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Adenovirus VAI RNA complexes with the 68 000 M_r protein kinase to regulate its autophosphorylation and activity

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We have investigated the interaction of VAI RNA with the interferon-induced, double-stranded (ds) RNA-activated protein kinase, P68, both of which regulate protein synthesis in adenovirus-infected cells. Previous work has shown that during infection by the VAI RNA-negative mutant, dl331, both viral and cellular protein synthesis are inhibited due to phosphorylation of the α-subunit of the eukaryotic initiation factor, eIF-2, by the P68 protein kinase. Utilizing monoclonal antibodies specific for P68, we demonstrated that the physical levels of P68 in dl331-infected, wild-type Ad2-infected and uninfected cells were all comparable suggesting that the elevated kinase activity detected during mutant infection was not due to increased P68 synthesis. To examine the basis of the increased activity of P68, the protein kinase was purified from infected-cell extracts using the monoclonal antibody. We found that P68 was heavily autophosphorylated during dl331 infection but not during wild-type or mock infection. The extent of autophosphorylation correlated with elevated P68 activity and the loss of the dsRNA requirements to phosphorylate the exogenous substrates, eIF-2α and histones. We also analyzed VAI RNA function *in vitro* and present evidence that purified VAI RNA can block the autophosphorylation of P68 in the ribosomal salt wash fraction of interferon-treated cells. Finally we suggest VAI RNA functions through a direct interaction with the P68 protein kinase, since we demonstrated that VAI RNA forms a complex with P68 both *in vitro* and *in vivo*.

Key words: protein kinase/adenovirus VAI RNA/translation/eIF-2 phosphorylation

Introduction

Late after adenovirus infection, a shift occurs from cellular to viral protein synthesis (Anderson, 1973; Bello and Ginsberg, 1976; Beltz and Flint, 1979; Babich *et al.*, 1983). Despite this shift, overall levels of protein synthesis during infection are maintained at high levels. The adenovirus-encoded RNA polymerase III product VAI RNA (Reich *et al.*, 1966) has been shown to play an important role in keeping protein synthesis levels high in infected cells. During infection by dl331, an adenovirus mutant which fails to synthesize VAI RNA, all protein synthesis, both cellular and viral, is drastically reduced (Thimmappaya *et al.*, 1982). These cells have been shown to be defective in functional eIF-2 (Reichel *et al.*, 1985; Schneider *et al.*, 1985; Siekierka *et al.*, 1985), the eukaryotic initiation factor that forms the ternary complex, eIF-2: GTP:Met-tRNA, which binds to the initiating $40S_n$ ribosomal subunit before mRNA is bound (Jagus *et al.*, 1981).

The inactivation of eIF-2 results from the phosphorylation of its α-subunit by a protein kinase activated during infection (Siekierka *et al.*, 1985; Schneider *et al.*, 1985). O'Malley *et al.* (1986) demonstrated that this protein kinase was identical to the protein kinase induced by interferon and activated by dsRNA (Lebleu *et al.*, 1976; Roberts *et al.*, 1976; Zilberstein *et al.*, 1976; Samuel *et al.*, 1977). The resulting phosphorylation of the α-subunit prevents the recycling of eIF-2:GDP to eIF-2:GTP by the recycling factor eIF-2B, the latter being trapped in an inactive complex with eIF-2:GDP (Konieczny and Safer, 1983; Pannier and Henshaw, 1983; Safer, 1983). Thus, during infection by the VAI RNA-negative mutant dl331, functional eIF-2 (eIF-2:GTP) is limiting and protein synthesis initiation is blocked, apparently non-selectively.

VAI RNA, when added to interferon-treated cell-free extracts, can block the phosphorylation of the α-subunit of eIF-2 (O'Malley *et al.*, 1986; Kitajewski *et al.*, 1986).It was suggested that VAI RNA functions, at least in part, by blocking the activation of the eIF-2α protein kinase. The protein kinase (referred to as P68 based on its M_r of 68 000 but also referred to by others as DAI or P1/eIF-2α kinase) is characterized by two distinct kinase activities: the first involves an autophosphorylation reaction, the second, a protein kinase activity on exogenous substrates, such as eIF-2α (Hovanessian and Galabru, 1986). Activation of P68 is dependent on dsRNA-induced autophosphorylation whereas, following activation, the kinase activity does not require dsRNA (Sen *et al.*, 1978; Hovanessian and Galabru, 1986). Both the autophosphorylation and kinase activities require ATP and are greatly stimulated by the divalent cation manganese (Mn^{2+}), although the autophosphorylation reaction requires ~10-fold more Mn^{2+} for optimal activity (Galabru and Hovanessian, 1985; Hovanessian and Galabru, 1986).

We have utilized monoclonal antibodies specific for P68 (Laurent *et al.*, 1985) to investigate the role of P68 and VAI RNA in the regulation of protein synthesis in adenovirus-infected cells. By immunoprecipitation analysis, we show that the physical levels of P68 are similar in mutant and wild-type virus-infected cells and are not induced above levels found in uninfected cells. However, the protein kinase becomes highly autophosphorylated and consequently highly activated only during dl331 infection. We also show that VAI RNA can prevent the autophosphorylation of P68 *in vitro*, in a crude ribosomal salt wash fraction prepared from interferon-treated cells. Finally we present data demonstrating that VAI RNA probably functions by directly complexing with the P68 protein kinase.

Results

The synthesis of P68 is not induced in mutant dl331-infected cells

To determine whether increased P68 synthesis accounted for the increased protein kinase activity in dl331-infected cells (Reichel

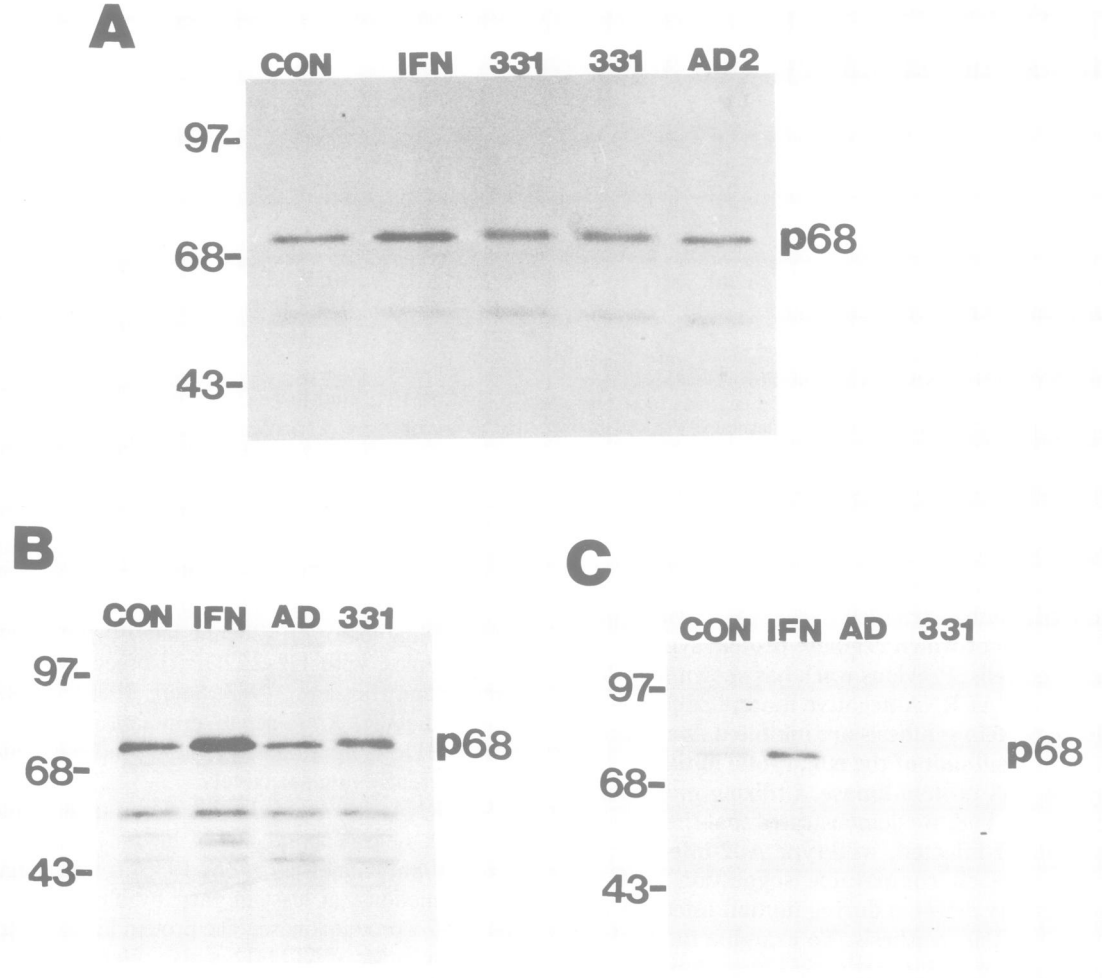

Fig. 1. P68 protein kinase synthesis is not induced during virus infection. The 293 cells were either uninfected (CON), treated with 1000 units/ml of interferon (IFN), infected with wild-type Ad2 (AD2), or dl331 (331). Cells were labeled with [^{35}S]methionine either for 1–16 h **(A)**, 11–16 h **(B)** or 15–16 h **(C)**, post-interferon treatment or post-infection. Two different dl331 virus stocks were tested in the experiment shown in **panel A**. Cell extracts were prepared and immunoprecipitated with the monoclonal antibody to P68 as described in Materials and methods. The samples were subjected to electrophoresis on a 10% SDS–polyacrylamide gel. Densitometer tracings of P68 revealed that P68 synthesis increased 4- to 5-fold in interferon-treated cells relative to control and infected cells. The position of mol. wt protein markers are shown on the left of each panel.

et al., 1985; Siekierka *et al.*, 1985; Schneider *et al.*, 1985), we compared P68 levels in virus-infected, mock-infected and interferon-treated 293 cells utilizing the monoclonal antibody specific for the protein kinase (Laurent *et al.*, 1985). The steady-state levels of P68 were measured by labeling the virus-infected, interferon-treated, and control cells continuously with [^{35}S]methionine from 1 to 16 h post-infection or post-treatment. Cell extracts were prepared and immunoprecipitation analysis performed as outlined in Materials and methods. The results (Figure 1A) show that P68 synthesis is not induced during infection: the levels of P68 are comparable in Ad2-infected and dl331-infected cells, as well as in uninfected cells. As previously shown, P68 levels do increase 4- to 5-fold after interferon treatment (Galabru and Hovanessian, 1985).

To examine the rate of P68 synthesis, cells were labeled for shorter periods, either from 11 to 16 h (Figure 1B) or 15 to 16 h (Figure 1C) post-infection or post-interferon treatment. The rate of P68 synthesis was found to increase relative to control levels only after interferon treatment. Similar results were obtained by pulse-labeling cells at 20 and 24 h post-infection (data not shown). As might be predicted, late after infection the rate of synthesis of P68 (a cellular protein) was even reduced compared with control levels because of the adenovirus-induced host shut-off and the translational defects existing in dl331-infected cells. We conclude from these results that the increased kinase activity found in dl331-infected cells cannot be due to increased P68 synthesis.

The P68 protein kinase is highly autophosphorylated in dl331-infected cells

Since the physical levels of P68 did not increase during dl331 infection, the detected rise in kinase activity was most likely due to the increased activation of P68 occurring during mutant virus infection. It has been demonstrated recently that P68 kinase activity, i.e. the ability of P68 to phosphorylate exogenous substrates, is directly proportional to its level of autophosphorylation (Hovanessian and Galabru, 1986). By utilizing the P68 monoclonal antibody, we quantitated the degree of P68 autophosphorylation in virus-infeced cells. Both uninfected and interferon-treated cells were included as controls. The 293 cells were labeled with [^{32}P]orthophosphate from 16 to 20 h post-infection or post-interferon treatment. Cell extracts were prepared and immunoprecipitation analysis performed. The results shown in Figure 2, demonstrate that P68 is significantly more autophosphorylated (>20-fold) in dl331-infected cells compared with Ad2-infected cells, although physical levels of P68 are identical (see Figure 1). Interestingly, the level of P68 autophosphorylation in wild-type infected cells is below levels found in uninfected cells, suggesting the kinase also is less activated than in control

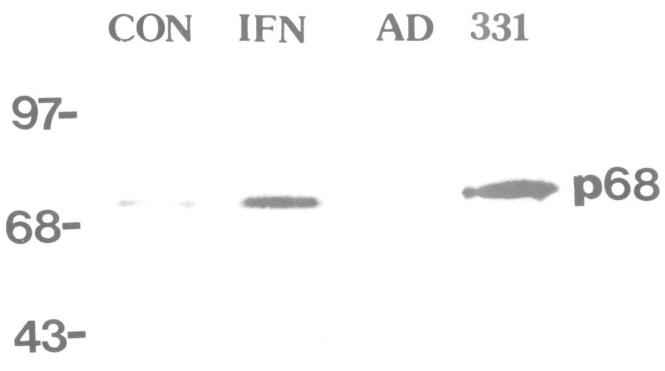

Fig. 2. The P68 protein kinase is heavily autophosphorylated during infection by the adenovirus mutant dl331. The 293 cells were mock-infected (CON), treated with 1000 units/ml interferon (IFN), infected with Ad2 (AD) or the mutant dl331 (331). Cells were labeled with [^{32}P]orthophosphate for 16–20 h post-treatment or post-infection. The labeled extracts were then reacted with the anti-P68 monoclonal antibody and the immunoprecipitates subjected to electrophoresis on a 10% SDS–polyacrylamide gel. Densitometer tracings revealed that P68 in dl331-infected cells was >20-fold more phosphorylated than the Ad2 P68 and 10-fold more phosphorylated than control P68. After correcting for the 4- to 5-fold induction of P68 occurring during IFN treatment, the phosphorylation levels of the IFN P68 were comparable with the control P68 levels. The position of mol. wt protein markers are shown on the left.

cells (see below). When normalized to correct for the 4- to 5-fold induction, the level of P68 phosphorylation in interferon-treated cells is comparable with levels in mock-infected cells.

The P68 protein kinase purified from dl331-infected cells is highly active and can efficiently phosphorylate exogenous substrates

To correlate the extent of P68 autophosphorylation with its functional activity, we purified the P68 on the monoclonal antibody column (Mab–Sepharose). It has been shown that the protein kinase remains functional when bound to the monoclonal antibody (Galabru and Hovanessian, 1985). The *in vivo* extent of P68 activation was measured in the absence of the activator dsRNA by the phosphorylation of the α-subunit of eIF-2 and the histones, HIIA. Both eIF-2α and the histones were heavily phosphorylated by the P68 purified from mutant-infected cells but not by the P68 from wild-type or uninfected cells (Figure 3). The kinase is highly activated as early as 11 h post-dl331 infection. We, and others, have found that prior to 11 h post-infection, P68 kinase activity is relatively low (O'Malley *et al.*, 1986; M.G.Katze, unpublished results). The P68, purified from wild-type Ad2-infected cells, which was found to be less autophosphorylated *in vivo* than the P68 from control cells (see Figure 2), was also less active as measured by its ability to phosphorylate these exogenous substrates. We confirmed that this reduced activity was not due to a reduction in the levels of P68 binding to the Mab–Sepharose (data not shown).

To better quantitate the kinase activity of P68, the bands representing histones (Figure 3B) were cut out of the gel and counted. The results (Table I) show the kinase from dl331-infected cells is ~7- to 8-fold more active than the P68 from control cells and 20- to 30-fold more active than P68 from Ad2-infected cells. As these assays were done in the absence of dsRNA, they measure the phosphorylating activity of P68 as it existed when the cells were harvested. When the assays were performed in the presence of dsRNA, the control and wild-type P68 kinase activities were elevated ~2- to 3-fold but the dl331 P68 kinase activity was raised only 15–25% (data not shown). This indicated that P68 from dl331-infected cells was almost fully activated *in vivo* prior to cell harvest. In contrast, P68 from control and Ad2-infected cells can be activated *in vitro* by dsRNA and can better phosphorylate the exogenous substrates.

To confirm the autophosphorylation of P68 occurring during dl331 infection directly correlated with its high kinase activity, the protein kinase purified from mutant infected cells was dephosphorylated with bacterial alkaline phosphatase (BAP) and tested for its kinase activity. The results, shown in Figure 4, demonstrate that the dephosphorylated P68 had a significantly reduced ability to phosphorylate histones (lanes C and D). This decrease was not due to a reduction in the physical levels of P68 which were analyzed and shown to be the same (data not shown). The kinase activity was not completely eliminated since the BAP treatment removes at best 50–75% of the phosphates from activated P68 kinase molecules (A.Hovanessian, unpublished results).

VAI RNA can block the autophosphorylation of P68 in the ribosomal salt wash fraction of interferon-treated cells

The data presented thus far suggests that in the absence of VAI RNA, i.e. during dl331 infection, the P68 protein kinase becomes highly autophosphorylated and activated. To directly show that VAI RNA can block the autophosphorylation of P68, it was necessary first to obtain a source of non-activated protein kinase. It has been demonstrated that high levels of functional P68, inactive in the absence of dsRNA, are present in the ribosomal salt wash (RSW) fraction of interferon-treated cells (Samuel *et al.*, 1977; Hovanessian and Galabru, 1986). We therefore prepared the RSW fraction from interferon-treated Daudi cells, and tested the activation (autophosphorylation) of P68 in the presence of increasing concentrations of reovirus dsRNA (Figure 5A). The P68 was activated at both 0.001 μg/ml (lane B) and 0.01 μg/ml dsRNA (lane C) but not at concentrations above or below. This strict optimum for kinase activation by dsRNA has been observed previously (Hunter *et al.*, 1975; Farrell *et al.*, 1977). Since the secondary structure of VAI RNA also has double-stranded regions (Akusjarvi *et al.*, 1980; Monstein and Philipson, 1981), the ability of highly purified VAI RNA to activate the P68 in the RSW was examined (Figure 5B). No activation of P68 was detected at concentrations of VAI RNA from 0.01 μg/ml to 10 μg/ml (lanes B–E). Similarly, purified 5S RNA (10 μg/ml) did not activate the kinase (lane F).

To test whether VAI can block autophosphorylation of P68, the RSW was pre-incubated with increasing concentrations of purified VAI RNA for 15 min at 30°C, after which reovirus dsRNA (0.01 μg/ml) was added in the presence of [γ-^{32}P]ATP for an additional 15 min incubation (Figure 5C). Purified VAI RNA at concentrations of 5 μg/ml (lane D) and 10 μg/ml (lane E) effectively prevented the reovirus dsRNA-induced autophosphorylation of P68 in the RSW. Indeed, after addition of 10 μg/ml VAI RNA, the resulting P68 phosphorylation levels are equivalent or even slightly below the levels found in the RSW in the absence of any added dsRNA (see densitometer scan, Figure 5D: compare lanes A and E). In contrast, purified 5S RNA at 10 μg/ml did not prevent the autophosphorylation of P68 (Figure 5C, D: lane F). Purified tRNA also failed to block P68 autophosphorylation (data not shown). These results demonstrate that physiological levels of VAI RNA specifically prevent the activation of P68 by reovirus dsRNA *in vitro*.

VAI RNA complexes with P68 in vivo and in vitro

To determine whether VAI functions via a direct interaction with P68, we examined both the *in vivo* and *in vitro* association of

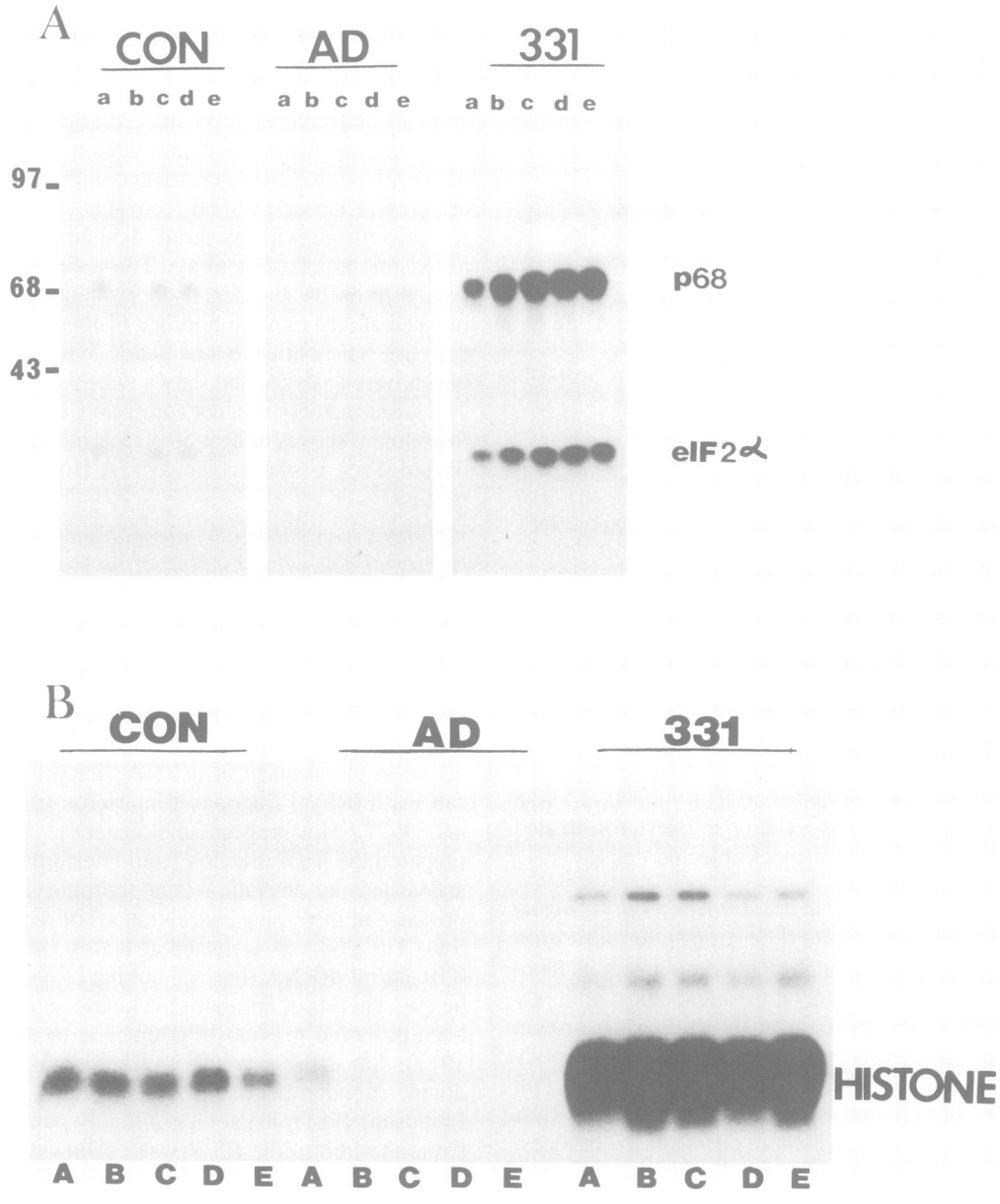

Fig. 3. The P68 protein kinase, purified from dl331-infected cells is highly activated and efficiently phosphorylates eIF-2α and histones. P68 was purified from uninfected cells (CON), from Ad2-infected cells (AD) or from dl331-infected cells (331) on Mab−Sepharose as described in Materials and methods. Cells were infected for 11 h (**lane A,a**), 13 h (**lane B,b**), 15 h (**lane C,c**), 17 h (**lane D,d**) or 21 h (**lane E,e**). In the top panel, **A**, 0.5 μg purified eIF-2 was added to the kinase assays and in the bottom panel, **B**, 25 μg calf thymus histones (HIIA) were added. The kinase assays were performed in the absence of dsRNA as described in Materials and methods. The samples were analyzed subsequently on a 10% SDS−polyacrylamide gel. Note that P68 was phosphorylated when incubated with eIF-2 but not when incubated with the highly basic histone proteins. It has been shown that P68 even in its fully autophosphorylated state can still bind ATP; it was also found that histones inhibited this ATP binding (Galabru and Hovanessian, 1985; Hovanessian and Galabru, 1986).

P68 and VAI RNA. If VAI forms a stable complex with P68 in adenovirus-infected cells, it should be co-precipitated with the protein kinase by the monoclonal antibody. This procedure has been utilized successfully to identify the association of VAI RNA with the cellular lupus antigen, La (Rosa *et al.*, 1981). The 293 cells were labeled with [^{32}P]orthophosphate from 18−22 h post-Ad2 infection. As controls, dl331 and mock-infected cells were similarly labeled. Cell extracts were prepared and immunoprecipitation performed. Rather than examining the labeled protein immunoprecipitated, the labeled RNA, specifically complexed with P68, was recovered from the immunoprecipitate by phenol−chloroform extraction and ethanol precipitation. The results, shown in Figure 6A, demonstrate that VAI RNA can be immunoprecipitated by antibody specific for P68 (lane E). No other RNAs, even the heavily labeled 5S and tRNAs, were

Table I. The phosphorylation of histones by P68 purified from Dl331, Ad2, and uninfected cells[a]

Hours post-infection	Control	Ad2	Dl331
11	6094[b]	2911	28 136
13	5516	1766	43 723
15	5470	1188	39 560
17	5089	1214	34 406
21	3917	1153	46 002

[a]The P68 was purified on Mab−Sepharose and incubated with histones, as described in the Materials and methods, and the legend to Figure 3.
[b]The figures represent c.p.m. per 25 μg histones which were excised from the polyacrylamide gel shown in Figure 3B and counted in a liquid scintillation counter.

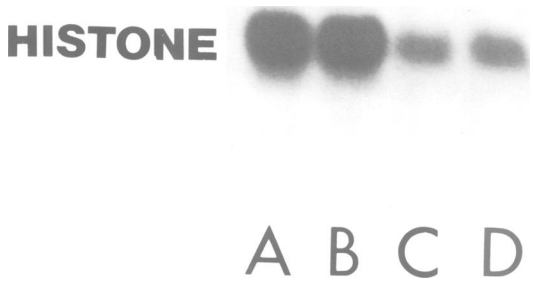

Fig. 4. P68 purified from dl331-infected cells has reduced kinase activity after dephosphorylation with bacterial alkaline phosphatase (BAP). The P68, purified from dl331-infected cells at 18 h post-infection utilizing the Mab−Sepharose, was pre-incubated for 30 min at 30°C in Buffer III containing: no BAP **(lane B)**; 5 units BAP **(lane C)**; and 10 units BAP **(lane D)**. **Lane A** depicts the kinase activity of P68 which was not pre-incubated nor treated with BAP. Following BAP or mock treatment, the P68 was washed twice with Buffer II and twice with Buffer III. The kinase assay was then performed in Buffer III with 2 mM $MgCl_2$, 1 mM $MnCl_2$, [γ -^{32}P]ATP as well as 25 μg histones per reaction. No dsRNA was added to the reactions. The samples were analyzed on a 10% SDS−polyacrylamide gel. The histone bands were also cut out from the gel and counted in a liquid scintillation counter: **lane A**: 23 456 c.p.m.; **lane B**: 24 606 c.p.m.; **lane C**: 10 760 c.p.m.; and **lane D**: 11 976 c.p.m.

found to bind to P68 during the immunoprecipitation reaction. In addition, we did not detect any binding of RNAs corresponding to VAII RNA. We consistently observed that only VAI RNA was specifically co-precipitated with the P68 monoclonal antibody.

To verify that VAI RNA forms a complex with P68, we developed the following *in vitro* binding assay. Cytoplasmic ^{32}P-labeled RNA from Ad2-infected cells, from dl331-infected cells and from uninfected cells was added to the P68 protein kinase previously purified on Mab−Sepharose (see Materials and methods). As a control against non-specific sticking, the binding of labeled RNA to Mab−Sepharose not exposed to the interferon-treated extracts was examined. After an appropriate incubation, possible RNA−P68 complexes were washed and bound RNA recovered by phenol−chloroform extraction and ethanol precipitation. As shown in Figure 6B, the purified P68 selectively bound the VAI RNA present in the cytoplasmic RNA prepared from wild-type infected cells (lane H). VAI RNA often migrates as a doublet as transcription can initiate at two different 5′ positions, the longer species beginning with an A residue, the shorter a G residue (Celma *et al.*, 1977). We also found that highly purified ^{32}P-labeled VAI RNA bound efficiently to purified P68 (data not shown). No detectable RNAs from either mutant or uninfected cells bound to the purified P68 (lanes I and G, respectively). Furthermore, the binding of VAI RNA was dependent on the presence of P68 since no detectable VAI RNA complexed with the Mab−Sepharose alone (lane E). As an additional control, cytoplasmic RNA from Ad2-infected cells was reacted with Mab−Sepharose which had been pre-incubated with an interferon-treated mouse cell extract. Again no VAI RNA bound to the Mab−Sepharose (data not shown) since monoclonal antibodies to the human P68 do not react with the protein kinase of other species (Laurent *et al.*, 1985). These results taken together strongly suggest that VAI RNA binds to P68 during adenovirus infection thereby preventing its autophosphorylation and activation. With the proper functioning of VAI RNA, eIF-2α is not heavily phosphorylated and the host translational machinery is not shut down.

Discussion

In cells infected by the VAI RNA-negative adenovirus mutant dl331 both viral and cellular protein synthesis are inhibited (Thimmappaya *et al.*, 1982). This shut down of protein synthesis is most likely due to the phosphorylation of the eIF-2α subunit by the interferon-induced P68 protein kinase. We have utilized a monoclonal antibody specific for P68 to help determine the role of the adenovirus-encoded VAI RNA and the P68 protein kinase in the regulation of protein synthesis late after viral infection. Although P68 protein kinase levels in dl331-infected cells do not increase relative to levels found in wild-type virus-infected or uninfected cells, P68 does become heavily autophosphorylated in mutant virus-infected cells. The phosphorylation state of P68 in dl331-infected cells correlated well with its elevated kinase activity, as measured by the phosphorylation of the exogenous substrates eIF-2α and histones. Experiments are now in progress to determine the phosphorylation levels of eIF-2 *in vivo*, in mutant and wild-type virus-infected cells.

In wild-type Ad2-infected cells, both the autophosphorylation of P68 and its kinase activity were below control levels. Interestingly, the kinase activity drops between 11 and 13 h post-infection (Figure 3B; Table I), a time when VAI RNA synthesis noticeably increases (O'Malley *et al.*, 1986). It may be that this reduction in kinase activity below basal levels is due to the presence of VAI which is complexing with the P68 during Mab−Sepharose purification (Figure 6). O'Malley *et al.* (1986), utilizing crude cell-free extracts, also have reported that kinase activity in Ad2-infected cells, when assayed in the presence of dsRNA, was below control levels. In contrast to our results, however, they found that the dsRNA-dependent kinase activity in mock-infected and dl331-infected extracts were almost equivalent. We have consistently found the kinase activity is several fold higher in mutant-infected cells compared with mock-infected cells even when assayed in the presence of dsRNA.

What is causing this tremendous activation of P68 during mutant virus infection? O'Malley *et al.* (1986) suggest that double-stranded RNA from the symmetrical transcription of the viral double-stranded DNA template (Petterson and Philipson, 1974) activates P68. However, Hovanessian and Galabru (1986) recently demonstrated that P68, purified from interferon-treated cells, can be activated efficiently by such compounds as heparin, dextran sulfate, chondroitin sulfate and poly(L-glutamine). These findings were unexpected since it was previously thought that maximal activation of P68 required a dsRNA structure 65−80 nucleotides long without mismatches (Minks *et al.*, 1979). The only feature these compounds share with dsRNA is that they are all polyanions. This, of course, does not rule out that dsRNA is responsible for the activation of P68 in adenovirus-infected

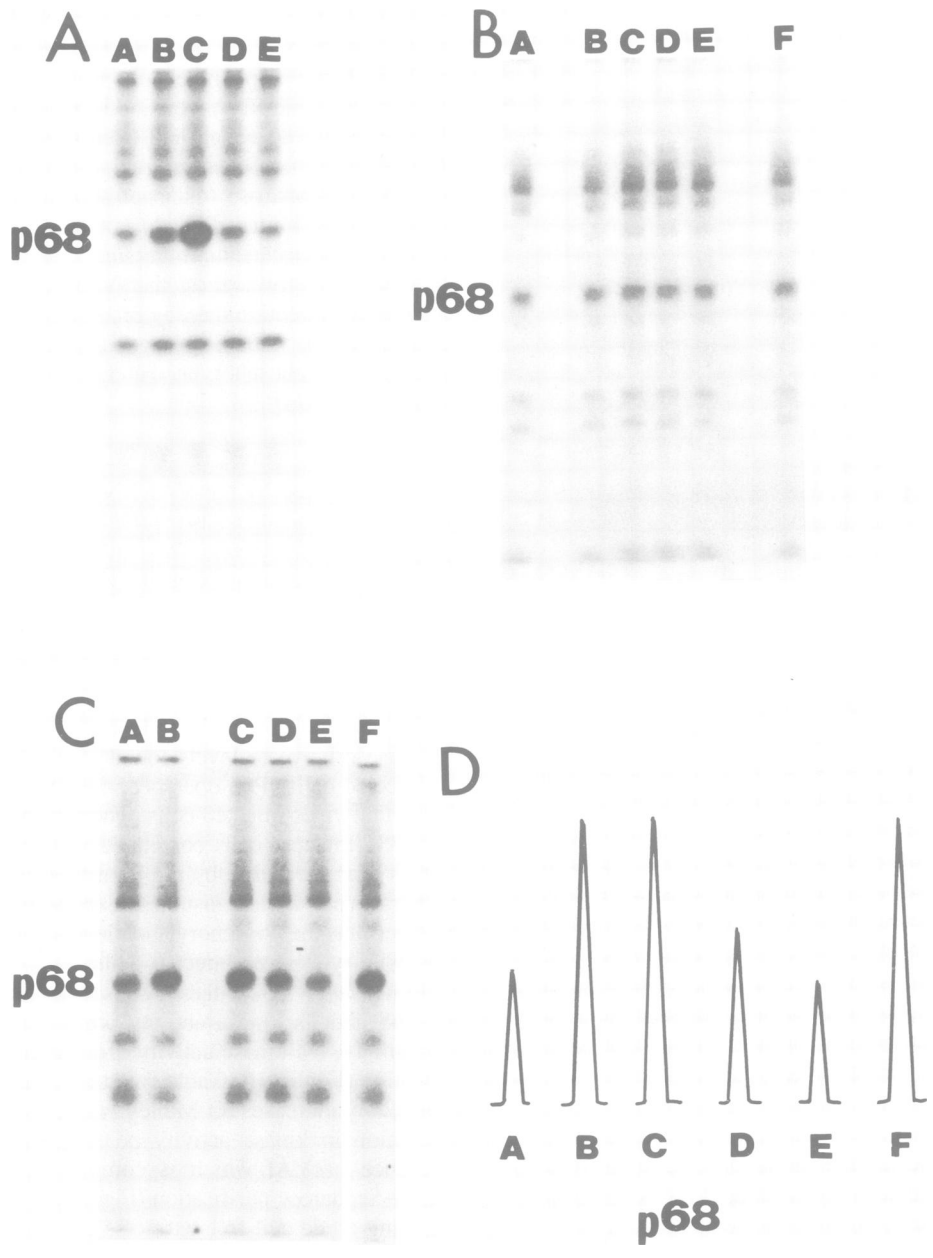

Fig. 5. VAI RNA blocks the autophosphorylation of P68 present in the RSW of interferon-treated cells. **Panel A:** the RSW fraction from interferon-treated Daudi cells was prepared as described in Materials and methods. Reovirus dsRNA was then added at a concentration of 0.001 μg/ml **(lane B)**; 0.01 μg/ml **(lane C)**; 0.1 μg/ml **(lane D)**; 1.0 μg/ml **(lane E)**; the reaction in **lane A** was performed in the absence of dsRNA. Protein kinase assays were done in Buffer III containing 2 mM $MgCl_2$, 0.4 mM $MnCl_2$, and [γ-^{32}P]ATP. Samples were analyzed on a 10% SDS−polyacrylamide gel. **Panel B**: purified VAI RNA was added to the RSW at a concentration of 0.01 μg/ml **(lane B)**; 0.1 μg/ml **(lane C)**; 1.0 μg/ml **(lane D)**; 10.0 μg/ml **(lane E)**. The reaction shown in **lane A** contained no VAI RNA. 10.0 μg/ml purified 5S RNA was added to the kinase reaction shown in **lane F**. The kinase activity was assayed as described in **Panel A**. **Panel C**: the RSW salt wash was pre-incubated in Buffer III containing 2 mM $MgCl_2$ and 0.4 mM $MnCl_2$ for 15 min at 30°C with buffer alone **(lanes A and B)**; 1.0 μg/ml purified VAI RNA **(lane C)**; 5.0 μg/ml VAI RNA **(lane D)**; 10.0 μg/ml VAI RNA **(lane E)**; and 10 μg/ml purified 5S RNA **(lane F)**. Following the pre-incubation, reovirus dsRNA (0.01 μg/ml) was added to samples B−F and [γ^{32}P]ATP to samples A−F for an additional 15 min incubation at 30°C. Samples were analyzed on a 10% polyacrylamide gel. **Panel D:** the P68 radioactive band in the autoradiogram shown in **panel C** was subjected to densitometer analysis: **lanes A−F** are identical to those described in **panel C.**

cells; it merely presents the possibility that activators other than dsRNA may exist in cells and under different physiological conditions (e.g. virus infection) may trigger kinase activation.

P68 has two distinct kinase activities, an autophosphorylation activity, and a protein kinase activity (Hovanessian and Galabru, 1986). In addition the levels of P68 autophosphorylation directly determined kinase activity, i.e. the more heavily phosphorylated P68 was, the more active it was. In the present report we show that VAI RNA blocks the initial activation process, the autophosphorylation reaction; the consequence of this action by VAI RNA is the prevention of eIF-2α phosphorylation by the P68 kinase. Based on our *in vitro* binding and *in vivo* co-precipitation data (Figure 6), it seems likely that VAI functions by directly interacting with the P68 molecules. These results do not rule out that other gene products or factors also interact with VAI RNA and P68 to participate in the blocking of kinase activation. For example it is known that VAI RNA complexes with the cellular La antigen in adenovirus-infected cells (Rosa *et al.*, 1981; Fran-

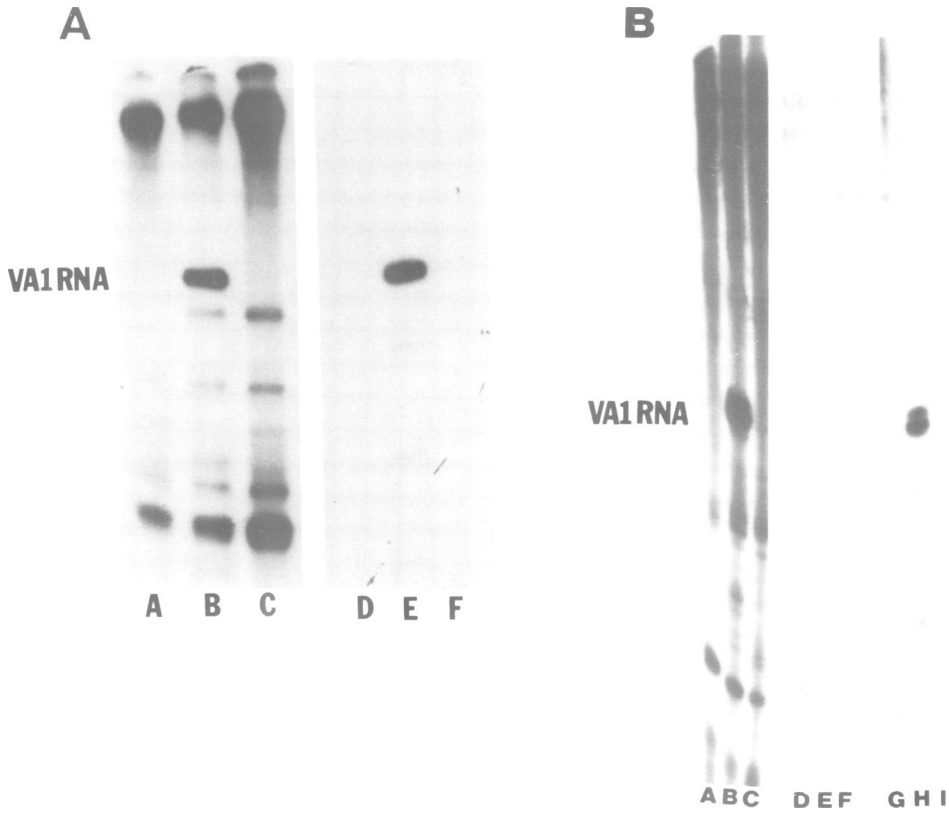

Fig. 6. The binding of VAI RNA to P68 *in vitro* and *in vivo*. **Panel A**: *in vivo* analysis: 293 cells were labeled with [^{32}P]orthophosphate for 18–22 h post-mock infection, Ad2 infection, and dl331 infection. Cell extracts were prepared and reacted with the monoclonal antibody to P68. Labeled RNA which co-immunoprecipitated with P68 was recovered by phenol extraction and ethanol precipitation. An aliquot of the total RNAs labeled after mock infection, Ad2 infection, and dl331 infection, are shown in **lanes A–C**, respectively. **Lanes D, E** and **F** show RNAs specifically immunoprecipitaed by P68 antibody from mock-infected, Ad2-infected, and dl331-infected cells, respectively. The RNAs were analyzed on a 20 cm 8% polyacrylamide gel containing 7 M urea. **Panel B:** *in vitro* analysis: ^{32}P-labeled RNA was prepared from mock-infected, Ad2-infected and dl331-infected cells; total small RNAs present in these cells are shown in **lanes A, B** and **C** respectively. The labeled cytoplasmic RNAs were then added either to Mab–Sepharose alone **(lanes D, E, F)** or to Mab–Sepharose which was pre-reacted with an interferon-treated 293 cell extract to purify P68 **(lane G, H, I)** as described in Materials and methods. RNA specifically bound was then recovered by phenol extraction and ethanol precipitation. **Lanes D** and **G**: RNA bound from mock-infected cells; **lanes E and H**: RNA bound from Ad2-infected cells; and **lanes F** and **I**: RNA bound from dl331-infected cells. The RNA was analyzed on a 40 cm 8% polyacrylamide gel containing 7 M urea. Note that VAI RNA did not migrate as a doublet in the gel shown in **A** since the shorter gel does not give the same resolution as the longer gel shown in **B**.

coeur and Mathews, 1982; Mathews and Francoeur, 1984). We have as yet not detected the presence of additional cellular proteins or RNAs specifically complexing with P68 during immunoprecipitation analysis. However, a more careful analysis of P68–VAI RNA interactions with other cellular gene products should now be possible utilizing the *in vitro* binding assay (Figure 6B).

As yet unresolved is whether VAI RNA functions in a virus-specific manner. Based on the data presented in this report and the fact that both viral and cellular protein synthesis are inhibited in dl331-infected cells (Thimmappaya *et al.*, 1982), it is difficult to understand how VAI functions specifically to enhance adenovirus translation. Indeed, Svensson and Akusjarvi (1984, 1985) convincingly demonstrated that VAI RNA enhanced the translation of non-viral as well as viral mRNAs. However, Kaufman (1985) has reported that only the translation of mRNAs containing almost the full adenovirus tripartite leader sequences was enhanced by the presence of VAI RNA. In addition, it has been reported that VAI RNA can bind to a cloned cDNA copy of the tripartite leader sequences of adenoviral mRNAs (Mathews, 1980). It is possible, therefore, that VAI RNA, through an as yet unidentified mechanism, can specifically promote adenoviral mRNA translation. Alternatively the function of VAI RNA may be only to prevent the overall shut-down of the host translational system. The selective shut off of host cell protein synthesis may be mediated by other mechanisms. For example, we have shown recently that after adenovirus infection, cellular mRNA translation is selectively blocked at the initiation and elongation steps of protein synthesis (Katze *et al.*, 1986a). In addition, there is accumulating evidence that the selective lack of translation of cellular mRNAs also may be linked closely to their lack of transport (Babich *et al.*, 1983; Flint *et al.*, 1983; Babiss *et al.*, 1985; Halbert *et al.*, 1985).

It has been suggested that the imperfect double-stranded structure of VAI RNA (Akusjarvi *et al.*, 1980; Monstein and Philipson, 1981), allows it to effectively block the activation of the P68 protein kinase (Kitajewski *et al.*, 1986; O'Malley, 1986). VAI RNA molecules which have mutations altering its dsRNA stem structures fail to function *in vivo* (Bhat *et al.*, 1985) and fail to block P68 activation *in vitro* (Kitajewski *et al.*, 1986). It would be of interest to determine what regions of VAI RNA are critical for P68 binding and whether these same regions confer functional activity. Even though we found that VAI RNA failed to activate P68 in crude extracts, we rather unexpectedly observed that purified VAI RNA can activate the purified kinase either in its bound state to the Mab–Sepharose or in solution after elution from the Mab–Sepharose (M.G.Katze and A.Hovanessian,

unpublished results). These results support the hypothesis that VAI RNA may require interaction with another cellular gene product to effectively function. When this factor is absent, i.e. when P68 has been purified, VAI RNA no longer blocks but actually stimulates P68 autophosphorylation (possibly due to its double-stranded structure, albeit imperfect). As an alternative explanation, VAI RNA may indeed function alone, but the activation requirements for a purified P68 are less stringent than for the P68 present in crude extracts. We are currently trying to distinguish between these two possibilities.

In addition to the adenoviruses, there are other viruses encoding gene products which suppress the P68 protein kinase activity, possibly as a defence against the anti-viral effects of interferon. For example, vaccinia virus codes for a factor (either RNA or protein) that prevents the phosphorylation of eIF-2α in interferon-treated cells (Whitaker-Dowling and Youngner, 1984; Rice and Kerr, 1984; Paez and Esteban, 1984). In addition, since the small EBER RNAs (Lerner *et al.*, 1981; Rosa *et al.*, 1981) are structurally similar and can functionally substitute for VAI RNAs in adenovirus-infected cells (Bhat and Thimmappaya 1983, 1985) it is likely that Epstein–Barr virus encodes products which block P68 activation. We also have found that influenza virus encodes a mechanism to suppress P68 kinase activity and are currently trying to identify the viral gene product(s) responsible (Katze *et al.*, 1986b).

Materials and methods

Cells and virus

The adenovirus 5-transformed human embryonic kidney cell line 293 (Graham *et al.*, 1977) were grown in monolayer in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (FCS). HeLa cells were grown in suspension in Joklik modified minimal essential media containing 5% FCS. Human Daudi cells were grown in suspension in RPM1-1640 medium containing 10^{-5} M β-mercaptoethanol and 10% FCS. Wild-type Ad2 was purified from infected cells as previously described (Petterson *et al.*, 1976). The adenovirus 5 deletion mutant dl331 (Thimmappaya *et al.*, 1982), kindly provided by Dr T.Shenk, was propagated on 293 cells. The titer of adenovirus stocks was determined by plaque assay on 293 cells (Lawrence and Ginsberg, 1967). Monolayer 293 cells were infected with 5–10 plaque forming units (p.f.u.) per cell of Ad2 or dl331 virus.

Labeling conditions and immunoprecipitation analysis

The 293 cells were labeled either with: (i) [^{35}S]methionine (250 μCi/ml) in DME containing one-tenth the normal amount of methionine; or (ii) [^{32}P]orthophosphate (250 μCi/ml) in DME lacking phosphate, for the times indicated in the Results section. After labeling, cells were washed with ice cold Hank's Balanced Salt Solution (HBSS) and then disrupted in Buffer I: 10 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1000 units/ml aprotinin, 1% Triton X-100. Following centrifugation at 12 000 *g*, the clarified extract was diluted with an equal volume of Buffer II: 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 100 units/ml aprotinin, 1 mM DTT, 0.2 mM PMSF, 20% glycerol, 1% Triton X-100. The labeled cellular extracts were then reacted at 4°C for 3–4 h with the monoclonal antibody to P68 which was previously coupled to CNBr-activated Cl-4B Sepharose (Mab–Sepharose) as earlier described (Laurent *et al.*, 1985; Galabru and Hovanessian, 1985). The immunoprecipitates were then washed five times with Buffer II followed by additional washings in Buffer III: 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 100 units/ml aprotinin, 20% glycerol. The immunoprecipitates were then boiled in 2-fold concentrated electrophoresis disruption buffer, and the samples were analyzed on a 10% SDS–polyacrylamide gel.

Assay of protein kinase activity utilizing the monoclonal antibody to P68

The P68 protein kinase to be analyzed for functional activity was first purified from cell extracts on the Mab–Sepharose as described above: 10 μl of Mab–Sepharose had the capacity to bind material from 2×10^7 interferon-treated cells (Galabru and Hovanessian, 1985). Following the washings in Buffer III, the immunoprecipitates were resuspended in Buffer III containing 2 mM $MgCl_2$ and 1 mM $MnCl_2$ (unless otherwise stated) as well as 5 μM [γ-^{32}P]ATP (50–100 Ci/mmol) and incubated at 30°C for 30 min (Galabru and Hovanessian, 1985). Again, when indicated, the activator, reovirus dsRNA, was added to the reaction. For the phosphorylation of exogenous substrates, ~25 μg of calf thymus histones (HIIA) or 0.5 μg purified eukaryotic initiation factor, eIF-2, was added to the reactions. The eIF-2 was purified by a modification of the procedure of Konieczny and Safer (1983) to be published elsewhere. The phosphorylation reactions were stopped by addition of 2-fold concentrated disruption Buffer and boiling. The samples were then analyzed on a 10% SDS–polyacrylamide gel.

Preparation of the ribosomal salt wash fraction

For the assay of VAI RNA function *in vitro*, ribosomal salt wash fractions were prepared from interferon-treated Daudi cells. Daudi cells, treated for 20 h with 1000 units/ml human lymphoblastoid interferon, were then homogenized in low salt Buffer A: 10 mM Tris-HCl pH 7.6, 10 mM KCl, 1 mM EDTA, 1000 units/ml aprotinin, 0.2 mM PMSF, 7 mM β-mercaptoethanol. Following centrifugations to remove the nuclei and the mitochondria, the extracts were centrifuged at 100 000 *g* for 2 h to pellet the ribosomes. The ribosomal pellet was rinsed in Buffer A and the ribosomes then resuspended in Buffer II. The suspension was left for 30 min at 4°C followed by dialysis overnight against Buffer II and centrifugation at 100 000 *g* for 90 min. The supernatant represented the ribosomal salt wash fraction which was assayed for protein kinase, P68, activity as described in the text.

Purification of VAI RNA

VAI RNA was purified according to a modification of the procedure of Mathews and Petterson (1978). Briefly, suspension HeLa cells were infected with Ad2 at a multiplicity of 25 p.f.u./cell. At 24 h post-infection total cytoplasmic RNA was extracted as previously described (Katze *et al.*, 1984). The cytoplasmic RNA was next precipitated with 2 M lithium chloride (LiCl) (Baltimore, 1966). We found that the majority of the VAI RNA could be precipitated by 2 M LiCl whereas the majority of the other small RNAs, e.g. tRNA and 5S RNA, remained in the supernatant after centrifugation. Without this LiCl step we found it difficult to obtain a purified VAI preparation free of contaminating tRNA. For the following purification steps the LiCl pellet served as our source for VAI RNA whereas the supernatant was the source of purified tRNA and 5S RNA. The RNA preparations were then fractionated according to size on 10–30% sucrose gradients containing 0.5% Sarkosyl. The regions representing RNA sedimenting between 4S and 10S were pooled and ethanol precipitated. Following electrophoresis of the RNA on an 8% polyacrylamide gel containing 7 M urea, the VAI RNA (and cellular tRNA and 5S RNAs) were recovered by electroelution. The RNA preparations were subjected to a final purification using a NENSORB 20 column (Dupont). A small aliquot of RNA was always tested for purity on an 8% polyacrylamide gel after the final purification.

In vitro binding assay of P68 and VAI RNA

The P68 protein kinase was purified from 293 cells treated for 16 h with 1000 units/ml human lymphoblastoid interferon, utilizing Mab–Sepharose as described above. Following the washings in Buffer III the immunoprecipitate was resuspended in Buffer III containing 2 mM $MgCl_2$, 2 mM $MnCl_2$, 7 mM β-mercaptoethanol and 10 μM ATP. Cytoplasmic RNA was prepared from mock-infected 293 cells or from 293 cells infected with wild-type adenovirus (or dl331 virus) labeled with [^{32}P]orthophosphate (500 μCi/ml) from 16 to 24 h post-infection. An aliquot of the total ^{32}P-labeled RNA preparation was added to the immunopurified P68 for a 20 min incubation at 30°C. As a control, RNA was added to Mab–Sepharose which was not previously reacted with an interferon-treated extract. The complex was then washed in Buffer III and subsequently resuspended in Buffer B: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40 (NP-40), carrier tRNA, and SDS (1%). After phenol–chloroform extraction and ethanol precipitation, cytoplasmic RNA, specifically bound to P68, was analyzed by electrophoresis on an 8% polyacrylamide gel containing 7 M urea.

In vivo analysis of the P68–VAI RNA complex

293 cells were labeled with [^{32}P]orthophosphate from 18 to 22 h post-mutant or wild-type infection. As a control, mock-infected cells were also prepared. The labeled extracts were then reacted with specific P68 antibody as described above except that Buffer II contained 50 mM not 400 mM NaCl. Following the washings in Buffer III, the immunoprecipitates were resuspended in Buffer B and the cytoplasmic RNA which co-immunoprecipitated with the P68 protein kinase was recovered by phenol–chloroform extraction and ethanol precipitation and analyzed on an 8% polyacrylamide gel containing 7 M urea.

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