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Critical Role of the Transcription Factor AP-1 for the Constitutive and Interferon-Induced Expression of IFI 16

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Abstract IFI 16 is a member of the HIN-200 family of transcriptional regulators that suppress cell growth, modulate the cell cycle and have been linked to cellular differentiation. We hypothesized that the activity of IFI 16 depends on its level of expression and therefore studied the transcriptional activity of the *IFI 16* promoter. A discrete sequence within the 5′ untranslated region was required for constitutive activity of the promoter and the functional motif within this region was shown to be a consensus AP-1 site. Interestingly, this AP-1 site was also critical for IFN-induced activation of the promoter and consistent with these observations, treatment of cells with IFNγ resulted in a rapid and robust induction of AP-1 activity that preceded expression of IFI 16. These experiments define the transcriptional mechanisms of *IFI 16* gene regulation and provide evidence suggesting that AP-1 activation may be an important event in IFN signaling. J. Cell. Biochem. 89: 80–93, 2003. © 2003 Wiley-Liss, Inc.

Key words: IFI 16; interferon; AP-1; transcription

IFI 16 is a member of the HIN-200 family of proteins that are believed to modulate cell growth, differentiation, and anti-viral responses [Johnstone et al., 1998; Landolfo et al., 1998; Johnstone and Trapani, 1999; Rolle et al., 2001]. IFI 16 is constitutively expressed in a range of tumor cell lines [Trapani et al., 1992] and a variety of normal human cell types [Wei et al., 2002]. In common with other family members, expression of IFI 16 can be potently induced by type I and type II IFNs [Dawson and Trapani, 1995a] and it is differentially regulated during hemopoietic differentiation [Dawson et al., 1998]. Much of our understanding of HIN-200 family biology is derived from studies of murine family members. Over-expression of the mouse family proteins p202 and p204 slows growth of some cell lines [Lembo et al., 1995; Gribaudo et al., 1999] and p202 may function as a tumor suppressor, as it can inhibit growth of human cancer cell lines both in soft agar and as tumors in immune-deficient mice [Wen et al., 2000; Wen et al., 2001]. HIN-200 family proteins may also play a role in cellular differentiation. p202 and p204 appear to play a reciprocal role in myoblast differentiation as p202 inhibits the expression of the differentiation-linked transcription factor myoD, but p204 is induced by myoD and enhances myoD DNA binding by inhibiting the activity of Id2 [Datta et al., 1998; Liu et al., 2000; Liu et al., 2002]. Furthermore, the human family members, IFI 16 and myeloid nuclear differentiation antigen (MNDA), are expressed at discrete stages during development of the myeloid lineage [Dawson et al., 1998].

The biological properties of HIN-200 proteins are believed to be a consequence of their ability to modulate transcription [Landolfo et al., 1998; Johnstone and Trapani, 1999]. Numerous interactions between HIN-200 proteins and other DNA binding factors such as those between p202 and c-fos, c-jun, NF-κB subunits, pRb, and E2F4/DP-1 have been recorded [Min et al., 1996; Choubey and Gutterman, 1997]. The growth suppressive effects of HIN-200 family proteins

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are proposed to be caused by inhibiting the binding of the rRNA specific transcription factor UBF1 to rDNA promoters [Liu et al., 1999] and/or by a mechanism involving the tumor suppressor protein pRb [Hertel et al., 2000]. IFI 16 can functionally interact with p53 [Johnstone et al., 2000] and is capable of acting as a transcriptional repressor when bound to a "target" promoter [Johnstone et al., 1998].

The expression of IFI 16 has been shown to correlate with the monocyte/macrophage population during myeloid differentiation [Dawson et al., 1998]. In addition, IFI 16 is specifically expressed in the basal layer epithelium of various human organs [Gariglio et al., 2002; Wei et al., 2002]. This distinctive pattern of IFI 16 expression suggests that transcription of *IFI 16* is tightly regulated. However, the molecular mechanisms that control either the constitutive or inducible expression of IFI 16 remain unknown. Understanding the regulation of IFI 16 expression should assist the characterization of its biological functions and provide some insight into the mechanisms of interferon-mediated gene expression. Therefore, we cloned a 2.5 kb fragment lying upstream of the IFI 16 gene and used this sequence to identify regions of the 5′ promoter that are important for the transcriptional control of this gene. We identified an AP-1 site at position +63 to +70, designated the IFI 16 activation element (IAE), that is necessary and sufficient for promoter activity in cells that constitutively express IFI 16. The integrity of this sequence was also necessary for normal activation of the *IFI 16* promoter by IFNγ and a single copy of the wild type IAE was sufficient to confer IFNγ-responsiveness upon a CAT reporter gene. The protein complex that bound to the IAE had the characteristics of a bona fide AP-1 complex. Interestingly, the DNA binding activity of AP-1 was augmented following treatment of cells with IFNγ and rapid, transient binding of AP-1 to the IAE after IFNγ stimulation preceded induction of the endogenous *IFI 16* gene. These data indicate that IFNγ may induce the activation of AP-1 and mediate the transcription of interferon-responsive genes through functional AP-1 binding sites.

METHODS

Cell Culture and Reagents

Primary fibroblasts derived from human neonatal foreskin were purchased from The Alfred Hospital (Melbourne, Australia). The human cervical cancer cell line HeLa, and HL-60 myeloid cell line were obtained from ATCC (Rockville, MD). All cell types were grown in DMEM (CSL, Parkville, Australia) supplemented with 10% FCS (CSL Parkville, Australia), 2 mM glutamine and 100 U/ml Penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY). Monoclonal antibody (mAb) SC-44X (Jun family) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-tubulin was purchased from Sigma Chemical Co. (St. Louis, MO). mAb 1G7 anti-IFI 16 was generated as previously described [Dawson and Trapani, 1995b]. 12-*O*-tetradecanoyl phorbol-13-acetate was purchased Sigma Chemical Co. (St. Louis, MO) and IFNγ was purchased from Amersham (Sydney, Australia).

Oligonucleotides and Plasmids

The following oligonucleotides were used to generate CAT reporter constructs:

1. 5′-CCGTAAAGCTTGTGTAGAAATTAGTAAATCA AG-3′;
2. 5′-CCGTAAAGCTTTGTGGTATGAATTATGGAGC-3′;
3. 5′-CCGTAAAGCTTGGTCAAGAGGACAGCTAG-3′;
4. 5′-CCGTAGTCGACAAATCAGGAACAGTACTCAC-3′;
5. 5′-CCGTAGTCGACTCACTTAGTTAGCTGACTAG-3′;
6. 5′-CCGTAAAGCTTCTAGTCAGCTAACTAAGTGA-3′;
7. 5′-CCGTAAAGCTTCTTTGCAGATACTTCATTTTC-3′;
8. 5′-CCGTAGTCGACCCCAGAAACGGAACCGC-3′;
9. 5′-CCGTAAAGCTTCCAAGGCCTTTTTTCCTTG-3′;
10. 5′-CCGTAGTCGACGGATGAGAAATTACTTTAATG-3′;
11. 5′-CCGTAAAGCTTCATTAAAGTAATTTCTCATCC-3′;
12. 5′-CTAACT**CC**GT**TC**CTCAACCAAGGCCTTTTTTC-3′;
13. 5′-GTTGAG**GA**AC**GG**AGTTAGCTGACTAGTGCT-3′.

Deletion mutants of the IFI 16 promoter were generated by PCR as follows: pCATIFI 16(−2484 to +279) was generated using primers 1 and 4; pCATIFI 16(−1471 to +279) was generated using primers 2 and 4; pCATIFI

16(−658 to +279) was generated using primers 3 and 4; pCATIFI 16(−658 to +65) was generated using primers 3 and 5; pCATIFI 16(+46 to +279) was generated using primers 6 and 4; pCATIFI 16(+94 to +279) was generated using primers 4 and 7; pCATIFI 16(+46 to +159) was generated using primers 6 and 8; pCATIFI 16(+71 to +159) was generated using primers 8 and 9; pCATIFI 16(−658 to +113) was generated using primers 3 and 10; pCATIFI 16(−134 to +65) was generated using primers 5 and 11. The production of the pCATIFI 16(−2484 to +279)ΔIAE was produced by splice overlap using primers 1 and 13 to produce fragment 1 and primers 12 and 4 to produce fragment 2. A mixture of fragments 1 and 2 was subsequently used as a template for a third PCR reaction using primers 1 and 4. PCR sense primers contain Hind III sites (5′AAGCTT3′) and antisense primers contain Sal I sites (5′GTCGAC3′) and these sites were used to sub-clone the promoter sequences into pCATBasic (Promega, Annandale, Australia) upstream of the CAT reporter gene. pCATIAE and pCATΔIAE were produced by digesting oligos IAE(A) and ΔIAE(A) respectively with Hind III and Sal I and sub-cloning the products into pCATBasic (Promega, Annandale, Australia). Each construct was sequence verified using ABI Big dye Terminator (Applied Biosystems, Melbourne, Australia) and sequencing gels were performed by AGRF (WEHI, Melbourne, Australia).

Calcium Phosphate Transfection

Cells were transfected as previously described [Shi et al., 1991]. Briefly, DNA was precipitated in a 1:1 mixture of HBS (140 mM NaCl, 5 mM KCl, 1.5 mM Na_2HPO_4, 6 mM dextrose, 25 mM HEPES, pH 7.05) and 1 M $CaCl_2$ for 30 min then added to petri dishes seeded with 2×10^5 cells. Media was changed after 24 h.

CAT Assays

CAT activity was assayed as previously described [Shi et al., 1991]. Briefly, cell pellets were resuspended in 0.25 M Tris, pH 8 (100 μl) and lysed by 3 cycles of freeze/thawing. The reaction was initiated by adding 4 mM acetyl coenzyme A (35 μl) and ^{14}C chloramphenicol 1 μl (Amersham, Sydney, Australia) and was performed at 37°C for 4 h. Products were extracted using ethyl acetate and separated by thin layer chromatography and identified and analyzed using a phospho-imager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) or QuantityOne software (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assays

The following oligonucleotides were used as probes in EMSAs. Prior to annealing, radiolabeling of these oligonucleotides was performed using T4 polynucleotide kinase (NEB, Inc., Beverly, MA). Oligonucleotides complementary and in reverse order to those listed below were produced in order to make double stranded oligonucleotide probes and competitors. The IAE is underlined and mutated nucleotides are in bold.

- IAE(A) 5′CCGTAAAGCTTTCAGCTAACTAAGTGACTCAACCAAGGTCGACTACGG3′;
- ΔIAE(A) 5′CCGTAAAGCTTTCAGCTAACTCCGT**TC**CTCAACCAAGGTCGACTACGG3′;
- IAE(B) 5′GCTAACTAAGTGACTCAACC3′;
- IAE(B)ΔIRF 5′GCTAACT**TT**GTGACTCAACC3′;
- IAE(B)ΔAP1 5′GCTAACTAAGTGACT**GT**ACC3′;
- AP-1 5′ATGGTTGACTCATCTGA CTC3′;
- ΔAP-1 5′ATGGTTGACT**GT**TCTGACTC3′;

GAS 5′TTTCAAGGATTTGAGATGTATTTCCCAGAAAAG3′

Nuclear extracts were prepared as previously described [Clarke et al., 1995] and nuclear proteins (5 μg) were mixed with ^{32}P labeled oligonucleotide probes (50,000 cpm) in buffer containing 20 mM Tris, pH 8, 6 mM KCl, 2 mM $MgCl_2$, 12% glycerol and incubated at room temperature for 30 min. For competition experiments, unlabeled oligonucleotides were added 10 min before the labeled probe. For supershift experiments, 2 μg of antibody was added 1 h prior to the labeled probe. Protein/DNA complexes were separated from unbound probe on a TBE non-denaturing gel that was subsequently dried and analyzed by autoradiography.

SDS–PAGE and Western Blotting

Nuclear extracts were prepared as previously described [Clarke et al., 1995] and nuclear proteins (5 μg) were mixed with five times Laemli sample buffer, boiled for 3 min, separated on a 10% polyacrylamide gel and proteins were transferred to PVDF membranes using a Transblot cell (Bio-rad, Hercules, CA). Membranes were blocked with 2% BSA in PBS +0.05%

Tween-20 and proteins were detected using specific monoclonal antibodies (1G7 supernatant was used at 1:100, anti tubulin was used at 1:3,000) and anti-mouse HRP (used at 1:5,000) (Amrad, Melbourne, Australia) and visualized by enhanced chemiluminescence (Amersham, Sydney, Australia).

RESULTS

Extensive analysis of the expression of IFI 16 in normal human tissues and revealed constitutive nuclear expression in epithelial cells of the skin, gastrointestinal tract, urogenital tract and in glands and ducts of the breast [Gariglio et al., 2002; Wei et al., 2002]. In addition, IFI 16 is expressed in fibroblasts found in the stroma of a range of different tissues. Consistent with these findings, all three mRNA splice variants of IFI 16 are constitutively expressed in primary human fibroblasts and in epithelial cell lines such as HeLa cells (Fig. 1A).

Identification of the Region Within the IFI 16 Promoter Necessary for Constitutive Promoter Activity in HeLa Epithelial Carcinoma Cells

We sought to identify regions in the IFI 16 promoter that were responsible for the constitutive expression of IFI 16 by testing the transcriptional activity of different regions of the promoter in HeLa cells using a reporter gene assay. A series of DNA sequences derived from the 5′ region of the *IFI 16* gene were produced and cloned into the pCATBasic reporter gene vector 5′ to the coding sequence of chloramphenicol acetyl transferase (CAT). These reporter gene constructs were transiently transfected into HeLa cells and after a 48-h incubation CAT activity in cellular extracts was assessed. The largest DNA fragment stretching from −2484 to +279 induced a large increase in CAT activity when compared with the promoter-less pCATBasic vector ($P = 0.003$) (Fig. 1B). The transcriptional activity of the promoter was unaffected by deletion of sequences at the 5′ end from −2484 to −658. However, removal of the sequence at the 3′ end between +65 and +279 caused a significant reduction in activity ($P = 0.03$) (Fig. 1B) suggesting that a major determinant of promoter activity lay in this region. Further studies showed that the fragment between +46 to +279 was transcriptionally active ($P = 0.002$) and deletions at the 3′ end of this sequence reduced activity by 50% (Fig. 1C). Strikingly, when the 5′ ends of this sequence were removed, in constructs pCATIFI 16(+94 to +279) and pCATIFI 16(+71 to +159), no significant transcriptional activity was detected ($P = 0.22$ and 0.146, respectively) (Fig. 1C). These results indicated that the region between +46 and +71 was critical for activity of the promoter.

IFI 16 Activation Element (IAE) Is Necessary and Sufficient for Promoter Activity

The sequence between +46 and +71 was analysed using Pattern Search software and the Transfac database [Heinemeyer et al., 1998]. It contained a consensus binding sequence for AP-1 complexes (TAACTAAGTGACTCAACC) and a partially overlapping core sequence of a site recognized by the IFN responsive factor (IRF)1/2 (TAACTAAGTGACTCAACC). A short sequence containing these sites (+50 to +75) was sufficient to mediate transcriptional activity when inserted into pCATBasic, but a 4 bp mutation that simultaneously disrupted both the AP-1 and IRF binding sites inhibited promoter activity ($P = 0.1$) (Fig. 1D). Importantly, when an analogous 4 bp mutation was introduced into pCATIFI 16(−2484 to +279), the transcriptional activity of the full length promoter was dramatically reduced ($P = 0.007$) (Fig. 1E). Taken together, these results indicated that a region between +50 and +75 was sufficient and necessary for the constitutive transcriptional activity of the 5′ promoter of *IFI 16* and therefore was designated the IFI 16 activation element (IAE).

DNA Binding Complex With Specificity for an AP-1 Sequence Binds to the IAE

Given that the IAE was sufficient to mediate transcription in HeLa cells, we considered it likely that nuclear extract from these cells would contain trans-acting factors capable of binding to this sequence. To investigate the nature of potential IAE binding proteins, electrophoretic mobility shift assays (EMSA) were performed using ^{32}P labeled double stranded oligonucleotides containing the IAE. In HeLa cell nuclear extracts, a single specific IAE binding complex (IBC) was observed. This was competed by unlabeled wild type IAE [IAE(A)], but not by unlabeled IAE containing the 4 bp mutation [ΔIAE(A)] that ablated the transcriptional activity of the IFI 16 promoter (Fig. 2A).

A more thorough analysis of the binding requirements for IBC was performed using

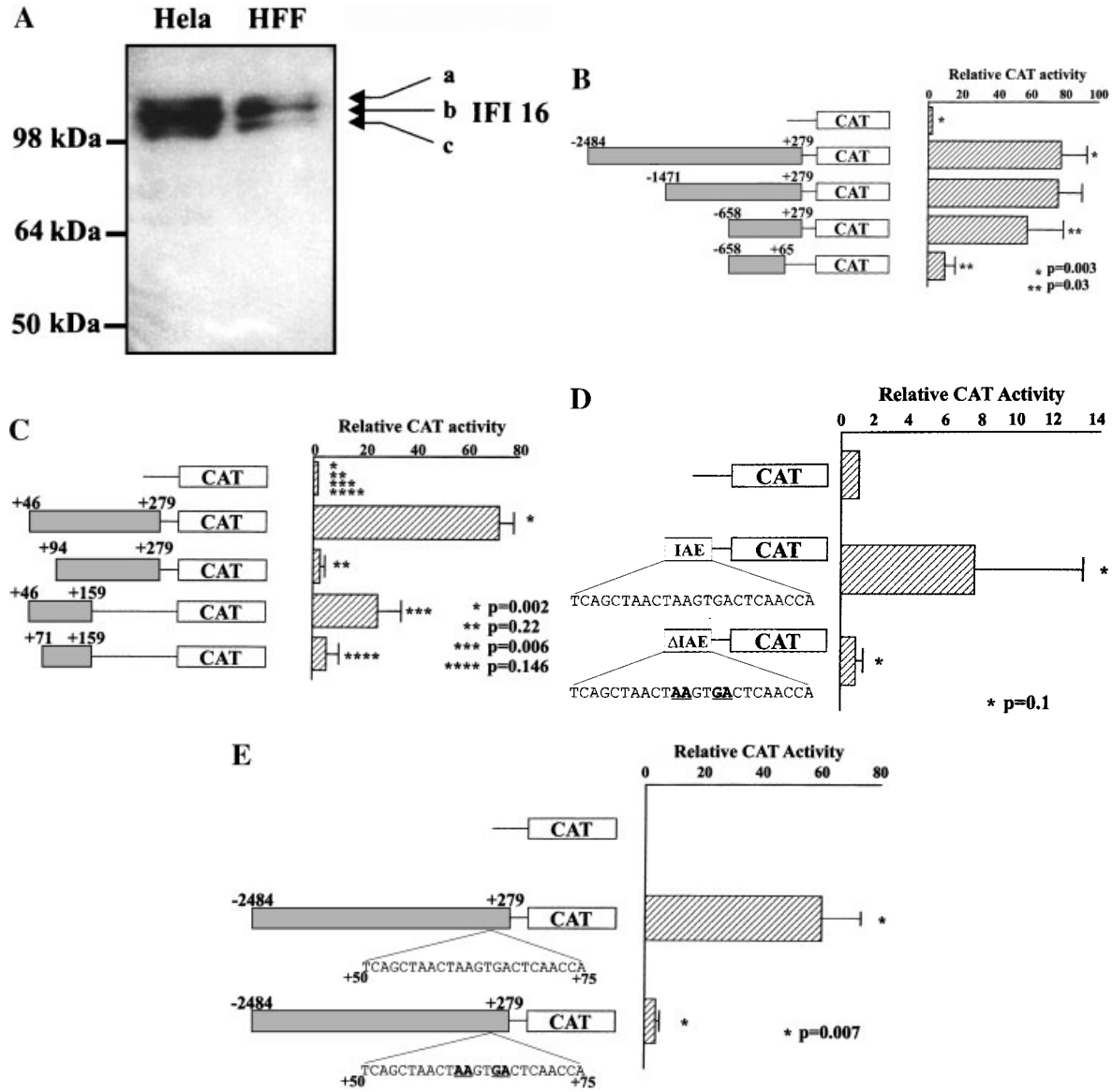

Fig. 1. Constitutive transcription of the *IFI 16* gene is controlled by a short sequence within the 5′ UTR. **A**: Nuclear extracts were prepared from HeLa cells and primary human fibroblasts (HFF) and analyzed by SDS–PAGE and Western blotting using mAb 1G7 specific for IFI 16. **B**, **C**: HeLa cells were transfected by calcium phosphate precipitation with pCATBasic reporter constructs containing a series of deletion mutants from a 2.7 kb fragment of the *IFI 16* gene promoter. Cells were incubated for 48 h before the lysates were assessed for CAT activity. **D**: Oligonucleotides containing wild type [pCATIAE(A)] and mutated [pCATΔIAE(A)] IAE sequences were inserted upstream of the CAT reporter gene in pCATbasic and used in transcription assays as above. The mutated sequences are in bold type. **E**: pCAT basic plasmids containing full length IFI 16 promoters with wild type [pCATIFI 16(−2484 to +279)] and point mutated [pCATIFI 16(−2484 to +279)ΔIAE] IAE sequences were used in transcription assays as above. The results are expressed relative to the pCATbasic construct and represent the mean and standard deviation of three independent experiments. Statistical differences were analyzed using Students paired *t*-test (Microsoft Excel).

separate oligonucleotides with targeted mutations that affected either the IRF-like site or the AP-1 consensus sequence within the IAE (Fig. 2B). IAE(B)ΔIRF, which contains a mutation only within the IRF site, retained strong IBC binding activity, but this activity was lost in IAE(B)ΔAP-1 that contained mutations in the AP-1 binding site (Fig. 2B). The ability of oligonucleotides to compete for wild type IAE binding was also tested. As expected, IBC binding was completely competed by a 100-fold excess of unlabelled wild type IAE(B). The ability to compete for IBC binding was unaffected by a mutation in the consensus IRF

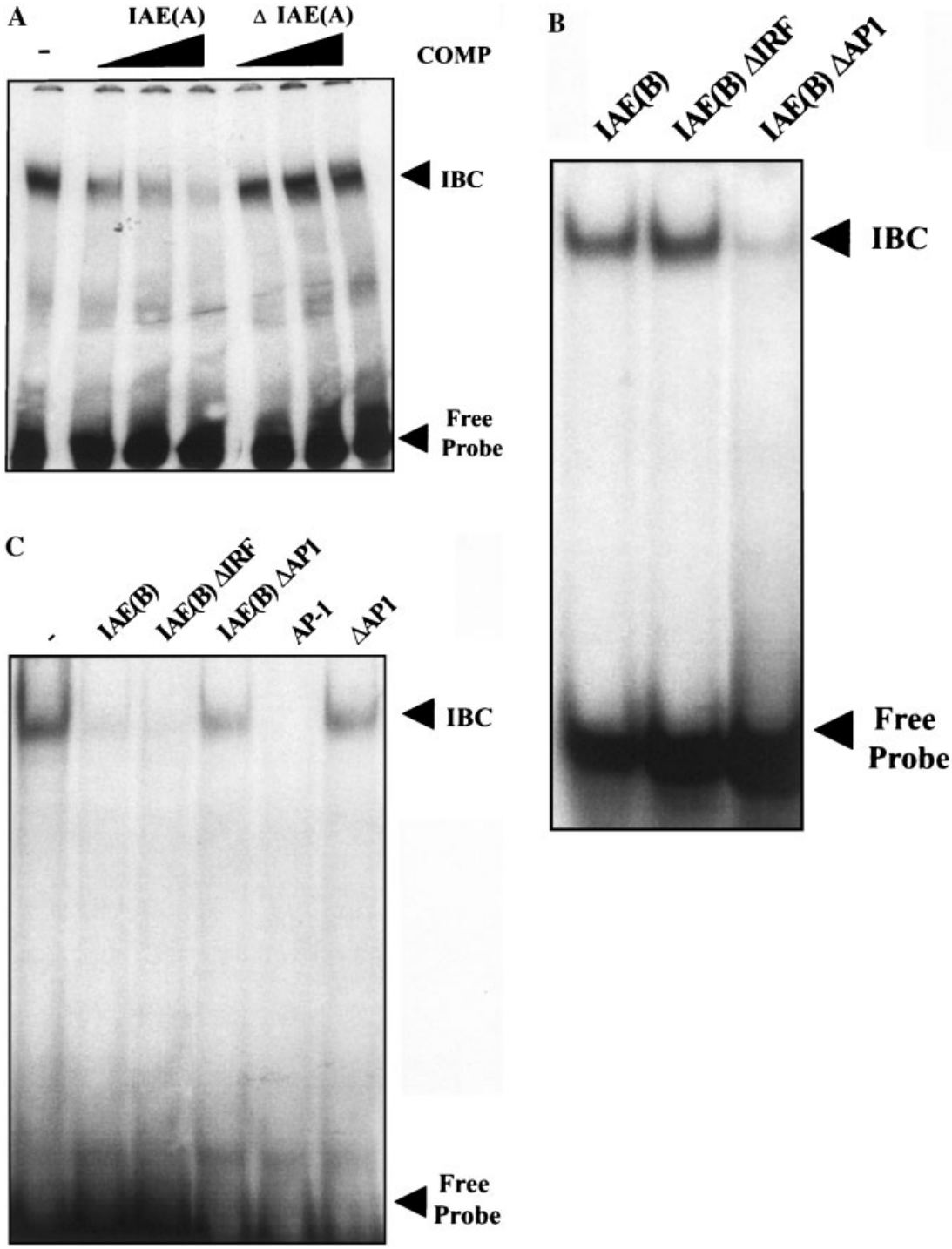

Fig. 2. HeLa cell nuclear extract contains a protein complex that binds specifically to the IAE. **A**: Nuclear extracts from HeLa cells were pre-incubated in the presence or absence of increasing concentrations (5–100-fold excess) of the unlabeled wild type IAE(A) or mutant ΔIAE(A) oligonucleotides and then incubated with ^{32}P labeled wild type IAE(A) containing oligonucleotides. **B**: Nuclear extract from HeLa cells was incubated with ^{32}P labeled wild type IAE(B) oligonucleotide, or oligonucleotides containing the IAE with mutations targeted to the IRF site [IAE(B)ΔIRF] or the AP-1 site [IAE(B)ΔAP-1]. **C**: Nuclear extracts from HeLa cells were pre-incubated in the presence or absence of unlabeled wild type IAE(B), oligonucleotides containing the IAE with mutations targeted to the IRF site [IAE(B)ΔIRF] or the AP-1 site [IAE(B)ΔAP-1] or oligonucleotides containing wild type [AP-1] or mutant [ΔAP-1] AP-1 sequences and then incubated with ^{32}P labeled wild type IAE(B) oligonucleotides. In each experiment, protein/DNA complexes were separated by electrophoresis and visualized by autoradiography.

binding site [IAE(B)ΔIRF], but was lost when mutations of the AP-1 site were introduced [IAE(B)ΔAP-1] (Fig. 2C). Most strikingly, the IBC could be completely competed by an unrelated oligonucleotide probe containing an AP-1 binding site [AP-1] but not by an oligonucleotide containing a mutated AP-1 site [ΔAP-1] (Fig. 2C). Together these data suggest the AP-1 site within the IAE is important for binding to the IBC from HeLa cell extract.

Mutation of the IAE Inhibits IFI 16 Promoter Activity in Primary Human Fibroblasts

We next tested whether the *IFI 16* gene was regulated in a similar fashion in primary human fibroblasts. As with HeLa cells, when pCATIFI 16(−2484 to +279) was transfected into primary fibroblasts it was found to be transcriptionally active, but this activity was lost when the IAE was mutated ($P = 0.01$) (Fig. 3). Furthermore, EMSA analysis demonstrated that similar IAE binding protein complexes could be detected in HeLa and primary fibroblast nuclear extract (data not shown). In each case, these complexes showed similar migration and binding characteristics to AP-1 binding complexes derived from the same extracts (data not shown).

IAE Binding Complex Contains Jun Family Members

Although we had established that the DNA binding requirements of the IBC were AP-1-like rather than IRF-like, we knew nothing of the composition of the complex. We attempted to identify potential DNA binding factors using specific antibodies to super-shift the IBC observed in EMSA. As Jun family members should be present in all AP-1 binding complexes, we used a pan-Jun family antibody to confirm the identity of the IBC. Complex formation was not affected when antibodies to several of the IRF family were added (data not shown), but the binding of the IBC to the IAE was markedly reduced when extracts of HeLa cells (Fig. 4) or primary fibroblasts (data not shown) were pre-incubated with pan-Jun family anti-serum. These data suggest that a member of the Jun family is present in the IBC and confirm that a sequence within the IAE is a functional AP-1 site capable of binding Jun family member(s).

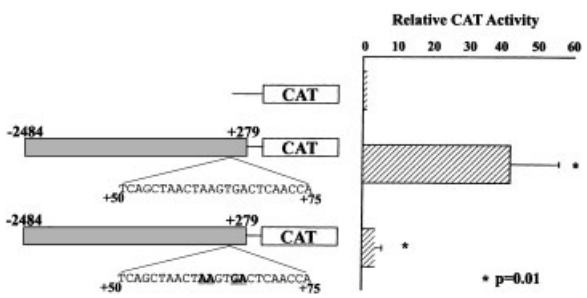

Fig. 3. *IFI 16* promoter activity is similar in HeLa cells and primary human fibroblasts. pCATbasic vector, pCATIFI 16(−2484 to +279) and pCATIFI 16(−2484 to +279)ΔIAE were transfected into primary human fibroblasts, incubated for 24 h before the lysates were assessed for CAT activity. The results are expressed relative to pCATBasic and represent the mean and standard deviation of three independent experiments. Statistical differences were analyzed using Students paired *t*-test (Microsoft Excel).

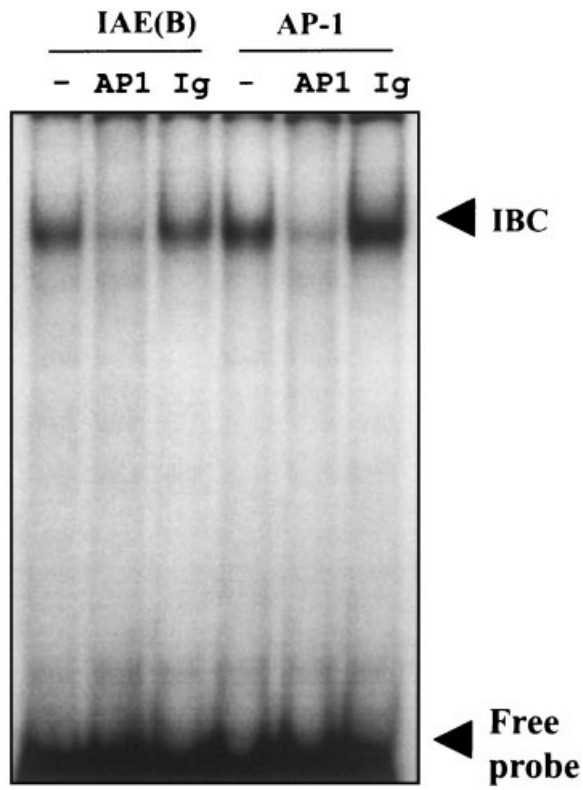

Fig. 4. IAE is supershifted by antibodies recognizing Jun family members. Nuclear extracts from HeLa cells were pre-incubated in the presence or absence antibody recognizing Jun family proteins (AP1) or control antiserum (Ig) then incubated with ^{32}P labeled wild type IAE oligonucleotides or wild type AP-1 oligonucleotides. Protein/DNA complexes were separated by electrophoresis and visualized by autoradiography.

Expression of IFI 16 in HeLa Cells Can Be Enhanced by Treatment With IFN and Phorbol Ester

We next tested whether the *IFI 16* gene regulation was controlled by similar mechanisms when expression was induced by IFN, as we had observed for constitutive expression. Although HeLa cells constitutively express IFI 16, they are also capable of responding to type I and II IFN treatment and we therefore investigated whether IFNγ would affect the expression of IFI 16 in these cells. In addition, as AP-1 sites are characterized as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) response elements (TREs), we reasoned that IFI 16 expression might also be induced by phorbol ester. Western blotting experiments revealed that, as in previous studies, both type I and type II IFNs were capable of inducing IFI 16 expression (Fig. 5A) and this occurred within 8 h of treatment? (data not shown). This experiment also showed that expression of IFI 16 was markedly increased following a 24-h treatment with TPA (Fig. 5A). Densitometric analysis of this data indicated that the increase in expression lay between 5- and 7-fold.

IAE Is Required for IFN-Induced IFI 16 Transcription

We now tested whether this IFN-mediated increase in IFI 16 expression required the IAE using a CAT reporter gene assay. As expected, the pCATIFI 16(−2484 to +279)ΔIAE was markedly less active than pCATIFI 16(−2484 to +279) in untreated cells and transcription from the wild type *IFI 16* promoter was increased 3.2-fold following treatment with IFNγ ($P = 0.003$) (Fig. 5B) which was of a similar order of magnitude to the observed changes in protein levels. Significantly, when cells transfected with the pCATIFI 16(−2484 to +279)ΔIAE were treated with IFNγ, no significant increase in CAT activity was observed ($P = 0.43$) (Fig. 5B). These experiments indicate that the IAE was absolutely required for IFNγ-induced activity of the *IFI 16* promoter.

The 2.5 kb fragment of the IFI 16 promoter used in this study contains three consensus GAS sites and a single ISRE sites, but these sites are intact in pCATIFI 16(−2484 to +279)ΔIAE and therefore they are insufficient to initiate IFI 16 transcription (Fig. 5B). This was further confirmed as systematic mutation of these sites had no effect on the activity of the promoter in response to IFNγ treatment (data not shown). Signal transduction mediated by JAK and STATs is the predominant mechanism for the activation of IFN-responsive genes, however IFN-mediated gene activation independent of STATs has recently been demonstrated [Ramana et al., 2002]. To determine whether the AP-1 pathway may be a component of this alternative IFN-stimulated signaling pathway, we investigated the effects of IFNγ on AP-1 DNA binding. AP-1 DNA binding was markedly enhanced within 20 min of IFNγ treatment (Fig. 5C; top panel) suggesting that activation through the IAE could be sufficient to cause the IFN-mediated increase in IFI 16 expression. The activity of IFNγ used in this study was confirmed in an EMSA using oligonucleotides containing a consensus GAS element (Fig. 5B; bottom panel). These experiments indicate that increased AP-1 DNA binding at the IAE could explain why the IAE is essential for IFNγ-responsiveness of the IFI 16 promoter.

IFNγ Activates AP-1 Driven Reporter Gene

To determine whether the IAE alone was sufficient to mediate IFNγ-induced promoter activation we tested the response of pCATIAE and pCATΔIAE to IFNγ. As expected pCATIAE could be activated by treating cells with TPA (Fig. 5D). Importantly, IFNγ stimulated the activity of pCATIAE reporter gene to a similar extent as TPA treatment (Fig. 5D) and the point mutations in pCATΔIAE, that we previously demonstrated could abolished binding to AP-1 (see Fig. 2), rendered this sequence completely unresponsive to both IFNγ ($P = 0.01$) and TPA ($P = 0.05$). This data extended our previous observation that IFNγ increased AP-1 DNA binding activity and suggests that activation of AP-1 by IFNγ is sufficient to initiate gene transcription.

IFNγ Can Also Activate AP-1 in HL-60 Promyelomonocytic Cells

Finally, we investigated the activation of AP-1 and its role in regulating the *IFI 16* gene using an alternative cell system. Unlike HL-60 pro-myelomonocytic leukemia cells do not express IFI 16, but mRNA [Dawson et al., 1995] and protein is strongly induced following IFNγ treatment (Fig. 6A). Consistent with a role for AP-1 in regulating the endogenous gene,

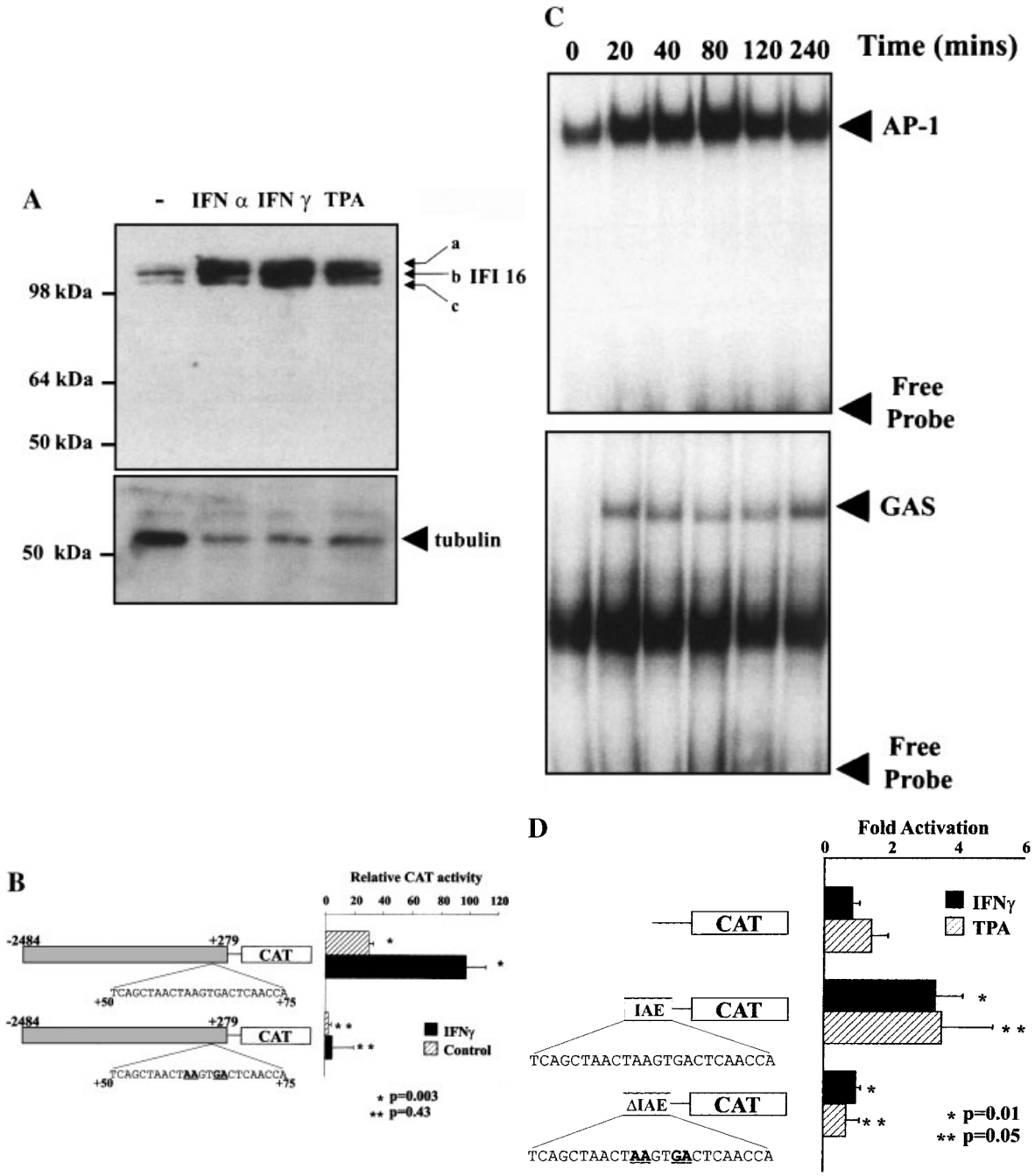

Fig. 5. The *IFI 16* promoter is activated by IFNs and phorbol esters. **A**: Nuclear extracts were prepared from HeLa cells treated for 24 h in the presence or absence of 500 U IFNα, 500 U IFNγ, or 200 nM TPA and analyzed by SDS–PAGE and Western blotting using mAb 1G7 specific for IFI 16 or mAb specific for tubulin. **B**: pCATIFI 16(−2484 to +279) or point mutated pCATIFI 16(−2484 to +279)ΔIAE were transfected into HeLa cells, incubated for 24 h and then treated for a further 24 h in the presence or absence of 500 U/ml IFNγ before the lysates were assessed for CAT activity. Statistical differences were analyzed using Students paired *t*-test (Microsoft Excel). **C**: Nuclear extracts were prepared from HeLa cells treated with 500 U IFNγ for different times and analyzed by EMSA using ^{32}P labeled AP-1 (**top panel**), GAS (**lower panel**) oligonucleotides. Protein/DNA complexes were separated by electrophoresis and visualized by autoradiography. **D**: pCATIAE and pCATΔIAE were transfected into HeLa cells, incubated for 24 h and then treated for a further 24 h in the presence or absence of 200 nM TPA or 500 U/ml IFNγ before the lysates were assessed for CAT activity. Sufficient extracts were used allow assays to be performed in the linear range, results are expressed as fold induction of treated vs. control CAT activity and represent the mean and standard deviation of three independent experiments. Statistical differences were analyzed using Students paired *t*-test (Microsoft Excel).

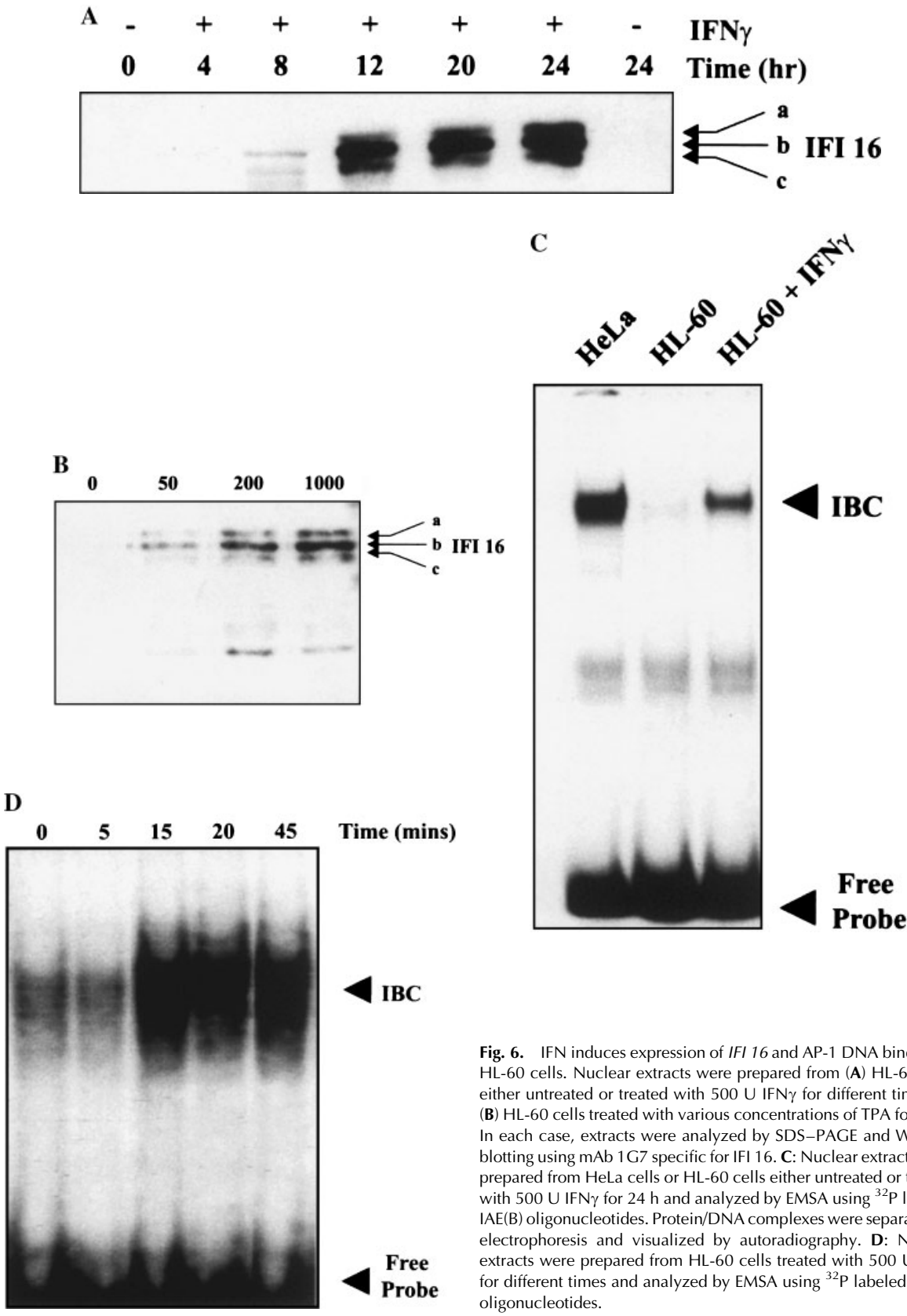

Fig. 6. IFN induces expression of *IFI 16* and AP-1 DNA binding in HL-60 cells. Nuclear extracts were prepared from (**A**) HL-60 cells either untreated or treated with 500 U IFNγ for different times; or (**B**) HL-60 cells treated with various concentrations of TPA for 14 h. In each case, extracts were analyzed by SDS–PAGE and Western blotting using mAb 1G7 specific for IFI 16. **C**: Nuclear extracts were prepared from HeLa cells or HL-60 cells either untreated or treated with 500 U IFNγ for 24 h and analyzed by EMSA using ^{32}P labeled IAE(B) oligonucleotides. Protein/DNA complexes were separated by electrophoresis and visualized by autoradiography. **D**: Nuclear extracts were prepared from HL-60 cells treated with 500 U IFNγ for different times and analyzed by EMSA using ^{32}P labeled IAE(B) oligonucleotides.

IFI 16 expression was also robustly induced following addition of TPA to the culture medium for 24 h (Fig. 6B). IFI 16 expression and AP-1 binding activity are well correlated in these cells because AP-1 activity was low or absent in extracts from untreated cells, but potently induced after treatment with IFNγ (Fig. 6C). The IAE-binding complex seen in IFNγ-treated HL-60 cells was indistinguishable from that seen in HeLa cells (Fig. 6C). A time course experiment was performed demonstrating that binding of AP-1 to the IAE was detectable 15 min after treatment with IFNγ (Fig. 6D) and these kinetics indicate that AP-1 activation is a primary response and is not a product of secondary gene induction. Together, these data suggest that activation of AP-1 binding factors may be a significant mechanism of IFN-mediated gene induction in different cell types and that AP-1 binding activity is correlated with IFI 16 expression in both HeLa and HL-60 cells.

DISCUSSION

HIN-200 proteins are reported to modulate cell growth, survival and the cell cycle and it is essential that such activities should be carefully controlled. One potential mechanism through which the activity of these proteins could be regulated is at the level of expression. This study has shown that the expression of a HIN-200 protein, IFI 16, is regulated at the level of transcription and identified the molecular events involved in the transcriptional control of gene expression. We have found that IFI 16 is constitutively expressed in a wide range of cell lines and tissues, including those of epithelial and fibroblast origin, and therefore investigated the regulation of *IFI 16* gene expression in these cell types. Using a deletion analysis and reporter gene approach we established that a 25 bp region in the 5′ UTR was necessary and sufficient for the constitutive activity of the *IFI 16* promoter. This sequence, designated IAE, formed a single specific complex when mixed with HeLa cell or primary fibroblast nuclear extract and the functional sequence was characterized as an AP-1 binding motif. The components of the AP-1 binding complex are not fully characterized, but the complex does contain a Jun family member and further studies are underway to thoroughly characterize all of the components.

Treatment of either HeLa or HL-60 cells with IFNγ enhanced expression of IFI 16 and this was associated with increased IAE/AP-1 DNA binding activity. In HeLa cells, activity of the IFI 16 promoter could be markedly enhanced by treatment with IFNγ suggesting that the increased level of protein expression was a consequence of increased transcription. Mutation of the IAE rendered the promoter unresponsive to IFNγ treatment, indicating that the AP-1 site is functional and contributes to IFNγ-mediated promoter activation. Similar conclusions were reached by those analyzing the regulation of IL-18 [Kim et al., 2000], and stromelysin-1 [Lee et al., 1998] promoters. The MAP kinase pathway has been linked to certain biological activities of IFN such as regulation of hematopoiesis, although the molecular mechanisms underlying these effects are unknown [Verma et al., 2002]. Our data indicate that IFNγ may activate transcription factors such as AP-1 that are downstream of the MAP kinase pathway, to induce expression of genes containing AP-1 binding sites.

The primary mediators of IFN-dependent transcription are the STAT family of transcription factors. However, other IFN-responsive transcription factors must exist because a subset of genes is induced by IFNγ regardless of STAT1 expression [Gil et al., 2001]. Such transcription factors are poorly characterized and therefore it is of considerable interest that the promoter of IFI 16, an IFN-inducible gene, can be regulated through an AP-1 binding site and that IFNγ can induce binding to AP-1 sites. This finding confirms other reports that have indicated AP-1 binding complexes may be transcriptional regulators of IFN-inducible genes [Lee et al., 1998; Kim et al., 2000] and that increases in expression of fos and Jun family members can be observed following IFN treatment [Rubio, 1997; Der et al., 1998]. Furthermore, signal transduction pathways that lead to activation of AP-1 binding factors are activated following IFNγ treatment [Liu et al., 1994; Sakatsume et al., 1998; Goh et al., 1999]. Together this evidence indicates that AP-1 activation may be a significant mechanism of IFN-mediated gene induction. Paradoxically, IFNγ has also been reported to antagonise AP-1 activity; c-fos transcription can be repressed following IFNγ treatment [Gil et al., 2001] and, in serum starved MEFs, c-jun is only induced in the absence of STAT1 [Ramana et al., 2000]. These contradictions are not easily explained, but one possibility is the differences in the cell

types that were used, as cell specific IFNγ signaling has been reported [Presti et al., 2001].

The most detailed analysis of IFI 16 expression has been performed using human CD34+ hemopoietic stem cells and their diverse progeny following programmed differentiation. These studies showed that IFI 16 was constitutively expressed in CD34+ cells, but following differentiation was associated with monocytic populations and the expression of the monocyte marker CD14. There is some correlation between AP-1 and hemopoietic differentiation, as agents that stimulate AP-1 binding activity can induce differentiation towards the macrophage lineage and Fos and Jun family expression increases during myeloid differentiation [Shabo et al., 1990; Szabo et al., 1991; Liebermann et al., 1998]. In our experiments, treatment of HL-60 with IFNγ, which causes this cell line to differentiate towards a monocyte-like phenotype [Sariban et al., 1987], concomitantly increased AP-1 binding activity and IFI 16 expression. However, increases in AP-1 binding activity also occurs in the neutrophil and erythroid lineages that do not express IFI 16 and therefore AP-1 DNA binding activity does not correlate with IFI 16 expression in this system [Liebermann et al., 1998]. AP-1 binding complexes are either homo-dimers of Jun family (c-jun, JunB, and JunD) proteins or hetero-dimers of these proteins with the Fos family (Fos, FosB, Fra1, and Fra2). The different complexes share DNA binding specificity, but they have distinct expression patterns and functions [Mechta-Grigoriou et al., 2001] (e.g., c-jun is a trans-activator, but JunD has no trans-activation domain). Therefore, variations in AP-1 complex composition between myeloid lineages may affect transcriptional responses in AP-1 containing promoters such as that of IFI 16. The composition of AP-1 binding complexes is certainly important during differentiation, as irradiated mice reconstituted with fetal liver cells from $JunB^{-/-}$ animals develop a myeloproliferative disorder characterized by increased numbers of granulocytic progenitors [Passegue et al., 2001]. It remains to be determined whether this mechanism explains the differential expression of IFI 16 in myeloid lineages or whether other lineage specific factors are involved.

The *HIN-200* genes in humans and mice are found at 1q21-23, a locus that has been linked to Systemic Lupus Erythromatosous (SLE) in humans, and a similar disease in mice. A recent study linked polymorphisms within the 5′ promoter region of *p202* to increased serum auto-antibody levels and susceptibility to a SLE-like autoimmune disease. Although the role played by p202 in this model remains unclear, it is possible that deregulation of the *IFI 16* promoter has similar pathological consequences in human disease. There is little sequence homology between the *p202* and *IFI 16* 5′promoter regions but functional studies of these two promoters have revealed some interesting parallels. Sequence analysis of the *p202* promoter has been used to identify ISRE sites [Gariglio et al., 1994], an AP-1 site [Geng et al., 2000], and p53 responsive consensus sequences [D'Souza et al., 2001] that are capable of binding to their respective transcription factors and are potentially functional. Interestingly, the p202 promoter can be activated by over-expressing JunD and the endogenous gene is induced under low serum conditions when JunD is the predominant Jun family isoform suggesting that the AP-1 sites in this sequence may be functional [Geng et al., 2000]. These observations and the IFN-responsiveness of the 5′ promoters of p202 and IFI 16, indicate that despite the lack of sequence similarity between them, there is a marked functional resemblance. In the light of these similarities, and given that IFI 16 is the human HIN-200 protein bearing the greatest structural similarity to p202, it will be of great interest to study the expression of IFI 16 in human patients suffering from SLE.

The studies described herein demonstrate that a functional AP-1 site within the 5′ untranslated region of IFI 16 is important for constitutive promoter activity in cells that endogenously express IFI 16. Importantly, our studies have revealed that AP-1 can be rapidly activated by IFNγ and that a functional IAE is sufficient to mediate promoter activation in response to IFNγ. These studies provide valuable information regarding the molecular mechanisms underpinning *IFI 16* gene expression and, importantly, identify AP-1 as a transcription factor that can mediate promoter activation following IFNγ stimulation.

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