Interferon-γ-mediated Inhibition of Cyclin A Gene Transcription Is Independent of Individual cis-Acting Elements in the Cyclin A Promoter

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Interferons (IFNs) affect cellular functions by altering gene expression. The eukaryotic cell cycle is governed in part by the periodic transcription of cyclin genes, whose protein products associate with and positively regulate the cyclin-dependent kinases. To understand better the growth inhibitory effect of IFN-γ on vascular smooth muscle cells (VSMCs), we compared the expression and activity of G1 and S phase cyclins in control and IFN-γ-treated VSMCs. IFN-γ treatment did not inhibit the G1 cyclins but did decrease cyclin A protein, mRNA, and associated kinase activity by 85, 90, and 90%, respectively. Nuclear run-on and mRNA stability determinations indicated that this decrease was the result of transcriptional inhibition. To investigate the molecular basis of this inhibition, we examined protein-DNA interactions involving the cyclin A promoter. Electromobility shift assays showed little change with IFN-γ treatment in the binding of nuclear proteins to isolated ATF, NF-Y, and CDE elements. In vivo genomic footprinting indicated that IFN-γ treatment changed the occupancy of chromosomal NF-Y and CDE sites slightly and did not affect occupancy of the ATF site. In a previous study of transforming growth factor-β1-mediated inhibition of the cyclin A promoter, we mapped the inhibitory effect to the ATF site; in the present study of IFN-γ treatment, functional analysis by transient transfection showed that inhibition of the cyclin A promoter persisted despite mutation of the ATF, NF-Y, or CDE elements. We hypothesize that IFN-γ inhibits cyclin A transcription by modifying co-activators or general transcription factors within the complex that drives transcription of the cyclin A gene.

The pleiotropic cellular effects of the interferons (IFNs) are thought to result from induction of gene expression. However, because IFNs increase expression of 50–100 different proteins, many without known function (1), the changes in cellular activity mediated by IFNs, including suppression of growth, are at best partially understood. Normal cellular replication is governed in part by periodic transcription of the cyclin genes, whose protein products associate with and positively regulate the cyclin-dependent kinases (cdks). The cdks are negatively regulated by phosphorylation or by association with a number of distinct proteins known as cdk inhibitors (reviewed in Refs. 2–5). Although growth inhibitory properties have been associated with several of the proteins induced by IFNs (6–8), there is little information about how genes induced by IFN signaling, particularly those affected by IFN-γ, interact directly with the machinery that regulates progression of the cell cycle.

IFN-α/β and IFN-γ are structurally dissimilar and bind to distinct cell-surface receptors. The pathways the IFN receptors use to signal the nucleus to induce gene expression share some components, and the various IFN types regulate expression of partially overlapping sets of genes (9). Although induction of gene expression by IFN-α/β is typically direct, requiring no new protein synthesis, that effected by IFN-γ, which targets a more diverse set of genes, can be direct or indirect (1). The precise subset of genes affected differs not only with the type of IFN but also with the type of cell (1).

In neoplastic hematopoietic cell lines, IFN-α treatment suppresses growth and is linked to decreased phosphorylation of retinoblastoma protein (10, 11) and CDC2 (12); decreased expression of c-Myc (11), cyclin D3 (13), cyclin A (11, 12), and CDC25A (13); and altered DNA binding by E2F (14). The independence of some of these effects may indicate the activation of parallel signaling pathways (11, 13). In primary bone marrow-derived macrophages, IFN-γ treatment inhibits phosphorylation of retinoblastoma protein (15) and expression of cyclin D1 coincident with its ability to inhibit DNA synthesis (16). In irreversibly arrested mammary epithelial cells, IFN-γ may mediate inhibition of cdk2 and cyclin E-associated kinase activity through p27kip1, which is up-regulated by a post-translational mechanism (17).

Because overlap among targets of IFN-γ and IFN-α/β is only partial, and because the profile of genes affected by individual IFNs varies by cell type, extrapolations about mechanisms among IFNs and cell types are uncertain. For example, cell

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### The abbreviations used are: IFN, interferon; cdk, cyclin-dependent kinase; VSMC, vascular smooth muscle cell; LM, ligand-mediated; PCR, polymerase chain reaction; DMS, dimethyl sulfate; CAT, chloramphenicol acetyltransferase; Rb, retinoblastoma protein; TGF, transforming growth factor; MHC, major histocompatibility complex.

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cycle arrest due to IFN-α treatment in hematopoietic cells corresponds to the G0 (13) or G0-G1 phase (11), whereas arrest due to IFN-γ treatment corresponds to the mid-G1 phase in mammary epithelial cells (18) and to the late G1 or early S phase in primary macrophages (15). These differences in the point of cell cycle arrest indicate functional differences in the underlying growth inhibitory mechanisms.

IFN-γ inhibits the growth of vascular smooth muscle cells (VSMCs). Because this effect occurs in vitro (19) as well as in vivo (20), IFN-γ may be clinically useful in vascular pathologies associated with high levels of VSMC proliferation (19). The growth inhibitory effect of IFN-γ on VSMCs has been linked with induction of 2′,5′-oligoadenylate synthetase (21), the first enzyme in the 2′,5′-adenylate IFN-regulated pathway of RNA degradation (22), and with suppression of c-myc mRNA and protein (23, 24). Little is known, however, about the effect of these changes in gene expression on components of the cell cycle machinery in VSMCs.

We evaluated the effect of IFN-γ on the expression of ckds and cdk inhibitors in VSMCs and the expression and activity of cyclins in VSMCs. IFN-γ treatment induced both proliferative (CDK6) and antiproliferative (P15) genes but did not inhibit kinase activity associated with the G1 cyclins. In contrast, cyclin A-associated kinase activity decreased markedly after IFN-γ treatment, in proportion to decreases in the expression of cyclin A mRNA and protein. Although this inhibition of cyclin A expression appeared to be caused by a decrease in transcription, electromobility shift assays, in vitro genomic footprinting, and functional analysis by transient transfection indicated that the decrease did not depend on individual cis-acting elements in the cyclin A promoter.

**MATERIALS AND METHODS**

**Cell Culture—**VSMCs were harvested from the aortas of male Sprague-Dawley rats (200–250 g) by enzymatic dissociation according to the method of Gunther et al. (25). The growth medium for these cells was Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10 μM HEPES (pH 7.4) (Sigma). Cells were cultured in quiescent by culture for 72 h in medium containing 0.4% calf serum. Rat VSMCs were used for experimentation within 4–6 passages from primary culture. Growth medium for human VSMCs (aortic; Clonetics) consisted of medium 199 (Life Technologies, Inc.) supplemented with 20% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml). These cells were used within 8 passages from primary culture. Recombinant rat IFN-γ (Life Technologies, Inc.) and human IFN-γ (Genzyme) were reconstituted in sterile water and stored in aliquots at –80°C until use. For some experiments, VSMCs were synchronized by incubation with medium containing 0.4% calf serum (HyClone) for 72 h before stimulation with growth medium (with or without IFN-γ). For in vitro footprinting experiments, human VSMCs were synchronized according to a two-step protocol as follows: cells in growth medium were first blocked in S phase for 20 h with hydroxyurea (1 mM) and then released and blocked in M phase for 20 h by adding new medium containing nocodazole (500 ng/ml). Synchronized monolayers were washed with phosphate-buffered saline, stimulated with fresh growth medium with or without IFN-γ, and harvested at defined intervals for DNA or RNA extraction.

**Protein Immunoblot and Cyclin-associated Kinase Assays—**Nuclear proteins were extracted as described (26), and protein concentration was determined by the Lowry method (DC protein assay, Bio-Rad). Total nuclear protein (30 μg) from control and IFN-γ-treated rat VSMCs was immunoprecipitated with antisera (2.5 μg) against cyclin A, D1, or E at 4°C for 2 h, followed by incubation at 4°C for 1 h with protein A-Sepharose beads (20 μl; Amersham Pharmacia Biotech). The beads were incubated for 20 min at 30°C in 40 μl of kinase buffer containing [γ-32P]ATP (6,000 cpm/pmol; 100 mM), with glutathione S-transferase-retinoblastoma protein (Rb) (40 μg/ml; Rb amino acids 769–921, Santa Cruz Biotechnology) used as phosphorylation substrate for anti-cyclin D1 immunoprecipitates, and histone H1 (200 μg/ml; Boehringer Mannheim) used as phosphorylation substrate for anti-cyclin A or E immunoprecipitates. Reaction products were resolved on 12% SDS-polyacrylamide gels and detected by autoradiography and phosphorimaging. Signals were quantified with the ImageQuant version 1.1 software analysis program (Molecular Dynamics).

**RNA Half-life Determination—**Quiescent rat VSMCs were stimulated with growth medium with or without IFN-γ for 24 h before addition of actinomycin D (5 μg/ml, Boehringer Mannheim). Cells were harvested for RNA extraction at intervals over the ensuing 8 h.

**RNA Blot Hybridization—**Total RNA was obtained from cultured cells by guanidinium isothiocyanate extraction followed by centrifugation through cesium chloride (28). The RNA was fractionated on 1.2% formaldehyde-agarose gels and transferred to nitrocellulose. Filters were hybridized with 32P-labeled cDNA probes (106 cpm/ml) in QuickHyb solution (Stratagene). Filters were washed in 30 mM sodium chloride, 3 mM sodium citrate, and 0.1% SDS at 55°C (probe and target RNA from single species) or at 25–42°C (probe and target RNA from different species), stored on phosphor screens for 12–16 h for phosphorimaging, and autoradiographed with Kodak XAR film for 48 h at –80°C. Previously described cDNAs used as probes in Northern analysis included rat cyclin A (29), human CDK2, CDK4, and CDK6 (30), which were provided by E. Harlow (Massachusetts General Hospital, Boston), and human Stat 1α (31), which was provided by X. Y. Fu (Yale University, New Haven, CT). Additional rat cDNA probes were generated by reverse transcription-polymerase chain reaction (PCR) from total rat lung RNA with primers derived from the corresponding reported rat or mouse sequences as follows: p15, 5′-ATGATGGTGGGACCCACCACCGGAGGAAAGGACGGATCC-3′ (32); p21, 5′-GGTTGACGTTGAGCTGGTAA-3′ and 5′-TGGTTGGCTCCCTTTTTT-3′ (33); and p27, 5′-ATGTCAAAAAAAAAGGAGAGCTGTT-3′ and 5′-GGAGGCCGCGGCTTCTGGGCG-3′ (34). 32P-End-labeled oligonucleotides complementary to 18S (5′-ACGATATGCTGATCGTCAGTCCAC-3′) or 28S (5′-CAGATTGACGATGCTCCACAC-3′) RNA were randomized and incubated with the filters to correct for differences in RNA loading. Phosphor screens were scanned, and radioactive signal intensity was determined with the ImageQuant version 1.1 software analysis program (Molecular Dynamics).

**Nuclear Run-on Analysis—**Quiescent rat VSMCs were stimulated for 24 h with growth medium with or without IFN-γ. The cells were lysed and mouse sequences as follows: p15, 5′-ATGATGGTGGGACCCACCACCGGAGGAAAGGACGGATCC-3′ (32). Nuclear suspension samples (200 μl) were incubated with 0.5 μg each of CTP, ATP, and GTP and 250 μCi of 32P-labeled UTP (3000 Ci/mmol, NEN Life Science Products) for 30 min. The samples were extracted with phenol/chloroform, precipitated, and resuspended in water for scintillation counting; equivalent amounts of radioactive probe were added to nitrocellulose filters bearing target cyclin A and control β-actin cDNAs. Filters were hybridized for 72 h at 40°C in the presence of formamide, washed, and subjected to autoradiography. Radioactivity hybridizing to cyclin A target cDNA was normalized to the activity of the corresponding β-actin control.

**In Vivo DNA Footprinting by Ligation-mediated (LM) PCR—**In vivo dimethyl sulfate (DMS) treatment was performed as described by Mueller and Wold (37) with some modification (38). Briefly, in vivo DNA methylation, intact human VSMC monolayers were exposed to 0.1% DMS at room temperature for 2.5 min. Cells were washed and lysed, and methylated DNA was recovered and deproteinized overnight at 37°C. For in vitro methylation, naked genomic DNA was treated with 1% DMS for 4 or 6 min at room temperature. Methylated DNA was cleaved by treatment with 1 μl papain. Human VSMCs were treated with DMS and papain in the method of Wilson et al. (39) with some modification. Cells in a monolayer were permeabilized with 0.05% saponin (Sigma) and then exposed to DNAse I (Boehringer Mannheim, 150 Kunitz units/ml) for 4 min at room temperature. Genomic DNA was treated in vitro with DNAse I (0.03 Kunitz units/ml) for 8 min at room temperature. LM-PCR was performed according to the method of Garrity and Wold.
IFN-γ Inhibits Cyclin A Gene Transcription

as described by Patterson et al. (38). The 5’ set of primers was derived from the published human cyclin A promoter sequence (40) and designed according to the criteria of Mueller et al. as described by Ausubel et al. (28). Primers 5’-1, 5’-2, and 5’-3, with their respective positions (40) and Tm values (42) as follows: 5’-CCCTAAATCTTCTCAGTCTCCC-3’ (214 to 195) (Tm 62.9 °C), 5’-TCCGGCCCGAGCACTGAGTCTCCC-3’ (174 to 150) (Tm 64.9 °C), and 5’-CCACGCTCGTCTCGGTGCCC-3’ (168 to 140) (Tm 68.5 °C).

The 3’ set of primers was described by Zwick et al. (41). For first-strand synthesis, DNA was denatured at 95.0 °C for 5 min, annealed at 55.0 °C for 30 min, and extended at 76.0 °C for 10 min. Ligation to asymmetric linkers was performed for 6 h at 17 °C. PCR cycling conditions for the 5’ primer set were 95 °C for 1 min, 65 °C for 2 min, and 76 °C for 3 min, for a total of 21 cycles. PCR cycling conditions for the 3’ primer set were 95.0 °C for 1 min, 68 °C for 2 min, and 76 °C for 3 min, for a total of 21 cycles. Two cycles of primer extension were performed. Primers were annealed at 68.5 °C with 32P-end-labeled dATP. Ligation and amplification reactions were resolved by electrophoresis through denaturing agarose gels and transferred to nitrocellulose filters, which were hybridized with 32P-labeled cDNA probes as indicated. Filters were washed as described under "Materials and Methods" and analyzed by exposure to radiography film and phosphor screens.

Electromobility Shift Assay—Electromobility shift assays were performed as described (42), with modifications. Sense strand sequences of NF-Y, 5’-GAGCGCCTTCATGCTCCATTTAATGC-3’ (65 to 36), and CDE-CHR, 5’-CAATGATCGAGCACTGCAAAG-3’ (43 to 16). (Underline indicates consensus protein-binding sites.)

The ATF 22-mer probe was described elsewhere (42). Typical binding reactions contained double-stranded DNA probe (20,000 cpm), 1 µg of poly(dI-dC)·poly(dI-dC), 50 mM KCl, 20 mM HEPES (pH 7.5), 10 mM MgCl2, 10% glycerol, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, and 10 µg of nuclear extract in a final volume of 25 µL. The specificity of binding interactions was assessed by competition with a 50-fold excess of unlabeled double-stranded oligonucleotide of identical sequence.

Transient Transfection Analysis of Promoter Activity—Reporter constructs were made by ligating wild-type (42) or mutated cyclin A promoter fragments into the promoterless pCAT basic vector (Promega). Deletion fragments and mutants were generated by PCR with Pfu polymerase (Stratagene) by using standard cycling conditions. Primers for the −83 to −5 fragment were forward 5’-GAATTCACGTTAACGGA-3’ and reverse 5’-CCGGCAGGACGCGTGTCC-3’. Site-directed mutagenesis of the reverse NF-Y site (ATGGG) was performed by PCR from a wild-type template with the forward primer 5’-GAGCGCCTTCATGCTCCATTTAATGC-3’ and CDE-CHR, 5’-CAATGATCGAGCACTGCAAAG-3’ (43 to 16). (Underline denotes mutant bases.) The ATF mutant was described elsewhere (42). Mutations were confirmed by dideoxy-sequencing with Thermosequenase (Amersham Pharmacia Biotech). Rat VSMCs in 6-well plates were transfected with reporter plasmid (2 µg) by using standard calcium phosphate precipitation. Rat p15, which contributes to and inhibits, respectively, activity associated with D-type cyclins (46), were both induced by approximately 2-fold in cells treated with IFN-γ in comparison with time-matched controls.

IFN-γ Inhibits Expression and Activity of Cyclin D1 but Not Those of Cyclin D1 or E—IFN-γ treatment is associated with arrest of the cell cycle in the G1 phase in fibroblasts (47, 48) and mammary epithelial cells (18). In myeloid cells, growth arrest associated with IFN-γ is linked to a decrease in expression of a principal G1 cyclin, cyclin D1, even when the cytokine is added late in the G1 phase (16). In light of these observations and the induction of CDK6 and p15 mRNAs in VSMCs that we observed (Fig. 1), we next assessed the effect of IFN-γ on G1 and G2-S phase cyclin expression and activity.

Cyclin D1 expression in rat VSMCs was also induced by IFN-γ at both the mRNA (data not shown) and protein levels, with immunoblot analysis showing a 3.8-fold increase in the amount of cyclin D1 (Fig. 2A). IFN-γ treatment had no significant effect on cyclin E protein levels, whereas it decreased cyclin A expression by 85% (Fig. 2A); this decrease was present also at the mRNA level (data not shown).

We then assayed cyclin-associated kinase activity to determine how these changes affected net cell cycle kinase activity. Despite the IFN-γ-mediated induction of p15, we found that cyclin D1-associated kinase activity increased 1.7-fold and that cyclin E-associated kinase activity changed little. Cyclin A-associated kinase activity, on the other hand, was inhibited by 90% (Fig. 2B).

These results are consistent with an IFN-γ-mediated arrest of the VSMC cycle that involves late G1 or G2-S phase regulatory mechanisms. Because cyclin A-associated kinase activity was inhibited in proportion to the decrease in cyclin A expression, and cyclins D1 and E were not inhibited, we investigated the means by which IFN-γ decreased cyclin A expression.

IFN-γ Inhibits Transcription of the Cyclin A Gene Without Affecting mRNA Stability—IFNs can inhibit gene expression by induction and activation of RNA degradation pathways (49)
by trans-post-translational mechanisms (50). To determine the underlying mechanism of the decrease in cyclin A mRNA mediated by IFN-γ, we directly assessed the mRNA half-life and transcriptional rate. The stability of cyclin A mRNA in the presence or absence of IFN-γ was evaluated by inhibiting transcription in rat VSMCs with actinomycin D. The calculated cyclin A mRNA half-life of 2.7 h was not affected by IFN-γ treatment (Fig. 3A). In nuclear run-on experiments, IFN-γ did not change transcription of the control β-actin gene, but it inhibited radioactive signal hybridizing to cyclin A sequences by 67% (Fig. 3B). These studies indicate that the decrease in expression of cyclin A resulting from IFN-γ treatment is due to inhibition of transcription.

Transcriptional Activity of the Proximal Cyclin A Promoter Is Inhibited by IFN-γ—Our finding that IFN-γ treatment decreased cyclin A transcription in VSMCs suggested that we could map the inhibitory effect to cis-acting elements in the cyclin A promoter and so identify downstream signaling pathways affected by the cytokine. In growing rat VSMCs transiently transfected with cyclin A promoter constructs driving expression of a CAT reporter gene (Fig. 4), the activity of the −406/+205 fragment was similar to that of the pCAT3 control plasmid. Deletion of 5′ sequences to yield the fragment −266/+205 had relatively little effect on overall activity, despite the removal of a consensus API1-binding site. The further removal of 3′ sequences including three consensus Sp1 sites and 3′ sequences including two consensus E2F sites, one consensus p53 site, and a potential composite Yt/Spi1 element (40), to yield the −83/+5 fragment, decreased activity to approximately 30% of the activity of the reference plasmid. Inhibition of cyclin A promoter activity by IFN-γ was retained in all constructs tested. The degree of inhibition by IFN-γ, ranging from 55 to 66%, was similar to that found in the nuclear run-on analysis (Fig. 3B). On the basis of these findings, we narrowed our search for an IFN-γ-responsive element to the −83/+5 fragment; further deletions within this fragment severely impaired promoter activity (data not shown).

Protein Occupancy of Proximal Cyclin A Promoter Elements Shows Little Change with IFN-γ Treatment—To determine whether IFN-γ treatment changed protein binding to elements in the proximal cyclin A promoter, we performed in vivo footprinting analysis of protein binding to the −266/+205 fragment. The footprint revealed a cluster of elements for the AP1 family, including two overlapping consensus AP1-binding sites and a potential composite Yt/Spi1 element (40), to yield the −83/+5 fragment. The footprinting analysis revealed no significant changes in protein binding to these elements upon IFN-γ treatment, indicating that the decrease in cyclin A mRNA transcription is not due to changes in protein binding to these elements.
IFN-γ Inhibits Cyclin A Gene Transcription

Proximal human cyclin A promoter. Competition assays with 50-fold excess of unlabeled probe were performed to verify specificity.

Quiescent human VSMCs were stimulated with growth medium with vehicle or human IFN-γ at 10 ng/ml for 24 h before harvesting. A, cellular monolayers were permeabilized with lysolactin and treated with DNase I in vitro. Purified human VSMC DNA that had been treated in vitro was used as a control. Protection of the coding strand from the action of DNase I was analyzed by LM-PCR with the 3′ set of primers described under “Materials and Methods.” The scale shows nucleotide positions within the human cyclin A promoter. Protected regions are indicated by heavy bars and consensus binding sites by thin bars. Asterisks correspond to hypersensitive residues. B, nuclear proteins were extracted from human VSMCs and used for electromobility shift analysis of protein binding to the ATF, NF-Y, and CDE-CHR elements in the proximal human cyclin A promoter. Competition assays with 50-fold excess of unlabeled probe were performed to verify specificity. Arrows indicate specific protein-DNA complexes.

Fig. 5. Protein interactions with the proximal cyclin A promoter defined by in vitro DNase I footprinting and electromobility shift assay. Quiescent human VSMCs were stimulated with growth medium with vehicle or human IFN-γ at 10 ng/ml for 24 h before harvesting. A, cellular monolayers were permeabilized with lysolactin and treated with DNase I in vitro. Purified human VSMC DNA that had been treated in vitro was used as a control. Protection of the coding strand from the action of DNase I was analyzed by LM-PCR with the 3′ set of primers described under “Materials and Methods.” The scale shows nucleotide positions within the human cyclin A promoter. Protected regions are indicated by heavy bars and consensus binding sites by thin bars. Asterisks correspond to hypersensitive residues. B, nuclear proteins were extracted from human VSMCs and used for electromobility shift analysis of protein binding to the ATF, NF-Y, and CDE-CHR elements in the proximal human cyclin A promoter. Competition assays with 50-fold excess of unlabeled probe were performed to verify specificity. Arrows indicate specific protein-DNA complexes.
In vivo DMS footprinting analysis of protein interactions with the proximal cyclin A promoter in human VSMCs, with and without inhibition by IFN-γ. Human VSMCs blocked in G₂ were released into growth medium containing vehicle (control) or human IFN-γ at 10 ng/ml and harvested at the indicated time points. A, Northern analysis was performed on RNAs from cells treated in parallel. Two cyclin A mRNA isoforms, 2.7 kilobases and 1.8 kilobases, are indicated. B and C, DNA in cellular monolayers was methylated with DMS in vivo for 2.5 min; purified DNA was methylated with DMS in vitro for 4 or 6 min (control). Methylated DNA was cleaved and used in LM-PCR. The coding strand (B) was analyzed with the 5’ primer set, and the noncoding strand (C) was analyzed with the 5’ primer set. In the vicinity of the ATF-binding site, diamond a indicates a hypersensitive guanine residue at −70, and asterisk b indicates a methylated adenine at −75 seen only in vivo. In the vicinity of the NF-Y site, diamond c and asterisk c indicate a protected guanine at −56 and a hypersensitive adenine at −58, respectively, and diamond d indicates a protected guanine at −60. In the region of the CDE, diamond e denotes guanines at −32 and −33 that show some variation in protection through the time course.

Inhibition of Cyclin A Promoter Activity by IFN-γ Is Not Affected by Mutation of Protein-bound Elements—The electromobility shift assays and in vivo footprinting with DNase I indicated little apparent change with IFN-γ treatment in protein binding to cis-acting elements in the proximal cyclin A promoter. In vivo footprinting with DMS showed complex and relatively subtle changes in binding through an extended time course. Whereas protection of the ATF site was relatively invariant, the changes in foot printing in the CDE and NF-Y elements suggested that they might be involved in mediating the inhibitory effect of IFN-γ. To determine the functional importance of these sites for promoter inhibition by IFN-γ, we performed further transient transfection experiments with mutated cyclin A promoter constructs. In transiently transfected growing cells, mutation of the ATF, NF-Y, and CDE-CHR sequences led in each case to a decrease in cyclin A promoter activity (Fig. 7) in comparison with the activity of the wild-type (parent) construct (−266/+205, Fig. 4). The greatest reduction in activity occurred with the ATF site mutant, and the activity of the NF-Y mutant was closest to that of the wild type. Nevertheless, in transiently transfected cells treated with IFN-γ, inhibition of promoter activity was not affected significantly by any of these mutations; with all three constructs, activity fell by 55–61% with IFN-γ treatment.

**DISCUSSION**

We have investigated the molecular basis of VSMC growth inhibition by IFN-γ. First we found that whereas most of the cdks and cdk inhibitors were not affected by exposure to IFN-γ, CDR6 and p15 were both induced at the mRNA level (Fig. 1). Despite these changes, however, cyclins D1 and E and the activities of their associated kinases were not inhibited (Fig. 2, A and B), although cyclin A protein and cyclin A-associated kinase activity decreased substantially. These findings suggest that IFN-γ treatment of VSMCs results in a relatively late G₁ or G₁-S junction cell cycle block. The simplest explanation for these findings is that the decrease in cyclin A protein leads to the decrease in cyclin A-associated kinase activity; this observation suggested that regulation of cyclin A expression might be a target of IFN-γ-mediated signaling in VSMCs. Cyclin A mRNA stability and nuclear run-on studies indicated in turn that the decrease in cyclin A expression resulting from IFN-γ treatment was due to inhibition of cyclin A gene transcription.

Cyclin A promotes cell cycle progression by associating with and stimulating cyclin-dependent kinases CDC2 and CDK2 and is a critical regulator of the cell cycle at both the G₁-S junction (53, 54) and during the G₂-M transition (53). Like that of other cell cycle molecules, the level of cyclin A protein is controlled primarily by cyclical changes in mRNA expression. The human cyclin A promoter has been cloned (40) and characterized in a number of growth-related contexts such as contact inhibition (42), adhesion dependence (55), and TGF-β1 treatment (56, 57). Inhibition of cyclin A promoter activity by TGF-β1 requires an intact ATF site, as we (57) and others (56) have reported previously; this effect involves decreased phosphorylation of CREB and ATF-1 (57). We hypothesized that it would be possible to identify the cis-acting elements in the cyclin A promoter required for its inhibition by IFN-γ. In turn, this identification would point to downstream targets of IFN-γ signaling that are critical to regulation of cell cycle progression,
as our analogous identification had pointed to downstream targets of TGF-β1 signaling.

Our initial transient transfections involved cyclin A promoter constructs driving expression of a luciferase reporter gene. With this system, however, we encountered a problematic nonspecific effect of IFN-γ on luciferase activity that extended even to control promoters. Others have also reported difficulties with using luciferase as a reporter for analysis of IFN-γ-mediated effects (58). The CAT system, in contrast, did not show nonspecific effects with IFN-γ, and we used it for all subsequent transfections.

Transient transfections with a CAT reporter showed that the inhibitory effect of IFN-γ was retained in an 88-base pair fragment of the proximal promoter. This fragment contained consensus binding sites for ATF and NF-Y transcription factors, as well as the CDE and CHR elements (40, 41, 52). One model of regulation of cyclin A promoter activity during the cell cycle involves phase-specific protein binding to the CDE and CHR elements to periodically repress transcriptional activation mediated through the upstream positive regulatory elements NF-Y, ATF, and Sp1 (41, 52). We anticipated that the inhibitory effect of IFN-γ would be mediated through one of the elements between bases −83 and +5.

However, complementary lines of evidence from in vivo DNase I footprinting (Fig. 5A) and electromobility shift assays (Fig. 5B) argued against a substantial change with IFN-γ treatment in the level of protein binding to cis-acting elements in the −85/+5 cyclin A fragment. Further investigation by in vivo DMS footprinting (Fig. 6, B and C) showed only limited changes in the protection pattern with IFN-γ treatment, despite confirmation of inhibition of cyclin A mRNA expression by as much as 95% (Fig. 6A). We then evaluated the functional significance of protein binding to these sites by transiently transfecting mutated promoter constructs. These experiments indicated the functional importance of the ATF, NF-Y, and CDE-CHR binding sites in the overall activity of the cyclin A promoter but showed further that inhibition by IFN-γ occurs regardless of mutations at these specific sites. The mechanism of down-regulation of cyclin A promoter activity by IFN-γ is thus not a simple extension of the repressive mechanism that normally operates in G1 (41, 52) nor does it involve the ATF site in a manner analogous to down-regulation by TGF-β1 (56, 57).

Change in protein-DNA binding is by no means required for altered transcriptional activity. For example, in vivo footprinting indicated no significant difference in protein interaction with the essential serum response element and flanking c-fos promoter sequences after A431 cells had been stimulated with epidermal growth factor, a treatment that resulted in dramatic induction and then repression of c-fos mRNA as determined by Northern analysis (59). In the case of the cyclin A promoter, we previously found that TGF-β1 treatment led to changes in transcription factor activity through modifications not reflected in protein abundance or DNA binding per se (57). These findings suggested that for transcription of certain genes, specific protein-DNA interactions are maintained and do not have to be disrupted to affect either activation or repression of transcriptional activity. In our analysis of TGF-β1 signaling, however, we identified a functional role for the ATF site in the cyclin A promoter, because its mutation eliminated the inhibitory effect of TGF-β1. With IFN-γ, we found that change in protein-DNA binding involving the proximal cyclin A promoter was quite limited and that mutation of the protein-bound DNA elements in the proximal promoter did not reduce the inhibitory effect of IFN-γ.

Indeed, although IFN-γ inhibits transcription of many genes (60–66), no IFN-γ-specific inhibitory elements have been identified. One proposed mechanism for inhibition of gene expression by IFN-γ, derived from studies of the macrophage scavenger receptor gene promoter, involves competition between positive regulatory factors (AP-1, ETS) and a direct target of IFN-γ signaling (activated Stat 1a). AP-1, ETS, and Stat 1a all depend on the essential co-activators CBP and p300 for full transactivation, so when cellular levels of these co-activators are limiting, the increase in activated Stat 1a due to IFN-γ treatment limits AP-1 and ETS-mediated transactivation by titrating away the co-activators (67). Such a mechanism, however, requires DNA binding by individual positive regulators, and thus inhibition should be susceptible to mutation of these individual cis-acting elements; our transfections with site-directed mutations of these elements showed that cyclin A promoter activity was still fully inhibited by IFN-γ. Moreover, co-transfection of a p300 expression plasmid in transient transfection analysis did not relieve IFN-γ-mediated inhibition of cyclin A promoter activity (data not shown).

In contrast to our limited understanding of how IFN-γ inhibits gene expression, functionally significant cis-acting elements mediating transactivation in response to IFN-γ signaling have been precisely identified in a number of genes (68, 69). Nevertheless, despite the directness of signaling through the JAK-Stat pathway, alternative mechanisms for gene induction by IFN-γ that do not require changes in cis element binding do exist. Class II MHC genes are strongly induced by IFN-γ, but expression of the transcription factors that bind directly to class II MHC promoters is not affected by IFN-γ (70). In vivo footprinting of class II MHC gene promoters showed that promoter occupancy in the class II MHC-negative mutant cell line RJ2.2.5 was no different from that in the class II MHC-positive Raji line, implicating a defect in a co-activator protein (71). This co-activator was subsequently identified as class II transactivator (CIITA) (72), an IFN-γ-inducible protein whose expression is strongly correlated with that of the class II MHC genes (73). CIITA has separable functional domains: the N-terminal domain provides transcriptional activation and the C-terminal domain confers promoter specificity (74). Mutation of any one of five distinct elements in the proximal promoter of the class II MHC DRA gene resulted in impaired CIITA-dependent transactivation (74), consistent with a model of CIITA as a second tier co-activator that does not bind DNA directly but activates transcription of specific promoters by a mechanism that involves multiple cis-acting elements.

IFN-γ can induce gene expression by direct and indirect mechanisms and can also inhibit gene expression, presumably through indirect pathways. Despite the opposite natures of these effects, induction of class II MHC transcription and inhibition of cyclin A transcription resulting from IFN-γ treatment both share a similar dissociation of promoter activity from individual protein-DNA interactions in the proximal promoter region. RNA polymerase II-mediated transcription involves a complex of factors that probably varies in composition from one promoter to the next (75). Evidence from yeast indicates that some cell cycle-regulated genes have unique requirements for particular components of the general transcription factor TFIIID complex (76). Indeed, the complex associated with cyclin A expression is known to be functionally distinctive, as a specific mutation in TAFIIU/250, the largest subunit of TFIIID, disrupts transcription from the cyclin A but not the c-fos or c-myc promoter (77). This suggests that transcription complexes assembling on growth-related genes may be selectively modified in response to changes in cellular growth status. Our findings about IFN-γ support the existence of a means of regulating transcriptional activity of the cyclin A promoter that does not involve direct DNA-protein interactions. More likely it
involves alteration of co-activators or other components of the transcription complex that assembles on the cyclin A gene. Given the array of cellular targets affected by IFN-γ, and its known growth inhibitory effects on a number of distinct cell types, we speculate that its regulatory capacity may extend to components of the transcriptional apparatus that drives expression of the cyclin A gene.

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