The family of eukaryotic initiation factor 2α (eIF2α) protein kinases plays an important role in regulating cellular protein synthesis under stress conditions. The mammalian kinases PKR and HRI and the yeast kinase GCN2 specifically phosphorylate Ser-51 on the α subunit of the translation initiation factor eIF2. By using an in vivo assay in yeast, the substrate specificity of these three eIF2α kinases was examined by substituting Ser-51 in eIF2α with Thr or Tyr. In yeast, phosphorylation of eIF2α inhibits general translation but derepresses translation of the GCN4 mRNA. All three kinases phosphorylated Thr in place of Ser-51 and were able to regulate general and GCN4-specific translation. In addition, both PKR and HRI were found to phosphorylate eIF2α-S51Y and stimulate GCN4 expression. Isoelectric focusing analysis of eIF2α followed by detection using anti-eIF2α and anti-phosphotyrosine-specific antibodies demonstrated that PKR and HRI phosphorylated eIF2α-S51Y on Tyr in vivo. These results provide new insights into the substrate recognition properties of the eIF2α kinases, and they are intriguing considering the potential for alternate substrates for PKR in cellular signaling and growth control pathways.

The human interferon-induced double-stranded RNA-activated protein kinase PKR, which functions in the cellular antiviral defense mechanism, is a member of a family of structurally related Ser/Thr kinases that specifically phosphorylate Ser-51 on the α subunit of the translation initiation factor eIF2α (1, 2). The binding of double-stranded RNA, thought to be generated during viral infections, is proposed to alter the conformation of PKR and activate the kinase to autophosphorylate (1, 2). The active, phosphorylated form of PKR can then phosphorylate eIF2α on Ser-51 and convert eIF2 into an inhibitor of its guanine nucleotide exchange factor eIF2B, resulting in the inhibition of translation initiation (1, 2). The other members of the eIF2α kinase family are the mammalian heme-regulated inhibitor of translation (HRI) that is activated by heme deprivation, the apparently ubiquitous kinase GCN2, first identified in yeast but also found in flies and mammals, which is activated under conditions of amino acid or purine nucleotide deprivation (1–4), and the newly identified mammalian kinase PERK or PEK, a transmembrane kinase located in the endoplasmic reticulum that is activated under conditions of endoplasmic reticulum stress (5, 6). In the yeast Saccharomyces cerevisiae, low level phosphorylation of eIF2α by GCN2 alters the pattern of translation reinitiation on the GCN4 mRNA and induces GCN4 expression (2). Increased synthesis of GCN4, a transcriptional activator of amino acid biosynthetic genes, enables cells to withstand amino acid starvation conditions. The mammalian eIF2α kinases PKR and HRI can substitute for GCN2 in yeast to phosphorylate eIF2α and stimulate GCN4 translation (7). In addition, high level phosphorylation of eIF2α in yeast by mutator hyperactivated alleles of GCN2 or by overexpression of PKR or HRI severely inhibits general translation initiation and impairs cell growth (7–10).

In addition to regulating translation by phosphorylating eIF2α, PKR has been proposed to play roles in cell signaling (11) and growth control (12). In addition, several reports have proposed additional substrates for PKR (13–16). In biochemical analyses, PKR has been shown to phosphorylate intact eIF2α or a 12-residue peptide containing the Ser-51 phosphorylation site (17). As part of our studies aimed to define the in vivo substrate recognition properties of PKR, we chose to examine the ability of PKR, HRI, and GCN2 to phosphorylate Thr or Tyr in place of Ser at residue 51 in eIF2α. In general, protein kinases phosphorylate either Ser/Thr or Tyr residues, and these two classes of protein kinases are mutually exclusive. In fact, when Ser or Thr was substituted for a Tyr autophosphorylation event, the apparent flexible active site that can accommodate Tyr as well as Ser/Thr phosphorylation.”

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The SU12-S51T and SU12-S51Y alleles encoding, respectively, the yeast eIF2α-S51T and eIF2α-S51Y proteins were constructed using the polymerase chain reaction. The 5’-TCC-3’ (Ser-51) codon of SU12 was changed to either 5’-ACC-3’ (Thr-51) or 5’-TAC-3’ (Tyr-51). The SU12 alleles were transformed to a low copy number LEU2/CEN4 vector to create pC107 (Ser-51), pC107 (S51T), and pC110 (S51Y) or to the high copy number LEU2 vector pRS425 (21) to create pC136 (S51Y). The wild-type SU12 (Ser-51) and SU12-S51A (22) alleles were subcloned into pRS425 to create the high copy number LEU2 SU12 (pC133) and SU12-S51A (pC135) plasmids. The low copy number URA3 plasmids.
carrying wild-type GCN2 (p722), GCN4-516 (GCN4-516-E523K-E522K, p1056), or GCN4-513 (GCN4-513-M719V-E1537G, p1052) have been described (9). The high copy number pEMBl14 ex2 plasmids that express human PKR (p1420) or PKR-K296R (p1421) and rabbit HRI (p1246) or HRI-K199R (p1247) under the control of a yeast GAL-CYC1 chimeric promoter were described previously (7).

**Strains**—Standard methods were used for culturing and transformation of yeast strains (23). The strains H1925 (MATa ura3-52 leu2-3 leu2-112 trp1-100 Δ312 geneΔ2, p919[SU2 URA3]), p1108[GCN4-lacZ TRP1] integrat dt1-56Δ3); H2057 (MATa ura3-52 leu2-3 leu2-112 trp1-100 Δ312 geneΔ2, p919[SU2 URA3]); and J101 (MATa ura3-52 leu2-3 trp1-100 Δ312 geneΔ2, p919[SU2 URA3]) were transformed with low or high copy number LEU2 plasmids containing the SU2 mutant alleles, and then the transformants were transferred to medium containing 5-fluorocytosine acid to evoke the URA3 plasmid carrying wild-type SU2. The various eIF2α kinase plasmids were introduced into the resultant strains by transformation selecting for Ura prototrophy.

**Assays of GCN4-lacZ Expression and Isoelectric Focusing (IEF) Gel Electrophoresis**—Identical cell growth conditions were used for GCN4-lacZ expression and IEF gel electrophoresis assays. For strains expressing HRI or HRI-K199R, pre-cultures were incubated 2 days in SD medium (2% raffinose in place of glucose in SD medium) and then inoculated 1:50 into fresh SD medium and harvested after overnight growth. For strains expressing PKR or PKR-K296R, pre-cultures were grown 2 days in SD medium, and then cells (∼0.1 A600 units) were inoculated into SGM medium (10% galactose plus 2% raffinose in place of glucose in SGM medium) and harvested after overnight growth. The methods for growing strains expressing GCN2 or GCN2* kinetics, cell harvesting and breaking, β-galactosidase assays, and IEF gel electrophoresis have been described previously (22).

**Immunodetection and Immunoprecipitation Methods**—To detect phosphoserylase on immunoblots, the membrane was probed with affinity purified rabbit anti-phosphoserylase monoclonal antibodies (2.3 μg/ml) in 1× TBS-T (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% Tween 20) containing 3% bovine serum albumin. The anti-phosphoserylase antibodies were a kind gift of Dr. R. Friesel, Holland Laboratory, American Red Cross, Rockville, MD. In addition, anti-phosphotyrosine antibodies were a kind gift of Dr. R. Friesel, Holland Laboratory, American Red Cross, Rockville, MD. Anti-phosphotyrosine antibodies were transformed with the plasmid p1420 carrying the authentic GCN2 promoter, whereas the mammalian kinases were expressed under the control of a yeast galactose-inducible promoter. Patches of transformants were grown to confluence on SD medium and replica-plated to SGal medium and SGal plus 3-AT (10 mM) medium. Plates were incubated at 30 °C for 3 days.

**PKR** was toxic in strains expressing either the Ser-51 or Thr-51 forms of eIF2α. This inhibition of eIF2B by phosphorylation of the Thr-51 form of eIF2α was suppressed by either the phosphatase CK2 or PKR kinases. Translational regulation by the eIF2α kinases is dependent on both phosphorylation of eIF2α and the ability of phosphorylated eIF2α to inhibit its guanine nucleotide exchange factor eIF2B. The fact that expression of PKR and the other kinases showed no toxicity in the eIF2α-S51Y strain suggests two possibilities as follows: 1) these kinases fail to phosphorylate eIF2α-S51Y, or 2) the phosphorylated form of eIF2α-S51Y is a weaker inhibitor of eIF2B than phosphorylated wild-type eIF2α. This inhibition of eIF2B by phosphorylated eIF2α is dependent on the α subunit of eIF2B, encoded by GCN3 in yeast (2, 7).

**RESULTS**

**Regulation of Translation in Yeast Cells Expressing eIF2α-S51T or eIF2α-S51Y in Place of Wild-type eIF2α**—To initiate an analysis of substrate recognition by the eIF2α kinases, we examined translational regulation in yeast strains in which Thr or Tyr was substituted for Ser-51 in eIF2α. Plasmids that express wild-type or inactive forms of PKR or HRI or containing wild-type GCN2 or hyperactivated GCN2* alleles were introduced into gen2a yeast strains expressing either eIF2α-S51T (Thr-51), eIF2α-S51Y (Tyr-51), eIF2α-S51A (Ala-51), or wild-type eIF2α (Ser-51). Expression of the GCN2*-513 kinase or PKR was toxic in strains expressing either the Ser-51 or Thr-51 forms of eIF2α; however, the toxicity of the GCN2*-513 kinase was slightly reduced in the Thr-51 strain (Fig. 1, A and B, GCN3 sectors). In contrast, the eIF2α-S51A and eIF2α-S51Y mutations completely suppressed the toxic effects of the GCN2*-513 and PKR kinases. Translational regulation by the eIF2α kinases is dependent on both phosphorylation of eIF2α and the ability of phosphorylated eIF2α to inhibit its guanine nucleotide exchange factor eIF2B. The fact that expression of PKR and the other kinases showed no toxicity in the eIF2α-S51Y strain suggests two possibilities as follows: 1) these kinases fail to phosphorylate eIF2α-S51Y, or 2) the phosphorylated form of eIF2α-S51Y is a weaker inhibitor of eIF2B than phosphorylated wild-type eIF2α. This inhibition of eIF2B by phosphorylated eIF2α is dependent on the α subunit of eIF2B, encoded by GCN3 in yeast (2, 7). Deletion of GCN3 suppressed the toxicity resulting from expression of GCN2*-513 or PKR in yeast strains expressing either the Ser-51 or Thr-51 forms of eIF2α (Fig. 1, A and B, GCN3 sectors). These results suggest that the GCN2* and PKR kinases efficiently phosphorylate eIF2α on Ser or Thr at residue 51 and that the phosphorylated forms of wild-type eIF2α and eIF2α-S51T inhibit translation via the same mechanism.

A more sensitive assay of translational regulation in yeast is to monitor GCN4 expression. In wild-type strains phosphoryl-
PKR-K296R, in eIF2 addition, expression of PKR, but not the catalytically inactive H1925 containing the various eIF2 proteins. The indicated kinases were expressed in derivatives of the gen2Δ strain H1925 containing the various eIF2α proteins.  β-Galactosidase activities expressed from an integrated wild-type GCN4-lacZ fusion were measured in whole cell extracts and are the averages of 2–3 independent transformants; S.E.s. were 32% or less. For strains expressing GCN2 or GCN2*-S51, cells were grown under either non-starvation conditions where GCN4 expression is repressed (R) or under amino acid starvation conditions imposed by the addition of 10 mM 3-AT, where GCN4 expression is derepressed (DR). The PKR and PKR-K296R proteins were expressed from a yeast GAL-CYC1 hybrid promoter. For assays, cells were grown exponentially in SD medium, where kinase expression is low, and then shifted to SGR-inducing medium. Cells were harvested after overnight growth in inducing medium. The GCN4-lacZ expression values obtained for GCN2 and PKR cannot be directly compared because the different media used for these cultures resulted in altered basal levels of GCN4-lacZ expression, as is apparent in the eIF2α-S51A strain.

FIG. 2. Regulation of GCN4 expression by GCN2 and PKR in yeast strains expressing various eIF2α mutant proteins. The indicated kinases were expressed in derivatives of the gen2Δ strain H1925 containing the various eIF2α proteins. The β-Galactosidase activities expressed from an integrated wild-type GCN4-lacZ fusion were measured in whole cell extracts and are the averages of 2–3 independent transformants; S.E.s. were 32% or less. For strains expressing GCN2 or GCN2*-S51, cells were grown under either non-starvation conditions where GCN4 expression is repressed (R) or under amino acid starvation conditions imposed by the addition of 10 mM 3-AT, where GCN4 expression is derepressed (DR). The PKR and PKR-K296R proteins were expressed from a yeast GAL-CYC1 hybrid promoter. For assays, cells were grown exponentially in SD medium, where kinase expression is low, and then shifted to SGR-inducing medium. Cells were harvested after overnight growth in inducing medium. The GCN4-lacZ expression values obtained for GCN2 and PKR cannot be directly compared because the different media used for these cultures resulted in altered basal levels of GCN4-lacZ expression, as is apparent in the eIF2α-S51A strain.

| GCN2  | GCN2*-S513 |       |       |       |       |
|-------|------------|-------|-------|-------|-------|
|       | R | DR | R | DR | R | DR | R | DR | R | DR |
| eIF2α | 24 | 95 | 100 | 130 | 38 | 8  | 38 | 8  | 38 | 8  |
| eIF2α-S51 | 21 | 83 | 140 | 130 | 38 | 8  | 38 | 8  | 38 | 8  |
| eIF2α-S51Y | 18 | 37 | 19  | 34  | 18 | 10 | 18 | 10 | 18 | 10 |
| eIF2α-S51A | 15 | 34 | 15  | 25  | 9  | 8  | 9  | 8  | 9  | 8  |

FIG. 3. IEF gel electrophoresis of eIF2α from yeast strains expressing various eIF2α proteins and the GCN2, GCN2*, or PKR kinase. Plasmids that express the indicated eIF2α kinases were introduced into gen2Δ yeast strains expressing the indicated eIF2α proteins from low copy number plasmids. A and B, strains expressing GCN2 and GCN2* kinases. Yeast strains expressing the indicated eIF2α proteins were transformed with plasmids that express wild-type GCN2 (p722), or the constitutively activated GCN2*-S516 (p1056), or GCN2*-S513 (p1052) kinases under the control of the natural GCN2 promoter. Cells were grown under nonstarvation conditions (R) or amino acid starvation conditions induced by the addition of 10 mM 3-AT (DR), as indicated. C, strains expressing PKR. Plasmids expressing wild-type PKR (p1420) or the inactive mutant PKR-K296R (p1421) under the control of a GAL-CYC1 hybrid promoter were introduced into gen2Δ yeast strains expressing the indicated eIF2α proteins. Extracts were prepared from cells grown exponentially in SD medium and then shifted to SGR medium to induce PKR expression. Immunoblot analysis using PKR monoclonal antibodies on 50-μg aliquots of the same extracts used for IEF-PAGE are aligned below the IEF data.

To confirm that PKR and HRI were phosphorylating eIF2α on Ser or Thr but not Tyr or Ala at residue 51 (Fig. 3, A and B), we tested the results of the genetic tests. In addition, phosphorylation of eIF2α was readily detected in strains expressing wild-type eIF2α, eIF2α-S51T, or eIF2α-S51Y and either PKR or HRI but not PKR-K296R or HRI-K199R (Fig. 3, C, upper panel, and data not shown).

To confirm that PKR and HRI were phosphorylating eIF2α-S51Y on Tyr, a second IEF-PAGE analysis was performed. In yeast strains lacking the endogenous eIF2α kinase GCN2 and expressing either wild-type eIF2α or eIF2α-S51Y, the expression of wild-type PKR or HRI resulted in eIF2α focusing as a doublet on IEF gels (Fig. 4, upper panel, lanes 2, 4, 5, and 7). When the same blot from Fig. 2 (upper panel) was probed with anti-phosphotyrosine antibodies (Fig. 4, lower panel), cross-reactive bands were only detected in samples from strains expressing eIF2α-S51Y and a wild-type kinase. When the two blots (Fig. 4, upper and lower panels) were overlaid, the anti-phosphotyrosine cross-reactive species aligned perfectly with the upper, hyperphosphorylated form of eIF2α. These results confirmed that PKR and HRI were phosphorylating eIF2α-S51Y on Tyr and demonstrated that in vivo these proteins possess Tyr kinase activity. Comparison of the ratio of the hyperphosphorylated to the basal form of wild-type eIF2α and eIF2α-S51Y in strains expressing PKR (see Fig. 4, lanes 4 and 5; also Fig. 3C, upper panel, 1st and 5th lanes) may suggest that eIF2α-S51Y is a poorer substrate for PKR than is wild-type eIF2α; however, alternative interpretations of these results are possible.
provided below. The inability to detect Tyr phosphorylation by GCN2 may suggest that GCN2 is an inherently less active kinase than HRI or PKR or that the GCN2 active site cannot accommodate a Tyr residue. Alternatively, it is likely that HRI and PKR were expressed at higher levels than GCN2 in these experiments, so it may be possible to detect Tyr kinase activity if we express GCN2 at higher levels in yeast cells.

The expression of PKR is subject to negative translational autoregulation in both yeast (7, 10) and mammalian (1) cells such that kinase expression is inversely related to its effects on cellular translational activity. For example, although PKR was expressed at lower levels than PKR-K296R in strains expressing wild-type eIF2\(a\) (Fig. 3C, lower panels), this autoregulation was relieved in eIF2\(a\)-S51T strains and abolished in eIF2\(a\)-S51A and eIF2\(a\)-S51Y strains (Fig. 3C, lower panel, last 6 lanes). In addition, whereas the GCN2-S51 kinase appeared to phosphorylate wild-type eIF2\(a\) and eIF2\(a\)-S51T to the same extent (Fig. 3B), the eIF2\(a\)-S51T strain grew significantly better (Fig. 1). This lack of correlation between eIF2\(a\) phosphorylation and growth rate was also observed in eIF2\(a\)-S51Y strains expressing PKR as noted previously. These results are consistent with a model in which the phosphorylated forms of eIF2\(a\)-S51T and eIF2\(a\)-S51Y are weaker inhibitors of eIF2B than phosphorylated wild-type eIF2\(a\). Finally, whereas the eIF2\(a\), eIF2\(a\)-S51T, and eIF2\(a\)-S51Y proteins were phosphorylated to similar levels, it cannot be concluded that PKR and HRI are equally efficient at phosphorylating these three different amino acids at residue 51, because in vivo phosphorylation levels are dependent on the balance between kinase and phosphatase activities. As we do not know the identity or the efficiency of the phosphatases that dephosphorylate these three eIF2\(a\) proteins, we cannot at this time evaluate the relative efficiencies of PKR and HRI to phosphorylate eIF2\(a\) in vivo on Ser versus Thr or Tyr at residue 51.

**Immunodetection of Human PKR Using Anti-Phosphotyrosine Antibodies**—Previously it has been reported that mouse PKR, also known as TIK, can be detected in immunoblot assays using anti-phosphotyrosine antibodies (25). To determine if human PKR is also immunoreactive with anti-phosphotyrosine antibodies, wild-type human PKR and the inactive PKR-K296R proteins were expressed in *E. coli*, and crude protein extracts were separated by SDS-PAGE followed by immunoblotting with anti-PKR or anti-phosphotyrosine antibodies. As shown in Fig. 5A, both wild-type PKR and the PKR-K296R mutant proteins were expressed in *E. coli*. When the same membrane was probed with affinity purified anti-phosphotyrosine antibodies, cross-reactive species were detected in extracts prepared from cells expressing wild-type PKR but not PKR-K296R (Fig. 5A, left panel). Similar results demonstrating that recombinant wild-type human and mouse PKR (TIK), but not catalytic mutants, cross-react with anti-phosphotyrosine antibodies were recently published during the course of these experiments (26). In addition to the prominent anti-phospho-
tyrosine antibody cross-reactive species co-migrating with PKR at ~70 kDa, several larger proteins were detected (Fig. 5A, left panel). This result suggests that PKR expressed in E. coli may autophosphorylate on Tyr and can also phosphorylate certain bacterial proteins on Tyr.

To confirm that PKR was the major species cross-reacting with anti-phosphotyrosine antibodies, we performed immunoprecipitation reactions. As shown in Fig. 5B (lanes 3 and 6), recombinant PKR could be immunoprecipitated from both yeast and bacterial cell extracts using affinity purified anti-phosphotyrosine antibodies. PKR was not precipitated when the anti-phosphotyrosine antibodies were omitted from the reactions (Fig. 5B, compare lanes 3 versus 2 and 6 versus 5). In addition, the precipitation was specific for functional PKR because the catalytically inactive PKR-K296R protein expressed in E. coli could not be precipitated using anti-phosphotyrosine antibodies (Fig. 5C, lanes 3 and 4). As shown in Fig. 5B (lanes 7–9), the endogenous PKR expressed in interferon-treated HeLa cells could also be immunoprecipitated using the anti-phosphotyrosine antibodies. Whereas these results suggest that PKR can autophosphorylate on Tyr, phosphoamino acid analyses of PKR isolated from in vivo labeled yeast and bacterial cells revealed phosphoserine and phosphothreonine, but not phosphotyrosine (data not shown). This latter result is consistent with the results of Icely et al. (25), who could only find phosphoserine and phosphothreonine in mouse PKR despite the fact that mouse PKR also cross-reacted with anti-phosphotyrosine antibodies. Icely et al. (25) speculated that the anti-phosphotyrosine antibodies may have recognized an unusual epitope on mouse PKR (25); however, negative results in phosphoamino acid analysis may reflect a low phosphorylation stoichiometry or a labile phosphotyrosine residue (27). In addition, it has been proposed that anti-phosphotyrosine antibodies may be over 100-fold more efficient at detecting phosphotyrosine than is phosphoamino acid analysis (28). Due to these conflicting results and the plausible explanations for failure to detect phosphotyrosine in the phosphoamino acid analyses, we are unable to conclude whether human PKR autophosphorylates on Tyr.

**DISCUSSION**

We have shown that the kinases PKR, HRI, and GCN2 can phosphorylate eIF2α on Ser or Thr at residue 51. In addition both PKR and HRI can phosphorylate eIF2α on Tyr at residue 51 in vivo. It is generally accepted that Ser and Thr kinases are structurally similar, and many kinases are known to phosphorylate both residues, so the finding that the eIF2α kinases could phosphorylate Thr in place of Ser-51 is not surprising. However, the phosphorylation of Tyr at residue 51 in eIF2α by PKR and HRI is unexpected.

Two proposals can account for the Tyr phosphorylation activity by PKR and HRI. In the first proposal PKR and HRI would recognize eIF2α with high affinity and simply phosphorylate any hydroxyl group present at residue 51. Based on a mutational analysis of the vaccinia virus K3L protein, a pseudosubstrate inhibitor of PKR with homology to eIF2α, we have proposed that PKR utilizes a sequence element over 30 residues from the site of phosphorylation to recognize eIF2α (29). According to this model, the ability of PKR to phosphorylate Tyr in place of Ser-51 in eIF2α simply reflects the strong contribution of this remote sequence for substrate recognition by PKR and the lack of specificity determinants around residue 51. Consistent with this idea, it has been reported that the PKR and HRI phosphorylation of intact eIF2α is roughly 3 orders of magnitude more efficient than phosphorylation of a 12-residue synthetic peptide containing the Ser-51 phosphorylation site (30). However, regardless of how PKR initially recognizes eIF2α, it is important to note that the kinase active site must be able to accommodate both the alkyl hydroxyl groups of Ser and Thr and the phenolic hydroxyl of Tyr. The crystal structures solved to date for both Ser/Thr and Tyr kinases suggest that these enzymes would be unable to phosphorylate substrates on the alternate phospho-accepting residue due principally to steric limitations (20). Indeed, it has previously been shown that the p130ag-fps Tyr kinase could not phosphorylate Ser or Thr in place of an authentic Tyr phosphorylation site (18). Therefore, it is reasonable to expect that PKR and HRI possess a unique and more flexible structure as compared with the traditional Ser/Thr or Tyr kinases.

The second proposal to account for the Tyr kinase activity of PKR and HRI is that these proteins are members of the class of dual specificity protein kinases. A number of kinases have been proposed to have dual specificity; however, the criteria used in making this assignment has not been standardized (27, 31). Several kinases autophosphorylate on both Tyr and Ser/Thr residues (27, 31), and the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase (32) phosphorylates the extracellular signal-regulated kinase kinases on both Tyr and Thr. The wee1 kinase, although structurally related to the Ser/Thr class of protein kinases, phosphorylates the cdc2 kinase on Tyr (27). The Myt1 kinase, identified in both Xenopus and humans and a member of the wee1 family of kinases, phosphorylates Cdc2 on both Thr and Tyr (33); and recently, Myt1 has also been shown to autophosphorylate in vitro on Ser, Thr, and Tyr (34). In contrast to these kinases in which phosphotyrosine and phosphoserine or phosphothreonine was readily detected in substrate phosphorylation or autophosphorylation reactions, other proposed dual specificity kinases, including TIK (25) and PYT/ESK/TTK (28), were identified based primarily on cross-reactivity with anti-phosphotyrosine antibodies. These kinases structurally resemble Ser/Thr kinases; however, when expressed in bacteria they cross-reacted with anti-phosphotyrosine antibodies. In this report we have demonstrated that human PKR (the TIK homolog) will also cross-react with anti-phosphotyrosine antibodies. Whereas these results may suggest that PKR is a dual specificity kinase, it will be necessary to identify an in vivo substrate that PKR phosphorylates on Tyr to conclude convincingly that PKR is a member of the class of dual specificity protein kinases.

The identification of Tyr and Thr kinase activity by PKR is very interesting in regards to alternative substrates and biological roles proposed for PKR. Although eIF2α is the only well characterized PKR substrate, and the regulation of translation is thought to be the primary function of PKR, recent reports suggest additional substrates and roles for PKR. PKR has been reported to phosphorylate LeuB (13), HIV Tat (14, 15), and NF90 (16) in vitro. In addition, overexpression of catalytically inactive mutants of PKR leads to heightened viral sensitivity (35), altered gene regulation (Ref. 36 and references therein) and malignant transformation (12). At least some of the effects of PKR on gene regulation do not appear to be mediated by changes in eIF2α phosphorylation, suggesting that phosphorylation of other proteins may mediate these effects (36). Finally, mice deficient in PKR are impaired in cell signaling pathways including their interferon-γ and double-stranded RNA-induced antiviral response (11), although these effects are not observed in all PKR null mice (37). Our studies on eIF2α phosphorylation by PKR raise the possibility that PKR may have other substrates that it naturally phosphorylates on Thr or Tyr. As Tyr phosphorylation is a common step in cellular signal transduction pathways, it is tempting to speculate that the defects in signal transduction pathways and transcriptional regulation of gene expression associated with reduced PKR function are due
to a loss of the PKR Tyr kinase activity. It will be interesting to identify these alternative PKR substrates and to determine if they are phosphorylated on Tyr, and thereby provide a physiological role for this unexpected Tyr kinase activity of PKR.

Acknowledgments—We are grateful to Bob Friesel for the anti-phosphotyrosine antibodies, Scott Shors for the HeLa cells, Mike Mathews for PKR polyclonal antiserum, and Julie Watson and Ribogene, Inc., for PKR monoclonal antibodies. We thank members of the Dever and Hinnebusch laboratories for helpful discussions, and especially Alan Hinnebusch, Graham Pavitt, Minerva Garcia-Barrio, and Jim Anderson for comments on the manuscript. Finally, we thank an anonymous reviewer for several critical insights and helpful suggestions.

REFERENCES

1. Clemens, M. J. (1996) in Translational Control (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., eds) pp. 139–172, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

2. Hinnebusch, A. G. (1994) Semin. Cell Biol. 5, 417–426

3. Santoyo, J., Alcalde, J., Mendez, R., Pulido, D., and de Haro, C. (1997) J. Biol. Chem. 272, 12544–12550

4. Olsen, D. S., Jordan, B., Chen, D., Wek, R. C., and Cavener, D. R. (1998) Genetics 149, 1495–1509

5. Harding, H. P., Zhang, Y., and Ron, D. (1999) Mol. Cell. Biol. 19, 3959–3974

6. Shi, Y., Vattem, K. M., Sood, R., An, J., Liang, J., Stramm, L., and Wek, R. C. (1999) Mol. Cell. Biol. 18, 7499–7509

7. Dever, T. E., Chen, J. J., Barber, G. N., Cigan, A. M., Feng, L., Donahue, T. F., London, I. M., Katze, M. G., and Hinnebusch, A. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4616–4620

8. Romano, P. R., Green, S. R., Barber, G. N., Mathews, M. B., and Hinnebusch, A. G. (1995) Mol. Cell. Biol. 15, 365–378

9. Yang, Y.-L., Reis, L. F. L., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B. R. G., Aguet, M., and Weissmann, C. (1995) EMBO J. 14, 6095–6106

10. Koromilas, A. E., Roy, S., Barber, G. N., Katze, M. G., and Sonenberg, N. (1992) Science 257, 1685–1689

11. Kumar, A., Haque, J., Lacoste, J., Hiscott, J., and Williams, B. R. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6288–6292

12. McMillan, N. A. J., Chun, R. F., Siderovski, D. P., Galabru, J., Toone, W. M., Samuel, C. E., Mak, T. W., Hovanessian, A. G., Jeang, K., and Williams, B. R. G. (1995) Virology 213, 413–424

13. Brand, S., Kebayashki, R., and Mathews, M. (1997) J. Biol. Chem. 272, 8388–8395

14. Langland, J., Kao, P., and Jacobs, B. (1999) Biochemistry 38, 6361–6368

15. Muller, H., and Proud, C. G. (1991) Biochem. Biophys. Res. Commun. 178, 430–437

16. Weinmaster, G., and Pawson, T. (1986) J. Biol. Chem. 261, 328–333

17. Hubbard, S. R., Wei, L., Ellis, L., and Hendrickson, W. A. (1994) Nature 372, 746–754

18. Taylor, S. S., Radzio-Andzelm, E., and Hunter, T. (1995) FASEB J. 9, 1255–1266

19. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) Gene (Amst.) 110, 119–122

20. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. D., and Hinnebusch, A. G. (1992) Cell 68, 585–596

21. Rose, M. D., Winston, F., and Hieter, P. (1989) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

22. Barber, G. N., Tomita, J., Hovanessian, A. G., Meurs, E., and Katze, M. G. (1991) Biochemistry 30, 10356–10361

23. Lawrence, L. L., Gross, P., Bergeron, J. M., Devault, A., Afar, D. E. H., and Bell, J. C. (1991) J. Biol. Chem. 266, 16073–16077

24. Abraham, N., Jaramillo, M., Duncan, P., Methot, N., Icely, P., Stojdil, D., Barber, G., and Bell, J. (1998) Exp. Cell Res. 244, 394–404

25. Lindberg, R. A., Quinn, A. M., and Hunter, T. (1992) Trends Biochem. Sci. 17, 114–119

26. Lindberg, R. A., Fischer, W. H., and Hunter, T. (1993) Oncogene 8, 351–359

27. Kawagishi-Kobayashi, M., Silverman, J. B., Ung, T. L., and Dever, T. E. (1997) Biochemistry 36, 5831–5838

28. Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992) Science 258, 478–480

29. Mueller, P. R., Coleman, T. R., Kamagai, A., and Dunphy, W. G. (1995) Science 269, 86–90

30. Liu, F., Stanton, J. J., Wu, Z., and Piwnica-Worms, H. (1997) Mol. Cell. Biol. 17, 511–518

31. Der, S. D., and Lai, A. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 92, 8841–8845

32. Koromilas, A. E., Cantin, C., Craig, A. W. B., Japu, R., Hiscott, J., and Sonenberg, N. (1995) J. Biol. Chem. 270, 25426–25434

33. Abraham, N., Stojdil, D. F., Duncan, P. I., Methot, N., Ichii, T., Dube, M., Vanderhyden, B. C., Atkins, H. L., Gray, D. A., McBurney, M. W., Koromilas, A. E., Brown, E. G., Sonenberg, N., and Bell, J. C. (1999) J. Biol. Chem. 274, 5953–5962