Viral infection results in transcriptional activation of the cellular interferon α/β-stimulated genes (ISGs) independent of the autocrine action of interferon α/β (IFN-α/β). Induction of ISG expression by virus appears to be mediated through production of viral double-stranded RNA (dsRNA). Previously, we identified two novel dsRNA-activated factors (DRAFs) that bind to the interferon-stimulated response element (ISRE), the DNA sequence that mediates transcriptional activation by IFN-α/β. In this report we define sequences that flank the classical ISRE as necessary for DRAF1 binding. More significantly, it is shown that the sequences required to bind DRAF1 correlate with the ability to mediate ISG induction by virus. These results strongly suggest that DRAF1 is a positive regulator of ISG transcription. DRAF1 is shown to bind selectively to the promoters of those ISGs which are strongly induced by viral infection, again suggesting the functional significance of this factor. UV cross-linking experiments indicate that DRAF1 and DRAF2 share a common DNA-binding subunit of approximately 70 kDa which is referred to as the DRAF binding component (DRAFb). DRAFb is shown to preexist in the cytoplasm of unstimulated cells. Consistent with this observation, both DRAF1 and DRAF2 are activated in the cytoplasm prior to nuclear translocation.

A cell responds to viral infection with the transcriptional induction of specific genes that can function in the defense against the invading virus. Expression of the type I interferon genes (IFN-α/β) during viral infection is thought to be stimulated by viral double-stranded RNA (dsRNA) generated during the course of infection (reviewed in Ref. 1). Once synthesized, the IFN-α/β cytokines are secreted from the cell, bind to cell surface receptors, and function in an autocrine or paracrine manner to confer resistance to viral infection (reviewed in Ref. 2). The protective effects of IFN-α/β are mediated through the activation of a specific set of genes known as interferon-stimulated genes (ISGs) whose products function in cellular defense (reviewed in Refs. 1–4). It has now become clear that transcription of many of the ISGs is also directly activated during viral infection, independent of the synthesis or action of IFNs (5–10).

The direct activation of the ISGs in response to viral infection allows cells to express the ISG-encoded proteins rapidly, before the synthesis, secretion, or action of IFNs. Such a response pathway may be critical to the survival of virally infected cells and an important component of the immune response against virus. In this study we examine the basis of direct activation of ISGs by viral infection and dsRNA, the apparent mediator of induction.

ISGs induced by IFN-α/β contain a promoter sequence known as the interferon-stimulated response element, or ISRE (reviewed in Refs. 3 and 4). Following IFN binding to its receptor, a multimeric transcription factor, the interferon-stimulated gene factor 3 (ISGF3), is activated by tyrosine phosphorylation in the cytoplasm of the cells and subsequently translocates to the nucleus to bind to the ISRE. Despite the potential significance of direct activation of the ISGs by viral infection, little has been characterized of this pathway at the molecular level. Preliminary experiments have suggested that a DNA sequence containing the ISRE can mediate a transcriptional response to virus (6, 11). However, ISG induction by viral infection is independent of ISGF3 activation (6). Consistent with these observations, we have identified two novel nuclear factors rapidly induced by viral infection or dsRNA transfection that recognize an ISRE-containing DNA sequence (6). These factors have been termed dsRNA-activated factor 1 and 2 (DRAF1 and DRAF2).

An important question concerns the DNA sequence transcriptionally responsive to viral infection or dsRNA. Experiments suggesting that the ISRE is sufficient to mediate the transcriptional response to virus have employed DNA elements that contain sequence flanking the classical ISRE (6, 11). In addition, certain ISGs are induced weakly or undetectably by virus although they are strongly induced by IFN-α/β (10). This suggests that the DNA sequence which determines a strong transcriptional response to virus is different from the classical ISRE. In this report, we examine the functional significance of the DRAFs by determining more precisely the DNA sequence required to mediate viral induction of ISGs and correlating it with the ability of the induced DRAFs to bind to this sequence. It is demonstrated that the DNA sequence required to mediate a response to virus correlates with binding to DRAF1. This is the first strong evidence that DRAF1 is a positive activator of ISG transcription during infection. In addition, we provide a characterization of DRAF1 and DRAF2 that indicates that they share a common DNA-binding protein which preexists in the cytoplasm of untreated cells. DRAF activation is also demonstrated to initiate in the cytoplasm of the cell. Thus, this report forms the basis of understanding the nature of a DNA element and transcription factors which mediate the direct activation of ISGs during viral infection.
EXPERIMENTAL PROCEDURES

Cell Culture—HEC-1B (ATCC HTB 113) and HeLa S3 cells (ATCC CCL2.2) were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum.

Viral Infections—Newcastle disease virus (NDV) was a gift from Dr. Paula M. Pitha-Rowe (The Johns Hopkins University, Baltimore, MD). HEC-1B cells were infected with NDV at 100 hemagglutination units per ml as follows: cells in 150-mm plates were washed with serum-free DMEM, and then overlaid with 4 ml of serum-free DMEM containing the appropriate amount of virus. After 1 h, the medium was removed and the cells were fed with DMEM containing 5% calf serum.

Transfections—dsRNA transfections were performed as described (6). Cells were transfected with DNA by the calcium phosphate coprecipitation technique (13). 24 h after transfection, cells were split into duplicate plates. Subsequently, one of the duplicate plates was infected with NDV and overlaid with 4 ml of medium (DMEM) containing 10% calf serum.

Gel Mobility Shift Assay—Nuclear and cytoplasmic extracts used in gel shift assays were prepared as described (18). The gel shift assay was performed as described (6). The gel shift probe was a double-stranded 27-mer corresponding to the sequence from −114 to −92 of the human ISG15 promoter: 5′-GGGAGGACGGAACCCGAAACTGAA-3′. The endogenous ISG15 promoter is an ISRE-TK plasmid that has been described previously (6). The endogenous ISG15 promoter is a construct that contains three copies of the ISRE (−114 to −94 of the human ISG15 promoter) cloned into the promoter of the herpes simplex virus thymidine kinase gene promoter. The ISG15/E1B plasmid contains various regions of the ISG15 promoter (−96, −108, or −115 to −44) fused to the 3′ region of the adenovirus E1B gene (15). The SVβi globin plasmid was used to control for transfection efficiency which has been described (16).

RESULTS

Viral Infection Activates ISG Expression through a DNA Sequence Containing the ISRE—We have demonstrated previously that infection of HeLa cells with adenovirus can induce expression of a reporter gene driven by a DNA sequence containing the ISRE (6). In that experiment, the infection was performed in the presence of cycloheximide to prevent virally infected cells from synthesizing IFN-α/β. However, subsequent experiments suggested that even in the presence of cycloheximide, virally infected HeLa cells can synthesize enough IFN-α/β to induce detectable ISGF3 activation (6). Therefore, it was possible that some or all of the ISRE-dependent reporter gene expression observed in response to adenovirus infection of HeLa cells was due to the action of IFN-α/β and not directly to the adenovirus. In order to establish definitively that virus can activate transcription through the ISRE, independently of IFN-α/β action, it was necessary to use a cell line (HEC-1B) which is resistant to IFN-α/β. HEC-1B is a human endometrial carcinoma cell line which does not respond to IFN-α/β (5, 9, 20). HEC-1B cells were transfected with either a plasmid containing 3 copies of the ISG15 ISRE cloned into the promoter of the herpes simplex virus thymidine kinase gene (ISRE-TK) or a plasmid containing the TK gene driven by the ISRE (Fig. 1A). A plasmid encoding β-globin was cotransfected to control for transfection efficiency (16).

Following transfection, cells were either untreated (lanes 2 and 4) or infected with Newcastle disease virus (NDV), an RNA virus that produces dsRNA during the course of infection (lanes 3 and 5). Cytoplasmic RNA was isolated from the cells, and the level of reporter gene (TK) expression was quantified. As a control, HEC-1B cells were treated with IFN-α to demonstrate the lack of response of these cells to IFNs (lane 1). NDV was chosen as an inducing agent for the transfected cultures since it is known to be a very efficient activator of ISG expression in HEC-1B cells (5, 9), and an infection will introduce viral dsRNA into the maximum number of the cells in the transfected culture. In our studies, we have found that NDV infection primarily induces the appearance of DRAF1 and less notably DRAF2 (data not shown). NDV infection of the transfected cultures was found to strongly induce expression of the TK gene driven by the ISRE (lane 4), but it did not induce expression of the control TK gene without the ISRE (lane 2).

The efficiency of the viral infection was equivalent in both cases, as judged by the induced expression of the endogenous ISG15 gene (lanes 2 and 4). This experiment conclusively demonstrates that a DNA sequence containing the ISRE can mediate gene expression in response to viral infection, and that the ISRE-dependent gene expression observed is not due to the action of IFN-α/β-induced ISGF3.

The ISRE-TK plasmid used in Fig. 1A contains an ISRE oligonucleotide corresponding to positions −111 to −94 of the ISG15 promoter. Therefore, the sequence from −111 to −94 is sufficient to support a transcriptional response to virus. To define more precisely the region of the ISG15 promoter which is required to mediate a response to virus, three plasmids containing various regions of the ISG15 promoter were tested. These plasmids, called dISG15-E1B, contain from −96, −108, or −115 to +44 of the ISG15 gene linked to the adenovirus E1B gene as a reporter. These plasmids have been used previously to define the region of the ISG15 promoter required to mediate a transcriptional response to IFN-α/β (15, 17). Each of these plasmids was transfected into HEC-1B cells along with a plasmid encoding β-globin as cotransfection control (Fig. 1B).

Following transfection, cells were either left untreated (lanes 1,
The −115 plasmid responded strongly to viral infection, with more than a 5-fold induction of reporter gene expression (lane 6). It should be noted that the actual induction is greater than it appears to be, since we have observed a general destabilization of mRNA following viral infection or dsRNA treatment (compare actin levels in lanes 2, 4, and 6 with those in lanes 1, 3, and 5). Thus, if reporter gene mRNA levels are normalized to actin levels, the viral induction is severalfold greater. The different responses mediated by the −108 and −115 plasmids is not due to a difference in the efficiency of viral infection, since the endogenous ISG15 gene was induced to similar levels in both cases (lanes 4 and 6). These results demonstrate that several nucleotides upstream of the consensus ISRE (−107 to −95) are required to mediate a maximal response to virus, although the ISRE alone (−108 plasmid) is able to mediate modest transcriptional activation.

Binding of DRAF to the ISG15 Promoter Correlates with Transcriptional Activation by Virus—Cells respond to dsRNA transfection in the absence of new protein synthesis with the activation of two DNA-binding factors, DRAF1 and DRAF2, that recognize a sequence containing the ISRE (6) (Fig. 2A). DRAF1 migrates very slowly in the electrophoretic gel shift assay and appears just below the gel well suggesting that it is a large molecular complex. Although both the DRAFs and ISGF3 bind to an oligonucleotide containing the ISRE, the DRAFs do not contain any of the protein components of ISGF3 as measured by antibody cross-reactivity (Ref. 6 and data not shown).

Since the −115 and −108 plasmids demonstrated a measurable difference in inducibility by viral infection, we determined if efficient binding of DRAF1 and/or DRAF2 to these sequences correlated with gene expression. To define more precisely the 5′ boundary of the sequence required to bind DRAF1 and DRAF2, a competitive gel mobility shift assay was performed (Fig. 2B). The DNA binding reactions were performed with a radiolabeled ISG15 ISRE oligonucleotide (−114/−92) in the absence (lane 1) or presence (lanes 2–4) of a 100-fold molar excess of various unlabeled competitor DNAs. The competitor DNAs corresponded to −115/−94, −111/−94, or −108/−94 of the ISG15 promoter. The −115/−94 and −111/−94 DNA competed efficiently for binding of both DRAF1 and DRAF2 (lanes 2 and 3). However, the −108/−94 DNA only competed efficiently for DRAF2 binding (lane 4). DRAF1 binding therefore requires a nucleotide(s) between −109 and −111, directly adjacent to the ISRE (AAA in the ISG15 promoter). Thus, the stronger induction of reporter gene expression observed for the −115 plasmid compared to that seen with the −108 plasmid (Fig. 1B) appears to be due to the ability of DRAF1 to bind much more efficiently to the −115 plasmid than to the −108 plasmid. This suggests that DRAF1 is the major positive transcriptional regulator, since DRAF1 binding to the ISG15 ISRE is tightly correlated with induced transcription of this gene during viral infection.

The DNA binding specificity of DRAF1 is distinct from ISGF3 and suggests that DRAF1 activates a subset of ISGs (14).

To define the 3′ boundary of the DRAF1 and DRAF2 binding sites within the ISG15 promoter, a similar DNA binding competition experiment was performed (Fig. 2C). Each DNA binding reaction contained radiolabeled ISRE probe in the absence (lane 1) or presence (lanes 2–4) of a 100-fold molar excess of unlabeled competitor DNA. The competitor DNAs contained −111/−94 of the ISG15 ISRE or a deletion of nucleotides −98 and −97 (df 98) or of nucleotides −96 to −94 (df 96) as shown. As expected, the −111/−94 DNA competed efficiently for binding of DRAF1 and DRAF2 (lane 2). However, neither the df 98 nor the df 96 DNAs competed for DRAF1 or DRAF2 binding (lanes 3 and 4). Thus, binding of DRAF1 and DRAF2 requires
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DRAF Binding to Various ISG Promoters Is Correlated with Potent Transcriptional Activation by Virus—The fact that DRAF1 and DRAF2 require slightly different sequences for efficient DNA binding suggested the possibility that these factors bind to distinct subsets of ISG promoters. Since efficient DRAF1 binding requires a nucleotide(s) upstream of the consensus ISRE (Fig. 2B), DRAF1 may bind selectively to ISG promoters, as these flanking nucleotides (AAA) are not strictly conserved. Since DRAF2 binds efficiently to an oligonucleotide containing just the consensus ISRE (Fig. 2B), DRAF2 may bind to the ISRE of most, if not all, ISGs. In order to test this, a gel mobility shift assay was performed using nuclear extract containing DRAF1 and DRAF2 activity (Fig. 3). The competitor DNAs contained the ISRE from the human ISG15 (15), ISG54 (22), 2'-5' oligoadenylate synthetase (22), or 6-16 (23) genes. Expression of the ISG15 and ISG54 genes has been shown to be induced strongly by virus and dsRNA, whereas the 6-16 gene is induced to a much lesser extent than ISG15 and ISG54 (5, 6, 9, 10, 12). Expression of the 2'-5' oligoadenylate synthetase gene is induced very weakly or not at all by virus, depending on the cell line used (10, 12). Each DNA binding reaction contained radiolabeled ISG15 ISRE probe in the absence (lane 1) or presence of a 100-fold molar excess of unlabeled competitor DNA. The ISRE of the ISG15 and ISG54 genes competed very efficiently for both DRAF1 and DRAF2 binding (lanes 2-5). The ISRE from the 2'-5' oligoadenylate synthetase gene efficiently competed for DRAF2 binding, but was unable to compete for DRAF1 binding (lanes 6 and 7). The ISRE from the 6-16 gene was able to compete very weakly for both DRAF1 and DRAF2 binding (lanes 8 and 9). Thus, DRAF1 is only competent to bind to a particular subset of ISG promoters. The two ISREs we tested (ISG15 and ISG54) that bind DRAF1 efficiently have three adenine nucleotides directly adjacent to the consensus ISRE which are not present in the promoters which do not bind DRAF1 (6-16 and 2'-5' oligoadenylate synthetase, lanes 6-9).
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This strongly suggests that these nucleotides are important for DRAF1 binding. Since the ISG15 and ISG54 ISREs differ in the two central nucleotides between the GAAA repeats (CC in the ISG15 ISRE and GT in the ISG54 ISRE), the sequences of these nucleotides do not appear to be critical for DRAF1 binding. DRAF2 bound to all of the ISRE's tested, although it bound to the 6-16 ISRE inefficiently. This may reflect a preference of DRAF2 for particular nucleotides between the GAAA repeats. These results reinforce the interpretation of data in Figs. 1 and 2 by suggesting that DRAF1 activity is responsible for ISG transcription. If DRAF1 can bind to a particular promoter (ISG15 and ISG54), then activation of the corresponding gene by viral infection is relatively strong. If DRAF1 cannot bind, or binds with poor affinity, to an ISG promoter (2'-5' oligoadenylate synthase and 6-16), then gene activation by virus is relatively weak or nonexistent.

DRAF1 and DRAF2 Share a Common DNA-binding Subunit of Approximately 70 kDa—In order to begin to characterize the molecular nature of DRAF1 and DRAF2, the factors were partially purified from dsRNA-treated HEC-1B cells. The purification involved ion exchange chromatography followed by DNA affinity chromatography (see "Experimental Procedures"). When fractions from the DNA-affinity column were tested for DRAF1 and DRAF2 activity by a gel shift assay, it was observed that a faster migrating ISRE binding activity copurified with DRAF1 and DRAF2 (Fig. 4, left). To determine the molecular weight of the protein(s) in DRAF1 and DRAF2 which contacts the ISRE and to determine the relationship of the novel faster migrating activity to DRAF1 and DRAF2, all three activities were UV-cross-linked to the ISRE (Fig. 4, right). A gel mobility shift assay was performed with a radiolabeled ISRE probe substituted with 5-bromodeoxyuridine on one strand to increase the efficiency of cross-linking. The entire gel was exposed to UV irradiation, and each protein-ISRE complex (DRAF1, DRAF2, novel activity) was excised and electrophoresed directly into an SDS-polyacrylamide gel. The protein contacting the ISRE is visible by virtue of its covalent attachment to the radiolabeled DNA. The cross-linked product (indicated by the arrowhead) derived from the faster migrating activity (lane 1), DRAF2 (lane 2), and DRAF1 (lane 3) appeared to be the same in each case. The cross-linked protein is specific for the ISRE since it is absent if the preparative DNA-binding reactions contain excess unlabeled competitor ISRE. When the molecular size of the cross-linked oligonucleotide is taken into account, the approximate molecular mass of the cross-linked protein is 70 kDa. Thus, it appears that a 70-kDa protein contained in DRAF1, DRAF2, and the novel activity is contacting the ISRE directly. The possibility exists that the cross-linked proteins are not the same in each case, but are distinct proteins of identical molecular mass. This seems to be unlikely, however, since the proteins have the added similarity of binding the ISRE, presumably a property shared by relatively few cellular proteins. Thus, this novel faster-migrating activity contains the 70-kDa DNA-binding subunit of DRAF1 and DRAF2. This activity is referred to as the DRAF DNA-binding component or DRAF (3). DRAF specifically binds to the ISRE of ISG15 and not to the inner core of the ISRE (−105/−97) (6). To determine the native molecular mass of the DRAF, a glycerol gradient sedimentation experiment was performed (Fig. 5A). Affinity-purified DRAF was sedimented in a 25-40% glycerol gradient. Fractions were assayed for DRAF activity by gel shift with a radiolabeled ISRE probe. In a parallel gradient, proteins with known sedimentation coefficients (s) were sedimented, and their positions were determined by SDS-PAGE analysis of the gradient fractions (data not shown). As shown in Fig. 5A, DRAF sedimented very closely to the 150-kDa marker protein. A standard curve was generated by plotting the s value of the marker proteins against distance traveled (fraction) (Fig. 5B). This allowed for a determination of the s value for DRAF, which was used to estimate its molecular mass as 140 kDa (19). This suggests that the DRAF activity exists in solution as a dimer of a 70-kDa protein. However, the possibility that DRAF activity consists of the 70-kDa cross-linked protein and a distinct protein of similar size cannot be excluded. Attempts to estimate the native molecular mass of DRAF1 and DRAF2 by glycerol gradient sedimentation were unsuccessful, since neither DRAF1 nor DRAF2 activity could be recovered in the gradients, suggesting dissociation of a multimeric complex (data not shown).

DRAF Preexists in the Cytoplast of Unstimulated Cells—Since DRAF appeared to be a component of DRAF1 and DRAF2, it was possible that DRAF preexists in cells or that its formation is an intermediate step in the formation of DRAF1 and DRAF2. To determine whether DRAF preexists in cells, cytoplasmic and nuclear extracts were prepared from unstimulated HEC-1B and HeLa cells and used in a gel mobility shift assay. DNA binding reactions were performed using the same number of cell equivalents of nuclear or cytoplasmic protein and a radiolabeled ISRE probe (Fig. 6A). This allows for an estimate of the percentage of an activity which is in the nuclear or cytoplasmic compartments. Using affinity-purified DRAF from dsRNA-treated nuclear extract as a marker for migration distance (lane 1), no DRAF activity was detectable in nuclear extracts from untreated HEC-1B or HeLa cells (lanes 2 and 3). In contrast, cytoplasmic extracts from untreated HEC-1B and HeLa cells had significant DRAF activity (lanes 4 and 5). The fact that this activity in untreated cytoplasmic extracts is DRAF was confirmed by UV-cross-linking. Thus, DRAF pre-

2 C. Daly and N. C. Reich, data not shown.
3 C. Daly, B. Weaver, and N. C. Reich, data not shown.
exists within the cytoplasm of unstimulated cells. This does not rule out the possibility that there is some DRAF in the nucleus of unstimulated cells, but clearly the great majority of this activity exists in the cytoplasm, even following dsRNA transfection. This observation holds true for two different cell lines, HEC-1B and HeLa.

**DRAF1 and DRAF2 Are Activated in the Cytoplasm of dsRNA-treated Cells**—Since DRAF exists in the cytoplasm of unstimulated cells, it seemed likely that DRAF1 and DRAF2 are activated in the cytoplasm prior to their accumulation in the nucleus. To test this hypothesis, and to examine the kinetics of the activation process, HEC-1B cells were treated with dsRNA for various times. At each time point, cytoplasmic and nuclear extracts were prepared and assayed for DRAF1 and DRAF2 activity by gel shift with a radiolabeled ISRE probe (Fig. 6B). DRAF1 activity is first detectable in cytoplasmic extracts by 20 min after treatment (lane 3), and the level of DRAF1 activity in the cytoplasm continues to increase through 60 min of treatment (lane 5). Significant DRAF1 activity appears in nuclear extracts by 60 min after treatment (lane 5). Thus, it appears that DRAF1 is activated in the cytoplasm, after which it translocates to the nucleus.

DRAF2 activity appears in the cytoplasm by 10 min after treatment (lane 7) and appears to peak by 20 min (lane 8). DRAF2 is first detectable in nuclear extracts 10 min after treatment (lane 7) and continues to increase through 30 min of treatment (lane 9). Therefore, DRAF2 also appears to be activated in the cytoplasm. Since the gel shifts were performed with 20 µg of cytoplasmic protein and 10 µg of nuclear protein, and not normalized to cell equivalents, the actual percentage of DRAF1 and DRAF2 in the cytoplasmic fraction is greater than shown. This strongly suggests that the presence of DRAF1 and DRAF2 in cytoplasmic fractions is not an artifact of the extract preparation, but that it accurately reflects the cytoplasmic activation of these factors.

**DISCUSSION**

The defense response of cells to viral infection or dsRNA transfection is a rapid activation of latent dsRNA-activated factors (DRAFs) that recognize a DNA sequence containing the IFN-α/β-stimulated response element (ISRE) (Fig. 2). Activation of the DRAFs occurs with concomitant induction of the IFN-α/β-stimulated genes (ISGs) (6). In this report, we have addressed several fundamental issues regarding the induction of ISG transcription by virus. We have defined the DNA sequence which mediates this induction, and we have shown that this sequence correlates with the sequence required to bind DRAF1. This result indicates that DRAF1 functions as a positive regulator of ISG transcription during viral infection. We also have provided an explanation for the observation that certain ISGs are transcriptionally activated to a greater extent than others by demonstrating differential ISRE binding of DRAF1. In addition, we have provided a preliminary biochemical characterization of DRAF1 and DRAF2, demonstrating that they contain a novel 70-kDa ISRE-binding subunit which...
preexists in the cytoplasm of unstimulated cells.

It is now apparent that viral infection can activate expression of ISGs directly through a DNA sequence containing the ISRE. This conclusion is based on the fact that NDV (which strongly activates DRAF1) induces expression of a reporter gene driven by the ISRE (Fig. 1A). Moreover, since this experiment was performed in the HEC-1B cell line, which does not respond to IFN-α/β, we can definitively rule out the possibility that the ISRE-dependent transcription observed during viral infection is due to ISGF3 function.

As further evidence that DRAF1 is the primary mediator of ISG transcriptional activation, we have shown that the region of the ISG15 promoter which is required to mediate a strong transcriptional response to NDV infection correlates with the region of the promoter required for efficient DRAF1 binding (Figs. 1B and 2B). Previous work by others has shown that viral infection selectively induces the expression of a subset of ISGs. In particular, expression of ISG15 and ISG54 is strongly activated by NDV infection, while expression of ISG16 and ISG2 is not bound efficiently to the 6-16 or 2'-5' oligoadenylate synthetase is induced very weakly (5, 9, 10, 12).

This suggests that the transcription factor(s) which induce ISG expression during viral infection binds selectively to certain ISG promoters. In fact, we have shown that DRAF1 binds efficiently to the promoters of both the ISG15 and ISG54 genes, but it does not bind efficiently to the 6-16 or 2'-5' oligoadenylate synthetase promoters (Fig. 3). Thus, ISG promoters which bind DRAF1 can support a strong transcriptional response to virus, providing additional evidence for the functional significance of this factor. The two ISREs analyzed that bind DRAF1 well (ISG15 and ISG54) are flanked upstream by a stretch of three adenine nucleotides that appear to be critical for DRAF1 binding (Figs. 2B and 3). Furthermore, the ISRE of another ISG that is strongly activated by virus, ISG56, is also flanked by three adenine nucleotides and therefore would be predicted to bind DRAF1 efficiently (24).

The fact that DRAF1 does not bind efficiently to all consensus ISREs can account for the observation that only a subset of ISGs are induced strongly by virus. However, the result that DRAF2 binding appears to require just the consensus ISRE suggests that DRAF2 potentially could bind to certain ISG promoters which DRAF1 cannot and activate gene expression (Fig. 2B). At the present time, however, the functional significance of DRAF2 is unclear, and further work is required to elucidate the role of this activity during viral infection. A recent report has shown that dsRNA can induce transcription of the IP-10 gene via an ISRE-containing sequence (11). However, the authors did not observe binding of DRAF1 to the IP-10 ISRE. This appears to be due to the fact that the IP-10 ISRE does not contain the flanking adenine residues which are important for DRAF1 binding. The factor(s) which are responsible for inducing ISRE-dependent IP-10 transcription remains to be identified and may require new protein synthesis for its appearance.

In an effort to begin to characterize the molecular nature of DRAF1 and DRAF2, the activities were partially purified from dsRNA-treated cells. UV-cross-linking analysis with these preparations indicates that the protein in both DRAF1 and DRAF2 which is in contact with the ISRE is approximately 70 kDa (Fig. 4). The size of this DNA-binding component is distinct from the 48-kDa DNA-binding component of ISGF3, consistent with our previous observation that DRAF1 and DRAF2 do not react with antibodies against the 48-kDa protein (6). Interestingly, although DRAF1 and DRAF2 appear to contain the same DNA-binding protein, their DNA binding specificities differ (Figs. 2B and 3). This suggests that additional proteins in the DRAF complexes influence their ISRE binding properties. However, the exact protein composition of DRAF1 and DRAF2 and their relationship to each other remain to be determined.

In the course of our purification of DRAF1 and DRAF2, we observed a faster migrating activity which copurified with the DRAFs (Fig. 4). UV-cross-linking of this activity indicated that it also contained the same 70-kDa DNA-binding protein as DRAF1 and DRAF2 (Fig. 4). The native molecular mass of this activity was estimated by glycerol gradient sedimentation to be approximately 140 kDa (Fig. 5). This result suggests that this novel activity exists in solution as a homodimer of the 70-kDa ISRE-binding protein. The activity has thus been termed DRAFβ (DRAF DNA binding component). Interestingly, we were able to detect DRAFβ activity in cytoplasmic extracts from untreated cells (Fig. 6A). Apparently, DRAFβ preexists in the cytoplasm and is capable of binding to the ISRE without requiring any modification induced by the presence of dsRNA. Consistent with the cytoplasmic localization of DRAFβ, both DRAF1 and DRAF2 are rapidly activated in the cytoplasm upon exposure of cells to dsRNA (Fig. 6B). The molecular details which underlie activation of the DRAFs remain to be characterized. Our previous studies have indicated that the protein kinase inhibitor, staurosporine, and the tyrosine kinase inhibitor, genistein, can block the activation of ISGs by virus, suggesting that DRAF activation involves a cytoplasmic kinase, possibly a tyrosine kinase, that is activated by dsRNA (6). It has not yet been determined if the 68-kDa dsRNA-dependent kinase (PKR) is involved in DRAF activation. A PKR inhibitor, 2-aminopurine, blocks ISG induction by viral infection; however, it also blocks ISG activation by IFNs and therefore it is not a specific inhibitor of PKR (12, 25). In addition, others have shown that 2-aminopurine does not inhibit PKR activity in vivo (26).

The fact that both a DNA virus (adenovirus) and an RNA virus (NDV) can activate DRAF1 suggests that this factor may be of widespread significance in mediating an important host defense against viral infection (6). The mediator of viral activation of the DRAFs is dsRNA, which can activate these factors within minutes of treatment. Direct activation of ISG expression in response to virus presumably allows the infected cell to rapidly establish an antiviral phenotype via production of ISG-encoded proteins. Viral infection does elicit the production of IFN-α/β, which eventually can activate ISG expression in the infected cell via an autocrine pathway and in neighboring cells via a paracrine pathway. However, since the IFNs must first be synthesized, these pathways induce ISG expression with significantly slower kinetics in comparison to direct viral activation (6). Thus, the direct activation of ISG transcription via DRAF1 may provide cells with an increased chance of survival during viral infection and appears to be a primary defense mechanism of the virus.

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