Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5447–5451, June 1992
Biochemistry

Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase

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Communicated by George R. Stark, February 12, 1992

ABSTRACT **The double-stranded RNA (dsRNA)-binding domain of the human p68 kinase has been localized to the N-terminal half of the enzyme by using progressive deletion analysis and *in vitro* binding assays. To further define the domains responsible for binding to dsRNA, we cloned the mouse dsRNA-activated p65 kinase and used sequence alignment to identify conserved domains in the N-terminal region. Deletions in either of two 12-amino-acid-long and arginine- or lysine-rich regions abrogated binding to dsRNA. Moreover, in an *in vivo* growth inhibition assay in the yeast *Saccharomyces cerevisiae*, these mutants failed to exhibit a slow-growth phenotype.**

The double-stranded RNA (dsRNA)-activated protein kinase is an interferon (IFN)-induced enzyme, existing as a 68-kDa protein in human cells (p68) and a 65-kDa protein in mouse cells (p65). It was originally discovered based on the inhibitory effect of dsRNA on protein synthesis in a cell-free system (see ref. 1 for a review). Once activated by dsRNA, the protein becomes autophosphorylated and in turn phosphorylates its specific substrate, the α subunit of eukaryotic initiation factor 2, eIF2α (1, 2), leading to a decline in the rate of protein synthesis in cells (3–6). dsRNA, in addition to being an activator of the p68 kinase, is also an effective inducer of IFN genes and IFN-inducible genes; this induction could be blocked by 2-aminopurine, an inhibitor of the p68 kinase (7–11). Furthermore, dsRNA has been shown to activate the nuclear transcriptional factor NFκB via the phosphorylation and deattachment of its inhibitor, IκB (12), and NFκB is involved in regulating IFN-β gene expression. Consequently, it has been suggested that the dsRNA-activated kinase may also phosphorylate other substrates that are involved in signaling or transcriptional activation of IFN and IFN-inducible genes.

We have reported (13) the isolation and characterization of the cDNA for human p68 kinase. This cDNA contains all of the conserved domains represented in protein kinases (14), and the sequence confirmed previous observations (15, 16) that p68 is a member of the serine/threonine kinases. The *in vitro* translated product of the cDNA is able to bind to dsRNA, be recognized by a monoclonal antibody to native p68 kinase, autophosphorylate, and phosphorylate eIF2α *in vitro* (13, 17, 18). When expressed in the yeast *Saccharomyces cerevisiae*, the human p68 kinase exhibits a growth-suppressing phenotype, which is correlated with phosphorylation of yeast eIF2α (18). Therefore, these studies provide a basis for further analysis of the relationship between structure and function of the p68 kinase.

To reveal the mechanism for the regulation of p68 kinase activity by dsRNA, we have performed a deletion analysis of the p68 kinase to reveal domains important for binding to dsRNA. We have cloned and sequenced[§] the murine p65 kinase and used sequence alignment between p68 and p65 to identify conserved regions in the N-terminal domain. Site-directed mutagenesis of these segments indicates that positively charged domains within the conserved regions are responsible for complexing to dsRNA and activating the kinase activity.

MATERIALS AND METHODS

Mouse p65 cDNA Cloning and Sequencing. A cDNA library in phage λgt11 made from the mouse mammary carcinoma FM3A cell line, obtained from J. Hassel (McMaster University, Hamilton, ON), was screened by using the *Xba* I–*Pst* I [1.8 kilobases (kb)] fragment of the human p68 cDNA (13) as a probe. Hybridization was done at 50°C in 5× SSC, 5× Denhardt's solution, 0.1% SDS, 9% dextran sulfate, and 0.1 mg of salmon sperm DNA per ml for 20 hr (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7; 1× Denhardt's solution = 0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll). The membranes were washed twice in 3× SSC/0.1% SDS, once in 1× SSC/0.1% SDS at room temperature, and once in 1× SSC/0.1% SDS at 50°C. The positive clones were isolated, and *Eco*RI inserts were subcloned into the vectors pGEM-3Z (Promega) and M13mp18 (New England Biolabs). Sequencing of both strands of deletion subclones in M13mp18 made with cyclone I biosystem kit (IBI) was done by Sanger's dideoxy chain-termination method with a kit from Pharmacia.

Mutagenesis of p68 Kinase. Different-size fragments of p68 cDNA were isolated and inserted into pGEM-3Z as shown in Fig. 1. For site-directed mutations (Table 1), the p68 cDNA cloned in pBluescript was used as a template. The template DNA was amplified in strain CJ236 (dut$^-$ ung$^-$ F′) with uracil incorporation into the strand at some thymidine positions. The mutant 27-mers (listed in Table 1) were used as primers for synthesis of mutant strands by following the protocol supplied by Stratagene. The deletion mutants were identified and confirmed by sequencing.

***In Vitro* Transcription and Translation.** Linearized plasmid DNA (5 μg) was transcribed *in vitro* with phage T7 RNA polymerase (Pharmacia) as described (Promega), and the complementary RNA yield was measured at OD_{260}. For *in vitro* translation, 1 μg of complementary RNA was mixed with 35 μl of nuclease-treated rabbit reticulocyte lysate (Promega), 1 μl of RNasin ribonuclease inhibitor (at 40 units/μl), 1 μl of 1 mM amino acid mixture (minus methionine), 4 μl of

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Abbreviations: dsRNA, double-stranded RNA; IFN, interferon; eIF2α, eukaryotic initiation factor 2 α subunit; C5 and C13, clones 5 and 13.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M93567).

Table 1. Characteristics of deletion mutants made in the N-terminal dsRNA binding region of the human p68 kinase

dsRNA binding region	Deleted positions*	AA sequence†	Net charges	dsRNA binding‡	Inhibition of yeast cell growth§
Mutant					
1	39–50	RRFTFQVIIDGR	+2	4%	+/−
2	58–69	RSKKEAKNAAAK	+4	3%	+/−
3	110–119	QKKRLTVNYE	+2	58%	++
4	150–159	KQEAKQLAAK	+2	39%	++
5	195–204	TSTLASESSS	0	106%	ND
Wild type	—	—	—	100%	++++

AA, amino acid; ND, not done.

*Oligonucleotides used for mutagenesis are: mutant 1, CCTCCACATGAT—GAATTTCCA-GAAGGT; mutant 2, GAAGGTGAAGGT—TTAGCTGTTGAGATA; mutant 3, AATAGAAT-TGCC—CAGTGTGCATCGGGG; mutant 4, ACAGGTTCTACT—CTTGCATATCTTCAG; mutant 5, AACTCTTTAGTG—GAAGGTGACTTCTCA, where the dash indicates the deleted positions.

†These amino acid sequences deleted are almost 100% homologous to the mouse corresponding regions.

‡The percentage is based on the density scanning of a typical binding experiment (see Fig. 4), with the binding efficiency of wild-type p68 as 100%.

§The inhibition is illustrated in Fig. 5.

[^{35}S]methionine at 10 μCi (370 kBq)/ml, and H_2O to 50 μl. The reaction was at 30°C for 60 min.

Poly(rI)·Poly(rC) Binding Assay. *In vitro* translated products were tested for poly(rI)·poly(rC) binding by using poly(rI)·poly(rC)-cellulose (a gift from R. H. Silverman) as described (19). dsRNA-bound and unbound proteins were analyzed by NaDodSO$_4$/PAGE through 10% gels. The percentage of p68 kinase bound was quantified by taking the binding efficiency [the ratio of dsRNA-bound and total protein applied to poly(rI)·poly(rC)-cellulose, estimated by scanning of autoradiographs] of wild-type p68 kinase as 100% and comparing to the different mutants.

Expression in Yeast. Mutant p68 kinase cDNA inserts were cut from pBluescript and cloned into the yeast expression vector pEMBLyex4 and expressed in *S. cerevisiae* as detailed (18). The expression plasmids were introduced into the haploid yeast strain W303-1a, and transformed strains were grown for 2 days at 30°C on agar containing synthetic medium that lacked uracil and contained 2% glucose or galactose as the sole carbon source.

RESULTS

Localization of the dsRNA-Binding Domain to the N-Terminal Region of the p68 Kinase. To define the functional domain for dsRNA binding by the p68 kinase, we cloned the full-length p68 cDNA, the 5′ and 3′ halves of the cDNA, and a set of fragments progressively deleted from the 3′ end in pGEM-3Z. These constructs were *in vitro* transcribed, and the derived cRNA was translated *in vitro* with the incorporation of [^{35}S]methionine. The ^{35}S-labeled translational products were tested for their capability of binding to

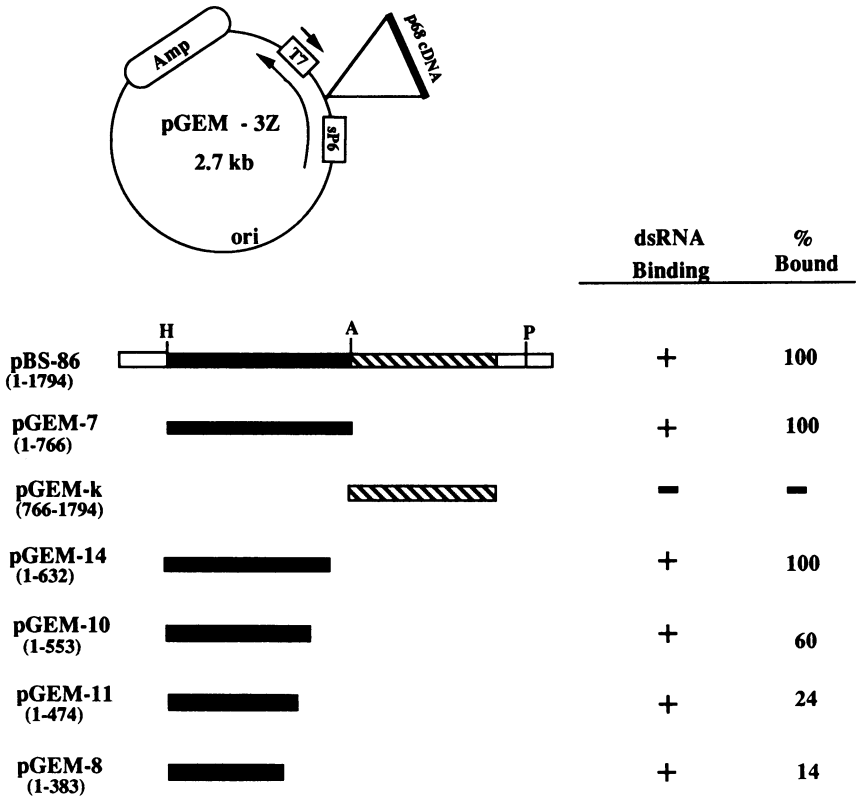

FIG. 1. Localization of dsRNA binding to the N-terminal region of p68 kinase. p68 cDNA fragments covering the full coding region or different lengths starting from the N terminus of the kinase were cloned in pGEM-3Z as shown. Linearized plasmids were *in vitro* transcribed, and the resulting cRNA was *in vitro* translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Poly(rI)·poly(rC) binding of the full-length and truncated kinase proteins was performed by using ^{35}S-labeled products as reported (13) and was analyzed on SDS/10% PAGE.

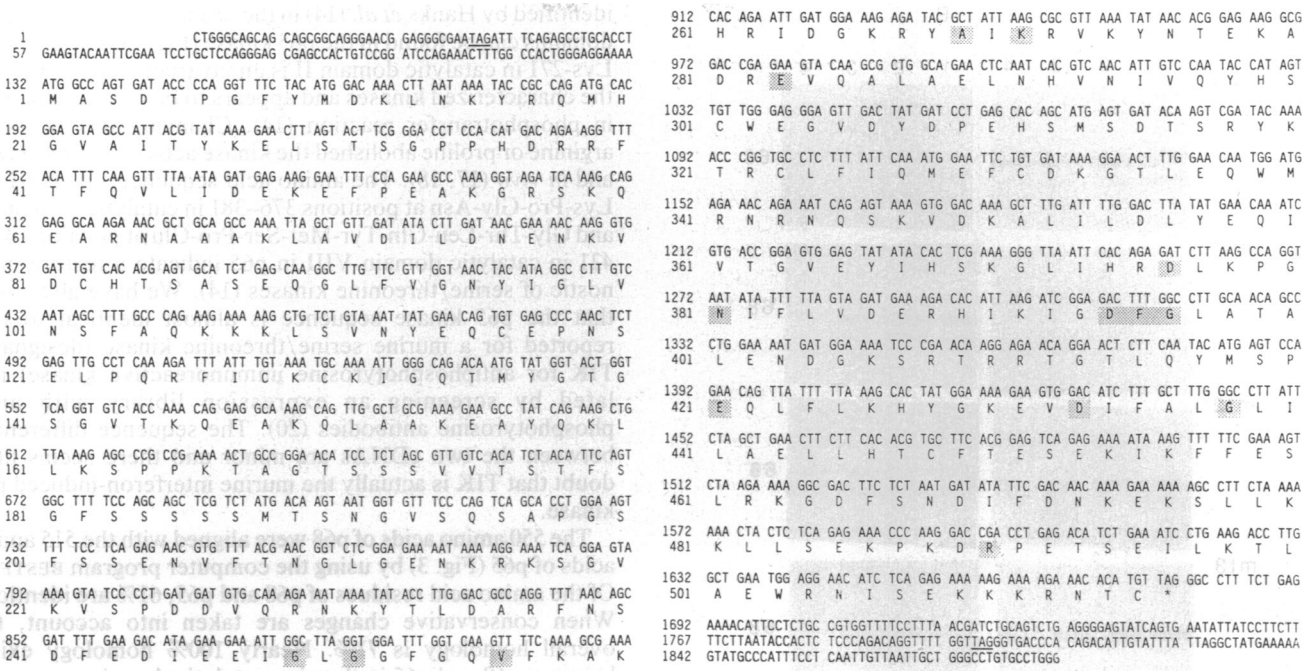

FIG. 2. The nucleotide sequence and deduced amino acid sequence of the mouse p65 kinase cDNA. The 1886-nucleotide sequence of C13 is shown together with its primary translation products of 515 amino acids. The single-letter designation is used for amino acids. The asterisk indicates the TAG stop codon. Two other TAG stop codons in the same reading frame within the 5′ and 3′ untranslated sequences are underlined. Fifteen conserved residues characteristic of kinase catalytic domains are highlighted.

poly(rI)·poly(rC), a synthetic dsRNA. The dsRNA-binding activity was located in the N-terminal portion of the protein (Fig. 1). Through the examination of a nested set of deletion mutants (Fig. 1), we were able to localize the dsRNA-binding function to within 120 amino acids of the N terminus, as pGEM-8 only contains the cDNA sequence of p68 from position 1 to 383 (Fig. 1). To further localize domains important for dsRNA binding, we undertook the cDNA cloning of the mouse p65 kinase. Since the mouse and human enzyme show virtually identical dsRNA-binding characteristics, we reasoned that sequence comparison between the two species might reveal conserved regions contributing to dsRNA binding.

Cloning and Characterization of the Mouse p65 Kinase. To isolate the cDNA for the mouse p65 kinase, we used the human p68 cDNA (13) as a probe and screened a cDNA library of mouse mammary carcinoma cells under low-stringency conditions. Two independent clones were isolated, clone 5 (C5), 1.2 kb, and clone 13 (C13), a 1.9-kb cDNA with an internal *Eco*RI site. Southern blot analysis of mouse genomic DNA probed with the 1.1-kb *Eco*RI fragment of C13 showed a simple pattern of bands, suggesting a single copy of this gene present in mouse genome (data not shown). Northern analysis of RNA from BALB/c 3T3 A31 cells using the C13a probe showed a major IFN-induced transcript of about 2.5 kb (data not shown).

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          1                   20                  40                  60                  80
p68       MAGDLSAGFFMEELNTYRQKQGVVLKYQELPNSGPPHDRRFTFQVIIDGREFPEGEGRSKKEAKNAAAKLAVEILNKEKK
p65       MASD.TPGFYMDKLNKYRQMHGVAITYKELSTSGPPHDRRFTFQVLIDEKEFPEAKGRSKQEARNAAAKLAVDILDNENK

                     100                 120                 140                 160
p68       AVSPLLLTTTNSSEGLSMGNYIGLINRIAQKKRLTVNYEQCASGVHGPEGFHYKCKMGQKEYSIGTGSTKQEAKQLAAKL
p65       ....VDCHTSASEQGLFVGNYIGLVNSFAQKKKLSVNYEQCEPNSELPQRFICKCKIGQTMYGTGSGVTKQEAKQLAAKE

                     180                 200                 220                 240
p68       AYLQILSEETSVKSDYLSSGSFATTCESQSNSLVTSTLASESSSEGDFSADTSEIIYSDSLNSSSLLMNGLRNNQRKAKR
p65       AYQKLL..............KSPPKTAGTSSSVVTSTFSGFSSSSSMTS.....NGVSQSAPGSFSSENVFTNGLGENKR

                     260                 280                 300                 320
p68       SLAPRFDLPDMKETKYTVDKRFGMDFKEIELIGSGGFGQVFKAKHRIDGKTYVIKRVKYNNEKAEREVKALAKLDHVNIV
p65       KSGVKVSPDDVQRNKYTLDARFNSDFEDIEEIGLGGFGQVFKAKHRIDGKRYAIKRVKYNTEKADREVQALAELNHVNIV

                     340                 360                 380                 400
p68       HYNGCWDGFDYDPETSDDSLESSDYDPENSKNSSRSKTKCLFIQMEFCDKGTLEQWIEKRRGEKLDKVLALELFEQITKG
p65       QYHSCWEGVDYDPEHS.............MSDTSRYKTRCLFIQMEFCDKGTLEQWMRNRNQSKVDKALILDLYEQIVTG

                     420                 440                 460                 479
p68       VDYIHSKKLIHRDLKPSNIFLVDTKQVKIGDFGLVTSLKNDGK.RTRSKGTLRYMSPEQISSQDYGKEVDLYALGLILAE
p65       VEYIHSKGLIHRDLKPGNIFLVDERHIKIGDFGLATALENDGKSRTRRTGTLQYMSPEQLFLKHYGKEVDIFALGLILAE

                     500                 520                 540
p68       LLHVCDTAFETSKFFTDLRDGIIS.DIFDKKEKTLLQKLLSKKPEDRPNTSEILRTLTVWKKSPEKNERHTC 550
p65       LLHTCFTESEKIKFFESLRKGDFSNDIFDNKEKSLLKKLLSEKPKDRPETSEILKTLAEWRNISEKKKRNTC 515
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FIG. 3. The identification of homology among human p68 and mouse p65. The 550 amino acids of human p68 kinase were aligned with the 515 amino acids of mouse p65 kinase. Amino acids identical between p68 and p65 are underlined under the p68 sequence. Total identity is 61%.

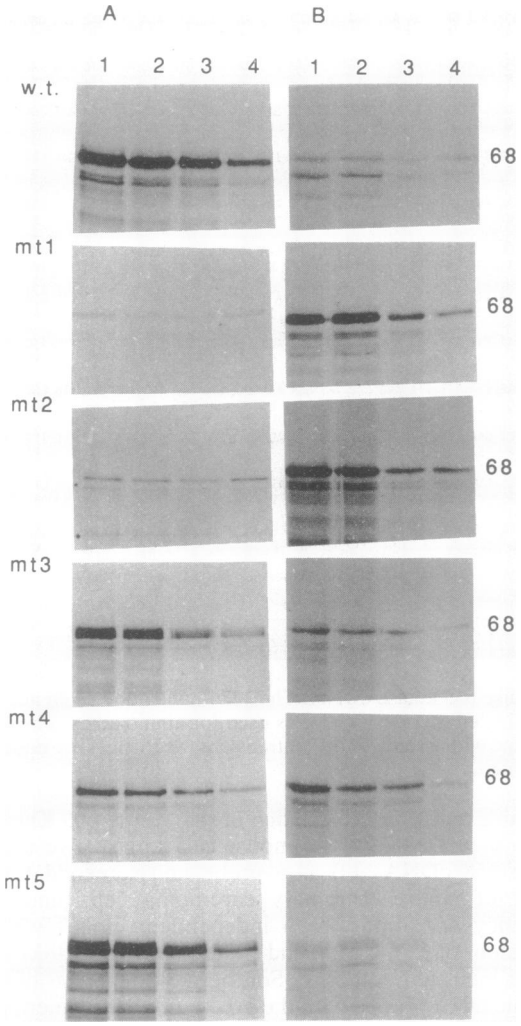

FIG. 4. Poly(rI)·poly(rC) binding capability of site-directed deletion mutants of p68 kinase. An excess amount (10 μl) of poly(rI)·poly(rC)-cellulose beads was mixed with 10, 7, 4, or 1 μl (lanes 1–4, respectively) of *in vitro* translated products of wild-type p68 (w.t.) and the five mutants (mt1–mt5). The dsRNA-bound proteins (*A*) and proteins remaining in the supernatants (*B*) after centrifugation of the poly(rI)·poly(rC)-cellulose beads were analyzed by SDS/PAGE. Sizes are shown in kDa to the right.

The 1886-nucleotide sequence of C13, although not a full-length clone, encodes a 515-amino acid protein (Fig. 2). This compares to the human p68 kinase, which comprises 550 amino acids (13). All of the 15 conserved amino acid residues identified by Hanks *et al.* (14) in the proposed kinase catalytic domains can be found in the p65 kinase (Fig. 2). For example, Lys-271 in catalytic domain II is an invariant residue in all of the characterized kinases and appears to be directly involved in phosphotransfer reaction (14). Changing this lysine to arginine or proline abolished the kinase activity of p68 *in vitro* and *in vivo* (17, 18). The amino acid sequences of Asp-Leu-Lys-Pro-Gly-Asn at positions 376–381 in catalytic domain VI and Gly-Thr-Leu-Gln-Tyr-Met-Ser-Pro-Glu at positions 413–421 in catalytic domain VIII in p65 indicate a feature diagnostic of serine/threonine kinases (14). We have also noted that the p65 kinase sequence is almost identical to that reported for a murine serine/threonine kinase (designated TIK for antiphosphotyrosine immunoreactive kinase) isolated by screening an expression library with antiphosphotyrosine antibodies (20). The sequence differences between the two cDNAs are minor and there seems little doubt that TIK is actually the murine interferon-induced p65 kinase.

The 550 amino acids of p68 were aligned with the 515 amino acids of p65 (Fig. 3) by using the computer program BESTFIT. Of the amino acid residues of p68 and p65, 61% are identical. When conservative changes are taken into account, the overall homology is 71%. Nearly 100% homology exists between p68 and p65 in the nine catalytic domains proposed by Hanks *et al.* (14) for kinases. Of particular interest are the homologous regions in the N-terminal domain between p68 and p65. We have noted (18) that there is sequence similarity in the catalytic domains between the human p68 kinase, mouse p65 kinase, heme-regulated inhibitor (HRI; ref. 21), and yeast kinase GCN2, specifically in a "kinase insert" region located between catalytic domains IV to VI. GCN2 is a ribosome-associated protein kinase whose substrate is yet to be identified. Ten amino acids (Leu-Phe-Ile-Gln-Met-Glu-Phe-Cys-Asp-Lys) in particular that are located in this "kinase insert" region could play a role in recognition of their putative common substrate, eIF2α.

Identification of dsRNA-Binding Regions in p68. Sequence comparison reveals highly conserved regions in the N-terminal domains between p68 and p65 (Fig. 3). Furthermore, these regions are enriched for positively charged amino acids, especially arginine, typical of the RNA-binding motif for RNA-binding proteins (22). To demonstrate a role in binding to dsRNA, we selected four arginine- or lysine-rich regions of p68 and p65 that are almost 100% homologous. In each area, we deleted 10–12 amino acids (Table 1). As a control, we made another mutant in which we deleted a serine/threonine-rich region. All these mutant constructs were efficiently transcribed and translated *in vitro* (Fig. 4). The radiolabeled translational products were then tested for their ability to bind to poly(rI)·poly(rC)-cellulose (Fig. 4). Mutant 5 showed

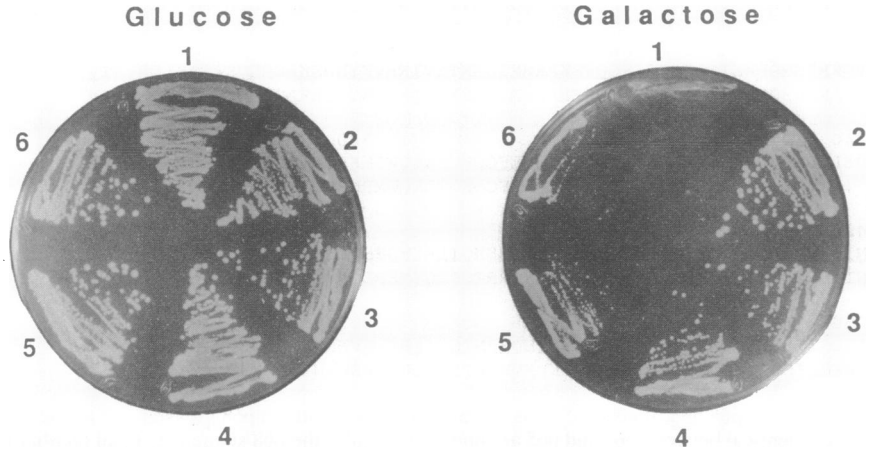

FIG. 5. Suppression of yeast cell growth by wild-type and mutant p68 kinase. Cloning of wild-type and mutant p68 kinase cDNAs into a yeast expression vector and transformation of yeast cells have been described (18). Transformants were picked up and grown for 2 days at 30°C on 2% agar plates of the synthetic medium for yeast lacking uracil with either 2% glucose or galactose as the sole carbon source. Numbers: 1, wild-type p68; 2, yex vector only; 3–6, mutants 1–4.

the same binding capability as the wild-type p68 kinase. Mutants 3 and 4 retained almost half of the binding capability, while mutants 1 and 2 lost most of their ability to bind poly(rI)·poly(rC) (Table 1).

Attempts were made to examine the effect of these deletions on autophosphorylation and, therefore, the activation of the kinase activity. However, as these mutants harbor deletions in the N-terminal region of the kinase, which contains the binding site for the monoclonal antibody used for *in vitro* kinase assay, this assay was not informative. Therefore, we took another approach by examining their inhibitory effect on yeast cell growth. Our previous work has shown that p68 kinase suppression of yeast cell growth results from activation of the kinase by an endogenous activator in *S. cerevisiae* and phosphorylation of yeast eIF2α (18). Thus, this provides an ideal experimental system to test if the p68 mutants deficient in binding to dsRNA *in vitro* are still active *in vivo*. While the wild-type p68 completely inhibited the growth of the transformed yeast cells (Fig. 5), mutants 1 and 2 showed little, if any, suppression, and mutants 3 and 4 exhibited an intermediate inhibitory effect compared with wild type (Fig. 4). This is consistent with their poly(rI)·poly(rC) binding phenotype as determined by the *in vitro* assay (Fig. 5 and Table 1). These observations suggest that the specific regions in the N terminus of the p68 kinase are responsible for dsRNA binding and activation of the kinase function.

DISCUSSION

The p68 kinase studied here together with another IFN-induced enzyme, 2′,5′-oligoadenylate synthetase, are regulated by dsRNA. However, the mechanism leading to the activation of the enzymes by dsRNA is not known. The cloning and sequencing of the mouse p65 kinase reported here enabled us to compare the homologous domains between the human and mouse kinase and study their potential roles in binding to dsRNA and in the regulation of kinase activity in an *in vivo* assay. We found two regions, deleted in mutants 1 and 2 (Fig. 4 and Table 1), that appear to be responsible for binding to dsRNA *in vitro* and for *in vivo* activation of the kinase in yeast cells. Although it is possible that these mutants were not expressed stably in yeast, we have previously shown that an inactive mutant of the p68 kinase can be expressed under similar conditions (18). Mutants 1–4 are not recognized by the monoclonal antibody (13) raised against the p68 kinase. At present, we do not know whether the sites we have identified are the actual binding sites or are critically involved in maintaining the conformation of the domain for binding. Studies from other RNA-binding proteins have demonstrated that an arginine-rich domain is a common RNA-binding feature (22). For example, mutations changing the two arginines at positions 38 and 39 and four arginine residues at positions 41–44 into asparatic acid and leucine abolished the binding of rev protein of the human immunodeficiency virus (HIV) to its RNA target sequence (22). In our mutant 1 of the p68 kinase (Table 1), the deleted region also contains three arginine residues and mutant 2 contains one arginine and four lysine residues. Thus, these regions all have net positive charges. Furthermore, these segments are almost 100% homologous between human and mouse, suggesting their importance in the regulatory functions of the kinase. Similar arginine-rich regions can be seen in the N-terminal domain of 2′,5′-oligoadenylate synthetase, and these have been found recently to be involved in binding to dsRNA (23). Further studies using point mutagenesis are needed to define the role of these amino acids in contacting dsRNA. Our results do not rule out the possibility that there are other dsRNA-binding sites existing in the kinase. In fact, several truncated molecules of the human p68 kinase that are 90 (*Eco*RI), 155 (*Eae* I), and 242 (*Ban* I) amino acids from the N terminus have been tested for binding to reovirus dsRNA. Only the *Ban* I peptide was able to bind to reovirus dsRNA, whereas both the *Eco*RI and *Eae* I peptides were deficient in binding (17). This result differs from our observations shown in Fig. 1. We note that the small peptides produced from pGEM-11 (comparable to *Eae* I) and pGEM-8 do bind to poly(rI)·poly(rC), but at much reduced levels. Perhaps different binding sites or affinities of various dsRNAs can explain the discrepancies in results. Taken together, we would suggest that, although the predominant dsRNA binding sites lie between amino acids 39 and 50 and 58 and 69 of the p68 kinase, other parts of the N-terminal domain are required to maintain the protein conformation for efficient binding of dsRNA. Further quantitative analysis of the binding affinity between these mutants and various dsRNAs is needed to test the hypothesis that several dsRNA binding sites with different affinities exist in the p68 kinase.

Equal contributions were made by G.-s.F. and K.C. We thank J. Hassel for the cDNA library of mouse FM3A cells, R. H. Silverman for poly(rI)·poly(rC)-cellulose, and A. Hovanessian for helpful discussions. This work was supported by grants from the Medical Research Council of Canada and National Cancer Institute of Canada to B.R.G.W. G.-s.F. is supported by a postdoctoral fellowship of the Medical Research Council of Canada, and K.C. is supported by an Ontario Ministry of Health Studentship.

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