2 Materials and Methods

2.1. Cell Culture.

All plastic materials used for cell culture were obtained from Sarstedt (Nümbrecht), cell culture media and sera, non-essential amino acids, HBSS, and trypsin/EDTA solution were purchased from Gibco/BRL (Eggenstein). Penicillin/streptomycin was from Boehringer Mannheim, and amphotericin B from Squibb-Myers Bristol. Cells were generally cultured at 37° C in a humid atmosphere under 5% CO_2.

At subconfluence cells were passaged by washing once with Mg^{2+}/Ca^{2+}-free HBSS, with subsequent incubation in trypsin (0.25% w/v)/EDTA solution for a time varying from 5 to 20 minutes, depending on cell type. Trypsin was then inactivated by addition of an equal volume of appropriate cell culture medium, cells were collected by centrifugation at 200 g for 5 minutes, and reseeded at a splitting ratio of 1:10. Frozen stocks were prepared in respective cell culture medium containing 10% v/v DMSO, and stored at –80° C for short periods (month), or in liquid nitrogen for longer storage times.

For cell culture experiments, cells were grown to confluence (or highest achievable density, where this was not possible), and kept in serum-reduced, hormone-free medium (DMEM supplemented with 0.5% v/v FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1% v/v non-essential amino acid mixture) for two days, with one exchange of medium, in order to reduce basal activation levels.

2.1.1. Cell lines.

The Fisher rat thyroid adenoma cell line FRTL-5 was originally established by Ambesi-Impiombato *et al.* (1980), and obtained from the American Type Culture Collection (ATCC; Rockville, USA). They were grown in Coom´s modified Ham´s F12-medium containing stable glutamine, 5% v/v newborn calf serum, 1% v/v H5-hormone cocktail, 10 mU/mL TSH (from bovine pituitary, Sigma, Taufkirchen), 100 U/mL penicillin, 100 µg/mL streptomycin, and 125 µg/mL amphotericin B. One week before experiments FRTL-5 cells were transferred to TSH-free medium.

FTC 133 cells were a gift from Prof. P. Goretzki (Düsseldorf) and stem from a patient with a follicular carcinoma. From theses the more homogenous subclone HTC was derived. Both cell

lines were grown in Coom´s modified Ham´s F12-medium with stable glutamine, supplemented with 10% v/v fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 125 µg/mL amphotericin B.

The XTC.UC1 cell line was derived from a breast metastasis of a Hürthle cell carcinoma, a variant of the follicular carcinoma. In contrast to follicular thyroid carcinomata, Hürthle cell carcinomata rarely take up radioiodine and frequently metastasize to the lymph nodes. XTC cells are highly differentiated, synthesizing and secreting thyroglobulin, and responding to TSH with an increase in cAMP, proliferation and invasion of reconstituted basement membrane (Zielke et al., 1998). They were grown in DMEM supplemented with 10 % v/v FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 125 µg/mL amphotericin B.

Two anaplastic carcinoma cell lines, HTh 74 and SW1736, were included in this study. Both were provided by Prof. N.-E. Heldin, Uppsala, Sweden. HTh 74 cells were shown to express some thyroid-specific genes like functional TSH receptor protein (Heldin, Cvejic et al., 1991), and mRNA for thyroglobulin, albeit both at very low levels (Heldin and Westermark, 1991). In addition, HTh 74 cells express functional, exogenous PDGF-α and PDGF-β receptors, normally a characteristic of mesenchymal cells (Heldin, Cvejic et al., 1991). HTh 74 cells were grown in Coom´s modified Ham´s F12-medium with stable glutamine, supplemented with 10% v/v fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 125 µg/mL amphotericin B. In contrast, SW1736 cells, which originally were isolated by Dr. Leibowitz (Memorial Sloan-Kettering Cancer Center, New York, USA), are highly dedifferentiated. Their epithelial origin, however, was confirmed by demonstration of cytokeratin expression (Heldin and Westermark, 1991). These cells were grown in Dulbecco´s Modified Eagle Medium with stable glutamine, supplemented with 10% v/v fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 125 µg/mL amphotericin B.

2.1.2. Isolation of thyroid primary cells.

Human thyroid tissue gained at thyroidectomy was provided by the surgical team of Prof. Zumtobel at the St. Josef Hospital Bochum. Tissue was classified as “nodular” or “paranodular”, and corresponding to the appearance on scintigrams as “hot”, “warm” or “cold”. Immediately after surgery tissue was transferred into ice-cold HBSS containing penicillin/streptomycin. Employing sterile techniques, visible capsule material was removed, and

in order to increase surface area, the remaining tissue was cut with a scalpel into small pieces, leaving a connecting string of connective tissue intact. The tissue was subjected to enzymatic digestion was carried out in fresh HBSS with dispase II (30 mg/mL) and collagenase A (5 mg/mL) (Boehringer, Mannheim) at 37 °C with gentle shaking. At intervals of 90 to 120 minutes, depending on progress of the digestion, the isolated follicles were collected by passing the digest was given through a sieve, the undigested tissue fragments being left behind. The flow-through was centrifuged at 200 g for 5 minutes. The supernatant was then returned to the remaining tissue to continue digestion, while the pellet containing the follicles was resuspended in primary cell culture medium (Coom´s modified Ham´s F12-medium with stable glutamine supplemented with 1% H5, 0.5 mU/mL TSH, 10% v/v fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 125 µg/mL amphotericin B) by repeated up-and-down pipetting. This procedure was repeated until only white connective tissue remained. Cultures were passaged after seven days into TSH-free medium and transferred to serum-reduced medium the day before they were used in experiments.

H5- hormone cocktail:

10 ng/mL glycyl-histidyl-lysine (Sigma)
10 µg/mL insulin (human, recombinant, Lilly or Hoechst)
10 ng/mL somatostatin (Sigma)
5 µg/ mL apo-transferrin (Sigma)
3.2 ng/mL hydrocortisone (Sigma)

2.2. Western Blotting

2.2.1. Preparation of total protein lysate.

Cells were treated as indicated, washed twice with ice-cold PBS and harvested by scrapping them off cell culture dishes using a rubber policeman. Cells were pelleted by centrifugation at 500 g for 5 minutes in a pre-cooled centrifuge at 4° C, washed once in PBS and resuspended in RIPA buffer containing phosphatase and protease inhibitors (NaF 1 mM, Na-molybdate 0.1 mM, ortho-vanadate 0.5 mM, Na-pyrophosphate 1 mM, aprotinin 2 µg/mL, leupeptin 5 µg/mL,

pepstatin A 10 µg/mL, PMSF 0.2 mg/mL) by repeated up-and-down pipetting. Lysis was completed by vigorous shaking on ice for 20 minutes. Cell debris was removed by centrifugation at 10,000 g for 5 minutes at 4° C. From the supernatant, 2 µL samples were preserved for subsequent determination of the protein concentration, the rest was immediately frozen at –80 °C.

RIPA-Buffer:

50 mM Tris-HCl, pH 7.4
1% w/v Nonidet P40
0.25% w/v desoxycholate
150 mM NaCl
1 mM EGTA

2.2.2. Determination of protein concentration.

To determine the protein concentration of cell lysates, the so-called BCA-assay from Pierce (Perbio Science Deutschland GmbH, Bonn) was used. This assay employs a modification of the biuret method using bicinchonic acid as originally described by Smith *et al*. (1985). For each determination, a new standard curve was run using BSA as protein standard. The assay was carried out as recommended by the supplier, apart from that, after the 30 minutes incubation step at 37 °C, the samples were brought quickly to room temperature by incubation in an ice water bath for further ten minutes in order to slow down the reaction. As the BCA method is not an end-point determination, this was found to give better results when large numbers of samples were run simultaneously. Each sample was run in duplicate, and the volume difference to the standard was made up by adding an appropriate amount of distilled water, which was also used as blank, since the small amount of lysis buffer was not found to interfere with the reaction. Measurements were done at 562 nm.

2.2.3. SDS-PAGE

Samples were analysed by SDS-PAGE corresponding to the principle of isotachophoresis originally described by Laemmli. Protein samples were mixed with an appropriate amount of loading buffer, heated to 95 °C for 8 minutes, spun down and kept on ice until loading. Wherever possible, 50 µg of total protein were loaded per lane. In cases where the low protein yield was too low, the amount loaded was reduced to 30 µg protein per lane. 5% stacking and 10% separating gels were employed, and run for about 4 hours at 150 V. Progress of separation was followed by migration of a prestained molecular weight standard (Sigma).

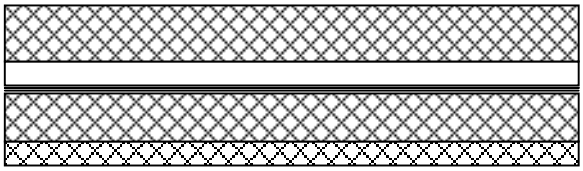
Loading Buffer (4x):	**Running Buffer:**
200 mM Tris-HCl pH 6.8	25 mM Tris-base
400 mM DTT	192 mM glycine
0.8% w/v SDS	0.1% w/v SDS
0.4% w/v bromophenol blue	
40% v/v glycerol	

	Stacking Gel (5%):	**Separating Gel (10%):**
H_2O	5 mL	11 mL
1 M Tris-HCl, pH 8.8	-	15 mL
1M Tris-HCl, pH 6.8	2.5 mL	-
Acrylamide solution 30%	2 mL	12.5 mL
SDS 10% w/v stock	200 µL	800 µL
TEMED	20 µL	26.8 µL
APS 10% w/v stock	100 µL	267.2 µL

2.2.4. Semi-dry blotting.

After gel electrophoresis, the front glass plate was removed, and the gel cut to size using a scalpel. Gel and nitrocellulose membrane (ECL-Hybond, Amersham, Freiburg) were equilibrated in cathode buffer and deionized water, respectively, for 10 minutes. Filter papers (3MM, Whatman, Brentford, UK) were soaked in anode or cathode buffer, and the blot build up as shown. Bubbles were removed by rolling with a pipette over the sandwich. Transfer was carried out at 3 mA per cm^2 of membrane for 30 to 40 minutes. Even transfer was controlled by staining the gel with Coomassie after end of electroblotting.

Cathode



6 x filterpaper in cathode buffer
gel
nitrocellulose membrane
4 x filterpaper in anode buffer II
2 x filterpaper in anode buffer I

Anode

Anode Buffer I:	**Anode Buffer II:**	**Cathode Buffer:**
300 mM Tris-base	25 mM Tris-base	100 mM L-arginine
20% v/v methanol	20% v/v methanol	0.01% w/v SDS
		20% v/v methanol

2.2.5. Immunodetection.

After protein transfer, nitrocellulose membranes were rinsed twice in deionized water and then detection of specific proteins was carried out according to the recommendations of the suppliers of the primary antibodies used:

CREB and phopsho-CREB-Blots:

Anti-CREB and anti-phospho-CREB antibodies were purchased from Upstate (Biomol GmbH, Hamburg). Blocking of membranes was achieved by incubation in 3% w/v skimmed milk powder (Glücksklee) in PBS and with agitation at room temperature. Anti-CREB and anti-phospho-CREB were diluted 1:1400 and 1:1700, respectively, in PBS with 3% w/v milk powder, and membranes incubated over night at 4 °C on a shaker. Antibody solutions were prepared freshly for each blot. On the second day, antibody solutions were decanted and membranes washed three times for 3 minutes in deionized water.

CREM-Blots:

Anti-CREM-1 antibody was from Santa Cruz Biotechnology (Heidelberg) and the blocking step was performed in 5% skimmed milk powder in PBS-Tween 20 (0.1% w/v) for 2 hours at room temperature. Primary antibody was diluted 1:100 in blocking buffer. The membrane was washed thrice in PBS-Tween to remove excess milk powder, and then incubated with primary antibody over night at 4 °C while undergoing gentle agitation. Antibody solution was used repeatedly until a clear loss in sensitivity was noticed.

After incubation with primary antibody CREM-blots were washed thrice for 5 minutes in TBS-Tween.

PKC-Blots:

Isozyme specific PKC antibodies were obtained from Gibco/BRL. Blocking was performed in NET-buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-base, 0.25 % w/v gelatine, 0.05 % v/v Triton X-100) for four times 10 minutes on a shaker at room temperature. Primary antibodies were diluted 1:1000 in NET-Buffer and blots incubated with antibody solutions at 4 °C over night on a shaker. Antibody solutions were found to be stable over several years. Washing was performed again in NET-buffer for four times 10 minutes.

Secondary anti-rabbit antibody conjugated to horse radish peroxidase was from Amersham and was used at a dilution of 1:2000. Antibody was diluted in the same buffers as the primary antibodies used, and washing steps were similarly repeated.

The enhanced chemiluminescence system (ECL, Amersham) was used for detection of bands recognized by antibodies. Membranes were rinsed briefly 4 to 5 times in deionized water to remove detergents, and then incubated with the ECL reagent for 1 minute. Excess detection

reagent was allowed to drip off, blots wrapped in SaranWrap™ and film exposed for times varying between a few seconds (anti-CREM-blots) to up to 45 minutes (anti-phospho-CREB and low protein load).

2.3. Polymerase Chain Reaction (PCR).

2.3.1. RNA isolation.

Total RNA from cell culture monolayers was purified according to the method described by Chomczynski and Sacchi. Cell cultures were washed twice with ice-cold PBS, and cells lysed by incubation in 5.4 mL lysis solution for five minutes on ice. The lysate was transferred to a 15 mL falcon tube, and 500 µL of 2 M sodium acetate pH 4.0, 5 mL phenol and 1 mL chloroform-isoamyl alcohol (49:1) were added, mixed and incubated for another 15 minutes on ice. The mixture was centrifuged in a pre-cooled centrifuge at 4000 g at 4 °C for 30 minutes. The upper aqueous phase was transferred to a fresh tube, and RNA precipitated by addition of two volumes of ice-cold ethanol and incubation at –20 °C for 1 hour. The solution was again centrifuged at 4000 g, 4°C for 30 minutes, the supernatant removed and the pellet dissolved in 1 mL of lysis solution. RNA was precipitated once more from this by addition of two volumes of ethanol and incubation at –20 °C. The centrifugation step was repeated, the pellet washed with 70% ethanol, and the pellet allowed to dry in the air and then suspended in DEPC-H_2O with 0.1% w/v SDS. To determine the RNA concentration, a dilution of 1:50 in DEPC-H_2O was measured at 260 and 280 nm with DEPC-H_2O serving as blank. Taking an OD_{260nm} of 1 as being equal to an RNA concentration of 40 mg/mL, the sample concentration was calculated. Samples were regarded as pure RNA when the OD_{260}/OD_{280} ratio lay close to 2.0. Measurements were done in duplicate. The purified RNA was stored at –80 °C.

RNA Lysis Solution:

4 M guanidinium-thiocyanate
25 mM sodium citrate, pH 7.0
0.55 % w/v N-lauryl sarcosine
11 mM ß-mercaptoethanol

2.3.2. Reverse Transcription.

To remove contaminating DNA, 1.6 µg of total RNA were treated with RNase-free DNase I (Boehringer, Mannheim) (10 U with 2 mM $MgCl_2$ in 10 µL end volume) for 1 hour at 37 °C. DNase I was subsequently inactivated by heating the sample to 90 °C. For reverse transcription 0.8 µg of the so-treated RNA was mixed with 100 pmol random hexamers (Boehringer, Mannheim) in a total volume of 10.5 µL, and the RNA denatured by heating the samples at 70 °C for 10 minutes. Samples were then put on ice immediately to prevent renaturation. Reverse transcription was carried out in an end volume of 20 µL containing 10 mM DTT, 4 mM of dNTP mixture (Pharmacia, Freiburg), 1 U/mL RNase-inhibitor (Pharmacia, Freiburg), 20 U/mL reverse transcriptase (Gibco BRL, Eggenstein), 50 mM Tris-HCl pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$. Binding of random hexamers occurred at 20 °C (10 minutes), the enzymatic reaction at 42 °C (1 hour), followed by an inactivation step (95 °C for 5 minutes). cDNA samples were stored at –20 °C.

2.3.3. ICER-PCR.

PCR was performed as first described by Saiki *et al.* in a Biometra Trio-Thermoblock. Species-specific primers recognizing all four previously described isoforms of ICER were chosen as follows: human CREM gene: forward 5`-TGGAACACTTTATGTTAACTGTGG-3` (-63 to –39) and reverse 5´-CAAACTTCCGGGCGATGCAGCCATC-3` (113 to 145); rat CREM gene: forward 5´-TGGAACACTTTATTTTGGACTGTGG-3` (-63 to –39) and reverse 5´-CAGGCTTCCTGGTGAGGCAGCCATC-3`(113 to 145). Oligonucleotides were synthesized by Pharmacia Biotech (Freiburg). 50 µL PCR volumes were used containing: 5 µL of cDNA, 20 mM Tris-HCl pH 8.4, 50mM KCl, 1 mM $MgCl_2$, 0.4 mM dNTP mixture, 25 pmol each of forward and reverse primer, and 1.5 U Taq polymerase (Gibco BRL, Eggenstein). Samples were overlaid with 50 µL mineral oil. Cycling conditions were as follows: denaturation: 95 °C for 1 minute; hybridisation: 52 °C for 1 minute; amplification: 72 °C for 2 minutes with 35 cycles, and extension: 72 °C for 10 minutes. PCR products were separated on 1% w/v agarose gels containing 0.5 µg/mL ethidium bromide (TBE running buffer, 100 V). Bands were visualized on a UV-transilluminator at 312 nm and documented with a digital system (Appligene oncor with “The Imager, version 2.04” software).

DNA-Loading Buffer (5x):

4 M urea
50% w/v sucrose
0.1 M EDTA, pH 8.0
0.1% w/v bromophenol blue

TBE Buffer (20x):

1.8 M Tris-base
1.8 M boric acid
25 mM EDTA
pH was adjusted to 8.3

2.4. Reporter Gene Assay.

2.4.1. Transformation of *E.coli* with plasmid DNA.

Transformation of *E. coli* cultures with reporter or ß-Gal control plasmid was carried out corresponding to the method of Mandel and Higa. Competent *E. coli* strain XL1-blue (Qiagen) were obtained by inoculating 10 mL of LB-medium with 1 mL of an overnight culture and growing the bacteria at 37 °C and 200 rpm until an OD_{550nm} of 0.3 was reached. Bacteria were then harvested by centrifugation at 2000 x g for 5 minutes. The pellet was washed by resuspension in 10 mL of 10 mM, ice-cold NaCl and the centrifugation step repeated. The bacterial pellet was then taken up in 10 mL of 100 mM, ice-cold $CaCl_2$ and incubated on ice for 20 minutes. Subsequently, bacteria were again pelleted by centrifugation and finally resuspended in 2 mL of ice-cold 100 mM $CaCl_2$. Competent *E. coli* were either used immediately for transformation or stored as glycerol stocks (50% v/v glycerol) at –80 °C. For transformation, 200 µL of competent bacteria was mixed with 100 ng plasmid DNA and the mixture incubated on ice for 45 minutes. Subsequently, cells were heat-shocked by incubation at 42 °C for 3 minutes. Then 2.5 mL of LB-medium was added and cells incubated at 37 °C on a bacterial shaker (200 rpm) for one hour to permit expression of ß-lactamase. Successfully transformed bacteria were selected by plating on ampicillin containing LB-agar plates (0.1 mg/mL ampicillin).

2.4.2 Isolation of plasmid DNA for transfection experiments.

Vector DNA for transfection was purified using the Qiagen Plasmid Mega kit with the protocol recommended by the supplier, which in principle constitutes a modification of the alkaline extraction methode described originally by Birnboim and Doly (1979). In brief, an overnight culture was used to inoculate 500 ml of LB medium containing ampicillin, and the culture grown at 37 °C for 12 hrs with vigorous shaking. Bacteria were harvested by centrifugation at 6000 g for 15 minutes at 4 °C. The pellet was resuspended in 50 mL of buffer P1 by repeated up- and downpipetting. To the lysate an equal volume of buffer P2 was added, and the sample mixed cautiously by inverting the tube a couple of times to avoid shear stress, and then incubated at room temperature for 5 minutes. Subsequently, 50 mL of ice-cold buffer P3 were added, and the sample mixed as before, then incubated on ice for another 30 minutes. The precipitated protein and genomic DNA were then removed by centrifugation at 20,000 x g at 4 °C. The supernatant containing supercoiled plasmid DNA was removed and re-centrifuged at 20,000 x g, 4 °C for 15 minutes. In the meantime a QIAGEN-tip 500 was equilibrated by application of 10 mL buffer QBT, and the column allowed to empty by gravity flow. Subsequently, the plasmid containing supernatant from the last centrifugation step was applied to the column. The column was washed twice with 30 mL of buffer QC, then DNA was eluted in 15 mL of buffer QF. DNA was precipitated by addition of 10.5 mL of isopropanol and subsequent centrifugation at 15,000 x g for 30 minutes at 4°C. The supernatant was decanted carefully and the pellet washed with 5 mL 70 % v/v ethanol, the centrifugation step repeated, the pellet dried in air, and finally resuspended in water. DNA concentration was determined photometrically with an optical density of 1.0 at 260 nm taken as 50 µg/mL of DNA. A ratio of OD_{260}/ OD_{280} nm of 1.8 was regarded as pure DNA solution.

2.4.3 Transient transfection of HTC-TSHr cells.

Electroporation was used to transiently transfect cells with the CRE-reporter plasmid 4xSCE1/2T81. 4xSCE1/2T81 was constructed from the general luciferase reporter plasmid pT81luc by insertion of a synthetic oligonucleotide comprising four copies of the somatostatin CRE consensus sequence into a *BamHI* site just upstream of the HSV tk promoter (Oetjen et al.,

1994). This construct was kindly provided by Prof. Knepel (Göttingen). As a standard for transfection efficiency, and to permit the exclusion of unspecific effects on transcription/translation, the control plasmid pSV ß-gal was co-transfected, and results standardized against ß-gal activity. Of five thyroid cell lines tested, only HTC-TSHr cells were found to give acceptable transfection levels in the absence of TSH. 5 x 10^6 cells from a subconfluent culture were mixed with 2 µg pSV ß-gal and 8 µg 4xSCE1/2T81 in an electroporation cuvette. The sample was exposed to a pulse of 100 µF and 260 V in an Electroporator II (Invitrogen). Cells were then seeded in full culture medium for 24 hours to allow recovery, before they were starved in serum-reduced (0.5% v/v FCS) medium for another 24 hours. On the third day cells were stimulated with the indicated agents for the times indicated.

.

2.4.4 Luminometric measurement of reporter gene activity.

At the end of the stimulation period, cultures were washed twice with ice-cold PBS, and then lysed on the plate in 200 µL lysis buffer. After an incubation period of 20 minutes, the lysate was scrapped off, transferred to microcentrifuge tubes, and cell debris removed by centrifugation (12,000 rpm, 2 minutes, 4 °C). The supernatant was conserved and stored at –70 °C until assayed. ß-galactosidase activity was determined using a commercial kit ("Galacto Light", Tropix, Weiterstadt) according to the instructions of the supplier. In brief: lysates were incubated at 48 °C for 15 minutes to inactivate endogenous ß-galactosidase. Per sample, 10 µL of lysate and 60 µL reaction mixture were used, the latter containing 1% v/v Galacton™ (chemoluminescent substrate) and 99% v/v Galacto-Light™ reaction buffer. Reaction samples were incubated at room temperature in the dark for one hour, and measured in a luminometer (AutoLumat LB 953, Berthold). Luciferase activity was determined with luciferin as substrate. 10 µL of sample was mixed with 45 µL of reaction buffer (111 mM K_2HPO_4, 5.6 mM ATP, 11.1 mM $MgCl_2$), and the reaction started by injection of 100 µL of luciferin solution (3.184 mg/mL luciferin, 10 mM KH_2PO_4, 90 mM K_2HPO_4). Measurements were again done in a luminometer (see above).