Unprocessed Proinsulin Promotes Cell Survival During Neurulation in the Chick Embryo

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We have chosen a vertebrate model accessible during neurulation, the chick, for analysis of endogenous insulin signaling and its contribution to early embryonic cell survival. Unlike rodents, humans and chickens have a single preproinsulin gene, facilitating its prepancreatic expression characterization. We show that in vivo interference with embryonic insulin signaling using antisense oligonucleotides against the insulin receptor increases apoptosis during neurulation. In contrast, high glucose administration does not increase the level of apoptosis in culture or in vivo. Exogenous insulin and, remarkably, proinsulin achieve similar survival protective effects at 10^{-8} mol/l. The low abundant preproinsulin mRNA from the prepancreatic embryo is translated to a protein that remains as unprocessed proinsulin. This concurs with the absence of prohormone convertase 2 (PC2) in the embryo, whereas PC2 is present later in embryonic pancreas. A C-peptide–specific antibody stains proinsulin-containing neuroepithelial cells of the chick embryo in early neurulation, as well as other cells in mesoderm- and endoderm-derived structures in the 2.5-day embryo. We have determined by 5′-RACE (rapid amplification of cDNA ends), and confirmed by RNase protection assay, that prepancreatic and pancreatic proinsulin mRNA differ in their first exon, suggesting differential transcriptional regulation. All these data support the role of endogenous proinsulin in cell survival in the chick embryo during important pathophysiologic periods of early development.
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Programmed cell death, with the cellular characteristics of apoptosis, is an essential, highly regulated process that occurs during normal embryonic development (1–3). Altered control of cell death is associated with an increasing number of pathologies, including embryonic abnormalities in the nervous system (2–4). We previously showed that balanced insulin signaling is required for normal chick development at early embryonic stages, since blockade with anti-insulin antibodies or an excess of insulin was deleterious to the embryo (5,6). In the chick embryo, before the emergence of pancreas, preproinsulin mRNA levels are developmentally regulated but are not increased by high glucose (7). An increase in apoptosis affecting the neural tube was elicited by preproinsulin mRNA antisense oligonucleotides in chick embryos at day 1.5 of development (equivalent to 3–4 weeks of gestation in humans) (8). Very low or very high prepancreatic insulin availability, due to altered preproinsulin mRNA regulation induced by factors related to or independent of hyperglycemia, may contribute to the appearence of congenital abnormalities.

Low insulin levels were reported to cause retarded growth and development in cultured rat embryos (9), but the inaccessibility of neurulating embryos in mammalian models limits in vivo studies. As an alternative, in the present study, we used the chicken embryo because it is accessible, has a well-established development pattern (10), and is isolated from maternal influences. In addition, as in humans, chickens have only one preproinsulin gene (11), unlike mice and rats, which have two preproinsulin genes (12,13). Here we report the effect of decreased insulin signaling and high glucose conditions on apoptotic cell death in the neurulating chick embryo. We characterize the survival effect of proinsulin relative to various insulins. We also show that the product of preproinsulin mRNA translation in the embryo remains as unprocessed proinsulin and that there are different 5′ leader regions of preproinsulin mRNA in the neurulating embryo compared with the embryonic pancreas.

RESEARCH DESIGN AND METHODS

Chick embryos. Fertilized White Leghorn eggs (Granja Rodríguez-Serrano, Salamanca, Spain) were incubated at 38.4°C and 60–90% relative humidity for the indicated periods. The embryos were staged according to Hamburger and Hamilton (HH stages) (14) and referred to by the corresponding day of embryonic development (E).

Whole embryo culture. E1.5 embryos together with the extraembryonic membranes immobilized by a nitrocellulose ring were cultured as described (7,8). Purified chicken insulin (Litron Lab, Rochester, NY), recombinant human insulin and proinsulin (gifts of Eli Lilly, Indianapolis, IN), or partially purified bovine insulin (Sigma, St. Louis, MO) was added to the culture medium at the indicated doses. Where indicated, the cultured embryos were treated with 38.5 μmol/l oligodeoxynucleotide (ODN) (12.5 nmol total) combined with 25 μg/ml Cytofectin (Glen Research, Sterling, VA) as described (15,16).

All ODNs were synthesized as phosphorothioate derivatives (Oligos Etc., Wilsonville, OR, or Roche Diagnostics, Mannheim, Germany). The antisense to the chicken insulin receptor (AS *IR*) was 5′-GGTGAACGAATCGGC-3′, and a random sequence of the same base composition (RAN *IR*) was 5′-GACG GCAGTACGAGT-3′. For glucose treatment, Dulbecco's modified Eagle's/F12 medium was replaced by glucose-free RPMI-1640 medium. D-Glucose (Sigma) was added to culture medium at the indicated concentrations.

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AS, antisense; E, day of embryonic development; IR, insulin receptor; ODN, oligodeoxynucleotide; P, day of postnatal development; PC2, prohormone convertase 2; RACE, rapid amplification of cDNA ends; RAN, random; SUR1, sulfonylurea receptor 1; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling; UTR, untranslated region.

Detection of apoptosis. Apoptotic cell death was determined by two methods: counting of pyknotic nuclei in dissociated embryonic cells, performed as described (8,16,17), and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) of the fragmented DNA, performed in whole-mount embryos as described (17,18). The two methods provide complementary information. The former allows for a more accurate scoring of the proportion of apoptotic cells, and the latter provides positional information about the location of the dead cells. Our previous reports have shown that both methods reflect the relative differences in cell death among the treatments (8,16–18).

Oligodeoxynucleotide and glucose treatment in vivo. At the desired incubation time, a lateral window was cut in the eggshell and the developmental stage of the embryos was assessed. When indicated, 12 nmol ODN with 25 μg/ml Cytofectin or 200 μl of 50 mmol/l D- or L-glucose were applied to the embryo. Eggs were sealed with cellotape and incubated further for the indicated period, after which the embryos were processed to quantify apoptotic cell death.

Immunoblotting. For proinsulin analysis, 100 E1.5-E2 embryos were homogenized, and protein was acid ethanol extracted as described (19). Proteins were fractionated in a Tris-tricine PAGE SDS-14% Prosieve (FMC, Rockland, ME) polyacrylamide reducing gel. For prohormone convertase 2 (PC2) analysis, embryos at E1.5 and pancreata from E13 were homogenized in 50 mmol/l Tris-HCl (pH 7.5), 300 mmol/l NaCl, 10 mmol/l EDTA, 1% (wt/vol) Triton X-100, and protease inhibitor cocktail (Roche). The homogenates were clarified by centrifugation, supernatant protein content was measured, and 80 μg was fractionated in a Tris-tricine PAGE SDS-7.5% gel and transferred to nitrocellulose membranes. These were analyzed for proinsulin by incubation with rabbit anti–C-peptide antibody (Rb40, 1/5,000; generated in our laboratory against synthetic chicken C-peptide [19]) and reprobed for insulin/proinsulin by incubation with guinea pig anti–porcine insulin/proinsulin antibody (lot HO1P-L; Bio-Teck, Winooski, VT). For PC2, membranes were incubated with rabbit anti-PC2 antibody (1/2,000, "Thumpa"; a gift of C. J. Rhodes, Seattle, WA). All membranes were reprobed with mouse anti–β-tubulin antibody (1/20,000; Sigma) and visualized by chemiluminescence.

Immunohistochemistry. Immunostaining was performed on cryosections of E1 and E2.5 embryos by sequential incubation at room temperature with 15% (vol/vol) normal goat serum (30 min), rabbit anti–C-peptide antibody (1/500 dilution [19]), or rabbit anti–IGF-I antibody (1/500; a gift of Dr. I. Torres-Alemán, Instituto Cajal, Madrid, Spain) for 1 h, biotinylated goat anti–rabbit Ig (1/200; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h, and Cy2-conjugated streptavidin (1/200; Amersham, Rainham, UK) for 45 min.

Isolation of RNA. Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Integrity of the RNA was verified by electrophoresis on formaldehyde-agarose gels. PolyA$^+$ RNA was isolated using the Micro-Fast Track kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

5′ RACE. The 5′ rapid amplification of cDNA ends (RACE) procedure was performed according to manufacturer's instructions (Life Technologies). Briefly, 5 μg total RNA from E1.5 embryos and 1 day postnatal (P1) pancreas were used as templates for a reverse transcriptase reaction with the chicken preproinsulin primer 5′-TTGACTTTCTCGTATTCCT-3′, located in exon 3, 244 bp downstream of the translation start site. The first PCR reaction was carried out with the abridged anchor primer (5′-GGC CACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) and the preproinsulin primer 5′-GACTGCTCACTAGGGGCTGC-3′, located 196 bp downstream of the translation start site, comprising the 3′ and 5′ borders of exons 2 and 3, respectively. A nested PCR was performed on the products of the first PCR reaction using the abridged universal amplification primer (5′-GGC CACGCGTCGACTAGTAC-3′) and the preproinsulin primer 5′-GCTCTCCACA CACCAGGTAG-3′, located in exon 2, 136 bp downstream of the translation start site. The PCR products were analyzed in a 2% agarose gel and subcloned into the pCRII-TOPO vector (Invitrogen). Several clones from E1.5 embryos and P1 pancreas were further characterized by sequencing analysis.

Solution hybridization-RNase protection assay. The preproinsulin exon 1_B cDNA probe was generated by subcloning the preproinsulin 5′ RACE product from E1.5 embryos, as described above. A [^{32}P]-labeled antisense riboprobe was generated using T7 polymerase in the presence of CTP, GTP, ATP, and [^{32}P]UTP. The resulting transcripts were purified through a 6-column Bio-Spin (Bio-Rad, Hemel Hempsted, UK). Solution hybridization-RNase protection assay was carried out as described (20), with minor modifications. Either polyA$^+$ or total RNA was hybridized with 2×10^5 cpm of preproinsulin exon 1 antisense riboprobe in a buffer containing 75% formamide (45°C, 16 h). After hybridization, RNA samples were digested with RNase A at 35 μg/ml and RNase T1 at 0.1 units/ml (both from Roche) for 1 h at 30°C. Protected hybrids were extracted with phenol/chloroform, ethanol precipitated, and separated on a 6% polyacrylamide gel containing 7 mol/l urea (Invitrogen). The gel was dried and exposed to film (Biomax MS; Eastman Kodak, Rochester, NY) for 1–8 days.

RESULTS

Blockade of insulin receptor in vivo induces cell death in the neurulating chick embryo. Insulin prevents apoptotic cell death during chick embryonic development (8,18). To further characterize the tissues and cells dependent on prepancreatic insulin in vivo, we determined the spatial distribution of apoptotic cells in embryos. We blocked the insulin receptor using specific antisense ODN, a strategy that has been successfully employed in the embryo (15,16).

Dead cells found in the neurulating chick embryo (E1.5) were globally scarce (1.4 ± 0.4% of total embryonic cells; see also Morales et al. [8]). E1.5 embryos treated in ovo with AS *IR* presented a greater proportion of apoptotic cells (3.8% ± 2.3; $n = 6$) than did control embryos incubated with RAN *IR* (1.9% ± 0.9; $n = 6$, $P < 0.025$ by Student's t test). Confocal microscopy analysis of TUNEL-stained whole embryos demonstrated that naturally occurring apoptotic cell death was prevalent along the neural tube, mainly in the anterior region of the prosencephalon and rhombomer 3, as well as in the cochleovestibular ganglia, the somites, and the dorsal lip of the neural tube (Fig. 1*A–C*). AS *IR* treatment (Fig. 1*D-F*) increased the density of apoptotic cell death in locations similar to those observed in untreated or RAN *IR*–treated embryos (Fig. 1*G– I*). We did not attempt to block insulin synthesis in the embryo in ovo, as the large volume of the egg provides a significant amount of insulin (21). Nonetheless, both the maternally derived egg insulin and the embryonic product of preproinsulin mRNA can be neutralized with antibodies or antisense ODN, disrupting normal development (5,17).

High glucose has no effect on apoptosis in neurulating chick embryos. Because hyperglycemia has been associated with diabetic embryopathy in mammals (22–25), we studied whether high glucose increased apoptosis in the neurulating chick embryo. E1.5 embryos were cultured in conditions of low glucose (5.5 mmol/l) or high glucose (50 mmol/l, moderate for the chick embryo surrounded by white, which contains 30 mmol/l glucose [26]). Culture of the neurulating embryo in growth factor–deprived medium induced time course– dependent apoptosis that was partially attenuated by insulin (8). Glucose had no additional effect on the number of apoptotic cells, independently of the presence or absence of insulin (Fig. 2). As expected, insulin exerted an anti-apoptotic effect in the presence of either glucose concentration. To further confirm the lack of high glucose effect in cell survival during neurulation, E1.5 embryos were treated in ovo. Embryos ($n = 16$) embedded in 50 mmol/l D-glucose presented a slight, nonsignificant increase in the proportion of apoptotic cells compared with embryos treated with 50 mmol/l L-glucose (a non–metabolically active control) (2.2 ± 1.5% vs. 1.8 ± 0.6%). Altogether, these data suggest that endogenous insulin prevents cell death by additional mechanisms independent of its possible effect on glucose homeostasis.

Exogenous insulin and proinsulin are cell survival–promoting factors in vitro. We previously showed that proinsulin, the unprocessed insulin precursor, is expressed in the neuroretina (19) and involved in regulating

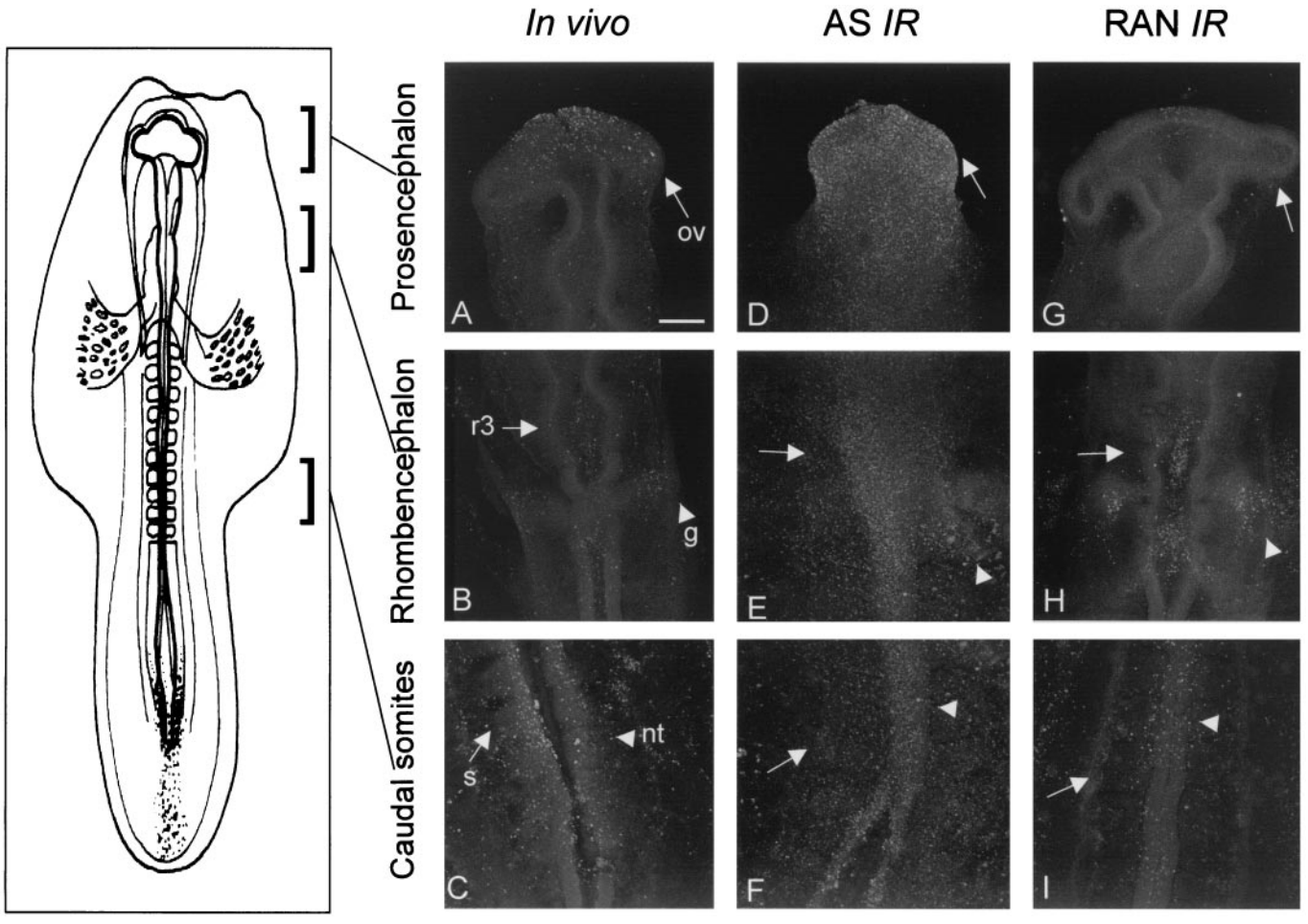

FIG. 1. Cell death distribution in neurulating chick embryos treated with insulin receptor antisense ODN. Apoptotic cells were visualized by TUNEL on freshly dissected whole E1.5 embryos (in vivo, *A–C*) or embryos treated with insulin receptor antisense ODN (AS *IR*, *D–F*) or control random ODN (RAN *IR*, *G–I*) for 10 h. Serial images were captured in a confocal microscope every 10 μm with a ×10 objective. Different regions are bracketed in the embryo scheme inserted: prosencephalon (*A*, *D*, *G*), rhombencephalon (*B*, *E*, *H*), and neural tube and caudal somites (*C*, *F*, *I*). The bright dots represent TUNEL-stained pyknotic bodies. ov, Optic vesicle; r3, rhombomere 3; g, forming cochleovestibular ganglion; nt, neural tube; s, somites. Bar, 200 μm.

proliferation, differentiation, and cell survival during early retinal development (17,18,27). To investigate whether proinsulin is also active in regulating cell survival during neurulation, we cultured E1.5 embryos in a defined medium in the presence of proinsulin or various insulins at the indicated concentrations. Proinsulin prevented apoptotic cell death with essentially the same potency as chicken insulin (Fig. 3). We used human proinsulin for these treatments; the stimulatory potency of chicken proinsulin may be even higher than that of chicken insulin. In contrast, bovine insulin, the most frequently used in standard culture media, was the least potent in preventing apoptosis in our system.

Endogenous proinsulin remained unprocessed in the neurulating embryo. To determine whether the endogenous molecule that activated the survival response of the insulin receptor in the embryo was insulin or proinsulin, we performed Western blot analysis with two different antibodies that permit distinction of insulin and proinsulin (19). Immunoblotting of E1.5 extracts with Rb40 (raised against synthetic chicken C-peptide) identified a protein with the electrophoretic mobility of recombinant chicken proinsulin (Fig. 4*A*, upper panel). There was no cross-reaction with purified chicken insulin, consistent with the antibody specificity. In contrast, a commercial polyclonal anti-insulin antiserum recognized insulin well and proinsulin with much lower affinity (Fig. 4*A*, bottom panel). Because unprocessed proinsulin is the only "insulin" detectable in embryo extracts, we then studied the expression of the proinsulin-processing convertase PC2 in the neurulating embryo and the mid-embryogenesis pancreas. The 62/63 kDa mature PC2 was found in E13 pancreas, whereas it was not detected in whole E1.5 embryos (Fig. 4*B*), despite similar β-tubulin levels in both extracts.

Proinsulin is present earlier than IGF-I in the embryo. The presence of proinsulin in the chick embryo was analyzed by immunohistochemistry in parallel with another member of the insulin family, IGF-I. In gastrulation (E1), proinsulin immunoreactivity was detected mainly in the neuroepithelial cells of the embryonic ectoderm (Fig. 5*A* and *B*). Proinsulin staining was also detected in scattered cells within the mesoderm and endoderm layers, coinciding with the proinsulin mRNA expression pattern observed by in situ hybridization studies (8,28). In contrast, IGF-I was not found in any of the three blastoderm layers at this stage (Fig. 5*F*). In early organogenesis (E2.5), proinsulin immunostaining was also detected in the three embryonic layers (Fig. 5*D* and *E*). Among the ectodermal

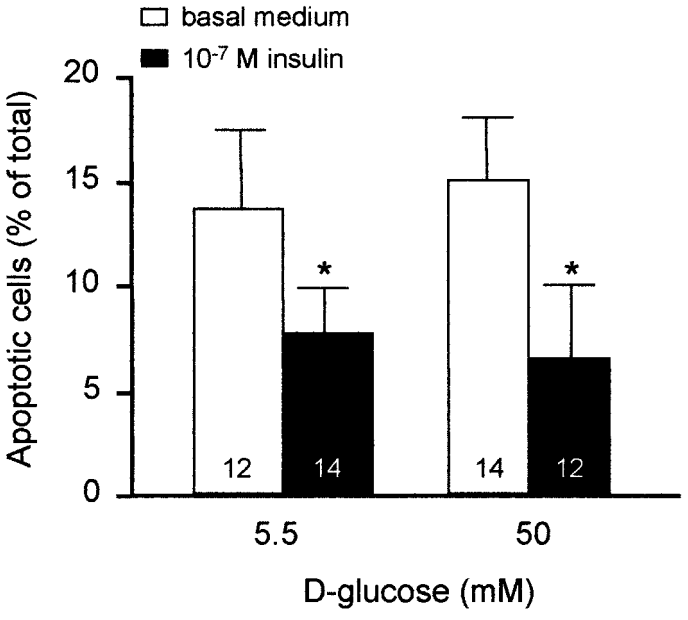

FIG. 2. Lack of effect of high glucose on apoptotic cell death in cultured embryos. E1.5 embryos were cultured in basal medium or supplemented with 10^{-7} mol/l chicken insulin in the presence of 5.5 mmol/l or 50 mmol/l D-glucose for 8 h. Apoptotic cells were scored in dissociated embryos by DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) staining of pyknotic nuclei. A minimum of 1,000 cells were counted in duplicate experimental points. The values represent the means ± SD of the total number of embryos indicated inside the bar, treated in three independent experiments. *$P \leq 0.001$ (Student's t test) vs. the respective control.

structures, proinsulin was more prevalent in the dorsal ectoderm, covering the neural tube and in the floor plate. Some mesodermal structures such as the notochord, dermamyotome, nephric tube, and nephrogenic mesenchyme

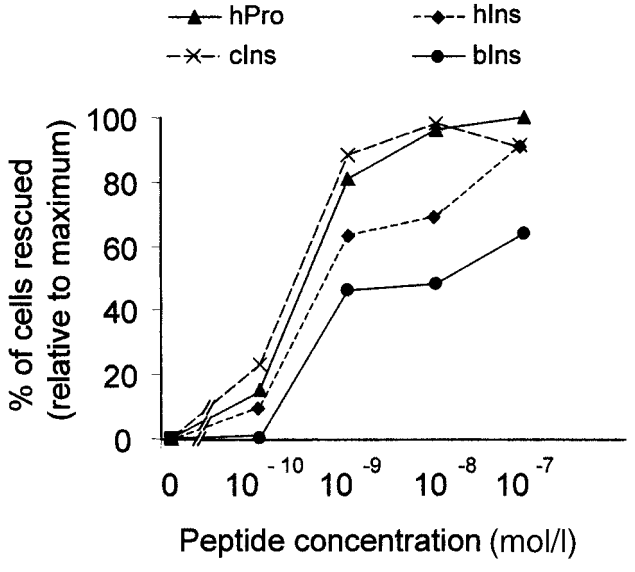

FIG. 3. Cell survival dose response to insulins and proinsulin. E1.5 embryos were cultured in basal medium supplemented with the indicated doses of human insulin (hIns), human proinsulin (hPro), chicken insulin (cIns), or bovine insulin (bIns) for 8 h. Apoptotic cells were scored in dissociated cells by DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) staining of pyknotic nuclei. A minimum of 1,000 cells were counted in duplicate experimental points. The values represent the means of duplicates of six embryos in three independent experiments. For better comparison, the values are represented relative to the maximal rescue, displayed by 10^{-7} mol/l human proinsulin (3.19% of apoptotic cells), with respect to basal medium (8.2% of apoptotic cells).

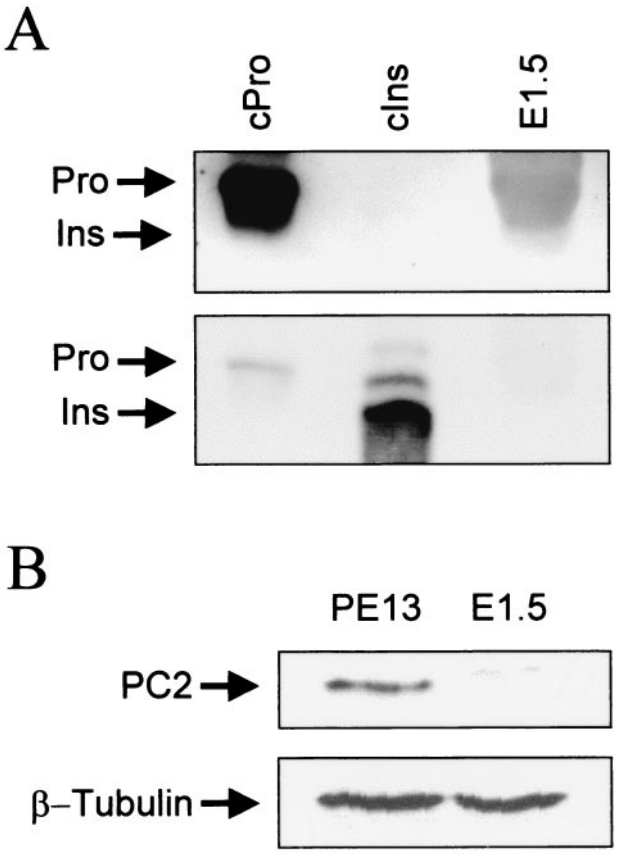

FIG. 4. Immunoblot analysis of insulin, proinsulin, and PC2 in E1.5 embryos. *A*: Embryo protein extracts were resolved in 14% SDS-PAGE and immunoblotted sequentially with anti–peptide C serum (upper panel) and anti-insulin serum (bottom panel). cPro, 0.5 μg recombinant chicken proinsulin; cIns, 1 μg purified pancreatic chicken insulin. In this reducing gel, insulin A and B chains resolve separately, creating a smear. *B*: E1.5 embryo and E13 pancreas protein extracts were separated in 7.5% SDS-PAGE and immunoblotted sequentially with anti-PC2 serum and anti–β-tubulin antibody.

showed positive proinsulin immunostaining. The prospective digestive endoderm was also positive. IGF-I displayed a very restricted staining pattern; at this caudal level, it was detected in only a few ectodermic cells, as well as in the prospective digestive endoderm (Fig. 5*G*). At the level of the rostral somites, IGF-I was strongly positive in sympathetic and dorsal root ganglia (data not shown), and in the head, it was positive in cranial ganglia (Fig. 5*H*), among other structures. A negative control with preimmune rabbit immunoglobulins showed no immunostaining (data not shown).

Prepancreatic and pancreatic preproinsulin mRNAs differ in their 5′-leader sequences. In previous studies, we demonstrated that preproinsulin mRNA levels are differentially regulated by glucose in embryonic pancreas and the neurulating embryo (7). Whereas glucose increases preproinsulin mRNA expression in E13 pancreas, it has no effect in E1.5 embryo preproinsulin mRNA levels. One mechanism by which to achieve developmental and tissue-specific differential gene expression regulation is to generate alternative transcripts. To characterize possible 5′-heterogeneity in the chicken preproinsulin mRNA, we used the 5′-RACE method, a sensitive procedure for amplifying and cloning the 5′ end of a specific mRNA, on RNA isolated from E1.5 embryos or P1 pancreas. A

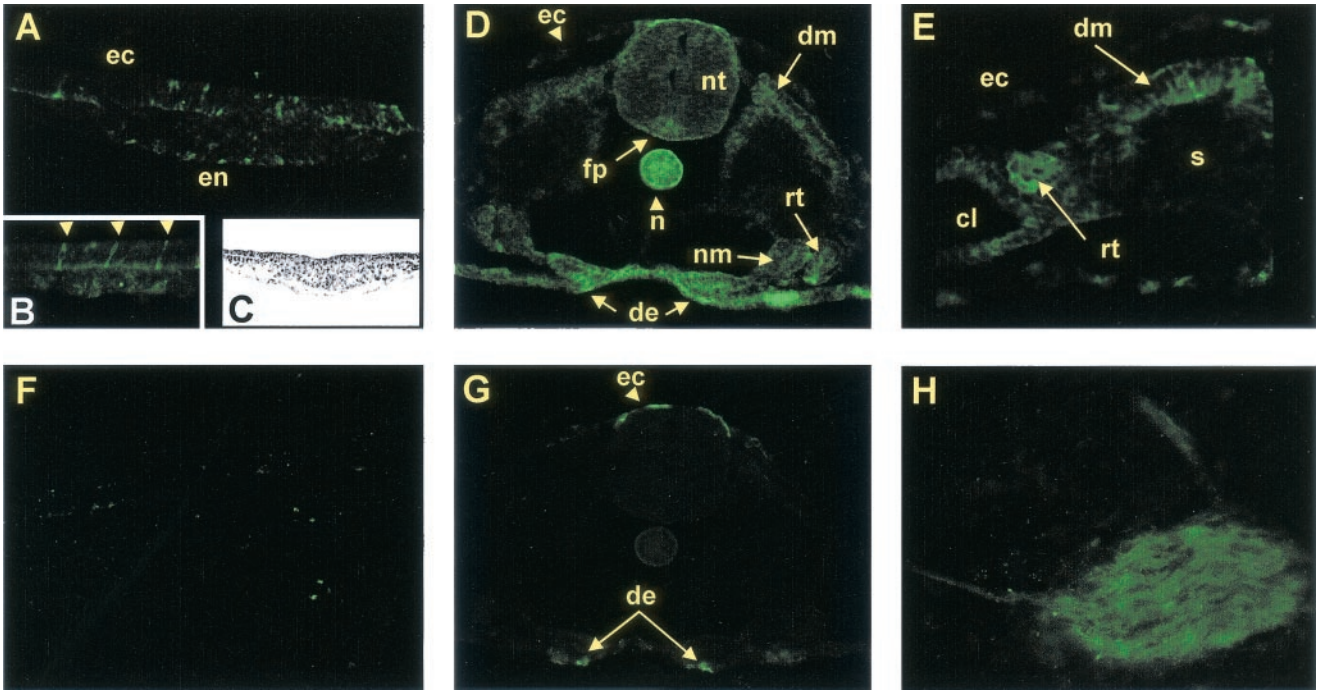

FIG. 5. Proinsulin precedes IGF-I expression during gastrulation and early organogenesis. *A*, *B*, *D*, and *E*: Proinsulin immunostaining. *F*, *G*, and *H*: IGF-I immunostaining. *A* and *F*: Midlevel transversal cryosections of E1 embryos corresponding to the micrograph in *C* (Bellairs and Osmond. *The Atlas of Chick Development*. San Diego, CA, Academic, 1998). *D*, *E*, and *G*: Transversal cryosections of E2.5 embryo caudal to vitelline arteries. *B* shows a higher magnification detail of the lateral area in *A*. Arrowheads indicate labeled neuroepithelial cells. *H*: Cranial ganglion. cl, Embryonic coelome; de, digestive endoderm; dm, dermamyotome; ec, ectoderm; en, endoderm; fp, floor plate; n, notochord; nm, nephrogenic mesenchyme; nt, neural tube; rt, nephric tube; s, somites. Original magnification in *A* and *F*, ×200; *D* and *G*, ×100; *E*, ×175; and *H*, ×300.

~300-bp initial PCR product was obtained in pancreas (Fig. 6*A*). The level of preproinsulin mRNA expression in E1.5 embryo was below detection limits by ethidium bromide staining. The subsequent nested PCR displayed a ~240-bp product in pancreas and a ~280-bp product in E1.5 embryos. Cloning and sequencing analysis of these PCR products revealed two different 5′-untranslated exon 1 sequences in the pancreatic and E1.5 embryo preproinsulin mRNAs (now termed exon 1_A and 1_B, respectively). Exon 1_A contained 57 nucleotides, and exon1_B comprised the complete exon 1_A plus 32 nucleotides at the 5′ end (Fig. 6*B*). Interestingly, both exons 1_A and 1_B differed from that predicted by Perler et al. (11) based on genomic cloning (see DISCUSSION). We confirmed the presence of each preproinsulin mRNA variant by RNase protection assay with a riboprobe generated using the 5′-RACE product from E1.5 embryos as a template. This probe is complementary to exon1_B and to 153 nucleotides of exon 2 (Fig. 7*A*), rendering a 210-bp protected band for pancreas and a 242-bp band for E1.5 (Fig. 7*B*). These results suggest that the preproinsulin gene has different transcriptional regulation in the neurulating embryo than in the pancreas. The alternative first exon, which is untranslated in both cases, does not change the primary protein product, preproinsulin.

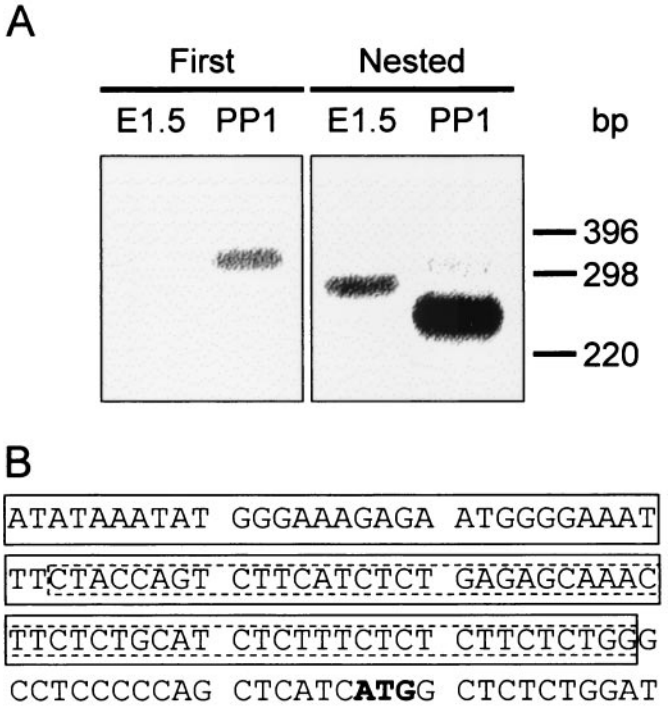

FIG. 6. Cloning and sequencing of the 5′-leader region of prepancreatic and pancreatic preproinsulin mRNA. *A*: 5′-RACE amplification was performed with 5 μg total RNA from E1.5 embryos and postnatal day 1 pancreas (PP1) as template for reverse transcriptase reaction, followed by a first PCR. A second nested PCR was subsequently performed (see RESEARCH DESIGN AND METHODS). The resulting PCR products were electrophoresed and stained with ethidium bromide. *B*: Sequence of exon 1_A, enclosed in dotted box, and 1_B, enclosed in solid box; the coding ATG is in bold.

DISCUSSION

Recent studies have demonstrated the involvement of disregulated cell death in the genesis of the diabetic embryopathy (22–25,29), a nonsurprising finding given the essential role of programmed cell death in embryonic development (1–3). High glucose levels induce apoptotic cell death in the mouse preimplantation embryo (24,29)

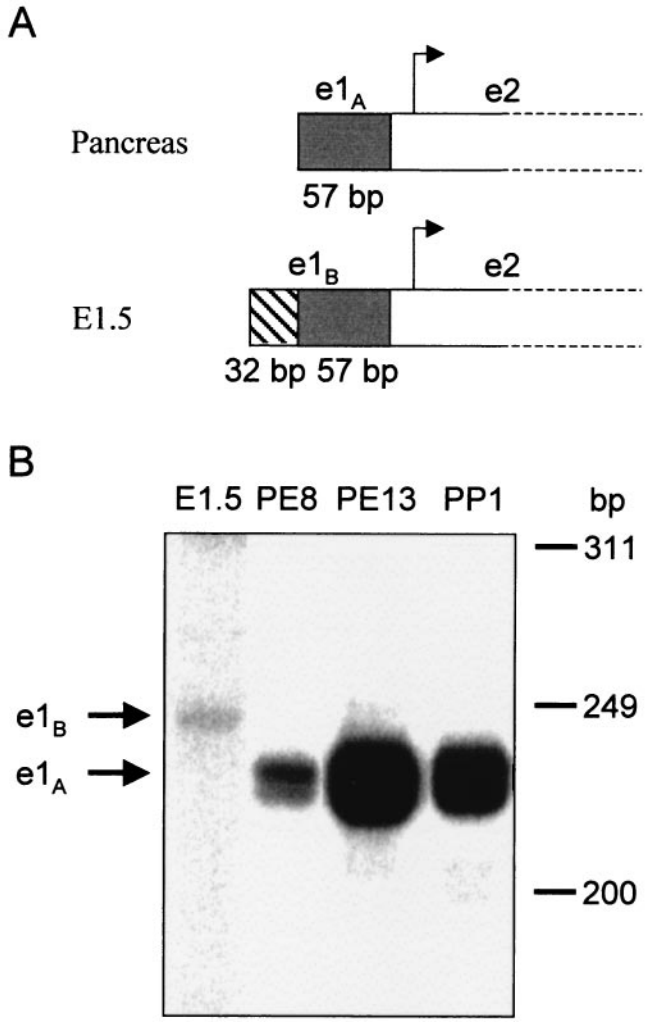

FIG. 7. RNase protection assay. *A*: Schematic representation of the 5′-leader regions of the E1.5 and pancreatic preproinsulin mRNAs. Arrows indicate the translation initiation site. *B*: RNase protection assay. Four micrograms of polyA$^+$ RNA from E1.5 embryos, polyA$^+$ RNA from four E8 pancreata (PE8), 20 μg total RNA from E13 pancreas (PE13), and 10 μg total RNA from postnatal day 1 pancreas (PP1) were hybridized with the exon 1_B antisense riboprobe. e, Exon.

and during neurulation (25). In addition, the levels of a novel gene, *dep*-1, which is regulated by the transcription factor Pax-3, are reduced in the diabetic embryopathy of the mouse (30). Nonetheless, it is likely that congenital malformations, including neural tube defects, are multifactorial processes (31,32) in which maternal hyperglycemia is an important, but not exclusive, etiology. The use of a nonmammalian model to analyze insulin action during neurulation may help to identify processes that eventually can be of importance for mammals as well. One should also consider that there are differences even between mice and humans concerning insulin action–related genes. Whereas the knockout mice for the insulin receptor present only a slight reduction of embryonic size, the spontaneous leprechaun human dwarfs show a more extreme phenotype (33). Another recent example is the basal normoglycemia of the sulfonylurea receptor 1 (SUR1) knockout mouse, strikingly different from the persistent hyperinsulinemia/hypoglycemia of infants who lack SUR1 action (34).

There are strong data supporting the role of prepancreatic insulin in cell survival during development in the maternally independent chick embryo model. During gastrulation and neurulation, the chick embryo is avascular and prepancreatic. Remarkably, it expresses preproinsulin mRNA, and the insulin receptor is functional at these stages, as indicated by its tyrosine kinase activity and anti-apoptotic effect, features shared in part by other species (8,28,35–38). Interference in vivo with insulin receptor synthesis using antisense oligodeoxynucleotides increases apoptosis in the neurulating embryo, demonstrating the physiological relevance of insulin signaling in the regulation of embryonic cell survival/death (this study and Morales et al. [8]). Here, we show that exogenous proinsulin is nearly as effective as insulin in preventing the increase in cell death caused by culture with growth factor deprivation, and this concurs with the observation that the product present in the embryo is proinsulin. Accordingly, we also show the absence of PC2 expression in the neurulating embryo. Both PC2 and PC3 function in the pancreatic regulated secretory pathway and are required for efficient proinsulin processing (39,40), which is essential for the postnatal demands of metabolic regulation. Because young embryos usually lack typical endocrine granules (41), proinsulin in the neurulating chick embryo is probably secreted by a constitutive secretory pathway. In the neuroretina cultured in hormone-free medium, we have observed that proinsulin secretion to the medium is rapid (27). The location of the proinsulin-producing cells in the embryo is consistent with its proposed role in neuroepithelial cell survival. During E1, scattered neuroepithelial cells are immunostained; when the neural tube is closed, at E2.5, some ectodermal cells on its dorsal side are still proinsulin-positive. This pattern is quite different from that of IGF-I, expressed later, and from that of IGF-II (not shown). Altogether, these observations are reminiscent of features in the chick embryo retina, another organ in which the anti-apoptotic role of proinsulin/insulin is relevant (17,18). In this tissue, embryonic proinsulin signals through low discriminating hybrid insulin-IGF receptors (42). Proinsulin not only has high affinity for the hybrid receptors (42) but also a slower dissociation rate than insulin (43), and signal duration may be important for the survival effect. A similar receptor study has not yet been possible due to the scarce tissue of the neurulating embryo. Although the mechanisms implicated in the protective anti-apoptotic effect of proinsulin remain to be studied in detail, we have proposed that the maintenance of chaperone heat shock cognate 70 (Hsc70) levels during neurulation is at least part of them (16). We anticipate that the antioxidant function of insulin may also be implicated. Insulin treatment prevents the increase in oxidative stress caused by diabetes (44). Insulin may also counteract the tumor necrosis factor (TNF)-α malformations increase linked to diabetic embryopathy (45) and help restore the inositol metabolism disturbed by the hyperglycemia (46) in rat models of embryopathy.

An additional important piece of evidence on an alternative growth-related role for embryonic proinsulin/insulin is the characterization that the 5′ untranslated region (UTR) of the early embryo preproinsulin mRNA contains a longer sequence than the pancreas mRNA, suggesting differential transcriptional and/or postrancriptional con-

trol of these alternative transcripts. The alternative exon 1_A or 1_B corresponding to the 5′UTR does not alter the amino acid sequence of either prepancreatic or pancreatic proinsulin. Surprisingly, none of these exons coincides with the previously predicted exon 1 based on the genomic sequence of the chicken preproinsulin 5′-flanking region (11). Thus far, only a partial chicken cDNA from pancreas had been reported (47). Moreover, we did not find the exon 1_A or 1_B sequences within the ~600 bp of the chicken 5′-flanking genomic preproinsulin provided by Soares et al. (48), suggesting that both exons are located upstream from this region. Further experiments will be required to address whether the two preproinsulin transcripts originate from the use of different transcription start sites under the control of a unique promoter or from the presence of two alternative promoters. The use of multiple and developmental-specific promoters or transcription start sites provides a flexible mechanism for developmental stage–specific regulation of individual regions by environmental signals and has been extensively described for both IGF-I and IGF-II (49). Indeed, we previously showed that preproinsulin mRNA did not change in response to glucose in the neurulating embryo, whereas preproinsulin mRNA from embryonic pancreas increased twofold (7). Analysis of the promoter sequences from the chicken preproinsulin gene should allow us to better understand how early embryonic cells regulate the expression of locally required proinsulin before the emergence of the pancreas. The alternative 5′ ends of the preproinsulin transcript may additionally confer differential stability or translatability of the mRNA.

The data presented here emphasize the impact of low insulin signaling independently of the deleterious effect of high glucose in the chick embryo model, as it develops in a 30 mmol/l glucose environment, and increasing this to 50 mmol/l glucose does not augment apoptosis. Embryos at a slightly earlier stage, nevertheless, display relatively high glucose metabolism rates (50), and after E2, high glucose is teratogenic, as indeed is very high insulin (6). Our results suggest that endogenous proinsulin prevents cell death, during a critical neurulation stage, by mechanisms other than its metabolic effect on glucose homeostasis.

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REFERENCES

1. Jacobson MD, Weil M, Raff MC: Programmed cell death in animal development. *Cell* 88:347–354, 1997
2. Vaux DL, Korsmeyer SJ: Cell death in development. *Cell* 96:245–254, 1999
3. de la Rosa EJ, De Pablo F: Cell death in early neural development: beyond the neurotrophic theory. *Trends Neurosci* 23:454–458, 2000
4. Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA: Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 384:368–372, 1996
5. De Pablo F, Girbau M, Gomez JA, Hernandez E, Roth J: Insulin antibodies retard and insulin accelerates growth and differentiation in early embryos. *Diabetes* 34:1063–1067, 1985
6. De Pablo F, Hernandez E, Collia F, Gomez JA: Untoward effects of pharmacological doses of insulin in early chick embryos: through which receptor are they mediated? *Diabetologia* 28:308–313, 1985
7. Perez-Villamil B, de la Rosa EJ, Morales AV, De Pablo F: Developmentally regulated expression of the preproinsulin gene in the chicken embryo during gastrulation and neurulation. *Endocrinology* 135:2342–2350, 1994
8. Morales AV, Serna J, Alarcon C, de la Rosa EJ, De Pablo F: Role of prepancreatic (pro)insulin and the insulin receptor in prevention of embryonic apoptosis. *Endocrinology* 138:3967–3975, 1997
9. Travers JP, Pratten MK, Beck F: Effects of low insulin levels on rat embryonic growth and development. *Diabetes* 38:773–778, 1989
10. Lillie FR: *Lillie's Development of the Chick: An Introduction to Embryology.* New York, Holt, 1952
11. Perler F, Efstratiadis A, Lomedico P, Gilbert W, Kolodner R, Dodgson J: The evolution of genes: the chicken preproinsulin gene. *Cell* 20:555–566, 1980
12. Lomedico P, Rosenthal N, Efstratidadis A, Gilbert W, Kolodner R, Tizard R: The structure and evolution of the two nonallelic rat preproinsulin genes. *Cell* 18:545–558, 1979
13. Wentworth BM, Schaefer IM, Villa-Komaroff L, Chirgwin JM: Characterization of the two nonallelic genes encoding mouse preproinsulin. *J Mol Evol* 23:305–312, 1986
14. Hamburger V, Hamilton HL: A series of normal stages in the development of the chick embryo. *J Dev Physiol* 9:79–88, 1951
15. Morales AV, De Pablo F: Inhibition of gene expression by antisense oligonucleotides in chick embryos in vitro and in vivo. *Curr Top Dev Biol* 36:37–49, 1998
16. de la Rosa EJ, Vega-Núñez E, Morales AV, Serna J, Rubio E, De Pablo F: Modulation of the chaperone heat shock cognate 70 by embryonic (pro)-insulin correlates with prevention of apoptosis. *Proc Natl Acad Sci U S A* 95:9950–9955, 1998
17. Díaz B, Serna J, De Pablo F, de la Rosa EJ: In vivo regulation of cell death by embryonic (pro)insulin and the insulin receptor during early retinal neurogenesis. *Development* 127:1641–1649, 2000
18. Díaz B, Pimentel B, De Pablo F, de la Rosa EJ: Apoptotic cell death of proliferating neuroepithelial cells in the embryonic retina is prevented by insulin. *Eur J Neurosci* 11:1624–1632, 1999
19. Alarcón C, Serna J, Pérez-Villamil B, De Pablo F: Synthesis and differentially regulated processing of proinsulin in developing chick pancreas, liver and neuroretina. *FEBS Lett* 436:361–366, 1998
20. Lowe WL, Roberts CT, Lasky SR, LeRoith D: Differential expression of alternative 5′ untranslated regions in mRNAs encoding rat insulin-like growth factor I. *Proc Natl Acad Sci U S A* 84:8946–8950, 1987
21. De Pablo F, Roth J, Hernández E, Pruss RM: Insulin is present in chicken eggs and early chick embryos. *Endocrinology* 111:1909–1916, 1982
22. Pampfer S, Vanderheyden I, McCracken JE, Vesela J, De Hertogh R: Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumor necrosis factor-alpha in vitro. *Development* 124:4827–4836, 1997
23. Phelan SA, Ito M, Loeken MR: Neural tube defects in embryos of diabetic mice: role of the Pax-3 gene and apoptosis. *Diabetes* 46:1189–1197, 1997
24. Moley KH, Chi MM, Knudson CM, Korsmeyer SJ, Mueckler MM: Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. *Nat Med* 4:1421–1424, 1998
25. Fine EL, Horal M, Chang TI, Fortin G, Loeken MR: Evidence that elevated glucose causes altered gene expression, apoptosis, and neural tube defects in a mouse model of diabetic pregnancy. *Diabetes* 48:2454–2462, 1999
26. Romanoff AL: Chemistry of non-embryonic portions of the egg. In *Biochemistry of the Avian Embryo*. New York, Wiley and Sons, 1967, p. 177–233
27. Hernández-Sánchez C, López-Carranza A, Alarcón C, de la Rosa EJ, De Pablo F: Autocrine/paracrine role of insulin-related growth factors in neurogenesis: local expression and effects on cell proliferation and differentiation in retina. *Proc Natl Acad Sci U S A* 92:9834–9838, 1995
28. Alarcon C, Morales AV, Pimentel B, Serna J, De Pablo F: (Pro)insulin and insulin-like growth factor I complementary expression and roles in early development. *Comp Biochem Physiol B Biochem Mol Biol* 121:13–17, 1998
29. Moley KH: Hyperglycemia and apoptosis: mechanisms for congenital malformations and pregnancy loss in diabetic women. *Trends Endocrinol Metab* 12:78–82, 2001

30. Cai J, Phelan SA, Hill AL, Loeken MR: Identification of Dep-1, a new gene regulated by the transcription factor Pax-3, as a marker for altered embryonic gene expression during diabetic pregnancy. *Diabetes* 47:1803–1805, 1998
31. Pregnancy: outcomes in the Diabetes Control and Complications Trial. *Am J Obstet Gynecol* 174:1343–1353, 1996
32. Chang TI, Loeken MR: Genotoxicity and diabetic embryopathy: impaired expression of developmental control genes as a cause of defective morphogenesis. *Semin Reprod Endocrinol* 17:153–165, 1999
33. Accili D: Insulin receptor knock-out mice. *Trends Endocrinol Metab* 8:101–104, 1997
34. Seghers V, Nakazaki M, DeMayo F, Aguilar-Bryan L, Bryan J: Sur1 knockout mice: a model for K(ATP) channel-independent regulation of insulin secretion. *J Biol Chem* 275:9270–9277, 2000
35. Girbau M, Bassas L, Alemany J, De Pablo F: In situ autoradiography and ligand-dependent tyrosine kinase activity reveal insulin receptors and insulin-like growth factor I receptors in prepancreatic chicken embryos. *Proc Natl Acad Sci U S A* 86:5868–5872, 1989
36. Shuldiner AR, De Pablo F, Moore CA, Roth J: Two nonallelic insulin genes in *Xenopus laevis* are expressed differentially during neurulation in prepancreatic embryos. *Proc Natl Acad Sci U S A* 88:7679–7683, 1991
37. Deltour L, Leduque P, Blume N, Madsen O, Dubois P, Jami J, Bucchini D: Differential expression of the two nonallelic proinsulin genes in the developing mouse embryo. *Proc Natl Acad Sci U S A* 90:527–531, 1993
38. Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E: An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr Biol* 11:213–221, 2001
39. Neerman-Arbez M, Sizonenko SV, Halban PA: Slow cleavage at the proinsulin B-chain/connecting peptide junction associated with low levels of endoprotease PC1/3 in transformed beta cells. *J Biol Chem* 268:16098–16100, 1993
40. Michels JE, Bauer GE, Johnson D, Dixit PK: Morphometric analysis of the endocrine cell composition of rat pancreas following treatment with streptozotocin and nicotinamide. *Exp Mol Pathol* 44:247–258, 1986
41. Kelly RB: Pathways of protein secretion in eukaryotes. *Science* 230:25–32, 1985
42. García-de Lacoba M, Alarcón C, de la Rosa EJ, De Pablo F: Insulin/insulin-like growth factor-I hybrid receptors with high affinity for insulin are developmentally regulated during neurogenesis. *Endocrinology* 140:233–243, 1999
43. De Meyts P: The structural basis of insulin and insulin-like growth factor-I receptor binding and negative co-operativity, and its relevance to mitogenic versus metabolic signalling. *Diabetologia* 37 (Suppl. 2):S135–S148, 1994
44. Eriksson UJ, Borg LA, Cederberg J, Nordstrand H, Siman CM, Wentzel C, Wentzel P: Pathogenesis of diabetes-induced congenital malformations. *Ups J Med Sci* 105:53–84, 2000
45. Pampfer S, Vanderheyden I, Wuu YD, Baufays L, Maillet O, De Hertogh R: Possible role for TNF-alpha in early embryopathy associated with maternal diabetes in the rat. *Diabetes* 44:531–536, 1995
46. Wentzel P, Wentzel CR, Gareskog MB, Eriksson UJ: Induction of embryonic dysmorphogenesis by high glucose concentration, disturbed inositol metabolism, and inhibited protein kinase C activity. *Teratology* 63:193–201, 2001
47. Hasegawa S, Honda K, Nata K, Yonekura H, Okamoto H, Hikami Y: Isolation of a cDNA encoding chicken insulin precursor. *Animal Scien Tech* 62:867–869, 1991
48. Soares MB, Schon E, Henderson A, Karathanasis SK, Cate R, Zeitlin S, Chirgwin J, Efstratiadis A: RNA-mediated gene duplication: the rat preproinsulin I gene is a functional retroposon. *Mol Cell Biol* 5:2090–2103, 1985
49. Rosenfeld RG, Roberts, CT: *The IGF System: Molecular Biology, Physiology and Clinical Applications*. Totowa, NJ, Humana, 1999
50. Baroffio A, Raddatz E, Markert M, Kucera P: Transient stimulation of glucose metabolism by insulin in the 1-day chick embryo. *J Cell Physiol* 127:288–292, 1986