Tokens of the different vocal expressions were digitized at 22 kHz. In all, there were 27 angry sounds, 20 disgust, 24 fear, 27 happy and 31 sad sounds. These were presented to 6 judges for categorization. From their classifications, ten stimuli per emotion were selected so that the six emotions were recognized at similar overall levels (happiness, 90%; sadness, 87%; anger, 95%; fear, 93%; disgust, 97%; surprise, 93%).

For the test of emotion-recognition from sounds, the digitized tokens selected were presented in a random order using PsyScope software[27] running on a Macintosh Powerbook 540v computer and two loudspeakers set to a comfortable loudness level. Six additional items (one for each emotion) were used as practice trials, following which the test sounds were presented in a random order, with each stimulus presented twice across two blocks of trials. D.R.'s performance is compared to 12 controls aged 50–64 years (mean, 57.17; s.d., 3.88) without formal education.

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Correspondence and requests for materials should be addressed to A.W.Y. (e-mail: andy.young@mrc-apu.cam.ac.uk).

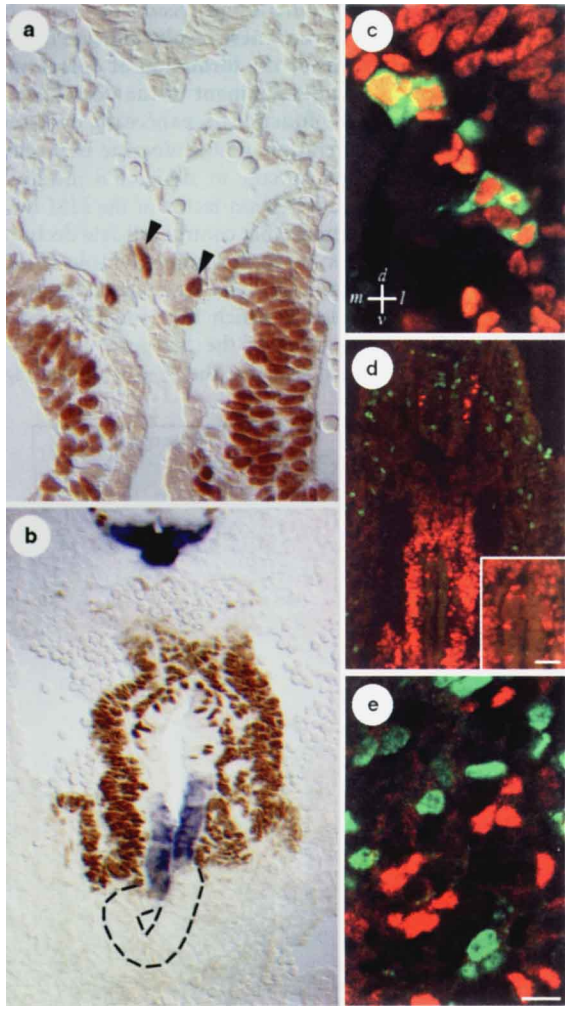

Figure 1 Expression of ISL1 in pancreatic epithelium and mesenchyme. **a**, In 16-somite embryos, ISL1$^+$ cells are detected in the dorsal pancreatic epithelium (arrowheads) and in the lateral mesenchyme. **b**, At the 25-somite stage, ISL1$^+$ mesenchyme has accumulated around the dorsal pancreatic bud. ISL1 is not expressed in the ventral pancreatic epithelium (broken line) or in the adjacent mesenchyme. *Shh* mRNA expression (dark blue) is used to visualize lateral endoderm[16]. **c**, Confocal image of the dorsal pancreatic bud of an e9.5 *Isl1*(+/+) embryo showing that all glucagon$^+$ cells (green) are also ISL1$^+$ (red). The image is representative of 5 embryos examined. **d**, No pancreatic epithelial cells in e9.5 *Isl1*(+/+) embryos are double-labelled with mpm2 (ref. 22) (green) and ISL1 (red) antibodies (insert) (n = 653 ISL1$^+$ cells analysed; 4 embryos). **e**, No ISL1$^+$ cells (red) incorporated BrdU (green) in explants of e12 dorsal pancreas cultured for 24 h and labelled with BrdU for 60 min to detect cells in the S and G2 phases of the cell cycle (n = 814 ISL1$^+$ cells; n = 1694 BrdU$^+$ cells analysed; 2 embryos). In **a**–**d**, dorsal epithelium is up. Scale bar in **a**, **d**, insert, 20 μm; in **b**, 40 μm; in **c**, **e**, 10 μm.

Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells

Ulf Ahlgren*, Samuel L. Pfaff†‡, Thomas M. Jessell†, Thomas Edlund* & Helena Edlund*

** Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden*
† Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Center for Neurobiology and Behavior, Columbia University, New York, New York 10032, USA

The mammalian pancreas is a specialized derivative of the primitive gut endoderm and controls many homeostatic functions through the activity of its component exocrine acinar and endocrine islet cells. The LIM homeodomain protein ISL1 is expressed in all classes of islet cells in the adult[1,2] and its expression in the embryo is initiated soon after the islet cells have left the cell cycle. ISL1 is also expressed in mesenchymal cells that surround the dorsal but not ventral evagination of the gut endoderm, which together comprise the pancreatic anlagen. To define the role of ISL1 in the development of the pancreas, we have now analysed acinar and islet cell differentiation in mice deficient in ISL1 function[3]. Dorsal pancreatic mesenchyme does not form in ISL1-mutant embryos and there is an associated failure of exocrine cell differentiation in the dorsal but not the ventral pancreas. There is also a complete loss of differentiated islet cells. Exocrine, but not endocrine, cell differentiation in the dorsal

‡ Present address: Gene Expression Laboratory, The Salk Institute, 10010 North Torrey Pines Road, LaJolla, California 92037, USA

pancreas can be rescued *in vitro* by provision of mesenchyme derived from wild-type embryos. These results indicate that ISL1, by virtue of its requirement for the formation of dorsal mesenchyme, is necessary for the development of the dorsal exocrine pancreas, and also that ISL1 function in pancreatic endodermal cells is required for the generation of all endocrine islet cells.

The *Isl1* gene was defined initially in studies on insulin gene regulation and encodes a transcription factor of the LIM homeodomain family, a class of proteins that control cell-fate decisions in invertebrates and vertebrates[3–10]. To determine the role of ISL1 in the differentiation of pancreatic islet cells, we analysed the development of the pancreas in mice in which ISL1 function had been eliminated by targeted disruption of the *Isl1* gene[3]. In wild-type mice, ISL1$^+$ cells can first be detected at the 15–16-somite stage, at embryonic day (e) 9, in the dorsal pancreatic epithelium and in cells of the lateral mesenchyme surrounding the gut epithelium (Fig. 1a). At the 20–25 somite stage, ISL1 expression is detected in the mesenchymal cells that have now enveloped the dorsal pancreatic epithelium (Fig. 1b). At no stage do mesenchymal cells flanking the ventral pancreatic epithelium express ISL1 (Fig. 1b), indicating that the mesenchyme surrounding the early pancreatic epithelium is patterned along the dorsal–ventral axis.

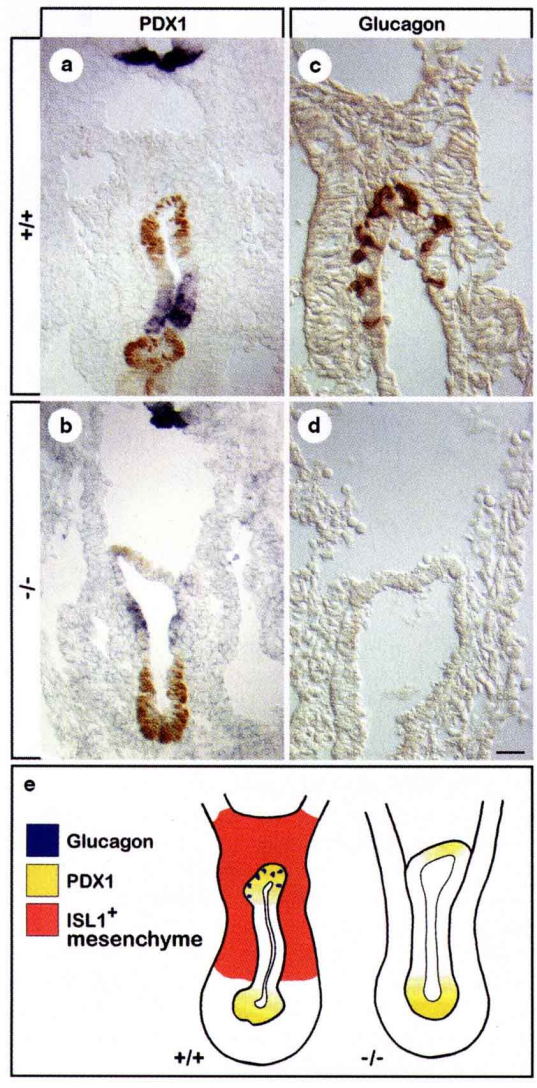

Figure 2 Loss of dorsal pancreatic mesenchyme and glucagon$^+$ cells in *Isl1*(−/−). Photomicrographs of transverse sections of *Isl1*(+/+) (**a**, **c**) and *Isl1*(−/−) (**b**, **d**) at the level of the pancreas. In **a** and **b**, *in situ* hybridization (dark blue) using a *Shh* probe was done to visualize the lateral endoderm[16]. **a**, Transverse section of an e9.5 *Isl1*(+/+) embryo stained with PDX1 antibodies to indicate the dorsal and ventral pancreatic buds. **b**, Transverse section of an e9.5 *Isl1*(−/−) embryo labelled with PDX1 antibodies. The dorsal pancreatic mesenchyme is absent, whereas the ventral pancreatic epithelium with its associated mesenchyme appears normal. **c**, At the 25-somite stage, glucagon$^+$ cells have appeared in the dorsal pancreatic epithelium in *Isl1*(+/+) embryos, whereas in somite-matched *Isl1*(−/−) embryos (**d**), no glucagon$^+$ cells are detected. **e**, Comparison of e9.5 wild-type and ISL1 mutant phenotype.

Glucagon-positive cells appear first at the 20–25-somite stage (e9.5) and they all express ISL1 (Fig. 1c), whereas cells expressing insulin, somatostatin and pancreatic polypeptide differentiate at later stages[11–14] and also express ISL1 when they appear (ref. 2, and data not shown). ISL1 expression in the ventral pancreatic epithelium can first be detected at embryonic day 11 (data not shown), consistent with the 1–2-day delay in the genesis of islet cells in the ventral pancreatic bud[12,15]. ISL1$^+$ endocrine cells analysed between e9.5–e13 did not express the M-phase-specific antigen detected by the anti-mpm2 antibody and did not incorporate BrdU (Fig. 1d, e). Together, these results provide evidence that ISL1 expression in islet cells is initiated after their final mitotic division but before the onset of hormone-gene expression.

Mice lacking ISL1 are arrested in development soon after e9.5 (ref. 3) and we therefore analysed cell differentiation in e9.5 *Isl1*(+/+) and *Isl1*(−/−) embryos using PDX1 expression to delineate specialized pancreatic epithelium and *Shh* expression as a more general marker of gut epithelium[16]. In *Isl1*(−/−) embryos, the pancreatic epithelium and its associated ventral and lateral mesenchyme appeared normal histologically, PDX1 expression in the ventral epithelium was normal and the lateral gut epithelium still expressed *Shh* (Fig. 2a, b). In contrast, there was a virtually complete depletion of the mesenchyme flanking the dorsal pancreatic epithelium (Fig. 2b) and PDX1 was expressed at markedly lower levels in the dorsal epithelium in *Isl1*(−/−) embryos than in *Isl1*(+/+) embryos (Fig. 2a, b). To determine whether ISL1 has an independent function in the generation of islet cells, we monitored the expression of glucagon in *Isl1*(+/+) and *Isl1*(−/−) embryos. Glucagon-positive cells were detected in *Isl1*(+/+) but not in *Isl1*(−/−) e9.5 embryos (Fig. 2c, d). Thus, as summarized in Fig. 2e, ISL1 is necessary for the formation of the dorsal pancreatic mesenchyme and for the generation of glucagon-expressing cells in the dorsal pancreatic epithelium.

To determine whether the absence of glucagon-position cells in e9.5 *Isl1*(−/−) embryos reflects a delay or a complete block in their differentiation, we isolated segments of gut containing the pancreatic anlagen from e9.5 *Isl1*(+/−) and *Isl1*(−/−) embryos and maintained them in culture for 7 days, which enabled us to analyse whether the differentiation of the later-appearing insulin-, and somatostatin-expressing cells is also impaired in *Isl1*(−/−) embryos. Explants derived from *Isl1*(+/−) embryos gave rise to endocrine cells positive for glucagon, insulin and somatostatin (Fig. 3a–c), and exocrine cells positive for amylase (Fig. 3d). Explants derived from *Isl1*(−/−) embryos, however, gave rise to cells that were negative for glucagon, insulin and somatostatin (Fig. 3e–g), although exocrine cells were present that were positive for amylase (Fig. 3h). These results indicate that ISL1 expression in pancreatic epithelium is required for the differentiation of islet but not exocrine cells.

The differentiation of pancreatic epithelium is thought to depend on signals provided by the surrounding mesenchyme[17]. In *Isl1*(−/−) embryos pancreatic mesenchyme surrounds only the ventral pancreatic epithelium, so the amylase-positive exocrine cells generated in gut explants from *Isl1*(−/−) embryos may have derived solely from the ventral pancreatic epithelium. In support of this, amylase-positive exocrine cells were generated in cultured ventral half-gut explants but not in dorsal half-gut explants isolated from *Isl1*(−/−) embryos (Fig. 4a, b). Both gut explant halves isolated from *Isl1*(+/−) embryos generated amylase-positive cells

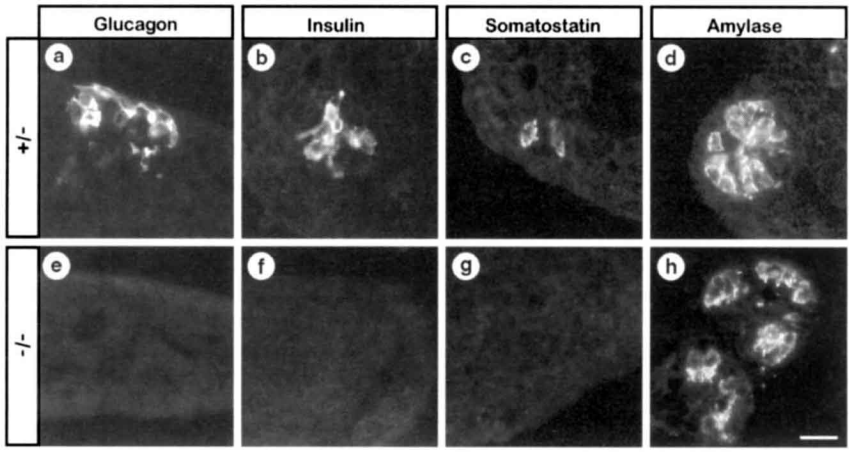

Figure 3 Differentiated islet cells are not generated in explants derived from *Isl1*(− / −) embryos. Fluorescence immunohistochemistry of alimentary tract explants derived from *Isl1*(+/ −) (**a**-**d**) and *Isl1*(− / −) (**e**-**h**) mice. Islet cell hormones, glucagon (**a**), insulin (*b*) and somatostatin (**c**) and the exocrine enzyme amylase (**d**) are detected in explants derived from *Isl1*(+/ −) embryos. In explants derived from *Isl1*(− / −) embryos there is no expression of glucagon (**e**), insulin (**f**) and somatostatin (**g**), whereas amylase$^+$ cells (**h**) are still present. Images are representative of at least 4 explants. Scale bar, 20 μm.

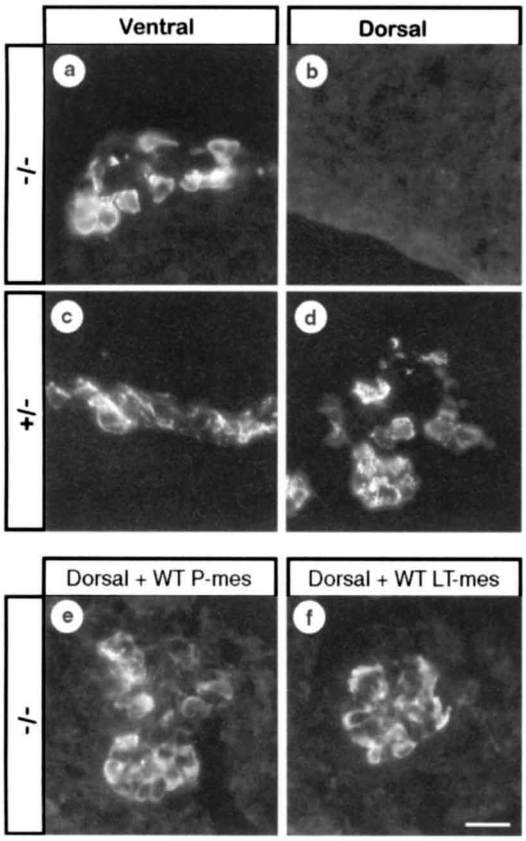

Figure 4 Dorsal pancreatic exocrine differentiation is impaired in explants derived from *Isl1*(− / −) embryos. Immunohistochemical analysis of marker expression of explants derived from the dorsal (**b**, **d**, **e**, **f**) or ventral (**a**, **c**) pancreatic anlagen. Amylase$^+$ cells are generated in ventral (**a**) but not dorsal (**b**) explants derived from *Isl1*(− / −) embryos. In contrast, amylase$^+$ cells are detected in both ventral (**c**) and dorsal (**d**) explants from *Isl1*(+/ −) embryos. Recombination of dorsal pancreatic anlagen from *Isl1*(− / −) (**e**, **f**) with dorsal pancreatic (**e**) or lung-tracheal region (**f**) mesenchyme from somite-matched (+/+) type embryos rescues the differentiation of dorsal exocrine cells. Images are representative of 5 (**c**, **d**), 8 (**a**, **b**) and 3 (**e**, **f**) explants. Abbreviations: P, pancreas; LT, lung-tracheal; mes, mesenchyme. Scale bar, 20 μm.

(Fig. 4c, d). To determine whether the loss of amylase-positive cells in the dorsal bud of *Isl1*(− / −) embryos reflected the absence of mesenchyme, we recombined the dorsal half gut of *Isl1*(− / −) embryos with dorsal pancreatic mesenchyme derived from wild-type e9.5 embryos. Amylase-positive cells were generated in such recombinants (Fig. 4e) and also when the dorsal half gut of *Isl1*(− / −) embryos was recombined with mesenchyme from the lung-tracheal region (Fig. 4f). The presence of wild-type mesenchyme did not restore the differentiation of endocrine islet cells in cultures of the dorsal half-gut region (data not shown).

These findings suggest that ISL1 functions at two independent steps in the development of the pancreas. First, ISL1 is expressed in, and is required for, the development of dorsal pancreatic mesenchyme, providing genetic evidence that the elimination of pancreatic mesenchyme blocks exocrine pancreas development. Second, ISL1 expression in pancreatic epithelial cells is required for the differentiation of islet cells, suggesting a cell-autonomous function similar to that reported in motor neuron generation[3]. The development of the pancreas seems therefore to be controlled through the sequential activities of distinct classes of transcription factors. The homeodomain transcription factor PDX1 seems to specify the early pancreatic epithelium, permitting its proliferation, branching and subsequent differentiation[16,18,19]. The basic helix–loop-helix protein *NeuroD/BETA2* (refs 20, 21) is expressed in pancreatic endocrine cells (refs 20, 21, and our own unpublished observations) and, by analogy with its neurogenic function[20], may act upstream of *Isl1* in the islet-cell differentiation pathway. More generally, the demonstration of a requirement for ISL1 in both islet-cell and motor-neuron development suggests that there are conserved elements in the transcriptional control of neuronal and pancreatic endocrine differentiation. □

Methods

Animals. The generation of *Isl1*(− / −) mice has been described elsewhere[3]; +/+, +/ − and − / − mice were obtained from our local breeding colony of *Isl1*(+/ −) mice. Mice and embryos were genotyped as described[3].

Immunohistochemistry. Primary antibodies, rabbit anti-ISL1[2], rabbit anti-PDX1[16] and guinea-pig anti-glucagon (Linco) were detected using the ABC immunoperoxidase system (Vector Labs). Guinea-pig anti-rat insulin-C peptide (Linco), guinea-pig anti-glucagon (Linco), rabbit anti-somatostatin (DAKO) and rabbit anti-human α-amylase (Sigma) was detected as described[16]. *In situ* hybridization using an *Isl2* probe[3] showed that no cells in the pancreatic epithelium express *Isl2* (data not shown). In addition, cells in the

pancreatic epithelium and in the dorsal pancreatic mesenchyme that are recognized by anti-ISL1/ISL2 ploYclonal antibody are also recognized by an ISL1-specific mouse monoclonal anti-ISL1 antibody[3] (data not shown), indicating that these cells express ISL1 but not ISL2 and can therefore be referred to as ISL1$^+$ cells. Double-label immunohistochemistry, *in situ* hybridization and Confocal microscopy analysis were done as described[16], as were ISL1/mpm2 and ISL1/BrdU double labelling[2]. Primary antibodies were mouse anti-mpm2 (Upstate Biotech), mouse anti-BrdU (Becton Dickinson), and rabbit anti-ISL1. When analysing transverse sections of e9.5 embryos, PDX1 was used on consecutive sections as a pancreatic marker.

Culture of pancreatic rudiments. Gut explants containing the dorsal and/or ventral pancreatic anlagen from e9.5 *Isl1*(+/−) and *Isl1*(−/−) embryos were isolated and cultured for 7 days[16]. Explants were then processed, sectioned and immunostained using standard procedures[16,18].

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Correspondence and requests for materials should be addressed to H.E. (e-mail: Helena. Edlund@micro.umu.se).

Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression

Giles E. Hardingham, Sangeeta Chawla, Claire M. Johnson & Hilmar Bading

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Calcium entry into neuronal cells through voltage or ligand-gated ion channels triggers neuronal activity-dependent gene expression critical for adaptive changes in the nervous system[1–5]. Cytoplasmic calcium transients are often accompanied by an increase in the concentration of nuclear calcium[6–9], but the functional significance of such spatially distinct calcium signals is unknown. Here we show that gene expression is differentially controlled by nuclear and cytoplasmic calcium signals which enable a single second messenger to generate diverse transcriptional responses. We used nuclear microinjection of a non-diffusible calcium chelator to block increases in nuclear, but not cytoplasmic, calcium concentrations following activation of L-type voltage-gated calcium channels. We showed that increases in nuclear calcium concentration control calcium-activated gene expression mediated by the cyclic-AMP-response element (CRE), and demonstrated that the CRE-binding protein CREB can function as a nuclear calcium-responsive transcription factor. A second signalling pathway, activating transcription through the serum-response element (SRE), is triggered by a rise in cytoplasmic calcium and does not require an increase in nuclear calcium.

Confocal laser scanning microscopy of the Ca^{2+} indicator Fluo-3 was used to measure changes in cytoplasmic calcium ($[Ca^{2+}]_c$) and nuclear calcium ($[Ca^{2+}]_n$) concentrations in AtT20 cells, a mouse pituitary cell line. Ca^{2+} influx through L-type calcium channels was triggered by KCl-induced membrane depolarization in the presence of the L-type calcium-channel agonist FPL 64176 (ref. 10) (KCl/FPL treatment). Upon KCl/FPL treatment, $[Ca^{2+}]_c$ increased from resting levels of 108 ± 5 nM to 783 ± 40 nM and $[Ca^{2+}]_n$ rose from 104 ± 6 nM to 979 ± 58 nM ($n = 47$). To block increases in $[Ca^{2+}]_n$ specifically without altering the rise in $[Ca^{2+}]_c$, we microinjected the Ca^{2+} chelator BAPTA into the nucleus. The BAPTA used was coupled to a 70K dextran molecule (BAPTA-D70), which prevented it from diffusing out of the nucleus into the cytoplasm. This was confirmed by microinjecting BAPTA-D70 together with a Texas red-labelled 70K dextran into the nucleus. The localization of the red dye, which reflects the subcellular distribution of the 70K dextran, was monitored and found to remain exclusively nuclear for up to 16 hours after injection (data not shown).

Nuclear microinjection of BAPTA-D70 specifically reduced KCl/FPL-induced increases in $[Ca^{2+}]_n$ by ~50% (558 ± 76 nM; $n = 8$) but did not affect the rise in $[Ca^{2+}]_c$ (864 ± 104 nM; $n = 8$) (Fig. 1a, b). In cells injected with the control solution, $[Ca^{2+}]_c$ and $[Ca^{2+}]_n$ increased from resting levels of 106 ± 17 nM and 79 ± 12 nM to 879 ± 93 nM and 1,105 ± 149 nM ($n = 12$), respectively, upon KCl/FPL stimulation (Fig. 1a, b). These Ca^{2+} levels are similar to those observed in uninjected cells, demonstrating that the injection process *per se* has no effect on Ca^{2+} levels. A time course of the $[Ca^{2+}]_n$:$[Ca^{2+}]_c$ (N/C) ratio, an indicator of the specificity of the effect of BAPTA-D70 on $[Ca^{2+}]_n$, demonstrates that the reduction of $[Ca^{2+}]_n$ in BAPTA-D70-injected, KCl/FPL-stimulated cells persisted for at least 30 min (Fig. 1c).

Having established a technique to reduce specifically increases in $[Ca^{2+}]_n$ without interfering with $[Ca^{2+}]_c$, we next determined whether nuclear Ca^{2+} is critical for the control of Ca^{2+}-activated transcription. The c-*fos* gene, a well characterized Ca^{2+}-inducible gene[1–3], was used as a model system. Nuclear microinjection of BAPTA-D70, but not of the control, reduced KCl/FPL-induced expression of the endogenous c-*fos* protein, detected by immunofluorescence and quantified using a confocal laser scanning microscope, to 55 ± 7% (136 cells analysed). In contrast, cAMP-induced endogenous c-*fos* protein expression in BAPTA-D70-injected cells was 113 ± 7% compared with that of control-injected cells (57 cells analysed), demonstrating the specificity of the inhibition by nuclear BAPTA-D70 of Ca^{2+}-dependent transcriptional activation. Because Ca^{2+}-activated expression of c-*fos* was only partially sensitive to inhibition of increases in $[Ca^{2+}]_n$, we considered that Ca^{2+} might control gene expression through several mechanisms, differing in their requirements for nuclear Ca^{2+}. Expression of c-*fos* by Ca^{2+} signals is regulated by two DNA regulatory elements, the CRE and the SRE[11]. To investigate whether the mechanisms that control CRE- and SRE-dependent Ca^{2+}-activated transcription differ in their dependency for increases in $[Ca^{2+}]_n$, we introduced into the cells plasmids containing the wild-type human c-*fos* gene (including either 711 base pairs (bp) in plasmid pF711myc, or 222 bp of upstream regulatory sequence in plasmid pF222myc), or the human c-*fos* gene in which the SRE or the CRE had been mutated in the context of an otherwise intact promoter. An oligonucleotide encoding a Myc-epitope tag was inserted in-frame into the c-*fos* coding region, enabling the expression of these c-*fos* gene constructs to be detected by immunofluorescence using an antibody against the Myc epitope (9E10) (Fig. 2a).