Developmental Expression and Distinctive Tyrosine Phosphorylation of the Eph-related Receptor Tyrosine Kinase Cek9

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Abstract. Cek9 is a receptor tyrosine kinase of the Eph subfamily for which only a partial cDNA sequence was known (Sajjadi, F.G., and E.B. Pasquale. 1993. *Oncogene.* 8:1807–1813). We have obtained the entire cDNA sequence and identified a variant form of Cek9 that lacks a signal peptide. We subsequently examined the spatio-temporal expression and tyrosine phosphorylation of Cek9 in the chicken embryo by using specific antibodies. At embryonic day 2, Cek9 immunoreactivity is concentrated in the eye, the brain, the posterior region of the neural tube, and the most recently formed somites. Later in development, Cek9 expression is widespread but particularly prominent in neural tissues. In the developing visual system, Cek9 is highly concentrated in areas containing retinal ganglion cell axons, suggesting a role in regulating their outgrowth to the optic tectum. Unlike other Eph-related receptors, Cek9 is substantially phosphorylated on tyrosine in many tissues at various developmental stages. Since autophosphorylation of receptor protein–tyrosine kinases typically correlates with increased enzymatic activity, this suggests that Cek9 plays an active role in embryonic signal transduction pathways.

RECEPTOR protein–tyrosine kinases play critical roles in signal transduction pathways by relaying signals from the cell exterior to the interior. They accomplish this by phosphorylating proteins on tyrosine. Receptor protein–tyrosine kinases are influenced by ligands, often polypeptide growth factors, that interact with the extracellular domain of the receptors, causing them to dimerize and autophosphorylate on tyrosine. Some of the autophosphorylated tyrosines and their flanking residues form binding sites for src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains of target molecules, such as other kinases, substrates, or adaptor proteins (Blaikie et al., 1994; Kavanaugh and Williams, 1994; van der Geer et al., 1994). This in turn leads to the downstream activation of signaling molecules, ultimately causing cell proliferation, differentiation, migration, or survival (Schlessinger and Ullrich, 1992; van der Geer et al., 1994), processes that are all important in embryogenesis. As a result, receptor protein–tyrosine kinases, by serving as enzymes that catalyze phosphorylation of tyrosine residues, are likely to be involved in the formation of complex anatomical structures in the developing embryo.

The Eph subfamily represents the largest group of receptor protein–tyrosine kinases, comprised of at least 13 distinct genes. The patterns of expression of members of the Eph subfamily during development suggest that these kinases are important in pattern formation (Becker et al., 1994; Ganju et al., 1994; Henkemeyer et al., 1994; Ruiz et al., 1994; Ellis et al., 1995; Xu et al., 1994; Patel et al., 1996) and axonal guidance (Cheng et al., 1995; Drescher et al., 1995; Holash and Pasquale, 1995; Henkemeyer et al., 1996). While the p85 subunit of phosphatidylinositol 3-kinase, (Src-like adapter protein) (SLAP), and the Src family kinase Fyn are the only known potential targets for Eph-related kinases (Pandey et al., 1994; 1995; Ellis et al., 1996), recently a number of related but distinct molecules have been identified as the ligands for receptors of the Eph subfamily (Bartley et al., 1994; Beckmann et al., 1994; Cheng and Flanagan, 1994; Davis et al., 1994; Shao et al., 1994; Bennet et al., 1995; Bergemann et al., 1995; Brambilla et al., 1995; Drescher et al., 1995). These ligands are unusual in that they are cell surface–associated proteins rather than soluble growth factors. Two of the ligands are transmembrane proteins (Beckmann et al., 1994; Shao et al., 1994; Bennett et al., 1995; Bergemann et al., 1995), while the others contain a consensus sequence for glycosyl phosphatidylinositol linkage. Each ligand interacts in vitro with more than one Eph-related receptor, but with varying affinities (Bartley et al., 1994; Cheng and Flanagan, 1994; Davis et al., 1994; Shao et al., 1994; Bergemann et al., 1995; Brambilla et al., 1995).

The study of the regulation of the catalytic activation of receptor protein–tyrosine kinases in tissues is likely to provide valuable insight into their in vivo functions. In the case of several receptor protein–tyrosine kinases, autophosphorylation correlates with increased enzymatic activ-

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ity towards substrates (Ellis et al., 1986; Kazlauskas et al., 1991; van der Geer and Hunter, 1991; Longati et al., 1993; Soans et al., 1994) and can be examined without knowledge of the activating ligand. Thus, the level of activation can be inferred from the level of autophosphorylation on tyrosine, which is presumably regulated by the availability of ligand. It is, therefore, important to examine not only the distribution of the kinase but also its level of tyrosine phosphorylation. The Eph-related receptor Cek5, for example, is highly expressed during retinal development in the chicken, and there is a dramatic increase in its level of tyrosine phosphorylation over a few days during retinal differentiation, suggesting an important role for Cek5 in retinal histogenesis (Pasquale et al., 1994).

Cek9 is a member of the Eph subfamily closely related to Cek5 (Sajjadi and Pasquale, 1993). Here we report the entire Cek9 cDNA sequence and the identification of Cek9', a variant form of Cek9. We have used specific polyclonal antibodies to characterize the expression pattern of Cek9 during embryonic development and examined its level of catalytic activation in various embryonic tissues, as determined by its phosphorylation on tyrosine. Assessing the expression pattern of the activated protein is important to understand the physiological function of Cek9 and facilitate the identification of its ligand and substrates.

Materials and Methods

cDNA Library Screening

Clones extending into the 5' region of the Cek9 cDNA were searched for by screening a 10-d chicken embryo λgt11 library (Clontech Labs, Palo Alto, CA) with a probe comprised of nucleotides 1309–1912 of Cek9 and corresponding to the 5' end of the Cek9 sequence previously published (Sajjadi and Pasquale, 1993). The probe was labeled with digoxigenin dUTP (Boehringer Mannheim Corp., Indianapolis, IN) by random priming and used for hybridization at high stringency as previously described (Pasquale, 1991; Sajjadi and Pasquale, 1993). A plaque identified as positive (clone L13B) was subjected to several rounds of purification before purifying λ phage DNA with gravity-flow columns (QIAGEN, Inc., Chatsworth, CA). The insert was subcloned into pBluescript and its sequence analyzed on both strands, using the dideoxynucleotide chain-termination technique with Sequenase (United States Biochem. Corp., Cleveland, OH).

PCR Amplification

1 μl intact phage (10^5–10^7 pfu) was used as template for PCR amplification of each cDNA library using the following program: one cycle for 1.5 min at 94°C, 1 min at 52°C, and 3 min at 72°C, followed by 34 cycles for 1 min at 94°C, 2 min at 52°C, and 3 min at 72°C. The following cDNA libraries were amplified by PCR: 10-d whole chicken embryo (Clontech Labs), 13-d chicken embryonic brain (gift of Dr. Barbara Ranscht, The Burnham Institute, La Jolla, CA), 11-d chicken embryonic retina (Novagen, Inc., Madison, WI), and adult chicken brain library (Clontech Labs). The region containing the initial ATG was amplified using the oligonucleotide GGTGGAGTCTACAGAGAATCT (corresponding to nucleotides 116–136) as the sense primer and CTGACCTGGTTCATCCATGTT (corresponding to nucleotides 378–358) as the antisense primer. The juxtamembrane region was amplified using the oligonucleotide ATGGCACAG-GACCGACTGCC (corresponding to nucleotides 1792–1811) as the sense primer and ATCAATGTAATACTTCACTC (corresponding to nucleotides 1974–1955) as the antisense primer. The PCR products were separated on 1.5% agarose gels and photographed. In some cases, amplified fragments were gel purified, ligated into the pCRII cloning vector (InVitrogen, San Diego, CA), and sequenced.

Antibodies

Anti-Cek9 antibodies were prepared using two different antigens: (1) a pep-

tide corresponding to the carboxy terminus of Cek9 and (2) a histidine-tagged Cek9 fragment corresponding to the 101 carboxy-terminal amino acids. To prepare the first antibody, the peptide KVHLNQLEPVEV, corresponding to the 12 carboxy-terminal amino acids of Cek9, was coupled to BSA using glutaraldehyde (Harlow and Lane, 1988) and injected in rabbits to raise immune serum. The antipeptide antibodies were affinity purified on a column in which the peptide antigen was coupled to N-hydroxysuccinimide–activated agarose (Affi-Gel 10, BioRad Labs, Hercules, CA). To prepare the second antibody, the cDNA coding for the COOH-terminal 101 amino acids of Cek9 was amplified by PCR using the oligonucleotides GCAAGCCATCTGCTCTCAAA (corresponding to nucleotides 2834–2853) as the sense primer and GAGTCTGGTGAAATAGAGAC (corresponding to nucleotides 3169–3150) as the antisense primer. A plasmid containing the appropriate region of the Cek9 cDNA was used as the template. The amplified segment was gel purified and cloned into the pCRII cloning vector (InVitrogen). Several of the resulting plasmids were sequenced to confirm that no mutations were introduced. One plasmid was digested in the polylinker region with the restriction enzymes BamHI and PstI to release the amplified fragment. The BamHI-PstI fragment was then cloned into the expression vector pQE31 (Qiagen, Inc.) to overexpress the Cek9 COOH-terminal fragment as a histidine-tagged fusion protein in M15 (pREP4) cells (Qiagen, Inc.). After induction, the bacteria were resuspended in 8 M urea, 0.1 M NaPi, and 0.01 M Tris, pH 8.0, and lysed by sonication. The lysate was centrifuged at 15,000 g for 20 min and the supernatant, containing the histidine-tagged Cek9 fragment, was run through a Ni-NTA column (Qiagen, Inc.). After washing with the same urea buffer at pH 6.3, the Cek9 polypeptide was eluted with the urea buffer at pH 4.5. After dialysis against PBS, the purified Cek9 polypeptide was used to inject rabbits to raise immune serum.

The antibodies to the histidine-tagged Cek9 carboxy terminus were affinity-purified using two different columns. One column was prepared by coupling the histidine-tagged Cek9 fragment, purified by SDS-PAGE, and eluted from the acrylamide to Affi-Gel 15. The second column was prepared by coupling to Affi-Gel 10 a glutathione-S-transferase (GST)[1]–Cek9 fusion protein purified with glutathione-agarose beads according to the manufacturer's recommendations (Pharmacia, LKB Biotechnology, Piscataway, NJ). To produce the GST–Cek9 carboxy terminus fusion protein in the XL1 Blue strain of Escherichia coli, the cDNA coding for the carboxy terminus of Cek9 was excised from the pQE31 vector by digestion at two EcoRI sites flanking it and cloned into the vector pGEX-1 (Pharmacia LKB Biotechnology). Each antibody was further purified by incubation with the insoluble material obtained after sonication of bacteria expressing a β-galactosidase–Cek5 fusion protein (Pasquale, 1991). This removes antibodies that may be present recognizing Cek5 or bacterial proteins. Anti-Cek9 carboxy-terminal peptide antibodies were used for the whole-mount immunolocalization experiments (see Figs. 4 and 5). The anti-Cek9 antibodies purified with the histidine-tagged Cek9 carboxy terminus were used in the immunoprecipitation experiments and for the immunoblotting experiments in Fig. 10 B (except for cerebrum and thigh muscle) and Fig. 11. The antibodies purified with the GST–Cek9 carboxy terminus were used for the remaining immunoblotting experiments and for immunohistochemistry, except for the whole-mount experiments. The different antibody preparations were compared in immunoblotting experiments and they produced similar results. However, when used to probe whole tissue extracts, the antipeptide antibodies produced a weak signal and the antibodies purified on the histidine-tagged antigen produced a higher background than those purified on the GST–Cek9 fusion protein. The preparation of antiphosphotyrosine, anti-Cek4, and anti-Cek8 antibodies have been described previously (Soans et al., 1994). Anti-Cek5 antibodies were prepared using the peptide QMNQIQSVEV, which corresponds to the 10 carboxy-terminal amino acids, coupled to BSA with glutaraldehyde (Harlow and Lane, 1988), and purified on an affinity column containing a GST–Cek5 carboxy terminus fusion protein comprising amino acids 897–995 of Cek5. Anti-GST antibodies were obtained from immune serum raised to the GST–Cek5 carboxy terminus fusion protein and affinity-purified using bacterially expressed GST coupled to N-hydroxysuccinimide–activated agarose (Affi-Gel 10).

Immunoblotting

Bacteria, dissected tissues, or whole chicken embryos were sonicated in PBS containing 1 mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitor

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1. *Abbreviation used in this paper: GST, glutathione-S-transferase.*

units of aprotinin/ml, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate. The samples were stored in aliquots at −70°C. Equal amounts of protein were loaded in each lane after boiling the lysates for 3 min in sample buffer containing SDS. The tissue extracts or bacterial lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose filters were stained with amido black to confirm that equal amounts of protein were present in each lane, blocked overnight in 3% BSA in TBS and incubated for 4 h in 3% BSA in TBS containing 0.1 μg/ml anti-Cek9 antibody (see Fig. 2), 3 μg/ml anti-Cek9 antibody, 3 μg/ml anti-phosphotyrosine antibody, or 0.8 μg/ml anti-GST antibodies. The immunoblots were incubated with 0.2 μg/ml protein A–peroxidase (Sigma Chemical Co., St. Louis, MO) in 3% BSA in TBS for 1 h, rinsed several times with TBS, and developed using enhanced chemiluminescence reagents (Amersham Corp., Arlington Heights, IL). The membranes were reprobed with different antibodies after drying for a few hours, followed by incubation in blocking solution containing 0.2% sodium azide to inhibit the peroxidase activity remaining on the filters.

Immunoprecipitation

Tissue extracts were diluted in RIPA buffer (150 mM sodium chloride, 10 mM sodium phosphate, pH 7.2, 1% deoxycholate, 1% Triton X-100, 0.1% SDS) and 0.1 mM sodium orthovanadate and precleared with Staph A. Whenever antiphosphotyrosine antibodies were to be used for immunoblotting of the immunoprecipitates, phosphotyrosine was added to the sonicated extract to a final concentration of 8 mM. The samples were then incubated for 40 min with 5 to 10 μg of antibodies that had previously been absorbed to 5–10 μl Staph A. The immunoprecipitated material was washed three times with RIPA buffer and once with PBS. Sample buffer was added and the immunoprecipitates were boiled for 5 min, separated on 7.5% polyacrylamide gels, and then transferred onto nitrocellulose for immunoblotting. Tissue extracts were used in excess, so that the amounts of immunoprecipitated receptors would not be dependent upon their expression levels.

To determine the fraction of tyrosine-phosphorylated Cek9, 50 μg of anti-Cek9 antibody was used to immunoprecipitate Cek9 from a 10-d embryonic retina extract. The immunoprecipitate, which contained both phosphorylated and nonphosphorylated Cek9, was boiled in 0.1 ml 1% SDS for 5 min, centrifuged for 3 min to remove the insoluble Staph A, and the supernatant was transferred to a fresh tube containing 0.9 ml of RIPA buffer lacking SDS. This was added to 50 μg of antiphosphotyrosine antibody preabsorbed to Staph A to immunoprecipitate the tyrosine phosphorylated fraction of Cek9. The sample was incubated on ice for 40 min with occasional vortexing and then centrifuged for 3 min. The supernatant, containing nonphosphorylated Cek9, was transferred to a different tube containing 50 μg of anti-Cek9 antibodies preabsorbed to Staph A and subjected to immunoprecipitation. The immunoprecipitates were then probed with antiphosphotyrosine antibodies or anti-Cek9 antibodies after separation on a 7.5% polyacrylamide gel.

Immunohistochemistry

Embryos were removed from the eggs, fixed in 4% paraformaldehyde and 0.1 mM sodium orthovanadate in PBS for 16 to 24 h, and then cryoprotected in 20% sucrose in PBS and 0.1 mM sodium orthovanadate. Embryos were then embedded in OCT compound (Miles Inc., West Haven, CT) and frozen in dry ice/2-methylbutane. Cryostat sections, 10-μm thick, were collected on polylysine-coated glass slides and stored at −70°C. The sections were treated with 0.3% H_2O_2 for about 10 min, followed by a blocking buffer containing 1.5% normal goat serum in PBS for 30 min. Sections were incubated in rabbit anti-Cek5 or anti-Cek9 antibodies (5–10 μg/ml) in 3% BSA for 30 min. After incubation with the primary antibody, the sections were rinsed several times with PBS and incubated with biotinylated goat anti–rabbit antibody (Vector Labs, Inc., Burlingame, CA) for 30 min. Antibody binding was visualized using the Vectastain ABC elite avidin-biotin-horseradish peroxidase technique (Vector Labs). After reacting in diaminobenzidine for ∼2–3 min, the sections were rinsed in PBS, air dried, mounted in Permount, and sealed with a No. 1 coverslip. Controls involved preincubation of the affinity-purified antibodies with the antigen before use. Specimens were photographed with an inverted microscope (model 405M; Carl Zeiss, Inc., Thornwood, NY).

Whole-mount immunohistochemistry was performed as described by Henkemeyer et al. (1994), with the following minor modifications: The concentration of Triton X-100 used throughout was 0.1%. The embryos were bleached in methanol-DMSO-30% H_2O_2 (4:1:1). Anti-Cek9 peptide

antibodies were used at a concentration of 1 μg/ml. The specificity of the anti-Cek9 antibody staining was confirmed by incubating the antibodies with the peptide antigen (50 μg/ml) for 30 min before addition to the embryos. As a further control, preimmune serum was used at a dilution of 1:10,000 to obtain a concentration of antibodies of ∼1 μg/ml. Antibody binding was visualized using the Vectastain ABC elite avidin-biotin-horseradish peroxidase technique (Vector Labs, Inc.). Embryos were incubated in diaminobenzidine for 20 min and then with diaminobenzidine, H_2O_2 and $NiCl_2$. Usually 2–3 min proved sufficient for color development. After staining, the embryos were washed in PBT (PBS containing 0.2% BSA, 0.1% Triton X-100), followed by PBS, and photographed in PBS. Alternatively, after washing in PBT, the embryos were dehydrated in a methanol/PBS series, cleared in (benzyl alcohol-benzyl benzoate [1:2])-methanol (1:1) for 20 min and photographed in (benzyl alcohol-benzyl benzoate [1:2]). Kodak Gold 100 print film (Kodak Co., Rochester, NY) was used for photography with a stereomicroscope (model Stemi SV11; Carl Zeiss, Inc.).

In Situ Hybridization

Embryos were fixed in 4% paraformaldehyde and embedded in paraffin. 5-μm sections were cut with a microtome and mounted on slides (model Superfrost Plus; Fisher Scientific, Pittsburgh, PA). In situ hybridization was performed according to standard procedures (Simmons et al., 1989). Briefly, deparaffinized sections were deproteinated in 0.2 M HCl and treated with 2.5 μg/ml proteinase K. After acetylation and dehydration in ethanol, the sections were air dried. The sections were then incubated overnight with the Cek9 ^{33}P-labeled riboprobes (about 10^4 dpm/μl) in hybridization buffer (300 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1× Denhardt's, 0.2% SDS, 10 mM DTT, 0.25 mg/ml tRNA, 50% formamide, 10% dextran sulfate) at 50°C. After hybridization, the slides were immersed in 4× SSC (150 mM NaCl, 15 mM Na_3 citrate·$2H_2O$), followed by two 15-min washes in 2× SSC at 63°C, RNaseA digestion, one 30-min wash in 0.1× SSC at 63°C, and one 10-min wash at room temperature. After dehydration, the sections were air dried, exposed to x-ray film for 2 d, and then dipped in photographic emulsion and exposed for 2 wk before development. The developed slides were then counterstained with cresyl violet and photographed.

The sense and antisense riboprobes were prepared by using a Cek9 fragment (nucleotides 490–710), amplified by using the polymerase chain reaction, and cloned in the pCRII vector (InVitrogen) as a template. After linearization of the plasmids with BamHI, the riboprobes were synthesized with T7 RNA polymerase using a Promega in vitro transcription system (Madison, WI) and [33P]UTP, according to the specifications of the manufacturer for the preparation of high specific activity riboprobes, with some modifications. The modifications were as follows: the reaction volume was reduced from 20 to 11.5 μl, and spin columns (model CHROMA SPIN-10; Clontech Labs) were used to remove unincorporated nucleotides.

Results

Cek9 and Cek9'

A partial Cek9 cDNA clone (comprised of nucleotides 1309–3712 in Fig. 1 A) was previously isolated by screening a 10-d chicken embryonic cDNA library using a Cek5 cDNA probe (Sajjadi and Pasquale, 1993). However, this clone encodes only a small portion of the Cek9 extracellular domain. By screening the same 10-d whole embryo cDNA library with a 0.6-kb probe corresponding to the 5′ end of the Cek9 cDNA previously isolated, a 2342-bp clone (L13B), corresponding to Cek9′ in Fig. 1 A, was identified. Clone L13B encodes the entire extracellular and transmembrane domains of Cek9 and a portion of the cytoplasmic domain. However, the ATG predicted to encode the initial methionine is not followed by a typical hydrophobic signal peptide sequence and corresponds to the region immediately following the signal peptide of other Eph-related receptors. Hence, clone L13B appears to encode a form of Cek9 lacking a signal peptide. To test
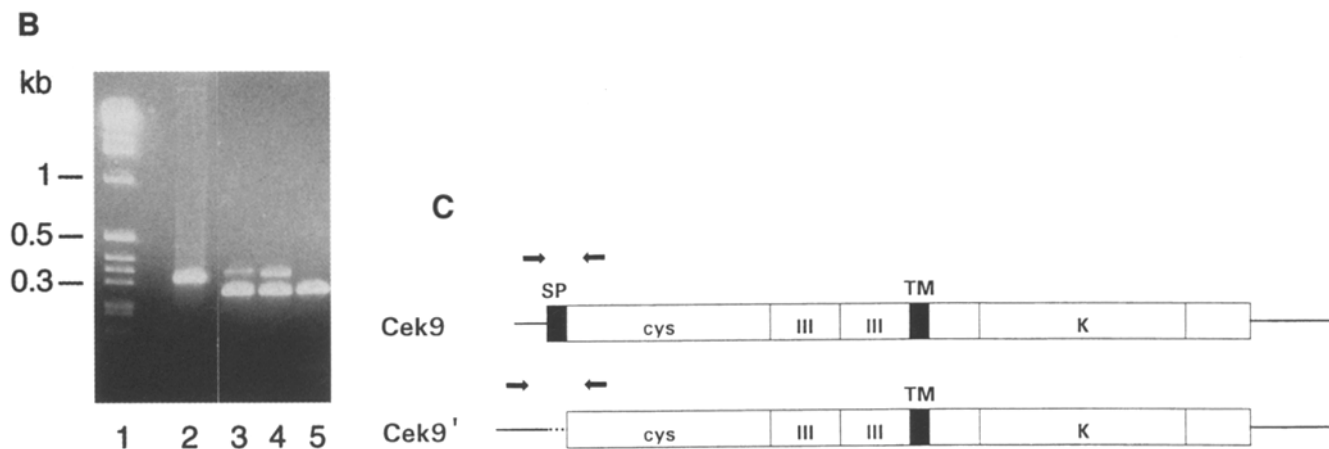
A

Cek9'

CGGCTCTGACTTTGTGTTAACGGTTTATGGACTGGTTCCAAAGAGCTCAAAGGTACCAAAACACTCCAAGCAACCTCTGAACCATTCAAGCAAGTAGTGTGTGTTTATTGGATATGGTGG
AGTCTACAGAGAATCTTCATGGATTCTAATGCTGACATCAGTGCAAGAAGAGTGTCAGGAATGGATTGGCTCTGGCTGGTTTGCTTCTTTCATCTAGTCACTTCACTAGAAGACCTGCAT

                 M T E I L L D T T G E T S E I G W T S H P P D G
CCTGACCAACCGGAAAGGTGAGCAGGATGAGGCCATTGGTGGTGCTGTCATGACTGAAATACTTCTGGATACAACTGGAGAAACCTCAGAGATTGGCTGGACCTCTCACCCTCCTGATGG

  W E E V S V R D D K E R Q I R T F Q V C N M D E P G Q N N W L R T H F..............
GTGGGAAGAAGTAAGTGTCCGGGATGATAAGGAGCGCCAGATCCGAACCTTTCAAGTTTGTAACATGGATGAACCAGGTCAGAATAACTGGTTGCGTACTCACTTC..............

Cek9

CGGCTCTGACTTTGTGTTAACGGTTTATGGACTGGTTCCAAAGAGCTCAAAGGTAC	56
M D S N A D I S A R R V S	13
CAAAACACTCCAAGCAACCTCTGAACCATTCAAGCAAGTAGTGTGTGTTTATTGGATATGGTGGAGTCTACAGAGAATCTTCATGGATTCTAATGCTGACATCAGTGCAAGAAGAGTGTC	176
G M D W L W L V C F F H L V T S L E ↓E I L L D T T G E T S E I G W T S H P P D G	53
AGGAATGGATTGGCTCTGGCTGGTTTGCTTCTTTCATCTAGTCACTTCACTAGAAGAAATACTTCTGGATACAACTGGAGAAACCTCAGAGATTGGCTGGACCTCTCACCCTCCTGATGG	296
W E E V S V R D D K E R Q I R T F Q V C N M D E P G Q N N W L R T H F I E R R G	93
GTGGGAAGAAGTAAGTGTCCGGGATGATAAGGAGCGCCAGATCCGAACCTTTCAAGTTTGTAACATGGATGAACCAGGTCAGAATAACTGGTTGCGTACTCACTTCATAGAGCGACGTGG	416
A H R V H V R L H F S V R D C A S M R T V A S T C K E T F T L Y Y H Q S D V D I	133
AGCCCACCGAGTCCATGTCCGCCTTCATTTCTCAGTGAGGGACTGTGCCAGCATGCGTACTGTGGCCTCTACTTGCAAAGAGACTTTCACACTCTACTACCACCAGTCAGATGTCGACAT	536
A S Q E L P E W H E G P W T K V D T I A A D E S F S Q V D R T G K V V R M N V K	173
AGCCTCTCAGGAACTGCCAGAGTGGCATGAAGGCCCCTGGACCAAGGTGGATACTATTGCAGCTGATGAAAGCTTTTCCCAGGTGGACAGAACTGGGAAGGTGGTAAGGATGAATGTTAA	656
V R S F G P L T K H G F Y L A F Q D S G A C M S L V A V Q V F F Y K C P A V V K	213
AGTACGCAGCTTTGGGCCACTCACAAAGCATGGCTTCTACCTGGCCTTCCAGGACTCAGGAGCCTGTATGTCCCTGGTGGCAGTCCAAGTCTTTTTCTACAAGTGTCCAGCTGTGGTGAA	776
G F A S F P E T F A G G E R T S L V E S L G T C V A N A E E A S T T G S S G V R	253
AGGATTTGCCTCCTTCCCTGAAACTTTTGCTGGAGGAGAGAGGACCTCACTGGTGGAGTCACTAGGGACGTGTGTAGCAAATGCTGAAGAGGCAAGCACAACTGGGTCATCAGGTGTTCG	896
L H C N G E G E W M V A T G R C S C K A G Y Q S V D N E Q A C Q A C P I G S F K	293
GTTGCACTGCAATGGAGAAGGAGAGTGGATGGTGGCCACTGGACGATGCTCTTGCAAGGCTGGTTACCAATCTGTTGACAATGAGCAAGCTTGTCAAGCTTGTCCCATTGGTTCCTTTAA	1016
A S V G D D P C L L C P A H S H A P L P G S I E C V C Q S H Y Y R S A S D N S D	333
AGCATCTGTGGGAGATGACCCTTGCCTTCTCTGCCCTGCCCACAGCCATGTCCCACTGCCAGGTTCCATTGAATGTGTGTGTCAGAGTCACTACTACCGATCTGCTTCTGACAATTCTGA	1136
A P C T G I P S A P R D L S Y E I V G S N V L L T W R L P K D L G G R K D V F F	373
TGCTCCCTGCACTGGCATCCCCTCTGCTCCCCGTGACCTCAGTTATGAAATTGTTGGCTCCAACGTGCTCCTGACCTGGCGCCTCCCCAAGGACTTGGGTGGCCGCAAGGATGTCTTCTT	1256
N V I C K E C P T R S A G T C V R C G D N V Q F E P R Q V G L T E S R V Q V S N	413
CAATGTCATCTGCAAGGAATGCCCAACAAGGTCAGCAGGGACATGTGTGCGCTGTGGGGACAATGTACAGTTTGAACCACGCCAAGTGGGCCTGACAGAAAGTCGTGTTCAAGTCTCCAA	1376
....
L L A R V Q Y T F E I Q A V N L V T E L S S E A P Q Y A T I N V S T S Q S V P S	453
CCTATTGGCCCGTGTGCAGTACACTTTTGAGATCCAGGCTGTCAATTTGGTGACTGAGTTGAGTTCAGAAGCACCCCAGTATGCTACCATCAACGTTAGCACCAGCCAGTCAGTGCCCTC	1496
A I P M M H Q V S R A T S S I T L S W P Q P D Q P N G V I L D Y Q L R Y F D K A	493
CGCAATCCCTATGATGCATCAGGTGAGTCGTGCTACCAGTAGCATCACACTGTCTTGGCCTCAGCCAGACCAGCCCAATGGGGTTATCCTGGATTACCAGCTACGGTACTTTGACAAGGC	1616
....
E D E D N S F T L T S E T N M A T I L N L S P G K I Y V F Q V R A R T A V G Y G	533
AGAAGATGAGGATAATTCATTTACTTTGACTAGTGAAACTAACATGGCCACTATATTAAATCTGAGTCCAGGCAAGATCTATGTCTTCCAAGTACGAGCTAGAACAGCAGTGGGTTATGG	1736
P Y S G K M Y F Q T L M A G E H S E M A Q D R L P L I V G S A L G G L A F L V I	573
CCCATACAGTGGAAAGATGTATTTCCAGACTTTAATGGCAGGAGAGCACTCGGAGATGGCACAGGACCGACTGCCACTTATTGTGGGCTCAGCACTTGGTGGTCTGGCATTCTTGGTAAT	1856
A A I A I L A I I F K S K R R E T P Y T D R L Q Q Y I S T R G L G V K Y Y I D P	613
TGCTGCCATTGCCATTCTTGCCATCATCTTCAAGAGTAAAAGGCGAGAGACTCCATACACAGACCGCCTGCAGCAGTATATCAGTACACGAGGACTTGGAGTGAAGTATTACATTGATCC	1976
o o o	
S T Y E D P N E A I R E F A K E I D V S F I K I E E V I G S G E F G E V C F G R	653
TTCCACGTATGAAGATCCCAATGAAGCTATTCGAGAGTTTGCCAAAGAGATAGATGTGTCCTTCATCAAAATTGAGGAGGTCATTGGATCAGGAGAATTTGGAGAGGTGTGCTTTGGGCG	2096
o	
L K H P G K R E Y T V A I K T L K S G Y T D E Q R R E F L S E A S I M G Q F E H	693
CCTAAAACACCCAGGGAAACGTGAATACACAGTAGCTATTAAAACCCTGAAGTCAGGTTATACTGATGAACAGCGTCGAGAGTTCCTGAGCGAGGCCAGCATCATGGGGCAATTTGAGCA	2216
P N V I H L E G V V T K S R P V M I V T E F M E N G S L D S F L R Q K E G Q F S	733
TCCCAATGTCATCCACCTGGAGGGGCGTGGTCACCAAAAGCCGACCAGTCATGATTGTCACAGAATTCATGGAGAATGGATCACTGGATTCCTTCCTCAGGCAGAAGGAGGGACAGTTCAG	2336
V L Q L V G M L R G I A A G M R Y L S D M N Y V H R D L A A R N I L V N S N L V	773
TGTGTTACAGCTGGTGGGAATGCTACGAGGGATTGCAGCAGGCATGCGCTACCTTTCAGACATGAACTATGTGCATCGTGATCTCGCAGCACGTAACATCTTAGTCAACAGTAACCTTGT	2456
●	
C K V S D F G L S R F L E D D A S N P T Y T G A L G C K I P I R W T A P E A V Q	813
ATGCAAGGTGTCAGACTTTGGTTTGTCTCGCTTTCTGGAAGATGATGCTTCAAATCCCACTTATACTGGAGCTCTGGGTTGCAAAATCCCCATCCGTTGGACTGCCCCTGAAGCTGTCCA	2576
Y R K F T S S S D V W S Y G I V M W E V M S Y G E R P Y W D M S N Q D V I N A I	853
GTATCGCAAGTTCACCTCCTCCAGTGATGTCTGGAGCTATGGCATTGTCATGTGGGAGGTGATGTCCTATGGTGAGAGACCTTACTGGGACATGTCCAACCAGGATGTAATTAATGCCAT	2696
D Q D Y R L P P P P D C P T V L H L L M L D C W Q K D R V Q R P K F E Q I V S A	893
TGACCAGGACTATCGCCTGCCACCACCCCCAGACTGCCCAACTGTTTTGCATCTGCTGATGCTTGACTGCTGGCAGAAGGATCGAGTCCAGAGACCAAAATTTGAACAAATAGTCAGTGC	2816
L D K M I R K P S A L K A T G T G S S R P S Q P L L S N S P P D F P S L S N A H	933
CCTAGATAAAATGATCCGCAAGCCATCTGCTCTCAAAGCCACTGGCACTGGGAGCAGCAGACCATCTCAGCCTCTCCTGAGCAACTCCCCTCCAGATTTTCCTTCACTCAGCAATGCCCA	2936
E W L D A I K M G R Y K E N F D Q A G L I T F D V I S R M T L E D L Q R I G I T	973
CGAGTGGTTGGATGCCATCAAGATGGGTCGTTACAAGGAGAATTTTGACCAGGCTGGTCTGATTACATTTGATGTCATATCACGCATGACTCTGGAAGATCTCCAGCGTATTGGAATCAC	3056
L V G H Q K K I L N S I Q L M K V H L N Q L E P V E V *	1000
CCTGGTTGGTCACCAGAAAAAGATTCTAAACAGCATCCAGCTCATGAAAGTTCATTTGAACCAGTTGAAGTGTGATGCTTTAAGTCTCTATTTCACCAGACTCAAATTCT	3176
GAAAGAGTCCTGAGGGGATTCAGAGGGATTGTCACTGTATGAAAAGGAAATGGCAAGATGCTCCTTGAAGACTTACTGCACCTAGAGAGTAGACATTACACATTCCATTCCACCAGCAAA	3296
AAGAGAATCTTGCCATCATTTAAAAGCAGAGTTAAATAGCTGGTGGTTAAATATGACTGCCATCATACACTAGGAGTAGGTCAGGGAGGGAAAGTTATAGTAATGCAGAGTGGAGCTGGT	3416
ATAATAGTTTGGACAGACCACAAGCACCTGCTAGCTCTTCTCCACTAAATAAAAAATCAGACAATTCTCCAGTGCCATCAGCAGGCTTTATCTCGTGACTGGGAACAAAGAAATCACAATT	3536
TTTCCAAGAGAGTATCAGCACATTGTGAGAGTTATCACTCAGTTGGAAATGGACATCACTTGCTATGCCAGATTTGTGAGAAACTGGAGTTCCACTGAGTGCACCCATATGTGGTAAACAA	3656
TAAGGTACATCACCTCGTAATTTTTACAGAGGTTGAGAGTAAAGGGCCCA	3706

Figure 1. Molecular characterization of the Cek9 and Cek9' cDNAs. (*A*) Nucleotide sequence and predicted amino acid sequence of the Cek9' and Cek9 receptor tyrosine kinase cDNAs (GenBank Accession No. U23783). The amino acids comprising the presumed signal peptide sequence and the putative transmembrane domain are underlined. Potential sites for N-linked glycosylation are marked by dotted overlines. Nucleotides marked by a double underline represent the insert present in the Cek9' cDNA, but not the Cek9 cDNA. An arrow marks the position of the insertion in the Cek9 sequence. Only the 5' end of the Cek9' sequence is shown, since the remaining portion (indicated by dots) is identical to the corresponding region of Cek9. Some amino acid residues within the kinase domain that are involved in ATP binding are labeled by open circles, and tyrosine 796, corresponding to a conserved site of autophosphorylation, is indicated by a filled circle. The sequences are in bold characters, except for the ATG codons in the 5' untranslated regions and a potential polyadenylation signal (AATAAA) in the 3' untranslated region. The nucleotides are numbered starting from the first residue following the EcoRI site of λgt11. The numbering refers to the Cek9 amino acid and nucleotide sequence. (*B*) PCR amplification of chicken cDNA libraries. Ethidium bromide staining of a 1% agarose gel reveals two bands corresponding to the two variant forms Cek9 and Cek9'. The larger bands result from the amplification of Cek9', which contains an insert. Lane *1*, 1 kb DNA ladder (GIBCO BRL); lane *2*, amplification of Cek9' cDNA subcloned in pBluescript; lane *3*, amplification of an adult chicken brain cDNA library; lane *4*, amplification of a 10-d chicken embryo cDNA library; and lane *5*, amplification of an 11-d embryonic chicken retina cDNA library. The positions of the primers used are indicated in (*C*). The positions of several DNA size markers are indicated at left. (*C*) Schematic representation of the domain structure of Cek9 and Cek9'. *SP*, signal peptide; *cys*, cysteine rich region; *III*, fibronectin type III repeats; *TM*, transmembrane domain; and *K*, kinase domain. Untranslated regions are indicated by a line, the 64-bp insertion in Cek9' is indicated by a dotted line. Arrows indicate the positions of the primers used for the PCR amplifications shown in (*B*).

whether this represents a possible variant form of Cek9, we used primers upstream and downstream of the putative initial ATG for PCR amplification using chicken cDNA libraries as templates. Two bands were obtained with the 10-d whole embryo library and an adult chicken brain library, while only the lower band was detected after amplification of an 11-d embryonic retina library (Fig. 1 *B*). By Southern analysis, both bands hybridized with a digoxigenin labeled probe prepared by amplification of clone L13B with the same primers (not shown). The two products obtained from amplification of the 10-d whole embryo library were subcloned and sequenced. The upper band (326 bp) corresponds to a fragment of clone L13B, as expected based on its size, and contains an insertion of 64 bp that is not present in the lower band (262 bp). In the absence of the 64-bp insertion, the open reading frame extends further to the 5' end and an upstream ATG is predicted to represent the site for translation initiation (Cek9 in Fig. 1 *A*). The initial methionine is followed by a hydrophobic sequence that is likely to represent a signal peptide. The sequences of Cek9 and Cek9' are shown in Fig. 1 *A*, and the structure of the two isoforms is schematically represented in Fig. 1 *C*.

To search for additional isoforms of Cek9, several chicken cDNA libraries were used as templates for PCR amplification of the Cek9 juxtamembrane domain, since variant forms of several other Eph-related kinases have insertions in the juxtamembrane domain (Sajjadi and Pasquale, 1993; Maisonpierre et al., 1993; Siever and Verderame, 1994; Connor and Pasquale, 1995). The libraries included the 10-d whole embryo library, 11-d embryonic retina library and adult brain library described above, and a 13-d embryonic brain library. cDNA prepared by reverse transcription PCR from 8-d retina mRNA was also used as template. These experiments did not provide evidence for the existence of isoforms of Cek9 containing inserts in the juxtamembrane domain (not shown).

Expression of Cek9 during Embryonic Development

The carboxy terminus of the Eph receptors comprises about 100 amino acids and is one of the least conserved regions (Sajjadi and Pasquale, 1993; Fox et al., 1995). In particular, the 10–12 amino acids at the extreme carboxy terminus exhibit very little conservation. As a consequence, the carboxy terminus has been the region targeted for the preparation of antibodies specific for a number of Eph receptors (Lindberg and Hunter, 1990; Lhoták et al., 1991; Henkemeyer et al., 1994; Soans et al., 1994; Zhou et al., 1994; Holash and Pasquale, 1995). Three different antibodies recognizing the carboxy-terminal region of Cek9 were prepared to study the expression pattern of Cek9 during embryonic development. One of the antibodies was prepared by using a synthetic peptide corresponding to the

12 carboxy-terminal amino acids of Cek9 both as the antigen and for affinity-purification. A second antibody was prepared by using both as the antigen and for affinity-purification a fusion protein containing the carboxy-terminal 101 amino acids of Cek9 tagged with six histidine residues. A third antibody was prepared using the same histidine-tagged fusion protein as the antigen, and a GST–Cek9 fusion protein comprising the carboxy-terminal 101 amino acids of Cek9 for affinity purification. After affinity purification, both antifusion protein antibodies were absorbed to a bacterial extract containing a β-galactoside–Cek5 fusion protein (Pasquale, 1991). This was to remove antibodies cross-reactive with sequences conserved within the Eph subfamily that may have been present. In fact, the regions in the Cek9 carboxy terminus that are homologous to Cek5 are the same that are homologous to other Eph receptors, except that the amino acid conservation with Cek5 is more extensive than that with other more distantly related receptors (Sajjadi and Pasquale, 1993). The specificity of the antibodies was then assessed by comparing their reactivity with Cek9, Cek5, and Cek8 (Fig. 2). All three anti-Cek9 antibody preparations preferentially detect the GST–Cek9 carboxy terminus fusion protein rather than GST fusion proteins containing similar carboxy-terminal regions of the closely related kinases Cek5 and Cek8. Because the anti-Cek9 antibodies do not recognize regions of Cek5 and Cek8 that share sequence homology with Cek9, they are unlikely to recognize the same conserved regions in other more distantly related receptors. In tissue extracts, all three anti-Cek9 antibodies recognize a 120-kD protein that undergoes autophosphorylation on tyrosine in vitro and after SDS-PAGE exhibits an apparent molecular weight lower than that of Cek5 (not shown).

Because other Eph receptors exhibit interesting distribution patterns during the early stages of embryonic development (Becker et al., 1994; Ganju et al., 1994; Ruiz et al., 1994; Henkemeyer et al., 1994; Ellis et al., 1995; Xu et al., 1995; Patel et al., 1996), whole chicken embryo extracts at days 1.5 to 3.0 of development in ovo were examined by immunoblotting with anti-Cek9 antibodies to examine Cek9 expression. Cek9 can be detected by immunoblotting as early as embryonic day 1.5 and its level of expression increases with time of development (Fig. 3). The spatial distribution of Cek9 at about 2 d of development (Hamburger and Hamilton stages 13–15) was examined by whole-mount immunohistochemistry (Figs. 4 and 5). Cek9 expression is most prominent in the eye, the brain and the posterior portion of the neural tube (Fig. 4). Cek9 immunoreactivity above control levels is also apparent in the somites, with higher levels in the posterior (more recently formed) somites, but not in the condensing somites. Comparison of the expression pattern of Cek9 in the posterior region of the 2-d embryo with that of Cek8 and Cek5 (Fig. 5) reveals marked differences. In the neural tube and somites, Cek8 immunoreactivity is concentrated in more posterior regions than Cek9 immunoreactivity (Fig. 5, A and B). Cek8 is highly expressed in the primitive streak, the four or five most recently formed somites, and the condensing somites. In addition, Cek8 appears to be expressed at higher levels in the somites than in the neural tube, whereas Cek9 immunoreactivity is more prominent in the neural tube. In contrast to Cek9 and Cek8, Cek5 appears evenly expressed at low levels in the posterior region of the embryo (Fig. 5 C), similar to its mouse homologue Nuk (Henkemeyer et al., 1994). In the hindbrain region, the expression patterns of Cek9 and Cek8 also differ. While Cek8 immunoreactivity is very prominent in rhombomeres 3 and 5, the otic vesicle, and the migrating neural crest cells adjacent to rhombomere 5 (Fig. 5 D), Cek9 immunoreactivity is evenly distributed (Fig. 5 E).

Immunoperoxidase labeling of frozen sections of embryos 4–8 d old revealed similar levels of Cek9 immunoreactivity in most tissues (not shown). In the 10-d embryo, Cek9 can be detected in all the tissues that we examined by immunoblotting, except for the blood (Fig. 6). Cek9 is present in the liver (at very low levels), gizzard, thigh, and, at higher levels, the intestine and brain. Cek9 is most highly expressed in the developing retina.

To determine whether Cek9 plays a role in the development of retinotectal projections, as has been proposed for
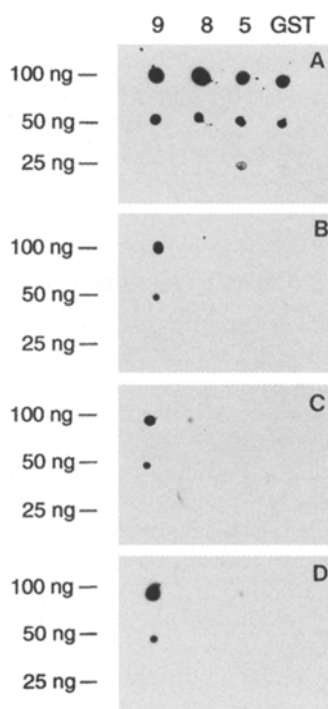
Figure 2. Specificity of the anti-Cek9 antibodies. Purified GST fusion proteins containing similar portions of the carboxy termini of Cek9 (9), Cek8 (8), and Cek5 (5), as well as GST, were spotted on nitrocellulose filters in the amounts indicated at left. They were probed with antibodies to: (A) GST; (B) the histidine-tagged Cek9 carboxy-terminal 101 amino acids, purified using the same histidine-tagged fragment of Cek9; (C) the histidine-tagged Cek9 carboxy-terminal 101 amino acids, purified using the same fragment of Cek9 as a GST fusion protein; and (D) the Cek9 carboxy-terminal peptide (see Materials and Methods section).
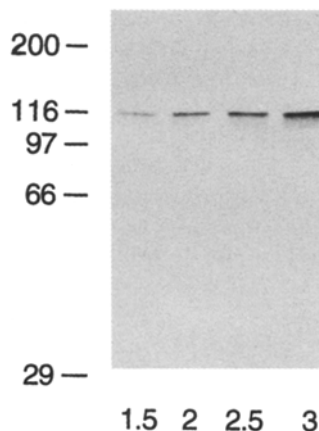

Figure 3. Developmental regulation of Cek9 expression in the early embryo. Extracts from embryos at the days of development in ovo indicated at the bottom and containing equal amounts of protein were loaded in each lane and probed with anti-Cek9 antibodies. The positions of molecular mass markers, in kD, are indicated at left.
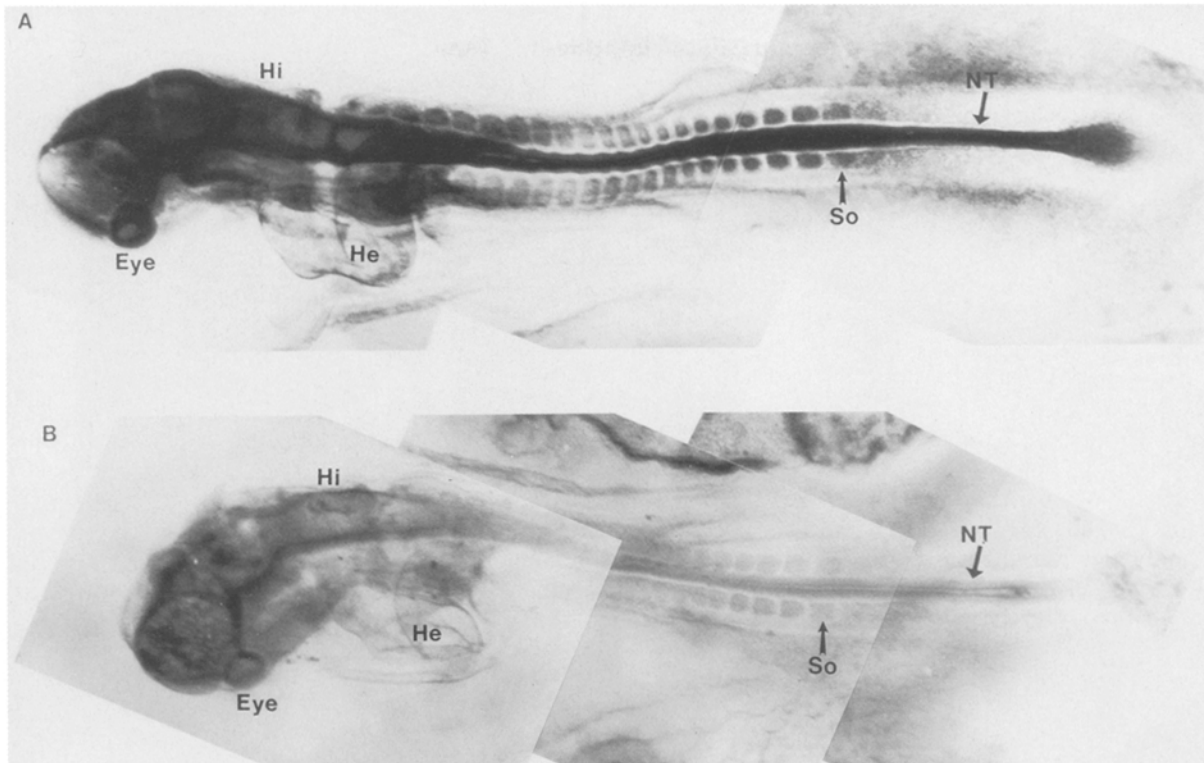
Figure 4. Expression pattern of Cek9 in the chicken embryo at 2 d of development in ovo. Dorso-lateral view of a 2-d chicken embryo stained with anti-Cek9 antibodies (*A*) or preimmune serum (*B*) by whole-mount immunohistochemistry. *Hi*, hindbrain; *He*, heart; *So*, somites; and *NT*, neural tube. The arrow next to "So" marks the most recently formed somite.

several other Eph-related receptors (Cheng et al., 1995; Drescher et al., 1995; Holash and Pasquale, 1995; Kenny et al., 1995), its spatial distribution in the visual system was examined by immunohistochemistry. Cek9 is evenly expressed throughout the thickness of the undifferentiated chicken retina (not shown). After the retina differentiates into distinct layers, Cek9 becomes most concentrated in the inner plexiform layer and the nerve fiber layer (Fig. 7 *A* and 8 *A*), both of which contain neuronal processes (Fig. 8 *B*). Consistent with the distribution of the Cek9 protein, Cek9 mRNA is detected in the retinal ganglion cell layer and the inner portion of the inner nuclear layer, which contains amacrine neurons. Neurons in these layers extend processes in the nerve fiber layer and the inner plexiform layer, where the Cek9 protein is concentrated. In addition to the nerve fiber layer of the retina, Cek9 immunoreactivity outlines all other structures in the visual pathway that contain the axons of retinal ganglion cells: the optic nerve, optic tract, and stratum opticum of the optic tectum (Fig. 7 *A*). The distribution of Cek9 in the retina and optic nerve is similar to that of Cek5, except that Cek5 is concentrated in the ventral aspect of the chicken retina and in only about half of the optic nerve, as previously reported (Fig. 7 *C* and Holash and Pasquale, 1995). Unlike Cek5, Cek9 appears to be evenly distributed along the dorsal-ventral axis of the retina. To determine whether Cek9 is expressed in a gradient along the anterior-posterior axis of the retina (Holash and Pasquale, 1995), extracts from the anterior and the posterior retina were probed with anti-Cek9 antibodies. As shown in Fig. 9, Cek9 appears evenly

distributed along the anterior-posterior axis. In the same samples the expression of another Eph receptor, Cek4, was examined for comparison. As expected (Cheng et al., 1995), Cek4 is concentrated in the posterior retina (Fig. 9).

Temporal changes in Cek9 expression throughout development were examined in the whole embryo as well as in a number of tissues (Fig. 10 *A*). In the whole embryo, Cek9 expression continues to increase between E3 and E4 and remains approximately constant thereafter. In the cerebrum, Cek9 expression is high at the earliest time examined (embryonic day 6), and it only slightly decreases during development. In the cerebellum, Cek9 expression is lower than in the cerebrum and does not change substantially between embryonic days 10 and 20, while it is somewhat lower at embryonic day 8. In the neural retina, Cek9 expression is high and decreases somewhat between embryonic days 8 and 19. Finally, in the skeletal muscle of the thigh, Cek9 is expressed highly between embryonic days 7 and 15, and at much lower levels at later stages.

Tyrosine Phosphorylation of Cek9 during Embryonic Development

The results shown in Fig. 10 *A* indicate that in many tissues, Cek9 expression does not change substantially during development. We next examined temporal changes in Cek9 phosphorylation on tyrosine (Fig. 10 *B*). Upon ligand binding, receptor protein–tyrosine kinases typically dimerize and autophosphorylate (van der Geer et al., 1994). This is thought to correlate with their catalytic activation,
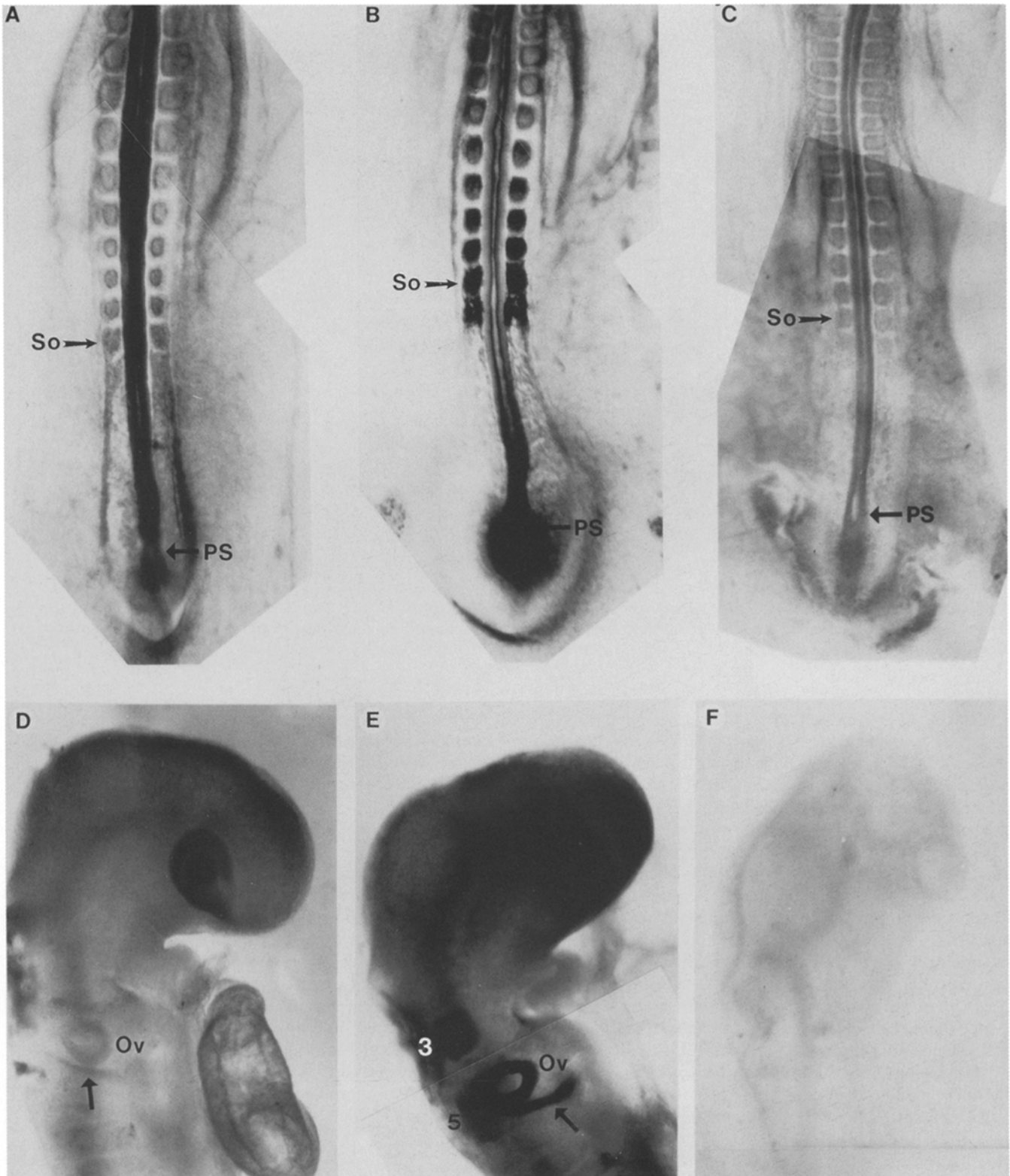
Figure 5. Expression patterns of Cek9, Cek8, and Cek5 in the chicken embryo at 2 d of development in ovo. The top three panels show dorsal views of the posterior regions of embryos stained with anti-Cek9 (*A*), anti-Cek8 (*B*), and anti-Cek5 (*C*) antibodies. The bottom three panels show lateral views of the anterior regions of embryos stained with anti-Cek9 (*D*) and anti-Cek8 (*E*) antibodies and anti-Cek9 antibodies that had been preincubated with the peptide antigen as a control (*F*). *So*, somites; *PS*, primitive streak; *Ov*, otic vesicle; *3*, rhombomere 3; *5*, rhombomere 5. The arrow next to SO in *A*, *B*, and *C* marks the most recently formed somite. The somite that is condensing posterior to that is prominently labeled with anti-Cek8 antibodies in *B*, but not with anti-Cek9 (*A*) or anti-Cek5 (*C*) antibodies. The arrow in *D* and *E* marks the migrating neural crest cells adjacent to rhombomere 5.

200 —

116 —
97 —

66 —

Br Re Le Lu He Li In Gi Th Bl
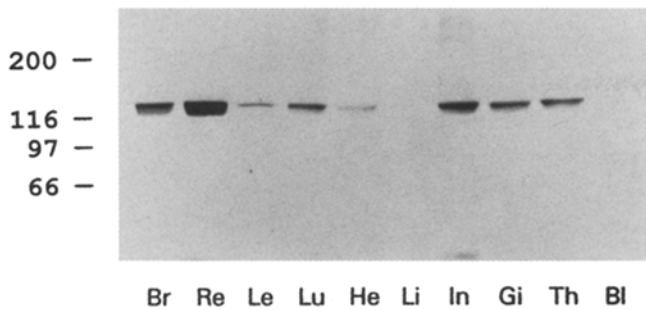
Figure 6. Cek9 expression in chicken embryonic tissues at embryonic day 10. Tissue extracts containing equal amounts of protein were loaded in each lane. The immunoblot was probed with antibodies to Cek9. *Br*, brain; *Re*, retina; *Le*, lens; *Lu*, lung; *He*, heart; *Li*, liver; *In*, intestine; *Gi*, gizzard; *Th*, skeletal muscle of the thigh; *Bl*, blood. The positions of molecular mass markers, in kD, are indicated at left.

which results in the phosphorylation on tyrosine of target molecules. We determined the extent of Cek9 phosphorylation on tyrosine in vivo by immunoprecipitation with anti-Cek9 antibodies, followed by SDS-PAGE and immunoblotting with antiphosphotyrosine antibodies. Protein tyrosine phosphorylation of Cek9 in the whole embryo was examined from day 4 to day 10. Phosphorylation of Cek9 was detected at every stage, with somewhat higher levels between days 7 and 9. Protein tyrosine phosphorylation of Cek9 in the cerebrum is high between embryonic days 5 and 11, and it decreases to barely detectable levels at later developmental stages. In the cerebellum, Cek9 phosphorylation on tyrosine is low and only detectable until embryonic day 11. Cek9 phosphorylation is highest in the retina, particularly before embryonic day 13. Cek9 is phosphorylated on tyrosine in the retina at every developmental stage examined. In the skeletal muscle of the thigh, Cek9 is also phosphorylated on tyrosine at every developmental stage that was examined, but at embryonic day 19 the level of phosphorylation is barely detectable.

Thus, Cek9 is phosphorylated on tyrosine in all the tissues examined during at least certain periods of embryonic development. Since our previous data indicated that other Eph-related receptors are not substantially phosphorylated on tyrosine in most embryonic tissues (Pasquale et al., 1994; Soans et al., 1994), we compared the phosphorylation of Cek9 in the 8-d embryo with that of Cek4, Cek5, and Cek8, other receptors of the Eph subfamily that are closely related to Cek9. Each receptor was immunoprecipitated with 20 μg of specific antibodies and probed by immunoblotting with antiphosphotyrosine antibodies. The results, shown in Fig 11 *A*, reveal that in both the body tissues and the brain of the 8-d chicken embryo, Cek9 is the most highly phosphorylated of the four Eph-related receptors examined, suggesting that it is the most catalytically active. Phosphorylation on tyrosine of Cek4 and Cek8 was low in the 8-d embryonic brain and not detectable in the body tissues. Phosphorylation of Cek5 was very low in both brain and body tissues at embryonic day 8.

To determine the fraction of Cek9 molecules that are phosphorylated on tyrosine in vivo in the 10-d embryonic retina, a tissue in which the level of Cek9 phosphorylation

on tyrosine is relatively high (Fig. 10 *B*), Cek9 was immunoprecipitated with anti-Cek9 antibodies. The resulting immunoprecipitate was reimmunoprecipitated using antiphosphotyrosine antibodies to separate the Cek9 molecules that were phosphorylated on tyrosine from those that were not. The nonphosphorylated Cek9 remained in the supernatant because it is not recognized by the antiphosphotyrosine antibodies. It was then reimmunoprecipitated using anti-Cek9 antibodies. Immunoblotting of the two immunoprecipitates with anti-Cek9 antibodies allowed the proportion of phosphorylated and nonphosphorylated Cek9 to be compared (Fig 11 *B*). While the fraction of Cek9 that is phosphorylated on tyrosine is easily detected with antiphosphotyrosine antibodies (*left panel*, lane *Y*), it cannot be detected with anti-Cek9 antibodies (*right panel*, lane *Y*) unless the film is overexposed (not shown). This indicates that only a small fraction of the Cek9 molecules are phosphorylated on tyrosine in vivo, as it would be expected since phosphorylation on tyrosine of receptor protein–tyrosine kinases after ligand binding is typically transient.

Discussion

Cek9 was, until now, one of the least characterized of the Eph-related kinases. Here we report the full coding sequence of Cek9. Similar to all other members of the Eph subfamily, Cek9 is comprised of an extracellular ligand-binding domain containing 20 conserved cysteines and two fibronectin type III repeats, a single membrane-spanning segment, and a cytoplasmic region containing a conserved kinase domain. Based on its amino acid sequence, Cek9 belongs to a subgroup within the Eph subfamily that includes Cek5/Nuk/HEK5, Cek6/Elk, Cek10/HEK2, and HTK (Sajjadi and Pasquale, 1993; Fox et al., 1995). Homologues of the Cek9 gene have not yet been identified in other species.

mRNAs encoding at least two isoforms of Cek9 exist. The variant mRNA that we have designated Cek9′ lacks a recognizable signal peptide in the predicted amino acid sequence. This is due to the presence of a 64-bp insertion near the 5′ end, which causes a shift in the reading frame. Since the insertion is precisely in the position where an intron is located in the related receptor Cek5 (Connor and Pasquale, 1995), it is likely that the two forms of Cek9 are the result of alternative splicing. Although variant forms have been reported for other Eph-related receptors (Connor and Pasquale, 1995 and references therein), none is similar to Cek9′. However, there are other examples of receptor tyrosine kinase isoforms that lack a signal sequence for membrane translocation. These include variant forms of TrkC (Garner and Large, 1994), fibroblast growth factor receptor 1, (Hou et al., 1991) and v-*erb*-B (Hayman and Beug, 1984; Privalsky and Bishop, 1984). Despite the prediction that these variants are likely to be localized to the cytoplasm, v-*erb*-B is expressed as a transmembrane protein. In contrast, it is not known whether the TrkC, fibroblast growth factor receptor 1, and Cek9 variant transcripts are efficiently translated into protein. Because Cek9, after cleavage of the signal peptide, and Cek9′ are expected to have the same sequence (with the exception of the two initial amino acids of Cek9′), we could not distin-
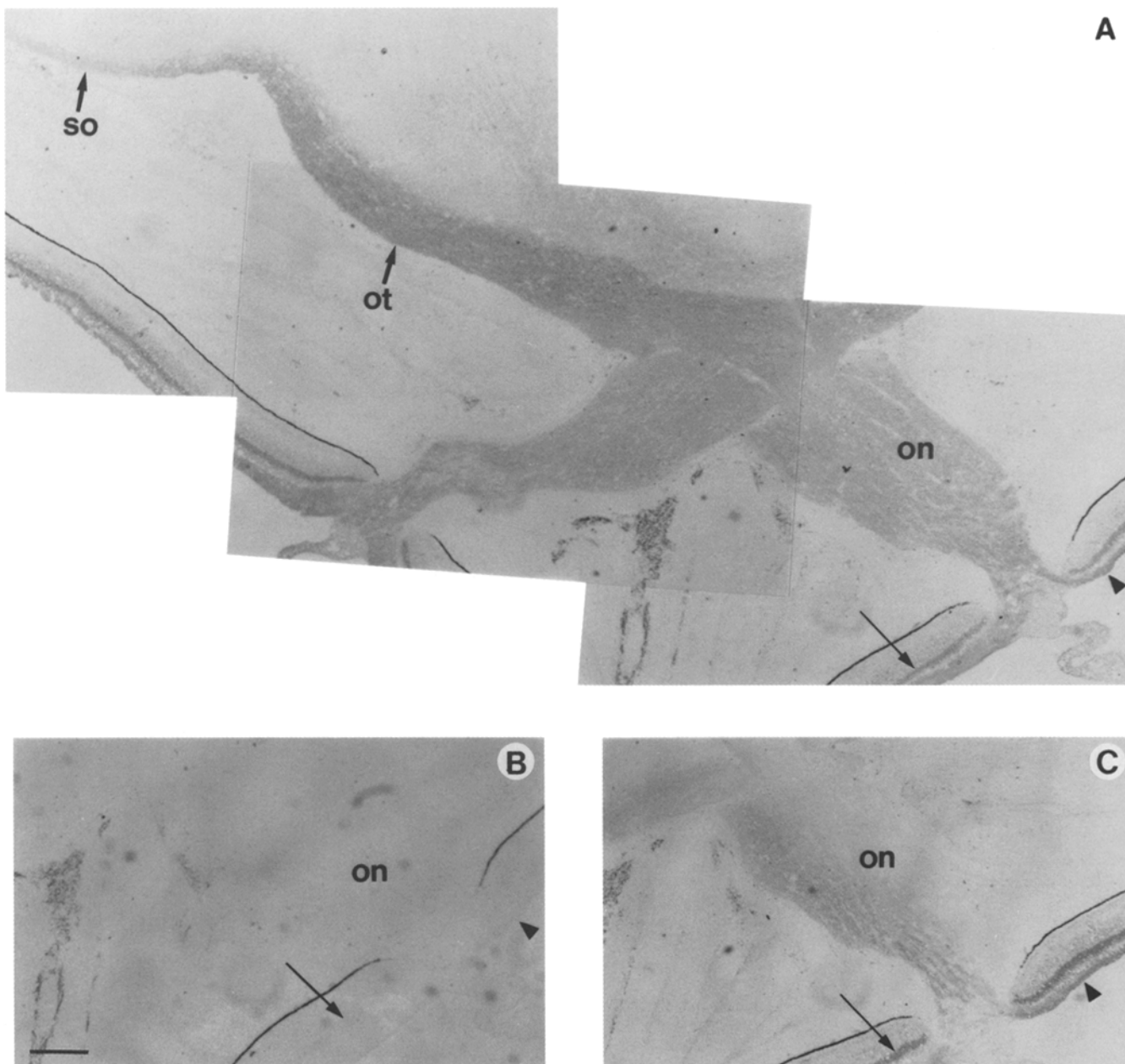
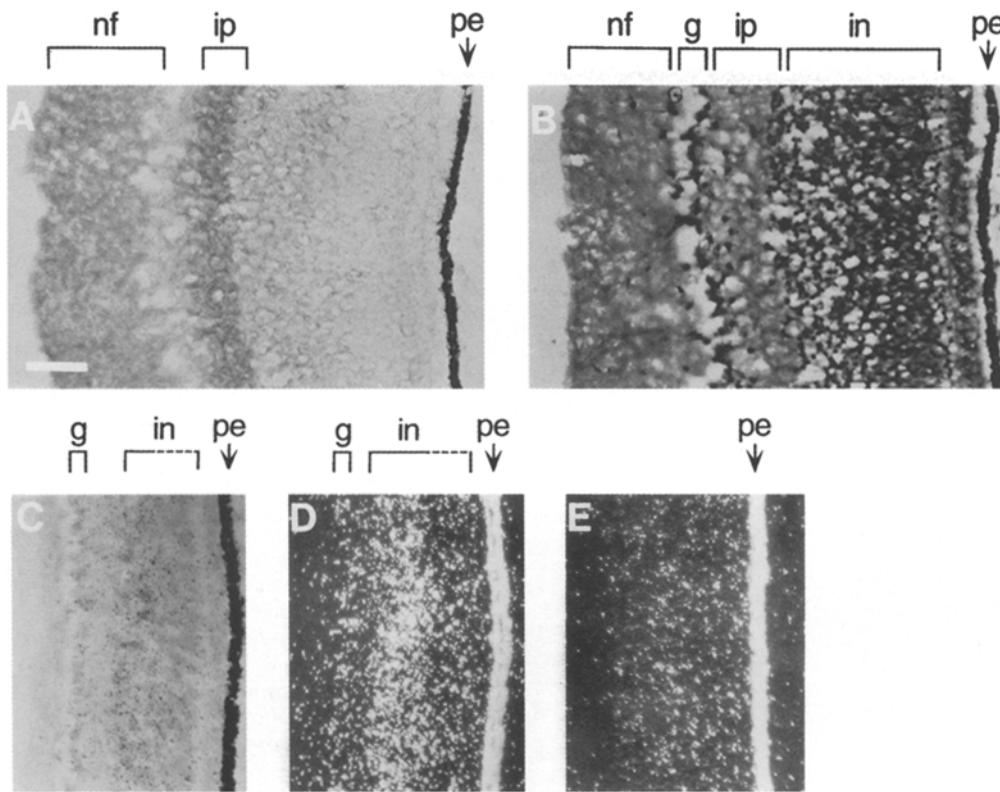*Figure 7.* Expression patterns of Cek9 in the visual pathway of the embryonic day 12 chicken embryo. (*A*) Immunoperoxidase labeling with anti-Cek9 antibodies resulted in the labeling of the ganglion cell processes. The entire pathway of the ganglion cell axons is labeled, including the nerve fiber layer (*arrowhead*), optic nerve (*on*), optic tract (*ot*), and stratum opticum of the tectum (*so*). Strong immunoreactivity is also observed in the inner plexiform layer of the retina (*arrow*). (*B*) The staining observed in *A* is abolished by preincubation of the anti-Cek9 antibodies with the GST fusion protein used as the antigen. (*C*) Similar to Cek9, Cek5 immunoreactivity is concentrated in the inner plexiform layer and nerve fiber layer of the retina. Unlike Cek9, Cek5 immunoreactivity is concentrated in only a portion of the optic nerve. Bar: (*B*) 300 μm.

guish Cek9′ from Cek9 with our antibodies nor assess whether the Cek9′ protein is expressed. However, it should be noted that six initial ATG sequences are located upstream of the predicted Cek9′ initiation codon (compared to two in the Cek9 cDNA). Translation starting from any of these ATGs would produce short polypeptides and inhibit the translation of Cek9′ (Kozak, 1992).

To fully appreciate the importance of the Eph-related receptors in development, their distribution patterns must be compared. This allows potential roles of these receptors

to be hypothesized and permits the assessment of whether members of the Eph subfamily are likely to function redundantly or uniquely. Cek9 expression was previously studied by Northern analysis. Among adult chicken tissues, Cek9 mRNA expression is most abundant in the retina and thymus (Sajjadi and Pasquale, 1993). In the 10-d chicken embryo, it was found that Cek9 mRNA is more highly expressed in the brain than in body tissues. In the current study we have used specific polyclonal antibodies to characterize the expression and spatial localization of

Figure 8. Expression patterns of Cek9 protein and mRNA in the neural retina of the embryonic day 12 chicken embryo. (*A*) Immunoperoxidase labeling with the anti-Cek9 antibodies resulted in labeling of the inner plexiform layer (*ip*) and nerve fiber layer (*nf*), shown here at higher magnification than in Fig. 7 *A*. The pigmented epithelium (*pe*) appears dark because it is pigmented. (*B*) Cresyl violet staining shows the layers of the retina, such as the inner nuclear layer (*in*) and retinal ganglion cell layer (*g*), which consist of cell bodies, and the inner plexiform layer and nerve fiber layer, which consist of neuronal processes. (*C* and *D*) Bright field and dark field photomicrographs, respectively, of retina probed with Cek9 antisense mRNA. Cek9 transcripts are concentrated in the retinal ganglion cell layer and the inner portion of the inner nuclear layer, which contains amacrine cells. Apparent labeling of the pigmented epithelium is nonspecific (compare *D* and *E*). (*E*) Dark field photomicrograph of retina probed with Cek9 sense mRNA, as a control. All the panels are at the same magnification; however, differences in tissue processing for immunohistochemistry or in situ hybridization experiments are responsible for the apparent different sizes of the retinas in the top and bottom panels. In addition, *A* and *B* represent sections of the retina close to the optic nerve, and thus contain a more prominent nerve fiber layer. Bar: (*A*) 50 μm.

the Cek9 protein during development. The widespread expression of Cek9 supports its playing roles in multiple developmental processes. Like Cek5 and Cek8, two other Eph-related receptors that were previously examined, Cek9 is expressed in the chicken embryo at early stages of development (Pasquale, 1991; Pasquale et al., 1994; Soans et al., 1994; Ohta et al., 1996). However, the distribution of Cek9 in the 2-d embryo suggests that Cek9 has a distinct functional role. Unlike Cek5, both Cek9 and Cek8 exhibit a graded distribution in the posterior region of the 2-d embryo, with higher levels in the more caudal regions of the neural tube and in the more posterior somites. A similarly

Figure 9. Cek9 and Cek4 expression in the anterior (*A*) and posterior (*P*) embryonic day 8 chicken retina. Extracts of anterior and posterior retina containing equal amounts of protein were probed with antibodies to Cek9 or Cek4. The positions of molecular mass markers, in kD, are indicated at left.

graded, but more restricted distribution in the developing somites was previously observed for the mRNA encoding Sek-1, the mouse homolog of Cek8 (Nieto et al., 1992). A similar expression pattern of the Cek8 protein was also detected in the stage 12 chicken embryo (Irving et al., 1996; Ohta et al., 1996). Because the posterior structures of the developing chicken embryo are less mature, the expression pattern of Cek9 suggests a function in the early patterning of neural tube and somites. Although the patterns of expression of Cek9 and Cek8 are partially overlapping, the Cek9 domain of expression is more anterior than that of Cek8, indicating a later onset of expression and a later downregulation in the somites and early neural tube. Furthermore, in contrast to Cek8, Cek9 is expressed more prominently in the neural tube than in the somites. Its even distribution in the developing hindbrain suggests a role for Cek9 different from regulating the segmental patterning of this structure, at least in the 2-d chicken embryo. In contrast, roles in patterning of the hindbrain have been attributed to several other Eph-related receptors (Becker et al., 1994; Ganju et al., 1994; Ruiz et al., 1994; Henkemeyer et al., 1994; Ellis et al., 1995; Xu et al., 1995).

At all developmental stages examined, Cek9 is prominently expressed in structures of the optic pathway (Figs. 6–9), suggesting a special involvement in the formation of the visual system. It has been recently reported that Cek4, Cek5/QEK5, Ebk, and several of the ligands for Eph-
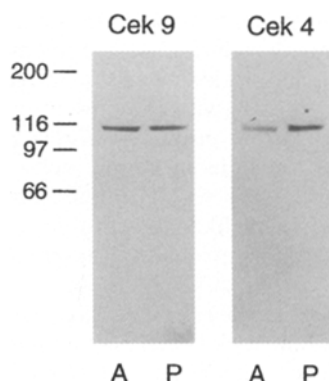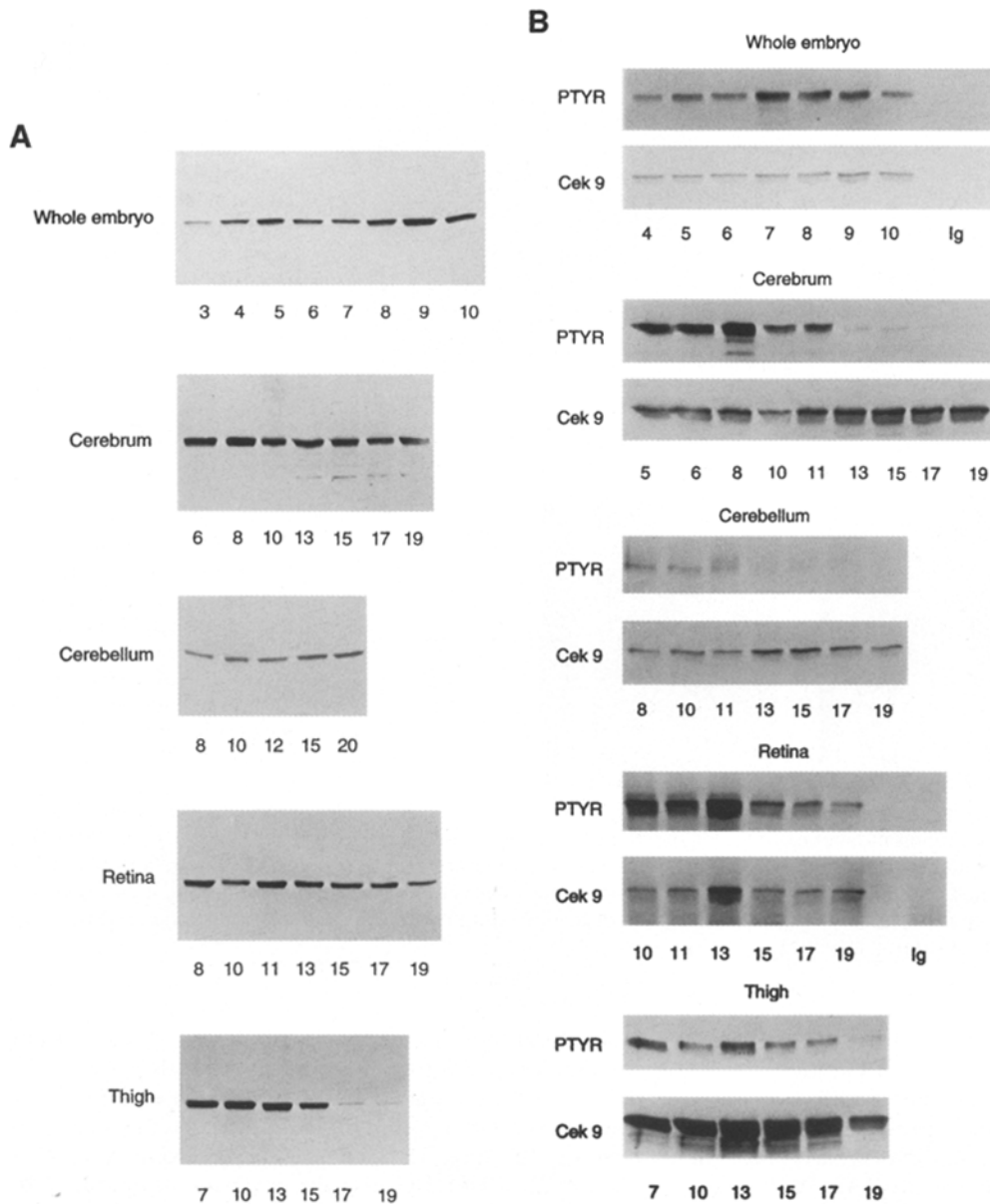
A

B

Figure 10. Developmental regulation of Cek9 expression and phosphorylation on tyrosine. (*A*) Whole embryo extracts or tissue extracts (cerebrum, cerebellum, neural retina, and skeletal muscle of the thigh) containing equal amounts of protein were loaded in each lane and probed with anti-Cek9 antibodies. (*B*) Whole embryo extracts or tissue extracts were subjected to immunoprecipitation with equal amounts of anti-Cek9 antibodies and analyzed by blotting with antiphosphotyrosine antibodies (*PTYR*), followed by reblotting with anti-Cek9 antibodies (*Cek9*). The lanes labeled Ig in the "Whole embryo" and "Retina" panels also contain control immunoprecipitations using nonimmune rabbit IgG. Days of development in ovo are indicated at the bottom of each panel. It should be noted that the levels of Cek9 that were immunoprecipitated in the experiments shown in *B* do not reflect expression levels, since an excess of tissue and limiting amounts of antibodies were used for the immmunoprecipitations.

related receptors exhibit polarized distributions in the retina, optic nerve, and/or optic tectum (Cheng et al., 1995; Drescher et al., 1995; Holash and Pasquale, 1995; Kenny et al., 1995; Ellis et al., 1995; Holash, J.A., and E.B. Pasquale, unpublished data). On the basis of these observations, it was proposed that these Eph-related receptors and their ligands confer positional information in the visual system, thereby regulating the specificity of retinotectal projections. Cek9 expression is highly enhanced in regions containing the axonal projections of retinal ganglion cells, but its expression does not appear to be in a gradient. This distribution of Cek9 does not support a role in regulating retinotectal specificity; rather, it suggests a more general function relating to the outgrowth of retinal ganglion cell axons to the optic tectum. However, it cannot be excluded that Cek9 functions in the visual pathway by acting in concert with other Eph-related receptors that have a partially overlapping polarized distribution. These include

Cek5, which is concentrated in the inner plexiform layer and the retinal ganglion cell axons from the ventral retina (Pasquale et al., 1994; Holash and Pasquale, 1995), and/or Cek4, which is concentrated in the retinal ganglion cell axons from the posterior retina (Cheng et al., 1995).

The widespread expression of Cek9 outside the visual system suggests multiple functions during development. For example, the downregulation of Cek9 expression in the skeletal muscle of the thigh coincides with the time of increased expression of muscle-specific proteins (Pasquale, 1991) and suggests that Cek9 functions in muscle before terminal differentiation. Further work will be required to establish whether activation of Cek9, like that of other receptor protein–tyrosine kinases (for review see Hauschka, 1994), interferes with skeletal muscle differentiation.

The differences in their spatiotemporal expression patterns suggest that Cek5, Cek8, and Cek9 each play unique roles in development. However, the feature that most
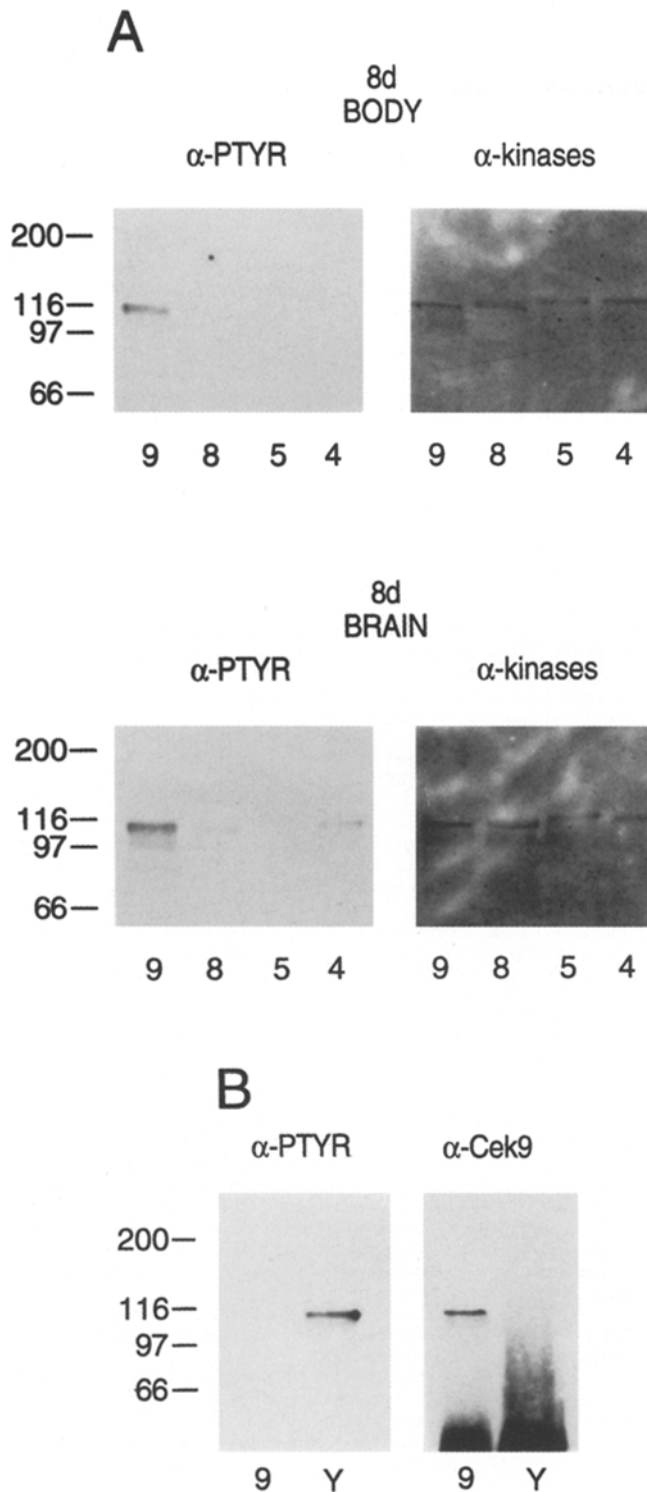
A

8d
BODY

α-PTYR   α-kinases

200—

116—
97—

66—

      9   8   5   4   9   8   5   4

8d
BRAIN

α-PTYR   α-kinases

200—

116—
97—

66—

      9   8   5   4   9   8   5   4

B

α-PTYR   α-Cek9

200—

116—
97—
66—

      9   Y   9   Y

Figure 11. Comparison of the in vivo phosphorylation on tyrosine of Cek9, Cek8, Cek5, and Cek4 and extent of in vivo Cek9 phosphorylation on tyrosine. (*A*) Cek9, Cek8, Cek5, and Cek4 (lanes *9, 8, 5,* and *4,* respectively) were immunoprecipitated from extracts of 8-d embryonic body tissues and 8-d embryonic brain using approximately equal amounts of anti-Cek9, anti-Cek8, anti-Cek5, or anti-Cek4 antibodies. The immunoprecipitates were probed with antiphosphotyrosine antibodies (*α-PTYR*) and re-probed with a mixture of antibodies to the four receptors (*α-kinases*). The use of a mixture of antibodies allowed the simultaneous visualization of all four receptors, but caused increased background.

clearly distinguishes Cek9 from other Eph-related receptors is its enhanced phosphorylation on tyrosine in embryonic tissues. Therefore, in many tissues at certain developmental stages, a fraction of the Cek9 present is likely to be activated. Although the fraction of the Cek9 molecules that are phosphorylated on tyrosine in vivo at any time is small, it is likely to be significant. Because uncontrolled tyrosine kinase activity may have pathological consequences (Bishop, 1991), protein tyrosine phosphorylation appears to be tightly regulated in normal tissues. The in vivo activation (phosphorylation on tyrosine) of several other Eph-related receptors that we have examined is less extensive than that of Cek9, suggesting a particularly widespread involvement of Cek9 in the processes of embryogenesis. The high Cek9 phosphorylation on tyrosine during the first half of development may implicate it in developmental processes such as cell division or migration.

Phosphorylation on tyrosine of receptor protein–tyrosine kinases is typically caused by their interaction with an extracellular ligand. Hence, the pattern of Cek9 phosphorylation on tyrosine that we have observed implies that the Cek9 ligand is widely expressed and its pattern of expression must at least partially overlap with that of Cek9 during the first half of chicken embryonic development. The decrease in Cek9 tyrosine phosphorylation during the second part of embryonic development, which was observed in all tissues examined, although to different extents, may result from the decreased expression of the Cek9 ligand(s). It is also possible that receptor and ligand may become confined to distinct regions, as would be required if Cek9 is involved in establishing boundaries between adjacent anatomical compartments. Such a role has been proposed for other Eph receptors (Cheng et al., 1995; Drescher et al., 1995; Xu et al., 1995). Alternatively, the persistent expression, but low activation levels, of Cek9 at later developmental stages may indicate a function that is not dependent on catalytic activation. Although Cek9 is most closely related to Cek5, Cek6/Elk, and Cek10, a ligand which activates the latter three receptors does not bind to Cek9 (Brambilla et al., 1995), suggesting the existence of a distinct Cek9 ligand. Interestingly, the extracellular domain of Cek9 is 57% identical at the amino acid level to that of Cek5, 53% identical to that of Cek6/Elk, and 48% identical to that of Cek10, whereas the extracellular domains of Cek5, Elk, and Cek10 are between 63 and 68% identical to each other. Amino acid sequence differences between the extracellular domain of Cek9 and those of Cek5, Cek6/Elk, and Cek10, which bind the same ligand, are presum-

(*B*) The tyrosine phosphorylated (lane *Y*) and nonphosphorylated (lane *9*) forms of Cek9 were immunoprecipitated separately from retina extracts at embryonic day 10, as described in the Materials and Methods, and both were probed with either antiphosphotyrosine antibodies (*α-PTYR*) or anti-Cek9 antibodies (*α-Cek9*). The lack of signal (at this level of exposure) in the lane containing Cek9 immunoprecipitated with antiphosphotyrosine antibodies and probed with anti-Cek9 antibodies (*right panel,* lane *Y*) indicates that only a small fraction of the Cek9 molecules are phosphorylated on tyrosine in the retina at embryonic day 10. However, longer exposure times allow detection of a band in this lane. The positions of molecular mass markers, in kD, are indicated at left.

ably responsible for the distinct ligand specificity of Cek9. The identification of physiological ligand(s) for Cek9 will greatly facilitate the study of the role of Cek9 in developmental processes.

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