3. METHODS

All molecular biology methods were performed using standard procedures (Sambrook et al. 1989). All solutions were prepared with bi-distilled water, which was autoclaved if necessary.

3.1 Generation of the reporter gene construct

3.1.1 Amplification of the human ALC-1 promoter by PCR

The human ALC1 promoter (hALC-1 promoter) was amplified from human genomic DNA by PCR (nucleotide -1942 to +75; cf. Rotter et al. 1991, EMBL accession number X55000 and X58851). Buffer conditions were 1x PCR buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 2.5 U Taq Polymerase (Invitrogen Life Technologies). An annealing temperature of 55°C and 35 cycles were appllied (PCR cycler from Perkin Elmer LAS GmbH). The following primers were used in a 0.5 μM concentration: 5'-attggtaccTTGCCTGTAAAACAGCATG-3'; 5'-ctcccatggTGTCTTGTTGGGATCTTTGGC-3' (sense and antisense, respectively; promoter sequence in capital letters, overhang in small letters; primers from BioTez).

3.1.2 Cloning of the reporter gene construct

After verification of the resulting PCR products by sequencing, they were cloned into the luciferase reporter vector pGL3-Basic (Promega GmbH) using the restriction enzyme site KpnI (and following restriction enzymes: Amersham Biosciences) in the multiple cloning site, and the NcoI site situated at the start codon of the luciferase reporter gene in pGL3-Basic, resulting in the construct hALC-1 promoter-luciferase. In order to obtain a selection marker for stable expression, the neomycin resistance cassette composed of SV40 promoter, neomycin resistance gene and SV40 polyadenylation signal was excised from the pcDNA3.1 vector (Invitrogen Life Technologies), using the restriction enzymes AseI at the 5' end and SalI at the 3' end. The hALC-1 promoter-luciferase plasmid was linearised with SalI and ligated (T4 DNA ligase, New England Biolabs) with the isolated neomycin resistance cassette using the compatible ends of the Sal I restriction enzyme site. After polishing of the remaining AseI and SalI ends with T4 DNA

polymerase (New England Biolabs) a second ligation was performed with blunt-ends. The hALC-1 promoter of the resulting hALC-1 promoter-Luciferase-SV40-Neo clone (reporter gene construct; cf. Fig. 5) was confirmed by sequence analysis and restriction enzyme analysis (cf. Appendix 1 and Fig. 5+6). This construct was then used to establish a stable cell line. The respective plasmid clones were propagated in Top10 bacteria (Invitrogen Life Technologies). Plasmid purification was done using either the QIAprep Mini Prep kit or the Qiagen Plasmid Maxi kit (Qiagen).

3.1.3 Sequence analysis

Sequence analysis (cf. Appendix 1) was performed by the company InViTek (Berlin-Buch) according to the cycle sequencing method (Innis et al. 1988) using the ABI PRISM BigDye Terminator Cycle Sequencing kit and the ABI PRISM 377 DNA sequencing system. The following primers were used: 5'-CTAGCAAAATAGGCTGTCCC-3', 5'-CTTTATGTTTTTGGCGTCTTCCA-3' and 5'-TGGCCAAGTTGTTCATCCAG-3'.

3.1.4 Restriction enzyme analysis

The reporter gene construct was digested with NcoI, NcoI and KpnI and NcoI and XbaI at 37°C for approximately 2 hours using the appropriate buffer conditions (Amersham Biosciences).

3.1.5 Gel electrophoresis

The complete reaction was loaded onto a 1% agarose (Biozym) gel (w/v) suspended in 0.5x TBE (Invitrogen Life Technologies) and run at 120V (Power supply ST 606, Invitrogen Life Technologies) in an electrophoresis chamber (Sub Cell GT, Bio-Rad GmbH) filled with 0.5x TBE. The gel was photographed using the gel documentation system Gel Doc 2000 (Bio-Rad GmbH).

3.2 Cell culture

3.2.1 Maintenance of the H9c2 cardiomyoblasts

H9c2 cardiomyoblasts are derived from embryonic rat heart tissue (cf. Kimes and Brandt 1976) and were obtained from the European Collection of Animal Cell Cultures (ECACC). The cells were maintained in the myoblast state in DMEM (PAA Laboratories) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies). Cells were grown to near confluency (not more than 80%) and incubated at 37°C and 5% CO_2 in a humidified atmosphere (incubator from Forma Scientific). To harvest the cells, they were washed with PBS and treated with Trypsin-EDTA (both Invitrogen Life Technologies). Analysis of the number of living cells was performed by trypan blue (Invitrogen Life Technologies) exclusion. Cells were not used above passage number 34. Typically, vasopressin experiments were performed in triplicates in 6-well plates (TPP) and immunofluorescence experiments were carried out in duplicates in 4-well plates (Nunc). All cell culture was performed under sterile conditions using a laminar flow (BDK Luft- und Reinraumtechnik GmbH).

3.2.2 Freezing of the H9c2 cardiomyoblasts

Since the H9c2 cardiomyoblasts were found to be sensitive upon freezing, a specific protocol was followed. The cells were harvested according to standard procedures (cf. 3.2.1) and centrifuged twice (10 min at 1500 rpm, Allegra^{TM} 6R centrifuge, Beckman Coulter GmbH) with intermediate washing in PBS. The cell pellet was carefully resuspended in DMEM without antibiotics and containing 40% FBS. The same volume of a second medium, consisting of 40% FBS and 20% DMSO (Serva), was then cautiously added and the final solution was gently mixed. In order to achieve a -1°C/min rate of cooling, aliquots were first stored in a cryo freezing container (Nalgene Nunc International) at -80°C. After one day, the cells were transferred into a liquid nitrogen container for long term storage.

3.3 Testing of different transfection methods

The Calcium Phosphate Transfection System (Invitrogen Life Technologies), the GeneShuttle™ Transfection Reagent (Q Biogene), the GenePorter™ Transfection Reagent (Gene Therapy Systems Inc.) and the Nucleofector Kit (Amaxa) were used for transfection of the H9c2 cardiomyoblasts according to the manufacturer protocols.

3.4 Dose response curve for selection of the H9c2 cardiomyoblasts with G418

10^5 H9c2 cardiomyoblasts were seeded per well of a 12-well plate (Costar). G418 (geneticin, Invitrogen Life Technologies) was solubilized in DMEM and sterilized by filtration. G418 was added to the cells in the following concentrations: 0 mg/ml (negative control), 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml and 0.8 mg/ml. After 3 days, the medium was changed and G418 was added to the cells as before. After another 5 days, the cells were washed with PBS and dyed in a tryphan blue (Invitrogen Life Technologies)/PBS mixture for 20 min at RT. Following 3 more washes with PBS, cells were inspected microscopically in order to assess the number of dead cells in response to G418. Untreated wells (negative control) were confluent and only included few dead cells. Wells containing cells treated with 0.1 and 0.2 mg/ml G418 showed a clear reduction of the number of living cells. G418 doses of 0.4 mg/ml and above led to overall cell death.

3.5 Generation of the stably transfected H9c2 cardiomyoblast lines (H9c2T1 and H9c2T2)

Two stably transfected clonal H9c2 cardiomyoblast cell lines (H9c2T1 and T2) were generated.

3.5.1 Preparation of DNA

To prepare the DNA for transfection, it was phenol-chloroform extracted, precipitated in 3 M sodium acetate and 100% ethanol, washed in 70% ethanol and dissolved in Tris-EDTA (TE, pH 8.0) under sterile conditions.

3.5.2 Calcium phosphate transfection

10^5 H9c2 cardiomyoblasts were seeded per well of a 6-well plate. The next day, the cells were transfected with 5.0 and 7.5 μg of the hALC-1 promoter-Luciferase-SV40-Neo reporter gene construct in a total cell culture volume of 1.2 ml using a calcium phosphate transfection kit (Invitrogen Life Technologies). Per sample, 2 tubes were prepared: a first tube containing 61.2 μl 1x Hepes buffered saline (HBS) buffer (10x HBS buffer, sterile H_2O, 1 N NaOH) mixed with 1.2 μl of a phosphate solution and a 2nd tube containing the DNA (supplemented with H_2O), which had been subsequently mixed with 1.2 μl and 6.1 μl of a 2 M $CaCl_2$ solution in a gentle manner. While oxygenizing the first tube, the contents of the second tube were slowly added. The final mixture was incubated for 20 min at RT. The resulting precipitate was added dropwise to each well while swirling the plate.

3.5.3 Selection with G418 and subcloning

A medium change on the next day stopped the overnight transfection. 48 hours later, the cells were split into selective medium using the antibiotic G418 in a concentration of 0.7 mg/ml. The transfected cardiomyoblasts (H9c2T) were kept under these conditions for the following 25 days, after which the cells were subcloned. A series of different dilutions was made from the surviving transfected cells, leading to the isolation of two different clones (T1 and T2). After further cultivation of each clone, the stably transfected cardiomyoblast cell lines H9c2T1 and H9c2T2 were obtained. The cells were cultured as the wild type H9c2 cardiomyoblasts with the addition of G418 (0.7-0.8 mg/ml) to the culture medium.

3.5.4 Microscopic analysis of H9c2 and H9c2T cardiomyoblasts

H9c2 wild type and H9c2T cardiomyoblasts were inspected with a light microscope (Leica) and photographed (Cannon) using a 10-fold magnification.

3.6 Genotyping of the stably transfected H9c2 cardiomyoblasts

3.6.1 Extraction of DNA

DNA extraction from the H9c2T1 and H9c2 cardiomyoblasts was performed using the Invisorb Twin Prep DNA/RNA Kit (InViTek). Briefly, cells were harvested, washed with PBS and homogenized in a lysis buffer containing chaotropic salt. After addition of adsorbin to the cell lysate, vortexing and incubation on ice for 5 min, the lysate was centrifuged (centrifuge 5417 R, Eppendorf). The adsorbin bound DNA pellet was washed several times with wash buffer (buffer concentrate supplied by the company, which was dissolved in distilled water and 96% ethanol; intermediate centrifugation at 10000 rpm for 1 sec). The DNA was eluted and stored at -20°C.

In order to determine the concentration of the DNA and to assess its quality, the optical density of the extracted DNA was measured at 260nm and 280nm using a spectrophotometer (Shimadzu Europa). The concentration was calculated using the standard formula. The ratio of OD_{260nm}/OD_{280nm} was between 1.8-2.0, indicating a protein-free DNA solution.

3.6.2 PCR

PCR conditions were 1x PCR buffer, 1.25 $MgCl_2$, 0.375 mM each dNTP, 0.5 μM each primer, 1U Taq Polymerase (Invitrogen Life Sciences). The following construct specific primers were used in a final concentration of 0.5 μM: sense 5'-ACACCCGAGGGGGATGATAA-3' (Luc sense: located in the luciferase reporter gene; BioTez); antisense 5'-CGGCACTTCGCCCAATAGCA-3' (Neo antisense: located in the neomycine resistance gene; BioTez). 30 cycles were performed with an annealing temperature of 65°C. The complete PCR reaction was applied for gel electrophoresis analysis (cf. 3.1.5) using a 1% agarose gel (w/v).

3.7 Cell culture treatments

During treatment of the cells with the various chemicals, G418 was omitted from the culture medium.

3.7.1 Stimulation with vasopressin

For induction of hypertrophy by vasopressin, 2.5 x 10^5 H9c2T1 cardiomyoblasts were seeded per well of a 6-well plate. After 24 hours, serum starvation was induced and maintained until the end of the experiment. After 24 hours, (Arg ^{8}) Vasopressin (Sigma), dissolved in water, was added in a concentration of 1 μM. The cells were cultured for 48 hours and harvested to assess the amount of luciferase using the luciferase assay.

3.7.2 Stimulation with ionomycin

1.0 x 10^5 H9c2T1 cardiomyoblasts were seeded per well of a 6-well plate. After 24 hours, ionomycin (Calbiochem, suspended in methanol) was added to the culture medium in a concentration of 0.5 μM for the duration of 30 min. The cells were then washed for 5 min with pure medium containing 5% BSA (AppliChem GmbH). Normal culture medium was added and the cells were harvested for luciferase analysis 48 hours later.

3.7.3 Stimulation with vasopressin under Ca^{2+}-free conditions

Hypertrophy was induced by vasopressin as described before (cf. 3.7.1). However, next to normal culture medium, DMEM lacking Ca^{2+} was also used (PAA Laboratories, nominally Ca^{2+}-free). 48 hours later cells were harvested and the amount of luciferase was analyzed.

3.7.4 Treatment with inhibitors

All inhibitors were purchased from Calbiochem except Cyclosporin A (Sigma). Cyclosporin A (CspA) was solubilized in ethanol. The CaMK inhibitor KN93 and its inactive analogue KN92 were solubilized in sterile H_2O and DMEM without FBS, respectively. Bisindolylmaleimide (BIM) was used as a PKC inhibitor and dissolved in H_2O. Cells were pretreated with inhibitors 30 min (CspA, 1 μM) or 1 hour (KN93 or KN92, 10 μM each; BIM, 200 or 400 nM) before addition of vasopressin and kept in the medium during the experiment.

3.8 Determination of protein in H9c2/H9c2T1 cardiomyoblasts

3.8.1. Extraction of protein

H9c2 and H9c2T1 cardiomyoblasts were harvested, homogenized in lysis buffer (50 mM Hepes, 200 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween-20, 10 mM βglycerophosphate, 1 mM NaF, 2 mM Na_3VO_4, 1 mM DTT, 0.2 mM PMSF, 5 μg/ml leupeptin) and incubated on ice for 90 min with intervening vortexing. After centrifugation of the homogenate (15 min at 14000 rpm), the supernatant was recovered and stored at -20°C.

3.8.2 Protein quantification

The protein content was analyzed with a modified lowry assay (DC Protein Assay, Bio-Rad GmbH). The sample supernatant was diluted in PBS. Serial dilutions of a bovine serum albumin (BSA) standard (Bio-Rad GmbH) were prepared in a lysis buffer (cf. 3.8.1)/PBS mix to give a standard curve. This mix was prepared in the same dilution as the samples. 10 μl of the series of standards, the samples and the lysis buffer/PBS mix (for background subtraction) were loaded onto a 96-well plate (Perkin Elmer LAS GmbH) in triplicates. 25 μl of detection solution 1 (alkaline copper tartrate solution mixed with SDS) was pipetted onto the plate, after which 180 μl of solution 2 (dilute folin reagent) was added. After incubating the plate for 18 min at RT and hidden from light exposure, it was measured at 655 nm in a microplate reader (Bio-Tek Instruments GmbH). Data was analyzed using the KC-4 software (Bio-Tek Instruments GmbH).

3.9 Luciferase assay

3.9.1 Cell harvest and extraction of luciferase

The amount of luciferase (Luc) was analyzed with a commercially available luciferase assay system (Promega GmbH) according to the manufacturer. The cells were harvested, washed with PBS and lysed in 1x reporter lysis buffer (RLB, 110 μl per pellet). To ensure complete lysis, one freeze-and-thaw cycle was carried out using liquid

nitrogen. After homogenisation of the cell lysate (vortexing for 10-15 sec) and subsequent centrifugation (12000g for 15 sec at RT or 12000g for 2 min at 4°C), the supernatant was collected and stored at -80°C.

3.9.2 Luminescence analysis

Luminescence analysis was carried out using the Fluoroskan Ascent FL Type 374 (Labsystems). The luminometer was programmed to perform a delay of 2 sec and a data acquisition interval of 10 sec. Serial dilutions of recombinant luciferase (Promega GmbH) were prepared as standards in a RLB mix (1x RLB, 1 mg/ml BSA). Measuring the luminescence of the recombinant protein (in relative light units (RLU)), a calibration curve for calculation of the amount of luciferase (μg/ml) was obtained (cf. Fig.10). The samples were measured undiluted or diluted (1:10 in a mix of 1x RLB, 1 mg/ml BSA). 20 μl of the samples, the standards and the RLB mix were loaded onto a 96-well plate in quadruplicates and luminescence was measured using 100 μl of the luciferase assay reagent per well. The empty 96-well plate was also measured to record background luminescence, which was subtracted from each standard or sample. Data was analyzed using the Fluoroskan Ascent FL software (Labsystems).

3.10 Western blot analysis

3.10.1 Generation of ALC-1 antibodies

Antibodies raised against ALC-1 were generated in New Zealand white rabbits by immunization with synthetic peptides coupled to keyhole limpet hemocyanin according to standard protocols. The isoform-specific ALC-1 antibody was raised against the peptide, PAPAPAPEPLRDSAFDPKS, corresponding to amino acids 21-39 of rat ALC-1 (Acc.No. P17209). The panspecific antibody was raised against the peptide, APKKPEPKKEAAK, corresponding to amino acids 2-14 of human ALC-1 (Acc.No. P12829). This region is highly homologous in rat and human ALC-1 as well as in VLC-1. The antibody-containing serum fractions were affinity purified on the respective peptide antigen columns.

3.10.2 Protein extraction from rat heart tissue

Frozen tissue of the right atrium and ventricle of a WKY rat heart was homogenized in ice-cold lysis buffer (10 mM Hepes, 0.1 mM PMSF, 0.2 mM DTT, 5 μl of a protease inhibitor cocktail) using an Ultra-Turrax TM homogenizer (Ika Werke GmbH, 3x5 sec at 50000 U/min). The resulting homogenate was kept at –20°C.

3.10.3 Electrophoresis

The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to a modified Laemmli gel method (Laemmli 1970). The gel was prepared using a Mini Protean 3 system (Bio-Rad GmbH; resulting gel dimensions: 82 mm width, 70 mm length, 1.5 mm thickness). In a first step, a 12% seperating gel was prepared (Table 1) and poured into the gel apparatus, covered with water and left to polymerise. After preparation of the 4% stacking gel, it was applied on top of the seperating gel. A comb was placed into the upper part of the stacking gel, which was then allowed to polymerise. 10 μg of protein was loaded per well. Before loading, the protein was mixed with sample buffer and heated at 95°C for 5 min. Electrophoresis was carried out at 100V (Model 1000/500 power supply, Bio-Rad GmbH) for 1 hour in a Mini Protean 2 chamber (Bio-Rad GmbH) filled with SDS electrophoresis buffer.

	12% Seperating gel	**4% Stacking gel**
30% acrylamid	8 ml	1.33 ml
Seperating gel buffer	10 ml	
Stacking gel buffer		5 ml
Distilled water	1.55 ml	3.43 ml
10x TEMED	140 μl	100 μl
APS	280 μl	200 μl

Table 1. Protocol for the preparation of seperating and stacking polyacrylamide gels.

- Seperating gel buffer (2x): 0.75 M Tris, 0.2 % (w/v) SDS; pH 8.8
- Stacking gel buffer (2x): 0.25 M Tris, 0.2 % (w/v) SDS; pH 6.8
- Sample buffer (1x): 50 mM Tris, 0.25 M saccharose, 75 mM urea, 10% (v/v) glycerol, 5% (w/v) SDS, 0.001% (w/v) bromophenol blue, 10 mM DTT; adjusted to pH 7.5 with HCl
- SDS electrophoresis buffer: 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS

3.10.4 Immunoblotting

The protein was transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) using a tank-blot system (Mini-Trans-Blot Cell, Bio-Rad GmbH). The membrane and blotting papers (Gel blot paper, Schleicher & Schuell Bioscience GmbH) were cut into the corresponding dimensions and suspended in transfer buffer together with the filterpads. The Gel was taken out of the electrophoresis chamber and the upper stacking gel was removed. The sandwich grid was opened and the following components were placed into it, avoiding air pockets: 1 filterpad, 1 gel blot paper, the gel (wrong way round), the membrane (wrong way round), 1 gel blot paper, 1 filterpad. The sandwich grid was placed into the transfer chamber (Mini Protean 2, Bio-Rad GmbH) accordingly. The blot was run at 280 mA for 90 min while beeing cooled with ice. At the end of the blotting procedure, the membrane was removed and suspended in Ponceau solution for visualization of the proteins. The resulting staining confirmed the presence of equal amounts of protein in all lanes. Extensive rinsing in water removed the colouring completely, preparing the membrane for immunochemistry processing.

- Transfer buffer: 0.04 M Tris, 0.306 M glycine, 20% (v/v) methanol, 0.01% (w/v) SDS
- Ponceau solution: 2% (w/v) Ponceau-S, 30% (v/v) trichloracetic acid, 30% (v/v) sulfosalicylic acid; for application the solution was diluted in water (1:10) and filtered

3.10.5 Immunodetection

An isoform-specific or a panspecific ALC-1 antibody (2 μg/ml each) was applied as a first antibody (for detailed description of antibodies, cf. 3.10.1). An anti-rabbit-POD antibody (BioGenes GmbH) was used in a 1/1000 dilution. The membrane was rinsed in

TBS buffer for 10 min, after which it was blocked overnight in a 5% solution of non-fat dry milk (Bio-Rad GmbH) at 4°C. It was then washed in TBS-Tween several times for 10 min. Incubation with the first antibody was overnight at 4°C. Subsequently the membrane was washed in TBS-Tween (2x 10 min each), blocked (1x 5 min in 5% non-fat dry milk) and washed again (2x 10 min TBS). The second antibody, dissolved in TBS and 0.1 % ovalbumin, was then applied and remained on the membrane for 60 min at RT. After two final washes (2x 10 min in TBS-Tween and 2x 10 min in TBS) the membrane was drained of excess fluid in order to carry out immunodetection.

- TBS buffer: 250 mM NaCl, 0.1 M Tris solution (pH 8.0)
- TBS-Tween: TBS buffer containing 0.05% Tween
- non-fat dry milk: non-fat dry milk powder dissolved in TBS-Tween
- TBS buffer for first antibody: 50 mM Tris, 120 mM NaCl, 1% BSA, 0.04% NaN_3; pH adjusted to 8.0 with HCl

Rinsing/washing of the membrane and incubation with antibodies was performed under constant rocking.

Immunodetection was performed with the ECL system (Amersham Biosciences). The membranes were incubated with sufficient volume of the detection mixture for 1 min at RT, after which excess reagent was drained off. The membranes were wrapped in saran foil, avoiding air pockets, and placed in an X-ray cassette protein-side up. A Hyperfilm ECL film (KODAK) was placed on top of the membranes, the cassette was closed and the film was exposed for 3 min and developed using a Curix 60 developing machine (AGFA).

3.11 Immunofluorescence analysis

For immunofluorescence analysis, H9c2T1 cardiomyoblasts were washed twice with PBS. Fixation of the cells was performed for 5 min in ice-cold methanol, followed by two more washes with PBS. The cells were then repeatedly (3x) incubated in blocking buffer (BB) for 10 min at RT with intermediate washing in PBS.

Overnight incubation at 4°C with primary antibodies followed: NFAT (NFATc4,1:500 dilution, Santa Cruz Biotechnology) also in combination with the corresponding NFAT blocking peptide (Santa Cruz Biotechnology, 5 or 10 μg blocking peptide/1 μg NFAT antibody), CaMKIIδ (3 μg/ml, cf. Hoch et al. 1998), or CaMKIV (1 μg/ml, BD Biosciences Pharmingen). After washing with BB, the cells were incubated for 30 min at 37°C with the secondary antibodies conjugated with Cy3 (1:100 dilution) or Alexa 594 (1:1000 dilution) (Molecular Probes). In case of NFAT localization, the secondary antibody was incubated together with DAPI (1:10000 dilution of a 5 mg/ml stock solution) for staining of the nuclei. Washing with BB (3x 10 min) and PBS (1x 10 min) was followed by embedding of the glas microchips (Menzel) in aqua polymount (Polysciences Europe GmbH) on top of a glas slide (Menzel).

- Blocking buffer: 130 mM NaCl, 0.05% Tween-20, 0.02% NaN_3, 20 mM Tris-HCl, 1% BSA

Fluorescence of cells was detected using an Axioplan II fluorescence microscope or an LSM 510 META confocal microscope (Zeiss) with appropriate filter systems. Images were captured with a digital camera. Excitation/emission maxima for visualization of DAPI fluorescence of nuclei, Cy3 fluorescence and Alexa 594 fluorescence were as follows: 340/488 nm, 550/570 nm, and 590/617 nm, respectively.

3.12 Determination of the percentage of nuclear NFAT staining

For evaluation of the percentage of nuclear NFAT localization, a number (n) of untreated (- V) or treated (+ V) H9c2T1 cardiomyoblasts from more than one experiment were analyzed by visual inspection. Cytoplasmic or nuclear localization of NFAT was an all- or none-phenomenon and could therefore be analyzed and quantitated by immnunofluorescence using an Axioplan II fluorescence microscope (Zeiss).

3.13 Statistics

Values are expressed as means +/- SEM. Luciferase expression experiments were performed in triplicate, each measured 3 to 4 times (i.e. 9 to 12 measurements per treatment). Significance analysis was performed using the unpaired Student's t-test or ANOVA with Bonferroni as post test.

3.14 Promoter consensus binding site analysis

Promoter sequence analysis for detection of consensus binding sites was performed using Genomatix Matinspector.