Developmental regulation of a proinsulin messenger RNA generated by intron retention

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Proinsulin gene expression regulation and function during early embryonic development differ remarkably from those found in postnatal organisms. The embryonic proinsulin protein content decreased from gastrulation to neurulation in contrast with the overall proinsulin messenger RNA increase. This is due to increasing levels of a proinsulin mRNA variant generated by intron 1 retention in the 5' untranslated region. Inclusion of intron 1 inhibited proinsulin translation almost completely without affecting nuclear export or cytoplasmic decay. The novel proinsulin mRNA isoform expression was developmentally regulated and tissue specific. The proportion of intron retention increased from gastrulation to organogenesis, was highest in the heart tube and presomitic region, and could not be detected in the pancreas. Notably, proinsulin addition induced cardiac marker gene expression in the early embryonic stages when the translationally active transcript was expressed. We propose that regulated unproductive splicing and translation is a mechanism that regulates proinsulin expression in accordance with specific requirements in developing vertebrates.

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INTRODUCTION

Insulin biosynthesis in the postnatal pancreas is considered a paradigm of tissue-specific expression by the islet β -cells, in which

it is synthesized as proinsulin and processed to insulin by specific convertases (Steiner *et al*, 1990). In postnatal organisms, insulin is an essential hormone that is responsible for maintaining glucose homeostasis. In recent years, other functions of insulin have emerged. Insulin and its precursor proinsulin are key regulators of cell survival during embryonic development (Díaz *et al*, 2000; Hernández-Sánchez *et al*, 2002). In parallel with this alternative function, new mechanisms for the regulation of proinsulin gene expression have been described. During early development, proinsulin is expressed before formation of the pancreatic primordium in several vertebrates (Serrano *et al*, 1989; Shuldiner *et al*, 1991; Deltour *et al*, 1993). In the prepancreatic chick embryo, proinsulin messenger RNA is expressed and translated to proinsulin by discrete cells located in the three embryonic layers (Morales *et al*, 1997; Hernández-Sánchez *et al*, 2002).

In contrast to postnatal regulation, glucose does not affect proinsulin mRNA levels in the prepancreatic embryonic stages (Pérez-Villamil *et al*, 1994), suggesting distinct mechanisms of gene expression regulation. We recently described a specific embryonic proinsulin mRNA (Pro1B) with a 32-nucleotide (nt) extension in its 5' untranslated region (UTR), but which shares its coding region with the pancreatic transcript (Pro1A). The translational activity of the embryonic proinsulin variant is repressed by the presence of two upstream AUGs (upAUGs) in the extended 5' UTR (Hernández-Sánchez *et al*, 2003). Alternative gene splicing generates mRNAs that encode distinct protein products, but has also been proposed as a mechanism to switch gene expression on and off (Smith & Valcarcel, 2000; Black, 2003). Here, we identified a new embryonic proinsulin transcript isoform generated by retention of the first intron in the 5' UTR. This large, structured 5' UTR nearly blocked proinsulin translation, although it did not affect mRNA transport or cytoplasmic stability. The proportion of embryonic proinsulin intron retention was developmentally regulated and tissue specific. We also observed that the addition of proinsulin to chick embryos induced heart tissue. Our results show a further level of post-transcriptional control of the proinsulin gene—by intron 1 splicing regulation.

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Fig 1 | Embryonic proinsulin protein and messenger RNA content. (A) Proinsulin was measured in extracts of pools of 18 embryos of stage (st.) 4 (12–18 h of incubation) and st. 10 (~36 h) by radioimmunoassay (RIA), and is expressed relative to total protein concentration. Results represent the mean $\pm$ s.d. of two replicates. Note that chick proinsulin protein levels might be underestimated as a rat insulin/proinsulin RIA kit was used. (B) Quantitative reverse transcription–PCR of RNA from st. 4 and st. 10 embryos, with primers P1 and P2 (indicated in Fig 2A) for the proinsulin coding region shared by the two embryonic transcripts. The levels of proinsulin mRNA were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Results represent the mean $\pm$ s.d. of two experiments each in duplicate.

RESULTS AND DISCUSSION

Divergent proinsulin protein and mRNA expression

Analysis of proinsulin expression in prepancreatic chick embryos during gastrulation (stage, st. 4) and neurulation (st. 10) showed a dissociated pattern between mRNA and protein levels. Radioimmunoassay of embryonic extracts showed 3.4-fold higher proinsulin protein content in the st. 4 embryos than in the st. 10 embryos (Fig 1A). Similar results were obtained by enzyme-linked immunosorbent assay (not shown). In contrast, the proinsulin total mRNA, measured by quantitative reverse transcription–PCR (RT–PCR; Fig 2A), increased almost twofold as development proceeded (Fig 1B).

A novel proinsulin mRNA generated by intron retention

To assess whether the discrepancy between the protein and mRNA levels was due to the presence of variant embryonic proinsulin transcripts, we carried out a detailed RT–PCR study during gastrulation, neurulation and early organogenesis (Fig 2). PCR with the P3 and P5 primers showed two amplification products (Fig 2A,B). Cloning and sequencing of these PCR products showed that the larger band corresponded to an alternatively spliced isoform (Pro1B1) of the embryonic proinsulin transcript; it retained intron 1 (717 nt) in the 5' UTR, but spliced out intron 2 (3,432 nt). Both spliced and intron-retained embryonic transcripts were polyadenylated, since oligo-dT was used in the RT reaction. The intron 1 splicing pattern is developmentally regulated; the intron 1-containing isoform was nearly undetectable during gastrulation (st. 4), and the percentage of intron 1 retention increased throughout neurulation (st. 8–10) and organogenesis (st. 12; Fig 2B,C). No PCR products were detected in the absence of reverse transcriptase, which indicated the lack of contaminating genomic DNA. The intron-retained embryonic

Fig 2 | Characterization of embryonic proinsulin transcripts.

(A) Schematic organization of the chicken proinsulin gene and embryonic transcripts: exons (E), introns (I) and primers (P) used in PCR are indicated. The intron 1 sequence is provided in the supplementary information online. (B,C). Proinsulin transcript expression during chick early development. Proinsulin intron 1 retention was assayed by reverse transcription–PCR (RT–PCR) with P3 and P5 primers, followed by Southern blot analysis. Percentage of intron retention was calculated as ((mRNA retaining intron 1)/(mRNA retaining intron 1 + mRNA with intron 1 processed) $\times$ 100). (D) RT–PCR with P4 and P5 primers of RNA from the pancreas of embryonic day 13 (E13) and stage (st.) 10 embryos. (E) Mouse proinsulin transcript expression. RT–PCR of E7.5 or E9 mouse embryo RNA and of adult mouse pancreatic RNA. (F) Proinsulin RT–PCR of total and cytoplasmic RNA from st. 10 embryos. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was carried out as RNA input and general splicing control. U6 small nuclear RNA (snRNA) amplification was carried out as nuclear contamination control; RT, control without reverse transcriptase. Products in (B) and (E) were cloned and sequenced to confirm that they corresponded to the spliced and intron 1-retained proinsulin transcripts.

transcript was not detected in the embryonic chick pancreas (Fig 2D). This finding is not unique to the chick embryo, as we also found the intron 1-retained proinsulin transcript in the embryonic day 9 (E9) mouse embryo, although it was undetectable in earlier embryos (E7.5) and in the adult mouse pancreas (Fig 2E). There are no similarities between the two species in the size of the retained intron 1 (717 nt in chicken versus 102 nt in mouse) or their sequence, and no consensus regulatory sequences have been found, although the intron splicing consensus sequences are highly conserved in both species (supplementary information online).

As partially spliced RNA is often retained and degraded in the nucleus (Dreyfuss *et al*, 2002), we analysed whether the proinsulin intron-containing transcript was exported to the cytoplasm. We analysed its expression in the RNA cytoplasmic fraction as compared with that in total RNA from st. 10 embryos. RT-PCR analysis showed the intron-containing transcript in the cytoplasm (Fig 2F). Moreover, the percentage of intron 1 retention in the cytoplasmic fraction was nearly identical to that in the total embryo (48% versus 50%). Splicing of the second intron might facilitate the nuclear export of the intron 1-retained transcript (Kataoka *et al*, 2000). We confirmed that the cytoplasmic RNA preparation was free of nuclear contamination, by testing for U6 small nuclear RNA (Boelens *et al*, 1995). RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with primers flanking intron 6 (167 nt) showed no intron retention in the GAPDH transcript, which suggests that there is no general splicing inhibition at that embryonic stage. Proinsulin intron 1 splicing sites are efficiently recognized in cultured cell lines, as intron 1 was constitutively spliced out when transfected into BHK-21 (baby hamster kidney) or NIH3T3 (murine fibroblast) cell lines (supplementary information online).

Altogether, these results indicate that intron 1-containing proinsulin transcript is a reliable splicing variant that is transported to the cytoplasm and developmentally regulated, rather than a splicing intermediate retained in the nucleus. These findings indicate a complex developmental regulation of embryonic proinsulin expression.

Proinsulin mRNA variants were previously reported in pancreatic cells. In the murine pancreatic β -cell line β TC3, a small proportion of the cytoplasmic proinsulin mRNA retained intron 1 (Wang *et al*, 1997). We did not detect the intron 1-retained variant in the chicken or mouse pancreas. In human pancreatic islets, an insulin mRNA was described with another intron 1 variant due to activation of a cryptic 5' splice site (Shalev *et al*, 2002). In addition, there are few examples of the regulated retention of introns, of which the best characterized are the Msl2 and P-element transcripts in *Drosophila* (Gebauer *et al*, 1998; Rio, 1991).

Intron retention does not affect mRNA stability

To determine whether the long leader sequence (806 nt) of the intron 1-retained isoform affected mRNA stability, we compared the decay rate of the spliced-out and intron 1-containing embryonic proinsulin transcripts. Proinsulin intron 1 is correctly spliced out in cell lines transfected with DNA constructs. To avoid nuclear splicing that would yield exclusively Pro1B RNA (supplementary information online), we transfected NIH3T3 cells with *in vitro*-synthesized mRNA, including the cap and poly(A)

Fig 3 | Stability of proinsulin embryonic messenger RNA. (A) Northern blot of NIH3T3 cells transfected with spliced-out (Pro1B) or intron-retained embryonic capped mRNA (Pro1B1). (B) Decay curves. RNA was extracted at the times indicated and equal amounts of RNA were subjected to northern blot analysis. Pro1B and Pro1B1 mRNA levels were corrected by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

tail structures. The half-life of the intron 1-retained isoform was estimated at 9 h, whereas that of the spliced-out isoform was only 10% longer (Fig 3). The decay curves for the two transcripts thus indicated that intron 1 retention does not influence mRNA degradation levels markedly.

In the direct mRNA transfection experiments described above, we did not address the susceptibility of the intron 1-retained proinsulin transcript to the nonsense-mediated mRNA decay surveillance mechanism, as the *in vitro*-synthesized mRNA did not have the exon-exon junction complexes needed to define premature termination codons (see the review by Maquat, 2004).

Embryonic proinsulin mRNAs translation

5' UTRs have an important impact on the translational activity of the downstream open reading frame (reviewed by Kozak, 2002; Gebauer & Hentze, 2004). We examined the effect of the long 5' UTR (806 nt) of intron 1-retained mRNA on the translational activity of the novel isoform. Translation of equal amounts of *in vitro*-transcribed proinsulin isoforms in a reticulocyte lysate system showed that the intron 1-containing isoform yielded much less proinsulin than the pancreatic or the spliced-out embryonic transcripts (Fig 4A). Differences in translation levels were then analysed *in vivo*. To avoid interference by nuclear splicing or cryptic promoter, NIH3T3 cells were infected before transfection with vaccinia virus VTF7-3 expressing the T7 RNA polymerase, to allow for cytoplasmic transcription of the transfected constructs. Proinsulin expression was nearly undetectable in cells transfected

Fig 4 | Translational activity of proinsulin transcripts. (A) *In vitro* translation of pancreatic (Pro1A), embryonic spliced-out (Pro1B) and intron-retained (Pro1B1) proinsulin-V5 transcripts. The amount of proinsulin generated by Pro1B1 messenger RNA was 2.5-fold lower than that generated by Pro1B mRNA. (B) Proinsulin expression in transfected NIH3T3 cells. Proinsulin expression was analysed by immunoblot using an anti-V5 tag antibody. β -Tubulin was used as a loading control. (C) Northern blot of transfected NIH3T3 cells. Membranes were probed for proinsulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Proinsulin mRNA level normalized by GAPDH mRNA was 2.64 for Pro1A, 2.72 for Pro1B and 6.58 for Pro1B1. (D) Assessment of internal ribosome entry site (IRES) activity. Diagram of the bicistronic reporter construct (upper drawing). The *SacI* cloning site in the intercistronic region is indicated. IRES activity is calculated as firefly luciferase activity/*Renilla* luciferase activity. Results represent the mean $\pm$ s.d. of three replicates.

with the intron 1-containing construct (Fig 4B). The spliced-out embryonic construct produced proinsulin—although much less than the pancreatic construct—as reported (Hernández-Sánchez *et al*, 2003; Fig 4B). Northern blot analysis confirmed that the

differences in proinsulin expression were mediated by different translational efficiencies rather than by mRNA content (Fig 4C).

To analyse further the translation capacity of the intron 1-containing transcript and discard possible internal ribosome entry site (IRES)-dependent initiation (reviewed by Stoneley & Willis, 2004), we examined the IRES activity of the spliced-out and intron 1-containing 5' UTR, using bicistronic constructs. In this system, expression of the first cistron indicated cap-mediated translation, and expression of the second cistron indicated IRES-driven translation (Fig 4D, upper panel). NIH3T3 cells were infected before transfection with vaccinia virus VTF7-3. Neither the spliced-out 5' UTR nor the intron 1-retained 5' UTR showed IRES activity (compare sense and antisense orientation of each construct; Fig 4D). Transfection with the bicistronic construct with the characterized IRES of the foot and mouth disease virus (Martínez-Salas *et al*, 1993) was used as a positive control. Similar results were obtained when BHK-21 or insulin-expressing quail embryonic retina cells (RTC5; Hernández-Sánchez *et al*, 2003) were transfected. The lack of IRES activity was not due to the absence of a nuclear event; NIH3T3 cells transfected with the bicistronic construct carrying the intron 1-retained 5' UTR, with the 5' and 3' splice sites mutated and under the control of the thymidine kinase promoter (an RNA polymerase II promoter), showed no IRES activity (supplementary information online).

Together, these results indicate that the extra 717 nt provided by intron 1 to the intron-retained proinsulin transcript imposes a further restriction on the translational activity, generating proinsulin mRNA that is unable to mediate either cap or IRES translation.

Intron-retained proinsulin transcript is tissue selective

As we found that proinsulin gene splicing is developmentally regulated (Fig 2B,C), we analysed whether it was distributed in a tissue-selective manner in neurulating embryos (Fig 5A, st. 10), which express both proinsulin isoforms. Proinsulin intron 1 was preferentially retained in the heart tube and the presomitic region, whereas it was efficiently spliced out in the optic vesicle (Fig 5B,C). We then studied the proinsulin splicing pattern at different stages of cardiac morphogenesis. At st. 4, when the bilateral precardiac regions appear, the precardiac region expressed mainly the spliced-out isoform. The percentage of intron 1 retention subsequently increased in the cardiac crescents (st. 7 and st. 8), reaching a maximum when the single heart tube was formed (st. 10; Fig 5D,E).

Proinsulin action in early cardiogenesis

As shown above, the precardiac region at st. 4 expressed mainly the embryonic transcript that was able to produce proinsulin, which suggests that proinsulin may have a role in the morphogenesis of the heart. We therefore implanted proinsulin-coated beads into the lateral limit of the precardiac region of st. 5 embryos (Fig 6A). Proinsulin led to formation of ectopic tissue adjacent to the beads. *In situ* hybridization showed that this tissue expressed *VMHC1*, a marker of differentiating cardiac myocytes, which was later restricted to ventricular myocytes (Fig 6B,C). These effects were observed with beads at various distances from the host heart (Fig 6B,C). Moreover, immunohistochemistry showed that the ectopic tissue contained cells positive for MF20—a myosin heavy chain normally expressed in muscle cells in the myocardium of the major chambers (Fig 6D,F). Proinsulin beads did not induce

Fig 5 | Modulation of the proinsulin splicing pattern. (A) Diagram of embryonic regions. (B,C) Proinsulin reverse transcription-PCR (RT-PCR) with P3 and P5 primers followed by Southern blot of RNA from different regions of stage (st.) 10 embryos. (D,E) RT-PCR with P3 and P5 primers followed by Southern blot of cardiac regions during cardiac morphogenesis. Part (D) includes three distinct embryonic RNA pools. CC, cardiac crescents; H, heart; OV, optic vesicle; PC, precardiac region; PS, presomitic region. Percentage of intron retention is calculated as in Fig 1. Results represent mean percentage $\pm$ s.e.m. of intron retention from three independent experiments.

expression of other mesodermal markers such as *Shh*, which is specific to the axial mesoderm (Fig 6E), or *paraxis*, which is specific to the lateral mesoderm (Fig 6F).

The above data indicate that proinsulin induced ectopic expression of cardiac lineage markers and that it could have a crucial role in cardiogenesis. Proinsulin did not act as an initial inducer of nonspecified mesoderm, which could later differentiate into cardiac mesoderm. These results are in accordance with studies in which insulin induced terminal cardiac differentiation (Lough & Sugi, 2000). Proinsulin administration to st. 8 embryos induced neither ectopic tissue nor expression of cardiac markers (not shown), which suggests that proinsulin is not effective once the process of cardiogenesis is defined. At this stage, the intron 1-retained mRNA isoform in the region that formed the heart became the foremost variant.

Speculations

This study demonstrates novel aspects of the physiological regulation of proinsulin expression in early embryonic development through the uncommon mechanism of stage- and tissue-specific intron retention. We have shown that the intron 1-retained mRNA is a bona fide transcript despite being translationally inactive. We propose that the unusual splicing pattern and the limited proinsulin cellular responsiveness in the precardiac region are related mechanisms that contribute to ensuring tight temporal control of cardiac morphogenesis. In addition, we speculate that the maintenance of proinsulin gene transcriptionally active but translationally inactive may provide a reversible (supplementary Fig 3 online) back-up mechanism for the embryonic tissues to adapt to environmental or developmental changes.

METHODS

General methods used were as described by Hernández-Sánchez *et al* (2003). Specific methods used are described in the supplementary information online.

Fig 6 | Proinsulin effect on cardiac expression markers. (A) Diagram of proinsulin bead (asterisk) implanted on one side at the lateral limit of the precardiac region in stage (st.) 5 embryos. Embryos at st. 10 (B,D,E) and st. 11 (C,F) treated with a proinsulin-coated bead, in a solution of 600 ng/ml (B,E) or 15 μ g/ml (C,D,F) are shown. *In situ* hybridization of *VMHC1* (B,C) and *Shh* (E). Immunohistochemistry for MF20 (D). *In situ* hybridization for *paraxis* followed by immunohistochemistry for MF20 (F). Ectopic tissue in the ventral face of the bead (arrowhead) located between the bead (asterisk) and the heart tube expressed *VMHC1* (B,C) and MF20 (D,F). No labelling was detectable around the bead for *Shh* (E) or *paraxis* (F). Insets in parts (C,D) correspond to heart level transversal sections. H, heart; N, neural tube. Scale bar, 120 μ m.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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