Repression of Transforming Growth Factor β1 Protein by Antisense Oligonucleotide-induced Increase of Adrenal Cell Differentiated Functions*

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Transforming growth factor β1 (TGFβ1) is a potent inhibitor of several differentiated functions in bovine adrenal fasciculata cells (BAC). In addition, these cells express and secrete this factor. To determine whether this peptide plays an autocrine role in BAC, cells were transfected with 10 μM unmodified sense (SON) or antisense (AON) oligonucleotide complementary to the translation initiation region of the TGFβ1 mRNA in an attempt to inhibit TGFβ1 protein synthesis. We investigated first, the cellular uptake, the stability, and the intracellular distribution of 32P-labeled TGFβ1 AON and SON; and second, the effects of both oligonucleotides on BAC specific functions. We have demonstrated that in BAC, the TGFβ1 AON uptake reached a plateau after 8 h of transfection (16% of the radioactivity added) and remained fairly constant for at least 24 h. In contrast, the uptake of TGFβ1 SON reached a plateau after 2 h of transfection (8% of the radioactivity added), remained stable for only 3 h, and then declined. After 8 h of transfection, followed by 44 h of culture without oligonucleotides, the intracellular level of TGFβ1 AON was still high with about 8% of the radioactivity added, whereas that of TGFβ1 SON represented only 1.2%. Moreover, AON was present in the cytoplasmic and nuclear fractions, and it was hybridized in both compartments. However, TGFβ1 SON was present mainly in the cytoplasmic fraction where it was not hybridized. Neither TGFβ1 AON nor SON modified TGFβ1 mRNA levels; however, TGFβ1 AON, but not SON, caused the disappearance of TGFβ1 immunoreactivity inside the cells. Finally, the steroidogenic responsiveness of BAC transfected with TGFβ1 AON increased about 2-fold, and this was associated with a 2-fold increase of the mRNA levels of both cytochrome P450 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase. Neither TGFβ1 SON nor a scrambled oligonucleotide containing the same number of G nucleotides as TGFβ1 AON had any effect on these parameters. Thus, these studies demonstrate that TGFβ1 has an autocrine inhibitory effect on BAC differentiated functions, an effect that can be overcome by TGFβ1 AON.

The transforming growth factor β (TGFβ) family of peptides

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1 The abbreviations used are: TGFβ, transforming growth factor β;
TGF\(\beta\) mRNA level, whereas AngI increases TGF\(\beta\) mRNA and protein levels. All these data suggest that TGF\(\beta\) local production could play an autocrine role on BAC differentiated functions.

Synthetic oligonucleotides represent a new tool to investigate the role of many proteins in cell growth and differentiation. Ideally, an antisense oligonucleotide is targeted in a sequence-specific manner to nucleic acids (RNA or DNA) to inhibit the expression of a specific protein involved in cellular signal transduction, growth, proliferation, or differentiation. Antisense oligonucleotide inhibition of cellular protein production has been used to study the actions of several growth factors including basic fibroblast growth factor (22, 23), insulin-like growth factor-I (24), insulin-like growth factor-II (25), platelet differentiating growth factor, and TGF\(\beta\) (23).

In the present study, using a TGF\(\beta\)1 antisense oligodeoxynucleotide complementary to a sequence that includes the translation start site of the human TGF\(\beta\)1 mRNA, we have inhibited TGF\(\beta\)1 synthesis in BAC and demonstrated an autocrine role for TGF\(\beta\)1 on BAC differentiated functions.  

**EXPERIMENTAL PROCEDURES**

**Materials—** \(\gamma\)-[\(^{32}\)P\]ATP (4000 Ci/mmol) and \(\alpha\)-[\(^{32}\)P\]CTP (3000 Ci/mmol), were purchased from ICN Biomedicals France (Orsay, France). Synthetic oligonucleotides were obtained from Eurogentec-France (Angers, France), and Protein A-Sepharose CL-4B from Sigma. Hu-Pontoise, France, and acroleine from Polysciences (Warrington, PA). Sandimmun (Sandimmun) from Sandoz (Rueil-Malmaison, France), synthetic unmodified 15-base deoxyribonucleotides from Eurogentec-France (Angers, France), Lipofectamine™ Reagent from Life Technologies, Inc. (Cergy Pontoise, France), and acroleine from Polysciences (Warrington, PA). Amplify and Hybond-N membrane were purchased from Amersham (Les Ulis, France), and Protein A-Sepharose CL-4B from Sigma. Human TGF\(\beta\)1 cDNA was donated by Dr. R. Derynck (Genentech Inc., San Francisco, CA). Bovine P450 17α-hydroxylase cDNA was donated by Dr. M. R. Waterman (Vanderbilt University School of Medicine, Nashville, TN). Human \(\beta\)2M cDNA was donated by Dr. F. Labrie and V. Luu (Centre Hospitalier Universitaire Laval, Québec, Canada). Polyol nuclease reaction (C-11-V) directed against a common C-terminal peptide of G\(_{\alpha}\)/G\(_{\beta/\gamma}\) proteins and polyol anti-TGF\(\beta\)1 rabbit antibody were prepared in our laboratory as described previously (29, 30). Goat anti-rabbit immunoglobulin G (IgG) conjugated to peroxidase was purchased from Nordic Immunology (Tilburg, The Netherland). Isolation and Culture of Bovine Adrenocortical Cells—BAC were prepared by sequential treatment of adrenals cortical slices with trypsin (0.16%) as described previously (31). Then, cells were purified on a discontinuous Percoll density gradient (d = 1.032, 1.048, and 1.082 g/ml) to eliminate cellular fragments and red blood cells. The purified fasciculata cells recovered on the Percoll gradient with a density of 1.048 g/ml were collected, washed and cultured in a chemically defined medium, Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1), containing 10 μg/ml transferrin, 10 μg/ml insulin, 10^{-4} M vitamin C, and antibiotics without serum. Oligonucleotides—Antisense, sense, and scrambled unmodified 15-base deoxyribonucleotides corresponding to the translation initiation region of human TGF\(\beta\)1 mRNA were used: antisense (AON) (5′-GGAGGGGCGCATGGG-3′); sense (SON) (5′-CCCATGCGCCCTTCC-3′); scrambled (SCR) (5′-AGTGAGCAGGCGG-3′). Cell Transfection and Viability Test—On day 2 of culture, cells were transfected with labeled and/or unlabeled TGF\(\beta\)1 AON or SON. To introduce the oligonucleotides into BAC, a cationic liposome-mediated transfection method was used. Oligonucleotides dissolved in one volume of antibiotic-free medium were mixed with Lipofectamine™ reagent dissolved in the same volume of antibiotic-free medium and incubated for 45 min at room temperature. Thereafter, the oligonucleotide-liposome complexes were diluted with eight volumes of antibiotic-free medium and then added to cells that had been washed twice with antibiotic-free medium. In the experiments reported, the concentration of oligonucleotides and Lipofectamine™ in the transfection medium was 10 μM (50 μg/ml) and 1.25%, respectively. For the viability test (trypan blue exclusion assay), the number of living cells was assessed at the end of the experimental period (8 h of transfection followed by 44 h of culture). Oligonucleotide Cellular Uptake and Degradation—Oligonucleotides were labeled with \(\gamma\)-[\(^{32}\)P\]ATP by use of bacteriophage T4 polynucleotide kinase and further purified by dialysis (specific activity 8 × 10^8 dpm/μg). The transfection medium containing 1 × 10^6 dpm/μg oligonucleotides and 10 μM bovine P450 17α-hydroxylase cDNA was used. The cells. At indicated times, the culture medium was removed and cells were washed three times with medium, and the cell washes were also removed and saved. Cells were lysed in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 1% sodium dodecyl sulfate) and extracted with phenol-chloroform-amy1 alcohol (25:25:1). After centrifugation (1000 × g, 15 min, 4°C), the aqueous phase was removed and saved. Then the phenol phase was extracted with water and centrifuged, and the aqueous phase was removed and pooled with the first one. Aliquots of the three fractions, i.e. combined aqueous phases corresponding to the intracellular radioactivity, cell washes, and culture medium, were counted. Oligonucleotide uptake was calculated as the percentage of the intracellular radioactivity over the counts recovered in the three fractions. To determine oligonucleotide degradation, aliquots containing equal amounts of radioactivity of the combined aqueous phases and of the culture medium fraction were analyzed by electrophoresis (10% polyacrylamide, 7 μm urea gel) and autoradiography.

Oligonucleotide Distribution and Hybridization—After transfection with \(\gamma\)-[\(^{32}\)P\]labeled oligonucleotide, a subcellular fractionation of the cells was carried out. Briefly, after washes, cells were removed from the culture plate with trypsin (100 × g, 10 min, 4°C) and washed twice with medium. The cells were lysed for 10 min at 4°C in buffer A (10 mM Tris HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl\(_2\), 0.5% Nonidet P-40, 1 mM dithiothreitol). After centrifugation (800 × g for 10 min, the upper cytoplasmatic fraction (combined cytosol and cell membranes) was removed and saved. The pellet (nuclei) was dissolved in a small quantity of buffer A and then laid over 1 ml of 25 mM Tris-HCl, pH 7.4, 2.5 mM MgCl\(_2\), 0.25 μm sucrose and centrifuged (800 × g for 10 min). The purified nuclei pellet was dissolved in buffer A. Aliquots of the cytoplasmic and nuclear fractions were counted. In order to determine whether the oligonucleotide formed duplexes with cellular RNA, an S1 nuclease protection assay was performed on aliquots of cell lysates, cytoplasmic, and nuclear fractions. As a control the medium was also treated with the enzyme. The nucleic acids of each fraction were precipitated at −20°C for 20 min and −70°C for 10 min with cold ethanol (2.2 volumes), 3 mM sodium acetate (0.1 volume), pH 5.2, and 10 μg of tRNA. After centrifugation (12,000 × g for 30 min at 4°C), the pellet was dissolved with cold ethanol (50% v/v) and lyophilized for 30 min, and then resuspended in 70 μl of hybridization buffer (400 mM PIPES, 400 mM NaCl, 1 mM EDTA). Then 30 μl of each aliquot was incubated without (control) or with 20 units of S1 nuclease. Then the samples were acetylated for 30 min and then resuspended in 0.2 μl of hybridization buffer (1.5 μM sodium acetate, 125 mM EDTA, 75 mM MgCl\(_2\)). Samples were precipitated at −20°C for 20 min and −70°C for 10 min with 20 μg of tRNA and 0.75 μl of ethanol, centrifuged, and washed as described above. Samples were analyzed by electrophoresis using 10% polyacrylamide, 7 μm urea gel. Protected fragments were visualized by autoradiography.

RNA Preparation and Northern Blot Analysis—Total RNA was isolated from cells by the method of Chomczynski and Sacchi (32). Samples (10–15 μg of RNA) were separated by electrophoresis through a 1% agarose gel containing 10% formaldehyde. RNA was then transferred to Hybond-N membrane. Prehybridization and hybridization solutions were as described previously (33). Labeled probes, DNA, bovine P450 17α-hydroxylase cDNA, and human β2M cDNA were used as probes (1 × 10^6 dpm/μl). Labeling of these probes in the presence of [\(^{32}\)P]CTP was performed with a Megaprime DNA labeling system (Amersham). The blots were washed with more or less stringency depending on the probes used and then exposed to photographic film. The relative intensity of hybridization signals was evaluated using a scanning densitometer (Precision Sebia, Paris, France). Equal loading of RNA samples was confirmed by scanning the 28 S RNA negatives.

Immunocytochemistry—BAC cells were plated at a density of 6.0 × 10^4 cells/chamber in eight-chamber tissue culture slides (Plastic Labtek) and transfected with antisense or sense oligonucleotide as described above. After the transfection medium was removed, cells were washed two times with fresh medium, cultured for 44 h and subjected to immunocytochemical analysis. For comparative studies, control cells were always run in the same immunocytochemical assay to reduce

2 C. Le Roy, P. Leduque, P. M. Dubois, J. M. Saez, and D. Langlois, unpublished results.
Experimental Procedures.

Fig. 1 shows the time-dependent 0.4% in control cells (n cells and in the medium during the cellular uptake, TGF stable for only 3 h, and then declined. At the plateau, 8% of the account the cellular volume and the specific activity, TGF plateau at 8 h, and remained stable for the next 24 h. At this first minutes of transfection, increased progressively to reach a higher than that in the medium. TGF for 1 h at room temperature in 2% acrolein in 10 mM phosphate buffer (pH 7.4), and washed overnight in 100 mM PBS (pH 7.6) at 4 °C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min, rinsed, and exposed for 1 h to a 1:4 dilution of nonimmune rabbit serum. The polyclonal anti-TGF β1 rabbit antibody was used as primary antibody at a dilution of 1:1000 overnight in a humidity chamber at 4 °C. The second antibody to rabbit IgGs conjugated to peroxidase was used at a dilution of 1:2000 for 1 h at room temperature. To localize the antigen-antibody complexes, cells were incubated for 2 min with 0.05% 3,3’ diaminobenzidine tetrahydrochloride, 0.01% H2O2, and 2.5% nickel ammonium sulfate. Next, the cultured cell preparations were mounted in PBS-glycerol (1:1). The specificity of the TGF β1 antibody has been tested previously (30).

Cortisol Production—It was measured in the medium by a specific radioimmunoassay (33).

Metabolic Labeling—For metabolic labeling, before the end of the culture, the cells were preincubated for 1 h in methionine-free medium, after which the medium was replaced by fresh methionine-free medium containing [35S]methionine (50 μCi/ml) during the last 4 h.

Immunoprecipitation—After metabolic labeling, cells were washed three times with phosphate buffered saline. Cells were then lysed by addition of 500 μl of ice-cold immunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). After centrifugation for 10 min at 10,000 × g, the supernatants from the cells extracts were incubated at 4 °C for 2 h with nonimmune rabbit serum at a final concentration of 5% and then with 50 μl of a 50% (v/v) suspension of protein A-Sepharose CL-4B. After 1 h of incubation, the beads were sedimented by centrifugation. The supernatants were collected and submitted to immunoprecipitation with anti-a-β121 IgG (1/50, v/v) at 4 °C for 2 h, followed by incubation with protein A-Sepharose CL-4B. After centrifugation, the beads were washed four times with ice-cold immunoprecipitation buffer and once with 0.1% SDS. The radiolabeled proteins in the final pellet were analyzed by electrophoresis using 7.5% polyacrylamide gels. Gels were fixed, soaked in Amplify, dried, and the labeled proteins were revealed by autoradiography.

Western Blot Analysis—After appropriate treatments, cells were lysed and submitted to Western blot analysis as described previously (34) except 10% polyacrylamide gels were without urea.

Statistical Analysis—Statistical analysis were performed with Student’s t test for comparison of two groups. Differences were considered significant when p < 0.05.

RESULTS

Cell Viability—Exposure of BAC to AON or SON or SCR oligonucleotides did not affect cell viability: 89.5 ± 1.0%, 87.4 ± 3.4%, 90.2 ± 2.7%, respectively, versus 91.3 ± 0.4% in control cells (n = 3).

Oligonucleotide Uptake and Degradation—To investigate the kinetics of oligonucleotide uptake and degradation, cells were transfected with 32P-labeled TGF β1 AON or SON (final concentration 10 μM) for different times as described under “Experimental Procedures.” Fig. 1 shows the time-dependent cellular uptake of TGF β1 AON (solid line) and TGF β1 SON (dotted line). TGF β1 AON cellular uptake started within the first minutes of transfection, increased progressively to reach a plateau at 8 h, and remained stable for the next 24 h. At this time, 16% of the radioactivity were inside the cells. Taking into account the cellular volume and the specific activity, TGF β1 AON intracellular concentration (500 μM) was about 50-fold higher than that in the medium. TGF β1 SON cellular uptake was also rapid. Uptake reached a plateau at 2 h, remained stable for only 3 h, and then declined. At the plateau, 8% of the radioactivity was inside the cells.

To examine the extent of oligonucleotide degradation within cells and in the medium during the cellular uptake, TGF β1 AON was analyzed by gel electrophoresis. The results of Fig. 1 show that, inside the cells, TGF β1 AON appeared to be intact for up to 24 h. However, in the culture medium, a progressive degradation of the TGF β1 AON was observed. The oligonucleotide was first transformed into another compound with higher mobility. This in turn was converted into another compound with faster mobility after 3 h. A similar pattern of degradation, but much more rapid, was observed with TGF β1 SON (data not shown).

Intracellular Stability of the TGF β1 AON and SON—To determine the intracellular stability of the oligonucleotides, cells were transfected with 32P-labeled TGF β1 AON or SON for 8 h (time at which TGF β1 AON uptake was maximum), the medium was then removed and replaced with fresh medium without oligonucleotides, and the culture continued for another 44 h (Fig. 2). As shown, after 8 h of transfection, the intracellular concentration of TGF β1 AON or SON represented 16% and 6%, respectively, of the radioactivity added. However, after 44 h of culture, the intracellular concentration of TGF β1 AON was still 8% of the radioactivity added, whereas that of TGF β1 SON represented only 1.2%.

Intracellular Distribution and Hybridization of TGF β1 AON and SON—In order to determine the intracellular distribution of both TGF β1 AON and SON, subcellular fractionation of the cells was performed after 8 h of transfection and 44 h after the oligonucleotides’ removal (Fig. 3). After 8 h of transfection, 60% and 40% of the intracellular TGF β1 AON were in the cytoplasmic and nuclear fractions, respectively. This distribution was the opposite after 44 h of culture. In contrast, at both times more than 85% of the intracellular TGF β1 SON were in the cytoplasmic fraction. Indeed, the TGF β1 SON present in the nuclear fraction after 44 h of culture without oligonucleotides...
Fig. 2. Intracellular stability of the TGFβ1 AON and SON. Cells were transfected with 32P-labeled AON or SON for 8 h. Several wells were harvested, while the medium of the other wells was removed and replaced with fresh medium without oligonucleotides. The culture was continued for another 44 h. All aliquots of cell extracts at 8 h and 8 h followed by 44 h of culture were taken and counted. The results are expressed as percent of total radioactivity. Results are the mean ± S.D. of triplicate measurements from two separate experiments for the TGFβ1 AON and from one experiment for TGFβ1 SON.

Fig. 3. Intracellular distribution of the TGFβ1 AON and SON. Cells were transfected with 32P-labeled AON or SON for 8 h or 8 h followed by 44 h of culture and subcellular fractionation was carried out as described under “Experimental Procedures.” Aliquots of the cytoplasmic and nuclear fractions were counted. The results are expressed as percent of intracellular radioactivity. Results are the mean ± S.E. of triplicate measurements from three separate experiments for the TGFβ1 AON and one experiment for TGFβ1 SON.

Fig. 4. Intracellular hybridization of the TGFβ1 AON and SON. Cells were incubated with 32P-labeled AON or SON for 8 h or 8 h followed by 44 h of culture. Aliquots of medium and cell lysates were precipitated by ethanol to recover the nucleic acids. For each condition, aliquots containing equal amounts of radioactivity were incubated for 30 min at 37°C in the absence of the presence of 20 units of S1 nuclease. All samples were analyzed by urea-PAGE and autoradiography. The intensity of the signal after S1 nuclease digestion is expressed as the percentage of the signal without S1 nuclease digestion (100%). Top, diagram; bottom, autoradiography of one representative experiment.

Fig. 5. Intracellular distribution of hybridized TGFβ1 AON. Cells were incubated with 32P-labeled AON for 8 h or 8 h followed by 44 h of culture. Subcellular fractionation was performed and aliquots of culture medium, cytoplasmic and nuclear fractions were subjected or not to partial S1 nuclease digestion. Aliquots were then analyzed by urea-PAGE and autoradiography. Results are expressed as described in the legend of Fig. 4. Top, diagram; bottom, autoradiography of one representative experiment.

Effects of TGFβ1 AON and SON on BAC TGFβ1 mRNA and Protein Content—To assess whether the oligonucleotides were able to modify the transcription and/or the translation of TGFβ1, we investigated their effects on TGFβ1 mRNA by Northern blot and on cellular TGFβ1 protein content by immunocytochemistry. The results of Fig. 6 showed that neither TGFβ1 AON nor SON modified the level of the 2.5-kilobase transcript of TGFβ1 mRNA. In contrast, the results of immunocytochemistry (Fig. 7) showed that all the control cells (A) or cells pretreated with TGFβ1 SON (C) were immunoreactive. However, the TGFβ1 immunoreactivity completely disappeared in TGFβ1 AON treated cells (D). In B, where the TGFβ1 antibody was saturated with the peptide used to produce this antibody, there was no TGFβ1 signal, thus showing the specificity of this antibody. Since it has been reported that cyclosporine increased the expression of TGFβ1 (35), we treated BAC for 44 h with this factor (1 μg/ml) in the absence or presence of TGFβ1 AON and we examined the TGFβ1 content by immunocytochemistry (Fig. 8). Although this method is only...
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Fig. 6. Effects of TGFβ1 AON and SON on TGFβ1 mRNA. Cells were incubated for 8 h without (control cells) or with AON or SON (10 μM). The medium was removed, replaced by fresh medium without oligonucleotides, and the culture continued for 44 h. TGFβ1 mRNA was extracted and analyzed by Northern blot. A representative Northern blot of one of the six experiments performed is shown.

Fig. 7. Effects of TGFβ1 AON and SON on cell TGFβ1 protein content. Cells were incubated for 8 h without (control cells) or with AON or SON (10 μM). The medium was removed, replaced by fresh medium without oligonucleotides, and the culture continued for 44 h. Immunocytochemical staining was performed using a specific TGFβ1 antibody as described under “Experimental Procedures.” A, control cells; B, control cells incubated with the antibody saturated with the peptide (10 μg/ml) used to produce this antibody; C, cells transfected with SON; D, cells transfected with AON.

Fig. 8. Effects of cyclosporine and/or TGFβ1 AON on cell TGFβ1 protein content. Cells were incubated for 8 h without (A and C) or with AON (B and D). The medium was replaced by fresh medium without (A and B) or with (C and D) 1 μg/ml cyclosporine and the culture continued for 44 h. Immunocytochemical staining was performed as described in Fig. 7.

semi-quantitative, the results of Fig. 8 suggest that cyclosporine increased the cellular TGFβ1 content, and this effect was blunted by TGFβ1 AON.

Effects of TGFβ1 AON and SON on BAC Functions—As described in the Introduction, exogenous TGFβ1 decreases the steroidogenic responsiveness of BAC; thus, we investigated the effects of both oligonucleotides, TGFβ1 AON and SON, on the steroidogenic responsiveness to AngII of control and cyclosporine treated cells (Fig. 9). In the absence of cyclosporine, TGFβ1 AON increased the cortisol response to AngII about 2-fold compared to either control cells or TGFβ1 SON-treated cells. Cyclosporine alone decreased the steroidogenic capacity of BAC by about 50%, compared to cells not treated with cyclosporine. However, the cortisol production of cells treated with cyclosporine and TGFβ1 AON was 2.3-fold higher than that of cells treated with cyclosporine alone. Again, TGFβ1 SON had no effect.

One of the mechanism by which exogenous TGFβ1 decreases the steroidogenic capacity of BAC is by decreasing the mRNA levels of P450 17α-hydroxylase and 3β HSD (14, 16). The results (Fig. 10) clearly show that TGFβ1 AON, but not SON, increased P450 17α-hydroxylase and 3β HSD mRNA levels (2- and 1.7-fold, respectively), which encode two key enzymes in the steroidogenic pathway.

Control Experiments to Demonstrate the Specificity of TGFβ1 AON Effects—To prove that all the TGFβ1 AON effects on BAC functions were specific, some additional controls were performed. First, cells were transfected with a scrambled oligonucleotide (SCR) containing the same number of G nucleotides (10 of 15) as TGFβ1 AON but in a scrambled order. The results (Fig. 11) showed that, in contrast to TGFβ1 AON, neither SON nor SCR modified the cortisol secretion and the mRNA levels of P450 17α-hydroxylase. Second, none of the transfected oligonucleotides did change the sensitivity of the cells to the inhibitory effects of TGFβ1, since exogenous TGFβ1 caused similar inhibition of both cortisol secretion and P450 17α-hydroxylase mRNA levels, in control and in transfected cells (Fig. 11).

Finally, to prove that transfection did not produce a general inhibition of protein synthesis, we investigated the effects of transfection with the three oligonucleotides on the rate of synthesis and on the steady-state levels of Goq/Gαq11 proteins, which are not affected by exogenous TGFβ1 (34). The results showed that neither the rate of synthesis (Fig. 12A) nor the steady-state levels (Fig. 12B) of Goq/Gαq11 proteins were affected in transfected cells regardless of the nucleotide used.

**DISCUSSION**

Antisense oligonucleotides have been used as specific inhibitors of target gene expression. The specificity of an antisense oligonucleotide is due to highly specific hybridization to its complementary target sequence on the mRNA by Watson-Crick base pairing. This is obtained by using an oligonucleotide of about 15 bases directed against a complementary sequence of target mRNA (21, 36, 37). One key parameter in the oligonucleotide antisense approach is its intracellular concentration, which is the result of two opposite processes: the rate of penetration of the antisense molecule across cell membrane, and its
and similar uptake of both TGF-β1 antisense oligonucleotides (41). Using a cationic liposome-mediated transfection method in cell culture, we demonstrated a rapid, high, and extensive intercellular distribution. First, after 8 h of transfection followed by 44 h of culture, intracellular TGF-β1 oligonucleotide concentration was still high (8%), whereas that of SON was only 1.2%. Second, at any time point between 0 and 44 h, most of the intracellular TGF-β1 SON was located in the cytoplasm, and was not hybridized. However, TGF-β1 AON was predominant in the cytoplasm after 8 h of transfection; it became prevalent in the nucleus at the end of the experimental period. In addition, in both compartments, TGF-β1 AON was hybridized. This hybridization was particularly intense in the nucleus, and it was higher after 8 h of transfection followed by 44 h of culture (without oligonucleotides) than immediately after transfection. These results agree with other data showing that c-Myb (40) and prorenin (41) antisense oligonucleotides were preferentially accumulated in the nucleus. However, these results differ from those of Tensamani et al. (44) showing preferential cytoplasmic localization of several antisense oligonucleotides. Although the exact mechanism of oligonucleotide transfer from cytoplasm to nucleus is not completely understood, a passive diffusion through the nuclear pores has been postulated (40).

The present studies also show that 44 h after transfection about 44% and 75% of the TGF-β1 AON present in the cytoplasm and nucleus, respectively, were resistant to S1 nuclease digestion. Since the target sequence is present in both primary transcript and mRNA, TGF-β1 AON could hybridize to both and interfere with pre-mRNA maturation and/or nucleocytoplasmic transport (36, 37, 46), but not with transcription, since the level of TGF-β1 mRNA was not modified by TGF-β1 AON. In contrast, TGF-β1 AON causes complete inhibition of TGF-β1 protein production. This inhibition could be the result of either degradation of RNA by RNase H, which selectively cleaves the RNA at the target sequence, or inhibition of the translation by AON hybridization to the translation initiation site of the TGF-β1 mRNA (49, 50). The first hypothesis is unlikely because no decrease of TGF-β1 mRNA was observed. Although recent data show that c-Myb AON was not associated with ribosomes or endoplasmic reticulum (40), our results strongly suggest that the main mechanism by which TGF-β1 AON blocked the synthesis of TGF-β1 protein is by translation arrest.

Although the potential autocrine role of TGF-β1 has been suggested in several cell types (1–3), only in two models, rat adrenal cells (42–45). The kinetic studies allowed us to determine the optimal time of transfection (8 h) and to investigate the stability and the distribution of both TGF-β1 AON and SON. Although, as indicated above, the intracellular level of both TGF-β1 AON and SON were different after the first hours of transfection, both appeared intact in the cells. However, degradation products of both TGF-β1 AON and SON appeared in the culture medium. This process was more rapid and marked for TGF-β1 SON than for AON. Whether the degradation occurred inside or outside the cells was not determined in the present study. However, on the one hand, our culture did not contain serum thought to have DNase activity (36). On the other hand, after transfection of the cells for 8 h followed by extensive washings, oligonucleotide degradation products appeared in the fresh medium during the next 44 h of culture (data not shown). Thus, it is likely that the degradation of both oligonucleotides takes place inside the cells.

In addition, our results revealed marked difference between TGF-β1 AON and SON concerning their stability and cellular distribution. First, after 8 h of transfection followed by 44 h of culture, intracellular TGF-β1 AON concentration was still high (8%), whereas that of SON was only 1.2%. Second, at any time point after 8 h, intracellular TGF-β1 SON was still high (8%), whereas that of SON was only 1.2%. Second, at any time point after 8 h, intracellular TGF-β1 SON was located in the cytoplasm. Thus, it is likely that the degradation of both oligonucleotides takes place inside the cells.

rate of degradation in the cells. The uptake is a saturable process thought to be mediated by both receptor endocytosis and fluid phase endocytosis (38–40). An increased uptake has been obtained by the use of cationic liposomes (41). Using a cationic liposome-mediated transfection method in cell culture, we demonstrated a rapid, high, and similar uptake of both TGF-β1 AON and SON during the first 2 h of transfection. Thereafter, the kinetics of TGF-β1 SON and AON were different. Indeed, whereas the intracellular concentration of SON, after a short lag period, declined, the concentration of AON continued to increase reaching a plateau at 8 h (16% of the radioactivity added) and remained stable for at least 24 h. This uptake is several times higher than that observed in other studies in which no cationic liposomes were used (42–45). These kinetic studies allowed us to determine the optimal time of transfection (8 h) and to investigate the stability and the distribution of both TGF-β1 AON and SON. Although, as indicated above, the intracellular level of both TGF-β1 AON and SON were different after the first hours of transfection, both appeared intact in the cells. However, degradation products of both TGF-β1 AON and SON appeared in the culture medium. This process was more rapid and marked for TGF-β1 SON than for AON. Whether the degradation occurred inside or outside the cells was not determined in the present study. However, on the one hand, our culture did not contain serum thought to have DNase activity (36). On the other hand, after transfection of the cells for 8 h followed by extensive washings, oligonucleotide degradation products appeared in the fresh medium during the next 44 h of culture (data not shown). Thus, it is likely that the degradation of both oligonucleotides takes place inside the cells.

Fig. 10. Effects of TGF-β1 AON and SON on P450 17α-hydroxylase and 3β HSD mRNA levels. Cells were incubated for 8 h without (control cells) or with AON, SON, or SCR (10 μM), and the culture was then continued for 44 h. P450 17α-hydroxylase and 3β HSD mRNA were extracted and analyzed by Northern blot. A, Top, mean ± S.E. of duplicate measurements from two separate experiments. B, in the same two experiments P450 17α-hydroxylase mRNA were analyzed by Northern blot (one representative autoradiograph).

Fig. 11. Effects of exogenous TGF-β1 on BAC steroidogenic responses. Cells were incubated for 8 h without (control cells, CNT) or with AON, SON, or SCR (10 μM), and the culture was then continued for 44 h in the absence or the presence of 2 ng/ml of TGF-β1. The medium was removed, and the cells were washed and then stimulated for 2 h with AngI 10−7 M. A, cortisol production was determined by RIA. Results, expressed as ng/106 cells, are the mean ± S.D. of duplicate measurements from two separate experiments. B, in the same two experiments P450 17α-hydroxylase mRNA were analyzed by Northern blot (one representative autoradiograph).

Fig. 12. Effects of transfection on Gαq/Gα11 synthesis and steady-state levels. Cells were incubated for 8 h without (control cells, CNT) or with AON, SON, or SCR (10 μM), and the culture was then continued for 44 h. [35S]Methionine (50 μCi/ml) was added during the last 4 h of incubation. A, immunoprecipitation of radiolabeled Gαq/Gα11, B, Western blot of the cell lysates using Gαq/Gα11 antibody.
vascular smooth muscle cells (23) and human colon carcinoma cell line (51), has this been proven by using the antisense approach. Our results show that the biological consequences of TGFβ1 protein synthesis inhibition in control as well as in cyclosporine treated cells were a significant increase of cortisol production in response to AngII and ACTH (data not shown).

Another demonstration of the autocrine role of TGFβ1 on BAC was obtained by showing that TGFβ1 AON, but neither SON nor SCR, increased about 2- and 1.7-fold the mRNA levels of P450 17α-hydroxylase and 3β HSD, respectively, an effect that was opposite to that induced by exogenous TGFβ1 in these cells (Refs. 14–17 and the present data). Moreover, the effects of TGFβ1 AON on steroidogenic responses of viable BAC were specific. First, they were not mimicked by SON or SCR; second, they could be reversed by addition of exogenous TGFβ1; and third, they did not modify the normal production of unrelated proteins.

Taken together our data demonstrate, for the first time, that constitutive expression of TGFβ1 by BAC has an autocrine inhibitory effect on the differentiated functions of these cells. Moreover, since TGFβ1 is expressed by many cell types, it is likely that this factor might also play an autocrine role in other models. Finally, these studies illustrate and confirm that antisense technology should find widespread application for investigating the exact role of many regulatory proteins on cell growth and differentiation.

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