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Characterization of Trex1 Induction by IFN-γ in Murine Macrophages

Maria Serra, Sonia-Vanina Forcales, Selma Pereira-Lopes, Jorge Lloberas, and Antonio Celada

3′ Repair exonuclease (Trex1) is the most abundant mammalian 3′→5′ DNA exonuclease with specificity for ssDNA. Trex1 deficiency has been linked to the development of autoimmune disease in mice and humans, causing Aicardi-Goutières syndrome in the latter. In addition, polymorphisms in Trex1 are associated with systemic lupus erythematosus. On the basis of all these observations, it has been hypothesized that Trex1 acts by digesting an endogenous DNA substrate. In this study, we report that Trex1 is regulated by IFN-γ during the activation of primary macrophages. IFN-γ upregulates Trex1 with the time course of an early gene, and this induction occurs at the transcription level. The half-life of mRNA is relatively short (half-life of 70 min). The coding sequence of Trex1 has only one exon and an intron of 260 bp in the promoter in the nontranslated mRNA. Three transcription start sites were detected, the one at −580 bp being the most important. In transient transfection experiments using the Trex1 promoter, we have found that two IFN-γ activation site boxes, as well as an adaptor protein complex 1 box, were required for the IFN-γ-dependent induction. By using EMSA assays and chromatin immune precipitation assays, we determined that STAT1 binds to the IFN-γ activation site boxes. The requirement of STAT1 for Trex1 induction was confirmed using macrophages from Stat1 knockout mice. We also establish that c-Jun protein, but not c-Fos, jun-B, or CREB, bound to the adaptor protein complex 1 box. Therefore, our results indicate that IFN-γ induces the expression of the Trex1 exonuclease through STAT1 and c-Jun.

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In this study, we show that, in macrophages, IFN-γ transcriptionally upregulates the expression of Trex1 with the time course of an early gene. We determined the transcription start site of Trex1, and that two GAS boxes and an adaptor protein complex 1 box in the promoter are required for this induction.

Materials and Methods

Cell culture

Bone marrow-derived macrophages were isolated from 8-wk-old BALB/C mice (Charles River Laboratories, Wilmington, MA) as described previously (16). Bone marrow cells from the femora, tibia, and humerus were flushed and cultured in plastic tissue culture dishes (150 mm) in DMEM containing 2% FCS (Invitrogen, Carlsbad, CA) and 1% sodium-pyruvate (Biochrome, Cambridge, UK). The cells were incubated at 37˚C in a humidified 5% CO2 atmosphere. After 7 d of culture, a homogeneous population of adherent macrophages was obtained (>99% Mac-1). Staphylococcal knockout mice were kindly provided by Dr. Anna Planas (Consejo Superior de Investigaciones Científicas-Institut d’Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain) and Dr. Robert D. Schreiber (Washington University School of Medicine, St. Louis). For the experiments with these mice, we used the corresponding background mouse controls. Animal use was approved by the Animal Research Committee of the University of Barcelona (Procedure No. 2523). The RAW264.7 macrophage cell line (American Tissue Type Collection) was maintained in DMEM 10% heat-inactivated FCS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Human PBMCs were obtained from buffy coat preparations from healthy donors (Hospital Clinic, Barcelona, Spain). Mononuclear cells were isolated by density gradient centrifugation through Ficoll-Histopaque (Sigma-Aldrich Quimica S.A., Madrid, Spain), and monocytes were purified by adherence to plastic (17). The bone marrow-derived dendritic cells were generated as described previously (18). For stimulation studies, saturating amounts of LPS (10 μg/ml) (Escherichia coli 026:B6; Sigma-Aldrich) were added to the cultures. Mac-1.

Reagents

rIFN-γ was obtained from R&D Systems (Minneapolis, MN), Actinomycin D, cycloheximide (CHX), LPS, and DBR (5,6-dichlorobenzimidazole-5′-O-D-ribofuranoside) were from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest purity grade available and were also purchased from Sigma-Aldrich (St. Louis, MO). Deionized water that had been further purified with a Millipore Milli-Q system (Bedford, MA) was used.

Anti–Trent1-specific sera were obtained by immunizing rabbits with the mutant Trent1 H195A (14), which was produced by Vivotect (Madrid, Spain). Abs against STAT1, c-Fos, and phospho-c-Jun were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti–diphospho-ERK-1/2 (clone MAPK-YT), anti–jun-B, and anti–b-actin Ab were from D.I. Agiba (Mortsel, Belgium). Anti–ubiquitin Ab was obtained from a mouse library (129 SVJ) from the Jackson Laboratory (Bar Harbor, ME). Anti–β-actin were from Upstate Biotechnology (Lake Placid, NY), whereas those against tubulin were from Sigma-Aldrich (St. Louis, MO). All other Abs were from Santa Cruz Biotechnology. The Abs used were from the indicated suppliers.

Western blot analysis

Total cytoplasmic extracts were made by lysing cells as described previously (20). SDS-PAGE was performed, and the gel was transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences, Buckinghamshire, UK.). After blocking with milk, incubation with Trex1-specific serum and anti-rabbit secondary Ab was performed. Detection was done using ECL (Amersham), and the membrane was exposed to x-ray films (Agfa, Mortsel, Belgium). β-actin was used as a loading and transfferring control.

Northern blot

Total RNA was extracted as described earlier. For mRNA extraction, polyATtract mRNA isolation system from Promega (Madison, WI) was used, following the manufacturer’s instructions. In brief, total RNA was heated at 65°C for 10 min and incubated with a biotinylated-oligo(dT) probe. Streptavidin-paramagnetic particles (SA-PMPs) were added to the suspension, and the mixture was incubated for 10 min. SA-PMPs were recovered using a magnetic stand, supernatant was removed, and SA-PMP pellet was washed four times with 0.1 × SSC. The mRNA was eluted by resuspending the final pellet in RNase-free water, capturing the SA-PMPs and recovering the supernatant.

Total RNA (20 μg) or mRNA (2 μg) was mixed with loading buffer (deionized formamide 0.5%, 1× buffer MOES/EDTA, formaldehyde 6%, glycerol 13%, bromophenol blue 0.01%), and the samples were separated by electrophoresis in an agarose/formaldehyde gel 1.5%. Samples were transferred by capillary to a nylon membrane Hybond XL (Amersham) overnight in the presence of 10× SSC buffer (NaCl 1.5 M, sodium citrate 0.15 M; pH 7). RNA was fixed in the membrane by UV irradiation (150 mJ) in a Stratalinker (Bio-Rad).

As a probe, Trex1 full-length CDNA (1300 bp) was cloned into pCST vector from Stratagene using MseIII and EcoRI enzymes. This procedure produced a 1021-bp fragment comprising the ATG translation initiation codon to the poly A site. Fifty nanograms of the probe was labeled with 50 μCi of [32P] dCTP from ICN Pharmaceuticals (Costa Mesa, CA) using 10 units of Klenow fragment (Pharmacia Biotech, Stockholm, Sweden) and the Oligolabeling Kit (Pharmacia Biotech). After the labeling, the probe was eluted in 0.5× SSC Column (Pharmacia Biotech), and the efficacy of the labeling was evaluated using the Packard TRICARB 1500 (Packard Instrument, Meriden, CT).

Membranes were prehybridized for 4 h at 65°C in hybridization buffer (deionized formamide 20%, 4× SSC phosphate/EDTA, 5× Denhardt’s solution, SS 5%, dextran sulfate 10%, and 0.2 mg/ml denatured salmon sperm DNA). The labeled probe was then denatured at 95°C for 5 min and added at 2 × 106 cpm/ml to the hybridization buffer. Hybridization was performed for 18 h at 65°C, after which the membrane was sequentially washed during 30 min with 3× SSC 0.1% SDS, 1× SSC SDS 0.1%, and 0.1× SSC 0.1% SDS. The membrane was sealed in hybridization bags from Life Technologies (Invitrogen, Carlsbad, CA) and visualized by exposure to a photographic film (Kodak).

RACE-PCR

The genomic DNA of Trex1 was obtained from a mouse liver (129 SVJ) using the cDNA of Trex1 as probe. The SMART RACE cDNA Amplification Kit from Clontech (Mountain View, CA) was used to recover the 5′ flanking regions of Trex1 cDNAs. Two rounds of PCR were performed following the manufacturer’s specifications. For cDNA synthesis, 1 μg total or poly A RNA and Superscript II (Life Technologies) enzyme were used. A first PCR was performed using the Universal Primer Mix as the forward primer, which consists of a Long Universal Primer: 5′-TCTATACGGTCTATGAGGCAACGCCTGTTAACAAGCGAGGAGTGGC-3′ and a Short Universal Primer: 5′-TCTATACGGTCTATGAGGCAACGCCTGTTAACAAGCGAGGAGTGGC-3′. As reverse primers, Trex1-specific oligonucleotides located at −305 (5′-Trex1 RACE nested) or −353 (rev st/61) were used from the ATG translation initiation codon. The product of this PCR was used as template for a second PCR reaction using a Nested Universal Primer: 5′-AAGACGGTGGTAAACACCGCAGAGTG-3′ as the forward primer and Trex1-specific rev st/61 (−353) as the reverse primer. The 3′ flanking regions were resolved by electrophoresis, purified using QuiaEx (Qiagen, Düsseldorf, Germany), cloned in the pCR2.1 vector (Clontech), and sequenced.

Reporter plasmids

The Trex1 promoter region was cloned from murine genomic DNA isolated from bone marrow-derived macrophages into plasmid pCR2.1 (Invitrogen) and subcloned directionally into pGL3 basic vector (Promega).

The oligonucleotides used as forward primers were as follows: for the 5′-ACTAGTGGCAGACCGGTCCTC-3′ and 5′-AGAGAGGAGGCTGGAAAGAGCCCGTCCAGACTAC-3′ the sense primer was defined by the nucleotide number at which the fluorescence corresponding to the amplified PCR product is detected. The PCR arbitrary units of each gene were defined as the mRNA levels normalized to the β-actin expression level in each sample.

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350-bp construct, 5′-GGTTGACCGAGGTATTCTTGC-3′; for the 850-bp construct, 5′-GCAGTCCCTTGATAGAACAC-3′; for the 1,4-kb construct, 5′-GCGAGGCTCAGCTCTGCT-3′; and for the 2.6-kb construct, 5′-GAGCCGAGCAATATCTCAATGTG-3′. In all cases, 5′-ATGCTGACCCTGGGAGAAGATG-3′ was used as the reverse primer.

The pG3-Trex1 vector was used to introduce mutations of individual binding sites using the QuickChange Mutagenesis Kit (Stratagene, Amsterdam, the Netherlands). The GAS box from +325 to +669 was mutated using oligonucleotides 5′-ACC TTC CTC AGC CAC ACT GAG C-3′ and 5′-CCCT TAC TCC ACA CTT ACC TCC-3′. Twenty-four hours later, cells were stimulated with IFN-γ for 24 h or left untreated. Firefly luciferase and renilla activity was determined using Promega Dual Luciferase reporter assay system and on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) following the manufacturer’s instructions.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation analysis was performed as described previously (22). Approximately 2 × 10⁶ of bone marrow-derived macrophages were grown on 15-cm² dishes and cross-linked to attached cells at room temperature for 20 min by addition of formaldehyde (to a final concentration of 1%). Cells were washed twice with PBS, then collected with scraper and centrifugation into 3 ml of 0.1 M Tris HCl, pH 9.4, 10 mM DTT. Cells were incubated at 30°C for 15 min and collected by centrifugation at 2000 × g for 5 min at 4°C, and the resulting pellet was resuspended sequentially in cold PBS, in buffer I (10 mM HEPES, pH 6.5, 0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and protease inhibitors; 1 mM PMSF, 1 mM iodoacetic acid, 1 mM orthovanadate, 10 µg/ml aprotinin, 1 µg/ml leupeptin) and buffer II (10 mM HEPES, pH 6.5, 20 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and protease inhibitors), and centrifuged at each step. Cells were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris HCl, pH 8.1, and protease inhibitors) and sonicated on ice using the Ika Sonik 1200S Control (Ika Labortechnik, Staufen, Germany) with 20 pulses of 10 s, 30% cycle, 30% amplitude. The size of the fragmented chromatin obtained (between 200 and 1200 bp) was confirmed by electrophoresis. The soluble chromatin was cleared by centrifugation during 10 min at 16,000 × g, and the supernatant was diluted 10 times in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris HCl, pH 8.1, and protease inhibitors). For the control of input chromatin, 100 µl was saved.

Chromatin was precleared by incubation with 20 µg sonicated salmon sperm DNA (Amersham), 3 µg unspecific rabbit IgGs (Sigma-Aldrich), and 10 µl preimmune rabbit serum at 4°C overnight with 100 µl protein A Sepharose beads at 50%. After a short spin, the supernatant (precleared chromatin) was incubated for 8 h with 2 µg of each specific Abs. After that, 100 µl of 50% protein A Sepharose beads was added and the mixture was incubated at 4°C overnight. The beads were collected and washed several times (for 10 min at 3000 × g) with lysis buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 1 mM EDTA). To wash the beads, TE buffer and eluted three times with 100 µl of 0.1 M NaHCO₃, 1% SDS. Cross-links were reversed by incubating samples and input chromatin at 65°C overnight, and DNA was purified with the GFX Purification Kit (Amersham), eluted with 30 µl H2O, and assayed by quantitative PCR using the following primers: 5′-CCTCTTCGGGATACCTCCTGAG-3′ and 5′-CCCTTTTTCCGCCTCCT-3′. In some control experiments, we used a fragment of 2035 bp of the I-Aβ gene that contains an adaptor protein complex I box at −1722 bp from the start of transcription (18). The following primers were used: CAGAGGACAGGAGGTGG and GCCCGGCTAC-CGACCTT.

Statistical analysis

The Student paired t test was used to detect statistical differences.

Results

IFN-γ induces Trex1 expression in macrophages

After interaction of IFN-γ with bone marrow-derived mouse macrophages, a large number of genes are upregulated or downregulated. In this study, we used quantitative real-time RT-PCR to determine the levels of Trex1 in bone marrow-derived macrophages. With the exception of transections, this type of macrophage was used in all the experiments. As a function of time after stimulation with IFN-γ, an increase in Trex1 was detected (Fig. 1A). Trex1 expression increased within the first hour of stimulation, reaching a maximum at 6 h, when it reached <5-fold the basal level. By 24 h of incubation, expression had begun to decrease. The induction of Trex1 mRNA was accompanied by increase in the protein level, as confirmed by Western blotting (Fig. 1A). Trex1 protein was detected in resting macrophages, but its levels started to increase at about 6 h of stimulation with IFN-γ.

The Journal of Immunology 2301

Transient transfection and dual reporter luciferase-renilla assays

For plasmid transfection, 1 × 10⁶ RAW264.7 cells were seeded in 1 ml DMEM 10% FCS in 24-well plates. Cells were cotransfected with a renilla luciferase expression plasmid to verify uniformity of transfection. Treatment with 1 µg plasmid DNA (pGL3 constructs and pRL-TK-Renilla at a 100:1 ratio) per well was carried out using the JetPei-Man Kit from Molecular Dynamics, Sunnyvale, CA. For supershift experiments, after the binding reaction, 2 µg Abs was added and incubated for 30 min for competition experiments, 100-fold excess of unlabeled primers was included in the binding reaction. The oligonucleotides used as probes in the assay were 5′-end labeled using T4 polynucleotide kinase (USB Corporation, Cleveland, OH). All of them were synthesized by Genotek (Barcelona, Spain).

Nuclear extracts

Nuclear extracts were prepared from bone marrow-derived macrophages as described previously (21), with some modifications. For gel shift experiments, confluent cultures of macrophages were scraped and centrifuged (5 min at 1500 rpm, 4°C), and the pellet was rinsed twice with ice-cold PBS. The pellet was resuspended in 5 volumes of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT) and centrifuged at 1500 rpm for 5 min at 4°C. The pellet was then resuspended in volumes of hypotonic buffer and allowed to stand on ice for 10 min. The lysates were homogenized in a potter, and the homogenate was centrifuged at 5000 rpm for 20 min at 4°C to pellet crude nuclei. The nuclear pellet was resuspended in 1/2 volume of low-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 25% [v/v] glycerol, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT), followed by the addition of 1/2 volume of high-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 25% [v/v] glycerol, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). The crude nuclei were extracted at 4°C for 30 min with continuous stirring, followed by centrifugation at 14,000 rpm for 30 min. Supernatants were dialyzed with the PlusOne Mini Dialysis Kit (Amersham Biosciences, San Francisco, CA) in dialysis buffer (20 mM HEPES, pH 7.9, 20% [v/v] glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM EDTA) at 4°C. The extracts were cleared by centrifugation at 14,000 rpm for 20 min, and the supernatant was collected in aliquots and stored at −80°C until use. Protein concentrations were measured using the Bio-Rad Protein Assay Kit (Bio-Rad).

EMSA

EMSA were performed as described previously (18). In brief, binding reactions were prepared with 10 µg of nuclear extracts and 20,000 cpm [32P]-labeled probe in the presence of 2 µg poly(denysinosine–deoxyctydyllic) acid sodium, in a final volume of 15 µl containing 1X binding buffer (12 mM HEPES, pH 7.9, 60 mM KCl, 5 mM MgCl₂, 0.12 mM EDTA, 0.3 mM PMSF, 0.3 mM DTT, 12% glycerol). Extracts and poly (deoxyinosinic–deoxyctydyllic) acid sodium were preincubated for 8 min. The radiolabeled probe was then added and incubated for 15 min at room temperature. Samples were loaded onto 4% acrylamide gel containing 5% glycerol and 200 mM PMSF, separated by electrophoresis (20 V, 2 h), and dried. Gels were dried and bands were visualized using a Phosphofager (Molecular Dynamics, Sunnyvale, CA). For supershift experiments, after the binding reaction, 2 µg Abs was added and incubated for 30 min. For competition experiments, 100-fold excess of unlabeled primers was included in the binding reaction. The oligonucleotides used as probes in the assay were 5′-end labeled using T4 polynucleotide kinase (USB Corporation, Cleveland, OH). All of them were synthesized by Genotek (Barcelona, Spain).
reaching a plateau at about 12 h. This maximum level was maintained during the 96-h time course studied. The decrease in mRNA was due to the short half-life; however, no decrease in protein levels was observed. This finding is attributed to the long half-life of the protein, as detected through chase and pulse experiments with radiolabeled thymidine (data not shown).

Bone marrow macrophages are difficult to transfet in some experiments. We therefore used the macrophage cell line RAW264.7. In these cells, IFN-γ induces Trex1 at mRNA (Fig. 1B) and protein level (Fig. 1B). Also, we tested the expression of Trex1 in human monocytes. Similar to murine cells, Trex1 was induced by IFN-γ (Fig. 1C).

To examine whether the increase in Trex1 expression induced by IFN-γ treatment occurred at the transcriptional level or was due to mRNA stabilization, we determined the half-life of Trex1 transcripts in cells treated or not treated with IFN-γ (Fig. 1D). Macrophages were treated with IFN-γ for 3 h, thereby inducing Trex1. Actinomycin D and DBR were then added at a concentration sufficient to block all further mRNA synthesis (5 and 20 μg/ml, respectively), as determined by [3H]UTP incorporation (23). Then isolated mRNA from aliquots of cells at distinct intervals. Measurement of Trex1 expression by quantitative RT-PCR allowed us to estimate that the half-life of this mRNA in resting cells is quite short, about 70 min (Fig. 1D). IFN-γ treatment did not modify the stability of Trex1, thus indicating that the induction of this gene in response to IFN-γ was at the transcriptional level.

Trex1 expression began to increase within the first hour of IFN-γ treatment. This observation suggests that Trex1 is an early gene, and thus its induction may not require new protein synthesis. To confirm this hypothesis, we treated cells with IFN-γ in the presence of 10 μg/ml CHX. This treatment produced a >90% reduction in the incorporation of [3H]leucine into trichloroacetic acid-precipitable material (23). Macrophages were treated with CHX 30 min before and during stimulation with IFN-γ; cells were then incubated in normal media and gene expression was analyzed. This short treatment with CHX was carried out because this substance is highly toxic for macrophages. CHX alone increased Trex1 expression, and the combined stimulation of IFN-γ and CHX had a synergistic effect on the induction of the gene (Fig. 1E). These observations imply that the two stimuli induce Trex1 by distinct mechanisms. Moreover, the finding that CHX treatment did not block IFN-γ-induced Trex1 synthesis provides evidence that this induction is not dependent on de novo protein synthesis.

To analyze whether the inhibition of protein synthesis affected the half-life of Trex1, we quantified the half-life of this gene in resting macrophages and in IFN-γ–treated macrophages pre-treated with CHX. Treatment with CHX dramatically increased the Trex1 half-life in both resting and IFN-γ–activated macrophages (Fig. 2). This observation demonstrates the presence of a protein factor with short half-life that requires continuous synthesis and is responsible for Trex1 destabilization. This observation would explain the synergistic effect of the combined stimulation with IFN-γ and CHX on Trex1 induction.

**Determination of a splicing process in Trex1**

By comparing the genomic and Trex1 cDNA sequences, we determined that the Trex1 coding region is localized in a single exon. Regarding the 5′-untranslated region (UTR), Trex1 cDNA lacked a 260-bp fragment, spanning from positions −267 to −7, which coincides with a splicing donor and a splicing acceptor site, respectively. Analysis of the genomic sequence revealed another possible splicing donor site at −243 and two possible splicing acceptor sites at −207 and +12 (Fig. 3A). To determine whether other cDNAs were produced by alternative splicing, we performed RT-PCR using a forward primer beginning at −370 (for St/d1) and a reverse primer beginning at +13 (rev Trex1-5′), and resolved by electrophoresis. Two bands were detected with calculated molecular masses of 400 and 120 bp. Both were cloned and sequenced.
PCR was designed using to determine the transcription start sites of Trex1. Determination of Trex1 transcription start sites was done by using total RNA and poly A+ RNA from resting and IFN-γ-treated macrophages treated with Actinomycin D (ActD) + DBR in relation to untreated cells. This assay is representative of at least four independent experiments showing similar results.

The 383-bp fragment corresponded to unprocessed RNA, whereas the 123-bp fragment corresponded to a molecule obtained by splicing using the donor site located at −267 bp and the acceptor site located at −7 bp. In conclusion, during Trex1 maturation, a splicing process in the noncoding region eliminates an intron of 260 bp.

FIGURE 2. Trex1 mRNA stability is dramatically increased by CHX treatment. Trex1 mRNA stability was determined in resting macrophages, macrophages treated with IFN-γ, macrophages treated with CHX, and both IFN-γ and CHX. Trex1 mRNA stability was determined as in Fig. 1C. A. Decay of Trex1 mRNA expression is shown. B. Data are represented as the percentage of remaining mRNA at each time point of actinomycin D (ActD) + DBR in relation to untreated cells. This assay is representative of at least four independent experiments showing similar results.

Determination of Trex1 transcription start sites
To determine the transcription start sites of Trex1 in the mouse promoter, we performed a RACE-PCR procedure. A two-round PCR was designed using Trex1 cDNA. The first reaction used the 5′ Trex1 RACE nested (−305) or the rev st/d1 (−353) as the reverse primer. In both cases, the Universal Primer Mix primer (a mix of the Long Universal Primer and the Short Universal Primer) was used as the forward primer. Using the product of these PCRs as the template, we performed a second PCR with the Nested Universal Primer and the rev st/d1 primer. The products of these PCRs were resolved by electrophoresis and seven bands were obtained, which were cloned and sequenced. Three transcription start sites were detected, corresponding to positions −429 (producing a 1154-bp cDNA), −580 (producing a 1305-bp cDNA), and −788 (producing a 1513-bp cDNA) (Fig. 3B).

To determine the relative abundance of these transcript variants, we analyzed Trex1 expression by Northern blotting. We purified total RNA and poly A+ RNA from resting and IFN-γ-stimulated macrophages, and used Trex1 cDNA as a probe. Total RNA transcripts of a calculated m.w. of 1.1, 1.3, 1.5, 4.6, and 7.8 kb were detected, with the 1.3-kb transcript clearly being the most abundant. Moreover, in poly A+ RNA samples, only the 1.3-kb transcript was detected. Similar results were obtained when RNA from spleen or thymus was used. These results are supported by the studies of primer extension (data not shown). In conclusion, the main transcription start site in Trex1 in macrophages is located at −580, whereas those located at −429 and −788 are minor (Fig. 3B).

Analysis of the Trex1 promoter sequence
To determine the putative elements responsible for the transcriptional regulation of Trex1, a fragment of ~3 kb upstream of the first ATG was sequenced and analyzed to detect possible regulatory boxes using the TFSEARCH and Transfac databases. The analysis of the 5′-proximal region reflects the absence of canonic TATA and CCAAT boxes. However, this region contains GC-rich sequences (Sp1 motives) typical of TATA-less promoters (Fig. 4). Sp1 boxes are implicated in the initiation of transcription in the absence of TATA boxes. The accumulation of various transcription start sites in a small region is also common in GC-rich sequences that promoted initiation of transcription. An initiation consensus element (Inr) was also detected between positions −180 and −185 bp (CCCTCA). Consensus binding sites for several transcription factors, such as GAS elements, NF-κB, upstream transcription factor, Lyf.1, PU.1, GATA, adaptor protein complex 1, and ISRE boxes, were localized between positions +1 and −1400 bp but not between −1400 and −3000 bp.

Functional analysis of the mouse Trex1 promoter in IFN-γ-mediated Trex1 induction
Next, analysis of the functional activity of the Trex1 promoter sequence using the TFSEARCH database showed that the promoter region contains various putative sites for the binding of transcription factors involved in IFN-γ signal transduction, such as three GAS boxes (one from −1260 to −1252, one from −690 to...
−679, and one from −273 to −263) and an ISRE box (from −337 to −325). In addition, we detected one NF-κB box (−1247 to −1237) and three adaptor protein complex 1 boxes (−1361 to −1353; −1135 to −1126; −675 to −669; Fig. 4).

To delimitate the region responsible for IFN-γ induction, we linked vectors varying in the lengths of the 5′ sequence of the Trex1 promoter to the luciferase reporter gene. These vectors were transfected into RAW264.7 macrophages and luciferase activity was measured. Each construct was cotransfected with the renilla expression vector. All luciferase activity values were normalized to the level of renilla expression to correct for any differences in transfection efficiency. In unstimulated macrophages, a construct comprising 350 bp of the promoter showed negligible activity (Fig. 5A). The longest fragment that we tested comprised 2.6 kb and showed no IFN-γ inducibility. However, the 1.4-kb construct and the 850-bp fragment were clearly induced by IFN-γ. Little induction was found in the 600-bp fragment. All these data suggest that the sequence between −350 and −600 is required for basal activity, whereas the region between −600 and −850 is needed for induction by IFN-γ.

Two GAS boxes and an adaptor protein complex 1 box are required for Trex1 promoter inducibility

To determine more precisely the areas in the Trex1 promoter responsible for its induction by IFN-γ, we mutated some of the putative IFN-γ responsive elements by site-directed mutagenesis of the 850-bp construct and assayed activity as described earlier. Also, the adaptor protein complex 1 box located at −675 bp was mutated.

Mutation of the ISRE box at −337 had almost no effect, whereas mutation of the GAS boxes located at −273 and −690 caused a decrease in the promoter induction from 3.7-fold to 1.5- and 1.7-fold, respectively (Fig. 5B). These results demonstrate that both GAS boxes are required for maximal induction by IFN-γ, and

FIGURE 4. Putative consensus binding sites for transcription factors in the Trex1 promoter. The sequence spanning 1500 bp upstream of Trex1 translation start site was analyzed using TFSearch program, and putative boxes sharing >85% similarity with the consensus sequence for the binding of each transcription factor are shown. The transcription start sites detected by RACE-PCR and the splicing donor and acceptor sites are indicated by arrows.

FIGURE 5. Characterization of the Trex1 functional promoter. A, RAW264.7 macrophages were transiently transfected with pGL3 plasmids containing distinct deletions of the Trex1 promoter region. At 24 h posttransfection, cells were stimulated with IFN-γ for a further 24 h and luciferase activity was tested. B, Plasmids containing the 850-bp promoter region of Trex1 were used for site-directed mutagenesis, transfected in RAW264.7 cells that were stimulated with IFN-γ. Fold induction by IFN-γ is represented. A and B, A plasmid coding for renilla enzyme under the control of the Bos promoter was cotransfected, and renilla activity was used to control transfection. All assays are representative of at least four independent experiments showing similar results. Each point was performed in triplicate, and the results are shown as mean ± SD. *p < 0.01 in relation to the controls when all the independent experiments had been compared.
are consistent with the low induction of the 600-bp construct, which holds only one GAS box. Considering these experiments, the ISRE box plays a minimum role in the induction by IFN-γ. This finding can be expected because this motif is usually more implicated in the response to type I IFNs. Surprisingly, mutation of the adaptor protein complex 1 box abolished the IFN-γ induction of the promoter. This observation suggests a critical and unsuspected role of adaptor protein complex 1 factors in the regulation of the Trex1 promoter (Fig. 5B).

**STAT1 binds to the GAS boxes in response to IFN-γ**

To determine the proteins that bind to the areas of interest in the promoter from BALB/c mice, we performed gel electrophoresis DNA binding assays. Nuclear extracts were prepared from cells treated with IFN-γ. When the extracts of these cells were incubated with a probe covering the GAS1 box located at −690 bp, a band was induced that was inhibited when anti-STAT1 Abs were added (Fig. 6A). This band is specific because it competed with an excess of cold probe and with a consensus GAS box, but not with an adaptor protein complex 1 box or with the mutated GAS box. All these observations demonstrate that STAT1 specifically binds the GAS box at position −690 bp in the Trex1 promoter of macrophages in response to IFN-γ.

Similarly, a probe covering the GAS box located at −273 bp was retarded by extracts from IFN-γ-stimulated but not from resting macrophages (Fig. 6A, lower panel). The same extracts did not have the capacity to bind to a probe with a mutated GAS box. Moreover, binding to the wild-type GAS box was abolished with anti-STAT1 Abs, coinciding with the observation made for the GAS box at −690 bp. These observations demonstrate that STAT1 binds in vitro to two GAS boxes located in the Trex1 promoter in response to stimulation with IFN-γ.

To confirm that the binding of STAT1 to the Trex1 promoter also occurred in vivo, we performed chromatin immunoprecipitation assays, amplifying the area −522 to −693 bp. Whereas low levels of amplification were detected in the samples immunoprecipitated using total Igs, enhanced amplification of the Trex1 promoter was observed in the sample from IFN-γ-stimulated macrophages immunoprecipitated using STAT1-specific Ab (Fig. 6B). The specificity of the reaction was checked by using unrelated Abs or a fragment of the promoter that does not contain the transcription start site (data not shown). This result confirmed that the binding of STAT1 to the Trex1 promoter occurs in vivo in response to IFN-γ stimulation.

To further confirm the involvement of STAT1 in the regulation of Trex1 by IFN-γ, macrophages from STAT1-deficient mice were stimulated with IFN-γ for a range of times. RNA was extracted and Trex1 expression was detected by Northern blotting and quantitative real-time PCR. In comparison with what was observed in control cells, the induction of Trex1 in macrophages derived from STAT1-deficient mice was impaired, once again confirming that STAT1 is required for Trex1 induction in response to IFN-γ (Fig. 6C).

**Phospho–c-Jun binds to the adaptor protein complex 1 box in response to IFN-γ**

To determine the factors that bind to the adaptor protein complex 1 box in the Trex1 promoter, we performed EMSAs. An induced band was detected; however, this band did not appear when the mutated adaptor protein complex 1 box was used (Fig. 7A, left panel). There was no inhibition or supershift when the binding was made in the presence of Abs against adaptor protein complex 1 factors CREB or jun-B, or with unspecific Igs, but the binding was eliminated with a phospho–c-Jun–specific Ab (Fig. 7A, right panel). This result confirms that a complex containing phospho–c-Jun binds the adaptor protein complex 1 box in the Trex1 promoter in vitro in response to IFN-γ stimulation. By Western blot, we observed that IFN-γ induces the phosphorylation of c-Jun, as well.
As a control for the c-Fos Ab, we performed experiments of chromatin immunoprecipitation using Abs against c-Jun and c-Fos, and the promoter of I-Aβ gene (18). The fragments of DNA were amplified and the amounts determined by quantitative PCR. The adaptor protein complex 1 box was precipitated in dendritic cells by Abs against both c-Jun and c-Fos (Fig. 7D). This observation suggests that the lack of binding of c-Fos to the Trex1 promoter is not due to a technical problem. Finally, the sequence alignment of the adaptor protein complex 1 boxes in the murine and human genes, as well as in the I-Aβ gene used as a control, showed perfect homology (Fig. 7E).

Discussion
In this study, we have shown that Trex1 exonuclease expression in macrophages is regulated at the transcriptional level by IFN-γ. Trex1 gene is located in position F2 in chromosome 9 of the mouse karyotype, and its protein coding region is found at a single exon, which is quite uncommon in eukaryote genes. The analysis of Trex1 cDNA reveals the presence of a single intron upstream of the open reading frame, in the 5′-UTR. By the RACE-PCR technique, we have determined that at least three transcription start sites are active in the Trex1 promoter region, and using Northern blotting, we have observed that the most abundantly expressed transcript in bone marrow macrophages is located —580 bp from the translation start site, and that it generates a transcript of 1.3 kb. Trex1 promoter spans 850 bp from the main transcription start site and lacks the basic elements for transcription initiation, such as TATA and CCAAT boxes. However, an Inr sequence and several Sp1 (GC-rich) and upstream transcription factor motifs are found, which can functionally replace the TATA box sequence (27–29). Although this structure is typical of housekeeping genes, it has also been described in genes modulated by IFN-γ (30–33).

By blocking de novo protein synthesis, we have demonstrated that Trex1 induction by IFN-γ does not require the synthesis of a protein factor. Instead, Trex1 mRNA induction occurs with the timing of an early gene in the first hour of IFN-γ treatment. Moreover, CHX treatment alone was able to induce Trex1 expression, thereby suggesting that a protein factor negatively regulates Trex1 levels. Furthermore, CHX and IFN-γ treatment synergistically induced Trex1, thus demonstrating that the two treatments act through different mechanisms. By blocking transcription with actinomycin D, we determined that Trex1 is unstable, showing a half-life of about 90 min in resting mouse macrophages. Treatment with IFN-γ did not enhance the half-life of this gene but rather caused a slight decrease. This observation implies that the increase in Trex1 expression caused by IFN-γ is not due to an enhancement of mRNA half-life but to an increase in the transcription rate.

The most widely studied regulatory elements controlling mRNA stability are adenylate-uridylate-rich elements found mainly in the 3′-UTR of many genes coding cytokines and stress-response proteins (34). However, Trex1 has a very short 3′-UTR spanning (area −522 to −693) was increased in samples from stimulated macrophages immunoprecipitated with c-Jun Ab, but not from unstimulated samples or samples immunoprecipitated with an irrelevant Ab (Fig. 7C). These observations thus confirm that the binding of c-Jun to the Trex1 promoter in response to IFN-γ occurs in vivo. On the basis of these experiments, we conclude that neither c-Fos, jun-B, nor CREB binds to the Trex1 promoter in vivo. Thus, c-Jun is probably bound to DNA as homodimer, as has been shown in other genes (25, 26). However, more experiments are required to confirm this hypothesis.
that they are also present in the human promoter. The observation that these elements are conserved between the human and mouse genomes reinforces their role as critical regulatory areas.

IFN-γ is the most potent activator of macrophages; therefore, the data shown in this study reflect the requirement for Trex1 upregulation during activation of these cells. IFNs induce about 300 genes with diverse functions, the fight against viral infection being one of the major functions (9). To our knowledge, this is the first report of the induction of a DNA-specific exonuclease by proinflammatory stimulation. It has been reported that the ssRNA-specific IFN-stimulated gene 20 nuclease is induced by type I and II IFN treatments (42). According to several studies, this protein shows antiviral activity against RNA genomic viruses, such as vesicular stomatitis virus, influenza virus, and encephalomyocarditis virus. This activity is dependent on its exonuclease activity; therefore, IFN-stimulated gene 20 may act directly by degrading viral RNA (43). Similarly, Trex1 could be involved in the antiviral response against DNA genomic viruses. However, increased sensitivity to viral infection has not been reported in Trex1 knockout mice or in AGS patients. In Trex1-deficiency models, autoimmunity caused by chronic production of IFN is observed in the absence of viral infection (3, 44). Moreover, mutations in Trex1 are associated with the development of systemic lupus erythematosus (45). These observations lead to the hypothesis that Trex1 substrate is an endogenous nucleic acid species rather than a virus. In this context, the capacity of Trex1 to digest DNA from retrotranscribed elements and the accumulation of these elements in Trex1-deficient cells has recently been reported (6).

In this study, we show that Trex1 protein expression is regulated at the transcriptional level by a proinflammatory stimulus such as IFN-γ. Although the induction of Trex1 by IFN may be explained by the need for Trex1 upregulation during viral infection or accumulation of endogenous nucleic acids, other proinflammatory stimuli such as LPS or TNF-α were found to induce Trex1 expression in macrophages (M. Tartakis, M. Serra, J. Lloberas, and A. Celada, manuscript in preparation). These observations suggest an unknown role for Trex1 during macrophage activation that may not be related to antiviral activity. We have recently demonstrated that Trex1 interacts in vivo with the WW domain of the CA150 factor, a negative regulator of transcription elongation (14). In addition, Trex1-deficient cells have an impaired proliferative capacity and abnormal cell cycle checkpoint activation as a result of the chronic activation of the ATM kinase and destabilization of the cell cycle checkpoint kinase CHK2 (5). Moreover, the observation that human Trex1 is coded in the same open reading frame as ATRIP (ATR interacting protein), a key component of the cell cycle checkpoint machinery that is activated in response to replication stress and the presence of cellular ssDNA, further supports the notion that Trex1 is involved in these processes. On the basis of all these observations, we propose that Trex1 exerts an as yet unidentified function in other cellular pathways involving nucleic acid processing, such as DNA replication, DNA damage repair, and DNA transcription, in the context of inflammation and macrophage biology.

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Disclosures
The authors have no financial conflicts of interest.
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