutations observed, however, resided within the previously characterized cis-acting consensus sequences such as the SP-1, NF-κB, Y box, or cytokine-1 motif. Nevertheless, these sequence variations were not randomly distributed among the different strains. Rather, they showed some specific localization; for example, four base substitutional events occurred in a short stretch of 46 bases in the 5'-UTR, suggesting that perhaps these variations play a significant role in regulating the TNF-α gene. To test this possibility, we prepared luciferase reporter constructs. These included the TNF 5' upstream sequences containing the promoter and 5'-UTR region from low TNF producer NZW and NOD mice, intermediate producer NZB mouse, and high-TNF producer SWR mouse. We then compared their inducibility by LPS and IFN-γ-stimulation in transient transfection into the RAW 264.7 macrophage-like cell line.

As shown in Figure 2, no significant differences in luciferase activity were observed among the constructs, suggesting that the variability in the upstream regulatory sequences is not sufficient to explain the differential production of TNF-α among the various mouse strains. A cooperation between the upstream regulatory region and the 3'-UTR in the regulation of the TNF-α gene expression has been demonstrated (20). This cooperation has been observed both in the process of induction with LPS, and in the response of the TNF-α gene to dexamethasone, pentoxifylline, and cAMP (20, 21). Based on these findings, we hypothesized that a possible interaction of 5' and 3'-UTR sequences may explain the differential TNF-α production we observed. A mutational analysis of the 3'-UTR of the TNF-α gene from the stop codon at position 6215–6217 downstream was performed in various mouse strains.

It is noteworthy that some of the mouse strain 3'-UTR sequences have been published previously (22). Our TNF-α 3'-UTR sequencing results are in agreement with the previous data but also extend it to additional mouse strains. As shown in Figure 3, nine sites within the 3'-UTR region of TNF-α were found to be associated
The competitor template was co-amplified using the same oligonucleotide primers of a specific competitor template served as the competitive substrate with the unknown amount of target cDNA of interest. PCR product of the constructs expressed in RAW 264.7 cells, we used augmented the induction of luciferase production, while the inserted. To estimate the luciferase mRNA levels of the different insertional mutations down-regulates the amount of luciferase produced in the RAW 264.7 cell line followed by activation with LPS and IFN-y, and the luciferase activity was measured. The results, depicted in Figure 4, show that the different TNF-α producers (Table II). By contrast, the high TNF-α producer (SWR and C3H mice) did not show these insertional mutations and differed from each other at one single site (position 6306). To probe the functional relevance of these mutations, we made constructs in which 5′ TNF fragments from the low TNF-α producer NZW mouse and high-TNF-α producer SWR mouse were linked to the luciferase reporter gene. In some constructs, we inserted the whole 3′-UTR fragment from the NZW mouse; in others, we inserted the whole 3′-UTR sequences from the SWR strain downstream of the luciferase open reading frame. These different constructs were transiently expressed in the RAW 264.7 cell line followed by activation with LPS and IFN-γ, and the luciferase activity was measured. The results, depicted in Figure 4, show that the different TNF-α 3′-UTRs, when inserted downstream of the luciferase open reading frame, significantly altered the LPS and IFN-γ-induced biosynthesis of luciferase. The 3′-UTR from the SWR mouse TNF-α gene augmented the induction of luciferase production, while the insertion of the 3′-UTR from the NZW TNF-α gene had no such effect. In fact, the results suggest that the NZW 3′-UTR containing the insertional mutations down-regulates the amount of luciferase produced. To estimate the luciferase mRNA levels of the different constructs expressed in RAW 264.7 cells, we used a competitive RT-PCR method (Figure 5A). The co-amplification of serial dilutions of a specific competitor template served as the competitive substrate with the unknown amount of target cDNA of interest. The competitor template was co-amplified using the same oligonucleotide primers as were used for the target cDNA, with virtually identical kinetics of amplification. The PCR product of the competitor template, however, could be differentiated from that of the target cDNA by size fractionation on agarose gels (Fig. 5B). This enabled the quantitation of target RT-cDNA present in the samples. As an additional control we quantitated the glyceraldehyde phosphate dehydrogenase (GAPDH) gene expression in each sample. Because GAPDH is constitutively expressed in all cells, the abundance of its transcripts correlates with the amount of mRNA in each sample. Thus, the values for luciferase RT-cDNA were adjusted by the amount of GAPDH RT-cDNA in each sample. This sample-specific correction negated any effects due to possible intersample variability in the amount of total RNA subjected to the reverse transcription step or nonuniformity of the reverse transcription efficiency.
The competitive PCR experiments (Fig. 5C) showed no significant difference in luciferase mRNA levels between the NZW-luc-NZW and the SWR-luc-SWR constructs expressed by RAW 264.7 cells. While luciferase activities were significantly reduced in the constructs with the 3'-UTR originating from the NZW mouse, the level of luciferase mRNA was not affected. In addition, the 3'-UTR of TNF-α containing an AU-rich fragment with or without the GAT insertional mutation had no effect on the stability of the luciferase mRNA levels in RAW 264.7 cells. Thus, luciferase mRNA levels remained stable within the time points tested, supporting previous observations that the TNF-α AU-rich sequence had no mRNA destabilizing effect in macrophage cell lines (23, 24).

These results also support the notion that there is no necessary correlation between RNA transcript level of TNF-α and actual protein production (23, 24). Thus, activated peritoneal macrophages from the NZW mouse show reduced levels of TNF-α protein production (as shown above, Table II) but there is no significant reduction in TNF-α mRNA levels. Figure 6 exemplifies this for the NZW and SWR strains. Although there is a 12-fold difference in production of TNF-α protein by activated peritoneal macrophages from SWR vs NZW strains, competitive PCR for TNF-α
mRNA showed no significant difference between the two strains (Fig. 6). We therefore believe that we have reconstructed the differential production of TNF-α in NZW vs SWR mice in transient transfection experiments.

Discussion

We carried out extensive mutational analyses and a functional reporter gene analysis of the TNF-α gene, in order to identify the molecular basis of low TNF-α production in NZW mice.

The mutational analysis of the upstream and downstream regions of TNF-α gene show that, among various mouse strains, mutations are not uncommon within these regions. The most interesting mutations are those present in the 3′-UTR. It is remarkable that mouse strains as different phylogenetically as the Mus musculus NZW and Mus spretus share very similar insertional mutations in the 3′-UTR region of their TNF-α genes. It is equally interesting that these mutations have been preserved throughout evolution and remain in contemporary Mus spretus and NZW mice. There are three levels of divergence between the Mus spretus and Mus musculus NZW strain, with at least 3% of DNA divergence and an estimated 1.1 million-yr interval during which their gene pools were separated (25). TNF-α alleles containing these insertional mutations in the 3′-UTR were present in certain contemporary Mus species, indicating that these polymorphisms arose in the ancestors of these Mus species, and were subsequently maintained across multiple speciation events. These findings can be interpreted to indicate that this evolutionary selection may possess intrinsic value for the regulation of these gene products and may exist to maintain specific regulatory capabilities for them.

In expression experiments using a macrophage cell line, we have presented evidence that 3′-UTR mutations have significant functional importance in regulating LPS and IFN-γ-induced biosynthesis of TNF-α. Our results clearly show that the 3′-UTR of the NZW mouse is involved in the reduced capability of the NZW mouse to produce TNF-α. Although we do not know the precise role of the 3′-UTR in the biosynthesis of the cytokine in this mouse strain, we can propose that an interaction between the 3′-UTR and 5′-UTR is responsible for the post-transcriptional regulation of the TNF-α gene.

In considering potential 3′-UTR sequences that may be responsible for the interaction with 5′-UTR elements in regulating the TNF gene, the AU element should be taken into account. As is the case with proto-oncogenes, most cytokines possess an AU-rich motif in their 3′-UTR (26). At least two possible functions for this AU-rich element have been suggested: mRNA destabilization (27) and the involvement in translational control (28, 29). The instability promoted by the AU-rich element has been found in some systems, but not in others (27). We have clearly excluded the possibility that the 3′-UTR of TNF-α in the NZW mouse is involved in mRNA destabilization in macrophages. As for the role of AU-rich element in translational control, in Xenopus oocytes, for example, a single octameric AU element was capable of suppressing the expression of β-IFN by about 80% (29). The AU element was active only when positioned downstream from the coding region. It could be inactivated by the insertion of G or C nucleotides into the AU sequence. Using constructs in which various segments of the human TNF-α 3′-UTR were attached to the chloramphenicol acetyltransferase reporter gene open reading frame, Han et al. (20, 21) revealed a marked translational, regulational effect of the 3′-UTR. Thus, the 3′-UTR sequences were able to suppress translation in resting cells and "de-repress" translation by endotoxin-stimulated cells. The presence of the AU-rich element downstream of the chloramphenicol acetyltransferase reporter gene was fundamental to the translational activation, but the presence of some additional 3′-UTR-flanking sequences seemed to be necessary as well. Our results presented here support and extend conclusions in these previous reports attributing an important regulatory role for the 3′-UTR of TNF-α. The functional experiments shown here took place in a macrophage cell line because macrophages are believed to be the main cell type responsible for TNF-α production.

This study represents part of a steadily growing body of literature that demonstrates the importance of the regulatory role of the 3′-UTR in eukaryotic genes. A recent report, for example, showed that myotonic dystrophy, an autosomal dominant degenerative disease of muscle tissue, results from a defect in the 3′-UTR of a muscle-specific kinase (30). Other studies relate to cancer. Cancer-associated translocations involve not only alterations in the protein coding region, but also the disruption of untranslated regions of RNA. The trk oncogene, isolated from a human carcinoma, was created by a translocation that juxtaposed tropomyosin with a cellular kinase, and eliminated the tropomyosin 3′-UTR (31). Certain human leukemias and lymphomas often result from translocations that disrupt the 3′-UTR of the scI, bc12, or myosin heavy chain transcripts, which cease to be expressed (32–34). In yet another example, 3′-UTRs recently have been reported to be associated with the growth and differentiation of myoblasts (35).

A deliberate substitution of human TNF-α 3′-UTR with a globin 3′-UTR expressed in transgenic mice resulted in the development of an unexpected arthritic phenotype in these mice (36). These findings, together with the results reported here on the maintenance of identical mutations in 3′-UTR sequences of TNF-α during the evolution of the Mus species, suggest that 3′-UTRs contain sequences with important regulatory functions. We propose that the effect of these regulatory functions of the 3′-UTR appear to significantly contribute to the reduced level of TNF-α and therefore to the pathogenesis of lupus-prone mice. Further experiments will be required to determine the exact 3′-UTR sequences and the molecular mechanism through which they exert their regulatory activities.
Molecular Basis of TNF-α Production

References


