THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 52, Issue of December 26, pp. 53082–53089, 2003
Printed in U.S.A.

Critical Role of the Transcriptional Repressor Neuron-restrictive Silencer Factor in the Specific Control of Connexin36 in Insulin-producing Cell Lines*

Received for publication, June 27, 2003, and in revised form, September 26, 2003
Published, JBC Papers in Press, October 16, 2003, DOI 10.1074/jbc.M306861200

David Martin, Thomas Tawadros, Laure Meylan, Amar Abderrahmani, Daniele F. Condorelli‡, Gérard Waeber, and Jacques-Antoine Haefliger§

From the Department of Internal Medicine, University Hospital, CHUV-1011 Lausanne, Switzerland and the ‡Department of Chemical Sciences, University of Catania, 95125 Catania, Italy

Connexin36 (Cx36) is specifically expressed in neurons and in pancreatic β-cells. Cx36 functions as a critical regulator of insulin secretion and content in β-cells. In order to identify the molecular mechanisms that control the β-cell expression of Cx36, we initiated the characterization of the human 5′ regulatory region of the *CX36* gene. A 2043-bp fragment of the human *CX36* promoter was identified from a human BAC library and fused to a luciferase reporter gene. This promoter region was sufficient to confer specific expression to the reporter gene in insulin-secreting cell lines. Within this 5′ regulatory region, a putative neuron-restrictive silencer element conserved between rodent and human species was recognized and binds the neuron-restrictive silencing factor (NRSF/REST). This factor is not expressed in insulin-secreting cells and neurons; it functions as a potent repressor through the recruitment of histone deacetylase to the promoter of neuronal genes. The NRSF-mediated repression of Cx36 in HeLa cells was abolished by trichostatin A, confirming the functional importance of histone deacetylase activity. Ectopic expression, by viral gene transfer, of NRSF/REST in different insulin-secreting β-cell lines induced a marked reduction in Cx36 mRNA and protein content. Moreover, mutations in the Cx36 neuron-restrictive silencer element relieved the low transcriptional activity of the human *CX36* promoter observed in HeLa cells and in INS-1 cells expressing NRSF/REST. The data showed that *cx36* gene expression in insulin-producing β-cell lines is strictly controlled by the transcriptional repressor NRSF/REST indicating that Cx36 participates to the neuronal phenotype of the pancreatic β-cells.

Adjacent cells share ions, second messengers, small metabolites, and signaling molecules with a weight as high as 1000 Da (1) through intercellular channels that form gap junctions. This type of intercellular communication permits coordinated cellular activity and allows cells to review the functional state of their neighbors, a critical feature for the homeostasis of multicellular organisms (1–4). Intercellular channels result from the association of two half-channels named connexons, which are contributed to separately by each of two adjacent cells. Each connexon is an assembly of membrane proteins named connexins, which are encoded by a gene family consisting of at least 20 members (5, 6).

It has been reported that Cx36[1] is the predominantly expressed connexin in rodent neuronal cells (7–9). Immunogold labeling using freeze-fracture replicas have shown that Cx36 was found only in neurons (10) and indicate that the specific expression of connexins by the different cell types of the nervous system may define separate pathways for neuronal *versus* glial gap junction communication (10–12). We recently found that Cx36 is the predominant if not the sole connexin isoform expressed in insulin-producing β-cells of rat and mouse pancreatic islets (13, 14). Recently, Cx36 content was modified in insulin-secreting cells by using an adenoviral gene transfer approach (15) or a lentivirus-mediated transduction of *cx36* cDNA (16) in order to evaluate the contribution of Cx36 in the control of insulin secretion and content. These experiments have demonstrated that tight levels of Cx36 are crucial for proper function of insulin-producing cells. Moreover, stably transfected insulin-secreting MIN6 cells with a *cx36* antisense cDNA impaired the synchronization of glucose-induced $Ca^{(2+)}$ oscillations and insulin secretion in response to glucose (17). These data provided evidence that proper storage of insulin as well as the regulation of insulin release required that the levels of Cx36 be maintained within native values.

The objective of the present study was to characterize the mechanisms of cell-specific expression of Cx36. The presence of several gene transcripts in pancreatic β-cells and neuronal cells was correlated with the absence in these cell types of a transcription factor named NRSF/REST (neuron-restrictive silencer factor/repressor element silencing transcription factor) (18). NRSF/REST binds to a 21-bp cis element named neuronal restrictive silencer element (NRSE). Through this NRSE, NRSF/REST has been identified as a negative regulatory system that silences neuronal gene expression in non-neuronal tissues, neuronal precursor cells, and certain differentiated

* This work was supported by Swiss National Science Foundation Grants 31-068036.02 (to J.-A. H.) and 32-66892.01 (to G. W.), the Juvenile Diabetes Research Foundation Grant 1-2001-555, the Placide Nicod and the Octav and Marcella Botnar Foundations (to G. W. and J.-A. H.), and by Telethon Foundation Grant E. 1283 (Italy) (to D. F. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY341000.

§ To whom correspondence should be addressed: Dept. of Internal Medicine, Laboratory of Molecular Biology 19-135S, University Hospital, CHUV-1011 Lausanne, Switzerland. Tel.: 41-21-314-09-26; Fax: 41-21-314-09-68; E-mail: jhaeflig@chuv.hospvd.ch.

[1] The abbreviations used are: Cx36, connexin36; RT, reverse transcriptase; NRSF/REST, neuron-restrictive silencer factor/repressor element silencing transcription factor; NRSE, neuron-restrictive silencer element; m.o.i., multiplicity of infection; RACE, rapid amplification of cDNA ends; TSA, trichostatin A; GFP, green fluorescent protein; Ad, adenovirus; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; MOPS, 4-morpholinepropanesulfonic acid.

```
-1730 TAGGGGATCACCATTCCATCTGGAAGCTAACTGGATAATAGGGTATCCATTCGTCCTCACCC
-1668 TTGAACCTCCTCCGTGTTCAGAAGGAAACTAATAAATCACTTTATCCCTCTCTCCATTGTTA
-1606 GTGGACTCTTTTACTCCTTTGCAGTGAAAGGCTGCTTTCTTGGGGTAAAGATGTCAACCCTA
-1544 GCAGAGGATTTCAGTGATCTTTCTGGCGGCGAAGCTTAGACCGTGTGTTTAAACATCATTTT
-1482 ATGAAGAAGTACACTGTGAGTGGACTGAGGGCCATGCCCTGCACCTTTCTTCCCAGTCCCTG
-1420 GGCCACCAACTACCCAACCACCTCGGTTGTTCCAGAAGTGAAGGCTCACCATGGTGTGGCTT
-1358 ATGTAATATACATATGTGCTATGATCCCTGTGGATAATTAAGGCTAGGTCCCTTTATATGCC
-1296 TAGGACAAGTTAGCAACCCTTTCAAGGTCGTGTTTTCGAAAGGATGACAATTACTTTCCCAT
-1234 TGGACTTTTGGTACATTTACATCAAACACTGCCAGCTACAGTGTCTCCTAATACTGACCCCT
-1172 GGGCTAATTAGCAGCTTAGTGAGGTTGTTTCAATTTTTCAGCAACCAATTAAGTAGTTGCTC
-1110 TAGACAGAACTGACAACACAATGTGAATTTTAAAAACCGGGATCTGCTGGTTGAGGGGAGAA
-1048 AGCCAAACCCAGTAAGGCAAGTGGGGGCAGGGCGAGGCTGTGAGTGGCTCTGTTTCTGCTAG
 -986 CAGAATACTGGGTCTTAAATTCAGATTAAATCACAGTTTACCTGACGAGTACCCCGCCCCCC
 -924 CTTCCCATTAGTAAAATAATAGCAATAATTTAAAAAGAGCCGAGCATCTGGGTTAGTGCAAG
 -862 GGGAAGCGAGACCTTTAACCATGGCCACCCCACTTGCTCTTTGCGAGGCACCTGTTAGATGC
 -800 GCTCGTGTGCCCTGACCCCTATACCGAGAGACAGCTCCACACCTGCATTTACCCTTCAGCCT
 -738 AAATGGATTCCCATCAAGCCTTTCTCACTTCACTGTCGGATCCACAGAGGCACCGAACCGCA
 -676 GCCACTCTGCAGAAGCACTCTCAGCCACTCTGTCCCCTAAGGGAGATTTAAAAGTGGTCGCC
 -614 CCCACTCGCCCTCCCTCAGCCAGCATCTCCATCTAGGGAAGGAATTTCAGTTGTCTCCAGGA
 -522 GGCAAAAAGCGAAAAGGTGAGGGATGCACCGAGAGAAGAGAAGATACCTACTCACTCCCCTC
 -490 CCCACTCACCCACGCGTCCGCTCCCGGCTCCCCCTCCGCCTGCTTGGGTCTGGCGGTCAGGT
 -428 GAGGTTACCTGGGCTCCCCGAGAAGCCCCGAGGGGCGCTGCAGTCCTTCCTTTCGAAATCGC
 -366 CCCGCTCCCCGCCGGGCCTCCAGGACTCTTGCCCCTCTCCCCCCGCGCGCGTCCCCGGCCCC
 -304 TCGGGCTCCGACGCGCAGCGACAGCCGCACGGCGCGCTCTGCTCCCCTCCGCCGACCTGGCG
 -242 TGACGCAGGCGGAGACTACTTAAGGGCGGATTAGAGGCGGCGGCCGCGGATCCCCGAGCCCA
 -180 GCATTCGGCTCCGCGCGCTCGGCCCTGGCCTCGCAGGCTGCCGCGCCGGAGCGCCAGGTGCC
 -118 CGCTGAAGTAGCAGGAACAGAGCCGGTGTTCGGACCCTCCCCTGGCCCCCAACCTGCGGCGG
                                                          +1 ──►
  -56 AGAGCGGCGCTGAGACTTCTCCTGGGCGGGCGAGCGCTGGAGGACCTGCAGGCTGCCACCCC
   +7 GCCCCGCCCCTCGTCCCGGCGTCCGCGGGATCCAG ACCCGAACCCCGGACGGCGCC CGAGGT
  +69 TCGTGCCCTCTCTCTAGACCGCGCCAACTGCCCCATCGGTCGGGACGCGCTTGGATTTCTTT
 +131 CCCCATTTCTTCTCTTTTAAGTACAATAAAAGGAAAGGGGGATTCGAGGATTTTTTAAAAAA
 +193 TTGCTTCTTTAATTGGGAGGCAATTCACTCCCATTCTGGAAGTGCGGCCCGGGGAGGGGCCA
 +255 GGAGCGGGAACGTGCCCGGTGCTGCCCAGTCTTTGTCTGCTGCCTCCGGATGCACAGCG ATG
```

FIG. 1. **Nucleic acid sequence of a fragment of the human *CX36* promoter.** The transcription start site (+1) was determined by a 5′-RACE reaction using mRNA from INS-1 (rat) and βTC3 (mouse) insulin-secreting cell lines. A putative NRSE-binding site is indicated. The ATG in *boldface* is the codon of translation initiation of the Cx36 protein.

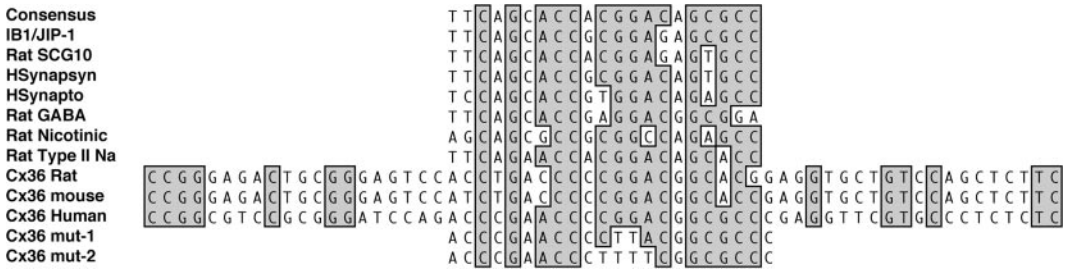

FIG. 2. **Sequence comparison of the rodent and human Cx36 NRSE with functional known NRSEs.** Conservation of NRSEs of human, mouse, and rat Cx36 NRSE. The sequences bordering the NRSEs are less conserved than the NRSE between the human and rodent Cx36 5′-untranslated region. Note that the CGGAC central region of the NRSE consensus is 100% conserved between the three species. *Cx36 mut-1* or *Cx36 mut-2*, mutants Cx36; *IB1/JIP-1*, scaffold protein termed JNK-interacting protein 1 (*JIP-1*) or Islet-Brain 1 (*IB1*); *GABA*, γ-aminobutyric acid; *Hsynapto*, human synaptophysin; *Rat nicotinic*, rat nicotinic acetylcholine receptor α7; *Rat type II Na*, rat type II sodium channel.

neurons (19–27). The repression effect induced by NRSF/REST required an interaction of NRSF/REST with the corepressor mSin3 and histone deacetylase I (28), which induces hypoacetylation of histone (29, 30). We have shown recently that the expression of the JNK-interacting protein-1 (IB1/JIP-1) gene is repressed by this mechanism in cells expressing REST (31). Many other genes specifically expressed in β-cells and neuronal cells, such as type II sodium channel and the rat NMDA receptor, were shown to be controlled by REST through the NRSE (18, 31, 32).

Here we identified a conserved NRSE sequence in the promoter of human and rodent *cx36* genes, and we demonstrated that NRSF/REST specifically binds to this human NRSE. Forced expression of REST in the nucleus of pancreatic β-cell lines decreases the *cx36* gene expression. After linking upstream to a luciferase gene reporter, the human Cx36 transcriptional activity was shown to be undetectable in REST-expressing cells, whereas it was highly detectable in insulin-secreting cells. Ectopic expression of REST decreased by 50% the transcriptional activity of *CX36* promoter in insulin-secreting cell lines. Introduction of mutated NRSEs into the human *CX36* promoter both abolished the silencing effect observed in INS-1-expressing REST and relieved the low transcriptional activity detected in HeLa cells expressing REST. This transcriptional activity was relieved and the *cx36* gene expression became detectable in trichostatin-treated HeLa cells expressing REST, indicating that the *cx36* gene silencing by NRSF/REST required histone deacetylase activity. Finally, these observations showed that the transcriptional repressor NRSF/REST controls *cx36* gene expression in pancreatic β-cell lines.

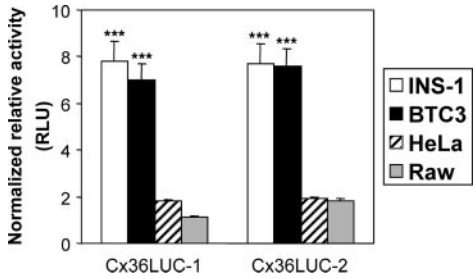

FIG. 3. **Transcriptional activity of the *CX36* promoter region.** The regions comprising bp −1730 to +313 (*Cx36LUC-1*) and bp −308 to +313 (*Cx36LUC-2*) of the human *CX36* promoter region drove high luciferase activity in the insulin-secreting INS-1 and βTC3 but not in unrelated HeLa and RAW cells. Luciferase activities were normalized using pRLCMVrenilla. RLU, relative light units. Columns represent mean ± S.E. of three independent experiments performed in triplicate. ***, $p < 0.001$.

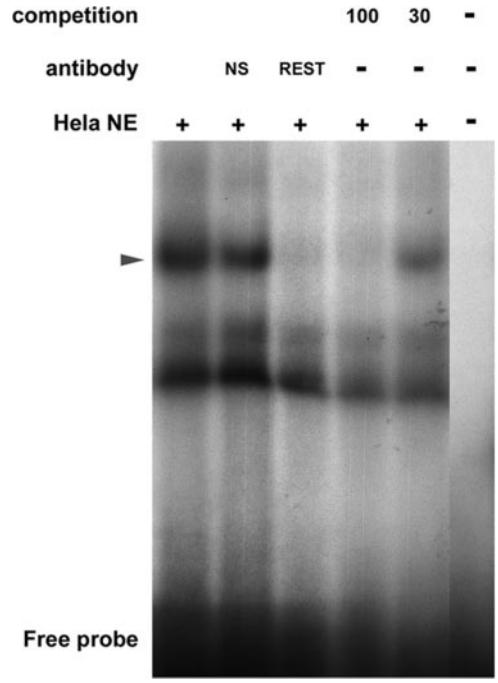

FIG. 4. **Specific binding activity of NRSF/REST to the human Cx36 NRSE.** EMSA with ^{32}P-labeled Cx36 NRSE using nuclear extracts (*NE*) from HeLa cells. A slow-migrating complex (*arrowhead*) was detected and compared with free-probe migration. NRSF/REST shift was competed with the incubation of polyclonal antibodies against REST but not with nonspecific antibody (*NS*). The DNA binding activity with human Cx36 NRSE was abolished by adding a 30- or 100-fold molar excess of unlabeled wild-type NRSE.

MATERIALS AND METHODS

Cell Lines—The rat insulinoma INS-1 cell line (33) was cultured in RPMI 1640 medium containing 11.1 mmol/liter glucose and supplemented with 10% fetal calf serum, 2 mmol/liter L-glutamine, 1 mmol/liter sodium pyruvate, 50 μmol/liter β-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin. The insulin-secreting cell line βTC3 was cultured in RPMI 1640 (11.1 mM glucose) supplemented with 10% fetal calf serum, 50 μg/ml streptomycin, 50 units/ml penicillin, 1 mmol/liter sodium pyruvate, and 2 mmol/liter glutamine (Invitrogen); Min6 cells (34) were cultured as described previously (17, 31). Cells were kept at 37 °C in a humidified incubator gassed with air and CO_2 to maintain medium at pH 7.4, fed at 2–3-day intervals, and trypsinized once a week. Human carcinoma HeLa and mouse macrophage-like RAW cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 2 mmol/liter L-glutamine, 1 mmol/liter sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Screening of a BAC Library of Human Genomic DNA and Plasmid Construction—A human genomic library in the pBeloBAC11 vector (Genome Systems, St. Louis, MO) was screened by PCR using specific rat Cx36 primers (forward, 5′-AACGCCGCTACTCTACAGTCTTCC-3′; 5′-GATGCCTTCCTGCCTTCTGAGCTT-3′). The identity of a single positive clone was first confirmed by Southern hybridization with the 269-bp rat Cx36 DNA probe (35). Four kilobases of the 5′-flanking sequence were sequenced. A 2043-bp fragment was PCR-amplified with primers 5′-GCGGTACCTAGGGGATCACCATTCCATCTG-3′ (sense) and 5′-GCAGATCTCGCTGTGCATCCGGAGGCAGCAGACAA-3′ (antisense), and a 621-bp fragment was amplified using a specific sense primer, 5′-GCGGTACCCCCCTCGGGCTCCGACGC-3′, and subcloned in pGL3 basic (Cx36LUC-1 and Cx36LUC-2, respectively) (Promega). Plasmid constructs were verified by DNA sequencing. The fragments were subcloned at KpnI/BglII sites into the promoterless expression vector pGL3 basic (Promega). Mutations in the NRSE sequence of the human *CX36* promoter were generated by PCR site-directed mutagenesis using high fidelity *Pfu* DNA polymerase according to the manufacturer's protocol (QuickChange, Stratagene, La Jolla, CA). *In vitro* mutagenesis was carried out on a human *CX36* promoter fragment of 621 bp (Cx36LUC-2) from double-stranded 5.5-kb plasmid DNA using oligonucleotide primers, each complementary to opposite strands of the vector (two forward primers, 5′-CCAGACCCGAACCCCTTACGGCGC-CCGAGG-3′ and 5′-CCAGACCCGAACCCTTTTCGGCGCCCGAGG-3′; and two reverse primers, 5′-CCTCGGGCGCCGTAAGGGGTTC-GGGTCTGG-3′ and 5′-CCTCGGGCGCCGAAAAGGGTTCGGGTCT-GG-3′. The mutated nucleotides are underlined. Both constructs (m1Cx36LUC-2 and m2Cx36LUC-2) were verified by DNA sequencing to confirm the integrity and the presence of the desired mutations in the constructs. The sequence of the fragment of the human *CX36* promoter (bases 1–2046) was assigned GenBank™ accession number AY341000.

RACE—For 5′-rapid amplification of cDNA ends (RACE), 3–4 μg of total RNA from INS-1 or βTC-3 were reverse-transcribed by avian myeloblastosis virus-reverse transcriptase according to the 5′/3′-RACE kit procedure (Roche Applied Science) by using an antisense Cx36-specific primer, 5′-AAGATGGTCCATTCCCCCAT-3′. The purified cDNA was then subjected to a terminal transferase tailing reaction, and the deoxyribosyladenosine-tailed cDNA was then used as a template of PCR using an oligo(dT) anchor primer provided by the 5′/3′-RACE kit and an antisense Cx36-specific primer (5′-ACGTTCTTGGTCCTG-GCCC-3′). A second nested-PCR round reaction was then performed using the PCR anchor primer provided by the race kit and a third antisense Cx36-specific primer 5′-ATTGCCCCCAATTAAAG-3′. The RACE products were subcloned into the T-Easy vector (Promega) according to the manufacturer's protocol, and individual clones were sequenced.

Transient Transfection and Luciferase Assays—Cells were transfected using the liposome-mediated Transfast Reagent (Promega) at a DNA/lipid ratio of 1:1 in RPMI1640 medium without fetal calf serum. Cells were plated in 12-well plates and grown to 60% confluence. On the day of the transfection, 1 μg of DNA was mixed with 3.0 μl of Transfast reagent and incubated at room temperature. After 15 min of incubation, plated cells were overlaid with DNA/Transfast mixture and incubated at 37 °C for 1 h. To each well, 2 ml of RPMI1640 with serum were added. Cells were incubated for 48 h and harvested with 110 μl of the passive lysis buffer (Promega). Luciferase activities (from Cx36LUC and normalization with *Renilla* pRLCMV [pRLCMVrenilla] vectors) were measured with 50 μl of protein extract solution by using the dual-luciferase reporter assay system (Promega) and a Turner TD-20/20 luminometer. All experiments were repeated three times in triplicate.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from cells were prepared according to the kit procedure (Activ Motif, CA). The protein concentration was measured using a Bio-Rad protein measurement kit. For EMSA, the double strand DNA probe corresponding to the Cx36 NRSE sequence sense (5′-GGATCCAGACCCGAACCCCGG-ACGGCGCCCGA-3′) and antisense (5′-CCTCGGGCGCCGTCCGGGG-TTCGGGTCTGGAT-3′) oligonucleotides was labeled with [α-^{32}P]dCTP by the Klenow fragment of DNA polymerase I (Roche Applied Science). The labeled probe was incubated with 5 μg of the nuclear protein, and 2 μg of poly(dI-dC) in a total volume of 20 μl containing 10% glycerol, 20 mM Hepes-NaOH, pH 6, 2.5 mM $MgCl_2$, 5 mM dithiothreitol, and 50 mM KCl. After incubation at 4 °C for 30 min, the reaction mixtures were resolved on a 4% non-denaturing PAGE in 0.25× TBE buffer (1× TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 4 °C for 2 h, and the products were detected by autoradiography. For the supershift experiments, affinity-purified REST polyclonal antibodies were added to the extracts, and the mixture was incubated for 10 min at room temperature prior to the addition of the labeled probe. Similar experiments were performed using nonspecific rabbit affinity-purified polyclonal antibodies. In the competition study, unlabeled specific double strand DNA competitors (30–100-fold in excess) were added into the reaction mixtures prior to incubation.

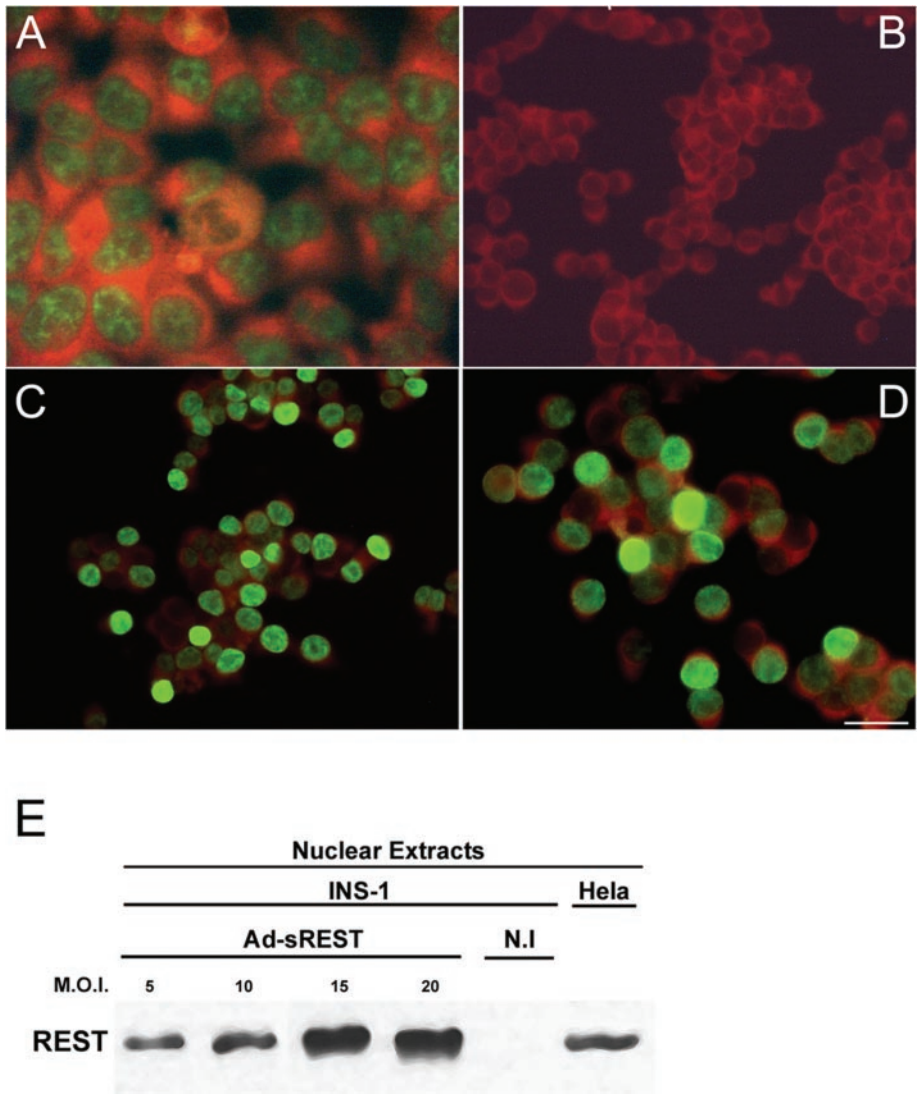

FIG. 5. **NRSF/REST is expressed in the nucleus of INS-1 and βTC-3 cells after gene transfer by using an adenoviral vector expressing REST.** *A–D*, immunostaining of REST-infected INS-1 (*C*) and βTC3 (*D*) cells (m.o.i. = 15) showed a nuclear labeling using antibodies directed against REST, whereas no protein could be detected in both insulin-secreting cell lines (INS- in *B*). The same antibodies resulted in a nuclear staining of wild-type HeLa expressing REST (*A*). The *bar* represent 20 μm in *A–C* and 30 μm in *D*. *E*, Western blot analysis of nuclear extracts further demonstrated the absence of REST in INS-1 cells and revealed a band at 200 kDa in HeLa cells or INS-1 cells transduced with four different doses (m.o.i. = 5, 10, 15, and 25) of Ad-sREST. The levels of this band increased with the adenoviral dosage.

Generation of Recombinant Adenoviruses and Cell Infection—To express REST in insulin-secreting cells, we generated recombinant adenoviruses (36–38) comprising the complete cDNA of human REST (31) in the sense orientation (Ad-sREST). Adenoviruses expressing the green fluorescent protein (GFP) (Ad-GFP) were used as control. The recombinant adenoviruses allowed for expression of REST and GFP were under the control of the immediate, early cytomegalovirus promoter (CMV). The cDNAs were inserted into plasmid pXC15 (39), and the adenoviruses were then generated by cotransfecting 293 cells with plasmids pJM17 and the pXC15 containing human REST cDNA, using a calcium phosphate procedure. Viruses were further plaque-purified three times on HER911 cells (IntroGene, Leiden, The Netherlands). Stocks were purified by two successive CsCl centrifugations, after which the virus band (1.5 ml) was collected and dialyzed at 4 °C against three changes (at least 200 volumes each) of 10 mM Tris-HCl buffer, pH 8.0, in a Slide-A-Lyzer (0.5–3.0-ml capacity), γ-irradiated 10K dialysis cassette (Pierce). INS-1 or βTC3 cells were seeded in 12-well dishes or 6-cm dishes (Falcon) and cultured for 48 h. For infection, cells were incubated for 60 min with different dilutions (multiplicity of infection (m.o.i.)) of a stock of the recombinant adenoviruses Ad-sREST or Ad-GFP and then cultured in RPMI 1640 medium for 24 h before any experiment.

Antibodies—Cx36 peptide corresponding to amino acids 289–303 (peptide B, AKRKSVYEIRNKDLP) (13, 15, 17) was conjugated to keyhole limpet hemocyanin and used to immunize rabbits (Eurogentec; Parc Scientific du Sart Tilman, Herstal, Belgium). Plasmid pQE containing the His_6 tag was used to direct the synthesis by *Escherichia coli* of a polypeptide comprising amino acids 7–355 of the human REST. An overnight culture of *E. coli* that had been transformed with the recombinant plasmid was diluted 1:10 in fresh medium and grown for 1 h at 37 °C before addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Four hours later, cells were pelleted and lysed on ice by sonication. The recombinant protein was incubated in the presence of nickel-nitrilotriacetic acid to purify the His_6-tagged REST fusion protein. Site-directed antibodies were affinity-purified by passing the sera diluted in PBS through HiTrap affinity columns (HiTrap NHS-activated Sepharose; Amersham Biosciences) that had been coupled with the Cx36 COOH-terminal tail peptide or recombinant REST protein. The columns were washed sequentially with PBS, 0.5% PBS/Tween 20, and PBS. Antibodies solutions were eluted with 100 mmol/liter glycine, pH 2.5, in a 1.5 mol/liter Tris-HCl buffer, pH 8.8, to reach neutrality (13, 40).

Immunofluorescence—For immunofluorescence labeling, cells were grown on glass coverslips, fixed 3 min in −20 °C acetone, air-dried, rinsed in PBS, and incubated in a buffer containing 2% bovine serum albumin and 0.1% Triton. Cells were then exposed for 20 h at room temperature in the presence of the polyclonal rabbit antibodies against human REST, diluted 1:200. After repeated washing, cells were incubated a second time for 1 h at room temperature, using fluorescein-conjugated antibodies against rabbit IgGs, diluted 1:500. After additional rinsing, cells were counterstained with Evans blue, coverslipped, and viewed on a microscope (Leica, Switzerland) fitted with filters for fluorescein detection. In these experiments, negative controls included INS-1 or βTC-3 cells.

RNA Isolation, RT-PCR, and Northern Blot Analysis—Cells were homogenized in the Tripure Isolation Reagent (Roche Applied Science), and total RNA was extracted using the kit procedure. 10–15 μg of total RNA were size-fractionated on 1% agarose gels containing 8% formaldehyde (Fluka, Switzerland) and 1× MOPS buffer (Fluka, Switzerland). Capillary transfer in the presence of 10× SSC transferred RNAs overnight to GeneScreen membranes (PerkinElmer Life Sciences). Membranes were UV cross-linked and vacuum-baked for 2 h at 80 °C. Total mRNA levels were determined by hybridization with random-primed (Roche Applied Science) cDNA probes labeled with [α-^{32}P]dCTP

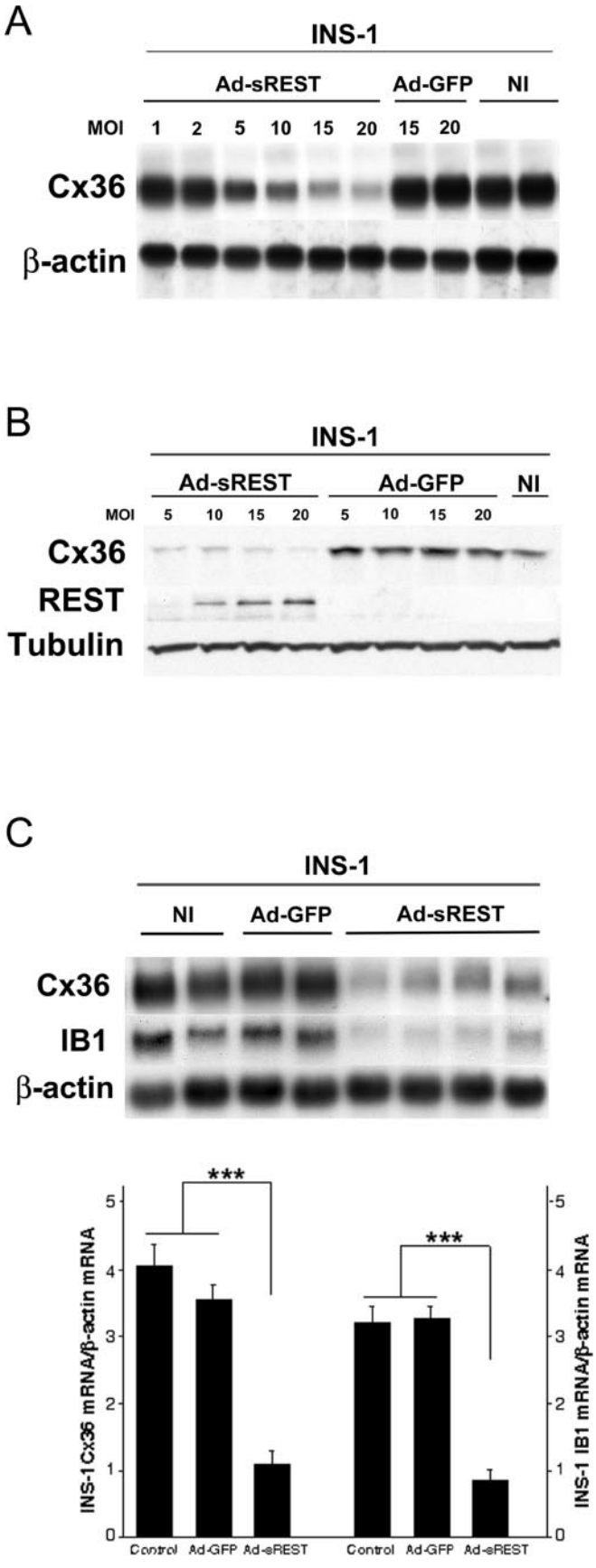

FIG. 6. **Expression of Cx36 mRNA and protein is decreased in REST expressing INS-1 cells.** *A*, Northern blot showed a dose-dependent reduction of Cx36 mRNA in INS-1 infected with Ad-sREST (m.o.i. = 1, 2, 5, 10, 15, and 20), 48 h after the infection. *B*, 48 h after infection with Ad-sREST, Western blot of total protein extracts showed a decrease of Cx36 levels in INS-1 cells. Non-infected cells (*NI*) and cells infected with Ad-GFP served as internal controls. Protein loading of each lane was evaluated by tubulin immunolabeling. *C*, after 48 h of infection, Northern blot revealed a marked decrease in the expression levels of Cx36 mRNA in INS-1 cells expressing REST (*upper panel*). IB1/JIP-1 mRNA expression in INS-1, known to be controlled by REST (31), is shown as a positive control. Quantitative assessment of 10 independent experiments (one experiment per lane, *lower panel*) showed that Cx36 mRNA or IB1/JIP-1 mRNA was significantly decreased (70–80%) in pancreatic β-cell lines infected with Ad-sREST compared with non-infected cells (control) or cells infected with Ad-GFP (m.o.i. = 20) ***, $p < 0.001$.

(Amersham Biosciences), which were specific for Cx36, IB1/JIP-1, and β-actin. Probes were prepared using the following cDNA clones for rat Cx36 (7, 13), for rat IB1/JIP-1 (38, 41), and for β-actin (42). Hybridizations were performed overnight at 42 °C in the presence of 5× SSPE, 50% formamide, 5× Denhardt's solution, 5% SDS, and 100 μg/ml purified salmon sperm DNA. Blots were washed 2 times for 10 min at 42 °C in 2× SSC supplemented with 1% SDS, and 2 times for 10 min in 0.1× SSC containing 1% SDS. Levels of transcripts were normalized relative to β-actin.

For reverse transcription PCR, total RNA was treated for 30 min in the presence of DNase I (DNA-free kit, Ambion, Cambridge, UK). One-μg aliquots of DNase-treated RNA were reverse-transcribed using Im-Prom-II reverse transcription system (Promega, Switzerland), as described by the manufacturer. One-half of the reverse-transcribed products was used for PCR in the presence of 20 ng of sense and antisense specific primers using recombinant Taq DNA polymerase (Invitrogen). The products obtained after 30 PCR cycles performed in a PCR machine (Biometra, Switzerland) with the different primers were analyzed by agarose gel. Negative controls included amplification of INS-1 samples that had not been reverse-transcribed. The following primers were used, a sense primer 5′-CACAGCGATGGGGGAATGGA-3′ and an antisense primer 5′-TGCCCTTTCACACATAGGCA-3′ to detect Cx36 mRNA. These primers amplified a fragment of 980 bp. The amplified DNA was visualized after ethidium bromide staining on agarose gel.

Western Blotting—INS-1 cells were lysed by sonication in a buffer containing 5% SDS supplemented with 5 mM EDTA, and the DC protein assay reagent kit (Bio-Rad) was used to determine protein content. Aliquots of cell lysates or nuclear extracts from INS-1 cells were heated at 95 °C in loading buffer and were fractionated by electrophoresis in a 12.5% polyacrylamide gel and immunoblotted onto Immobilon polyvinylidene difluoride membranes (Millipore) overnight at a constant voltage of 20 V. Membranes were incubated for 1 h at room temperature in PBS containing 5% milk and 0.1% Tween 20 (blocking buffer) and then incubated overnight at 4 °C with antibodies directed against rat Cx36 in blocking buffer. Antibodies directed against β-tubulin (Sigma) were incubated overnight at 4 °C in blocking buffer. Specific antigen-antibody complexes were detected with the horseradish peroxidase Western blot detection system (Amersham Biosciences).

Statistical Analysis—Densitometric analysis of immunolabeled proteins (Western blots) and mRNA signals (autoradiograms) was performed using an Amersham Biosciences scanner that integrates areas and corrects for background. Data were expressed as mean ± S.E. Differences between means were assessed by Student's t test and/or analysis of variance t test. Statistical significance was defined at a value of $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

Identification of the NRSE within the CX36 Promoter Region—A 2043-bp fragment of the human *CX36* promoter was PCR-amplified using specific human Cx36 primers. The nucleic acid sequence of this region is shown in Fig. 1. The transcriptional start site in insulin-secreting cells was determined using a 5′-RACE reaction with several primers. For this analysis, we used as template total RNA isolated from INS-1 and βTC3 cells, and the most prominent 5′ nucleotide identified the start site (bp +1 of the sequence shown in Fig. 1) located −313 bp from the 1st base of the translational start codon ATG in INS-1 and βTC3 cells. Sequence comparison between human, rat, and mouse *cx36* promoter region indicates high homology in the region of the start site between the three species (data not shown). Different putative sequences for the binding of a variety of transcription factors were identified within the 2043 bp by computer analysis

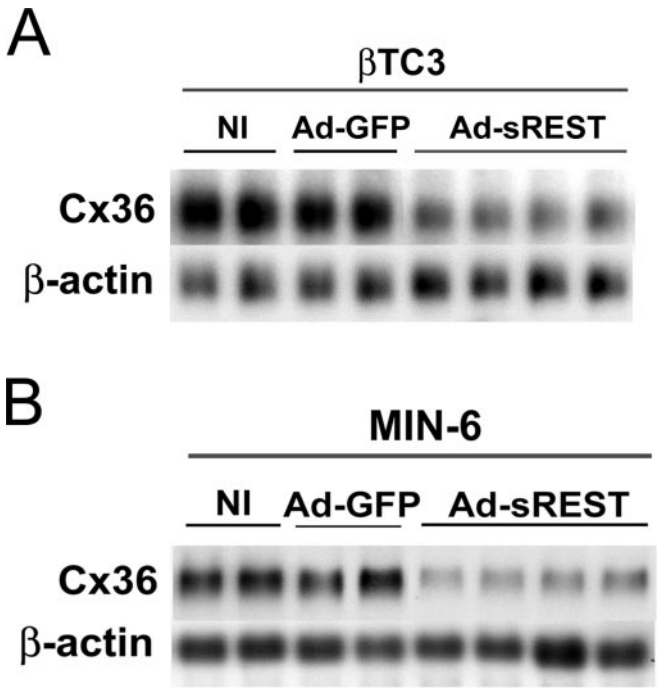

FIG. 7. **Expression of Cx36 mRNA is decreased in REST expressing insulin β-cells.** *A* and *B,* after 48 h of infection, Northern blot (one experiment per lane) revealed a marked decrease (60–80%) in the expression levels of Cx36 mRNA in βTC3 and Min-6 cells expressing REST. *NI*, non-infected cells.

(bioinformatics.weizmann.ac.il/transfac/), in particular an NRSE. As shown in Fig. 2, the 21-bp NRSE identified in human (bp 42–62) is highly conserved in mouse and rat (bp 40–60) sequences as well as with other known NRSE sequences and is homologous to the consensus NRSE sequence (23). Moreover, these Cx36 NRSE sequences are highly homologous compared with adjacent *cx36* promoter sequences suggesting a functional role of these elements for REST activity.

The Promoter Region of Cx36 Drives High Transcriptional Activity in Insulin-secreting β-Cell Lines but Not in Unrelated Cell Lines—We evaluated the transcriptional activity of two fragments comprising bp −1730 to +313 (Cx36LUC-1) and −308 to + 313 (Cx36LUC-2), respectively, of the promoter region of the human *CX36* gene in insulin-secreting β-cells and in unrelated cell lines (Fig. 3). As shown in Fig. 3, both constructs drove high transcriptional activity in INS-1 and βTC3 cells, whereas no significant reporter luciferase activity was detected in HeLa and Raw cells compared with the promoterless pGL3 basic vector (normalized to 1). These data indicate that important cis regulatory elements are present within the human *CX36* promoter to confer the β-cell-specific expression of the reporter gene.

REST/NRSF Interacts with Human NRSE of CX36 Promoter—To investigate whether the NRSF/REST was able to bind to the newly identified human Cx36 NRSE, we performed an EMSA using nuclear extracts prepared from HeLa cells expressing endogenous NRSF/REST and the putative NRSE of the human *CX36* promoter as the labeled probe (Fig. 4). To determine the specificity of this binding activity, competition experiments were performed using a 100- or 30-fold molar excess of unlabeled NRSE. Two DNA binding complexes were observed; the fast migrating complex reflects nonspecific binding as this complex is only slightly blocked in the presence of REST antibodies, whereas the upper complex disappeared. This DNA-binding complex was not detected using nuclear extracts obtained from βTC3 (not shown). These data indicate that NRSF/REST is able to bind to the human Cx36 NRSE.

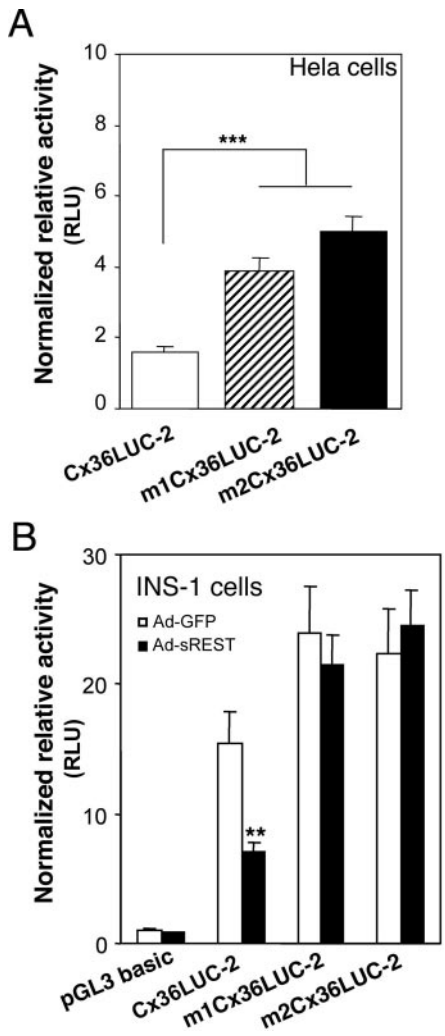

FIG. 8. **Mutations of the human Cx36 NRSE motif relieved transcriptional activity in HeLa cells while REST represses *CX36* promoter activity in INS-1 cells.** *A*, Cx36LUC-2 containing a region of the *CX36* promoter comprising bp −308 to +313 and mutated constructs (m1Cx36LUC-2 and m2Cx36LUC-2) were transiently transfected into REST-expressing HeLa cells. The luciferase activity was relieved with the mutants constructs. *Columns* represent mean ± S.E. of three independent experiments performed in triplicate; ***, $p < 0.001$. *RLU*, relative light units. *B*, the *CX36* promoter containing wild-type or mutated NRSE, Cx36LUC-2 and m1Cx36LUC-2 and m2Cx36LUC-2, respectively, were transfected in the INS-1 cells infected or not with Ad-sREST. The luciferase activity of Cx36LUC was decreased by 50% in REST-expressing cells, whereas REST expression has no effect on the Cx36LUC-2 mutants activities. *Columns* represent mean ± S.E. of three independent experiments performed in triplicate; **, $p < 0.01$.

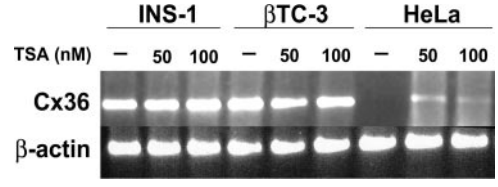

FIG. 9. **Endogenous *CX36* gene expression is relieved in TSA-treated cells.** Total RNAs from HeLa INS-1 and βTC3 cells treated in the presence of dimethyl sulfoxide (−) 50 or 100 mM of histone deacetylase inhibitor TSA (+) were analyzed for *cx36* gene expression by RT-PCR using specific primers. RNAs from INS-1 and βTC3 cells were used as a positive control for *cx36* gene expression. In NRSF/REST-expressing cells, RT-PCR showed one PCR fragment, which is only present in the TSA-treated cells.

Ectopic Expression of REST Decreased Cx36 Expression in Pancreatic β-Cell Lines—NRSF was detected by immunofluorescence in wild-type HeLa cells using specific NRSF/REST

antibodies but not in INS-1 cells (Fig. 5, *A* and *B*). In contrast, the protein was detected in the nucleus of INS-1 or βTC3 cells infected with Ad-sREST (Fig. 5, *C* and *D*). Western blots of nuclear extracts, using the same REST/NRSF antibodies, confirmed the absence of REST/NRSF in INS-1 cells and revealed a band at 200 kDa in HeLa cells or INS-1 cells infected with three different doses (m.o.i. = 5, 10, 15, 20) of Ad-sREST (Fig. 5*E*). The levels of this immunodetectable band increased with the adenoviral dosage.

Northern blot analysis of total RNA extracted from INS-1 cells infected with Ad-sREST showed a progressive decrease in the levels of Cx36 mRNA after 48 h of infection with six different doses of Ad-sREST (m.o.i. = 1, 2, 5, 10, 15, and 20) (Fig. 6*A*). Maximum reduction of Cx36 transcript expression occurred with an m.o.i. of 20. Western blot further demonstrated that the expression of Cx36, detected as a single immunoreactive form of about 36 kDa, is decreased due to ectopic expression of REST in INS-1 cells (Fig. 5*B*). Quantitative assessments for Cx36 mRNA showed that levels of these Cx36 transcripts were markedly decreased (70%) (Fig. 6*C*). As positive control, we confirmed the reduction of IB1/JIP-1 (31) by the heterologous expression of REST (Fig. 6*C*). Quantification of multiple blots showed that Cx36 mRNA levels were also markedly decreased (50–80%) in different β-cell lines such as βTC3 and Min-6 cells (Fig. 7, *A* and *B*). These results demonstrate the functional role of REST in the control of the cell-specific expression of *IB1/JIP-1* and *cx36* genes.

Transcriptional Activation of the CX36 Promoter Mutated in the NRSE in INS-1 and HeLa Cells—By using site-directed mutagenesis, the NRSE of the human *CX36* promoter was modified to the mutated NRSEs (substitution of the two, respectively, four nucleotides) described in Fig. 2. In transient transfection (Fig. 8*A*) the reporter gene activity mediated by the mutated NRSEs in the *CX36* promoter (m1Cx36LUC-2 and m2Cx36LUC-2) was relieved compared with Cx36LUC-2 activity in HeLa cells. This implies that the NRSE contributes to the repression of *CX36* promoter activity in non-β pancreatic HeLa cells. To assess whether NRSF/REST could repress human *CX36* promoter activity, the insulin-secreting INS-1 cells were infected with Ad-sREST and then transfected with the Cx36LUC-2 or mutated NRSEs constructs m1Cx36LUC-2 and m2Cx36LUC-2 (43). REST significantly decreased the Cx36LUC relative activity by 50%, whereas it has no effect on the mutated constructs. These data demonstrated that forced expression of REST in INS-1 was sufficient to repress, through the NRSE, the human *CX36* promoter activity in the non-REST-expressing INS-1 cells (Fig. 8*B*).

Induction of Endogenous CX36 Transcription in TSA-treated Cells—We assessed whether repression of the transcription of the endogenous *cx36* gene could be relieved in REST-expressing cells after trichostatin A (TSA) treatment (Fig. 9). Total RNA from HeLa, INS-1, and βTC3 cells treated with the vehicle dimethyl sulfoxide or with TSA were analyzed by RT-PCR for expression of the Cx36 transcript using a Cx36-specific primers set. The reaction yielded a PCR fragment detectable in insulin-secreting cells and only in NRSF/REST expressing cells treated with TSA. This observation indicates that deacetylase activity is required for the repression of endogenous *cx36* gene transcription.

DISCUSSION

The neuron-restrictive silencer factor (NRSF/REST) functions as a transcriptional repressor of neuronal genes in non-neuronal cells (21, 22). Target genes of NRSF/REST are the genes encoding synaptophysin, synapsin I, brain-derived neurotrophic factor, choline acetyltransferase, the type II sodium channel, SCG10, the m4 muscarinic acetylcholine receptor, the *N*-methyl-D-aspartate receptor subunit NR2C, the adhesion protein L1, NgCAM, and others (19, 23, 44–46), as a Blast search of the CELERA mouse data base identified more than 300 potential NRSF/REST-regulated genes (44).

The present study provides new insight into the transcriptional mechanisms that control the cell-specific expression of the *cx36* gene (9, 10, 12). Each of the gap junction proteins is characterized by a cell type-specific but overlapping expression pattern; cell-specific expression of connexin genes can take place at the transcriptional levels (47). The human Cx43 promoter contains regulatory sequences including the putative responsive element as SP1, E box, estrogen responsive element half-sites, AP-2 cAMP-responsive element-binding protein, and Ets-1 sites, which can serve as transcription factor targets (48, 49). In rat Cx43, a putative regulatory element-binding thyroid hormone receptor has been described as well as elements responsive to parathyroid hormone (50). Recently, it has been shown that the transcriptional up-regulation of Cx43 by Ras-Raf-MAPK is mediated by the interaction of a novel Cx43 promoter element with a protein complex that contains both HSP90 and c-Myc (51). Overexpression of Nkx2.5, a member of the NK2 homeodomain proteins was associated with a decrease in Cx43 expression, suggesting that Nkx2.5 may function as a transcriptional repressor (52). SP1 has also been found involved in the transcriptional regulation of Cx26, in rat mammary gland and uterus, as well as in the activation of Cx40 and Cx32 proximal promoter (53–55). Moreover, it has been demonstrate that Sox10, a transcription modulator, activates Cx32 expression (56).

We have identified a fragment of the human *CX36* promoter and characterized its initiation start site in insulin-secreting cells. This fragment drives high transcriptional activity in pancreatic β-cell lines but not in unrelated cell lines. We next identified an NRSE highly conserved between rodent and human. This cis element binds the transcription factor NRSF/REST prepared from HeLa cells. REST was shown to silence *CX36* promoter activity in non-β-cell lines. NRSF/REST contains two transcriptional repression domains on its NH_2 and COOH termini that recruit histone deacetylases (29, 57–59). Likewise, repression mediated by NRSF was shown to be sensitive to inhibitors of histone deacetylases such as TSA. Moreover, forced expression of REST in INS-1, βTC3, and Min-6 was associated with a marked decrease in Cx36 mRNA expression as well as in the mRNA expression of the c-Jun NH_2-terminal interacting protein-1 (IB1/JIP-1), known to be regulated by NRSF (31).

In pancreatic β-cells, the scaffold protein termed JNK-interacting protein 1 (JIP-1) or islet-brain 1 (IB1) (41, 60) is required for proper JNK activation and may play a critical role in the pro-apoptotic program observed during diabetes (38, 60, 61). We demonstrated recently (62) that IB1-JIP-1 content in insulin-producing β-cells is critical for β-cell function (38) and that an IB1/JIP-1 promoter variant was associated with Alzheimer's disease. We have shown recently that the absence of REST allows the cell-specific expression of the human *IB1* gene (31). This study further demonstrated that endogenous IB1 mRNA expression may be efficiently inhibited in β-cells expressing REST, validating the use of REST gene transfer approach to inhibit genes regulated by NRSF/REST and further indicating the importance of REST to repress *IB1* gene expression in non-β or non-neuronal cells. β-Cells and neuronal cells share similarities as both cell types are electrically excitable and may respond to hormonal stimuli and glucose by depolarization and exocytosis in a process similar to neurotransmitter release from synaptic vesicles (18, 63). Among genes that are linked to a direct pancreatic β-cell function, Cx36 (13–17, 38, 41, 60, 61)

and IB1/JIP-1 (38, 61) are the first found to be regulated by NRSF/REST (31).

It has been shown that NRSF/REST represses a subset of neuronal genes such as SCG10, synapsin I, the brain type II voltage-dependent sodium channel, the dopamine β-hydroxylase, synaptophysin, and the *IB1/JIP-1* genes (18, 31, 44, 64) which are also detected in pancreatic β-cells. Here we confirm that NRSF/REST is a potent repressor that contributes to the preferential expression of Cx36. The endogenous Cx36 transcriptional activity was relieved in trichostatin-treated cells, and the mechanism would be that NRSF/REST silences Cx36 transcription through histone acetylation, indicating that the expression of *cx36* gene is controlled by alterations of the chromatin structure. REST-induced hypoacetylation around the NRSE may change the nucleosomal structure and therefore could have a direct effect on TFIID-RNA polymerase II holocomplex access to the *CX36* promoter (29, 65). Indeed, the NRSE at the *CX36* promoter is located 41 bp downstream from the transcription start site, and so local changes to nucleosomal structure may affect the basal transcription complex.

In conclusion, the data provide evidence that NRSF/REST is a critical transcriptional factor that contributes to the cell-specific expression of the human and rodent *cx36* genes and demonstrate that Cx36 participates to the neuronal phenotype of the pancreatic β-cells.

REFERENCES

1. Bruzzone, R., White, T. W., and Goodenough, D. A. (1996) *BioEssays* **18,** 709–718
2. White, T. W., Bruzzone, R., and Paul, D. L. (1995) *Kidney Int.* **48,** 1148–1157
3. Bruzzone, R., White, T. W., and Paul, D. L. (1996) *Eur. J. Biochem.* **238,** 1–27
4. Spray, D. C. (1998) *Circ. Res.* **83,** 679–681
5. Richard, G. (2000) *Exp. Dermatol.* **9,** 77–96
6. Willecke, K., Eiberger, J., Degen, J., Eckardt, D., Romualdi, A., Guldenagel, M., Deutsch, U., and Sohl, G. (2002) *Biol. Chem.* **383,** 725–737
7. Condorelli, D. F., Parenti, R., Spinella, F., Trovato Salinaro, A., Belluardo, N., Cardile, V., and Cicirata, F. (1998) *Eur. J. Neurosci.* **10,** 1202–1208
8. Condorelli, D. F., Belluardo, N., Trovato-Salinaro, A., and Mudo, G. (2000) *Brain Res. Brain Res. Rev.* **32,** 72–85
9. Belluardo, N., Mudo, G., Trovato-Salinaro, A., Le Gurun, S., Charollais, A., Serre-Beinier, V., Amato, G., Haefliger, J. A., Meda, P., and Condorelli, D. F. (2000) *Brain Res.* **865,** 121–138
10. Rash, J. E., Yasumura, T., Dudek, F. E., and Nagy, J. I. (2001) *J. Neurosci.* **21,** 1983–2000
11. Rash, J. E., Staines, W. A., Yasumura, T., Patel, D., Furman, C. S., Stelmack, G. L., and Nagy, J. I. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7573–7578
12. Rash, J. E., Yasumura, T., Davidson, K. G., Furman, C. S., Dudek, F. E., and Nagy, J. I. (2001) *Cell Adhes. Commun.* **8,** 315–320
13. Serre-Beinier, V., Le Gurun, S., Belluardo, N., Trovato-Salinaro, A., Charollais, A., Haefliger, J. A., Condorelli, D. F., and Meda, P. (2000) *Diabetes* **49,** 727–734
14. Calabrese, A., Guldenagel, M., Charollais, A., Mas, C., Caton, D., Bauquis, J., Serre-Beinier, V., Caille, D., Sohl, G., Teubner, B., Le Gurun, S., Trovato-Salinaro, A., Condorelli, D. F., Haefliger, J. A., Willecke, K., and Meda, P. (2001) *Cell Adhes. Commun.* **8,** 387–391
15. Le Gurun, S., Martin, D., Formenton, A., Maechler, P., Caille, D., Waeber, G., Meda, P., and Haefliger, J. A. (2003) *J. Biol. Chem.* **278,** 37690–37697
16. Caton, D., Calabrese, A., Mas, C., Serre-Beinier, V., Charollais, A., Caille, D., Zufferey, R., Trono, D., and Meda, P. (2003) *J. Cell Sci.* **116,** 2285–2294
17. Calabrese, A., Serre-Beinier, V., Zhang, M., Caton, D., Mas, C., Satin, L. S., and Meda, P. (2003) *Diabetes* **52,** 417–424
18. Atouf, F., Czernichow, P., and Scharfmann, R. (1997) *J. Biol. Chem.* **272,** 1929–1934
19. Kraner, S. D., Chong, J. A., Tsay, H. J., and Mandel, G. (1992) *Neuron* **9,** 37–44
20. Mori, N., Schoenherr, C., Vandenbergh, D. J., and Anderson, D. J. (1992) *Neuron* **9,** 45–54
21. Chong, J. A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., Altshuller, Y. M., Frohman, M. A., Kraner, S. D., and Mandel, G. (1995) *Cell* **80,** 949–957
22. Schoenherr, C. J., and Anderson, D. J. (1995) *Science* **267,** 1360–1363
23. Schoenherr, C. J., Paquette, A. J., and Anderson, D. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9881–9886
24. Myers, S. J., Peters, J., Huang, Y., Comer, M. B., Barthel, F., and Dingledine, R. (1998) *J. Neurosci.* **18,** 6723–6739
25. Chen, Z. F., Paquette, A. J., and Anderson, D. J. (1998) *Nat. Genet.* **20,** 136–142
26. Timmusk, T., Palm, K., Lendahl, U., and Metsis, M. (1999) *J. Biol. Chem.* **274,** 1078–1084
27. Kallunki, P., Edelman, G. M., and Jones, F. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3233–3238
28. Rooprа, A., Sharling, L., Wood, I. C., Briggs, T., Bachfischer, U., Paquette, A. J., and Buckley, N. J. (2000) *Mol. Cell. Biol.* **20,** 2147–2157
29. Huang, Y., Myers, S. J., and Dingledine, R. (1999) *Nat. Neurosci.* **2,** 867–872
30. Grimes, J. A., Nielsen, S. J., Battaglioli, E., Miska, E. A., Speh, J. C., Berry, D. L., Atouf, F., Holdener, B. C., Mandel, G., and Kouzarides, T. (2000) *J. Biol. Chem.* **275,** 9461–9467
31. Abderrahmani, A., Steinmann, M., Plaisance, V., Niederhauser, G., Haefliger, J. A., Mooser, V., Bonny, C., Nicod, P., and Waeber, G. (2001) *Mol. Cell. Biol.* **21,** 7256–7267
32. Tapia-Ramirez, J., Eggen, B. J., Peral-Rubio, M. J., Toledo-Aral, J. J., and Mandel, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1177–1182
33. Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A., and Wollheim, C. B. (1992) *Endocrinology* **130,** 167–178
34. Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. (1990) *Endocrinology* **127,** 126–132
35. Belluardo, N., Trovato-Salinaro, A., Mudo, G., Hurd, Y. L., and Condorelli, D. F. (1999) *J. Neurosci. Res.* **57,** 740–752
36. Vozzi, C., Ullrich, S., Charollais, A., Philippe, J., Orci, L., and Meda, P. (1995) *J. Cell Biol.* **131,** 1561–1572
37. Tawadros, T., Formenton, A., Dudler, J., Thompson, N., Nicod, P., Leisinger, H. J., Waeber, G., and Haefliger, J. A. (2002) *J. Cell Sci.* **115,** 385–393
38. Haefliger, J. A., Tawadros, T., Meylan, L., Gurun, S. L., Roehrich, M. E., Martin, D., Thorens, B., and Waeber, G. (2003) *J. Cell Sci.* **116,** 1463–1469
39. Schaack, J., Langer, S., and Guo, X. (1995) *J. Virol.* **69,** 3920–3923
40. Haefliger, J. A., Demotz, S., Braissant, O., Suter, E., Waeber, B., Nicod, P., and Meda, P. (2001) *Kidney Int.* **60,** 190–201
41. Bonny, C., Nicod, P., and Waeber, G. (1998) *J. Biol. Chem.* **273,** 1843–1846
42. Haefliger, J. A., Tissieres, P., Tawadros, T., Formenton, A., Beny, J. L., Nicod, P., Frey, P., and Meda, P. (2002) *Exp. Cell Res.* **274,** 216–225
43. Kuhnel, F., Zender, L., Paul, Y., Tietze, M. K., Trautwein, C., Manns, M., and Kubicka, S. (2000) *J. Biol. Chem.* **275,** 6421–6427
44. Lietz, M., Hohl, M., and Thiel, G. (2003) *Eur. J. Biochem.* **270,** 2–9
45. Chin, L. S., Li, L., and Greengard, P. (1994) *J. Biol. Chem.* **269,** 18507–18513
46. Nishimura, E., Sasaki, K., Maruyama, K., Tsukada, T., and Yamaguchi, K. (1996) *Neurosci. Lett.* **211,** 101–104
47. Duval, N., Gomes, D., Calaora, V., Calabrese, A., Meda, P., and Bruzzone, R. (2002) *J. Cell Sci.* **115,** 3241–3251
48. Geimonen, E., Jiang, W., Ali, M., Fishman, G. I., Garfield, R. E., and Andersen, J. (1996) *J. Biol. Chem.* **271,** 23667–23674
49. Fernandez-Cobo, M., Stewart, D., Drujan, D., and De Maio, A. (2001) *J. Cell. Biochem.* **81,** 514–522
50. Stock, A., and Sies, H. (2000) *Biol. Chem.* **381,** 973–979
51. Carystinos, G. D., Kandouz, M., Alaoui-Jamali, M. A., and Batist, G. (2003) *Mol. Pharmacol.* **63,** 821–831
52. Kasahara, H., Wakimoto, H., Liu, M., Maguire, C. T., Converso, K. L., Shioi, T., Huang, W. Y., Manning, W. J., Paul, D., Lawitts, J., Berul, C. I., and Izumo, S. (2001) *J. Clin. Investig.* **108,** 189–201
53. Morsi, A. S., Godfrey, R. E., Chipman, J. K., and Minchin, S. D. (2003) *Toxicol. In Vitro* **17,** 191–199
54. Tu, Z. J., Pan, W., Gong, Z., and Kiang, D. T. (2001) *Mol. Reprod. Dev.* **59,** 17–24
55. Teunissen, B. E., van Amersfoorth, S. C., Opthof, T., Jongsma, H. J., and Bierhuizen, M. F. (2002) *Biochem. Biophys. Res. Commun.* **292,** 71–78
56. Bondurand, N., Girard, M., Pingault, V., Lemort, N., Dubourg, O., and Goossens, M. (2001) *Hum. Mol. Genet.* **10,** 2783–2795
57. Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., Burger, C., Moniwa, M., Davie, J. R., Bowers, W. J., Federoff, H. J., Rose, D. W., Rosenfeld, M. G., Brehm, P., and Mandel, G. (2001) *Neuron* **31,** 353–365
58. Naruse, Y., Aoki, T., Kojima, T., and Mori, N. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13691–13696
59. Thiel, G., Lietz, M., and Cramer, M. (1998) *J. Biol. Chem.* **273,** 26891–26899
60. Bonny, C., Oberson, A., Steinmann, M., Schorderet, D. F., Nicod, P., and Waeber, G. (2000) *J. Biol. Chem.* **275,** 16466–16472
61. Waeber, G., Delplanque, J., Bonny, C., Mooser, V., Steinmann, M., Widmann, C., Maillard, A., Miklossy, J., Dina, C., Hani, E. H., Vionnet, N., Nicod, P., Boutin, P., and Froguel, P. (2000) *Nat. Genet.* **24,** 291–295
62. Helbecque, N., Abderrhamani, A., Meylan, L., Riederer, B., Mooser, V., Miklossy, J., Delplanque, J., Boutin, P., Nicod, P., Haefliger, J. A., Cottel, D., Amouyel, P., Froguel, P., and Waeber, G. (2003) *Mol. Psychiatry* **8,** 413–422
63. De Camilli, P., and Jahn, R. (1990) *Annu. Rev. Physiol.* **52,** 625–645
64. Thomas-Reetz, A., Hell, J. W., During, M. J., Walch-Solimena, C., Jahn, R., and De Camilli, P. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5317–5321
65. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990) *J. Biol. Chem.* **265,** 17174–17179

Critical Role of the Transcriptional Repressor Neuron-restrictive Silencer Factor in the Specific Control of Connexin36 in Insulin-producing Cell Lines

David Martin, Thomas Tawadros, Laure Meylan, Amar Abderrahmani, Daniele F. Condorelli, Gérard Waeber and Jacques-Antoine Haefliger

J. Biol. Chem. 2003, 278:53082-53089.
doi: 10.1074/jbc.M306861200 originally published online October 16, 2003
