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Transcriptional Inhibition of Stromelysin by Interferon-γ in Normal Human Fibroblasts Is Mediated by the AP-1 Domain

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Abstract The expression of the major matrix-degrading metalloproteinase, stromelysin (SL), is modulated by a variety of cytokines and growth factors. Interferon-γ (IFN-γ) is a potent modulator of SL expression, either inhibiting or activating expression in a cell-specific manner. We have investigated the mechanisms involved in the regulation of SL gene expression in cultured human fibroblasts by IFN-γ. Reverse transcription-polymerase chain reaction (RT-PCR) assays confirmed the previously reported profound inhibitory response of SL mRNA expression to IFN-γ [Amaldi et al., 1989]. For evaluation in transient gene expression assays, 1.2-kilobase (kb) pairs (−1214 to +14 relative to the transcription start site), and shorter, deletion mutant fragments of the SL promoter were cloned into appropriate chloramphenicol acetyltransferase transferase (CAT) expression vectors. The SL promoter along this region contains an active polyomavirus enhancer A-binding protein-3 (PEA-3) site at −216 and an activator protein-1 (AP-1) site at −70. Treatment of transfected neonatal foreskin fibroblasts with 300–500 U/ml IFN-γ resulted in down-regulation of both basal and IL-1β-induced CAT gene expression. IFN-γ also decreased CAT expression when placed under the control of a synthetic multimeric AP-1 site construct. Gel-shift assay data indicate a decrease in specific binding to AP-1 oligonucleotide of nuclear extract from IFN-γ and PMA/IFN-γ-treated cells. The suppression of SL expression by IFN-γ, in human fibroblasts therefore is mediated through the AP-1 element. J. Cell. Biochem. 72:373–386, 1999.

Key words: interferon-gamma; stromelysin; transcriptional regulation; AP-1 domain

Interferon-γ (IFN-γ), a member of a highly pleiotropic family of cytokines produced by activated T lymphocytes and natural killer (NK) cells, exerts immunomodulatory and antiproliferative effects on a wide variety of cell types [Amaldi et al., 1989; Ono et al., 1989; Garbe and Krasagakis, 1993; Rosa and Fellous, 1988; Satoh et al., 1995]. In addition, IFN-γ manifests potent regulatory effects on the expression of extracellular matrix-related molecules. IFN-γ has been shown to inhibit the basal and transforming growth factor-β (TGF-β)-stimulated synthesis of type I, II, and III interstitial collagens in mesenchymal cells [Goldring et al., 1986; Granstein et al., 1989; Amento et al., 1991; Kahari et al., 1990; Varga et al., 1990; Farrar and Scheiber, 1993]. IFN-γ is also a potent inhibitor of both basal and IL-1β-induced expression of the matrix metalloproteinase (MMP) stromelysin (SL) and of the related enzyme, collagenase (CL), in human dermal fibroblasts, rheumatoid synovial fibroblasts, and human articular chondrocytes [Unemori et al., 1991; Andrews et al., 1989; Varga et al., 1995]. SL is an important member of the matrix metalloproteinase family of enzymes with a wide range of substrates, including collagen IV, fibronectin, and laminin, and plays a major role

Abbreviations used: SL, stromelysin; CL, collagenase; IFN-γ, interferon-γ; γRE, gamma response element; CAT, chloramphenicol acetyltransferase; AP-1, activator protein-1; PEA3, polyomavirus enhancer A-binding protein-3; MMP, matrix metalloproteinase; IL-1β, interleukin-1β; EGF, epidermal growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; TGF-β, transforming growth factor-β; ICAM-1, intracellular adhesion molecule-1; CBP, CREB binding protein; PB, polybrene; RT-PCR, reverse transcription-polymerase chain reaction; FFb, foreskin fibroblasts; TAE, tris acetate EDTA electrophoresis; LH, lactalbumin hydrolysate; PE, phycoerythrin; FACS, fluorescence activated cell sorting; EMSA, electrophoretic mobility shift assay.

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toward full activation of interstitial collagenase [Unemori et al., 1991; Knauper et al., 1993]. It is consequently a major mediator of extracellular matrix turnover, and its overproduction, which leads to an imbalance in the equilibrium of deposition and degradation essential to normal tissue development and repair, is associated with pathologic conditions, such as rheumatoid arthritis, tumor invasiveness, and metastasis.

The current understanding of the role of IFN-γ as regulator of MMPs in dermal and epidermal cells is that it is cell-type specific and that repression may involve both transcriptional and post-transcriptional mechanisms [Varga et al., 1995; Tamai et al., 1995]. Induced transcriptional activation [Benech et al., 1985; Figueiredo et al., 1989; Gupta, 1990; Bikoff et al., 991; Caldenhoven et al., 1994; Anderson et al., 1994] is mediated by the temporal activation of positive and negative transactivating factors through an IFN-γ activation site (GAS); whereas the consensus contains only a 9-bp core sequence, the activation unit also includes 10–20 bp of flanking sequence [Strehlow and Decker et al., 1992]. Positive transactivating factors have been shown to include the IFN-γ activation factor (GAF) [Darnell et al., 1994], consisting of a homodimer of the STAT-1α protein and IFN-γ response factor-1 (IRF-1) [Harroch et al., 1994]. While IRF-2 [Harada et al., 1989] and interferon consensus sequence binding protein (ICSBP) [Nelson et al., 1993] have been identified as factors that down-regulate genes that have been stimulated by IFN-γ, negative regulatory mechanisms attributable to IFN-γ in the absence of prior IFN-γ stimulation [Nelson et al., 1993; Heckmann et al., 1989; Besancon et al., 1994] have not been well characterized. IFN-γ has recently been reported to block gene transcription of colony-stimulating factor-1 (CSF-1) in macrophages [Xu et al., 1995], bullous pemphigoid antigen gene in keratinocytes [Tamai et al., 1995], and macrophage scavenger receptor A-gene (SR-A) in rat fibroblasts [Horvai et al., 1997]. With the exception of the SR-A gene, little is known about the specific sites within these genes that mediate these effects.

Activation of expression of the SL gene is regulated by various cytokines, including interleukin-1β (IL-1β), interleukin-10, tumor necrosis factor-α, nerve growth factor, epidermal growth factor (EGF), leukoregulin, and the phorbol ester TPA [Saus et al., 1988; Quinones et al., 1989; Buttice et al., 1991; Reitamo et al., 1994; Sciavolino et al., 1994; Jasser et al., 1994; Machida et al., 1991; Delany et al., 1992; Mauviel et al., 1992; Buttice and Kurkinen, 1993]. Inhibition of SL gene expression has been demonstrated by transforming growth factor-β (TGF-β), glucocorticoid, and retinoid hormones, and androgens [Saus et al., 1988; Delany and Brinkerhoff, 1992; Kerr et al., 1990; Mauviel, 1993; Shapiro et al., 1991; Nicholson et al., 1990; Schule et al., 1991; Schneikert et al., 1996]. SL gene regulation by all of these agents is mediated by the first 1,300 base pairs (bp) of the promoter 5′ of the transcription start site [Saus et al., 1988; Buttice et al., 1991]. Within this region lie the PEA-3 DNA binding element for the ets family of transcription factors [Wasylyk et al., 1990; Gutman and Wasylyk, 1991; Wasylyk et al., 1991] at position −216 (with a PEA-3-like element at −1078) and the AP-1 binding site at −70 [Gentz et al., 1989; Distel and Spiegelman, 1990; Vogt and Bos, 1990; Morgan et al., 1992; Radler-Pohl et al., 1993; Piechaczyk and Blanchard, 1994; Kerr et al., 1988] (with an AP-1-like element at −552). While the AP-1 site is absolutely required for basal expression [Xu et al., 1995], the PEA-3 site plays the major role in the induction of SL expression after treatment of cells with cytokines and growth factors [Buttice et al. 1991; Mauviel et al., 1992]. However, the primary target site for transcriptional suppression, unlike induction, appears to be the AP-1 site [Kerr et al., 1990; Shapiro et al., 1991; Nicholson et al., 1990]. A singular exception, identifying the PEA3 site at −216 as a site for SL down-regulation by androgens, has recently been documented [Schniekert et al., 1996]. In considering potential sequences that may be acting as targets for IFN-γ-mediated suppression, there are no known IFN-γ-related consensus sequences within this 1,300-bp region.

To clarify the role of IFN-γ on SL repression in human dermal fibroblasts at the transcriptional level and to identify the sequences necessary for the inhibition, we examined the ability of the SL promoter and fragments of the promoter to transduce the IFN-γ effect in transient expression experiments. Our data demonstrate unambiguously that the predominant negative regulatory effect of IFN-γ on SL transcription resides with the AP-1 factor-binding domain of the gene.

MATERIALS AND METHODS

Cells

Human neonatal foreskin fibroblasts (FFb) were obtained after tissue explant. Briefly, the

epidermal layer was removed from neonatal foreskin tissue. The tissue was then placed onto the surface of a 100-mm culture dish, the surface of which had been scored to allow for greater adhesion of tissue segments to the plastic. Cells were then allowed to migrate out of the tissue, at 37°C, with 5% CO_2, into Dulbecco's Modified Eagle Medium (DMEM)/10% fetal bovine serum (FBS) (Gemini BioProducts, Calabasas, CA) and 100 U/ml penicillin G, 100 µg/ml streptomycin (Gemini BioProducts). Cells were passaged when confluent.

Reagents

DMEM was supplied by Bio-Whittaker (Walkersville, MD) and lactalbumin hydrolysate by Gibco-BRL Life Technologies (Gaithersburg, MD). Hexadimethrine bromide (polybrene) and EGF were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human IL-1β and recombinant human IFN-γ were purchased from R&D Systems (Minneapolis, MN). PCR reagents were purchased from Perkin-Elmer (Foster City, CA). Restriction enzymes *Hin*dIII and *Kpn*I were purchased from Promega (Madison, WI). Other DNA-modifying enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). Ethidium bromide (ultraPURE, 10 mg/ml) was purchased from Gibco-BRL Life Technologies.

Polymerase Chain Reaction Amplification

RNA was extracted from FFb cells using the RNazol procedure (Tel-Test, Friendswood, TX). After spectrophotometric quantitation at 260 nm, 1 µg of total RNA was reverse transcribed using 50 pmol of oligo d(T) (Perkin-Elmer) priming with 100 U of Moloney murine leukemia virus reverse transcriptase (United States Biochemical, Cleveland, OH). PCR amplification of either genomic DNA or cDNA was carried out with AmpliTaq polymerase (Perkin-Elmer) in the presence of 75 pmol of respective primer pairs (sequences listed in Table I). All PCR amplifications were of 35-cycle duration and each used annealing temperatures 2°C below the lowest respective primer Tm°C values. When examining for the presence of specific mRNA, RT-PCR amplifications used conditions to ensure that products were within a linear amplification range for the product generated (data not shown). Amplified DNA was visualized on 1% TAE/agarose gels and the images captured and presented in negative photographic format, on an IS 1000 digital imaging system (Alpha Innotech, San Leandro, CA).

Oligonucleotides

Oligonucleotides (Table I) were synthesized by either Ana-Gen Technologies (Palo Alto, CA) or Keystone Laboratories (Menlo Park, CA). Oligonucleotides were designed for the amplification of mRNA of various genes (SLcd5′, SLcd3′, GAPDH1, and GAPDH2), for the preparation of promoter deletion-mutation constructs (SL-P1, SL-P2, SLM1, SLM2, SLM3, and SLM4), for the preparation of DNA for probe (SL1 and SL2), for the construction of synthetic gene sequences (SL-6xAP-1S and SL-6xAP-1AS), or for use in sequencing protocols (pCAT+43 and pCAT-59). Double-stranded oligonucleotides for the AP-1 and Sp-1 DNA binding domain consensus sequences were generated by Promega.

Promoter Constructs

Oligonucleotides, SL1 and SL2, were used to generate a 300-bp fragment of the SL coding region from +21 to +330. The fragment was purified, random-prime labeled, and used as a probe to screen 1×10^6 plaque-forming units (pfu) of a human lymphocyte genomic library (Stratagene). One of six positive clones was chosen to provide DNA for cloning of the SL promoter. A resultant 2,004-bp fragment spanning the blunt-end generated *Spe*I restriction endonuclease site at −1214 to the blunt-end generated *Ava*I site at −11, was cloned into the blunt-end generated *Sal*I site of the CAT reporter vector, pCAT.Basic (Promega), to form pCAT.SL-1214 (Fig. 1). pCAT.Basic contains only the ampicillin-resistance gene, the CAT gene and the multiple cloning site region directly 5′ of the CAT gene. RT-PCR amplification was also used to generate a fragment spanning positions +14 to −284 of the SL gene from the isolated genomic clone DNA. This PCR fragment was cloned into pCAT.Basic, generating pCAT.SL-284 (Fig. 1). The DNA sequence of the reporter vector inserts was verified by dideoxy-sequencing (the Sequenase™ protocol, United States Biochemical).

Deletion mutant CAT constructs were then generated. Construct pCAT.SL-755 was produced by digesting pCAT.SL-1214 with the restriction endonucleases *Hin*dIII and *Kpn*I (removing the region, −1214 to −756), blunt-ending the digested DNA sites and re-ligating

TABLE I. Synthetic Oligonucleotide Sequences[a]

Primer	Sequence	Vendor
SL1	ATAGAATTCGCAAGGCATAGAGACAACATAGAG	AGT
SL2	GCATCACCTCGAGAGTGTCGGAGT	AGT
SLcd5′	GCCTATCCATTGGATGGAGCTGCA	AGT
SLcd3′	GCCAATTTCATGAGCAGCAACGAG	AGT
SL-P1	GGGTCTAGTGAATTCCAGTCAATT	AGT
SL-P2	GCCTGCCTCCTTGTAGGTCCAACC	AGT
SL-M1	TCTCCTCTACCAAGACAGGAAGC	AGT
SL-M2	TGTATCATCCTACTTTGAATTTGG	AGT
SL-M3	CAAGGATGAGTCAAGCTGCGGG	AGT
SL-M4	CGGGTGATCCAAACAAACACTGTC	AGT
pCAT + 43	GTGTGGAATTGTGAGCGG	AGT
pCAT-59	TGGTATATCCAGTGATTT	AGT
SL-6xAP-1S	AAGCTTGGATGAGTCACTGTGAGTCACTGTGAGTC ACTGTGAGTCACTGTGAGTCACTGTGAGTCACTGC AGAAGCTTTCTGAAGGGCAAGGACTCTATATATAC AGAGCACCTCCTTACTAGCTGGGATCCAG	KL
SL-6XAP-1AS	CTGGATCCCAGCTAGTAAGGAGGTGCTCTGTATAT ATAGAGTCCTTGCCCTTCAGAAAGCTTCTGCAGTG ACTCACAGTGACTCACAGTGACTCACAGTGACTC ACAGTGACTCACAGTGACTCATCCAAGCTT	KL
AP-1	CGC TTG ATG AGT CAG CCG GAA GCG AAC TAC TCA GTC GGC CTT	
Sp-1	ATT CGA TCG GGG CGG GGC GAG C TAA GCT AGC CCC GCC CCG CTC G	
GAPDH1	ACCACAGTCCATGCCATCAC	KL, AGT
GAPDH2	TCCACCACCCTGTTGCTGTA	KL, AGT

[a]SL1 and SL2 primers were used to generate a DNA probe from reverse-transcribed SL mRNA; SLcd5′/SLcd3′, and GAPDH1/GAPDH2 were used for RT-PCR experiments; SL-P1/SL-P2, SL-M1 through SL-M4 for PCR-generated deletion constructs; SL-6xAP-1S/SL-6xAP-1AS to generate an AP-1-specific CAT construct; and pCAT+43 and pCAT-59 to sequence all pCAT constructs. AGT, Ana-Gen Technologies; KL, Keystone Laboratories. AP-1 and SP-1 double-stranded DNA oligonucleotides were purchased for EMSA from Promega.

(Fig. 1, Table II). PCR amplification, using pCAT.SL-284 as the PCR template, was used to generate constructs pCAT.SLM1, pCAT.SLM2, pCAT.SLM3, and pCAT.SLM4.

pCAT.SL6xAP-1 was constructed by ligating the product of annealing primers SL6xAP-1S and SL6xAP-1AS (Table I) into the blunt-ended *Sal*I restriction endonuclease site of pCAT.Basic. SL6xAP-1S and SL6xAP-1AS are 120-bp "sense" and "anti-sense" oligonucleotides, respectively, containing six multimeric AP-1 repeats, and the 5′ sequence for the TATA box region of the promoter for the CL gene. Sequence acting as "spacer" regions between the AP-1 repeats bore no homology to either SL or collagenase AP-1 site flanking region sequences.

Transient Transfections and CAT Reporter Assays

At 24 h before transfection, 2×10^5 cells were seeded in 35-mm dishes in DMEM and 10% FBS. Media were then removed and cells were washed twice with room temperature PBS (Ca^{2+} and Mg^{2+} free). Serum-free DMEM media supplemented with 0.2% lactalbumin hydrolysate (DMEM/LH) was then added to the cells for 30 min at 37°C. Media were removed and 2.5 µg of DNA was added to the cells in 0.75 ml DMEM/LH containing 10 µg/ml of hexadimethrine bromide (polybrene) [Kawai and Nishizawa, 1984]. Cells were then returned to 37°C/5% CO_2 for 4 h. The media and DNA were aspirated off and replaced in each well with 1 ml DMEM/LH + 26% DMSO for exactly 3 min. Cells were then washed three times with PBS (Ca^{2+} and Mg^{2+} free) and DMEM/LH added to cells alone or containing 10 µg/ml IL-1β or 300 U/ml IFN-γ, or both, for 48 h. Cells were washed three times with PBS (Ca^{2+} and Mg^{2+} free) and lysed with 1x reporter lysis buffer (Promega). Cell extracts were then fractionated as outlined

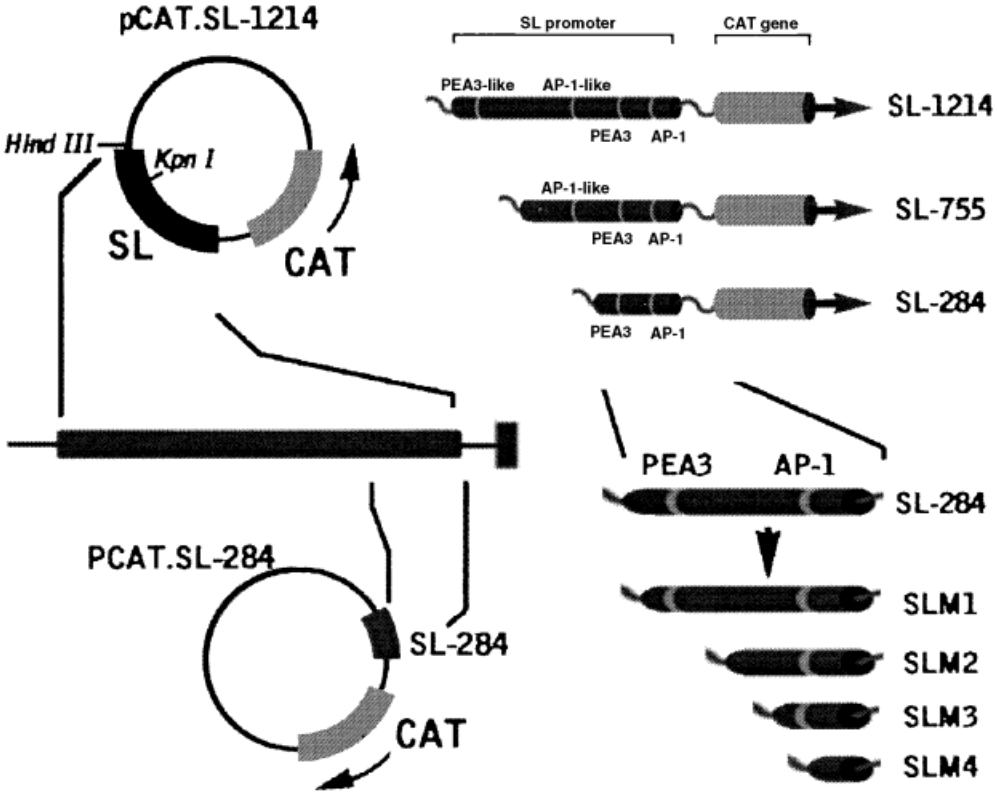

Fig. 1. SL promoter constructs. The SL promoter construct pCAT.SL-1214 was made by cloning the SL promoter region spanning position −1214 to −11, from a phage clone isolated from a human lymphocyte genomic DNA library. Subsequent SL promoter truncations were generated by deleting promoter regions using restriction endonucleases, as for construct pCAT.SL-755 (using *Hin*dIII and *Kpn*I), or by using polymerase chain reaction (PCR), as for construct SL-284. PCR primers were designed to produce varying length truncations using either pCAT.SL-1214 or pCAT.SL-284 as templates for the reactions. SL promoter regions, −1214 to −11, and −284 to +14 were inserted into the pCAT. Basic vector, generating pCAT.SL-1214 and pCAT.SL-284, and using these constructs, the subsequent truncated promoter regions, SLM1-SLM4 were produced. The schematic shows the relative regions of the SL promoter included in constructs pCAT.SL-1214, pCAT.SL-755, and pCAT.SL-284, the corresponding deletion constructs using the pCAT.SL-284 construct, and the potential binding domains and consensus sequences found within each region of SL promoter.

TABLE II. Deletion Constructs Generated by Restriction Digest or PCR From SL Promoter Constructs pCAT.SL-1214 and pCAT.SL-284[a]

Deletion constructs	Template	Restriction digest cloning	PCR cloning primer pairs	Consensus sequence deleted
pCAT.SL-755	pCAT.SL-1214	*Hin*dIII/*Kpn*I fragment deleted	—	PEA3-like@-1078
pCAT.SLM-1	pCAT.SL-284	—	SL-M1/SL-P2 (−231–+14)	AP-1-like@-552
pCAT.SLM-2	pCAT.SL-284	—	SL-M2/SL-P2 (−138–+14)	PEA3@-216
pCAT.SLM-3	pCAT.SL-284	—	SL-M3/SL-P2 (−76–+14)	—
pCAT.SLM-4	pCAT.SL-284	—	SL-M4/SL-P2 (−58–+14)	AP-1@-70

[a]Deletion construct pCAT.SL-755 was produced by a *Hin*dIII/*Kpn*I restriction digest of CAT.SL-1214, 37°C for 2h. Digests were treated with T4, DNA polymerase and 200 μM dNTP's for 5′ at 37°C, producing blunt-ends for cloning, with a further incubation period of 15′ at 65°C to destroy polymerase activity. pCAT.SL-284 was constructed using PCR and pCAT.SL-1214 as a template. PCR generated deletion constructs pCAT.SLM1/M2/M3/ and M4 were generated using pCAT.SL-284 as a PCR template. PCR-generated fragments were treated with polynucleotide kinase T4 and T4 DNA polymerase before gel separation. Gel-eluted DNA was then ligated: the resulting clones were analyzed for sequence integrity before using for transient transfections.

in the Promega CAT Reporter Assay protocol. CAT assays were performed on 50 µl of each extract. Briefly, 50 µl of extract was mixed with 120 mM Tris.HCl (pH 8.0), 0.25 µCi ^{14}C-chloramphenicol (Ci/mmol, Amersham Life Sciences, Arlington Heights, IL) and 25 µg n-butyryl Coenzyme A (Sigma) for 18 h at 37°C. Samples were then extracted three times with mixed xylenes (Eastman Kodak, New Haven, CT) and counted by liquid scintillation (Wallac Microbeta Plus, Turku, Finland). Total protein content was determined in equal volumes of extract using the BCA assay protocol (Pierce Biochemicals, Rockford, IL). CPM values were then normalized to reflect CAT activity/µg total protein.

Fluorescence Activated Cell Sorting (FACS)

Neonatal FFb were plated in 35-mm wells of a 6-well plate, at a density of 2.5×10^5 per well, 24h before cytokine treatment. Cells were washed twice with serum-free DMEM and then incubated with or without 300 U/ml of IFN-γ in DMEM/LH for 24 h, at 37°C with 5% CO_2. Cells were washed twice in PBS (no calcium or magnesium) and then treated with 2 mM EDTA to remove the cells from the plate. Cells were aliquoted into the wells of a 96-well round-bottom plate at 0.5×10^6 cells/well and centrifuged at 1,200 rpm for 2 min at 4°C. The supernatant was removed and pelleted cells were resuspended in 50 µl of chilled staining buffer (PBS without calcium or magnesium, containing 0.5% BSA and 0.01% of sodium azide). To the cell suspensions was added either phycoerythrin (PE)-conjugated mouse IgG or mouse monoclonal PE-conjugated human CD54 antibody (CD54-PE) (Becton Dickinson), which recognizes intracellular adhesion molecule-1 (ICAM-1). Antibodies were added to a final concentration of 100 µg/ml and incubated for 30 min at 4°C. The cells were then washed three times by centrifuging at 1200 rpm for 2 min, at 4°C, each wash. Cells were thoroughly resuspended in 100 µl of staining buffer and transferred to 12×75-mm polystyrene tubes (prechilled to 4°C). The volume was increased to 500 µl, and the labeled cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from neonatal FFbs as described by Nicolas and Goodwin [1994]. Microdialysis of nuclear extracts was carried out using the Microdialyzer System 100 (Pierce) and framed microdialysis membrane with a 12,000-MW cutoff. EMSA were carried out using the Promega gel-shift assay system. Briefly, 10 µg of dialyzed nuclear extract was mixed with 20–30 fmol of ^{32}P-labeled AP-1 consensus oligonucleotide (Table I); reactions were electrophoresed on a 5% nondenaturing polyacrylamide gel at a constant 50 V. Gels were then dried under vacuum at 80°C. Autoradiography film was then exposed to the gel for an appropriate length of time. For competition assays, the reactions included 3 pmol of unlabeled AP-1 oligonucleotide or Sp-1 consensus sequence oligonucleotide.

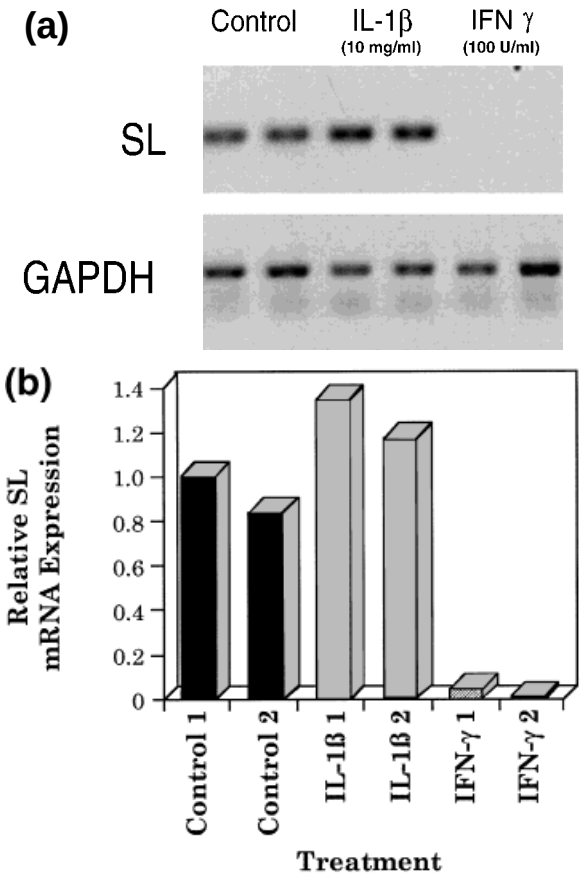

Fig. 2. Changes in SL expression after treatment of neonatal FFb with either interleukin-1β (IL-1β) or interferon-γ (IFN-γ). Total RNA was extracted from FFb, untreated or treated for 24 h with either 10 ng/ml IL-1β or 100 U/ml IFN-γ in duplicates using RNazol reagent. Reverse transcription-polymerase chain reaction (RT-PCR) was used to generate PCR products from 1 µg of total RNA using primers specific for SL and GAPDH. A representative ethidium bromide gel is presented as a reverse exposure image **(a)**. The densitometric analysis **(b)** of data depicted in (a) demonstrates that while IL-1β increases SL expression by approximately 20%, IFN-γ down-regulates SL expression by >90%.

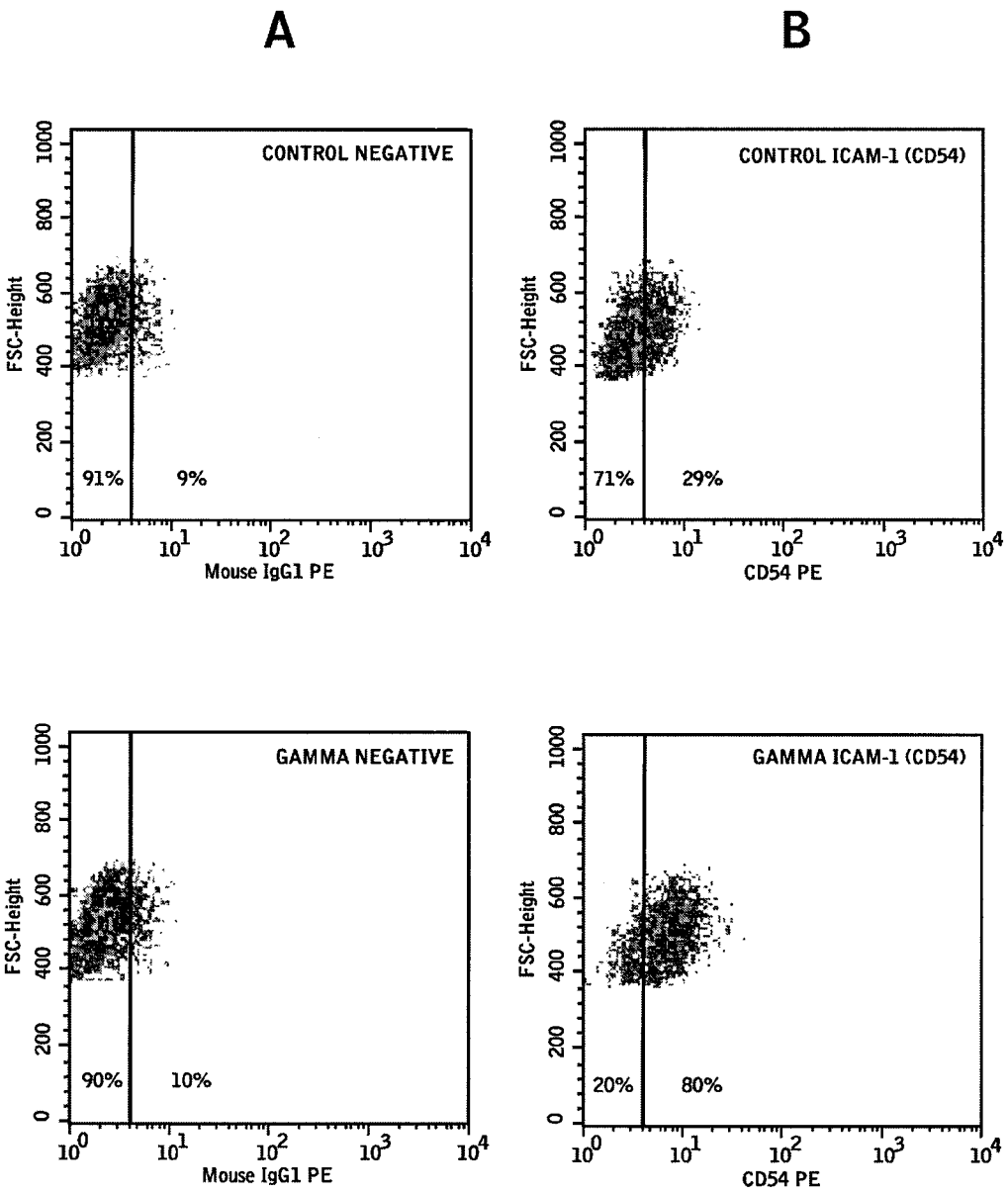

Fig. 3. Cytofluorographic analysis of neonatal FFb for intracellular adhesion molecule-1 (ICAM-1) induction by interferon-γ (IFN-γ). Cells were incubated for 30 min at 4°C with either phycoethrin (PE)-conjugated mouse IgG or a mouse monoclonal PE-conjugated anti-human CD54/ICAM-1 antibody. Dead cells were stained with propidium iodide (5 μg/ml) and gated out. Each plot represents the results from 10,000 cells. **A:** Background staining profile generated by mouse PE-IgG without (control negative) (9% staining) and with 300 U/ml of IFN-γ treatment (γ-negative) (10% staining). **B:** Fluorescence activated cell sorting (FACS) profile generated by staining of cells with anti-human CD54/ICAM-1 antibody, in the absence (control ICAM-1 [CD54]) (29% staining) and presence of 300 U/ml IFN-γ treatment (γ ICAM-1 [CD54]) (80% staining).

RESULTS

IFN-γ Treatment Specifically Down-Regulates SL

IFN-γ has been shown previously to down-regulate SL mRNA in normal human dermal and synovial fibroblasts [Unemori et al., 1991; Buttice and Kurkinen, 1993]. In the present study, human neonatal FFb were tested for similar responsiveness to IFN-γ. Steady-state SL mRNA levels were examined by RT-PCR in untreated cells, or 24 h after treatment with either IL-1β or IFN-γ. RT-PCR was performed on 1 μg of total RNA, per primer pair, to determine the effect of the cytokines on SL expression levels in these cells. As shown in Figure 2a, the expression of the GAPDH mRNA did not

change markedly in the presence of either IL-1β or IFN-γ, while SL mRNA levels increased slightly with IL-1β and were significantly down-regulated upon addition of IFN-γ (Fig. 2a,b).

To ascertain whether the effect of IFN-γ was specific to SL or reflected a nonspecific, down-regulatory cellular response, cells were treated with IFN-γ for 24 h and were then analyzed for the presence of a cell-surface marker, ICAM-1 (CD54), which is known to be up-regulated by IFN-γ on various cells [Farrar and Schreiber, 1993; Dustin et al., 1988; Frohman et al., 1989]. Cells were washed, isolated from culture and then stained with a phycoerythrin (PE)-conjugated antibody (CD54PE) to the cell-surface protein ICAM-1. Analysis by flow cytometry demonstrated that IFN-γ increased ICAM-1-specific staining on FFb by approximately 50% over untreated cells (Fig. 3). In view of the marked up-regulation of ICAM-1 expression by IFN-γ, the down-regulation of SL expression by IFN-γ is consistent with a specific regulatory event in neonatal foreskin fibroblasts.

SL Promoter Deletion Analysis

SL promoter fragments, spanning positions −11 to −1214 , −11 to −755 and +14 to −284 were cloned into CAT reporter vectors (pCAT.SL-1214, pCAT.SL-755, and pCAT.SL-284, respectively) (Figs. 1, 4), and transfected into FFb cells to examine transient expression of the CAT gene under the control of the SL promoter constructs. Cells were untreated or treated with IL-1β and IFN-γ either alone or in combination, and incubated for 48 h after transfection. Whole cell extracts were then examined for CAT activity. Baseline levels of CAT expression varied among the three constructs; expression through the pCAT.SL-1214 construct was approximately twofold that of pCAT.SL-755 and approximately fivefold that of pCAT.SL-284 (data not shown). Baseline levels of CAT expression of all constructs were normalized to 1.0 for ease of data presentation (Fig. 4). An approximately 90% elevation of CAT expression above baseline was observed with both the pCAT.SL-1214 construct and pCAT.SL-284 construct, after IL-1β treatment. Both constructs include the consensus PEA3 and AP-1 sites at −216 and −70, respectively, while pCAT.SL-1214 also contains the "upstream" "PEA3-like," and "AP-1-like" sequences, at −1078 and −552, respectively. Interestingly, little or no effect of IL-1β on CAT expression was seen for pCAT.SL-755, despite the presence of the PEA3 and AP-1 sites (−216 and −70, respectively). All constructs showed similar decreases in CAT expression, relative to control, after FFb treatment with IFN-γ alone (approximately 35%–50%) or in combination with IL-1β (40%-60%). Therefore, basal and IL-1β-induced regulation of SL,

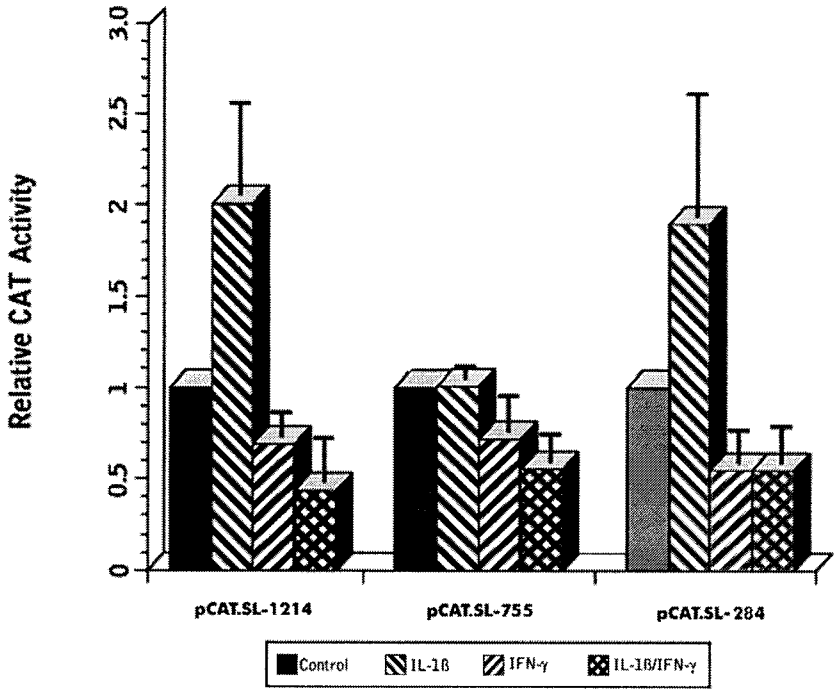

Fig. 4. Modulation of CAT expression through SL promoter constructs. pCAT reporter vectors were transiently transfected into neonatal foreskin fibroblasts using the polybrene transfection protocol. Cells were then either untreated or treated with interleukin-1β (IL-1β), interferon-γ (IFN-γ) or IL-1β + IFN-γ at 37°C for 48 h. Cells were lysed and extracts assayed for CAT activity. Values are relative to the untreated, transfected cells within each transfected group and are equalized based on the total protein content of each of the extracts. IL-1β caused an approximate twofold induction in CAT activity with constructs pCAT.SL-1214 and pCAT.SL-284, but no induction in CAT expression from pCAT.SL-755. Treatment of cells with IFN-γ caused down-regulation of basal expression (35–50%) for all constructs, and IL-1β-induced down-regulation with constructs pCAT.SL-1214 and pCAT.SL-284 (50%). Results are the mean from three experiments ±SEM (error bars).

through both distal and proximal transcription elements, is down-regulated by IFN-γ. On the basis of these findings, the gamma-responsive element (γRE) was deduced to lie within 284 bp of the transcription start site.

Deletion Construct CAT Assays

To identify the γRE sequence responsible for producing the negative regulatory effect, deletion mutant constructs were made using PCR and pCAT.SL-284 as a template (Table II, Figs. 1, 5). The construct pCAT.SL-284 contained the PEA3 site at −216, and the AP-1 site at −70 (Fig. 1). Constructs contained sequential deletions of this segment of the SL promoter. The deletion constructs, pCAT.SLM1, pCAT.SLM2, pCAT.SL-M3, and pCAT.SLM4, in addition to pCAT.SL-1214, and pCAT.SL-284, were transfected into FFb and cell extracts were analyzed for the expression of the CAT gene product. Basal levels of CAT expression, as driven by the promoter fragments, were varied. Absolute cpm values, as a measurement of CAT expression, decrease in the order pCAT.SLM1 > pCAT.SLM2 > pCAT.SLM3 > pCAT.SLM4. For example, basal CAT expression values obtained from a single representative experiment decreased accordingly: 89,620 cpm (M1), 35,699 cpm (M2), 14,677 cpm (M3), and 1,376 cpm (M4) (pCAT.Basic alone, 933 cpm). However, as shown in Figure 5, the response to IFN-γ was retained by all constructs, except pCAT.SLM4 in which IFN-γ enhanced CAT expression (as previously described the basal level of CAT expression from the pCAT.SLM4 construct was significantly lower than all other constructs).

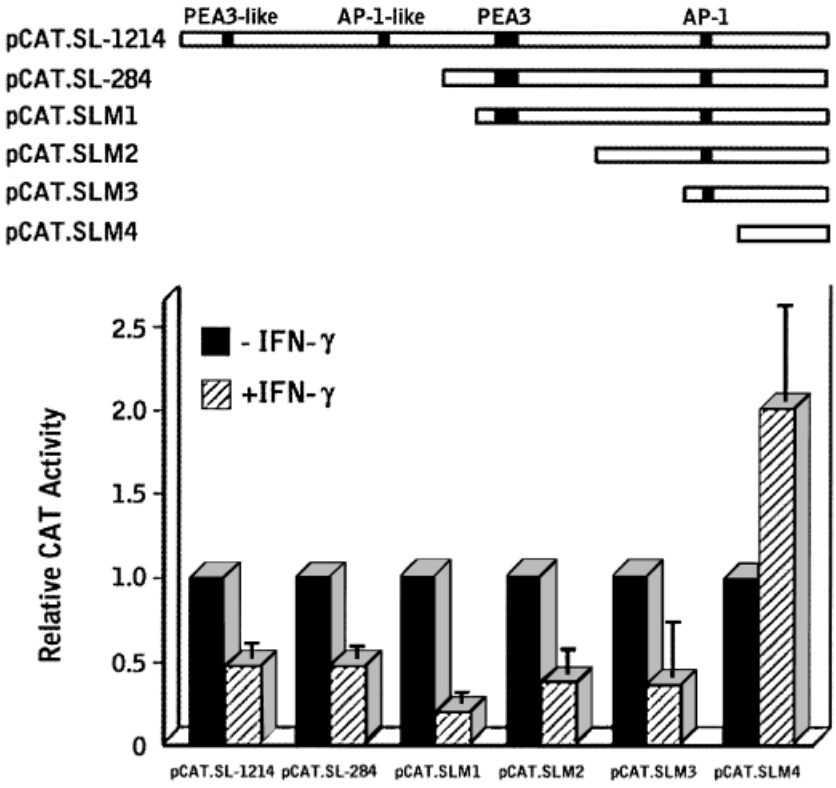

Fig. 5. Polymerase chain reaction (PCR) generated deletion mutant constructs of the pCAT.SL-284 construct. DNA fragments, generated by PCR using the pCAT.SL-284 construct as a template, were cloned into a CAT reporter vector and transiently transfected into FFb. Transfected cells either remained untreated or were treated for 48 h with interferon-γ (IFN-γ) (500 U/ml). Whole cell extracts were then assayed for CAT activity (duplicate data points from duplicate extracts). Results are the mean from 10 experiments ±SEM (error bars). Values are relative to the untreated, transfected cells within each transfected group and are equalized based on the total protein content of each of the extracts. IFN-γ down-regulates CAT activity in cells transfected with constructs pCAT.SL-1214, pCAT.SL-284 and pCAT.SLM1/M2/M3, within a range of 50%–80%. While pCAT.SLM4 activity is shown to be induced by IFN-γ, absolute cpm data values relating to CAT activity for this construct were approximately 80% lower than those observed for pCAT.SL-M3 (not shown). The diagram above the graph shows the corresponding SL fragment and relative positions of potential and active consensus sequences in a given promoter construct (fragment lengths are not to scale).

pCAT.SLM4 contained a 58-bp segment of the promoter containing only sequences 3′ of the AP-1 site to the transcription start site; there was no identifiable consensus DNA binding response element within this sequence. As the IFN-γ-induced down-regulation in transcription occurred only in the presence of the AP-1 site, we then tested the possibility that the γRE in the SL promoter was the AP-1 site itself.

IFN-γ Modulates CAT Expression from Synthetic AP-1 Constructs

To assess the possibility that the γRE was the AP-1 binding domain, constructs containing multiple AP-1 sequences in tandem were generated and tested for IFN-γ responsiveness. A 120-bp fragment (produced by annealing oligonucleotides 6xAP-1S (sense strand) and 6xAP-AS (antisense strand) (Table I) contained six AP-1 consensus sequence copies, which were arranged in tandem. The TATA box-region introduced into the oligonucleotide was that of the collagenase promoter [Vogt and Bos, 1990]. The transcriptional response of the constructs pCAT.Basic pCAT.SL-1214, pCAT.SLM3, and pCAT.SL6xAP-1 to IFN-γ or EGF after transient transfection into neonatal FFb was measured as CAT activity (Fig. 6). As previously observed (Figs. 4, 5), cells transfected with pCAT.SL-1214 and pCAT.SLM3 showed a decrease (approximately 85% and 50%, respectively) in CAT expression after treatment with IFN-γ. In pCAT.SL6xAP-1-transfected cells, CAT expression was significantly down-regulated (50%) after IFN-γ treatment. EGF treatment was used as a control to demonstrate that the construct acted in a functionally qualitative manner similar to that observed for endogenous AP-1 sites found within both the SL and collagenase promoter. CAT expression increased 230% after exposure of the cells to EGF.

IFN-γ Decreases Binding of Nuclear Protein to the AP-1 Domain

Nuclear protein extracts were isolated from neonatal FFb after treatment of cells for 24 h with IFN-γ alone, PMA alone, or IFN-γ and PMA together. Microdialyzed nuclear extract, containing 10 μg of protein, was then mixed with labeled AP-1 consensus sequence specific oligonucleotide, in the absence or presence of, 100-fold excess unlabeled AP-1 or Sp-1 consensus sequence oligonucleotide. Figure 7 indicates that IFN-γ caused a decrease in the basal level of binding of nuclear proteins to labeled AP-1 oligonucleotide. While PMA treatment dramatically increased binding, IFN-γ significantly inhibited this binding as well. Extracts incubated with labeled AP-1 oligonucleotide, in the presence of excess unlabeled AP-1 oligonucleotide, demonstrates that the binding of nuclear proteins to the consensus sequence was AP-1 specific. This is further indicated by the inability of excess unlabeled oligonucleotide, specific for the Sp-1 DNA-binding domain consensus sequence, to compete with bound, labeled AP-1 consensus sequence oligonucleotide.

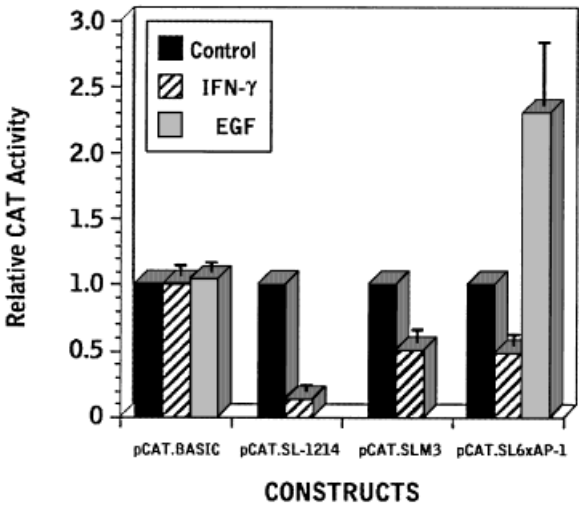

Fig. 6. CAT reporter activity through the synthetic, multimeric AP-1 construct. Cellular extracts of FFb transiently transfected with the pCAT.Basic, pCAT.SL-1214, pCAT.SLM3, and pCAT.SL6xAP-1 construct and treated with either interferon-γ (IFN-γ) (300 U/ml) or epidermal growth factor (EGF) (10 ng/ml) were assayed for CAT activity. No IFN-γ or EGF-responsive (striped bars and cross-hatched bars, respectively) CAT activity is observed from cells transfected with pCAT.BASIC. Data obtained from cells transfected with pCAT.SL,1214, pCAT.SLM3, and pCAT.SL6xAP-1 all exhibit inhibition of CAT expression (85%, 50%, and 50%, respectively), when treated with IFN-γ. Induction of CAT expression through the 6xAP-1 construct is demonstrated by a 2.3-fold increase in expression after treatment of transfected cells with EGF. Results are the mean from three experiments (two experiments for EGF-generated data) (with duplicate data points from duplicate extracts) ±SEM (error bars).

DISCUSSION

The present study was aimed at identifying a potential IFN-γ response element (γRE) within the SL promoter responsible for mediating SL suppression. Initial experiments using the RT-PCR technique confirmed the ability of IFN-γ to significantly inhibit SL expression at the mRNA level in human dermal fibroblasts. The specific-

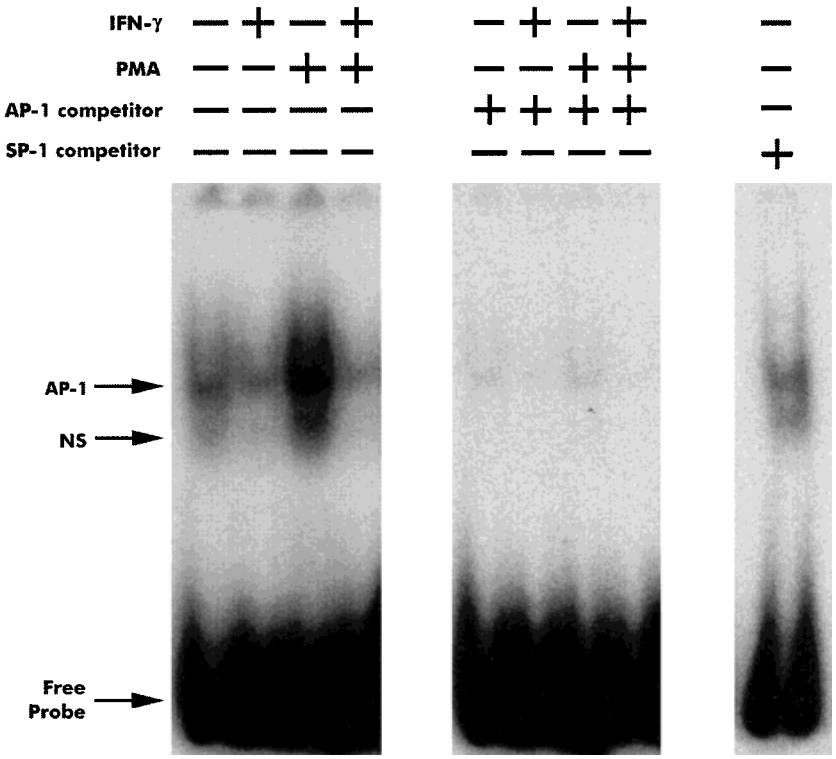

Fig. 7. Electrophoretic mobility shift assay (EMSA) of nuclear proteins from FFb treated with interferon-γ (IFN-γ) and phorbol myristate acetate (PMA). A total of 10 μg of nuclear proteins isolated from FFb treated with IFN-γ (300 U/ml) alone, PMA (50 ng/ml) alone, or a combination of both, was incubated with ^{32}P-labeled AP-1 double-stranded DNA oligonucleotide. Reactions were then electrophoresed and binding products examined after exposure of autoradiogram film to the dried gel. In the absence of competing oligonucleotide basal binding levels are significantly decreased by IFN-γ and increased by PMA. IFN-γ also decreases PMA-induced binding of nuclear proteins to AP-1 oligonucleotide. In the presence of a 100-fold excess of the AP-1, but not Sp-1 oligonucleotide, binding of proteins to labeled AP-1 oligonucleotide is competable.

ity of the effect of IFN-γ on SL was confirmed by a contrasting inductive effect of IFN-γ on ICAM-1 protein expression [Farrar and Schreiber, 1993; Dustin et al., 1988; Frohman et al., 1989] in these same cells, consistent with previous reports. CAT reporter assays, using various promoter sequences spanning position −1214 to +14 along the SL gene, confirmed that the regulation was transcriptional. On the basis of numerous transfection experiments using these promoter constructs, it was clear that sequences along the entire length of the promoter play a significant role in the expression of SL. This was illustrated by the fact that absolute transcriptional activity, driven by the the constructs, decreased in the order of the size of the constructs, pCAT.SL-1214 > pCAT.SL-755 > pCAT.SL-284 (data not shown). Despite this, IFN-γ was able to suppress basal and IL-1β induced expression from the pCAT.SL-1214 and pCAT.SL-284 constructs by similar relative amounts; therefore, the γRE inhibited not only gene expression activated through the well-characterized PEA3/ets (−216) and AP-1 (−70) sites, but also probably that driven by other, functional induction elements within the first 1,200 bp of the SL promoter.

Using deletion constructs of the SL promoter, we found that the regulatory element responsible for the IFN-γ effect lay within 284 bp of the transcriptional start site of the SL gene. This region of the SL promoter contained the consensus sequences for the PEA3/ets and AP-1 DNA binding domains, at −216 and −70, respectively. Both binding domains have been shown to be essential participants in the regulation of SL gene expression [Quinones et al., 1989; Buttice et al., 1991; Mauviel et al., 1992; Mauviel, 1993; Wasylyk et al., 1991], with the AP-1 site essential for basal SL expression and the PEA3 required in the induction of SL expression by PMA and IL-1β. Other constructs, containing progressively smaller segments of the SL promoter, 3′ from position −284, which retained the AP-1, but not the PEA3/ets consensus sequence site, showed no diminution in the IFN-γ effect. On the basis of these observations, the AP-1 site was considered to be the most likely γRE. However, it was necessary to consider the potential influence of sequences immediately flanking the AP-1 consensus as others have shown that differences in the flanking sequences surrounding an AP-1 site influence gene transactivation [Ryseck and Bravo, 1991; Kerppola et al., 1993]

Therefore, to confirm the specificity of the AP-1 site as the γRE, experiments were conducted using a multimeric AP-1 consensus se-

quence CAT construct. The multimeric consensus sequence was placed 5′ of the CL TATA region, with random sequence included between each AP-1 site. While SL and CL expression have been shown to be regulated coordinately, the regions lying between the AP-1 and transcriptional start sites of the two genes show only ~30% similarity. The CL TATA region was therefore selected to eliminate any potential influence of specific sequences 3′ of the SL AP-1 site. AP-1 domain flanking regions were excluded to eliminate their potential regulatory contribution, as well. IFN-γ caused a 50% reduction in expression of the CAT gene through the multimeric pCAT.SL6xAP-1 construct, similar in magnitude to that seen using pCAT.SLM3, a construct that includes the SL AP-1 site and flanking sequences. As the effect might be expected to be manifested in changes in binding of nuclear proteins to the AP-1 element EMSA were performed for detection of such alterations. Nuclear proteins, extracted from FFb after IFN-γ treatment, showed decreased binding to an AP-1 oligonucleotide sequence, suggesting that the down-regulatory effect of IFN-γ is mediated by a specific decrease in binding of transcriptional activators using the AP-1 domain binding. While these data suggest an important role for the AP-1 domain as a major site of IFN-γ activity, the fact that reporter construct experiments do not exactly mirror the magnitude of suppression of endogenous SL mRNA expression, suggests the possibility of additional IFN-γ effects. It is possible that, in the intact cell, a maximal down-regulatory effect on SL expression may require the coordinate involvement of other elements, upstream of position −1214 in the SL promoter, with the AP-1 site.

The AP-1 complex consisting of jun family homodimers or heterodimers with c-*fos* is an obvious candidate. A decrease in the activity of AP-1 could be caused by changes in (1) phosphorylation patterns of fos or jun or their respective pathway kinases, (2) the abundance of coactivators, or (3) competition by other members of the c-jun family that interfere with transcriptional activation [Mauviel et al., 1996]. A recent report suggests that the suppressive effect of IFN-γ on the expression of the macrophage scavenger receptor A gene (SR-A) in human fibroblasts occurs through the ability of IFN-γ-induced, activated STAT1-α to compete with AP-1/ets transcription factors, for limiting amounts of the co-activating regulatory factors CREB binding protein (CBP) or its CBP functional homologue p300 [Horvai et al., 1997]. The CBP/p300 proteins have been shown to interact with c-Fos [Bannister and Kouzarides, 1995] and c-Jun [Arias et al., 1994], an interaction that may be essential for AP-1 transactivating ability.

In summary, the work described here shows that down-regulation of SL gene expression by IFN-γ occurs almost exclusively due to transcriptional suppression via the AP-1 site, and that the down-regulatory IFN-γ effect dominates over activation signals present along the SL promoter region spanning 1,200 bp from the start site of transcription. The formation of complex(es) between an AP-1 consensus sequence and nuclear proteins appears to be abrogated by IFN-γ treatment of FFb. Whether this abrogation occurs because of differential changes in the expression of fos/jun family members or because of decreased binding of regulatory coactivation proteins to the AP-1 factor is the subject of further investigation.

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