Supplementary information

Figure legends

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Previous studies showed that neurulating embryos upregulated proinsulin mRNA levels when cultured without a source of growth factors (Pérez-Villamil et al., 1994). We studied each embryonic proinsulin isoform in these conditions, and found that the levels of both isoforms increased inversely to insulin concentration in culture medium. In the absence of growth factor, the spliced out isoform showed the largest increase. In the absence of a source of growth factor, intron 1 splicing regulation was also tissue-specific.
While the rate of intron 1 retention decreased in heart, it was not significantly affected in the optic vesicle.

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Methods

Embryos and whole chick embryo culture

Fertilized White Leghorn eggs (Granja Rodriguez-Serrano, Salamanca, Spain) were incubated at 38.4 °C and 60-80% relative humidity. Embryos were staged according to Hamburger and Hamilton (1951). E7.5 mouse embryos (C57BL/6) were removed from the uterus of pregnant females and subsequently dissected from the deciduum. E9 mouse embryo RNA was kindly provided by the UK HGMP Resource Center (Babraham, Cambridge). Animals were handled according to European Union Guidelines for animal research.

Proinsulin radioimmunoassay

A total of 36 embryos at each stage were pooled and homogenized in a volume of 100 ul of 10 mM Hepes pH 7.9, 1.5 mM MgCl₂ and 10 mM KCl through a syringe needle. The lysates were clarified by centrifugation for 5 min at maximum speed in a microfuge. The supernatants were split in two and the RIA was performed with a rat insulin/proinsulin RIA kit (Linco Research, St. Charles, MI) according to the manufacturer’s instructions. Serial dilutions of recombinant chicken proinsulin (obtained in our laboratory expressing a chicken proinsulin cDNA) were used as specific positive control.

RNA isolation, RT-PCR studies and Northern blot

Total RNA from whole embryos and NIH3T3 cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). The embryo cytoplasmic fraction was obtained as described (Herold et al., 2003) and RNA was subsequently extracted using the Nanoprep Kit (Stratagene, La Jolla, CA). RNA from heart, optic vesicle and presomitic mesoderm was purified using the Nanoprep Kit. The reverse transcriptase reaction (RT) was typically
performed with 5'g RNA (pretreated with DNase), Superscript III Kit and oligo-dT primer (all from Invitrogen). Primers for PCR amplification are listed below. When indicated, a subsequent Southern blot was performed. Briefly, amplification reactions were transferred to nylon membranes, hybridized with a 32P-labeled chicken proinsulin probe (Hernández-Sánchez et al., 2003) and quantified using a Fuji image analyzer.

Quantitative PCR for proinsulin coding region was performed with LUX™ fluorogenic primers and Platinium Quantitative PCR SuperMix-UDG (all from Invitrogen). For proinsulin isoforms, Sybr Green and Kit Core Master Mix (all from Applied Biosystems) were used. PCR was carried out in the real time PCR apparatus ABI Prism 7700 (Applied Biosystems).

Northern blot was performed using 32P-labeled chicken proinsulin probe as described (Hernández-Sánchez et al., 2003). After stripping, membranes were hybridized with a 32P-labeled mouse GAPDH probe (Hernández-Sánchez et al., 2003). Proinsulin and GAPDH mRNA levels were quantified with a Fuji image analyzer.

**Plasmids**

The pancreatic (Pro1A) and the spliced embryonic (Pro1B) proinsulin expression constructs were derived from the pCPro1A and pCPro1B vectors, respectively (Hernández-Sánchez et al., 2003). The intron-retained embryonic cDNA (Pro1B1) was generated in several steps. First RT of st. 10 embryo RNA with oligo-dT, followed by amplification with the chicken proinsulin primers described below, was cloned into the pCR1I TOPO shuttle vector, subsequently excised with SpeI and cloned in similarly digested proinsulin genomic pCR1I TOPO construct, described by Hérmandez-Sánchez et al., 2003. This construct was used as a template for a second PCR with the 5'ATATAATATGGAAAGAGAATG3'
(sense) and 5’GTTGCAGTAGTTCTCCAGTT3’ (antisense) primers, and the product cloned in-frame 5’ to the V5 epitope into pcDNA3.1/V5-His TOPO vector (Invitrogen) giving the pCPro1B1 expression construct.

For in vitro translation experiments, the HindIII/PmeI fragments from pCPro1A, pCPro1B and pCPro1B1 corresponding to each proinsulin cDNA were placed between the HindIII/SmaI sites of the PGEM-3Zf(+) vector (Promega, Madison WI). For in vivo translation, the HindIII/SmaI fragments from the from pCPro1A, pCPro1B and pCPro1B1 were cloned between the HindIII/SmaI sites of the pGEM-2 vector (Promega). For RNA transfection, a poly d(A) tail of 73 residues from the pBluescript vector (Gebauer et al., 1994) was added to each pGEM3 proinsulin construct. In all proinsulin-expressing constructs, the proinsulin cDNA was fused in-frame, 5’ to the V5 epitope.

The bicistronic constructs were based on a modified pBIC vector (Martinez-Salas et al., 1993), in which the first cistron corresponding to the chloramphenicol acetyl transferase was replaced by renilla luciferase cDNA (pBICR). The renilla luciferase coding region was generated by PCR of the pRL-CMV vector (Promega). The proinsulin transcript 5’ UTR were amplified by PCR and cloned in sense or antisense orientation into the SacI site of the intercistronic sequence of the pBICR. The 5’ and 3’ splice sites double mutant intron was generated by the ExSite PCR-based site-directed mutagenesis method (Stratagene, La Jolla, CA). The GT 5’ splice site was mutated to CA, and 25 nt including the polypyrimidine tract and the 3’ splice site) were deleted. The identity and orientation of all constructs was verified by sequencing.

In vitro transcription, translation and immunoprecipitation.

Capped RNAs were synthesized as described (Gray and Hentze, 1994), including m7G(5’)ppp(5’)G (Promega) in the transcription reaction. Equal amounts of capped RNA
(0.2 µg/l) were in vitro-translated using the rabbit reticulocyte lysate system and [35 S]Cys (Amersham Pharmacia Biotech, Essex, UK). The translation reaction was immunoprecipitated with anti-V5 epitope antibody (Invitrogen) and resolved by electrophoresis (Hernández-Sánchez et al., 2003).

**Transfections and Western blot**

NIH3T3 cells were cultured as described (Hernández-Sánchez et al., 2003). Cells were infected with vaccinia virus VTF7-3 expressing T7 RNA polymerase (Fuerst et al., 1986; López de Quinto and Martínez-Salas, 1999) 1 h prior to transfection of DNA constructs with Lipofectamine Plus (Invitrogen). Cells were processed 48 h after transfection for immunoblot as described (Hernández-Sánchez et al., 2003). Cells transfected with bicistronic constructs were lysed 24 h post-transfection and firefly and renilla luciferase activities were determined using the Dual Luciferase Kit (Promega). For RNA stability experiments, cells were transfected with capped and polyadenylated RNA using the TransMessenger reagent (Qiagen, Valencia, CA) and RNA was extracted at the times indicated.

**Proinsulin treatment**

Heparin acrylic beads (Sigma, St. Louis, MO) were soaked in proinsulin (600 ng/ml or 15 µg/ml) or PBS, and implanted into the lateral limit of the heart forming region of embryos at stage 5, as described (López-Sánchez et al., 2002). After 12 h further incubation, embryos were fixed and processed for whole-mount in situ hybridization and immunocytochemistry.

**In situ hybridization**

Embryos were processed for whole mount in situ hybridization as described (López-Sánchez et al., 2004). Probes were described in López-Sánchez et al. (2002).
Immunohistochemistry

Immunocytochemistry was performed essentially as described (Patel et al., 1989), using the MF20 antibody (Hybridoma Bank, Developmental Studies) (Sato and Yost, 2003). When it was performed after in situ hybridization the procedure of López-Sánchez et al. (2004) was followed.

Primers

For chicken proinsulin amplification, we used the 5’-GAATGGGGAAATTCTACCAGT-3’ (P3) or 5’-CCAGTCTCTCATCTCTGAGAG-3’ (P4) sense primers and the 5’-GCTAGTTGCAGTAGTTCTCCAGTT-3’ (P5) antisense primer.

Mouse proinsulin cDNA was amplified with the 5’-TAAGTGATCCGCTACAATCA-3’ (sense) and 5’-CAGTAGTTCTCCAGCTGGTA-3’ (antisense) primers.

Chicken U6 snRNA was amplified with 5’-GTGCTCGCTTCGGCAGCACA-3’ (sense) and 5’-CTTCACGAATTTGCGTGTCA-3’ (antisense) primers.

In quantitative PCR, Pro1B1 cDNA was amplified with the 5’-AGCTTTATTCCTCCCTTGCAAC-3’ (sense) and 5’-GACTGCTCAGTAGGGCTGC-3’ (antisense) primers; Pro1B with the 5’-TCTCTCTTCTGCGCCTCC-3’ (sense) and 5’-GCTCTCCACACACCAGGTAG-3’ (antisense) primers. LUX™ fluorogenic primers for proinsulin coding region were 5’-CCCTGGAACCAGCTATGCAG-3’ (P1, sense) and 5’-ctacctgCACGCTCTCCACACCCAGGTAG-3’ (P2, antisense). LUX™ fluorogenic primers for GAPDH were 5’-CCATGTTTGTGAGGTCGTCC-3’ (sense) and 5’-caactgAAGGGGTGCCAGGCAGTTG-3’ (antisense). In standard and quantitative PCR using Sybr Green, the endogenous reference GAPDH was amplified with the 5’-
GCAATGCATCGTGCACCACC-3’ sense and 5’-TGTGATGGCATGGACAGTGG-3’ antisense primers.

The renilla luciferase coding region was generated by PCR of the pRL-CMV vector (Promega) using 5’-CTCACTGCAAGGCTAGCCACC-3’ (sense) and 5’-GCTCGAAGCGGCCGCTGCAGAATT-3’ (antisense) primers that carry PstI sites (underlined). The 5’ UTR of the proinsulin transcripts were amplified with 5’GTCTTGAGCTCTGAGAGCAAAC-3’ and 5’-CAGAGCTGCTGATGAGCTGGG3’ primers, introducing SacI sites (underlined).
References used in the Supplementary Material


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