DYRK1 is a dual specificity protein kinase presumably involved in brain development. Here we show that the kinase belongs to a new family of protein kinases comprising at least seven mammalian isoforms (DYRK1A, DYRK1B, DYRK1C, DYRK2, DYRK3, DYRK4A, and DYRK4B), the yeast homolog Yak1p, and the *Drosophila* kinase minibrain (MNB). In rat tissues, DYRK1A is expressed ubiquitously, whereas transcripts for DYRK1B, DYRK2, DYRK3, and DYRK4 were detected predominantly in testes of adult but not prepuberal rats. By fluorescence microscopy and subcellular fractionation, a green fluorescent protein (GFP) fusion protein of DYRK1A was found to accumulate in the nucleus of transfected COS-7 and HEK293 cells, whereas GFP-DYRK2 was predominantly detected in the cytoplasm. DYRK1A exhibited a punctate pattern of GFP fluorescence inside the nucleus and was co-purified with the nuclear matrix. Analysis of GFP-DYRK1A deletion constructs showed that the nuclear localization of DYRK1A was mediated by its nuclear targeting signal (amino acids 105–139) but that its characteristic subnuclear distribution depended on additional N-terminal elements (amino acids 1–104). When expressed in *Escherichia coli*, DYRK1A, DYRK2, DYRK3, MNB, and Yak1p catalyzed their autophosphorylation on tyrosine residues. The kinases differed in their substrate specificity in that DYRK2 and DYRK3, but not DYRK1A and MNB, catalyzed phosphorylation of histone H2B. The heterogeneity of their subcellular localization and substrate specificity suggests that the kinases are involved in different cellular functions.

DYRK1 is a dual specificity protein kinase that catalyzes its autophosphorylation on serine/threonine and on tyrosine residues (1). The *Drosophila* homolog of this gene, minibrain, encodes three splicing variants of the protein kinase MNB. Mutant flies with a reduced expression of minibrain show specific behavioral defects (2, 3).

Due to the high sequence similarity of the rat DYRK1 cDNA with a human expressed sequence tag (EST) that had previously been mapped to chromosome 21, we were able to localize the gene of a human homolog of DYRK1 to 21q22.2 (1). By correlation of phenotype with genotype in patients with partial trisomies, this region has been defined as the “Down’s syndrome critical region”; its triplication appears to be responsible for many features of Down’s syndrome including mental retardation (4–6). In sequencing projects of the Down’s syndrome critical region, several groups have independently identified the human *DYRK1* gene (7–11). Because of its high similarity with *Drosophila* MNB, DYRK1 is currently considered a candidate gene for the aberrant development of the brain that underlies mental retardation in Down’s syndrome. The product of the human *DYRK1* gene is nearly identical with rat DYRK1 (3 of 763 amino acids differ). Recently, Smith et al. (12) reported that transgenic mice with a 180-kb fragment of human chromosome 21 including the *DYRK1* gene exhibit defects in learning tasks.

In *Saccharomyces cerevisiae*, Yak1p is the protein kinase with the highest sequence similarity with MNB and DYRK1. The *YAK1* gene was identified as a functional antagonist of the RAS/protein kinase A pathway and has been characterized as a negative regulator of growth (13, 14). These kinases share several sequence motifs in the catalytic domain that distinguish them from all other known protein kinases, e.g. a conserved sequence motif in the activation loop including putative regulatory tyrosine residues.

To define the family of DYRK-related kinases further, we report the identification and characterization of new members of the family. Our data indicate that DYRK-related kinases constitute a distinct family of protein kinases characterized by the structural similarity of their kinase domains and their capability to autophosphorylate on tyrosine residues. However, members of the DYRK family have unrelated sequences outside the catalytic domain and differ in their substrate specificity, tissue distribution, and subcellular localization.

**MATERIALS AND METHODS**

RNA Preparation and PCR Cloning of DYRK-related Kinases—Total RNA was prepared from differentiated 3T3-L1 cells (15) and from various rat tissues by the method of Chirgwin et al. (16). First-strand...
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cDNA was synthesized with oligo(dT) as primer (First-strand cDNA Synthesis Kit, Amersham Pharmacia Biotech). Partial cDNAs of DYRK-related kinases were amplified by nested PCRs. Degenerate oligonucleotide duplexes that included nonaligned subdomains VIB and IX of protein serine/threonine kinases were used as primers in the first PCR reaction. The resulting forward and reverse primers are described in the Supplementary Text. The PCR products were cloned into a plasmid vector and sequenced.

Identification of DYRK-related Kinases by PCR Cloning—DYRK-related kinases were amplified by nested PCRs. Degenerate primers from yeast genomic DNA. Restriction sites (lytic domain (amino acids 338–713 (13)) was amplified with specific primers for each of the kinases. The forward and reverse primers are described in the Supplementary Text. The PCR products were cloned into a plasmid vector and sequenced.

Expression of Green Fluorescent Protein (GFP) Fusion Proteins and Subcellular Fractionation of COS-7 Cells—cDNAs with full open reading frames for rat DYRK1A and human DYRK2 were inserted into the expression vector pEGFP-C1 (CLONTech) to generate the respective COS-7 cell lines. Exponentially growing cell lines were transfected by calcium phosphate precipitation. Cells were harvested 48 h after transfection using a plasmid transfection kit (Boehringer Mannheim) or by calcium phosphate precipitation. No effect of the transfection method on the subcellular localization of the GFP fusion proteins was observed. 48 h after transfection (48 h after GEF-DYRK1A transfection), cells were washed 2 × with phosphate-buffered saline (138 mM NaCl, 2.6 mM KCl, 10 mM NaHPO4, 1.8 mM KH2PO4, pH 7.4), fixed 20–30 min in 3% formaldehyde, pelleted by centrifugation, and resuspended in phosphate-buffered saline and 1 × with H2O, embedded in fluoromount-G (Southern Biotechnology Associates), and visualized with a Zeiss Axiophot equipped for epifluorescence. 2–5 independent transfections were performed with each fusion construct, and at least two different samples were evaluated microscopically in each experiment.

RESULTS

Identification of DYRK-related Kinases by PCR Cloning—Based on a comparison of Coomassie-stained histone bands, 70–90% of the chromatin was extracted from nuclei by this method. Aliquots of the protein samples corresponding to an equivalent portion of the original cell homogenate were separated by SDS-PAGE, transferred onto PVDF membranes, and probed with a GFP-specific antiserum (CLONTECH). Reactions bands were detected using a peroxidase-based chemiluminescence kit (Boehringer Mannheim).

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addition, a reverse primer corresponding with the sequence of a human EST was used (GenBank™ accession number T93981). PCR products were cloned and characterized by sequencing.

As illustrated in Fig. 1, cDNA sequences encoding seven different DYRK-related kinases were cloned. Six of the cDNAs encoded protein kinases with a sequence motif specific for DYRK-related kinases (DYRK, MNB, and Yak1p), i.e. the amino acids "Ser-Ser-Cys" following subdomain VII. The designation of the kinases was based on the different degree of similarity among the sequences. Four kinases that shared less than 70% identical amino acids in this region were designated DYRK1, DYRK2, DYRK3, and DYRK4. Two pairs of closely related sequences (more than 95% of identical amino acids) were classified as isoenzymes (DYRK1A/B and DYRK4A/B).

DYRK1A corresponds with the rat and murine "DYRK" as described previously (1, 9). The amino acid sequence of DYRK1B is identical with DYRK1A in this region, whereas the cDNA sequences are only 78% identical. In addition to DYRK1A and DYRK1B, the partial sequence of a third murine cDNA was classified as isoenzymes (DYRK1C, DYRK1A and DYRK1B, the partial sequence of a third murine cDNA sequence are only 78% identical). In addition to DYRK1B is identical with DYRK1A in this region, whereas the described previously (1, 9). The amino acid sequence of DYRK1, DYRK2, DYRK3, and DYRK4. Two pairs of closely related sequences (more than 95% of identical amino acids) were classified as isoenzymes (DYRK1A/B and DYRK4A/B).

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Both DYRK2 and DYRK3 contain a canonical kinase domain (22) that is located between a large N-terminal domain (149 and 173 amino acids, respectively) and a short C-terminal extension (66 amino acids). The sequences exhibit the specific features of DYRK-related kinases (DYRK1, MNB, and Yak1p), including the motif Ser-Ser-Cys (SSC) in subdomain VII, a cysteine residue in subdomain VIB, and the Tyr-X-Tyr motif in the activation loop.

Functional Characteristics of DYRK2 and DYRK3—Cloned PCR products of DYRK2 and DYRK3 were used to isolate full-length cDNA clones from a human fetal brain cDNA library. Seven cDNA clones of DYRK2 differing in the length of the 3′-untranslated region were isolated. In three clones, the poly(A) tail began only 184 bases after the stop codon (Fig. 2), whereas 4 of the cDNAs contained 1461 additional nucleotides of 3′-untranslated region. The existence of additional transcripts with an even longer 3′-untranslated region can be predicted from the sequences of four ESTs that were identified in the database (GenBank™ accession numbers R63190, R63622, R25341, and Z25301). Furthermore, of three cDNA clones that contained the 5′-end of the transcript, one differed from the others by the presence of a 149-bp insertion (Fig. 2). Due to the shift of the reading frame, the protein encoded by this cDNA clone may start at a different initiator codon and thus contain 73 additional amino acids at its N terminus.

The deduced amino acid sequence of DYRK2 appears to be identical with a human kinase that has previously been presented as a partial sequence (PSK-H2) in an alignment of catalytic domains of kinases (22). Differences of 5 amino acids may represent errors in the sequence of PSK-H2. However, in the absence of information on the nucleotide sequence of PSK-H2, it cannot be excluded that PSK-H2 is encoded by a different gene than DYRK2.

A 2170-bp cDNA clone of DYRK3 was isolated that comprised an open reading frame of 550 codons and a poly(A)-tail (Fig. 3). There is no in-frame stop codon before the first ATG codon. However, the sequence preceding this putative initiator codon appears “non-coding” as judged from the unusually high proportion of rare codons when compared with “average” human proteins. According to the codon usage table for human proteins (23), 9 codons (11%) in the first 252 nucleotides were considered rare, in contrast to only 20 in the 550 codons of the open reading frame starting with nucleotide 253 (3.6%; rare codon threshold 0.1). We therefore conclude that one of the two consecutive ATG codons at nucleotides 253–255 (Fig. 3) is most likely the initiator codon of DYRK3. Recently, a partial cDNA sequence of human DYRK3 was published as a database entry (GenBank™ accession number U69558). This clone contains an insertion of 95 nucleotides relative to the clone presented in Fig. 3 and most probably represents an alternatively spliced version of the transcript. This longer transcript contains an ATG codon that initiates an open reading frame encoding 15 additional N-terminal amino acids relative to the sequence shown in Fig. 3.

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immediately preceding the catalytic domain. DYRK2 and DYRK3 lack the striking sequence motifs that were identified in DYRK1, e.g. a nuclear targeting sequence, a PEST region, and a stretch of 13 consecutive histidine residues (1).

Genomic Localization of the Human Genes for DYRK2 and DYRK3—Data base searches identified a number of ESTs for DYRK2 and DYRK3. One of the ESTs for DYRK2 (amino acids 174, 324, 437, 444) has been mapped to human chromosome 12 in the Genexpress program (24). For DYRK3, the map position of an EST clone of the I.M.A.G.E. consortium (clone 23329; GenBank™ accession number R38268 (25)) was determined by fluorescence-based in situ hybridization to be 1q32 (26). The gene for the Van der Woude syndrome, an autosomal dominant craniofacial disorder, has been mapped to this region of chromosome 1 by linkage analysis (27).

Expression of DYRK-related Kinases in Different Rat Tissues—Tissue distribution of the transcripts for DYRK-related kinases was studied by Northern blot analysis. As previously observed (1), a specific probe for DYRK1A hybridized with two bands of approximately 2.8 and 5.4 kb which represent alternatively polyadenylated mRNA species (Fig. 4A). DYRK1A is ubiquitously expressed in all rat tissues studied, although mRNA levels varied widely (weak bands in RNA from liver were detected in parallel experiments). In contrast, the probes specific for DYRK1B, DYRK2, and DYRK4 hybridized only with RNA from testis. After hybridization with the DYRK3 cDNA, very weak signals were found in organs other than testis (spleen and adrenal gland). All detected bands corresponded reasonably well with the transcript sizes of the known cDNA clones.

**FIG. 2.** Nucleotide and derived amino acid sequence of the cDNA of human DYRK2. The catalytic domain is boxed. Amino acids that are conserved in the DYRK family but rarely found in other kinases are highlighted by shaded boxes. Residues of a partially sequenced protein kinase, PSK-H2 (22), that are different from DYRK2, are shown above those of DYRK2 (amino acids 174, 324, 437, 444). An insertion found in one cDNA clone is shown in lowercase letters, and the resulting N-terminal extension of the protein sequence is given in thin print. Additional sequence information of the 3'-untranslated region in the alternatively polyadenylated transcripts has been submitted to the EMBL data base (accession number Y13493).
We also studied expression of DYRK-related kinases in testes of prepuberal (2 weeks of age), puberal (8 weeks), and mature rats (16 weeks). As shown in Fig. 4B, low levels of the large transcript of DYRK1A (arrow) were detected that did not change during puberty. A strongly hybridizing transcript of about 3 kb (asterisk) appeared only in puberal and adult rats; this band most likely represents the cross-hybridizing mRNA of DYRK1B. The transcripts for DYRK1B, DYRK2, DYRK3, and DYRK4 were detected only after onset of spermatogenesis.

Subcellular Localization of DYRK1A and DYRK2—DYRK1A harbors a bipartite nuclear localization signal in its N-terminal region (1), whereas no targeting signal was identified in DYRK2. In order to evaluate the functional significance of this difference, we expressed GFP fusion proteins of DYRK1A and DYRK2 in two different mammalian cell lines (COS-7 cells and HEK293 cells). The subcellular localization of the recombinant proteins was determined by fluorescence microscopy of intact cells (Fig. 5) and by biochemical fractionation of transfected COS-7 cells (Fig. 6). In both cell lines, GFP-DYRK1A was found to be present in the nucleus (Fig. 5). The nuclei were not stained homogeneously in that the fluorescent signal was excluded from the nucleolus and appeared concentrated in discrete, irregularly shaped speckles. Consistent with the microscopic evaluation, GFP-DYRK1A was found mainly in the nuclear fraction (600 × g pellet) of transfected COS-7 cells (Fig. 6). In contrast, GFP-DYRK2 transiently expressed in COS-7 cells was not restricted to the nucleus. Moreover, in a small fraction of COS-7 cells (~2%, not shown in Fig. 5) and in the majority of HEK293 cells transfected with GFP-DYRK2, staining was largely excluded from the nucleus. Furthermore, after biochemical fractionation of COS-7 cells GFP-DYRK2 could not be detected in the nuclear fraction (Fig. 6). Possibly, the ab-
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Fig. 4. Expression of DYRK-related kinases in different rat tissues. Northern blots containing total RNA (15 μg/lane) from different tissues of adult rats (A and B; brain; H; heart; M; skeletal muscle; Lu; lung; Th; thymus; S; spleen; Li; liver; I; intestine; O; ovary; Te; testis; F; adipose tissue; A; adrenal gland) or from testes of rats of different ages (B, 2, 8, and 16 weeks of age) were hybridized with 32P-labeled cDNA probes for the indicated protein kinases. Because of the very high sequence similarity of DYRK4A and DYRK4B (Fig. 1A), transcripts hybridizing with the human DYRK4A probe may represent either one or both isoforms. Migration of 18S rRNA (1.9 kb) and 28S rRNA (2.5 kb) is marked by an arrow. Ethidium bromide staining of a gel loaded with the same RNA samples as the blots.

Expression of DYRK-related kinases in different rat tissues.

Subnuclear Distribution of DYRK1A—A series of deletion constructs of GFP-DYRK1A was prepared in order to identify the domain(s) of DYRK1A that direct nuclear localization and confer its distinct subnuclear distribution (Fig. 7A). As shown in Fig. 7B, a GFP fusion protein containing the N-terminal region of DYRK1A (GFP-DYRK1A1–176) exhibited exactly the same punctate pattern of nuclear staining as GFP-DYRK1A. A short fragment of DYRK1A containing the bipartite nuclear localization signal (DYRK1A105–139) was sufficient to direct the fusion protein to the nucleus. In contrast to GFP-DYRK1A, this construct appeared homogeneously distributed in the nucleus with no indication of a specific subnuclear localization. A construct comprising amino acids 1–104 of DYRK1A was only slightly enriched in the nucleus but appeared to be excluded from nucleolus similar to GFP-DYRK1A (see also next paragraph). This result suggests that GFP-DYRK1A1–104 is able to bind to specific intranuclear target structures once it enters the nucleus by random diffusion due to its small size (30).

The subcellular and subnuclear localization of the GFP-DYRK1A deletion constructs was also studied by biochemical fractionation of transfected COS-7 cells. To characterize the subnuclear distribution of DYRK1A, a “nuclear matrix” fraction was prepared by treatment of nuclei with a nonionic detergent, digestion with nucleases, and extraction with a buffer of high ionic strength (19–21) (Fig. 8A). By this method, most of the chromatin and all soluble and loosely bound proteins (“chromatin” fraction) are removed from an insoluble fraction (“matrix”) containing the peripheral nuclear lamina and an intranuclear skeletal network. As shown in Fig. 8B, GFP-DYRK1A and GFP-DYRK1A1–176 were found in the nuclear matrix, with no signal being detected in the soluble fractions (S, Ch). Reacting bands in the insoluble cytoplasmic fraction (100,000 × g pellet, P) probably represent contaminating nuclear proteins, since the first centrifugation step at 600 × g favors purity of the nuclear fraction rather than complete sedimentation of nuclei. In contrast, the majority of GFP-DYRK1A105–139 was found in the soluble fractions. This result indicates that GFP-DYRK1A105–139 lacks the domain necessary for the interaction of DYRK1A with the nuclear matrix, corresponding with the lack of the punctate subnuclear distribution of GFP fluorescence in COS-7 cells transfected with this construct (Fig. 7B). GFP-DYRK1A105–139 was mainly found in the “cytoplasmic” fractions (600 × g supernatant, S and P in Fig. 8), although its nuclear localization was clearly demonstrated by fluorescence microscopy (Fig. 7B). As observed with other proteins, this lack of correlation between data obtained by microscopy and cell fractionation is likely to result from leakage of soluble proteins during fractionation (32, 33). Interestingly, GFP-DYRK1A1–104 essentially resembles GFP-DYRK1A in its pattern of distribution. This result suggests that this region of DYRK1A (amino acids 1–104) mediates its association with an insoluble target structure, i.e. the nuclear matrix. Taken together, there is a strong correlation between the copurification of a GFP-DYRK1A construct with the nuclear matrix fraction (Fig. 8) and its association with the irregularly shaped subnuclear speckles visualized by fluorescence microscopy (Fig. 7B).

Protein Kinase Activity of DYRK-related Kinases—To characterize the enzymatic properties of DYRK-related kinases, we prepared recombinant proteins of DYRK1A, DYRK2, DYRK3, MNB, and Yak1p. Of the latter one, only the catalytic domain was expressed as a fusion protein with glutathione S-transferase (GST). The other constructs contained the full open reading frames of the kinases either fused with GST (GST-DYRK1, GST-DYRK2, and GST-DYRK3) or with maltose-binding protein (MBP-MNB, see “Materials and Methods”). Fusion proteins were expressed in E. coli and partially purified by affinity adsorption. Under these conditions, GST-DYRK1A is isolated as a truncated protein of 90 kDa (1). Migration of GST-DYRK2 in denaturing polyacrylamide gels corresponded with the expected size of 84 kDa, whereas bacterial expression of GST-DYRK3 yielded a band of 97 kDa (calculated molecular mass,
and a fragment of 46 kDa. MBP-MNB was detected as a single band of 103 kDa which also appears to represent a truncated product (calculated molecular mass, 132 kDa). The GST-Yak1p fusion protein migrated at the expected position (67 kDa). Fig. 9 illustrates that all recombinant proteins contained phosphotyrosine as detected with a specific antibody. A catalytically inactive point mutant of DYRK1A (DYRK-K188R) did not react with the antibody, indicating that tyrosine phosphorylation of the recombinant kinases reflects indeed their intrinsic tyrosine kinase activity.

Fig. 10 illustrates the protein kinase activity of the recombinant proteins toward different preparations of histone in vitro. GST-DYRK1A, GST-DYRK2, GST-DYRK3, and MBP-MNB catalyzed the incorporation of $^{32}$P into histone H3 and histone type II-S, a fraction of calf thymus histones commercially available from Sigma. In contrast, histone H2B was only phosphorylated by DYRK2 and DYRK3 but not by DYRK1A and MNB. As determined by thin layer chromatography of phosphoamino acids, all histones phosphorylated in these experiments contained phosphoserine/threonine with no phosphotyrosine being detectable (data not shown). This result is discordant with our previous report that DYRK1A catalyzed phosphorylation of tyrosine residues in histone IIS (1). In these experiments (Figs. 5D and Fig. 6 in Ref. 1), a radioactively labeled product of hydrolysis was misidentified as phosphotyrosine. Under improved conditions of phosphoamino acid analysis, this unidentified molecule was clearly separated from the phosphotyrosine standard as well as from phosphotyrosine in hydrolysates of histone phosphorylated by the insulin receptor tyrosine kinase (data not shown). Thus, at present there is no evidence that DYRK1A has tyrosine kinase activity toward exogenous substrates.

We failed to detect in vitro kinase activity of GST-Yak1p toward histone, casein, or in autophosphorylation reactions (data not shown), although this construct was obviously able to catalyze tyrosine autophosphorylation in E. coli (Fig. 9). Thus, GST-Yak1p might have lost its enzymatic activity during the partial purification of the protein. It should be noted that an equivalent DYRK1A construct, containing only the catalytic domain fused to GST, is still an active protein kinase.

**DISCUSSION**

The human DYRK1A gene on chromosome 21 has received considerable attention because of its potential role in the mental retardation of Down’s syndrome (7–12, 34). It is shown here that DYRK1A is a member of a small family of protein kinases that comprises at least seven different genes in mammals. These kinases exhibit characteristic common structural and biochemical features as well as striking functional differences. In particular, DYRK1A differs from other members of the family by three criteria as follows: 1) ubiquitous expression versus predominant expression in testis (DYRK1B and DYRK2–4); 2)
localization in specific subnuclear structures versus cytoplasmic localization (DYRK2); and 3) substrate specificity in histone kinase assays (DYRK2 and DYRK3).

The most striking common biochemical property of the DYRK kinases is their capability to autophosphorylate tyrosine residues (Fig. 9). Conservation of this feature in kinases from mammals, Drosophila, and yeast suggests that tyrosine autophosphorylation is important for the function of DYRK-related kinases. Toward exogenous substrates, e.g. histone (Fig. 10), the DYRK-related kinases exhibited different substrate specificity. Although the physiological substrates are not yet known, it is likely that DYRK1A/MNB and DYRK2/DYRK3 phosphorylate different substrates in vivo and may thus control different cellular processes.

Strong evidence for different cellular functions of DYRK1A and DYRK2 is also provided by their different patterns of subcellular distribution. A GFP-DYRK1A fusion protein was found in the nucleus of transfected COS-7 or HEK293 cells, whereas GFP-DYRK2 was localized in the cytoplasm. It has been shown by Song et al. (35) that a GFP fusion protein similar to GFP-DYRK1A1–176 (Fig. 7A) is targeted to the nucleus of transiently transfected NIH 3T3 cells. This result was confirmed and extended by our analysis of GFP-DYRK1A deletion constructs. The presumed nuclear localization signal is indeed sufficient to direct a GFP fusion protein to the nucleus. Furthermore, the present data show that GFP-DYRK1A is associated with a distinct subnuclear compartment, microscopically visualized as irregularly formed speckles. In cell fractionation experiments, GFP-DYRK1A was co-purified with the nuclear matrix, originally defined by Berezney and Coffey (19, 20) as the insoluble skeletal framework within the nucleus. This specific subnuclear localization of DYRK1A was not conferred by the nuclear localization signal alone but depended on the presence of the N-terminal region of the protein (amino acids
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![Diagram](image)

**FIG. 8.** Association of GFP-DYRK1A with the nuclear matrix. *A,* scheme of the fractionation procedure. *B,* immunochromic detection of GFP fusion proteins in cytoplasmic and nuclear fractions of COS-7 cells. Protein samples corresponding to the same fraction of the cell homogenate were separated by SDS-PAGE (GFP-DYRK1A, 8% gel; other constructs, 16% gel) and blotted onto PVDF membranes. GFP fusion proteins were detected with a specific antiserum and peroxidase-mediated chemiluminescence. *S* or *Sup.*, supernatant; *Ch,* chromatin; *Ma,* matrix; *P,* pellet.

**FIG. 9.** Tyrosine autophosphorylation of DYRK family kinases. Five different kinases of the DYRK family were expressed in *E. coli* as fusion proteins with either GST (DYRK1A, DYRK2, DYRK3, Yak1p) or MBP (MNB). To control for antibody specificity, a catalytically inactive mutant of DYRK1A (GST-DYRK1A-K188R) was analyzed in parallel. Fusion proteins were partially purified by affinity adsorption and separated by gel electrophoresis (8% acrylamide gel). Phosphotyrosine was detected immunochemically with a specific monoclonal antibody and 125I-protein A.

**FIG. 10.** Histone kinase activity of DYRK1A, DYRK2, DYRK3, and MNB. The indicated histone preparations (histone H3, histone H2B, and histone type II-S) were incubated with GST-DYRK1, GST-DYRK2, GST-DYRK3, or MBP-MNB in the presence of [γ-32P]ATP. Reaction products were separated by SDS-PAGE (16% gel), and the dried gel was subjected to autoradiography. A Coomassie-stained gel loaded with aliquots of the different histones is shown at the right.

In addition to substrate specificity and subcellular distribution, DYRK-related kinases differ strikingly in their tissue distribution. Preponderant expression of MNB has been found in *Drosophila* embryonic and larval brain. Several authors have reported that mRNA of DYRK1A is present in all human, murine, and rat tissues studied (1, 7–9). In contrast, all other kinases identified in the present study (DYRK1B are DYRK2–4) are predominantly expressed in the testes of adult rats. Transcripts for the new DYRK-related kinases were not detectable in testes of prepuberal rats, suggesting that these kinases are involved in spermatogenesis. It should be noted that their transcripts are not strictly testis-specific, since we have isolated cDNA clones for DYRK2, DYRK3, and DYRK4 (but not for DYRK1B)

Furthermore, the dbEST data base (39) contains a considerable number of expressed sequence tags for DYRK2–4 that were isolated from other predominantly fetal tissues (brain, liver, spleen). Thus, in addition to their function in testis, kinases of the DYRK family may play a role in embryonal development.

Sequence comparisons of DYRK1, DYRK2, DYRK3, MNB, and Yak1p allowed a definition of the common structural features of the DYRK family. Throughout the catalytic domain, members of the DYRK family share characteristic residues that are rarely found in other kinases (highlighted by shaded boxes in Figs. 2 and 3). The most striking common motifs are located in the core of the catalytic domain as follows: the conserved sequences of subdomains VI (HCDLKPEN), VII (DFGSSC), and VIII (YXYIQSRFYR/S/APE, where X is Q, H, or T). Mutational analysis has shown that tyrosines in the latter motif (Y319XY321 in DYRK1A) are essential for the activity of DYRK1A (1). In analogy with the mitogen-activated protein kinases, these tyrosines are likely to represent the site of an activating phosphorylation of DYRK1A. Since all DYRK-related kinases share the YXY motif in the activation loop, we suggest that their activity is regulated through a similar mechanism. Outside the catalytic domain, only a small region (20 amino acids) preceding the catalytic domain is conserved in the DYRK family. These sequence motifs are found in all members of the DYRK family that are presently accessible in the database, including several sequences that were recently identified in genome sequencing projects of fission yeast (*Schizosaccharomyces pombe*), the roundworm *Caenorhabditis elegans,* and *Drosophila melanogaster* (GenBank accessions Z54354; Z50142 and Z69885, Z70308, L43478, and L43480, respectively). In addition, two ESTs from *Arabidopsis thaliana* (accession numbers N65563 and R84412) apparently encode protein kinases with the Ser-Ser-Cys motif and a cysteine in

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3 S. Leder and W. Becker, unpublished data.

4 S. Leder and W. Becker, unpublished data.
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subdomain VI B. The conservation of DYRK-related kinases in plants, fungi, and animals suggests that these enzymes regulate fundamental functions of the eukaryotic cell.

In view of the interest focused on the DYRK1 gene on human chromosome 21, it is noteworthy that there are three very similar murine isoforms of DYRK1 (DYRK1A, -B, and -C). cDNA fragments for DYRK1B were also amplified from human testis, whereas the existence of a third isoform of DYRK1 can presently only be inferred from the data base entry of a partial murine cDNA clone (Fig. 1). The presence of multiple isoforms of DYRK1 points to an important role of this enzyme. However, the different patterns of expression of DYRK1A and DYRK1B indicate that these kinases are not functionally redundant but may play similar roles in the regulation of nuclear functions in different organs.

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