

Translational regulation by the interferon-induced double-stranded-RNA-activated 68-kDa protein kinase

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ABSTRACT Activation of the interferon-inducible 68-kDa protein kinase (referred to as P68) by double-stranded RNA catalyzes phosphorylation of the α subunit of eukaryotic protein synthesis initiation factor 2. We have analyzed the transient expression of mutant and wild-type kinase molecules in transfected COS cells to examine the effects of the kinase on gene expression in the absence of other interferon-induced gene products. The wild-type P68 kinase was expressed inefficiently whereas a catalytically inactive P68 was expressed at 30- to 40-fold higher levels. Protein stability measurements and primer-extension analysis of human kinase-specific mRNA levels provided evidence that kinase expression was regulated at the level of mRNA translation. Further, cotransfection experiments revealed that the domain II catalytically inactive mutant could stimulate reporter gene protein synthesis in a transdominant manner. We also examined the expression of mutants with deletions in the N-terminal double-stranded RNA binding domains and found that a kinase construct lacking aa 156–243 was expressed at levels comparable to the wild type whereas a P68 construct lacking aa 91–243 was expressed at levels 70-fold higher. Both the inactive domain II P68 mutant and the deletion mutant lacking aa 91–243 were less inhibitory to growth in yeast due to the reduced ability to phosphorylate initiation factor 2 α *in vivo*. In conclusion we have demonstrated that the P68 kinase can regulate mRNA translation primarily of its own mRNA and to a lesser extent of a heterologous mRNA and that this regulation is notably affected by mutations in either the catalytic or N-terminal regulatory domains.

The double-stranded (ds) RNA-activated kinase is a 68-kDa protein in human cells [thus referred to as P68 but also known as PKR (protein kinase-RNA activated), DAI (dsRNA-activated inhibitor), and P1/eukaryotic protein synthesis initiation factor 2 (eIF-2)] and is one of >30 genes induced by interferon treatment (1–5). Activation by dsRNA or polyions causes P68 to be autophosphorylated and in turn to catalyze phosphorylation of its natural substrate, the α subunit of eIF-2, culminating in an inhibition of protein synthesis initiation (6–9). Early studies have suggested that the P68 kinase plays an important role in the cellular response to viral infection (1, 2, 4), and there is now direct evidence for the antiviral role of P68 (10). It is the activation of P68 by viral-specific RNAs that leads to the inhibition of protein synthetic rates in infected cells (11–17). However, many viruses are capable of downregulating the activity of the P68 protein kinase, presumably to circumvent the deleterious effects on replication (1, 4, 18).

It also has been postulated that the P68 kinase may play a pivotal role in the general regulation of protein synthesis and growth control in the absence of virus infection or interferon treatment (1–3). For example, it has been suggested that P68

regulates the induction of certain protooncogenes and growth factors (19, 20) and adipocyte development (21, 22); further, cellular regulators of the kinase have now been identified (23–26). Direct evidence that the P68 kinase regulates cell growth has been provided by studies that show that kinase expression is growth-suppressive and/or toxic in insect and mammalian cells (27, 28) and in yeast (29, 30, 53). Further, we have recently demonstrated that catalytically inactive kinase molecules, when introduced into NIH 3T3 cells, can cause malignant transformation (28, 31). To elucidate the cellular physiological role of the P68 protein kinase and determine whether the kinase functions by regulating cellular gene expression at the level of mRNA translation, we analyzed expression of wild-type and mutant kinase variants *in vivo* in transfected COS-1 cells. We report an inactive mutant kinase with a Lys \rightarrow Arg substitution in catalytic domain II and an N-terminal P68 mutant with a deletion in a critical dsRNA binding regulatory domain were expressed at levels >35- to 70-fold higher than the wild-type P68. We further show that this expression was regulated at the level of protein synthesis.

MATERIALS AND METHODS

Construction of Vectors. The cDNAs encoding the entire human P68 gene (wild type, PK-WT, or PK-M1—the domain II mutant) were inserted into pcDNA1/NEO (Invitrogen) and it is important to note that the only differences in the kinase constructs were in the coding region (32). For construction of pcDNA1/NEO-SEAP, a *HindIII*–*Xho* I fragment containing the complete coding region for the secreted embryonic placental alkaline phosphatase (SEAP) product was isolated from pBC12/SEAP (a gift of Bryan Cullen, Duke University; ref. 33) and inserted into similarly treated pcDNA1/NEO. pcDNA1/NEO plasmids encoding kinase molecules with aa 91–243 deleted (PK-M3) and aa 156–243 deleted (PK-M2) in the N-terminal region have been described (32).

Transfection Analysis. COS-1 cells were maintained and transfected essentially as described (34).

Protein Analysis of Transfected Cells. Western blot analysis was performed as described (27) with a mouse monoclonal antibody specific for the human P68 protein kinase (35). For the measurement of the half-lives of the P68 kinase constructs, replicative transfected cultures were treated with cycloheximide (100 μ g/ml) to block *de novo* protein synthesis.

Assay of Protein Kinase Activity. Protein kinase activity was measured as described by Katze *et al.* (36, 37).

SEAP Reporter Gene Enzyme Assay. SEAP assays were performed as described by Berger *et al.* (33) and Garfinkel and Katze (38).

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Abbreviations: eIF-2, eukaryotic protein synthesis initiation factor 2; ds, double stranded; SEAP, secreted embryonic placental alkaline phosphatase; IEF, isoelectric focusing.

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Preparation and Analysis of mRNAs. Total cytoplasmic RNA was isolated as described (39, 40). Cytoplasmic poly(A)⁺ RNAs were isolated by oligo-d(T) chromatography. For primer-extension analysis, the following oligonucleotides were used to hybridize to polyadenylated mRNA: P68, 5'-TTGCACATGCCGAGCCGTTGTCGACGACG; Actin, 5'-CTGAGTGCTTGCGGCAGCGTGAAGCTAGGAG; Neomycin, 5'-GTATGTATTAAGTTCTCCATGAAGAAACC. For primer extension, primers were end-labeled with [³²P]ATP by using T4 polynucleotide kinase with 2 × 10⁵ cpm of the radiolabeled oligonucleotide hybridized with 5 μg of poly(A)⁺ mRNA (41).

Expression of Protein Kinase Constructs in Yeast and Analysis of eIF-2α Phosphorylation. This analysis was performed essentially as described in Dever *et al.* (42). Purified transformants were streaked on SD medium supplemented with tryptophan (SD), SD medium supplemented with tryptophan, leucine, isoleucine, and valine (SD+ Leu, Ilv), or SGAL medium supplemented with tryptophan (SGAL). On SD and SD+ Leu, Ilv media, the *GAL-CYC1* promoter driving P68 expression will be inactive. For the isoelectric focusing (IEF) analysis of eIF-2α phosphorylation, the yeast strains containing the P68 alleles were grown to exponential phase in SD+ Leu, Ilv medium after which they were transferred to SD medium and harvested after 13 hr. The IEF gels were analyzed as described (42).

RESULTS

Differential Protein Expression of Wild-Type and Mutant Protein Kinases in Transfected COS-1 Cells. We first com-

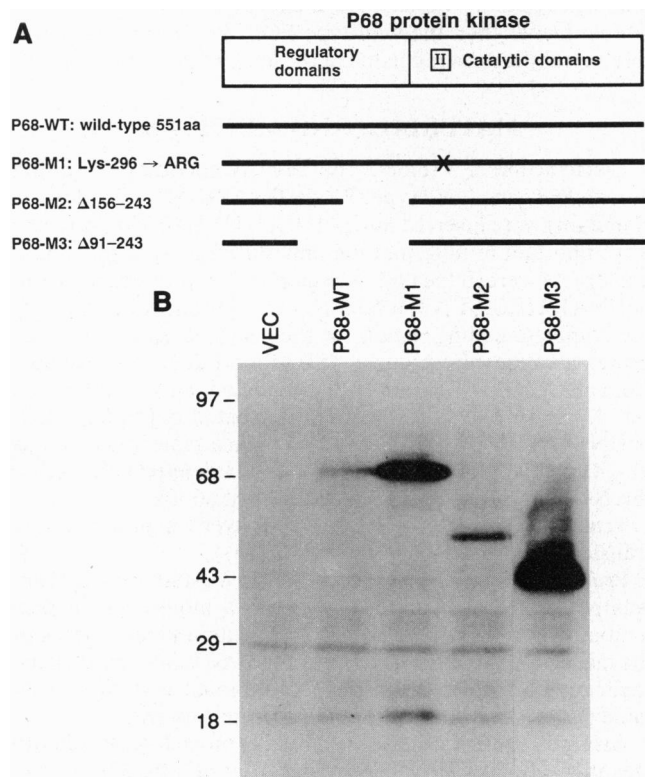


FIG. 1. Western blot analysis of wild-type and mutant protein kinases in transfected COS-1 cells. (A) Description of the P68 protein kinase: wild type, regulatory N-terminal deletions, and C-terminal domain II mutations. (B) Western blot analysis. After transfection of COS-1 cells with the constructs (4 μg) shown in A (including the empty vector, VEC), cytoplasmic extracts were fractionated by SDS/PAGE on 14% gels, blotted onto nitrocellulose, and probed with P68-specific monoclonal antibody.

pared the expression of the wild-type P68 (PK-WT) with kinases mutated in either their dsRNA binding regulatory region or catalytic domains, located at the N and C termini, respectively (27, 30, 32, 43-48). The catalytically inactive P68 (PK-M1) contained a Lys → Arg change at position 296 in kinase domain II (32). The two regulatory domain mutants contained deletions of aa 156-243 (PK-M2) or aa 91-243 (PK-M3); both of these kinases were previously found to be defective in the binding of activator dsRNA *in vitro*, particularly the latter (32). After DEAE-dextran-mediated transfection, cytoplasmic extracts were prepared and protein kinase expression was analyzed with a human-kinase-specific P68 monoclonal antibody (Fig. 1). Western blot analysis identified wild-type and mutant protein kinases with the expected molecular masses and human kinase-specific proteins were not detected in cells transfected with vector alone. Although expression levels of the different constructs varied dramatically, PK-M2 was expressed at levels comparable to the P68-WT. In contrast, PK-M1 and PK-M3 were expressed at levels ≈35- and 70-fold higher than P68-WT, respectively. The different levels of expression observed by Western blot analysis could arise from differential protein stabilities or from differences in the transcriptional or translational efficiencies of the kinase genes. To distinguish between these possibilities, we compared the half-lives of PK-WT and the "overexpressed" PK-M1 and PK-M3 proteins (Fig. 2). Extracts were prepared at the time of cycloheximide addition (0 time, lane A) or at 3 hr (lane B), 6 hr (lane C), and 9 hr (lane D) after drug addition. In accordance with earlier observations (11, 49), all the kinase proteins had a $t_{1/2}$ of 6-9 hr, showing that the expression differences probably are not due to differences in protein stability.

The P68 Protein Kinase Regulates Gene Expression at the Level of mRNA Translation. We next examined the levels of kinase-specific mRNAs in the transiently transfected COS-1 cells by using primer-extension analysis. In the first experiment, we compared the mRNA levels of PK-WT and PK-M1, the domain II mutant (Fig. 3A, arrow). Equivalent levels of P68-specific mRNA were detected in both cases, strongly suggesting that transcription rates of the two constructs were identical. As control in the primer-extension analysis, we examined mRNA levels in cells transfected with the empty vector alone and found only a slower migrating unidentified band that was also observed in kinase-transfected cells. To

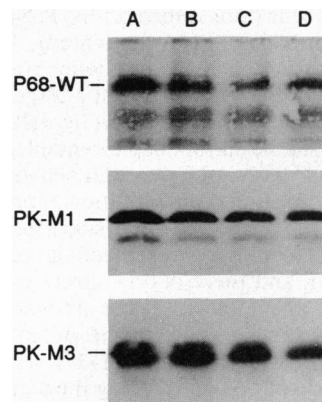


FIG. 2. $t_{1/2}$ analysis of the PK-WT, PK-M1, and PK-M3 protein kinases. Replicate cultures of COS-1 cells were transfected for 48 hr with PK-WT, PK-M1, or PK-M3 and then treated with cycloheximide (100 μg/ml). At 0 hr (lane A), 3 hr (lane B), 6 hr (lane C), and 9 hr (lane D), cytoplasmic extracts were prepared and analyzed on a Western blot as described in Fig. 1. The blots containing the P68-WT were overexposed due to the low expression of the wild-type kinase relative to the other constructs. Laser densitometry revealed a 50% reduction in P68-WT levels at 6 hr and PK-M1 and PK-M3 at ≈9 hr.

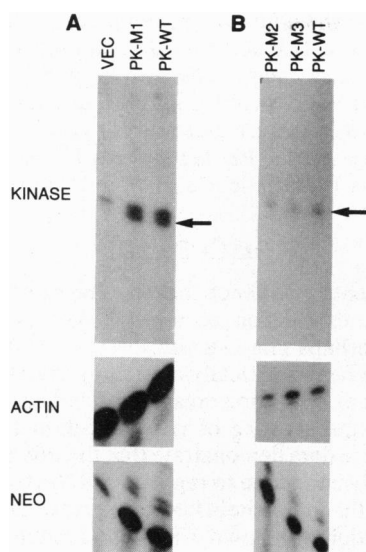


FIG. 3. Primer-extension analysis of protein kinase mRNA levels in transiently transfected COS-1 cells. (A) After transfection with the empty vector (VEC), PK-WT, or PK-M1, poly(A)⁺ mRNA was prepared and 5 μ g was analyzed by primer-extension analysis utilizing oligonucleotides specific for the protein kinase, actin, and neomycin-resistance (NEO) gene mRNAs. The mRNA specific for protein kinase mRNA is indicated by the arrow. (B) The poly(A)⁺ mRNA, prepared from cells transfected with PK-WT, PK-M2, and PK-M3, was analyzed as described in A.

demonstrate that similar amounts of mRNA were analyzed from each transfection, actin mRNA was quantitated using the appropriate probe (Fig. 3A). To ensure that transfection efficiencies were similar for the PK-WT and PK-M1 constructs, we measured the expression of the neomycin-resistance gene present in the pcDNA1/NEO vector. Comparable results were obtained in a separate experiment examining kinase mRNA levels in cells transfected with PK-WT, PK-M2, or PK-M3 (Fig. 3B, arrow). Thus these results allow us to conclude that the mutant kinase expression is primarily regulated at the level of mRNA translation.

To provide further evidence for this translational regulation, we cotransfected the PK-WT or mutant kinases with a reporter gene encoding SEAP (33). SEAP enzyme is secreted at high levels into the medium, where it can be readily assayed and quantitated (33, 38). The results, representative of at least five experiments, showed that P68-WT, PK-M2, and PK-M3 lowered SEAP activity to a small degree compared to the control transfection of the SEAP construct with empty vector (Fig. 4). In contrast, cotransfection with PK-M1 did not cause a decrease in SEAP enzyme levels but, rather, enhanced the synthesis of SEAP \approx 2-fold compared to the vector/SEAP cotransfection and \approx 3-fold relative to the cotransfections with the other kinase constructs. Finally, Northern blot analysis revealed that these changes in SEAP levels were not due to changes in SEAP mRNA levels but were rather due to alterations in protein synthetic rates (data not shown). The decrease (30–35%) in SEAP activity in cells cotransfected with PK-WT, PK-M2, and PK-M3 may be due to enhanced eIF-2 α phosphorylation in transfected cells resulting either from the contribution of the exogenously introduced kinases or from activation of the endogenous monkey kinase by kinase-specific RNAs transcribed from the incoming plasmids (50). That the less-active PK-M3 also slightly downregulated SEAP activity argues more for the latter explanation. Clearly, the downregulation of SEAP by P68-WT in trans was not as dramatic as the decreased expression of the protein kinase itself. We speculate that either the structure of the SEAP mRNA made its translation

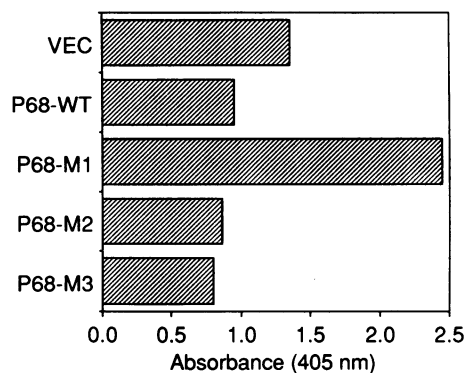


FIG. 4. Analysis of SEAP enzymatic activity in cotransfected COS-1 cells. COS-1 cells were cotransfected with 1 μ g of SEAP cDNA and 2 μ g of the protein kinase cDNA constructs shown (VEC is the empty vector). SEAP activity, secreted into the culture medium, was measured as A_{405} .

less susceptible to decreases in functional eIF-2 levels or, alternatively and perhaps more likely, that the extreme declines in kinase expression arose from a localized sequestration of phosphorylated eIF-2 α , which in turn resulted in sharp reductions only in kinase protein synthetic rates (see Discussion).

Activity of Wild-Type and Mutant P68 Kinases Immunopurified from Transfected COS-1 Cells. We next determined whether the expression of the wild-type and mutant P68 constructs correlated with protein kinase activity (Fig. 5). The following several points can be made concerning these results. (i) The PK-WT was readily activated by heparin and by poly(I)-poly(C) at 1.0 and 10.0 μ g/ml whereas the PK-M1 domain II mutation, which we showed (32) was inactive when synthesized *in vitro*, was completely inactive after *in vivo* synthesis; (ii) PK-M2, which was synthesized at levels comparable to that of PK-WT (Fig. 1), gave only 2- to 3-fold less activity than PK-WT; and (iii) PK-M3, which was made at least 70-fold more efficiently than PK-WT, was only 3-fold more active, demonstrating that PK-M3 was 20- to 25-fold less active than the wild type per mole of kinase synthesized. Both PK-M2 and PK-M3 required higher levels of dsRNA for activation than PK-WT, in accord with the fact that both these mutants are severely defective in dsRNA binding (32). These data indicate that the enhanced expression of the catalytic domain II mutant and the regulatory domain mutant PK-M3 is directly correlated with their reduced function as protein kinases.

Expression of PK-WT, PK-M1, and PK-M3 in Yeast. We next expressed P68-WT, PK-M1, and PK-M3 in a yeast strain lacking the endogenous eIF-2 α kinase GCN2 (42) and measured growth rate, eIF-2 α phosphorylation, and kinase expression *in vivo*. We have shown that expression of P68-WT in yeast H1894 inhibited growth and led to high levels of eIF-2 α phosphorylation whereas expression of PK-M1 had no effect on yeast cells (53). If the overexpression of PK-M3 in COS-1 cells was due to its defective function, we reasoned that PK-M3 would be less toxic in yeast, with a phenotype more similar to PK-M1 than PK-WT. We examined the growth of yeast containing the different P68 alleles on three media that dictated various expression levels of the kinase proteins: SD + Leu, Ilv, where expression would be the lowest; SD, where expression would be higher due to increased gene dosage; and SGAL, where the promoters would be the most active and kinase levels would be the highest (Fig. 6A). As predicted, none of the kinase constructs were growth-suppressive on SD + Leu, Ilv, whereas on SD, P68-WT was growth-suppressive but PK-M1 and PK-M3 were not, presumably due to their reduced function. In contrast, on SGAL medium, the PK-M3 construct was toxic

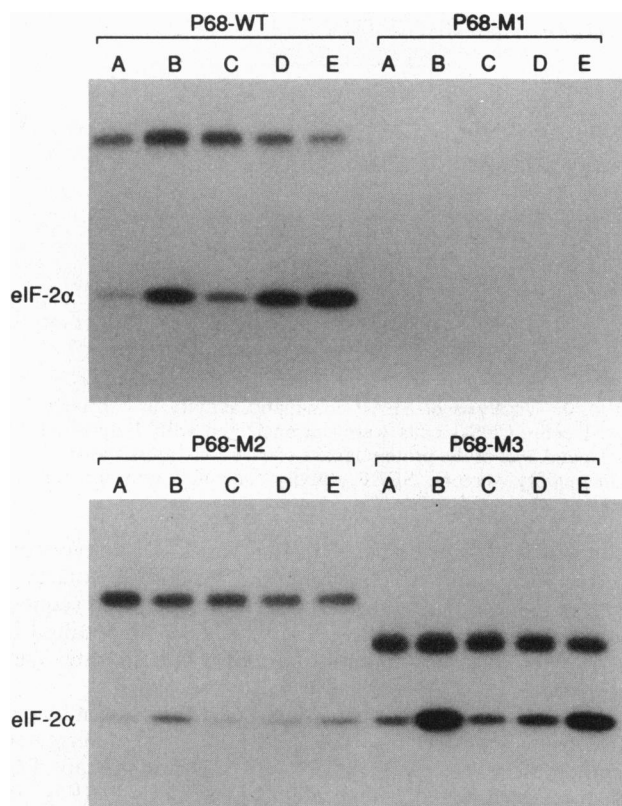


FIG. 5. Protein kinase activity of PK-WT, PK-M1, PK-M2, and PK-M3 immunopurified from transfected COS-1 cells. After transfection of COS-1 cells with P68-WT, PK-M1, PK-M2, or PK-M3, the protein kinases were immunopurified with P68 monoclonal antibody, incubated with 0.5 μg of purified eIF-2 in the absence of activator (lane A) or with heparin at 10 units/ml (lane B), poly(I)poly(C) at 0.10 $\mu\text{g}/\text{ml}$ (lane C), poly(I)poly(C) at 1.0 $\mu\text{g}/\text{ml}$ (lane D), or poly(I)poly(C) at 10.0 $\mu\text{g}/\text{ml}$ (lane E). The position of eIF-2 α is indicated; the slower-migrating phosphorylated band represents the autophosphorylated kinase molecules. It is relevant to note that in these *in vitro* kinase assays, the levels of kinase autophosphorylation, which is dependent on the P68 phosphorylation state when the cells are harvested, do not consistently correlate with eIF-2 α phosphorylation and as such are not a reliable measure of kinase activity (11, 36).

but P68-M1 was not. These results agree with our findings that PK-M3 is not catalytically dead, but only 20- to 25-fold

less active than the wild-type protein kinase. In accordance with these results, IEF gels revealed that the level of eIF-2 α phosphorylation in yeast cells grown in SD medium was highest for P68-WT, lower for PK-M3, and undetectable for PK-M1 (Fig. 6B). Western blot analysis revealed that protein expression levels of the PK-M1 and PK-M3 were similar but higher than the P68-WT levels (data not shown).

DISCUSSION

We have presented evidence that the P68 protein kinase can regulate gene expression *in vivo* at the level of mRNA translation. Perhaps more significantly, we showed that this regulation can be dramatically altered by mutations in either the regulatory or catalytic domains. Since these experiments were done in the absence of virus infection and interferon treatment, these data demonstrate that the P68 protein kinase can act directly and alone to regulate mRNA translation. We conclude that the P68 protein kinase exerts its greatest effects on the translation of its own mRNA and much lower effects in trans on other cellular mRNAs. Thus we can only speculate that such autogenous regulation by the kinase may lead to a more global type of protein synthesis regulation in the cell. There are now several reports that suggest a critical physiological role for the protein kinase possibly as a result of its translational regulatory properties (19–22, 27–31, 50). A major question that arises from the present report is why such dramatic differences exist between the expression and activity of PK-M2 and PK-M3 in the transfected COS-1 cells? We (27, 32, 46) and others (29, 30, 44, 45, 47) have shown that the N terminus of P68 contains regulatory domains that bind RNA activators and inhibitors of the kinase. Exactly what regions of the N-terminal half of the molecule are critical for binding has not yet been determined although domains between aa 11–77 and aa 101–167 may be important considering the sequence conservation that exists between these regions and other RNA binding proteins (45, 47). In fact, a family of RNA binding proteins of which the murine and human dsRNA-activated protein kinases are members is now emerging (45, 47). The P68 RNA binding motifs have thus far been identified only by their ability to promote dsRNA binding in a variety of *in vitro* studies. What may be more revealing are studies that correlate structural features with kinase function *in vivo* as we have attempted in this report and as has been reported by Thomis and Samuel (51) who also demonstrated that a catalytically inactive kinase was overexpressed in transfected cells. Although both PK-M2 and PK-M3 were

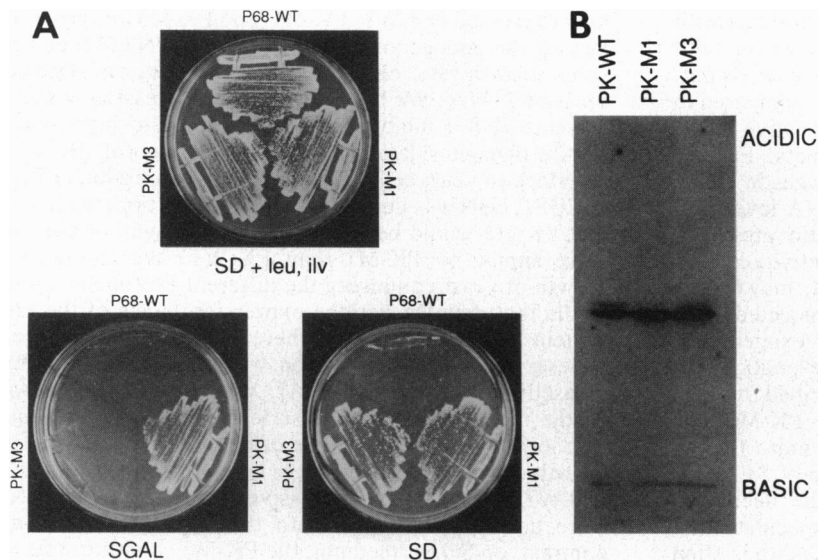


FIG. 6. Growth rate and IEF analysis of eIF-2 α phosphorylation levels in yeast strains expressing PK-WT, PK-M1, and PK-M3. (A) Transformants of yeast strain H1894 containing the indicated P68 alleles were streaked out on SD + Leu, Ilv, SD, or SGAL medium and incubated at 30°C for 3–4 days. (B) Whole cell extracts prepared from yeast strains expressing the P68 alleles were prepared and eIF-2 α phosphorylation levels were measured. The basic and acidic ends of the gel are indicated; the more acidic hyperphosphorylated form of eIF-2 α focuses higher in the gel than the more basic form, phosphorylated at basal levels.

deficient in binding dsRNA (32), the latter is clearly more defective for kinase function, resulting in dramatically higher expression levels. These results suggest that aa 91–155, which encompasses one of the two conserved regions, contain or span a domain important for kinase function *in vivo*.

Based on the data presented in this report, we propose a hypothetical model that explains P68 protein kinase regulation of mRNA translation in eukaryotic cells. We suggest that, for the P68 kinase to be fully functional (i.e., to become autophosphorylated and fully active to phosphorylate eIF-2 α), it must dimerize with another P68 molecule using dsRNA as a bridge. We would therefore predict that P68-WT would inhibit its own expression (at the level of protein synthesis; Fig. 1) because it could readily form active dimers with itself or even potentially with the endogenous monkey kinase. PK-M2, though defective in dsRNA binding, would still be able to dimerize and become nearly fully functional, reducing its own expression (Fig. 5). In contrast, PK-M1 would be overexpressed because, although able to dimerize, it is catalytically inactive and unable to phosphorylate eIF-2 α . We propose that the minimally functional PK-M3 is overexpressed since this kinase molecule cannot dimerize as it lacks the critical domains necessary for efficient interaction with dsRNA. In support of this hypothesis, there is now biochemical evidence for P68 protein kinase dimers in mouse L cells (52). This model also could explain why only the PK-M1 mutant functioned transdominantly to upregulate SEAP mRNA translation (Fig. 4): PK-M1, but not PK-M3, can form inactive dimers via dsRNA with the endogenous monkey kinase and thus prevent its activity on eIF-2 α .

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