Interferon Regulatory Factor-7 Synergizes with Other Transcription Factors through Multiple Interactions with p300/CBP Coactivators*

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Interferon regulatory factor (IRF)-7 is activated in response to virus infection and stimulates the transcription of a set of cellular genes involved in host antiviral defense. The mechanism by which IRF-7 is activated and cooperates with other transcription factors is not fully elucidated. Activation of IRF-7 results from a conformational change triggered by the virus-dependent phosphorylation of its C terminus. This conformational change leads to dimerization, nuclear accumulation, DNA-binding, and transcriptional transactivation. Here we show that activation of IRF-7, like that of IRF-3, is dependent on modifications of two distinct sets of Ser/Thr residues. Moreover, we show that different virus-inducible cis-acting elements display requirements for specific IRFs. In particular, the virus-responsive element of the ISG15 gene promoter can be activated by either IRF-3 or IRF-7 alone, whereas the P31 element of the interferon-β gene is robustly activated only when IRF-3, IRF-7, and the p300/CBP coactivators are all present. Furthermore, we find that IRF-7 interacts with four distinct regions of p300/CBP. These interactions not only stimulate the intrinsic transcriptional activity of IRF-7, but they are also indispensable for its ability to strongly synergize with other transcription factors, including c-Jun and IRF-3.

Viral infection of vertebrate cells results in the early secretion of a number of cytokines. These include interferons (IFNs), tumor necrosis factors, and several chemokines and interleukins that signal the occurrence of the infection and orchestrate the innate immune response directed against the invading virus (1–4). The production and action of these cytokines depends in large part on specific modulations of gene expression.

Expression of type I IFN genes is controlled at least in part by IFN regulatory factors (IRFs), such as IRF-3, IRF-5, and IRF-7. These IRFs differ in their pattern of expression: IRF-3 is a constitutively and ubiquitously expressed protein, whereas IRF-5 and IRF-7 are expressed at different levels in various tissues, and their synthesis can be further stimulated following exposure to IFNs. In cells where the constitutive levels of IRF-7 are low, this IFN-dependent up-regulation of IRF-7 is crucial for expression of its target genes (e.g., most IFN-α genes) (5). A similar situation may exist for IRF-5 (6). The activation of these IRFs involves the phosphorylation of a stretch of serine (Ser) and threonine (Thr) residues at their C-terminal ends. This phosphorylation results in a conformational change that allows nuclear accumulation, DNA-binding, and transcriptional activation of target genes (5–16).

Normally, IRF-3 and IRF-7 associate with each other, and on virus infection they further interact with the coactivators p300 and CBP (CREB-binding protein) to form a stable complex termed VAF (virus-activated factor). VAF alone is sufficient to activate the transcription of IFN-α and virus-inducible genes through their ISRE (IFN-stimulated response element), where it binds with high affinity. However, it is necessary but not sufficient to activate the IFN-β gene promoter, where it binds with much less affinity. In this case, two other virus-inducible activator proteins ATF-2/c-Jun and NF-κB are required, and together with VAF they assemble into a unique complex at the IFN-β gene promoter (13).

The activity of IRF-7 is modulated by virus-dependent post-translational modifications, but the exact mechanism by which it activates transcription is not fully elucidated. In particular, it would be of interest to identify the amino acids involved in IRF-7 activation, and to determine whether IRF-7 can activate transcription independently from other transcription factors or from coactivators. In this study, we examined IRF-7-dependent transcription in mammalian cells and in insect cells (S2 cells, derived from Drosophila melanogaster embryos). Insect cells have a number of advantages that alleviate limitations associated with the use of mammalian cells: 1) they do not have any apparent IRF ortholog (IRF family members have overlapping binding specificity and can associate with each other); 2) the role of mammalian coactivators can be assessed (Drosophila CBP ortholog is functionally different enough that it cannot coactivate transcription with IRF-3 (16)); 3) the IFN-dependent feedback loop that leads to IRF-1 and IRF-7 up-regulation cannot operate in insect cells (no IFN or IRF orthologs); 4) there is no stimulation of virus-activated signal transduction pathways by DNA transfection alone.

Here, we show that, unlike IRF-3, IRF-7 exhibits intrinsic transcriptional activity (16). Nevertheless, binding of the mammalian p300/CBP coactivators further stimulates the ability of IRF-7 to activate transcription. Whereas IRF-3 and IRF-7 can independently activate transcription from the ISRE, efficient
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EXPERIMENTAL PROCEDURES

Plasmid Constructs and Sequence Analysis—Effector constructs for transient transfections of mammalian and insect cells were cloned into pcDNA3 and pPac vectors, respectively, using standard methods (17). In these constructs, the coding sequence of human IRF-7B is preceded by a histidine tag (HisIRF-7) or a sequence encoding three flag tags, MDYKDHDGYDIDDKHDYDIDH (F3IRF-7). Alternatively, the coding sequence of IRF-7 was fused to the Gal4 DNA-binding domain (aa 1–147). Mutants of IRF-7 were generated by PCR and were all verified by sequencing. Reporter constructs have been described (13, 18), and multiple copies of cis-acting elements as indicated.

Cell Culture and Transfections—HEC-1B (HTB-113, ATCC) cells were derived from a human endometrial carcinoma and are resistant to IFN; San cells were derived from a human glioblastoma and are lacking type I IFN genes; 293T cells are a SV40 large T antigen expressing strongly transfactional derivative of 293 cells, which are derived from human embryonic kidney cells transformed with human adenovirus type 5. These cell lines were grown at 37 °C, 5% CO₂, in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Sendai virus was obtained from SPAFAS and used at 200 hemagglutinating units/ml. S2 cells were grown at 26 °C, in Schneider’s Drosophila medium containing 12% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Transfections using the calcium phosphate coprecipitation technique were as described (17). Mammalian cells in 100-mm dishes were transfected with 1 ml of a precipitate containing 10 μg of reporter, 5 μg of effector plasmid (except for Gal4, 1 μg), 5 μg of pCMV-lacZ and pSP72 to a total of 25 μg (5–9 μg) for 18 h, trypsinized, aliquoted for further treatments, and harvested 3 days after transfection. S2 cells were seeded in 6-well plates (3 million cells in 3 ml), transfected the next day with 0.3 ml of a precipitate containing 250 ng of hsp82lacZ, 500 ng of reporter plasmid, and effector plasmid mixtures as indicated in the figure legends (with pPac added to a total of 5.75 μg), and harvested 2 days after transfection. CAT activity and β-galactosidase activity were measured in extracts of transfected cells (17), and CAT activity was expressed in arbitrary units after normalization to β-galactosidase activity to control for transfection efficiency.

In Vitro Translation, Cell Extract, EMSA, and Western—In vitro translation in rabbit reticulocyte lysates were performed exactly according to the manufacturer’s recommendations using the T7 kit (Promega), linearized pcDNA effector plasmids, and T7 RNA polymerase. Whole cell extract preparation, binding, and PAGE conditions for EMSA were as described (13), except that 0.5 μg of poly(dI·dC) were added for EMSA involving in vitro translated IRF-7. Immunoblotting, after SDS-PAGE was performed as described (19), using M2 (anti-FLAG, Sigma) as primary antibody, and anti-mouse horseradish peroxidase conjugates as secondary antibody. The chemiluminescence detection system was from PerkinElmer Life Sciences.

Pull-down Experiments—GST-CBP-N, -M, -C, p300-N, -M, and C were described previously (20, GST-CBP-N1, -N2, -N3, -C1, -C2, -C3, and GST-p300-C1, -C2, -C3) were generated by subcloning PCR products and verified by sequencing. GST and GST fusions were expressed in Escherichia coli BL21 and purified as recommended by the manufacturer (Amersham Biosciences), and dialyzed against phosphate-buffered saline, 10% glycerol. 32P-labeled proteins were incubated with immobilized GST fusion proteins immobilized on glutathione-Sepharose beads in 150 mM KCl, 20 mM Tris, pH 8.0, 0.5 mM dithiothreitol, 50 μg/ml ethidium bromide, 0.2% Nonidet P-40, and 0.02% bovine serum albumin (binding buffer) for 1 h at 4 °C, followed by two washes with binding buffer and two washes with binding buffer without bovine serum albumin. Extracts from HEC-1B cells labeled in vivo with [32P]orthophosphate were prepared and treated with deoxycholate and Nonidet P-40 exactly as described (13). After 50-fold dilution with binding buffer and preclarifying on glutathione-Sepharose beads, the

RESULTS

Transcriptional Activity of IRF-7 Mutants in Insect Cells—In mammalian cells, IRF-7 is constitutively associated with IRF-3, which is present ubiquitously, and it is thus unclear whether IRF-7 can activate transcription on its own. The ability of IRF-7 to activate transcription independently from other transcription factors or from coactivators was tested in S2 cells. Because S2 cells lack the IFN system, IRF-7 cannot be activated through the virus-dependent pathway. However, IRF-7 can be made constitutively active in mammalian cells either by deletion of an internal inhibitory domain or by glutamic acid (Glu) substitutions of several critical Ser residues in the C terminus (5, 21). These residues have been proposed to be the targets of virus-dependent phosphorylation, and they fall into two sets based on sequence comparison (Fig. 1A). We used constructs expressing human IRF-7B wild-type (WT), IRF-7E6, IRF-7E6, and IRF-7E8 (mutants of one or both sets of target residues), and IRF-7Δi (lacking an internal inhibitory domain) with N-terminal FLAG tags. These plasmids were cotransfected with a reporter driven by three copies of the ISRE of the ISG15 gene (i.e. a high affinity binding site for IRFs), in the presence or absence of mammalian p300/CBP (Fig. 1B).

Expression of IRF-7B WT, IRF-7E6, or IRF-7E8 alone resulted in minimal activity of the ISREx3CAT reporter, whether the p300/CBP coactivators were coexpressed or not. By contrast, expression of IRF-7Δi significantly stimulated ISREx3CAT activity in the absence of transfected p300/CBP (Fig. 1B). Coexpression of p300/CBP further stimulated IRF-7Δi activity by about 40%. This stimulation was even more significant when the concentration of IRF-7Δi was low (see below).

The unexpected lack of activity of IRF-7E6 in insect cells might reflect poor DNA binding in the absence of IRF-3 with which it is normally associated. We tested this possibility using Gal4-IRF-7B fusions and a reporter, G5E1bCAT, driven by five copies of a Gal4-binding site. Gal4-IRF-7WT, E2, E6, and E8 had very low level activity in S2 cells, which was minimally stimulated by p300/CBP coexpression (Fig. 1C, middle panel). The activity of E6 was slightly stronger than that of Gal4-IRF-7 WT, which could be accounted for by the difference in their expression levels (Fig. 1C, right panel). By contrast, Gal4-IRF-7Δi was a very strong activator, which was further stimulated by p300/CBP coexpression (3–4-fold). The fact that Gal4-IRF-7Δi was much more potent than IRF-7Δi in S2 cells (compare Fig. 1, B with C, left panel, and note the different scales), suggests that IRF-7Δi bound DNA relatively weakly.

Thus, IRF-7Δi activated transcription in insect cells, demonstrating that IRF-7 can activate transcription independently from other IRFs. However, IRF-7E6 failed to do so, despite the ability of a very similar construct, IRF-7(D475–487), to function as a constitutive activator in 293T cells. IRF-7(D475–487) is a mutant form of HIRF-7A, where the Ser residues modified in IRF-7E6 have been mutated to aspartic acid (Asp) instead of Glu (21); IRF-7A differs from IRF-7B used in this study by an extra 29 aa after position 226. To understand the basis for this unexpected difference, we examined the activity of a variety of IRF-7B mutants in human cells.

Transcriptional Activity of IRF-7 Mutants in Human Cells—We first tested our IRF-7E6 mutant in 293T cells and confirmed that, as described for IRF-7(D475–487) (21), it was constitutively active and virus infection did not appreciably
stimulate its activity (data not shown). However, the use of 293T cells for such experiments might be problematic (limitations 1–4, see Introduction). Therefore, we examined the phenotypes of a panel of hIRF-7B mutants by doing cotransfections in SAN cells. These cells lack type I IFN genes, avoiding the complication of a feedback loop, and transfection per se does not activate virus-dependent pathways as effectively as in 293T cells (limitations 3 and 4). We used the P31x2CAT reporter, which is only weakly inducible by virus alone but strongly stimulated by WT IRF-7, thus allowing the phenotypes of mutations to be clearly assessed (13).

The sequence and phenotype of each IRF-7B point mutants are shown in Fig. 2A. If a residue is normally phosphorylated in response to viral infection, mutations to Ala are expected to decrease virus-dependent activity. By contrast, mutations to Glu could lead to constitutive activity or to a decrease in virus-dependent activity depending on how effectively Glu functions as a phosphoserine mimic. Consistent with previous observations, expression of full-length IRF-7B strongly stimulated the activity of P31x2CAT, in both uninfected and infected cells (13). Mutation of either set of Ser residues to Ala (IRF-7-A2, -A6, and -A8) drastically reduced the ability of IRF-7 to stimulate P31x2CAT activity in virus-infected cells, suggesting that both sets of residues are involved in virus activation. Mutation of the first set to Glu (IRF-7-E2) also strongly suppressed IRF-7 activity in virus-infected cells. By contrast, mutation of part or all of the second set of residues to Glu (IRF-7-E6 and E8) had no effect on virus-activated levels, but substantially increased IRF-7 constitutive activity. Combining the IRF-7E6 mutation with either Ala or Glu substitution in the first set (IRF-7A2E6 and -E8), however, led to a strong decrease in the ability of IRF-7 to stimulate P31x2CAT activity both in uninfected and virus-infected cells, suggesting that Glu residues do not serve as good phosphomimetics in the first set.

In addition, we also assayed each of these mutants as fusion proteins with the Gal4 DNA-binding domain by doing cotransfections with G5E1bCAT. This approach minimized the possibility of endogenous IRF proteins being associated with the ectopically expressed IRF mutants (limitation 1). Most mutants displayed no transcriptional activity (Fig. 2A, right panel), suggesting that the activity detected with the P31x2CAT reporter was the result of their association with endogenous IRF-3 or IRF-7 molecules. Gal4-IRF-7B WT activity was still inducible but the basal activity was significantly higher than for the native protein. Intriguingly, Gal4-IRF-7E6 showed very strong basal activity and virus infection enhanced it to levels even higher than those achieved with Gal4-IRF-7-Δi (see Fig. 3C).

The fact that the activity of IRF-7E6 or Gal4-IRF-7E6 could be further stimulated upon virus infection in SAN cells unless the first set of residues were mutated to Ala or Glu strongly suggests that residues within both sets were phosphorylated in response to viral infections. Furthermore, the constitutive activity displayed by IRF-7E6 or Gal4-IRF-7E6 in SAN or 293T cells is best accounted for by phosphorylation of the first set of residues upon transfection because this constitutive activity was absent when these residues were mutated or when IRF-7E6 or Gal4-IRF-7E6 were expressed in insect cells.

We also took advantage of the activity of IRF-7Δi in insect cells to test the effect of Ser residue substitutions in the context of this construct. Interestingly, all the substitutions tested were more active than IRF-7-ΔiWT in S2 cells (Fig. 3A). When the IRF-7Δi mutants were tested in mammalian cells, we observed the same pattern of activity as in insect cells (Fig. 3B for
HEC-1B cells, we obtained very similar data for SAN cells, not shown). Thus, whereas our data indicate that Glu substitutions in IRF-7 cannot sufficiently mimic the virus-activated form of IRF-7 in insect cells, these mutations can still increase the transcriptional activity of IRF-7 in the context of the internal deletion mutant, underscoring the importance of both sets of residues in IRF-7 activation.

The phenotypes of IRF-7B deletion mutants are shown in Fig. 3C. The shortest truncation (aa 449) led to a complete loss of virus responsiveness, and further deletions removed inhibitory sequences and uncovered a weak constitutive activation domain (aa 388, 308, and 266). By contrast, expression of an internal deletion mutant, IRF-7Δi, where aa 267–438 were removed, led to a strong activation of P31x2CAT in uninfected cells that was further increased upon virus infection (about 2-fold). It should be noted that the level of activation by IRF-7Δi is comparable with that achieved with virus-activated IRF-7B in this assay, whereas similar internal deletion constructs described by other investigators are much more active than WT IRF-7 (21, 22). As observed in insect cells, Gal4-IRF-7Δi had very strong transcriptional activity, about 20 times stronger than that of IRF-7Δi, consistent with relatively weak IRF-7Δi DNA binding.

**IRF-7 Interacts with Multiple Domains of p300/CBP**—The functional interaction between IRF-7 and p300/CBP as seen in the transfection experiments suggest that these proteins may also interact physically. We first examined the interaction between IRF-7 and the coactivators using metabolically 32P-labeled protein and three non-overlapping fragments of CBP fused to GST (see “Experimental Procedures”). As shown in Fig. 4B, IRF-7 associates specifically with the C-terminal 550 aa of murine CBP in a virus-dependent manner, although the interaction is much weaker than what was observed with IRF-3 in a similar experiment (16). Thus, endogenous virus-activated IRF-7 can interact with the C-terminal domain of CBP.

We next used in vitro translated IRF proteins in pull-down experiments to further map this interaction (a representative experiment is shown in Fig. 4C, and binding values referred to in the text below correspond to the average of at least three independent experiments). Both IRF-7BWT and IRF-7Δi proteins bound preferentially to the N-terminal region of CBP (–18 and ~57% of input, respectively) as compared with the C-terminal region (~0.3 and ~1% of input, respectively), whereas IRF-3 displayed a reversed preference (16). As was the case for IRF-3, however, binding to the N and C regions of CBP was further mapped to the N2 and C2 domains, and interestingly binding to C (~0.9 and ~6% for IRF-7WT and IRF-7Δi, respectively).

For p300, IRF-7WT and IRF-7Δi also bound to the N (~12 and ~30% of input, respectively) and C (~3 and ~12% of input, respectively) regions, but binding to the C region was further mapped to two independent domains, C1 and C2. For both p300 and CBP, weak (~2% of input) but reproducible binding to IRF-7 proteins was also detected with the middle part of each coactivator, M. Importantly, binding of IRF-7Δi to either the N or C region, but not the M region of p300/CBP was consistently stronger than that of IRF-7WT (about 3 times). Thus, internal deletion of aa 267–438 in IRF-7 favors a conformation that cannot only bind DNA and activate transcription on its own, but also interact more strongly with p300 and CBP. Moreover, IRF-7 proteins made more distinct contacts with the coactivators (3 for CBP and 4 for p300) than IRF-3 proteins (2 distinct contacts, (16)). Furthermore, the fact that the IRF-coactivator interactions were detected using protein produced in vitro (in rabbit reticulocyte lysates, Fig. 4C) and GST fusions produced in bacteria suggests that these interactions were direct and in particular did not involve IRF-3. It is noteworthy that the pull-downs using recombinant proteins were more sensitive than those using native proteins. The more stringent pull-down immunoprecipitation experiments shown in Fig. 4B failed to detect the interaction with CBP-N, presumably because this interaction is affected by the presence of detergent.

**Mapping IRF-7 Domains**—Latent IRF-7 does not bind DNA but virus activation or truncation of the C-terminal domain results in DNA binding activity (13). We conducted EMSA experiments using the ISRE of the ISG15 gene as a probe, WT or mutant IRF-7B proteins expressed in vitro, and GST-CBP fusion proteins. We detected very little ISRE binding for IRF-7B or IRF-7Δi in the presence of GST alone (Fig. 5A, *lanes* 4 and 7), and the same held true for IRF-7ΔE6, ΔE2, ΔE3, and ΔE5 (data not shown). By contrast, truncation to aa 388 led to detectable ISRE binding (Fig. 5A, *lane* 10), despite a
significantly lower expression level in rabbit reticulocyte lysate (Fig. 5A, right panel). No supershift was detected in the presence of GST-CBP-C2, despite the ability of this fusion protein to interact with IRF-7 (Figs. 4C and 5B).

In the presence of GST-CBP-N, a slow migrating complex was detected with IRF-7B, IRF-7Δi, and IRF-7B1–388. The CBP-N-supershifted IRF-7Δi complex showed an intensity similar to that of the supershifted IRF-7 WT complex, despite its lower expression level (right panel), indicating that this construct indeed interacts more strongly with this region of CBP (compare Fig. 5, A and B). Truncation to aa 388 lead to a small increase in binding to those regions of p300/CBP as compared with WT, whereas further truncation to aa 308 or 266 resulted in weaker binding to these coactivators.

These interactions were also assayed independently from DNA binding by pull-down assays (Fig. 5B). Interestingly, IRF-7Δi, which corresponds to the last 37 aa of IRF-7B spliced immediately after aa 266, bound much more strongly to p300/CBP in this assay than either IRF-7 WT or 1–266, whereas the removal of these last 37 aa (in IRF-7B1–388) had relatively little impact on the ability of IRF-7 to interact with the coactivators (Fig. 5B). As was the case for the EMSA assay, the properties of IRF-7E6 and -E8, and of IRF-7ΔiE2, -ΔiE3, and -ΔiE5 in the pull-down assay were indistinguishable from that of IRF-7 WT and IRF-7Δi WT, respectively (data not shown).

When tested in S2 cells, IRF-7B1–388 displayed no transcriptional activity, whereas further deletion to 308 showed some activity (Fig. 5C), as was the case in mammalian cells (Fig. 2B). Taken together, these results indicated that there is no straightforward correlation between the ability of IRF-7B to bind DNA, interact with the p300/CBP coactivators, and activate transcription in either mammalian or insect cells. Indeed, IRF-7B1–388 bound DNA most efficiently, interacted robustly with p300/CBP, and yet completely failed to activate transcription. Rather, these results suggest that IRF-7 functional domains are arranged in a complex pattern, where the domains involved in DNA binding, transcriptional activity, and interac-

![Fig. 3. Effect of point mutations in the context of IRF-7Δi and mapping of IRF-7 activation domains.](image-url)
tions with coactivators appear to be distinct and partially overlapping (Fig. 5D).

IRF-3, IRF-7, p300, and CBP Synergistically Activate Transcription—We have shown that constitutively active forms of either IRF-3 or IRF-7 can each activate transcription in insect cells, i.e. independently from the presence of any endogenous IRF (Fig. 1 and Ref. 16). In mammalian cells, however, IRF-7 is found to be constitutively associated with IRF-3, and ectopic expression of both proteins leads to maximal transcriptional activation (13), suggesting these proteins have evolved to regulate the expression of virus-inducible genes in a cooperative manner. We therefore examined the effect of cotransfecting IRF-3 and IRF-7 on two reporters, one driven by the ISRE of the ISG15 gene, and the other driven by the IRF-binding P31 element of the IFN-β gene promoter (Fig. 6).

In these experiments, we used a constitutively active form of IRF-3, IRF-3E7, where both sets of Ser/Thr residues have been changed to Glu residues (16). When equal amounts of IRF-7Δi or IRF-3E7 expression plasmids were transfected into S2 cells, IRF-3 proved a stronger activator than IRF-7 on the ISREx3CAT reporter, consistent with the higher affinity of IRF-3 for this sequence (13). In the absence of p300/CBP, cotransfection of IRF-3 and IRF-7 did not lead to any synergy in activation (Fig. 6A). In the presence of p300/CBP, however, the levels of activation achieved by the combination of IRF-3 and IRF-7 was much higher than by either activator alone. The extent of the synergy was even more dramatic for the P31x4CAT reporter: each constitutive activator, IRF-7Δi or IRF-3E7, activated transcription about 2.5-fold over background, whereas together they activate transcription about 300-fold above background.

We also examined the effect of increasing amounts of IRF-3E7, IRF-7Δi, p300, and CBP on their ability to synergistically activate transcription from the P31x4CAT reporter (Fig. 6C). The amounts of transfected plasmids were increased by a factor of two from 100 ng to 1.6 μg for each of IRF-3E7, IRF-7Δi, and p300/CBP. The upper and lower curves on the graph were computed from the highest or lowest levels of activation, respectively, assuming a linear relationship between amount of transfected plasmid and transcriptional activation, i.e. as if there was no cooperative association of the factors involved in activation of the reporter. Remarkably, there was a dramatic threshold effect, a sharp increase in transcriptional activity over the last 2-fold increase in amounts of expression plasmids transfected (Fig. 6C).

Thus, we conclude that IRF-3 and IRF-7 can strongly synergize in the presence of p300/CBP, as the combination of these transcription factors was 60 times more potent than what would be expected if their effects were purely additive. This synergy and the dramatic increase in transcription over a small
increment in amounts of transfected plasmids further suggest that the assembly of IRF-3, IRF-7, p300, and CBP into an activating complex on the P31 sequence is highly cooperative.

IRF-7, c-Jun, and CBP Synergistically Activate Transcription—A reporter driven by a single copy of the P31 element fails to respond to virus infection in mammalian cells. However, P431, which includes the AP-1-binding site of the IFN-β gene promoter, PRDIV, next to P31 (Fig. 6F), can confer virus inducibility to a reporter as a single copy (18). We tested the ability of IRF-7 and c-Jun to synergize in activating the P431x3CAT reporter in S2 cells (Fig. 6D). Transfection of c-Jun alone activates a PRDIVx6CAT reporter, and the transcriptional activity of c-Jun is only modestly stimulated by cotransfection of p300/CBP (1.5 μg of a 1:1 mixture) on the ISREx3CAT reporter. D, schematic of IRF-7 deletions and mapping of domains involved in dimerization, intrinsic transcriptional activity, and coactivator interactions.
These results suggest that the ability of IRF-7 and c-Jun to interact with CBP might be more important for their ability to synergize with other transcription factors than to activate transcription by themselves. Interfering with the interaction between these transcription factors and CBP by coexpressing a N- or C-terminal GST-CBP fusion protein inhibited the synergistic...
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activation of P431x3CAT by c-Jun/IRF-7Δi/CBP. GST-CBP1-1100 was a more potent inhibitor than GST-CBP1892-2441, consistent with the observation that both IRF-7 and c-Jun interact more strongly with CBP N terminus (Fig. 4). Either fusion protein alone, or in combination, had little effect on the level of activation achieved by IRF-7Δi/c-Jun, indicating that an intact CBP is required for maximal transcriptional synergy.

DISCUSSION

IRF-7 is a transcription factor found associated with the promoter of virus-inducible genes only in virus-infected cells and IRF-7 is critical for the full activation of type I IFN genes (5, 13). Because of a number of limitations in the experimental systems used so far, several aspects of the mechanism by which IRF-7 activates transcription remain unresolved (see Introduction). Complementing our studies with experiments in insect cells that bypass these limitations, we were able to address some of these issues.

IRF-7 Activated Transcription Directly—First, it was unclear whether IRF-7 could activate transcription independently from other transcription factors, because it is known to be constitutively associated with the IRF-3 protein, which is ubiquitous in mammalian cells, and it has the potential to interact with other IRFs. Here we showed that a constitutively active form of IRF-7, IRF-7Δi, could activate transcription in cells lacking any IRF family member (Fig. 1). The contribution of Drosophila transcription factors to IRF-7 activity is unlikely as we used reporters containing as regulatory elements only a minimal TATA box and IRF-binding sites.

It was also unclear whether IRF-7 activity is dependent on coactivators, as no physical interactions had been detected between these proteins (21), and elimination of the coactivators by gene targeting results in embryonic lethality. We found that IRF-7Δi activity in insect cells was further stimulated by coexpression of the mammalian p300/CBP coactivators, up to 3–4-fold (Figs. 1 and 3). This is in contrast to IRF-3E7, whose activity in insect cells is entirely dependent on the expression of the mammalian coactivator (16) (Fig. 6). These results suggest that IRF-7 had intrinsic transcriptional activity or that Drosophila CBP was able to partially coactivate with IRF-7.

We favor the former possibility because mapping experiments showed no direct correlation between interactions with coactivators and transcriptional activity (Fig. 5D).

IRF-7 Activation Depends on Modifications within Two Distinct Sets of Residues—We have shown that in the case of IRF-3, two sets of residues cooperate in achieving maximal transcriptional activation (16). However, the identity of phosphorylatable amino acids are still robustly virus-inducible, while mutants with both sets mutated are not, strongly suggests that residues within both sets of Ser were phosphorylated following viral infection. The constitutive activity of IRF-7E6 is relatively weak in SAN cells, very strong in 293T cells, and completely absent in insect cells. These results suggest that IRF-7E6 must be further modified upon transfection of mammalian cells. These differences in activity could best be accounted for by phosphorylation of the first set of residues by a mammalian-specific kinase, possibly activated by transfection (limitation 4), as suggested for IRF-3 (16).

Thus, Glu residues only partially mimicked the effect of Ser phosphorylation, which could be expected because of the intrinsic differences between their side chains. In the case of IRF-3, the Glu substitution was least efficient for the first set, but IRF-3E7 (where both sets were substituted with Glu residues) was nevertheless active (16). In the case of IRF-7, phosphorylation of the first set of residues was essential for activity as substitution to non-phosphorylatable residues blocked activity. Unlike what was observed for IRF-3, substitution of both sets with Glu residues in IRF-7 failed to activate transcription (Figs. 1 and 2). This failure to activate was not because of lack of DNA binding, as Gal4 fusions of the same mutants displayed as little activity as Gal4-IRF-7WT (Fig. 1). Rather, phosphorylation of the first set seems to be essential for the conformational change of IRF-7 that leads to dimerization, DNA-binding, and transcriptional activation. Nevertheless, in the context of IRF-7Δi, Glu substitution of residues within both sets increased transcriptional activity (Fig. 3), further suggesting that residues within both sets are modified upon virus infection. Whereas our data identify both sets of residues as functionally important for virus-dependent activation of IRF-7, further studies will be required to determine exactly which Ser residues within each set are phosphorylated upon virus infection.

IRF-3, IRF-7, p300, and CBP Activate Transcription Synergistically—The fact that IRF-3 and IRF-7 can act independently does not mean that they do so in physiological conditions. In fact, IRF-3 and IRF-7 can associate with each other, and this association is constitutive, unlike their virus-dependent association with p300/CBP (13, 21).

When the ISREx3CAT reporter was used, IRF-3 was a more potent activator than IRF-7 (Fig. 6), in agreement with the observation that IRF-3 binds more efficiently than the ISRE than IRF-7 (whereas the reverse is true for P31) (13). The combination of IRF-3 and IRF-7 was more potent than each activator alone, and these results are consistent with the analysis of ISG15 gene expression in embryonic fibroblasts derived from IRF-3 and IRF-9 double knock-out mice (23).

Activation from the P31x4CAT reporter was very low with IRF-3 or IRF-7 alone, but the combination of these two factors strongly synergized (60-fold, Fig. 6), as they do in mammalian cells (13). Synergy between IRF-3 and IRF-7, like IRF-3 transcriptional activity, was entirely dependent on the presence of the coexpressed mammalian coactivator. Synergy is the functional counterpart of cooperativity in physical association, which is itself dependent on multiple contacts between interaction partners. Multiple protein-protein interactions are indeed involved in the formation of VAF, a complex containing IRF-3, IRF-7, p300, and CBP. IRF-3 and IRF-7 each interact with p300/CBP through two and four distinct domains, respectively (Ref. 16 and this study); both IRF-3 and IRF-7 homodimerize; IRF-3 further interacts with IRF-7 through two distinct domains (24); and p300/CBP also interact with each other through multiple domains. These multiple interactions may account for the unusual stability of VAF as compared with most other transcription factor-coactivator complexes, which typically cannot be detected in mobility shift assays. Cooperativity in VAF formation is further suggested by the observation that a small increment in amounts of plasmids expressing

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2 H. Yang, C. H. Lin, G. Ma, and M. G. Wathelet, unpublished data.
INTRODUCTION

IRF-3, IRF-7, p300, and CBP resulted in a dramatic increase in transcription of the P31-driven reporter in insect cells.

Synergy between IRF-7 and c-Jun Is CBP-dependent—The presence of mammalian coactivators is essential for IRF-3 activity. Therefore, the possible mechanisms by which these coactivators promote synergy between IRF-3 and IRF-7, such as scaffolding a nucleoprotein complex or recruitment of the transcriptional machinery, could not be independently ascertained. By contrast, both IRF-7Δi and c-Jun have intrinsic transcriptional activities that were only moderately stimulated by coexpression of the mammalian coactivators. IRF-7Δi or c-Jun had little activity alone, or in the presence of CBP, but coexpression of IRF-7Δi, c-Jun, and CBP resulted in very strong synergy on the P431x3CAT reporter. Interfering with the interaction between these transcription factors and CBP by coexpressing fragments of CBP inhibited this synergy. These results are not due to squelching, i.e. interference with CBP transcriptional activity, as we previously demonstrated (16). Rather, these results suggest that the ability of IRF-7Δi and c-Jun to interact with CBP is more important to their ability to synergize with other transcription factors than to activate transcription by themselves. Taken together, these data suggest that in the activation of the P431x3CAT reporter, CBP does not only serve as an adaptor between the general transcription machinery and the activators, but it can stabilize the formation of an IRF-7-c-Jun-P431 nucleoprotein complex through simultaneous interactions with both IRF-7 and c-Jun. The flexible nature of CBP may be crucial for accommodating the specific arrangement of activator proteins on the IFN-β promoter as well as on other complex gene regulatory elements (25).

CONCLUSIONS

Our data indicate that IRF-7 could activate transcription independently from other transcription factors and coactivators, and are most consistent with virus infection leading to the phosphorylation of IRF-7B within two sets of Ser residues at its C terminus. We show that IRF-7 interacted with four distinct regions of the p300/CBP coactivators and that these interactions were playing an essential role in the ability of IRF-7 to strongly synergize with other transcription factors. Such synergy presumably reflected the cooperative assembly of nucleoprotein complexes where coactivators could be playing a scaffolding role. VAF is uniquely activated by virus infection or double-stranded RNA treatment and its cooperative assembly would ensure that p300/CBP molecules, which are thought to be present in the cell at limiting concentrations, would be redirected from other signaling pathways to participate in the antiviral transcriptional response required for the organism to survive a viral infection.

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REFERENCES

1. Sen, G. C. (2001) Annu. Rev. Microbiol. 55, 255–281
2. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) Annu. Rev. Biochem. 67, 227–284
3. Stewart, W. E. I. (1979) The Interferon System, Springer-Verlag, Wien, Austria
4. Bogdan, C. (2000) Curr. Opin. Immunol. 12, 419–424
5. Marie, I., Durbin, J. E., and Levy, D. E. (1998) EMBO J. 17, 6660–6669
6. Barnes, B. J., Moore, P. A., and Pitha, P. M. (2001) J. Biol. Chem. 276, 2382–2389
7. Au, W. C., Moore, P. A., LaFleur, D. W., Tombal, B., and Pitha, P. M. (1998) J. Biol. Chem. 273, 29210–29217
8. Lin, R., Heybrueck, C., Pitha, P. M., and Hiscott, J. (1998) Mol. Cell. Biol. 18, 2988–2996
9. Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998) FEBS Lett. 441, 106–110
10. Sato, M., Tanaka, N., Hata, N., Oda, E., and Taniguchi, T. (1998) FEBS Lett. 425, 112–116
11. Sato, M., Suenori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000) Immunity 13, 539–548
12. Schafer, S. L., Lin, R., Moore, P. A., Hiscott, J., and Pitha, P. M. (1998) J. Biol. Chem. 273, 2714–2720
13. Waterbeet, M. G., Lin, C. H., Parekh, B. S., Bence, L. V., Howley, P. M., and Maniatis, T. (1998) Mol. Cell 1, 507–518
14. Weaver, B. K., Kumar, K. P., and Reich, N. C. (1998) Mol. Cell. Biol. 18, 1359–1368
15. Yasunuma, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E., and Fujita, T. (1998) EMBO J. 17, 1087–1095
16. Yang, H., Lin, C. H., Ma, G., Orr, M., Inffi, M. O., and Waterbeet, M. G. (2002) Eur. J. Biochem. 269, 6142–6151
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Flavé, J. V., Parekh, B. S., Lin, C. H., Fraenkell, E., and Maniatis, T. (2000) Mol. Cell. Biol. 20, 4814–4825
19. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY
20. Gerritzen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2927–2932
21. Lin, R., Maman, Y., and Hiscott, J. (2000) J. Biol. Chem. 275, 34230–34237
22. Marie, I., Smith, E., Prakash, A., and Levy, D. E. (2000) Mol. Cell. Biol. 20, 8803–8814
23. Nakaya, T., Sato, M., Hata, N., Asagiri, M., Suenori, H., Noguchi, S., Tanaka, N., and Taniguchi, T. (2001) Biochem. Biophys. Res. Commun. 283, 1150–1156
24. Au, W. C., Yeow, W. S., and Pitha, P. M. (2001) Virology 290, 273–282
25. Lin, C. H., Hare, B. J., Wagner, G., Harrison, S. C., Maniatis, T., and Fraenkell, E. (2001) Mol. Cell 8, 581–590
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