Materials and methods

Probes and labeling

The PIT-1 (POU1F1) probe (specific sequence, with the binding site indicated in bold: 5′-TGTCTTCCAGAATTGTAATAAAGMAATTTA-3′) is based on the 3P binding site in the rat PRL proximal promoter, and the AP-1 probe (specific sequence, with the binding site indicated in bold: 5′-CGCTTAGACTCAGCCCGAAA-3′) was designed after the Santa Cruz reference sc-2501. (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Correct?

Classical probes were labeled with a single biotin or Cy5. LUEGO have been tested with biotin, IRDye700 or double Cy5 labels. Biotinylated and unmodified oligonucleotides were obtained from Eurogentec (Seraing, Belgium), and LI-COR IRDye700-, Cy3- and Cy5-labeled oligonucleotides were obtained from DNA Technologies (Leven, Belgium).

Probe annealing

Oligonucleotides were mixed in TE/NaCl (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl). After mixing, annealing was performed in a thermocycler with the following program: 2 min at 94°C, a rapid ramp down to 70°C at maximal speed (5°C/s), followed by slow (0.02°C/min) cooling to 18°C. The mixture was then diluted to 0.1 µM of the complete annealed probe in the same TE/NaCl buffer.

Gel shift assays

Pit-1

Purified PIT-1 protein (6×His N-tagged) was mixed at 7, 14 or 28 nM with 8.3 nM PIT-1—labeled probe (classic or LUEGO-based) in a reaction buffer containing 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM MgCl₂, 0.1% NP40, 5 mM DTT, 10% glycerol, and 0.5 µg/µL BSA. The reaction was incubated for 20 min at room temperature and 10 µL were loaded onto an 8.3% acrylamide mini-gel (37.5:1 acrylamide/bis-acrylamide) and run for 40 min at 100 V in 0.5× Tris-borate-EDTA (TBE). Biotin-labeled gels were electrotransferred onto an Amersham Hybond N+ membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and treated with the Thermo Scientific Pierce chemiluminescent nucleic acid detection module (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal was acquired with a GBOX XT camera (Syngene, Cambridge, UK). IRDye700 LUEGO gels were directly scanned after electrophoresis (inside the casting plates) with an Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA) and Cy5 was visualized using a Typhoon FLA9000 scanner (GE Healthcare). TIFF images (16-bits) were processed with ImageJ (http://rsbweb.nih.gov/ij/) to set the minimum and maximum display range values without saturation in the bands of interest and flattened to 8-bits for the figures.

To compare the classic PIT-1 probe labeled with a single Cy5 and a LUEGO-based PIT-1 probe labeled with 2×Cy5 in a protein titration assay, the two gels were scanned using the same parameters, and the images were set with the same display range (192 to 22805 gray levels). The scan was quantified using ImageJ, and the intensity of the bound probe (sum of the intensities of the monomer and dimer bands) relative to that of the total (bound plus free) probe was plotted against the concentration of PIT-1 added to the reaction mixture.

AP-1

Nuclear extracts were prepared using Pierce NE-PER reagents (ref. sc89833; Thermo Fisher Scientific) from control GH4C1 rat pituitary cells (ct) or cells treated at semi-confluency for 3 h with 10 µM forskolin (ft). The binding reaction was set with 0.25 µg/µL nuclear extract, 8.3 nM AP-1 consensus DNA [classic biotinylated or LUEGO (2×Cy5)-based probe], in a reaction buffer composed of 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 1 mM DTT, 0.7% Ficoll, 8.3 ng/µL, and poly(dI-dC). Unlabelled wild type (wt) or mutant (mt) (Santa Cruz sc-2514) competitor DNA (without the LUEGO tail) was added with a 10-fold excess where indicated. Rabbit polyclonal antibodies (Santa Cruz Biotechnologies) directed against FRA-2 (ref. sc-604) or PIT-1 (ref. sc442) were added where indicated (Fra, Pit) at 8.3 ng/µL. The reactions were then processed as described above for the PIT-1 assay.

LUEGO: a cost and time saving gel shift procedure

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Keywords: electrophoretic mobility shift assay (EMSA); gel shift