Diabetes Publish Ahead of Print, published online October 17, 2007

Long-Range Enhancers are Required to Maintain Expression of the Autoantigen IGRP in Adult Mouse Islets *In Vivo*

Yingda Wang[1], Brian P. Flemming[1], Cyrus C. Martin[1], Shelley R. Allen[1], Jay Walters[2], James K. Oeser[1], John C. Hutton[2] and Richard M. O'Brien[1]

[1]Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, Tennessee 37232

[2]Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, Aurora, Colorado 80045

Running Title: Islet-Specific Gene Expression

Corresponding Author:
Richard M. O'Brien
Department of Molecular Physiology and Biophysics
761 PRB
Vanderbilt University Medical School
Nashville, TN 37232-0615
E-mail: richard.obrien@vanderbilt.edu

Received for publication 19 January 2007 and accepted in revised form 11 October 2007.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

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ABSTRACT

Objective: Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) is selectively expressed in islet beta cells and is a major autoantigen in both mouse and human Type I diabetes. This study describes the use of a combination of transgenic and transfection approaches to characterize the gene regions that confer the islet-specific expression of IGRP.

Research Design and Methods: Transgenic mice were generated containing the IGRP promoter sequence from -306, -911 or -3911 to +3 ligated to a LacZ reporter gene. Transgene expression was monitored by XGal staining of pancreatic tissue.

Results & Conclusions: In all these transgenic mice robust LacZ expression was detected in newborn mouse islets but expression became mosaic as animals aged, suggesting that additional elements are required for the maintenance of IGRP gene expression. VISTA analyses identified two conserved regions in the distal IGRP promoter and one in the third intron. Transfection experiments demonstrated that all three regions confer enhanced luciferase reporter gene expression in βTC-3 cells when ligated to a minimal IGRP promoter. A transgene containing all three conserved regions was generated by using a bacterial recombination strategy to insert a LacZ cassette into exon 5 of the IGRP gene. Transgenic mice containing a 15 kbp fragment of the IGRP gene were then generated. This transgene conferred LacZ expression in newborn mouse islets, however, expression was still suppressed as animals aged, suggesting that long-range enhancers 5' or 3' of the IGRP gene are required for the maintenance of IGRP gene expression in adult mice.

KEY WORDS
glucose-6-phosphatase; autoantigen; diabetes; pancreas; transcription; promoter

ABBREVIATIONS
G6PC, glucose-6-phosphatase catalytic subunit; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; UGRP, ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein; G6P, glucose-6-phosphate; NOD, Non Obese Diabetic; CAT, chloramphenicol acetyltransferase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HIT, hamster insulinoma tumor; TK, thymidine kinase; TSS, transcription start site; IRES, internal ribosome entry site; BAC, bacterial artificial chromosome; EGFP, enhanced green fluorescent protein; FRT, FLP recombination target; Tet, tetracycline-resistance cassette.

The glucose-6-phosphatase catalytic subunit (G6PC) gene family is comprised of three members; G6PC, which is predominantly expressed in liver and kidney and plays a major role in glucose homeostasis (1); G6PC2, an islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) of uncertain function (2-4) and G6PC3, a ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein (UGRP) that confers low glucose-6-phosphatase activity to a broad range of tissues (5-8). The encoded proteins exhibit around 50% sequence identity, exhibit a common 9 transmembrane domain topology and are all localized to the endoplasmic reticulum (5; 6).

IGRP is principally confined to the β cells of the islet (9) and is a candidate for the low glucose-6-phosphatase enzyme activity detected in this tissue (2; 4). In combination with glucokinase, IGRP could create a substrate cycle and thus modulate β cell glycolytic flux and therefore glucose-stimulated insulin secretion (10). Indeed, a global knockout of the IGRP gene results in a mild metabolic phenotype characterized by a ~15% decrease in fasting blood glucose (4), consistent with a role for IGRP in glucose cycling. However, overexpression of IGRP by transient transfection of fibroblast or endocrine cell lines seems to have little (11), if any (2; 3; 12), impact on G6P hydrolysis in cell homogenates. This raises the question as to whether IGRP requires other cellular factors to exhibit activity or whether it is a catalytically-inert regulatory subunit of a metabolic process.

IGRP was recently identified as a target of cell-mediated autoimmunity in Type 1 diabetes both in mice (13-16) and humans (17). Up to 40% of the CD8 positive T cells infiltrating islets in Non Obese Diabetic (NOD) mice recognize an immunodominant peptide epitope (aa 206-214) within IGRP (18) and it is also a target of CD4 positive T cells (19). Significantly, *in vivo* administration of select IGRP epitope peptides to NOD mice has been shown to abrogate or delaythe disease process (14). However, whether there is a causal relationship between autoimmunity toward IGRP and type 1 diabetes is unclear. Indeed, a recent study in mice suggests that autoimmunity toward IGRP is a secondary event (20).

Our work has focused on identifying the transcription factors that control IGRP gene expression with the goal of identifying novel, islet-enriched transcription factors important for pancreatic development and/or function (3; 21-23). We believe this approach is reasonable given that similar work, focused on other islet-specific genes, has led to the identification of such proteins (24-26). A key step in the identification of such factors is the definition of the minimal IGRP gene sequence necessary for mimicking the expression pattern of the endogenous IGRP gene. We have previously demonstrated that the -306 to +3 region of the mouse IGRP promoter confers high fusion gene expression in islet-derived cell lines (21-23; 27) and is sufficient to initiate transgene expression in mouse islets, predominantly in β cells, at the expected time in development, around embryonic day 14 (28). However, unlike the endogenous IGRP gene, expression of this transgene was markedly suppressed in adult mouse islets suggesting that additional *cis*-acting elements in the IGRP gene are required for the maintenance of IGRP gene expression in adult mice. In this study we describe the identification of enhancers in the IGRP promoter and third intron, however, the expression of a transgene containing these enhancers was still suppressed in adult mice. These data suggest that long-range enhancers 5' or 3' of the IGRP gene are required for the

maintenance of IGRP gene expression in adult mice.

RESEARCH DESIGN AND METHODS

Fusion gene plasmid construction. The construction of IGRP-LacZ fusion genes containing mouse promoter sequence between -306, -911 and -3911 and +3, relative to the transcription start site (TSS), is described in the Online-Only Appendix (available at http://diabets.diabetesjournals.org) as is the use of bacterial recombination to introduce a LacZ-containing cassette into a bacterial artificial chromosome (BAC) clone containing the IGRP gene. The isolation and ligation of the putative IGRP enhancers A, B, C, E & F (Figs. 5 & 7) to a minimal IGRP-luciferase fusion gene is also described in the Online Only Appendix.

Animal care. The animal housing and surgical facilities used for the mice in these studies met American Association for the Accreditation of Laboratory Animal Care standards. All animal protocols were approved by the Vanderbilt University Medical Center Animal Care Committee. Food (Purina Mouse Chow 5001; Ralston-Purina, St. Louis, MO) and water were provided *ad libitum*.

Generation, breeding and genotyping of transgenic mice. Transgenic mice were generated by the joint Vanderbilt Cancer Center/Diabetes Research and Training Center Transgenic Animal/Embryonic Stem Cell Core Facility. The generation of transgenic mice expressing the -306 IGRP-LacZ fusion gene has been described (28). The -911 IGRP-LacZ, -3911 IGRP-LacZ fusion genes and the IGRP-BAC transgene were isolated as described in the Online-Only Appendix. Transgenic mice were produced by microinjection of 3-10 picoliters of a 3 ng/μl solution of the transgenes into the pronuclei of one cell embryos derived from B6D2F1 females. Embryos were then transferred into the oviducts of pseudopregant ICR females. Transgenic founders were bred with B6D2F1 mice (The Jackson Laboratory, Bar Harbor, ME) and offspring genotyped using real-time PCR in conjunction with the following primers: 5'-ACGCTGATTGAAGCAGAAGCC-3' and 5'-ATCGGTCAGACGATTCATTGGC-3' and the Bio-Rad iQ SYBR Green Supermix (Hercules, CA).

The detection of LacZ expression by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining of mouse tissues was performed at 4° C as previously described (28).

Cell culture, transient transfection and luciferase assays. Mouse pancreatic islet β cell-derived βTC-3 cells were cultured and co-transfected with 0.5 μg of an expression vector encoding SV40-Renilla luciferase (Promega) and 2 μg of the indicated firefly luciferase plasmids using the lipofectamine reagent (GibcoBRL) as previously described (22). Following transfection βTC-3 cells were harvested by trypsin digestion and then resuspended in passive lysis buffer (Promega). After two cycles of freeze/thawing, firefly and *Renilla* luciferase activity were then assayed using the Dual Luciferase Assay Kit (Promega). To correct for variations in transfection efficiency, results are expressed as the ratio of firefly:Renilla luciferase activity. Fusion gene expression was assessed in three separate experiments each using an independent preparation of each plasmid, transfected in triplicate.

RESULTS

The proximal IGRP promoter is insufficient for the maintenance of transgene expression in adult mice. Previous studies with transgenic mice in which LacZ gene expression was under the control of the -306