JOURNAL OF INTERFERON AND CYTOKINE RESEARCH 22:87–93 (2002)
Mary Ann Liebert, Inc.

REVIEW

Enhancement and Diversification of IFN Induction by IRF-7-Mediated Positive Feedback

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ABSTRACT

Interferons (IFN) are potent components of the innate immune response to microbial infection. The genes for type I IFN (IFN-α and IFN-β) are rapidly induced in response to viral infection through a mechanism that involves latent cellular transcription factors that are activated in response to innate recognition of viral components. IFN regulatory factor (IRF) proteins are key to this regulation, and their conversion from latent to active involves virus-induced serine phosphorylation. Differential utilization of distinct IRF proteins by different members of the type I IFN gene family produces a graded induction of gene expression, resulting in tight control of these cytokines through a positive feedback mechanism. Early response to virus causes secretion of a subset of IFN genes through the action of IRF-3 in conjunction with additional transcription factors, such as NF-κB and activator protein-1 (AP-1) (c-jun/ATF). This early IFN acts in an autocrine manner to stimulate production of IRF-7, a transcription factor capable of activating the many additional members of the IFN-α gene family. The dependence of IRF-7 on virus-induced phosphorylation for its activity insures that IFN production is limited to virus-infected cells. Characterization of the cellular components involved in viral detection and IRF activation will further delineate this vital mechanism of innate immune response.

INTERFERON AND INNATE IMMUNITY

SINCE THE DISCOVERY OF INTERFERONS (IFN) in 1957 as endogenous inhibitors of viral infection,[1] considerable evidence has accumulated revealing that these host defense molecules play a vital role in the innate resistance to a wide variety of infectious agents. Most notably, model studies in infected mice in which distinct aspects of the IFN system were ablated by treatment with neutralizing antibodies or by disruption of individual signalling components have provided dramatic evidence of the essential nature of this host defense system. Moreover, such studies have revealed unexpected synergies between IFN signalling and more cellular aspects of innate immunity, as well as potent regulatory interactions with the adaptive immune system, demonstrating roles for IFN as immunomodulatory cytokines in addition to being antimicrobial agents (for reviews, see refs. 2–6). Given the potency of IFN-mediated host defense, it is understandable that this family of cytokines is tightly regulated, being expressed at nearly undetectable levels in uninfected individuals but rapidly induced to high abundance after infection. This rapid induction of IFN gene expression in virus-infected cells has captured the imagination of molecular biologists, making the regulation of expression of these genes a model for transcriptional control of inducible eukaryotic genes.[7,8]

IFN comprise two families of genes, type I IFN (IFN-α/β), consisting of a group of closely related IFN-α species and a single IFN-β gene, and the structurally unrelated type II IFN (IFN-γ), encoded by a single gene. The expression of IFN-γ is largely restricted to activated T lymphocytes and is regulated by transcription factors tied to T cell activation and proliferation. In contrast, type I IFN, which are primarily responsible for innate inhibition of viral replication, are inducible in a wide variety of cell types. Although this induction most commonly occurs in response to viral infection, other inflammatory mediators, such as bacterial products, also induce IFN. Regulation of IFN-γ gene expression has been reviewed,[9] as has the molecular biology of the IFN-β enhanceosome.[10] Unique aspects of the regulation of the IFN-α multigene family, in particular the involvement of the transcription factor IFN regulatory factor-7 (IRF-7), is the subject of this review.

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INDUCTION OF TYPE I IFN AND STRUCTURE OF IFN PROMOTERS

Induction of IFN gene expression in response to viral infection occurs primarily at the transcriptional level, and the kinetics of IFN gene transcription closely parallel the rapid increase in IFN protein. Whereas the rapidity and transcriptional basis of IFN production have been long recognized, there has been controversy about to what extent this response is an immediate-early type induction, independent of ongoing host protein synthesis. Although discrepancies in the literature can be explained by differences between the distinct cell types or species examined, it has recently become clear that there are both immediate-early aspects and protein synthesis-dependent components to the regulation of IFN transcription.

Induction of the human and mouse IFN-β genes has been most intensely studied. It is clear that immediate-early gene induction depends on the coordinated action of a series of transcription factors, each of which is activated from a latent state in response to viral infection. These factors include the classically inducible transcription complex, NF-κB, which is activated by phosphorylation-dependent destruction of its cytoplasmic inhibitor, I-κB. Other activated transcription factors include the c-jun/ATF (activator protein-1 [AP-1]) complex and one or more IRF proteins, all activated by virus-induced phosphorylation. Phosphorylation is the common thread in response to infection, and the underlying mechanisms leading to phosphorylation-dependent transcriptional activity are distinct and depend, at least in part, on distinct cellular kinases. As mentioned, phosphorylation-dependent activation of NF-κB involves targeted proteolysis of an inhibitor, although there is also evidence that NF-κB itself is positively regulated by direct phosphorylation.[11] In contrast, the c-jun/ATF complex displays basal activity that is increased by direct phosphorylation, largely by enhanced recruitment of necessary transcription coactivators.[12] Similarly, IRF proteins are directly modified by virus-induced phosphorylation, resulting in either conversion from a latent to an active state or enhancement of basal transcriptional activity, depending on the particular IRF species.[13,14] Once activated, these three transcription factor complexes interact with the IFN-β gene transcriptional control region in a concerted and highly cooperative fashion, leading to stable interaction on DNA and efficient recruitment of transcription coactivators, the basal transcriptional machinery, and RNA polymerase holoenzyme. Elucidation of this elegant transcriptional induction mechanism clearly illustrates that immediate-early response to virus is due to the posttranslational activation of preexisting protein components.

Interest in the regulation of IFN-α gene expression has been stimulated by superficial similarities with the kinetics and pattern of IFN-β induction that occurs in spite of the absence of some of the mechanistic aspects critical for IFN-β transcription. For instance, the kinetics of IFN-α gene induction in virus-infected cells largely parallels that of IFN-β, and expression is at least in part independent of ongoing cellular protein synthesis. Moreover, the virus-derived inducing signals are at least similar. Both gene families respond to the virus-mimetic, double-stranded RNA (dsRNA). In spite of these clear parallels, the distinct structures of the IFN-α and IFN-β gene promoters, the dispensability of characterized IFN-β enhanceosome proteins, differences in the optimal cell types producing the different IFN, and the multigene nature of IFN-α raise the possibility of increased levels of complexity. There is no evidence for a requirement for NF-κB or c-jun/ATF complexes to induce IFN-α genes. However, a binding site for IRF proteins and a requirement for IRF protein activation is a common feature of both IFN-α and IFN-β genes, and it has become clear that IRF proteins are involved in both the coordinate induction and differential expression of distinct IFN genes.[14]

Immediate-early induction of IFN genes has become a model for rapid induction of mammalian genes.[15] It is interesting that the products of IFN gene expression are secreted cytokines that activate a second cascade of immediate-early gene expression, stimulating production of IFN-stimulated genes (ISG). However, there are considerable mechanistic differences between these two gene induction pathways. Virus-stimulated IFN induction relies on a phosphorylation cascade stimulated by an unknown component of viral replication, involving at least in part a dsRNA intermediate. Targets of phosphorylation are serine residues of the enhanceosome-binding proteins or their inhibitors, and activation initiates nuclear translocation and increased transcriptional activity. In contrast, ISG induction is initiated through a cellular plasma membrane receptor that associates with protein tyrosine kinases of the Jak family. Targets of IFN-induced tyrosyl phosphorylation are members of the Stat gene family, primarily Stat1 and Stat2. Tyrosyl phosphorylation is required for transcriptional activity of these proteins, regulating their ability to dimerize, translocate from cytoplasm to nucleus, and bind DNA, providing a relatively direct regulatory circuit from extracellular signal to nuclear gene expression. There are also interesting points of convergence in these two gene-inducing pathways.[16] First, the Stat1-Stat2 complex activated by type I IFN is recruited to DNA through the action of an IRF protein, IRF-9,[17] forming a trimolecular complex, IFN-stimulated gene factor 3 (ISGF3).[18] Second, another IRF gene, IRF-7, is a target for induction by IFN-activated ISGF3, providing a regulatory link between these two pathways.[19,20]

IRF-3, IRF-7, AND THE DIFFERENTIAL EXPRESSION OF IFN-α GENES

The IRF family, as discussed elsewhere in this issue, consists of nine mammalian proteins and several viral homologs characterized by an amino-terminal DNA-binding domain (DBD) containing a tryptophan repeat motif distantly related to c-myb.[21] IRF-3 and IRF-7 are closely related to each other and share several structural and functional characteristics. Both proteins bind similar DNA sequences, and their activity and subcellular localization are governed by virus-induced phosphorylation at a related, carboxyl-terminal regulatory domain. Although additional regions of both IRF-3 and IRF-7 are phosphorylated, it is phosphorylation within this common regulatory domain that is dependent on viral infection and required for the full activity of the proteins. Phosphorylated IRF-3 and IRF-7 activity can be mimicked by substitution of individual serine residues with phosphomimetic aspartate residues.[22] However, the exact sites of virus-induced phosphorylation within the regulatory domains have yet to be definitively established.

Similarity in sequence between the regulatory phosphorylation sites of IRF-3 and IRF-7 prompted the question of whether they are modified by the same virus-induced kinase cascade. However, these two proteins are phosphorylated at distinct times during infection, largely because IRF-3 is constitutively expressed in cells and, therefore, always present, including at early stages of viral infection, whereas IRF-7 must be induced in abundance through the action of autocrine IFN produced in response to the initial infection. This observation raised the question of whether differential utilization of IRF-3 and IRF-7 was solely due to the delayed accumulation of IRF-7 or was also due to involvement of distinct virus-activated pathways. To address this issue, IRF-7 was ectopically expressed by transfection, and the kinetics and pharmacologic profile of IRF-3 and IRF-7 activation were compared following viral infection.[23] This protocol allowed the activation event to be studied in isolation from the inducible production of IRF-7, and comparisons between IRF-3 and IRF-7 phosphorylation and activation revealed no differences. The kinetics of endogenous IRF-3 and transgenic IRF-7 phosphorylation were quite similar, occurring 4–5 h after infection. Similarly, the profile of agents capable of inducing IRF phosphorylation or inhibiting this event in infected cells was fully concordant between IRF-3 and IRF-7. Therefore, it is likely that a single virus-activated kinase (VAK) cascade targets both IRF-3 and IRF-7 activation, the difference in their natural kinetics being fully explained by the delayed appearance of IRF-7 protein.[23,24]

The requirement for IFN-dependent induction of IRF-7 abundance was a key to understanding its role in IFN-α gene expression. Because IFN is synthesized and secreted during viral infection, IRF-7 is induced and can contribute to further IFN gene expression. In the absence of IFN signalling (e.g., in cells lacking IFN receptors or the Stat1 transcription factor), IFN production is solely dependent on preexisting components, such as IRF-3. It was noted that in IFN signalling-deficient mouse cells, not only was the response to IFN impaired but also the production of IFN, particularly IFN-α, was severely curtailed.[19,20] The quantity of IFN produced in IFN signalling-deficient cells was reduced, and additional qualitative differences in the pattern of IFN-α subtype distribution characterized the absence of IRF-7.[19] Specifically, only IFN-β and IFN-α4 were induced by viral infection of Stat1-deficient or IFN receptor-deficient mouse cells.[19] These findings led to the hypothesis that induction of IFN-β and IFN-α4 occurs through an immediate-early signalling pathway dependent entirely on preexisting protein components, such as IRF-3.[25–27] In contrast, induction of other species of IFN-α, such as IFN-α2, IFN-α5, IFN-α6, and IFN-α8, required the additional induction and activation of IRF-7.[19] Induced accumulation of IRF-7 appears to be solely responsible for the synergistic effects of autocrine IFN, as ectopic expression of recombinant IRF-7 was fully capable of substituting for the requirement of IFN signalling for induction of the delayed class of IFN-α genes.[19,20,28]

The distinct use of trans-acting factors by IFN-β and IFN-α4 compared with other IFN-α genes reflects the distinct DNA-binding specificities of IRF-3 and IRF-7.[29] Although both IRF-3 and IRF-7 contain a typical IRF DBD, they each exhibit distinct patterns of interaction with target sequences. Selection of optimum DBD from a pool of random sequences showed that IRF-3 prefers a more restricted target sequence that does IRF-7. IRF-3 preferred an accurate direct repeat of the motif GAANN, which is found in type I IFN promoters. IRF-7 also bound a similar direct repeat, but it was much more tolerant to sequence variations in the core sequence elements.[29] Both IFN-α4 and IFN-β promoters contain perfect GAANN repeat motifs, allowing them to bind and be activated by IRF-3. Other IFN-α genes display more variability within their promoters, making them targets for IRF-7 but not for IRF-3.[30] It was also found that IRF-3 efficiently recruited coactivator proteins, such as CREB binding protein (CBP), and IRF-7 did not. It is possible that concerted recruitment of coactivator proteins is important for stable transcription complex formation within the IFN-β enhanceosome, facilitating IRF-3, but not IRF-7 incorporation into a functional transcription complex.

These observations led to the model that IFN-α gene expression could be divided into two distinct phases (Fig. 1). The first, immediate-early phase involves induction of IFN-β and IFN-α4 through activation of IRF-3 and other latent transcription factors by virus-induced phosphorylation. Following the rapid but low-level synthesis of these IFN proteins, positive feedback through autocrine IFN secretion and response leads to induction of IRF-7 protein production because of IFN-dependent Jak-Stat signalling. Should viral infection still be present in the cell, phosphorylation of newly synthesized IRF-7 would lead to its activation, resulting in induction of the remaining members of the IFN-α gene family. This model provides a mechanism for tightly regulating production of IFN. Initial production of IFN requires sensing a viral infection by the innate immune system. If the low levels of primary IFN initially produced early during infection prove sufficient to successfully block viral replication, further IFN production would be curtailed because IRF-7 protein accumulated in this phase would remain latent. However, given continued viral infection, the newly synthesized latent IRF-7 can undergo phosphorylation and subsequent activation, resulting in potent induction of numerous IFN-α species and high levels of IFN-α secretion.

The conservation of the IFN-α multigene family during vertebrate evolution has raised the question of the importance of so many distinct but seemingly functionally equivalent genes. One hypothesis would be that in spite of their similar functions and their ability to operate through a common receptor and signalling cascade, the distinct IFN-α proteins nonetheless produce unique biologic outcomes. The model of biphasic induction through positive feedback suggests an alternative hypothesis. The maintenance of a large block of individual genes may be selected as a means of modulating different levels of IFN when needed. Rather than the perhaps more complex mechanism of regulating the level of expression of a single gene by several distinct rates of transcription, increasing amounts of IFN can be achieved by activating differential numbers of individual genes, each as a relatively simple on–off switch. As activation of transcription is always linked to the presence of ongoing virus through the need to stimulate IRF phosphorylation, IFN is produced only when needed. The definition of a subset of IFN-α genes sensitive to the level and activation of another IRF protein, IRF-5, lends further credence to this model.[31] By the same token, the distinct actions of IRF-5 and IRF-7 on subsets of IFN-α genes raise the intriguing possibility of much greater complexity, perhaps because of distinct functions of individual IFN-α isoforms.

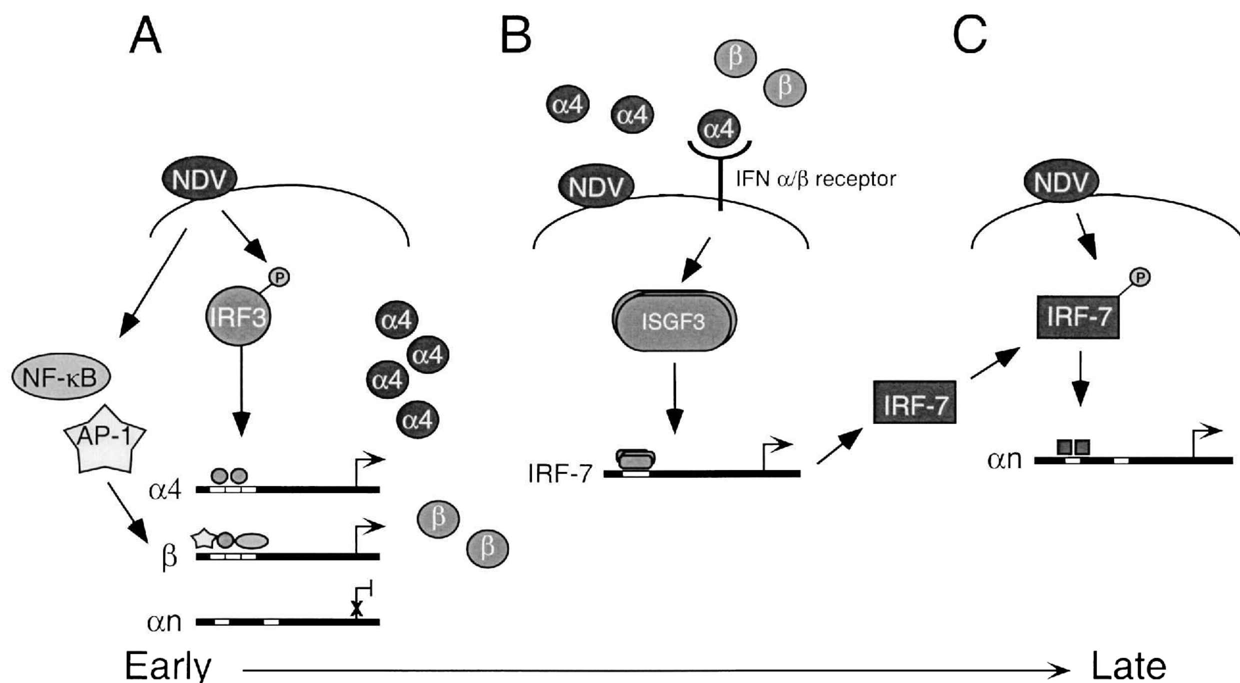

FIG. 1. Multiphasic induction of type I IFN genes. (**A**) Early induction of type I IFN gene expression is governed by an immediate-early phase dependent exclusively on preexisting cellular components. Viral infection stimulates a phosphorylation cascade, leading to activation of at least three families of transcription factors, including NF-κB, AP-1, and IRF-3. Activation of the IFN-β promoter requires all three transcription factors, whereas the mouse IFN-α4 promoter appears to require only activated IRF-3. Additional members of the IFN-α gene family (αn) are insensitive to these transcription factors and, therefore, remain dormant. (**B**) Secretion of early IFN produces an autocrine response through stimulation of the Jak-Stat pathway by the IFN receptor system, leading to activation of the trimeric transcription factor complex, ISGF3. Among the genes activated by ISGF3 is IRF-7, leading to accumulation of this protein only in response to IFN production. (**C**) IRF-7 is produced as a latent protein, but the continued presence of viral infection leads to its phosphorylation and subsequent activation. Many members of the IFN-α gene family (αn) possess promoter binding sites for IRF-7 and become transcriptionally active during this late phase of IFN induction.

REGULATION OF IRF-7 ACTIVITY

The switch of IRF-7 from latent to active form correlates with the accumulation of the phosphorylated protein in the nucleus, its acquisition of DNA-binding ability, and its competence for transcriptional induction of target genes. Similar to other IRF proteins, such as IRF-3, activated IRF-7 appears to exist as a dimer. Dimerization of active IRF-7 has been detected by velocity centrifugation,[32] coimmunoprecipitation,[33] and interaction with recombinant bacterial fusion proteins.[34] The question of whether it exists as a homodimer or as a heterodimer with IRF-3 remains controversial, and variations in the literature may reflect different species of origin, different cell types, or different activating viruses. Ablation of the IRF-3 gene in transgenic mice showed that IRF-7 was still capable of inducing IFN gene expression without IRF-3.[35] Similarly, IFN receptor-deficient, Stat1-deficient, and IRF-9-deficient mice that are incapable of upregulating IRF-7 expression are capable of expressing IFN-β and IFN-α4 genes, presumably through IRF-3 alone.[19,35] These results support a dichotomy in the action of IRF-3 and IRF-7 on distinct gene subsets, suggesting that these factors act primarily as homodimers. In contrast, IRF-3 ablation by using ribozyme technology showed loss of all IFN-α expression, and the spectrum of IFN-α gene transcription appeared to be sensitive to the ratio of IRF-3 and IRF-7.[30] These results would suggest cooperation between IRF-3 and IRF-7 that might be explained by formation of functional heterodimers.

The functional importance of dimerization for IRF-7 activity was demonstrated by forcing artificial dimerization in the absence of phosphorylation or other virally activated changes. To this end, mouse IRF-7 was fused with the hormone-dependent dimerization domain of estrogen receptor (ER). Although wild-type IRF-7 was inactive in the presence of estrogen agonists and was unable to bind DNA or stimulate target gene transcription as expected, the IRF-7-ER fusion protein shifted from cytoplasm to nucleus, bound DNA, and stimulated appropriate IFN-α gene transcription when expressed in hormone-treated cells.[32] Activity of IRF-7-ER required the transactivation domain of IRF-7 but not phosphorylation of its regulatory domain, suggesting that the sole function of regulatory phosphorylation is to induce dimerization.

Structure-function studies of IRF-7 (Fig. 2) have delineated an amino-terminal DBD plus two regions required for full transcriptional activity separated by a protein segment that inhibits IRF-7 function in the absence of phosphorylation.[32,34,36,37] The distal transactivation domain is also the site for inducible phosphorylation and, therefore, serves a second function as a

regulatory domain. Regulation of IRF-7 by virus-induced phosphorylation functions to relieve the constraints imposed by the internal inhibitory segment, which include inhibition of DNA binding and inactivation of the dual transactivation domains. The tight correlation between IRF-7 activity and induced dimerization and the ability of forced dimerization to substitute for virus-induced phosphorylation led to a model for IRF-7 regulation involving conformational change. It is thought that latent IRF-7 folds in a manner that allows its internal inhibitory segment to contact both the amino-terminal DBD and the bipartite transactivation domain, effectively occluding the activity of both domains. Conformational changes induced by dimerization must reorient the protein in such a manner as to uncover the DBD, allowing access to DNA, and derepressing the bipartite transactivation domain, allowing recruitment of the necessary coactivating machinery. It is interesting to note that the internal inhibitory region shows homology to a region present in other IRF proteins shown to be involved in protein-protein interactions, thus acquiring the name IRF association domain (IAD).[38,39] Therefore, it is likely that the IRF-7 inhibitory IAD region is involved in internal, heterotypic protein-protein interactions, causing the latent protein to fold as an inactive monomer. It may also facilitate dimerization of activated IRF-7 through homotypic interactions between the individual components of the dimer, a process dependent on carboxyl-terminal serine phosphorylation. A similar model has been proposed for regulation of IRF-3, which contains analogous structural domains,[40] and the IAD has been implicated in the formation of IRF-3-IRF-7 heterodimers.[34]

Although it is likely that dimerization brought about by phosphorylation-dependent conformational changes in IRF-7 is responsible for its activity as a transcription factor, the questions of how phosphorylation promotes dimerization and how virus signals phosphorylation remain unresolved. One possibility is that phosphorylation of IRF-7 destabilizes intramolecular contacts, thereby favoring intermolecular interactions involved in dimer formation. As for signalling, there is considerable evidence that dsRNA, an intermediate or by-product of many viral replication cycles, is an element of viral signalling.[41–43] However, the cellular components necessary for sensing dsRNA intermediates and other viral components necessary for signalling to IRF-7 have yet to be identified. One known target for dsRNA is the dsRNA-dependent protein kinase, PKR. However, although PKR is an important component of the IFN-dependent antiviral response,[44] gene-targeting experiments and the use of pharmacologic inhibitors have shown that it is not required for IRF activation in virus-infected cells.[23,24] In contrast, PKR has been implicated in dsRNA-induced and virus-induced expression of IFN-β, in particular, for induction of NF-κB, suggesting that at least two distinct virus-activated cellular kinases are required for complete activation of IFN genes. Interestingly, PKR is also required for dsRNA-induced expression of IFN-α genes but not for induction of these genes by virus, suggesting that dsRNA is only one component of the virus-activated signal.[45]

DISCUSSION AND PERSPECTIVES

Induction of IFN gene expression is an essential component of innate immunity. Molecular mechanisms that allow rapid and robust induction of these genes in response to viral infection while still keeping them under precise control are now being elucidated. Cross-talk between induction of IFN genes and subsequent IFN induction of an antiviral state through induction of IRF-7 accumulation is one example of this precise control, us-

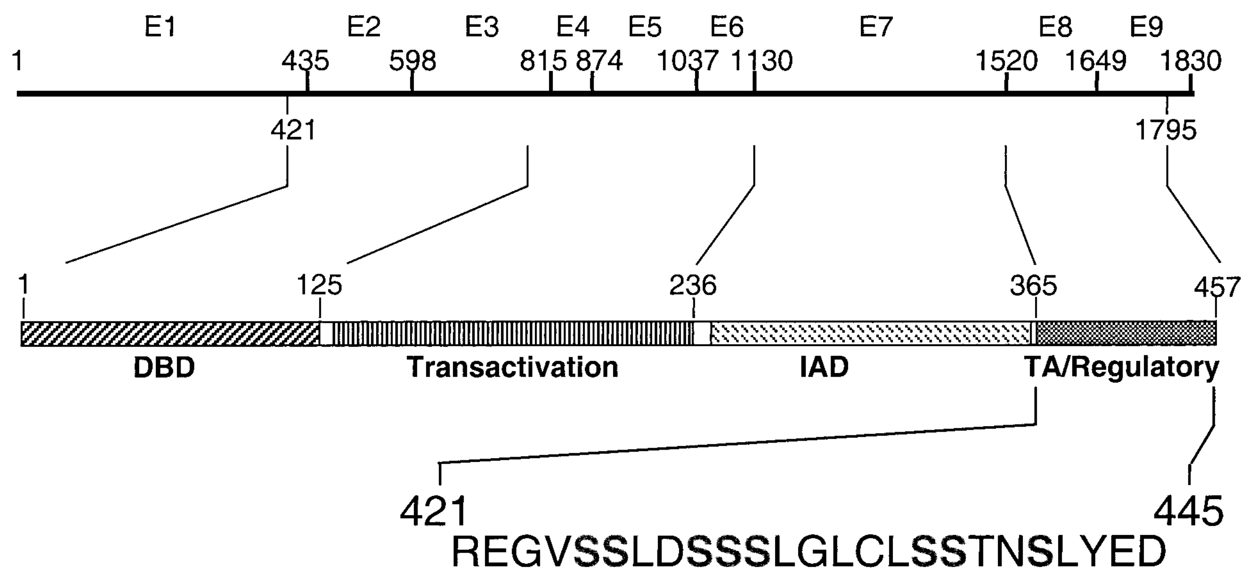

FIG. 2. Structure of IRF-7. The mouse IRF-7 gene consists of nine exons (**top**), encoding an mRNA 1830 nt in length and a protein 457 aa in length. Intron-exon junctions are indicated by the nucleotide position in the processed RNA. IRF-7 protein (**bottom**) is composed of four domains, including an amino-terminal DBD, an internal transactivation domain, an IAD, and a distal domain required for both regulation and transactivation (TA/Regulatory). This distal domain becomes phosphorylated following viral infection. Potential serine phosphorylation sites between aa 421 and 445 are highlighted.

ing positive feedback and multiple IFN-α target genes to achieve highly regulated levels of active IFN. The nature of the signal produced by viruses that initiate the cascade of events leading to IFN gene expression remains to be elucidated. It is expected that identification of the cellular components of this signalling pathway will define important aspects of how the mammalian innate immune system recognizes the presence of pathogens.

ACKNOWLEDGMENTS

Work in the authors' laboratory was supported by grants from the American Heart Association, the Arthritis Foundation, and the National Institutes of Health.

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Accepted 29 August 2001