

Letter to the Editor

Differences in human gingival and dermal fibroblasts may contribute to oral-induced tolerance against nickel

To the Editor:

The prevalence of allergic contact dermatitis has increased steadily with nickel (Ni) still as the main sensitizer.^{1,2} In this context, it seems contradictory that orthodontic materials contain high amounts of Ni, which lead to the inevitable release of Ni ions (Ni^{2+}) into the oral cavity.³ However, intraoral allergic reactions are rare and current evidence indicates that orthodontic treatment before piercings may even prevent Ni hypersensitivity probably via oral-induced tolerance against Ni.⁴ The detailed mechanisms

still remain unclear. Previous studies imply that immunological differences between mucosal and dermal dendritic cells (DCs) contribute to oral-induced immune tolerance.⁵ Thereby, mucosal DCs display a protolerogenic character, whereas dermal DCs have a proallergenic phenotype. Interestingly, DCs are strongly influenced by their microenvironment determining the subsequent phenotype of DCs.⁵⁻⁷ Mediators such as transforming growth factor (TGF)- β , IL-10, and certain innate immune receptors such as Toll-like receptor (TLR) 4 seem to play a pivotal role in these events.⁵⁻⁸

As fibroblasts primarily constitute tissue structure and are in direct contact with immune cells such as DCs, we determined whether dermal fibroblasts promote a proallergenic inflammatory microenvironment in contrast to oral gingival (mucosal) fibroblasts, which may favor a protolerogenic anti-inflammatory surrounding. Therefore, primary human oral/gingival fibroblasts (HGFs) and primary human dermal fibroblasts (HDFs) were

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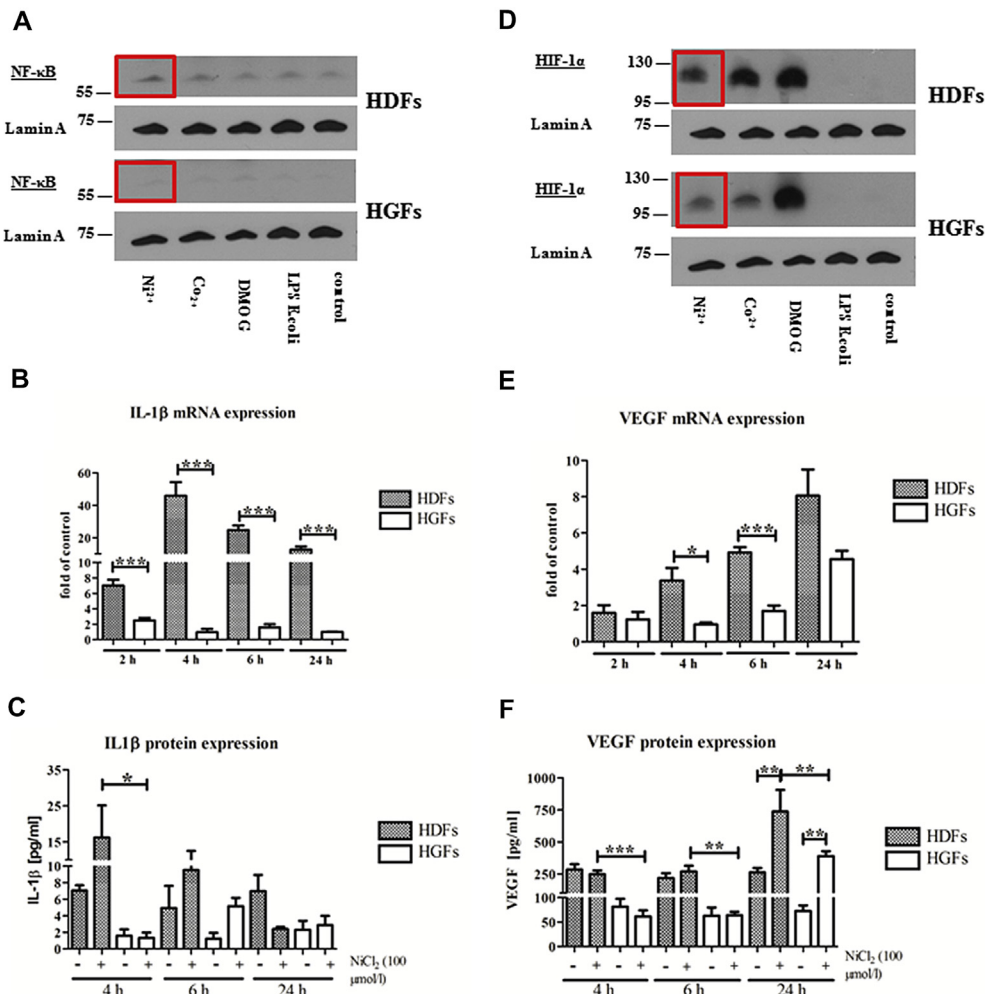


FIG 1. Altered NF- κ B and HIF-1 α activation. Activation of the transcription factors NF- κ B (**A**) and HIF-1 α (**D**) was determined followed by the analysis of mRNA and protein of their target genes IL-1 β (**B** and **C**) and VEGF (**E** and **F**) in dermal (HDFs) and gingival fibroblasts (HGFs) stimulated with 100 $\mu\text{mol/L}$ Ni^{2+} . Means $\pm$ SEMs are depicted (* $P < .05$, ** $P < .01$, *** $P < .001$).

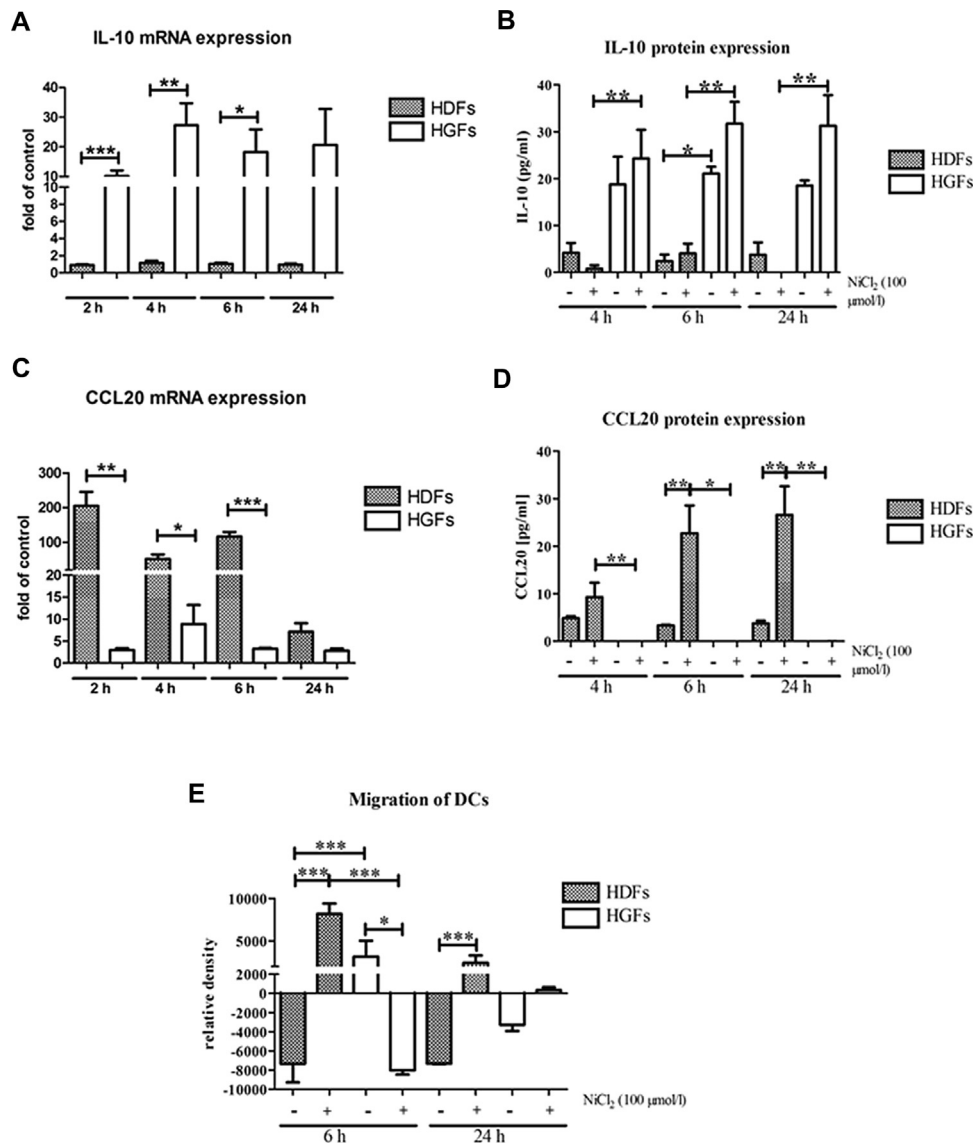


FIG 2. IL-10 and CCL20 expression and DC migration. IL-10 (**A** and **B**) and CCL20 (**C** and **D**) mRNA and protein expression was determined in dermal (HDFs) and gingival fibroblasts (HGFs) after 100 μmol/L Ni²⁺ challenge (**E**). Induction of DC migration by supernatants of HDFs or HGFs was determined after 24 hours. Means ± SEMs are depicted (**P* < .05, ***P* < .01, ****P* < .001).

challenged with Ni ions (Ni²⁺) and subsequent cell signaling and the expression of certain mediators responsible for immune cell activation and recruitment were evaluated (for details, see this article's Online Repository at www.jacionline.org).

Because Ni²⁺ exerts some effects via TLR4-binding and nuclear factor kappa B (NF-κB) is a key regulator of inflammation,⁸ downstream activation of the transcription factor NF-κB and the expression of its target gene IL-1β were investigated. Nuclear accumulation of NF-κB was markedly induced after Ni²⁺ stimulation in HDFs than in HGFs (Fig 1, A). In line with these findings, IL-1β mRNA expression was significantly elevated in HDFs (Fig 1, B). In contrast, reduced nuclear NF-κB levels were found in HGFs. Accordingly, Ni²⁺ provoked significantly higher expression levels of IL-1β mRNA in dermal cells than in gingival cells (45-fold, Fig 1, B). In good agreement with mRNA results, Ni²⁺ challenge induced an increase in IL-1β protein release by HDFs. Instead, in

HGFs IL-1β protein level was only slightly elevated and significantly less compared with that in HDFs (22-fold, Fig 1, C).

Because divalent cations such as Ni²⁺ are known for their hypoxia-mimicking capacities,^{E1,E2} we determined the activation of the transcription factor hypoxia-inducible factor (HIF)-1α and its target gene vascular endothelial growth factor (VEGF), which is liable for vascularization, endothelial permeability, and immune cell invasion.^{E3,E4} Notably, HIF-1α mRNA expression was not altered by Ni²⁺ stimulation in HDFs or HGFs (data not shown). However, HIF-1α activation measured as nuclear accumulation was increased in HDFs. Similar results were detectable in the cytoplasm of HDFs after Ni²⁺ challenge (data not shown). Notably, nuclear HIF-1α accumulation in HGFs was less intense than in HDFs (Fig 1, D). Accordingly, VEGF mRNA levels were more than twice as high in HDFs than in HGFs, which was statistical significant (Fig 1, E). Regarding VEGF protein, results were

in line with mRNA data because significantly higher protein levels were detected in HDFs than in HGFs (4-fold, Fig 1, F).

To antagonize inflammatory processes, anti-inflammatory mediators linked to the induction of immune tolerance are secreted.^{E5,E6} Therefore, we investigated IL-10 and TGF- β mRNA and protein expression after Ni²⁺ challenge. A low constitutive expression of IL-10 mRNA could be demonstrated in HDFs, which was not affected by Ni²⁺ incubation (Fig 2, A). In contrast, in HGFs Ni²⁺ incubation increased the expression levels of IL-10 significantly (10-fold to 30-fold). Accordingly, we found very low IL-10 protein levels in HDFs and significantly higher amounts in HGF supernatants (30-fold; Fig 2, B). Notably, TGF- β was not altered in HDFs or HGFs (data not shown).

Next, we determined chemokine (C-C motif) ligand 20 (CCL20); macrophage inflammatory protein-3 (MIP-3 α) because of its chemoattractant and inflammatory capacities leading to DC recruitment.^{E7} We could demonstrate that CCL20 mRNA was induced 200-fold by Ni²⁺ in HDFs (Fig 2, C). In contrast, Ni²⁺ provoked only minor changes in HGF CCL20 expression. Regarding CCL20 protein release, a time-dependent increase in HDF supernatants was detectable (35-fold). In contrast, CCL20 protein concentrations in HGF supernatants were below the detection limit (Fig 2, D).

Finally, to investigate the recruitment of DCs, a migration assay was performed using supernatants of each fibroblast type (Fig 2, E). Supernatants of HDFs cultured under control condition reduced DC migration, whereas control supernatants of HGFs induced an increase. Most interesting, supernatants of HDF challenged with Ni²⁺ significantly stimulated DC migration, whereas supernatants of similarly treated HGFs caused a pronounced inhibition of their migration (Fig 2, E).

Taken together, increased IL-1 β , CCL20, and VEGF protein levels as well as decreased IL-10 expressions predispose to an imbalanced inflammatory microenvironment with subsequent immune cell recruitment and invasion in dermal tissue, which seem to be inhibited in gingival/oral tissue. In line with present data, previous studies demonstrated that in contrast to dermal DCs, oral DCs respond with an increase in anti-inflammatory IL-10 secretion following TLR4 activation, which then stimulate the induction of regulatory T cells.^{6,7} Regulatory T cells are key players for the induction of immune tolerance. Usually, activation of TLR4 initiates a potent proinflammatory response accompanied by the release of proinflammatory cytokines and chemoattractants recruiting predominantly effector T cells, which aggravate inflammatory processes and allergic reactions.⁹ DCs as antigen-presenting cells regulate the initiation of hypersensitivities as they determine T-cell priming and activation. The tolerogenic function of different subsets of DCs depends on their maturation stage, certain ontogenics, and immunomodulating agents. As the microenvironment of DCs seem to play a crucial role in their maturation and fibroblasts primarily constitute the *intestinal lamina propria* in which DCs reside, we hypothesize that gingival fibroblasts contribute to their protolerogenic

phenotype via altered NF- κ B and HIF-1 α regulation and subsequent expression profile.

To conclude, present results unveil new aspects of oral-induced tolerance and provide additional information for current knowledge, which indicates that oral Ni²⁺ challenge before piercings may prevent Ni hypersensitivity.⁴ Accordingly, our findings may open the door for new therapeutic approaches solving current difficulties in allergic contact dermatitis and oral-induced hyposensitivity.

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METHODS

Isolation of human primary gingival fibroblasts

The present study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (Lfd. Nr 318/11). Written consent was obtained from all patients and if necessary from their parents before inclusion. HGFs were harvested from 9 donors who underwent extraction of wisdom teeth. Exclusion criteria were acute or chronic inflammation or medication. Cells were collected as previously described.^{E8} In brief, gingival tissues were washed twice with PBS (Life Technologies, Darmstadt, Germany) supplemented with 1% antibiotic and antimycotic solution containing penicillin/streptomycin and amphotericin B (Life Technologies). For tissue dissection, gingiva was incubated with collagenase 2 solution (PAA Laboratories, Cölbe, Germany) at 37°C for 2.5 hours. Afterward, connective tissue was separated from the epithelial layer and incubated with Dulbecco modified Eagle medium culture medium supplemented with 10% FCS and 1% antibiotic and antimycotic solution (all from Gibco, Life Technologies), in 37°C humidified atmosphere of 5% CO₂ in air.

HDFs were purchased from Promocell (Heidelberg, Germany) because an elective operation to obtain dermal fibroblasts was not ethical.

Characterization of human primary gingival and dermal fibroblasts

Human primary gingival and dermal fibroblasts were characterized before experiments with specific mucosal and dermal markers using PCR (Table E1). After phenotyping cell lines were cryoconserved and used for experiments at third or fourth passage.

Cell stimulation

Initially, we determined the dose-response of both cell types with respect to alteration in cell proliferation and viability by nickel(II)-chloride-hexahydrate (Ni²⁺) (Sigma-Aldrich, Taufkirchen, Germany) treatment using the Promo-Kine XTT Cell Proliferation Kit (Promocell). Therefore, 5000 fibroblasts per well from 3 donors were seeded in 96-well plates followed by Ni²⁺ stimulation with different concentrations (1–100,000 µmol/L) for 24 hours. To prevent nonspecific cell stimulation via TLR4 activation,^{E9} Ni²⁺ solution was tested for endotoxin contamination before use. After 24 hours of Ni²⁺ stimulation, XTT reaction solution was added for 2 hours followed by measurement of absorbance at 490 nm with correction wavelength 670 nm in a microplate reader. Ni²⁺ concentrations below 1 mM neither affected the proliferation nor the cell viability of fibroblasts (data not shown).

For final experiments, cells were seeded (100,000 cells/well) on 6-well plates and grown to 80% confluence followed by stimulation with 100 µmol/L Ni²⁺ for various time points (2, 4, 6, and 24 hours) because this concentration was far below the lethal dose and induced the highest mRNA expression levels. Unstimulated fibroblasts served as control. In addition, we used LPS derived from *Escherichia coli* (1 µg/mL LPS *E coli* [Invivogen, Darmstadt, Germany]; TLR4 agonist that generally induced a nuclear accumulation of NF-κB), dimethylalloylglycine (1 mM dissolved in culture medium; inhibitor of prolyl hydroxylase and the asparaginyl hydroxylase factor inhibiting HIF causing an upregulation of HIF-1α), and another divalent cation (100 µmol/L cobalt chloride [Co²⁺]) as positive controls for immunoblots.

RNA isolation and quantitative PCR

Fibroblasts were lysed with 4 mol guanidiniithiocyanat (Roth, Karlsruhe, Germany) and total RNA was extracted using the acid guanidinium thiocyanate/phenol/chloroform extraction method. One microgram of total RNA was reverse transcribed into cDNA by the use of 200 U of Maloney Murine Leukemia Virus reverse transcriptase (Bio-Rad, Munich, Germany) as previously described.^{E10,E11} cDNA expression was detected by real-time PCR using specific TaqMan probes. Amounts of cDNA were normalized to the housekeeping gene 18S, and cDNA expression was calculated as relative expression of respective controls.

Whole-cell lysate preparation

For whole-cell lysate preparation, fibroblasts were lysed with 150 µL lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.9, 1 mM EDTA, 0.1% Igepal, 1× Protease Inhibitor Cocktail [Roche Diagnostics, Mannheim, Germany])

for 20 minutes on ice. Afterward, lysates were centrifuged at 3600 rpm, 4°C for 5 minutes, and supernatants containing cellular protein were collected and stored at –80°C. Protein concentrations were quantified using the Bio-Rad protein assay reagent (Bio-Rad).

Nuclear extract preparation

To determine activated NF-κB and HIF-1α, nuclear protein accumulation was evaluated. Therefore, nuclear proteins were prepared using a NE-PER nuclear extraction kit (Pierce, Rockford, Ill) following manufacturer's instructions. Nuclear proteins were stored at –80°C until use and protein concentrations were determined with the Bio-Rad kit (Bio-Rad).

Immunoblots

After addition of 4× sample buffer (50 mM Tris, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.0125% bromophenol blue, and 1% glycine), 50 µg of total cell lysate or 20 µg of nuclear extract per lane was subjected to 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane (0.2 µm pore size; Schleicher & Schuell Microscience, Dassel, Germany). The efficiency of protein transfer and equal loading was confirmed by staining the membrane with Ponceau S (Sigma-Aldrich). Membranes were blocked overnight at 4°C with 5% (w/v) nonfat milk in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.05% Tween-20) and washed with TBS-T before incubation at room temperature for 2 hours with a rabbit polyclonal antibody directed against the p65 subunit of the NF-κB complex (Santa Cruz Biotechnology, Santa Cruz, Calif) or HIF-1α (Novus Biologicals, Littleton, Colo). Lamin A (Abcam, Cambridge, UK) served as marker for equal protein loading. After washing with TBS-T, horseradish peroxidase-conjugated antirabbit IgG antibody (New England Biolabs, Frankfurt am Main, Germany) was added. Immunoreactive proteins were visualized by chemiluminescence (Amersham Chemiluminescence Kit) and exposure to X-ray films (Agfa, Mortsel, Belgium).

ELISAs

To investigate the release of IL-1β, VEGF, IL-10, CCL20/MIP-3α, and TGF-β protein, cell-free supernatants were collected at the end of the respective incubation periods and kept at –80°C until further analysis. For the protein measurement, Quantikine ELISAs were used according to the manufacturer's instruction (R&D Systems, Wiesbaden, Germany).

DC isolation

Initially, PBMCs were purified from buffy coats using Ficoll-Paque density gradient. Afterward, CD14⁺ cells were separated by magnetic-activated cell sorting using CD14-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Then, human DCs were isolated using the DC isolation kit (human Diamond Plasmacytoid Dendritic Cell Isolation Kit) from Miltenyi Biotec. Afterward, DC purity (>90%) and cell viability (dead cells < 10%) were determined by flow cytometry and trypan blue staining, respectively. DCs were resuspended in Gibco Advanced RPMI 1640 supplemented with 10% heat-inactivated FCS, 1× GlutaMAX and 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Life Technologies).

Migration assay

To evaluate DC migration, the CultureCoat BME Cell Invasion Assay (R&D Systems) was used following manufacturer's instructions. Briefly, DCs were cultured in the migration chamber (inserted top chamber) of a 96-well microplate at a density of 50,000 cells/well in 50-µL medium. To induce cell migration, 150-µL supernatants of HDFs or HGFs preincubated with 100 µmol/L of Ni²⁺ solution for 6 and 24 hours, respectively, were added to the assay chamber (bottom chamber). After 24 hours of incubation with supernatants at 37°C and 5% CO₂, migrated DCs in the bottom chamber were dissociated and fluorescence labeled with Calcein AM for 1 hour followed by measurement of excitation at 485 nm with emission at 520 nm in a fluorescence plate reader. The induction of DC migration via supernatants of HGFs and HDFs was calculated in relation to DC migration incubated with medium only or medium containing Ni²⁺, respectively.

Statistical analysis

All experiments were performed in triplicate except for DC migration, which was performed in duplicate and repeated at least 2 to 3 times. Differences in mRNA expression between HGFs and HDFs were analyzed by unpaired *t* test, whereas differences in protein levels and DC migration between control groups of HGFs and HDFs as well as each Ni²⁺ group were determined by 1-way ANOVA followed by Bonferroni comparison test using GraphPad Prism 5.0 Software (GraphPad Software, La Jolla, Calif). Data are shown as means $\pm$ SEM, and *P* values of less than .05 were considered to indicate significance.

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TABLE E1. Primer sequences for characterization

Gene	Primer sequence (fwd/rev)
Integrin $\alpha 10$	fwd: AGAAAACACAGCCCAGACC rev: TGAACCAGCCTCAATAGTCC
Collagen, type 4, alpha 1 (COL4A1)	fwd: GCACAGCCAGACCATTGAGA rev: CTATGGTGGCGAGCCAAAAG
Collagen, type 10, alpha 1 (COL10A1)	fwd: AATGCCGAGTCAAATGGCCT rev: TCAGGGGGAAGGTTTGTGG
Osteopontin	fwd: CACTCCAGTTGTCCCCACAG rev: AACGGGGATGGCCTTGATG
Periostin	fwd: CAGCAGACACACCTGTTGGA rev: TTGTTAGTGTGGGTCCTTCAGT
Superoxide dismutase (SOD)3	fwd: CTCTGAGGTCTCACCTTCGC rev: AGTCTCAGGGCTTATGGGGT
TLR4	fwd: GAAGGGGTGCCTCCATTTC rev: GGGTCTTCTCCACCTTCTGC
Chemokine (C-X-C motif) 12 (CXCL12)	fwd: TGAGCTACAGATGCCCATGC rev: TAGCTTCGGGTCAATGCACA
Fibronectin	fwd: TCTGACAGGCCTCACCAGAG rev: GTGTAGGGGTCAAAGCACGA

fwd, Forward; *rev*, reverse.