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Modulation of Interferon Signaling in Human Fibroblasts by Phorbol Esters

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Phorbol esters activate the expression of a variety of early-response genes through protein kinase C-dependent pathways. In addition, phorbol esters may promote cell growth by the inhibition of expression of cellular gene products regulated by antiproliferative agents such as interferons (IFN)s. In human diploid fibroblasts, phorbol 12-myristate 13-acetate (PMA) selectively inhibits the IFN-α-induced cellular gene ISG54. Using transient transfection assays, we have delineated two elements in the promoter of this gene that are necessary for the inhibitory actions of PMA. These elements include (i) the IFN-stimulated response element (ISRE) which is necessary for IFN-α-induced cellular gene expression, and (ii) an element located near the site of transcription initiation. IFN-α treatment resulted in the rapid induction of ISGF3, a multisubunit transcription factor which binds to the ISRE. PMA caused a substantial reduction in IFNα-induced ISGF3 in both nuclear and cytoplasmic extracts, as determined by electrophoretic mobility shift assays with the ISRE as a probe. In vitro reconstitution experiments revealed that IFN-α activation of the ISGF3α component of ISGF3 was not affected by PMA. Further experiments were consistent with the possibility that PMA regulated the activity of a cellular factor which competed with ISGF3γ for binding of the activated ISGF3α polypeptides. Electrophoretic mobility shift assays using the cap site of ISG54 as a probe demonstrated the formation of a specific complex whose DNA binding activity was not affected by treatment of cells with PMA or IFN-α. Competitive inhibition studies were consistent with the DNA-protein complex at the cap site of ISG54 containing proteins with DNA binding sites in common with those which also interact with the ISRE. These data suggest a unique regulatory mechanism by which phorbol esters can modulate IFN signaling.

Interferons (IFNs) comprise a family of polypeptides that exhibit diverse biological activities, which include inhibition of cell growth and viral infection (28). Although the immediate responses following IFN-α receptor binding remain poorly defined, some of these early signaling events are beginning to be elucidated. Both type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) rapidly induce the transcription of several cellular genes (20). Interferon-stimulated genes (ISGs) ISG54 and ISG15 (the numbers indicated molecular weights in thousands), as well as all other IFN-α-activated cellular genes thus far studied, contain within their promoters similar elements (IFN-stimulated response elements [ISREs]) which are necessary and sufficient to induce their transcription by IFN-α. Two IFN-α-induced protein complexes (ISGF2 and ISGF3) and one constitutive complex (ISGF1) have been defined by their ability to bind to the ISRE in an electrophoretic mobility shift assay (EMSA) (7, 22). ISGF2, also referred to as IRF1, has been purified, and cDNA clones corresponding to the protein have been obtained (25, 30). The role of ISGF2 in the transcriptional induction of IFN-α-induced genes is still poorly defined. However, increased amounts of this protein are correlated with the transcriptional activation of IFN-β and IFN-α genes by viruses (25). ISGF3, a multisubunit protein complex, is the primary positive regulator of IFN-α-induced gene transcription (11, 20). One part of the complex, ISGF3α, becomes activated in the cytoplasm of cells within minutes after treatment with IFN-α. ISGF3α subsequently translocates to the nucleus, where it complexes with the other component of the complex (ISGF3γ), which binds to the ISRE (7, 22). The concentration of ISGF3γ (48 kDa) can be increased by extended treatment of cells with IFN-γ (22), and its constitutive concentration varies from one cell type to another.

Phorbol esters are well-described growth promoters which can also antagonize IFN's activity as an antiviral agent (31). We have recently shown that phorbol 12-myristate 13-acetate (PMA) inhibits IFN-α-induced transcription of ISG54 (3, 4). The actions of PMA are not only cell specific, in that their effects are seen primarily in fibroblasts and other terminally differentiated cells, but also gene specific, in that not all IFN-α-induced cellular genes are inhibited by PMA (3). To explore the mechanisms whereby PMA inhibits IFN-α-induced ISG54 expression, we have mapped two regions within the promoter of this gene that are necessary for the inhibitory effects of phorbol esters. Further experiments were performed to examine the effects of PMA on IFN-α-induced ISGF3 formation. Finally, evidence is presented for a cellular factor which can compete for the binding of IFN-α-activated ISGF3α and which may represent a common control mechanism whereby IFN-α signaling can be modulated by a variety of growth promoters and/or oncogenic proteins.

MATERIALS AND METHODS

Cells and culture. Human foreskin diploid fibroblasts GM00468 (from the National Institute of General Medical Sciences) and BUD8 (from the American Type Culture Collection) were grown and passaged in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Both sources of

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human fibroblasts demonstrated phorbol ester inhibition of IFN-α-induced ISG54 expression.

IFNs and reagents. Recombinant human IFN-α was a generous gift from Hoffmann-La Roche. Recombinant human IFN-γ was provided by Genentech Corp.

Measurement of RNA by using antisense RNA probes. RNA was prepared as described by Chirgwin et al. (5). RNAs in all experiments were quantitated by RNase protection assays. Antisense RNA probes were synthesized with SP6 RNA or T7 RNA polymerase (Bethesda Research Laboratories). Plasmid pIFN-IND-1 contains a 367-bp *Eco*RI restriction fragment of exon 2 of the ISG54 gene (3) subcloned into pGEM1. The size of the protected fragment for pIFN-IND-1 is 367 bp. ISG15 contains a 240-bp *Pst*I fragment of the cDNA also in pGEM1. The size of the protected fragment is 241 bp. A *Hin*dIII-*Pst*I fragment of exon 2 of adenovirus E1B was subcloned into pGEM1 and linearized with *Hin*dIII. Antisense transcripts were made by using SP6 RNA polymerase. The size of the protected fragment is 197 bp (21). α-Globin RNA was analyzed by RNase protection, using the probe described by Sheng et al. (33). The protected band is 130 bp. As an internal control, samples were hybridized to a ^{32}P-labeled antisense RNA probe for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This 1,400-bp *Pst* fragment of the cDNA was subcloned into pGEM1 and linearized with *Sau*3A. The protected fragment is 300 bp.

Solution hybridization and RNase mapping. Total cellular RNA (5 to 10 μg) was hybridized with 10^5 cpm of ^{32}P-labeled antisense RNA probe in 80% (vol/vol) formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)–0.4 M NaCl–1 mM EDTA at 56°C for 16 to 20 h, and the protected RNA was analyzed as described previously (4).

Measurement of transcriptional activity. Run-on transcription assays were performed by using isolated human fibroblast nuclei in the presence of [α-^{32}P]UTP as described by Nakagawa et al. (26). DNA probes were immobilized to Nytran membranes (Schleicher & Schuell) and hybridized to [^{32}P]RNA, using conditions described by Church and Gilbert (6). DNA probes included pGEM2 (to determine background), GAPDH, ISG54 800-bp *Eco*RI fragment from exon 2 (21), and ISG15 (32).

Transfection assays. Confluent human diploid fibroblasts (GM00468; less than passage 14) were passaged (1:5) the day before transfection. Transfections were performed as described by Akai et al. (2) except that 75 ng of simian virus 40 (SV40) α-globin per ml was cotransfected as an internal control (33). At 48 to 72 h after transfection, cells were exposed to PMA (5×10^{-8} M) for 2 h prior to the addition of 300 U of IFN-α per ml for 4 h. RNA was isolated as described by Chirgwin et al. (5), and poly(A) RNA was selected. E1B and α-globin RNAs were assayed by RNase protection (2). All constructions which used the ISG54 gene were derived from a genomic clone which contained bp −800 to +330 relative to the initiation site of transcription. The construction displayed in Fig. 3, line 6, was made by ligation of a plasmid which contained bp −44 to −800 with a set of four oligonucleotides which contained bp −125 to +8 of the human α1-globin gene (18). The construction in line 7 used two of the same oligonucleotides as in line 6 (bp −125 to −23 of the α-globin gene) and two other oligonucleotides (bp −23 to +8 of ISG54). Both of these constructions also contained the splice donor site of the intron of the ISG54 gene. All constructions were confirmed by sequencing.

Preparation of nuclear and cytoplasmic cell extracts. Nuclear and cytoplasmic cell extracts were essentially prepared as described by Levy et al. (22), with the following modifications. Nuclei were extracted with 0.3 M NaCl, and cytoplasmic extracts consisted of that fraction remaining after 1,500 × *g* isolation of the nuclei. The cytoplasmic extract was adjusted to 0.3 M NaCl prior to use in EMSAs. Selected extracts were alkylated with 10 mM *N*-ethylmaleimide (NEM) for 20 min at room temperature and then quenched by the addition of 15 mM dithiothreitol.

EMSAs and exonuclease protection assays. Gel shift assays were performed essentially as described by Levy et al. (22), using a ^{32}P-end-labeled synthetic oligonucleotide (1.0 ng) with the sequence (double-stranded) 5′-GATCCACGTTA CAAAAGGAAAGTGAAACTAGAAGTG-3′, which corresponds to the ISRE of the ISG54 gene. Other oligonucleotides or cloned double-stranded DNA fragments used in these assays included ISRE of ISG15 (5′-GATCCATGCCTC GGGAAAGGGAAACCGAAACTGAAGCC-3′, Sp1 (5′-GA TCCGGGGCGGGGCGGGGCGGGGC-3′), cyclic AMP (cAMP) response element (CRE)/ISRE (5′-AATTCGTTGA GCCCGTGACGTTTACACTCATTCATAAG-3′), and cap site (−42- to +28-bp fragment of the ISG54 gene subcloned into the BlueScript plasmid). This fragment was either labeled at both ends by digestion with *Eco*RI and *Xba*I or labeled only at the 3′ end by digestion with *Xba*I. Binding reaction mixtures containing 5 μg of protein were incubated for 5 min at 22°C and analyzed on 6% acrylamide gels. Reconstitution experiments were performed under the same conditions except that 10 μg of protein was used in each binding reaction. Exonuclease protection assays were performed as described previously (8).

RESULTS

Determination of the elements in the ISG54 promoter required for PMA inhibition. We chose to study two well-characterized IFN-α-inducible genes, ISG54 and ISG15, in human fibroblasts. The relative rates of transcription of each gene were measured by nuclear run-on assays. Compared with untreated cells or cells exposed only to PMA, IFN-α dramatically enhanced the rate of transcription of both ISG54 and ISG15 (Fig. 1A). Incubation of cells with IFN-α and PMA resulted in about a 75% inhibition in the rate of ISG54 transcription. In contrast, the transcription rate of ISG15 was not significantly changed by treatment with phorbol esters. RNA concentrations corresponding to ISG15 and ISG54 correlated with the transcription rates (Fig. 1B). These results were consistent with a gene-specific effect of PMA on IFN-α-induced gene transcription.

Transient expression assays were next done to define regions within the promoter responsible for the actions of PMA. Selected deletions and/or substitutions in the ISG54 promoter were ligated to the E1B gene (21), and IFN-α-activated E1B RNA was analyzed 48 to 72 h after transfection. As an internal control, cells were cotransfected with a SV40-driven human α-globin gene (33). As shown previously (21), a construction containing 800 bp 5′ and 330 bp 3′ to the start site of transcription showed IFN-α-inducible expression of E1B and also PMA-mediated inhibition (Fig. 2). A deletion of the promoter containing bp −800 to +8 of ISG54 also was inhibited by IFN-α plus PMA. However, when the TATA box and sequences 3′ to it were replaced with the human α globin TATA and the α-globin cap site, PMA was no longer effective. Figure 3 summarizes the results from several other constructions used to map other regions in the promoter of this gene necessary for PMA to inhibit IFN-α-induced RNA expression in fibroblasts. Three inferences

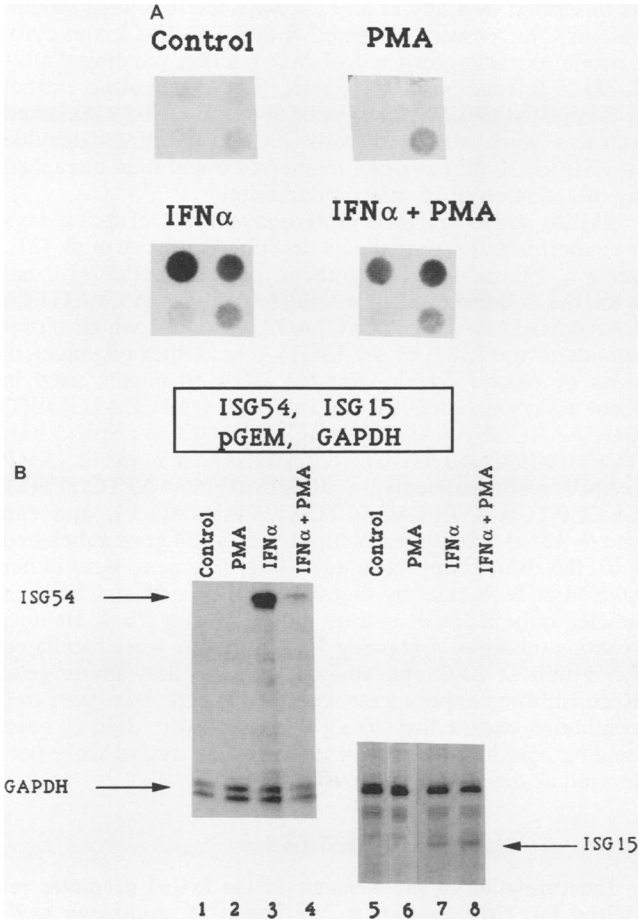


FIG. 1. Selective inhibition by phorbol esters of IFN-α-induced transcription of ISG54. Confluent human diploid fibroblasts were incubated with 5×10^{-8} M PMA for 2 h prior to the addition of IFN-α (250 U/ml). Nuclei were isolated 60 min after addition of IFN-α. (A) Nuclear run-on assays were performed as described in Materials and Methods, and ^{32}P-labeled RNA was hybridized to the cDNA probes indicated. (B) Abundances of ISG54 and ISG15 cytoplasmic RNAs were determined by using RNase protection. Cells were treated with PMA and IFN-α as in panel A. As an internal control, GAPDH RNA was simultaneously analyzed in each sample.

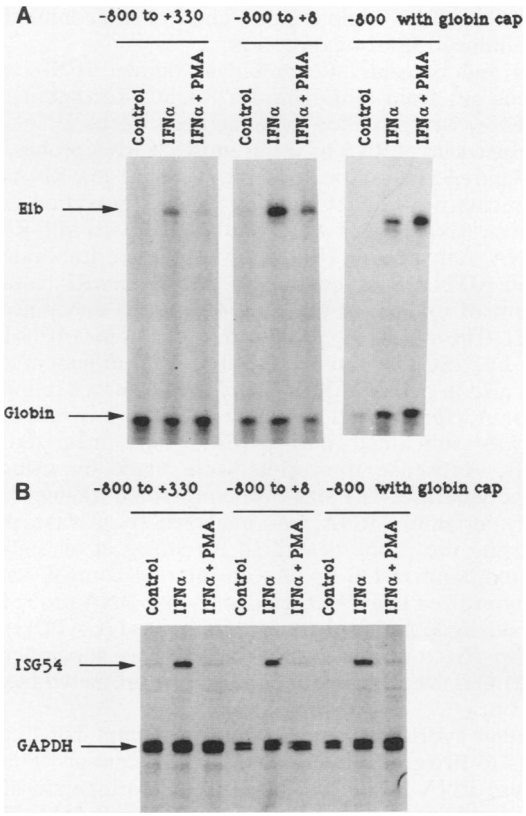


FIG. 2. Evidence that the cap region of the ISG54 promoter is required for phorbol esters to inhibit IFN-α-induced expression of the gene. (A) Human fibroblasts were transfected with the three ISG54-E1b DNA constructions containing either bp −800 to +330 of ISG54, −800 to +8 of ISG54, or −800 to −42 of ISG54 and −42 to +8 of the human α-globin gene (see Materials and Methods). SV40 α-globin was cotransfected as an internal control. At 72 h after transfection, cells were either untreated, incubated with IFN-α (300 U/ml) for 4 h, or incubated for 2 h with 5×10^{-8} M PMA prior to the addition of IFN-α for 4 h. RNA was harvested, and E1B and α-globin RNAs were analyzed by RNase protection (2). Cells transfected with the plasmids indicated here or in Fig. 3 that were treated with PMA alone showed no accumulation of E1B RNA (data not shown). (B) Endogenous ISG54 RNA concentrations were determined from the same RNA samples in which E1B and α-globin expression were analyzed.

could be drawn from these experiments. (i) Sequences in the ISG54 promoter 5′ to the ISRE did not contribute to the inhibitory effects of PMA (Fig. 3, lines 1 to 3), nor did sequences between bp −75 and −44 (data not shown). (ii) Since the TATA box of α-globin can be used in the context of the ISG54 cap site and PMA inhibition is restored, PMA is not exerting its effects solely through the ISG54 TATA element (line 7). (iii) A construction which contains a CRE as well as an ISRE showed PMA inhibition of IFN-α-induced E1B RNA expression but not PMA inhibition of forskolin-induced E1B expression (line 4). (Forskolin was used in these experiments to increase intracellular concentrations of cAMP.) These latter results suggested that the ISRE is also necessary for the inhibitory effects of PMA, since this construct contained the ISG54 cap site. Thus, at least two regulatory elements are needed together for PMA to inhibit IFN-α-induced transcription of this gene: one at the cap site (bp −25 to +8) and the other at the ISRE.

Specific DNA-binding proteins interact with the core promoter of ISG54. Using a combination of EMSAs and exonuclease protection assays, we examined whether any proteins interacted with sequences surrounding the cap site of ISG54. Although previous studies (24, 29) indicated the presence of an IFN-α-inducible DNase-hypersensitive site located in the region of the cap site of ISG54, there has been no further characterization of any potential regulatory function ascribed to this region of the promoter. Nuclear extracts were incubated with a labeled DNA probe (bp −42 to +28), and EMSAs were then performed (Fig. 4A). A probe of this length was used because three direct repeats of 5′-GAGAAG-3′ are present between bp −25 and +28, and such repeats are often associated with specific DNA-protein interactions. The binding of specific complexes to the cap site was not altered when nuclear extracts were prepared from cells treated with IFN-α and/or PMA under the conditions used for the transfection experiments as well as those used

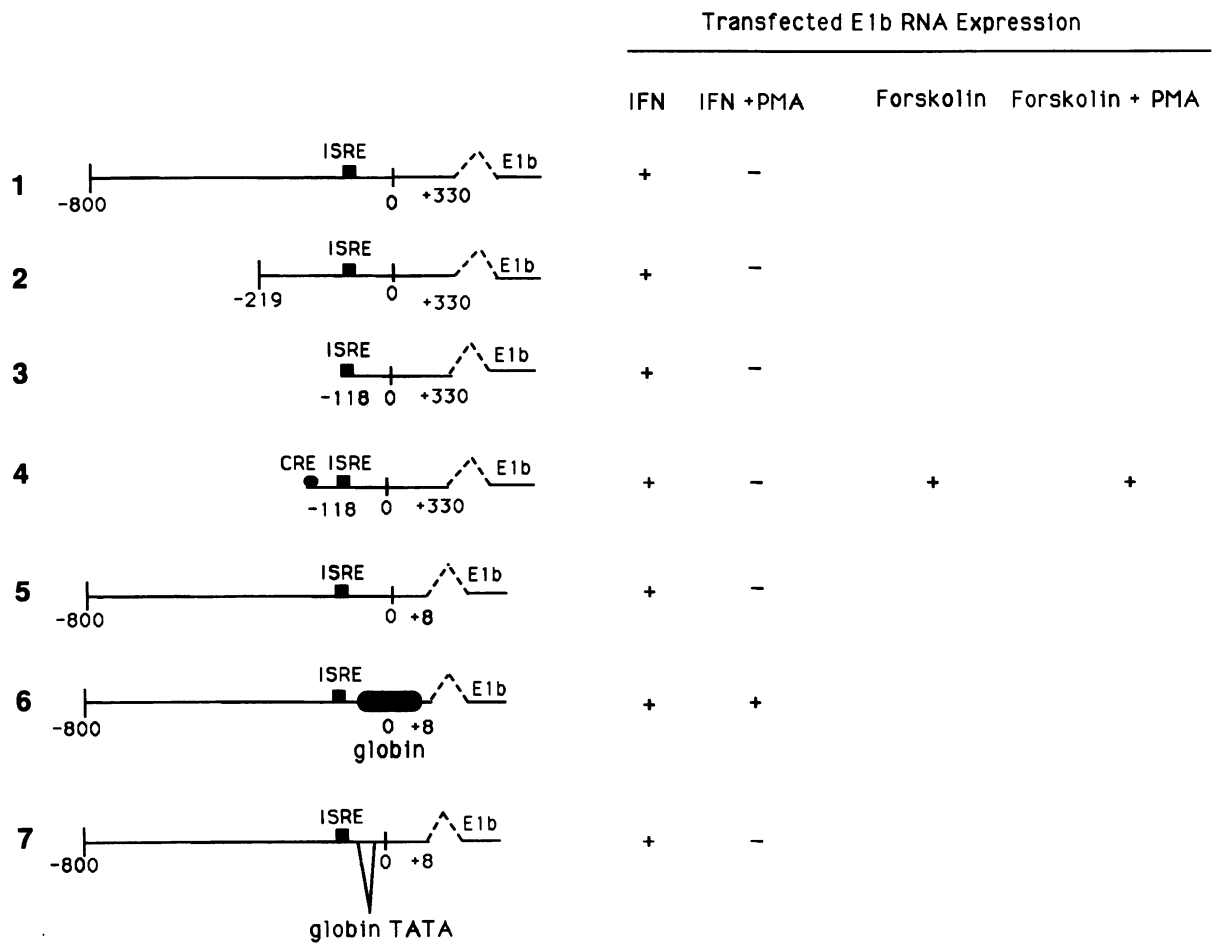


FIG. 3. Effect of PMA on IFN-α or forskolin induction of E1B expression from ISG54 promoter deletions. Human fibroblasts were transfected with the indicated ISG54 promoter deletions and the SV40 α-globin plasmid. At 72 h after transfection, cells were treated with IFN-α, IFN-α plus PMA, forskolin (50 μM), or forskolin plus PMA. Cells were incubated for 2 h with PMA prior to the addition of either IFN-α or forskolin for 4 h. Control plates received no growth factors. RNA corresponding to E1B or α-globin was measured by RNase protection and quantitated either by densitometry or direct counting. The amount of E1B RNA in any given sample was normalized to the expression of α-globin. For the IFN- and forskolin-treated samples, + indicates that IFN-α or forskolin induced E1B RNA; for the PMA-treated samples, −, indicates that PMA inhibited IFN-α-induced expression of E1B an average of at least 60% compared with IFN-α alone and + indicates that the inhibitory effects of PMA were less that 10%. Each deletion was transfected a minimum of three times. The constructs used in lines 2 and 3 were made by exonuclease digestion of the construct shown in line 1. The construct in line 4 is the same as that in line 3 with the addition of a CRE (9). Plasmids in lines 5 to 7 were constructed with oligonucleotides that were ligated into the 3′ *Hin*dIII site of a plasmid which contained bp −44 to −800 of ISG54.

for the gel shift assays shown in Fig. 5 to 7. Exonuclease protection assays were performed to define more accurately the binding site in the cap region (Fig. 4B). A specific stop site was observed at −17 bp relative to the transcriptional start site and adjacent to the opposite strand sequence 5′-GAGAAG-3′. The specific complex extends from about bp −17 to −5. Assays using a probe labeled on the upper (coding) stand confirmed the presence of a specific stop site at −5 bp (data not shown). Competitive binding studies using the oligonucleotides used in gel shift assays confirmed that the exonuclease stop sites and the complex seen by gel shift assays were equivalent (Fig. 4B, lanes 2 and 3). In addition, gel shift assays using oligonucleotides spanning this region confirmed the approximate boundaries of this complex as determined by exonuclease protection (data not shown). The sequences at the cap site and the ISRE and their relative positions in the promoter are diagramed in Fig. 4C.

PMA inhibits ISGF3 formation. Transfection studies indicated that both the ISRE and the cap region of the ISG54 were necessary for PMA inhibition of IFN-α-induced E1B RNA. To determine whether PMA altered the binding of the proteins known to interact with the ISRE, nuclear and cytoplasmic extracts were prepared from fibroblasts which had been incubated in medium alone, PMA, IFN-α, or IFN-α and PMA. EMSAs were then performed by using probes for the ISRE of either ISG54 or ISG15 (Fig. 5). As observed by others (7, 22), exposure of fibroblasts to IFN-α for 1 h induced the formation of ISGF3 in both nuclear (Fig. 5, lane 3) and cytoplasmic extracts (lane 7), while neither untreated cells nor cells exposed to PMA alone contained ISGF3 (lanes 1, 2, 9, and 10). Cells which were incubated simultaneously with IFN-α and PMA contained decreased ISGF3 (60 to 90%) in both cytoplasmic (lane 7 versus lane 8) and nuclear (lane 3 versus lane 4) fractions. Interestingly, the inhibitory effect of PMA was seen with either the ISG54 or ISG15 ISRE probe (lane 11 versus lane 12). Since ISG15

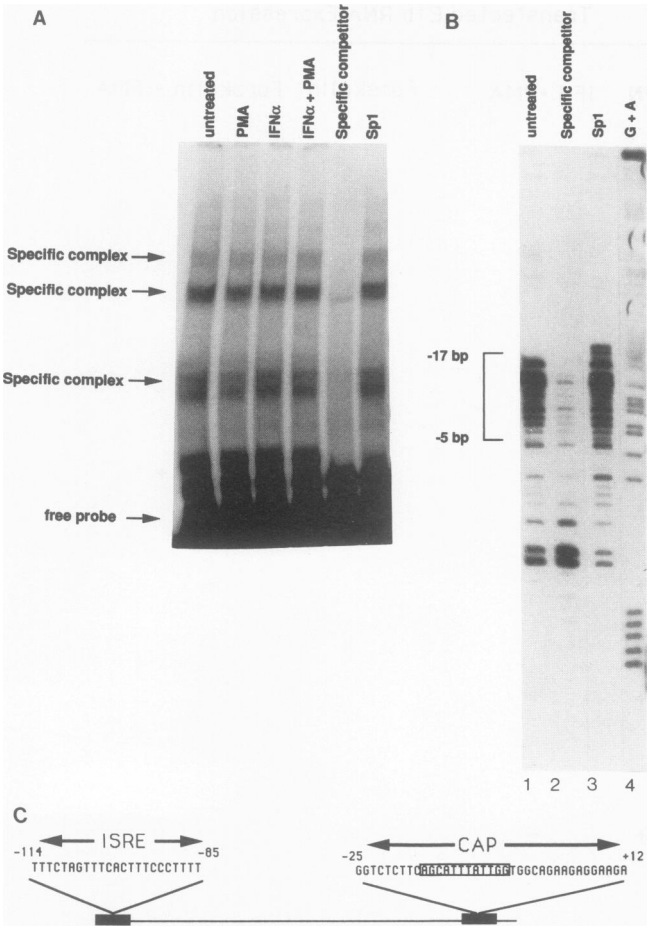


FIG. 4. Specific interaction of a DNA-binding protein(s) with the cap site of the ISG54 gene. (A) EMSAs were performed by using nuclear extracts made from human fibroblasts. Cells were untreated or exposed to PMA (5×10^{-8} M) for 75 min, IFN-α (300 U/ml) for 60 min, or PMA and IFN-α. The ^{32}P-labeled DNA probe was a fragment (bp −42 to +28) of the ISG54 gene (see Materials and Methods for details). The specific competitor and a nonspecific Sp1 oligonucleotide competitor were used at a 100-fold molar excess. (B) Untreated nuclear extracts were incubated with the same probe which had been labeled at the 5′ end of the noncoding strand after digestion of the plasmid with *Xba*I. Exonuclease assays were done as described by Decker et al. (8). Competitions were done as described for the EMSAs. To define the protected region, a G+A sequencing reaction was run with use of the same probe. (C) The sequence of the probe used for these assays is shown; the nucleotides which have been enclosed with a rectangle correspond to the region which is protected from exonuclease III digestion.

transcription was not inhibited by PMA, this result suggests that a combination of at least two regulatory elements is necessary for PMA to confer its inhibitory effects.

IFN activation of the ISGF3α proteins is not altered by PMA. We next determined whether PMA decreased IFN-α-induced ISGF3 formation by diminishing activation of the ISGF3α proteins or altering ISGF3γ (22). Studies were performed to determine whether increasing the intracellular concentration of the DNA-binding component, ISGF3γ, by prolonged exposure to IFN-γ reversed PMA inhibition of IFN-α-induced ISGF3 formation (Fig. 6A). Although pretreatment with IFN-γ did not reverse the inhibitory effect of PMA (Fig. 6A, lane 5 versus lane 6), there was more ISGF3 present in extracts prepared with IFN-γ–IFN-α–PMA than in cells treated with only IFN-α–PMA (lane 4 versus lane 6). These results suggested that regulation of ISGF3γ might play a role in the inhibitory effects of PMA. Although ISGF2 was also increased by IFN-γ, it is not involved in the formation of ISGF3 (30), and antibodies against ISGF2 do not inhibit the binding of ISGF3 to the ISRE (see Fig. 8) or reverse PMA inhibition (data not shown).

In vitro reconstitution experiments were used to investigate whether PMA decreased IFN-α activation of the ISGF3α proteins. NEM treatment destroys ISGF3 binding to the ISRE because ISGF3γ is NEM sensitive. However, the ISGF3α proteins have been shown to be relatively NEM resistant (22). Addition of nuclear extracts from IFN-γ-treated human fibroblasts (an enriched source of ISGF3γ) to NEM-treated IFN-α- or IFN-α–PMA-treated cell extracts reconstituted equal amounts of ISGF3 (Fig. 6B, lanes 5 and 6). Similar results were also seen with extracts not treated with NEM (data not shown). These results suggested that PMA did not inhibit the activation of the ISGF3α polypeptides, the earliest defined signaling event after IFNα binds its receptor.

The stability of the ISGF3 complex is not affected by PMA. Although PMA did not inhibit IFN-α activation of ISGF3α, PMA might modify ISGF3γ such that it no longer binds either the ISRE or activated ISGF3α. Alternatively, a preexisting protein in the cytoplasm might be modified by PMA so that it competes with ISGFγ for binding to activated ISGF3α. PMA had variable effects on binding of ISGF3γ to the ISRE in the absence of the ISGF3α, but in many experiments PMA had no effect on ISGF3γ binding under conditions in which ISGF3 is clearly decreased (Fig. 6A, lane 5 versus lane 6). Furthermore, the addition of excess activated ISGF3α proteins (made by NEM treatment of an extract prepared from IFN-α-treated cells) to an IFNα–PMA-treated extract did increase the amount of the ISGF3 complex, suggesting that there was functional ISGF3γ activity in IFNα–PMA-treated extracts (Fig. 6B, lane 2 versus lane 7). Therefore, reconstitution of the ISGF3 complex could be accomplished by increasing either the ISGF3α or ISGF3γ concentration. These results are consistent with PMA activation of a protein which can compete with ISGF3γ for binding of an activated ISGF3α protein(s), and this equilibrium can be shifted in vivo or in vitro to favor ISGF3 formation simply by increasing the concentration of either the ISGF3γ or ISGF3α protein.

If this hypothesis is correct, then the stability of ISGF3 in extracts prepared from either IFN-α- or IFN-α–PMA-treated cells would be identical. If PMA altered the affinity of ISGF3γ for ISGF3α or the affinity of ISGF3γ for the ISRE, then the stability of the ISGF3 present in IFN-α–PMA-treated cells would be decreased. To address this possibility, the relative rates of dissociation of ISGF3 were measured (Fig. 6C). This assay is particularly sensitive for detecting changes in ISGF3 stability, since the affinity of ISGF3γ for the ISRE is much weaker (about 25-fold) when it is not complexed with ISGF3α (17). Therefore, if PMA decreased the affinity of ISGF3α for ISGF3γ, a large difference should be detectable in the off rates for ISGF3 prepared from cells exposed to PMA. Using the ISRE of ISG54 as a probe, it was clear that the off rate of ISGF3 was the same whether or not cells were exposed to PMA. Similar results were obtained with use of the ISRE of ISG15 (data not shown). These results reinforce those shown in Fig. 6B and are consistent with a mechanism of a PMA-activated cytoplasmic protein

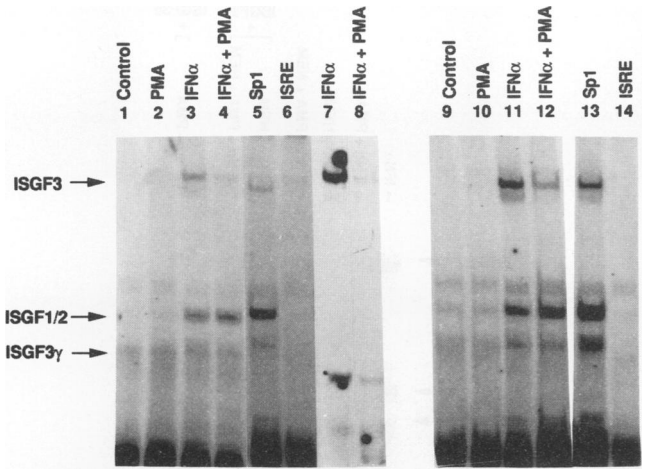


FIG. 5. Decrease of the IFN-α-induced transcription complex ISGF3 in human fibroblasts treated with IFN-α and PMA. Nuclear and cytoplasmic extracts were prepared from human fibroblasts treated with medium alone, PMA (5×10^{-8} M) for 75 min, IFN-α (300 U/ml) for 1 h, or PMA for 15 min prior to the addition of IFN-α for 1 h. EMSAs were then performed, using an ISRE probe to ISG54 (lanes 1 to 8) or ISG15 (lanes 9 to 14). Cytoplasmic extracts were assayed in lanes 7 and 8 (20 μg of protein was used for these gel shift assays, and the autoradiograph was exposed 10 times longer). The previously defined complexes known to interact specifically with ISRE probes (ISGF1/2 ISGF3 and ISGF3γ) are indicated. Specificity of the complexes is indicated by the fact that an oligonucleotide probe corresponding to the Sp1 consensus DNA binding site did not compete (lanes 5 and 13). (Cytoplasmic extracts from untreated or PMA-treated cells showed no ISGF3; data not shown).

which can compete with ISGF3γ for binding to the ISGF3α proteins.

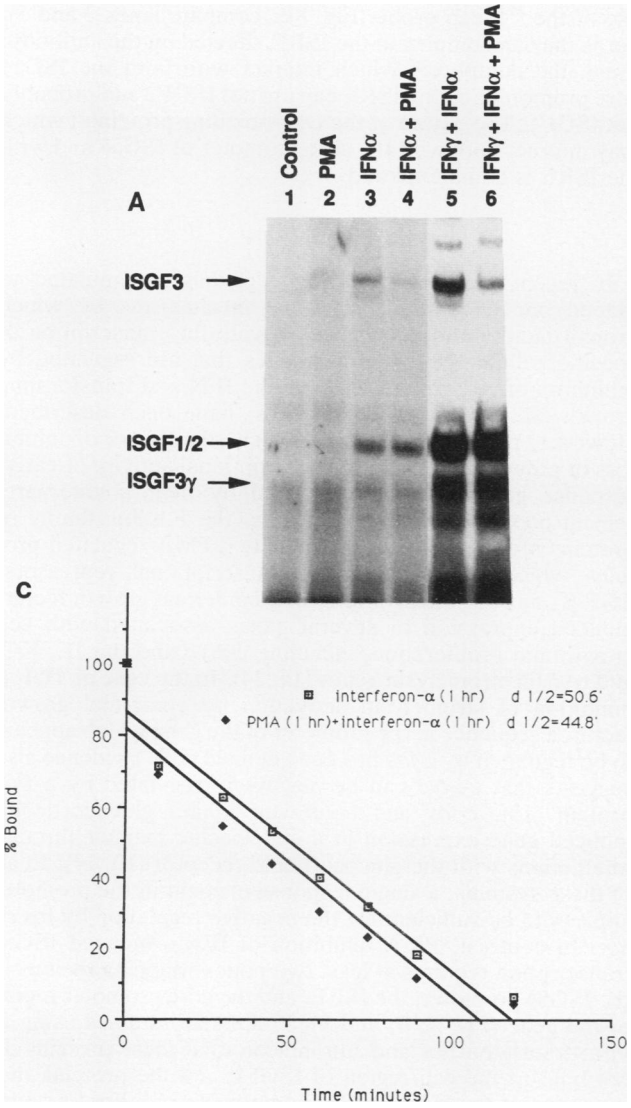
Proteins which interact with the core promoter region of ISG54 and with the ISRE have similar DNA binding specificities. Transfection studies suggested that two regulatory regions in the ISG54 promoter were required for PMA to inhibit IFN-α-induced expression of this gene. To explore whether there was any relationship between the proteins which bind to the core promoter of ISG54 and to the ISRE, competitive inhibition experiments were performed, using the ISRE as a competitor and the cap region as a ^{32}P-labeled probe or vice versa (Fig. 7). EMSAs using the ISRE as a probe indicated that the cap region of the ISG54 competed for binding to a single complex which has a slightly slower mobility than does ISGF1/2. This complex is labeled "CAP" in Fig. 7 (lane 1 versus 3). In a reciprocal fashion, the ISRE of ISG54 competed for the complexes that interacted with the cap site (lanes 6 to 10). When the core region of the ISG54 ISRE (which binds ISGF1/2) was replaced with a consensus binding site for the CRE, it partially competed for binding of ISGF1/2, ISGF3γ, and ISGF3 but not the cap complex (lane 5) or the specific complexes that interacted with the ^{32}P-cap probe (lane 10). Two assays further substantiated that ISGF1 and 2 do not interact with the ISG54 core promoter or the cap complex at the ISRE. First, ISGF1 and ISGF2, like ISGFγ, are sensitive to NEM, while the cap complex at the ISRE and the factors that bind to the cap oligonucleotide probe are NEM resistant (Fig. 8A). Second, a polyclonal antibody against ISGF2 (which also has some cross-reactivity with ISGF1) clearly gives a supershift complex in EMSAs using IFN-α-treated extracts and the ISRE probe (Fig. 8B). However, no complex can be detected with use of the ^{32}P-cap probe (Fig. 8B; compare lanes 2 and 5), nor is the cap complex at the ISRE affected by the antibody. Thus, the complexes which interact with both the ISG54 core promoter and the ISRE cap are not ISGF2 and probably not ISGF1. The nature of the DNA-binding protein(s) which may interact both with the core promoter of ISG54 and with the ISRE is being explored.

DISCUSSION

In recent years, much knowledge has accumulated to extend our understanding of the mechanisms by which growth factors and oncogenes activate the transcription of specific cellular genes. Other genes that are regulated by inhibitors of cell growth, such as the IFN and transforming growth factor beta (TGF-β) genes, have been described. However, the mechanisms by which stimulators or inhibitors of growth modulate the transcriptional activity of early-response genes that are activated by their counterparts remain poorly understood. To date, the Fos/Jun family of proteins are the only well-documented PMA-regulated proteins which can function as transcriptional repressors. TGF-β1 has been shown to inhibit epidermal growth factor-induced expression of several genes associated with cell growth and proliferation, including c-*myc* and the JE, KC, and transin/stromelysin genes (16, 34). In the case of TGF-β inhibition of stromelysin activation by epidermal growth factor, a sequence in the promoter of the gene which appears to be regulated by c-*fos* has been defined (16). Evidence also suggests that c-*myc* can be negatively regulated by a Fos protein (13). c-*fos* and c-*jun* can inhibit glucocorticoid-induced gene expression in a cell-specific manner through interactions with the glucocorticoid receptor (10, 14). In all of these systems, a single response element in the promoter appears to be sufficient for the negative regulation by *fos* or *jun*. In contrast, PMA inhibition of IFN-α-induced ISG54 transcription requires at least two nonoverlapping regions in the ISG54 promoter, the ISRE, and the core promoter region of the gene (Fig. 2, 3, and 9). Preliminary studies using in vitro-translated Fos and Jun indicate that these proteins do not bind to the cap region of ISG54, and the proteins that interact with the ISRE are not homologous to c-Fos or c-Jun. We therefore believe that the mechanisms by which PMA is exerting its negative regulatory actions in this system do not involve the Fos/Jun family of transcription factors.

The mechanism by which the cap region and the ISRE of ISG54 contribute to the PMA inhibition of this gene in a cell-specific and gene-specific manner is unclear. Recent investigations in this laboratory have demonstrated that in human peripheral blood monocytes, PMA dramatically inhibited IFN-α-induced transcription of the cellular genes ISG54, ISG15, IFN-IND2, and 9-27 as well as IFN-γ-induced transcription of IP-10 (unpublished data). This observation underlines the cell and gene specificity of this phenomenon and implies that the ISRE, which is present in all of these genes, maybe sufficient under certain circumstances for PMA to exert its inhibitory actions. Alternatively, these other IFN-regulated genes may contain a regulatory domain which functions in a similar manner to the cap region of ISG54. Examination of the core promoter regions of several IFN-activated genes has not revealed any sequence homology (data not shown). Cell-specific isozymes of protein kinase C are well documented (27), and it is also possible that these isozymes display different capabilities to inhibit IFN-α-activated ISGF3 formation.

The complexes as determined by EMSA which bind to the



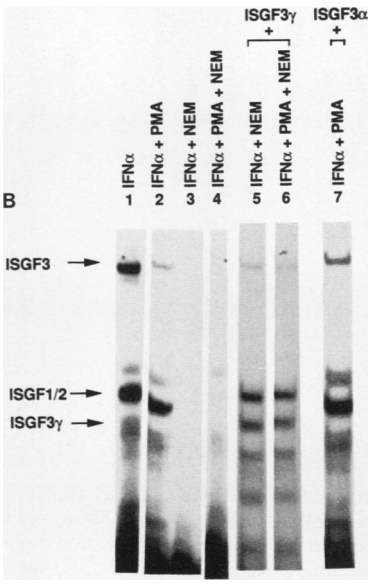


FIG. 6. Regulation of ISGF3 formation by PMA. (A) IFN-γ partially reverses the inhibitory effects of PMA on ISGF3 formation. Nuclear extracts were made from human fibroblasts, and ISGF3 complex formation was analyzed by gel shift, using the ISG54 ISRE. Lanes: 1, untreated cells; 2, PMA, 60 min; 3, IFN-α, 60 min; 4, IFNα plus PMA; 5, IFN-γ for 18 h and then IFN-α for 60 min; 6, IFN-γ for 18 h and then IFN-α plus PMA. (B) ISGF3γ and ISGF3α proteins reconstitute ISGF3 in IFNα–PMA-treated nuclear extracts. Nuclear extracts were prepared as described for panel A. As a source of ISGF3γ, nuclear extracts were made from fibroblasts treated for 18 h with IFN-γ. ISGF3α was prepared from nuclear extracts of cells treated with IFN-α for 60 min and then exposed to 10 mM NEM for 10 min prior to quenching with dithiothreitol (see Materials and Methods for details). Lanes: 1, IFN-α, 60 min; 2, IFNα plus PMA; 3, same as lane 1 treated with NEM; 4, same as lane 2 treated with NEM; 5, same as lane 3 with 10 μg of IFN-γ-treated nuclear extract; 6, same as lane 4 with 10 μg of IFN-γ-treated nuclear extract; 7, same as lane 2 plus lane 3. It should be noted that reconstitution of ISGF3 by using NEM-treated ISGF3α usually does not restore the full activity seen in extracts which have not been exposed to NEM, which accounts for the relatively weaker signals in lanes 5 and 6. (C) Relative dissociation rates of ISGF3 from nuclear extracts prepared from IFNα- or IFNα–PMA-treated human fibroblasts. Nuclear extracts were prepared as described for panel A. A standard binding reaction was performed by using the ^{32}P-labeled ISRE of ISG54 at room temperature for 10 min. A 200-fold molar excess of unlabeled ISG54 ISRE was then added, and aliquots were taken at the indicated times and loaded directly onto a running mobility shift gel. ISGF3 was directly quantitated by counting and plotted against time after the addition of unlabeled oligonucleotide. The indicated $T_{1/2}$s are similar to those observed by Kessler et al. (17).

core promoter of ISG54 are similar in specificity to the cap complex that binds to the ISRE. This observation leads to speculation that PMA may modify the phosphorylation of proteins which constitutively bind both to the ISRE and to the core promoter of ISG54 such that under certain physiologic conditions, direct interactions may occur between these two domains. The proteins which have similar specificities for the ISRE and for the core promoter do not appear to be ISGF1 or ISGF2 because these complexes are NEM resistant, and they do not interact with a polyclonal antibody specific for ISGF2 (Fig. 8). The multicomponent nature of the proteins which interact with the core promoter is suggested by the presence of several specific complexes seen by gel shift (Fig. 4). Since the cap region in the ISG54 gene is adjacent to the TATA box, it is possible that the TATA-binding protein TFIID or factors which interact with TFIID may be responsive to PMA. A recent report indicated that PMA can stimulate the transcription of the tumor necrosis factor gene through its core promoter region, and the level of this effect depends on the TATA box structure (19). Reports from several laboratories have also suggested that accessory proteins are needed for or influence the ability of TFIID to form an optimal preinitiation complex (23, 35). To date, there is no evidence that these accessory proteins are influenced by protein kinases. Purification of the proteins that bind to the cap site of ISG54 will allow us to determine whether there is a physical interaction between TFIID and the core promoter region, as well as to examine whether the ability of PMA to decrease the amount of ISGF3 is influenced by the cap-binding protein(s) (Fig. 5 and 6).

An understanding of the mechanisms by which IFN-α-induced signaling through binding of its receptor and subsequent activation of proteins (ISGF3αs) has significantly improved with the purification and characterization of the

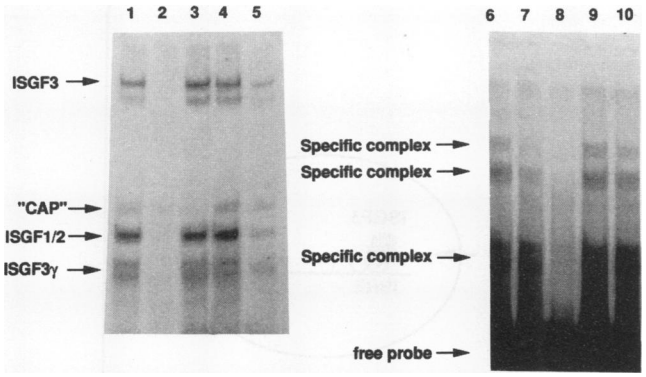


FIG. 7. Evidence that the protein which interacts with the cap site is similar in binding specificity to complexes that interact with the ISRE. Nuclear extracts were prepared from fibroblasts treated with IFN-α for 60 min; 10 μg of these extracts was then assayed for binding to the labeled ISG54 ISRE probe (lanes 1 to 5) or to the labeled cap (bp −42 to +28) probe (lanes 6 to 10). Competitions were performed with a 100-fold molar excess of ISRE (lanes 2 and 7), cap oligonucleotide (lanes 3 and 8), Sp1 (lanes 4 and 9), or an oligonucleotide in which the ISRE core equivalent was replaced by a consensus binding site for CRE (lanes 5 and 10). Specific complexes which interact at the cap site are indicated.

ISGF3 complex (11). Using our present understanding of this process, we can define one of the mechanisms by which PMA is able to inhibit IFN-α-induced expression of ISG54, i.e., through inhibiting the formation of ISGF3 (Fig. 5 and 6). Although PMA decreased ISGF3 formation with the ISRE of ISG15 as a probe (Fig. 5), IFN-α-induced transcription of ISG15 is not inhibited by PMA (Fig. 1), reinforcing our conclusion that in fibroblasts, a combination of both the ISRE and the cap region of ISG54 is necessary for PMA to inhibit ISG54 expression. This finding also suggests that there is not a strict correlation between IFN-α-induced transcription rates for a given gene (i.e., ISG15) and the presence of ISGF3 as measured by EMSA.

PMA does not inhibit IFN-α activation of the ISGF3α proteins (Fig. 6B), suggesting that the initial signaling events regulated by IFN-α binding to its cell surface receptor are not altered. Two possible mechanisms by which PMA could decrease IFN-α-activated ISGF3 include PMA modification of a fraction of ISGF3γ such that it cannot bind to the ISGF3α proteins (or DNA) and PMA modification of a cellular protein which then competes with ISGF3γ for binding to ISGF3α. Several independent assays favor the latter mechanism (Fig. 9). The fact that the amount of ISGF3 in IFN-α–PMA-treated cells can be increased in vitro by the addition of either ISGF3γ or activated ISGF3α demonstrated that an excess of either of these proteins can shift the equilibrium toward the formation of ISGF3 and indicated that ISGF3γ is functional in PMA-treated cells. Furthermore, the rates of dissociation of ISGF3 from the ISRE of cells exposed to either IFN-α or IFN-α–PMA are essentially the same (Fig. 6C), indicating that the intrinsic stability of this complex is not affected by PMA. These observations are all consistent with a mechanism whereby PMA activates a competitive antagonist. In addition, we have recently characterized a cervical carcinoma cell line in which IFN-α-induced ISG54 expression is constitutively antagonized in a similar manner to the inhibitory effects of PMA in fibroblasts. Initial purification of the factor which inhibits ISGF3 formation in this cell line clearly indicates that it is not ISGF3γ (see below).

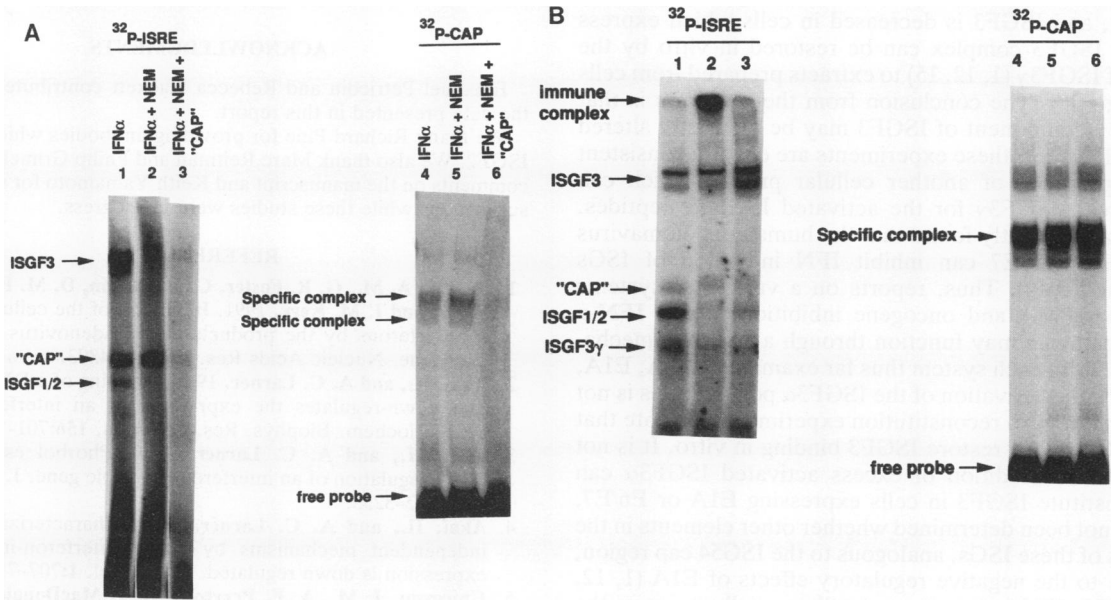


FIG. 8. Evidence that the complex which interacts with the cap site is NEM resistant and does not appear to be ISGF2. (A) The complex which binds to the cap site and its counterpart which binds the ISRE are NEM resistant. Nuclear extracts were prepared from IFN-α-treated cells, and EMSAs were done by using a labeled ISRE or cap probe described in Fig. 7 (lanes 1 and 4). NEM treatment destroyed ISGF1/2 and ISGF3 binding activity but did not affect the complex which bound to the cap probe or the complex labeled "CAP", which binds the ISRE (lanes 2 and 5). This NEM-resistant complex was competed for with excess unlabeled oligonucleotide corresponding to the cap site (lanes 3 and 6). (B) A polyclonal antibody raised against purified ISGF2 does not form an immune complex with the protein(s) which interacts with the cap site. The extract above was incubated with buffer (lanes 1 and 4), a 1:200 dilution of control rabbit serum (lanes 3 and 6), or serum containing anti-ISGF2 antibodies (lanes 2 and 5). Incubations were at 4°C for 3 h prior to analysis by EMSA.

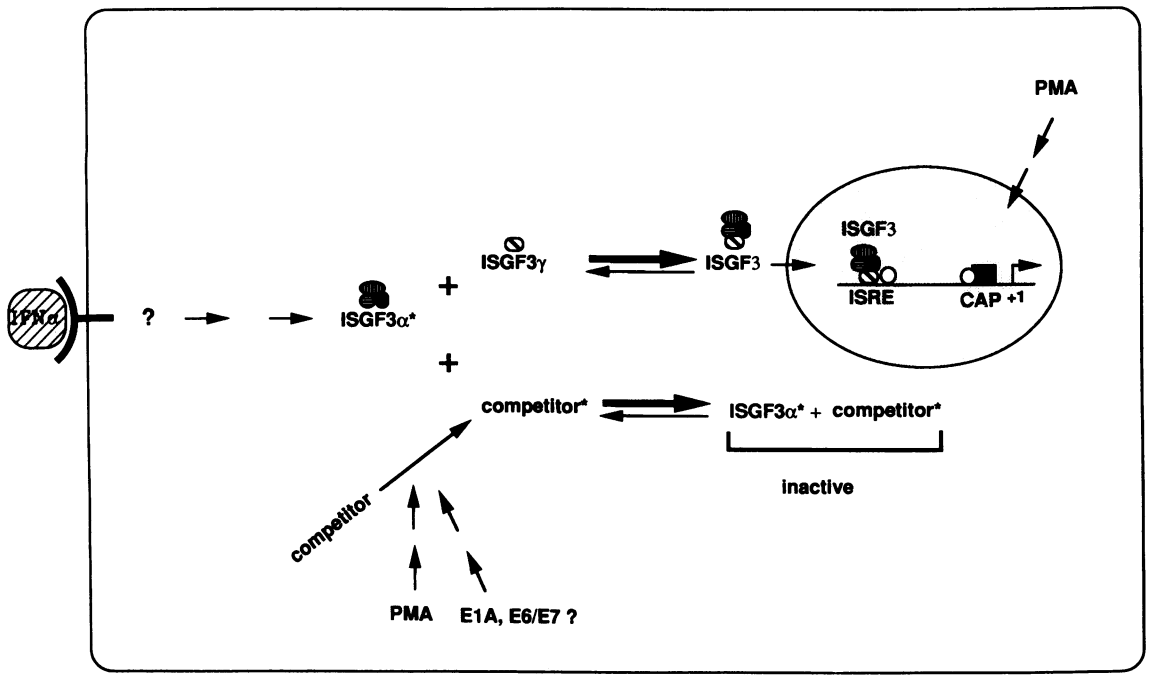


FIG. 9. Representation of the effects of PMA on IFNα-induced ISG54 transcription in human fibroblasts. IFN-α induces the activation of the ISGF3α peptides through an unknown mechanism. The activated ISGF3α* normally interacts with ISGF3γ, forming ISGF3, which can subsequently activate the expression of ISGs. PMA inhibits the induction of ISG54 in fibroblasts by two interdependent mechanisms: (i) a competitor is activated in the cytoplasm (denoted competitor*), which favors an equilibrium whereby the ISGF3α protein(s) are less available to interact with ISGF3γ; and (ii) the core promoter of ISG54 is altered by PMA such that the transcription of ISG54 is inhibited.

Recent reports indicate that oncogenic proteins in addition to PMA inhibit IFN-induced ISGF3 formation. Adenovirus E1A protein inhibits IFN-α-induced cellular gene expression (1, 12, 15), and ISGF3 is decreased in cells which express E1A. The ISGF3 complex can be restored in vitro by the addition of ISGF3γ (1, 12, 15) to extracts prepared from cells expressing E1A. The conclusion from these reports is that the ISGF3γ component of ISGF3 may be indirectly altered by E1A. However, these experiments are equally consistent with the presence of another cellular protein which can compete with ISGF3γ for the activated ISGF3α peptides. We have also recently found that the human papillomavirus proteins E6 and E7 can inhibit IFN induction of ISGs (unpublished data). Thus, reports on a variety of systems suggest that PMA and oncogene inhibition of this IFN-α signaling pathway may function through a common mechanism (Fig. 9). In each system thus far examined (PMA, E1A, and E6/E7), the activation of the ISGF3α polypeptides is not altered, and in vitro reconstitution experiments indicate that excess ISGF3γ can restore ISGF3 binding in vitro. It is not known whether addition of excess activated ISGF3α can also reconstitute ISGF3 in cells expressing E1A or E6/E7, and it has not been determined whether other elements in the promoters of these ISGs, analogous to the ISG54 cap region, contribute to the negative regulatory effects of E1A (1, 12, 15) or E6/E7. IFN-γ treatment of cells partially reverses the inhibitory effects PMA. Whether the inhibitory effects of E1A or E6/E7 can be reversed in vivo by extended incubation with IFN-γ may depend on either distinct competitor proteins which have different inhibitory potencies, or, alternatively, a single competitor which can be activated to various degrees (i.e., by PMA, E1A, or E6/E7). Fractionation and characterization of this inhibitor activity is needed so that a detailed understanding of this important control regulatory mechanism by which PMA and possibly other growth promoters modulate IFN signaling can be elucidated.

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