Brief Genetics Report

Wolcott-Rallison Syndrome

Clinical, Genetic, and Functional Study of *EIF2AK3* Mutations and Suggestion of Genetic Heterogeneity

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Wolcott-Rallison syndrome (WRS) is a rare autosomal-recessive disorder characterized by the association of permanent neonatal or early-infancy insulin-dependent diabetes, multiple epiphyseal dysplasia and growth retardation, and other variable multisystemic clinical manifestations. Based on genetic studies of two inbred families, we previously identified the gene responsible for this disorder as *EIF2AK3*, the pancreatic eukaryotic initiation factor 2α (eIF2α) kinase. Here, we have studied 12 families with WRS, totalling 18 cases. With the exception of one case, all patients carried *EIF2AK3* mutations resulting in truncated or missense versions of the protein. Exclusion of *EIF2AK3* mutations in the one patient case was confirmed by both linkage and sequence data. The activities of missense versions of EIF2AK3 were characterized in vivo and in vitro and found to have a complete lack of activity in four mutant proteins and residual kinase activity in one. Remarkably, the onset of diabetes was relatively late (30 months) in the patient expressing the partially defective *EIF2AK3* mutant and in the patient with no *EIF2AK3* involvement (18 months) compared with other patients (<6 months). The patient with no *EIF2AK3* involvement did not have any of the other variable clinical manifestations associated with WRS, which supports the idea that the genetic heterogeneity between this variant form of WRS and *EIF2AK3* WRS correlates with some clinical heterogeneity. *Diabetes* 53:1876–1883, 2004

The identification of susceptibility genes for multifactorial disorders, such as type 1 diabetes, presents many challenges. To date, only two susceptibility loci for type 1 diabetes have been unambiguously identified, *IDDM1* (*HLA* locus) and *IDDM2* (insulin gene locus). Genetic studies of monogenic forms of diabetes-related disorders can significantly increase our knowledge of β-cell function and may point to potential candidate genes for the common forms of diabetes. Much information has been gained on studying syndromes such as maturity-onset diabetes of the young (1,2), Wolfram syndrome (3), and, more recently, Wolcott-Rallison syndrome (WRS) (4). WRS is a very rare syndrome, with less than 20 cases described in the world literature (4–17). It associates permanent neonatal or early-childhood insulin-dependent diabetes and epiphyseal dysplasia. Other clinical features that show variability between WRS cases include mental retardation, hepatic and kidney dysfunction, cardiac abnormalities, exocrine pancreatic dysfunction, and neutropenia. Based on genetic studies of two consanguineous families, we previously identified the gene responsible for this disorder as *EIF2AK3* (or *PEK*), the pancreatic eukaryotic initiation factor 2α (eIF2α) kinase (4). In addition, two independent knockout mice have been produced and studied that show a phenotype remarkably similar to human WRS, with neonatal diabetes, skeletal defects, and small size with delayed growth (18,19). Recent reports have begun to identify mutations in EIF2AK3 genes linked with WRS (4,16,17). Together, these

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eIF2α, eukaryotic initiation factor 2α; GST, glutathione S-transferase; WRS, Wolcott-Rallison syndrome.

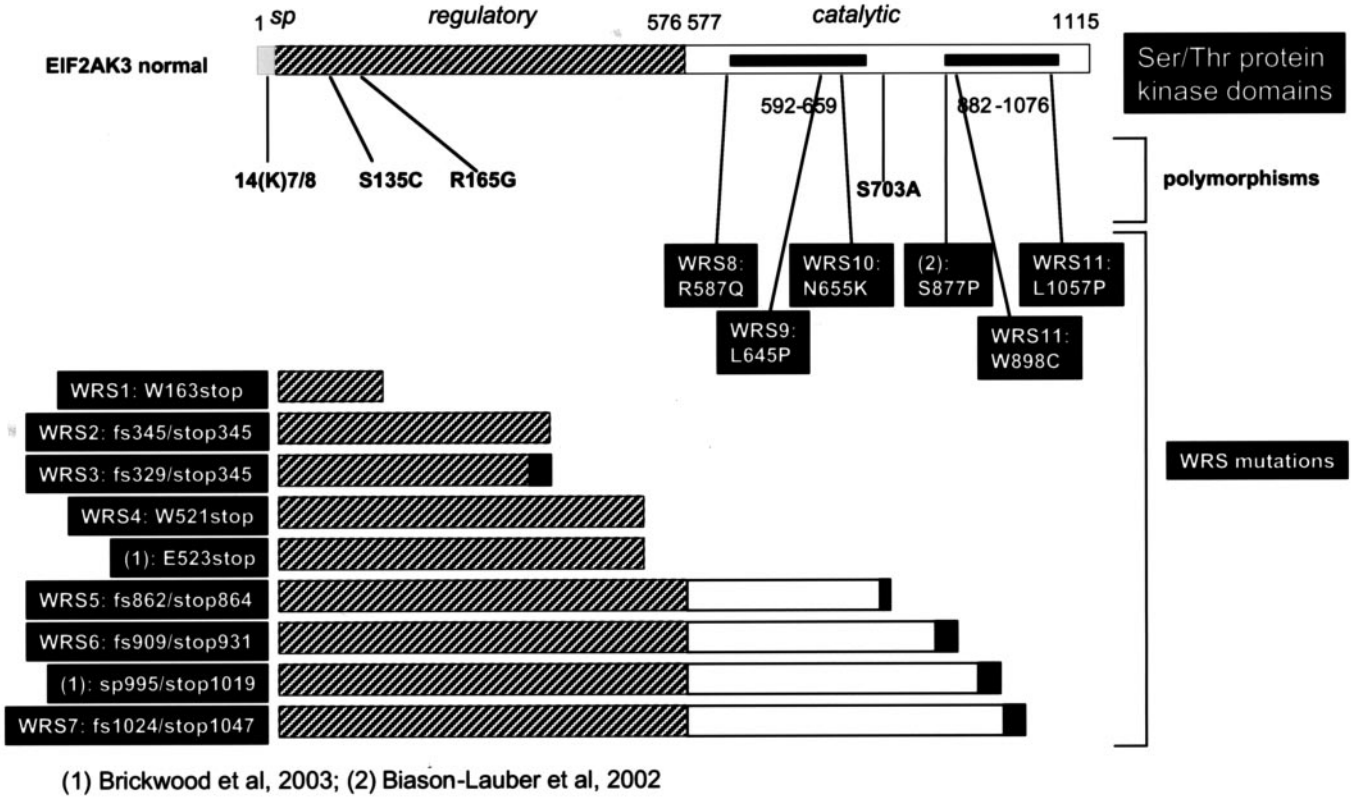

FIG. 1. EIF2AK3 polymorphisms and WRS mutations. The structure of EIF2AK3 is illustrated as a box, with the signal peptide (sp; dotted), regulatory domain (hatched), and a catalytic domain (white) indicated. Within the EIF2AK3 catalytic domain is a 222–amino acid residue insert between kinase subdomains IV and V (gap between black bars). Such inserts are characteristic of the eIF2α kinase family. The four frequent EIF2AK3 polymorphisms that have been identified in the Caucasian population are shown (black). EIF2AK3 mutations are shown (white on black background). Three of the mutations presented here have been reported in previous studies, referred to as 1 (17) and 2 (16). The bars indicate the extent of the EIF2AK3 mutant proteins. The black portions of the bars represent abnormal amino acid sequences, as a consequence of frameshift or splice mutations.

studies definitively establish that *EIF2AK3* mutations are responsible for WRS. However, the variability of the clinical manifestations observed in WRS remains unexplained. In the present study, we have assembled and studied a large collection of cases and families with WRS in order to extend the clinical and genetic investigation of this syndrome and to explore the determinism of its clinical variability.

A total of 12 families (18 patients with WRS) were studied, and detailed clinical information was obtained on these patients. This is the largest collection of WRS patients studied to date. The summary description of these patients is shown in Table 1. Patients were from various population origins, and, in most cases, their parents were known to be consanguineous (families WRS1, -2, -3, -4, -5, -6, -7, -8, -10, and -12) or likely to be since they lived in the same village (WRS9). There was no evidence of consanguinity in one case (WRS11). The age at onset was generally very young: 15 had an onset between the ages of 5 weeks and 6 months, but 2 had a significantly older age at onset, at 18 months (WRS12-1) and 30 months (WRS10-1). Excluding these two outliers, the mean age at onset was 3 months. Most of these cases were still alive (aged between 3 months and 13 years), and some had died (aged between 3 months and 35 years). Diabetes required insulin therapy from the onset of the disease. With one possible exception, there was no evidence of autoantibodies (islet cell antibody, insulin autoantibody, IA-2 antigen, and GADA) in the patients at the onset of disease when this was tested. Slightly elevated values were found for patient WRS12-1 at disease onset (GADA: 2.3 IU) but within normal range (0.94 IU) 3 months later. In addition to the multiple epiphyseal dysplasia common to all patients, there were various degrees of osteopenia, ranging from undetected or mild (possibly detected by bone densitometry) to severe, with multiple fractures. The mental and psychomotor development was assessed based on the sitting/walking age, speech and communication, and school performance. There was slight to severe mental retardation or developmental delay in the majority of the patients, but some were within the normal range (WRS9-1, 10-1, 11-1, and 12-1). Epilepsy was not reported in any of these patients, in contrast with other reports (5). Recurrent episodes of hepatitis occurred in the majority of the patients. These were major events, requiring hospitalization, often accompanied by acute renal failure and sometimes resulting in death. Hepatic and renal functions returned to normal for those surviving these episodes. In all cases where it was explored, no causative viral infection could be identified. In addition, progressive chronic renal insufficiency was noted in three patients, WRS8-1, WRS9-1, and WRS9-2, who were also the ones who lived to older ages (between 11 and 35 years). Four patients showed signs of exocrine pancreas dysfunction, WRS2-2, WRS4-1, WRS5-1, and WRS10-1, with pancreatic hypotrophy in WRS10-1 (12) and fibrosis infiltrations in pancreas

TABLE 1
Description of WRS patients

Patient code	Country of origin/country of living	References	Consanguinity	Age at onset of diabetes	Age at death	Osteopenia	Mental retardation/ developmental delay	Hepatic dysfunction (chronic)	Hepatitis episode (acute)
WRS1-1	S. Arabia	13,14	+	1.5 months	Alive (6 years)	+	+	−	+
WRS1-2*				2 months	Alive (3 months)	NA	NA	NA	NA
WRS2-1	Tunisia/France	11,4	+	2 months	3.5 months	+	NA	−	+
WRS2-2				2 months	5 years	+	+	+	+
WRS2-3				6 months	Alive (5 years)	+	+	−	−
WRS3-1	Quebec	New	+	5 months	Alive (5 years)	+	+/−	−	+
WRS4-1†	Turkey	New	+	3 months	3 months	+	NA	NA	+
WRS5-1	Iran/Belgium	New	+	Unknown‡	Alive (10 years)	+	+/−	−	+
WRS6-1	Pakistan/U.K.	New	+	6 months	Alive (2 years)	+	+	+	+
WRS7-1	S. Arabia	15	+	3 months	5 years	−	+	−	+
WRS7-2§				2.5 months	Alive (6 years)	−	+	−	+
WRS8-1	Pakistan/U.K.	4	+	4 months	Alive (13 years)	−	+	−	−
WRS8-2				2.5 months	Alive (4 years)	−	+	−	+
WRS9-1	Germany	6	Suspected	5 weeks	35 years	+	−	−	−
WRS9-2				2.5 months	11 years	+	+	−	−
WRS10-1	Mauritania/France	12	+	30 months	Alive (6 years)	+	−	+	+
WRS11-1‖	Slovakia	New	−	4 months	Alive (8 years)	+	−	−	−
WRS12-1	Italy	New	+	18 months	Alive (2 years)	+	−	−	−

*Patient WRS1–2 was very recently diagnosed. Other phenotypic manifestations are not yet apparent at this age and are therefore recorded as NA (not applicable), as they may express later. †Despite early death, signs of osteopenia and bone dysplasia were visible on X-ray examination. Two previous siblings of WRS4-1 died around the same age with the same clinical presentation, but samples were not available. ‡Family WRS6 was living in Iran at the onset of disease in the child, and the precise age at onset was not available. §A third sibling in this family was just diagnosed with diabetes (age at onset: 2 weeks); other phenotypic manifestations are not yet apparent. ‖Two other siblings with WRS died earlier, both from acute hepatic failure. Samples were not available. +, presence; −, absence; +/−, mild.

biopsy in WRS2-2 (11). Central hypothyroidism was noted in four patients (WRS1-1, WRS4-1, WRS7-1, and WRS7-2) (14,15), but thyroid function was normal in the others. Neutropenia was reported in nine patients (WRS2-1, WRS2-2, WRS2-3, WRS3-1, WRS4-1, WRS5-1, WRS6-1, WRS10-1, and WRS11-1), who also tended to suffer from frequent infections (bacterial, viral, and fungic). The phenotype of the parents and heterozygous siblings was not remarkable; in particular, none had diabetes.

Sequencing of the coding regions of *EIF2AK3* was performed in all the WRS cases and in available parents and siblings. *EIF2AK3* mutations were identified in 11 of the 12 families, in the homozygous state in the patients of 10 of them, and as a compound heterozygote in WRS11-1, whose parents were not consanguineous. In this last family, segregation of the alleles was consistent with linkage to *EIF2AK3*, since an unaffected sibling received different parental *EIF2AK3* alleles than the affected case, based on microsatellite genotyping and sequence analysis (data not shown). None of these 12 mutations were found in a control population of 95 Caucasian individuals (4). The nature, position, and consequences of these mutations are shown in Table 2 and displayed in Fig. 1, together with other previously reported mutations (16,17) and polymorphisms (4). Two novel mutations and a previously reported one were nonsense mutations, resulting in truncated proteins 162, 520, and 522 amino acid residues in length. Five novel mutations were frameshift mutations resulting in truncated proteins, with length varying between 344 and 1,024 correct amino acid residues compared with 1,115 for the full-length EIF2AK3. One *EIF2AK3* mutation was a splice-site mutation, resulting in a truncated protein with 994 correct residues. The other five novel mutations, as well as a previously reported one, were missense mutations, all located within the catalytic domain of the protein (Fig. 1). In contrast, the four frequent amino acid variants (polymorphisms) that we have identified in the Caucasian population (4) are located at various positions of the protein, but all outside of the kinase domain: one in the predicted signal peptide, two in the regulatory domain, and one in the insert region in the kinase domain.

All of the *EIF2AK3* mutations that resulted in truncated proteins are missing all or part of the kinase domain, which would be expected to lead to a complete loss of function (Fig. 1). To evaluate the functional consequence of the missense mutations, we measured the activity of EIF2AK3 mutant proteins using the well-characterized yeast translation assay (20). The EIF2AK3 catalytic domain was fused to an NH_2-terminal glutathione S-transferase (GST) tag and expressed in yeast strain H1894, which was deleted for its sole endogenous eIF2α kinase. Expression of the wild-type version of EIF2AK3 using a galactose inducible promoter blocked growth of the H1894-derived cells in the inducing SGAL medium (Fig. 2*A*). No growth defect was observed in the glucose medium (SD), in which EIF2AK3 protein is poorly expressed in yeast. This growth deficiency was due to hyperphosphorylation of eIF2α, as measured by immunoblot using a polyclonal antibody that recognizes eIF2α phosphorylated at serine-51 (Fig. 2*B*). By contrast, expression of a mutant version of EIF2AK3-K621M altered in the invariant lysine required for kinase catalytic activity did not induce eIF2α phosphorylation or elicit a growth defect in the SGAL medium.

We next measured the activities of the newly identified WRS missense mutants using the yeast translation system. Yeast strains expressing each of the five EIF2AK3 mutants

TABLE 1
Continued

Kidney dysfunction (chronic)	Kidney dysfunction (acute)	Neutropenia	Frequent infectious episodes	Hypothyroidism	Exocrine pancreas dysfunction	Autoantibodies	EIF2AK3 mutation	Nature of mutation
−	+	−	−	+	−	−	+	W163STOP
NA	NA	NA	NA	NA	NA	−	+	W163STOP
−	+	+	+	−	−	−	+	fs345/STOP345
−	+	+	+	−	+	−	+	fs345/STOP345
−	−	+	+	−	−	−	+	fs345/STOP345
−	+	+/−	−	−	−	NA	+	fs329/STOP345
NA	+	+	+	+	+	−	+	W521STOP
−	−	+/−	−	−	+	NA	+	fs862/STOP864
−	+	+	−	−	−	NA	+	fs909/STOP931
−	+	−	−	+	−	−	+	fs1024/STOP1047
−	+	−	−	+	−	−	+	fs1024/STOP1047
+	−	−	−	−	−	NA	+	R587Q
−	−	−	−	−	−	NA	+	R587Q
+	−	−	−	−	−	NA	+	L645P
+	−	−	−	−	−	NA	+	L645P
−	−	+/−	+	−	+	−	+	N655K
−	−	+/−	+	−	−	−	+	W898C, L1057P
−	−	−	−	−	−	+/−	−	—

showed efficient growth in the SGAL-inducing medium and no detectable eIF2α phosphorylation as measured by immunoblot (Fig. 2*A* and *B*). Levels of the mutant EIF2AK3 proteins in yeast were in fact higher than the wild-type counterpart, indicating that the residue substitutions did not impact their expression or stability. It is noted that the mutant EIF2AK3 proteins migrated faster than the wild-type version in the SDS-PAGE analysis preceding the immunoblot, due to autophosphorylation of EIF2AK3 that accompanies its activation (21). Phosphorylation of EIF2AK3 occurs at multiple residues. Such autophosphorylation can be measured in wild-type EIF2AK3 by immunoblot using polyclonal antibody that specifically recognizes EIF2AK3 phosphorylated at Thr-980 in the kinase activation loop between kinase subdomains VII and VIII (Fig. 2*B*). By contrast, no autophosphorylation was detected in the mutant versions of EIF2AK3, with the exception of *EIF2AK3-N655K*, which showed a modest autophosphorylation in vivo. Finally, an in vitro assay was carried out using the purified EIF2AK3 proteins prepared from yeast. Four of the mutant proteins showed a complete absence of autophosphorylation and of eIF2α phosphorylation activity, and the N655K mutant showed a fivefold reduced eIF2α phosphorylation compared with wild type, despite significant autophosphorylation in this assay (Fig. 2*C*). These results indicate that four of the missense mutations (R587Q, L645P, W898C, and L1057P) result in a complete loss of EIF2AK3 function in vivo and in vitro, while one (N655K) retains some kinase activity.

No homozygous or heterozygous mutations were detected in patient WRS12-1 or in the *EIF2AK3* exons and 3′ untranslated and promoter regions. Genotyping of five adjacent microsatellite polymorphisms encompassing the *EIF2AK3* gene excluded linkage to WRS in this family (Fig. 3). In addition, the sequence of *EIF2AK3* exons in this family showed that the patient was heterozygous at four genetic variants within this gene (data not shown). Although it is formally possible that this patient has inherited independent *EIF2AK3* mutations from each par-

TABLE 2
Nature, position, and consequence of mutations

Patients	Nucleotide alteration (AF110146)	Predicted amino acid change
WRS1-1,2	560G/A	W163STOP
WRS2-1,2,3	1103insT	fs345/STOP345
WRS3-1*	1. 1059insT	fs329/STOP345
	2. Intron 3, del(aggtg) −7 to −3 from exon 4	Possible effect on splicing
WRS4-1	1634G/A	W521STOP
WRS5-1	2658-2662del(AAGTT)	fs862/STOP864
WRS6-1	2791-2794del(GAGA)	fs909/STOP931
WRS7-1,2	del(184 bp) in exon 15/intron 15	fs1024/STOP1047
WRS8-1,2	1832G/A	R587Q
WRS9-1,2	2006-7TT/CC	L645P
WRS10-1	2037T/A	N655K
WRS11-1†	2766G/T	W898C
WRS11-1†	3242T/C	L1057P

*Patient homozygote for two mutations; †patient compound heterozygote for two mutations. fs, frameshift mutation, starting at the position shown.

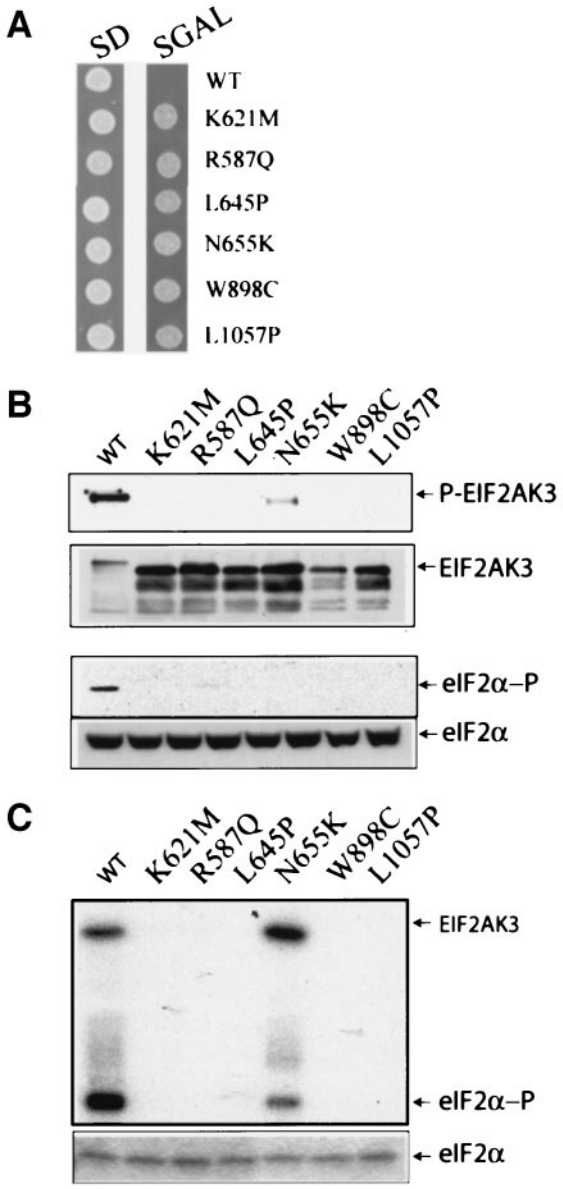

FIG. 2. Functional studies of EIF2AK3 mutations and controls. *A*: EIF2AK3 activity in yeast. The different EIF2AK3 mutant and control constructs were transformed into the H1894 yeast strain, and cell growth was measured in SGAL (galactose) medium and SD (glucose) medium. Only the cells expressing wild-type EIF2AK3 showed a slow growth phenotype in the galactose-inducible condition. *B*: Immunoblot analysis of EIF2AK3 and eIF2α. *Top panel*: Steady-state levels of wild-type and mutant versions of EIF2AK3 were measured in yeast cells grown in SGAL medium. GST-tagged EIF2AK3 proteins were purified using glutathione sepharose, and bound proteins were separated by electrophoresis in a 10% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose filters and immunoblot analysis was performed using either a polyclonal antibody that recognizes total levels of the GST-tagged EIF2AK3 or an antibody specific for EIF2AK3 phosphorylated at T980. *Bottom panel*: Strain H1894 expressing the indicated EIF2AK3 proteins was grown in SGAL medium, and the levels of eIF2α phosphorylation were measured by immunoblot analysis. Phosphorylated eIF2α was visualized using affinity-purified antibody that specifically recognizes eIF2α phosphorylated at serine-51 (Biosource International), and total eIF2α protein levels were measured with polyclonal antibody prepared against total yeast eIF2α. *C*: In vitro assay for *EIF2AK3* activity. GST-tagged EIF2AK3 proteins expressed in yeast were purified using glutathione agarose pancreatic eIF2 kinase and incubated with [γ-^{32}P]ATP and purified recombinant eIF2α. Radiolabeled proteins were separated by electrophoresis in a 12.5% SDS-polyacrylamide gel, and the gels were fixed by Coomassie staining, dried, and visualized by autoradiography (*top panel*). *Bottom panel*: Coomassie staining of the eIF2α substrate in each reaction mixture.

ent, which would both be located in distant regions of the gene or in introns, this is highly unlikely given the fact that the 15 *EIF2AK3* mutations identified so far in WRS affect the amino acid sequence. Together, these observations support the idea that the WRS in this patient is not caused by EIF2AK3 mutations. Since his parents are consanguineous, his syndrome is likely to result from a recessive mutation in another single gene, although we cannot exclude that the observed diabetes and bone dysplasia occurred by coincidence in this patient. In the first hypothesis, the responsible gene may be one of the alternative eIF2α kinase, another gene involved in this pathway, or interacting with *EIF2AK3*. Using microsatellite markers encompassing *GCN2*, *HRI*, and *PKR* genes, we could exclude linkage of WRS in WRS12 family to the regions of these three alternative eIF2α kinases (data not shown).

Other than the bone dysplasia and the presence of neonatal or early-infancy onset of insulin-dependent diabetes, present in all patients, there was marked variability in the other features frequently associated with WRS (Tables 1 and 2). We questioned whether this variability may be explained in part by the nature of the gene (*EIF2AK3* or not) or the type and location of the *EIF2AK3* mutation. Most patients had a very early age at onset (<6 months), but two of them (WRS10-1 and 12-1) had a relatively old age at onset (30 and 18 months, respectively). Remarkably, WRS10-1 carried the N655K mutation, which was the only one with residual kinase activity, while all of the other mutant versions of *EIF2AK3* either resulted in truncated proteins or had no catalytic activity; and WRS12-1 was the only patient with no implication of the *EIF2AK3* gene. In addition to the late age at onset, WRS12-1 was the only patient lacking all of the other frequent complications observed in WRS patients. Borderline positivity for GADA autoantibodies was also found in this patient at the onset of the disease, which did not persist after 3 months. HLA typing showed that this patient was *DR3* positive and *DR4* negative (not shown), which does not allow us to make any conclusions on the autoimmune nature of diabetes in this patient. Based on these observations, the WRS12-1 patient appears to have a variant form of WRS, with genetic and slight clinical heterogeneity compared with *EIF2AK3*-caused WRS.

Apart from age at onset, there was poor concordance overall between the nature of the *EIF2AK3* mutation and the variable features of the disease, suggesting that other genetic factors (modifier genes) or environmental factors may be involved. In particular, some affected siblings from the same family were discordant for mental retardation (family WRS9), the presence of acute or chronic hepatic or kidney dysfunction (families WRS2 and WRS8), and exocrine pancreas dysfunction (family WRS2). The patients who had chronic kidney dysfunction were the older living patients (age >11 years), suggesting that this feature may be a long-term complication of this syndrome. However, the absence of acute episodes or chronic conditions may not be a significant phenotype in young children, who may develop these at a later age. Possible familial aggregation was found for neutropenia/frequent infections, osteopenia, and hypothyroidism; however, it is difficult to conclude whether these complications are related to specific mutations because of the limited number of observations.

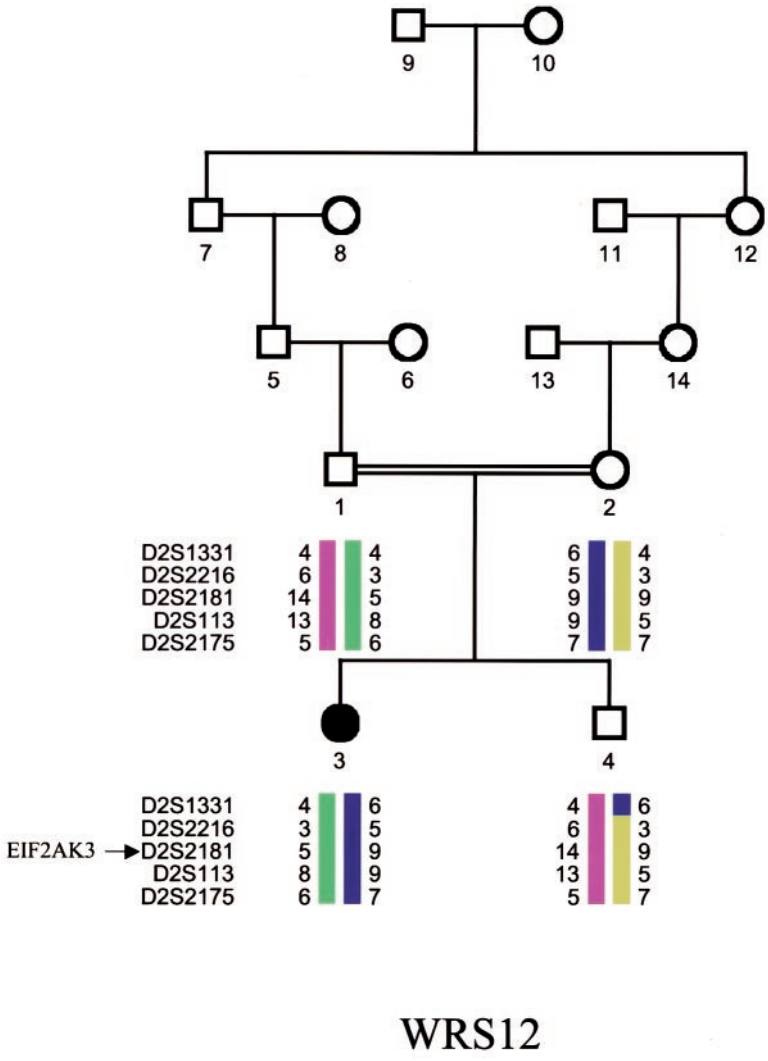

FIG. 3. Linkage analysis of the EIF2AK3 region in family WRS4. The approximate genetic map, based on combined information from the University of California at Santa Cruz (http://genome.ucsc.edu/cgi-bin/hgGateway) and deCODE (25) is as follows: D2S1331 (~110.50 cM), D2S2216 (111.48 cM), D2S2181 (112.01), EIF2AK3 (180 kb from D2S2181), D2S113 (~113.11 cM), and D2S2175 (113.11 cM).

Overall, our data suggest that factors unrelated to *EIF2AK3* gene contribute to a large extent to the clinical variability of WRS. Variation in environmental conditions that lead to endoplasmic reticulum stress or genetic variation in other genes involved in the response to endoplasmic reticulum stress are likely to modulate the severity and clinical characteristics of the disease. Such an effect of environmental stress factors on disease severity and progression has been shown in a rare recessive neurological disorder, leukoencephalothay with vanishing white matter, which is caused by mutations in subunits of the eIF2B translation initiation factor (22,23).

Parents and heterozygous siblings of WRS patients from this study had no remarkable features; in particular none had type 1 or type 2 diabetes or bone disorder. This suggests that *EIF2AK3* may not play a major role in the susceptibility to frequent forms of diabetes, at least as a single gene, despite our observation of linkage of the *EIF2AK3* region to type 1 diabetes in the Scandinavian population (24).

Our studies suggest the existence of a variant form of WRS, which does not present any of the complications frequently observed in this syndrome and may be associated with an older age at onset. In addition, the only patient who carried a homozygous *EIF2AK3* mutation with a slight residual activity in our functional studies was found to have a significantly delayed age at onset. Therefore, we propose to extend the age-at-onset definition in WRS to "early-infancy onset" instead of the generally used "neonatal" diabetes. Based on our observations, a diagnosis of WRS should be considered in patients presenting with insulin-dependent diabetes starting at older ages, in case of an association with epiphyseal dysplasia, and the *EIF2AK3* gene screened for mutations in these patients. In addition to these genetic heterogeneity factors, part of the variability of WRS is likely to be due to additional factors, such as modifier genes or factors related to the environment or patient's management, or to the natural progression of the disease.

RESEARCH DESIGN AND METHODS

A total of 12 families (18 patients with WRS) were studied, including the two original families that we previously reported (4), and other previously reported cases from Germany (6), Mauritania/France (12), and Saudi Arabia (13–15). Six other families from Quebec, Iran/Belgium, Pakistan/U.K., Slovakia, Italy, and Turkey have not been previously reported. All patients and families gave their informed consent for genetic studies, which were approved by local institutional committees. WRS diagnosis was made on the basis of the minimum association of neonatal or early-childhood insulin-dependent diabetes and multiple epiphyseal dysplasia, as described (5). A detailed question-

naire was provided to all the clinicians in order to collect additional clinical and familial information. A description of these cases is provided in Table 1.

Mutation screening. DNA was extracted from peripheral blood collected on EDTA, and *EIF2AK3* mutation screening was performed on genomic DNA on all the coding regions of the gene in the cases and their parents (when available), as previously described (4). Alternatively, RNA was extracted from peripheral blood and sequencing performed on the cDNA (4). Additional sequencing was performed in one patient and his parents (family WRS12) to cover the 3′ untranslated region of the gene, as previously described (4), and exon 1 and >1,500 bp of sequence 5′ of the starting ATG of the gene, using three sets of overlapping primers: 5p3f/73-377R, 5p2f/5p2r, and 5p1f/5p1r (template sizes: 962, 554, and 574 bp, respectively), with 5p3f: 5′-GTCAGAATC CGCCACGTAGT-3′, 73–377R: 5′-CGCGCGTAAACAAGTTG-3′; 5p2f: 5′-AGT TCAAATGCCTTGGCTGA-3′, 5p2r: 5′-GTCTGCGCTAACTGCCTCTT-3′; and 5p1f: 5′-ACCCATATTGCCAACACCTT-3′, 5p1r: 5′-GGGCAGAGTGGAGAA GACTG-3′.

Sequencing reactions were performed using the amplification primers and an additional primer in the case of 5p3f/73-377R: 5′-CGAGATAGGCTGTCACT CAGG-3′.

Microsatellite genotyping. Microsatellite genotyping was performed using fluorescence-labeled primers on an ABI3700 sequencer, using standard methods. For assessing linkage to the *EIF2AK3* region, the following microsatellite markers were used: *D2S1331*, *D2S2216*, *D2S2181*, *D2S113*, and *D2S2175*.

Analysis of EIF2AK3 activity. Functional assays for EIF2AK3 were performed using the yeast strain H1894 (*MAT*a *ura3–52 leu2–3*, −112 gcn2Δ *trp1*Δ*-63*), which is deleted for its sole endogenous eIF2α kinase. The *EIF2AK3* gene was inserted downstream of a galactose-inducible promoter in the *URA3*-marked low-copy plasmid p416. Each plasmid construct consisted of an NH_2-terminal GST tag fused in-frame with the EIF2AK3 catalytic domain, including residues 551–1,115. Five mutant constructs were generated, corresponding to WRS mutations R587Q, L645P, N655K, W898C, and L1057P. Positive and negative controls were wild-type EIF2AK3 and the kinase-deficient K621M mutant, respectively (21). Plasmids were transformed into H1894 cells using uracil prototrophy, and equal volumes of the yeast cultures at A_{600} = 0.25 were spotted onto agar plates containing synthetic minimal medium–containing glucose (SD) or in synthetic medium containing galactose (SGAL), which induces EIF2AK3 expression. Strains were grown for 3–4 days at 30°C on the agar plates and electronically imaged.

H1894 cells expressing the indicated EIF2AK3 proteins were grown in SGAL medium for 4 h at 30°C, and the levels of eIF2α phosphorylation were measured by immunoblot analysis. Phosphorylated eIF2α was visualized using an affinity-purified antibody that specifically recognizes eIF2α phosphorylated at serine-51 (Biosource International), and total eIF2α protein levels were measured by using polyclonal antibody prepared against total yeast eIF2α. The eIF2α-antibody complex was visualized by using horseradish peroxidase–labeled anti-rabbit secondary antibody and chemiluminescent substrate. To measure the steady-state levels of wild-type and mutant versions of EIF2AK3 in yeast, cells were inoculated into SGAL medium at A_{600} = 0.1 and incubated with constant shaking at 30°C for 20 h. Cells were collected, broken with glass beads, and lysates were clarified by centrifugation as described (20). The GST-tagged EIF2AK3 proteins were purified from 50 μg yeast lysate by using glutathione sepharose. Proteins associated with the sepharose were removed by boiling in SDS sample buffer and separated by electrophoresis in a 10% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose filters, and immunoblot analysis was performed with either an antibody that recognizes total levels of the GST-tagged EIF2AK3 or an antibody specific for EIF2AK3 phosphorylated at T980. Kinase reactions were carried out by using EIF2AK3 expressed in yeast strain J82, which contains a mutant version of eIF2α-S51A that blocks EIF2AK3 inhibition of translation in the galactose-inducing medium (20). The GST-tagged EIF2AK3 proteins were purified by using glutathione sepharose and assayed for phophorylation of recombinant eIF2α in a reaction containing 10 μCi [γ-^{32}P]ATP in a final concentration of 50 μmol/l, as described (20). Reaction mixtures were incubated at 30°C for 4 min, a time point that was found to be in the linear range of the assay.

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