# Correction of the neuropathogenic human apolipoprotein E4 gene (*APOE4*) to *APOE3 in vitro* using synthetic RNA/DNA oligonucleotides (chimeraplasts)

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## Abstract

Apolipoprotein E (apoE) is a multifunctional circulating 34-kDa protein, whose gene encodes single nucleotide polymorphisms linked to several neurodegenerative diseases. Here, we evaluate whether synthetic RNA/DNA oligonucleotides (chimeraplasts) can convert a dysfunctional gene, *APOE4* (C→T, Cys112Arg), a risk factor for Alzheimer's disease and other neurological disordersatherosclerosis, into wild-type *APOE3*. In preliminary experiments, we treated recombinant Chinese hamster ovary (CHO) cells stably secreting apoE4 and lymphocytes from a patient homozygous for the ε4 allele with a 68-mer apoE4-to-apoE3 chimeraplast, complexed to the cationic delivery reagent, polyethylenimine. Genotypes were analysed after 48 h by routine polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and by genomic sequencing. Clear conversions of APOE4 to APOE3 were detected using either technique, although high concentrations of chimeraplast were needed (≥800 nM). Spiking experiments of PCR reactions or CHO-K1 cells with the chimeraplast confirmed that the repair was not artefactual. However, when treated recombinant CHO cells were passaged for ten days and then subcloned, no conversion could be detected when >90 clones were analysed by locus-specific PCR-RFLP. We conclude that the apparent efficient repair of the APOE4 gene in CHO cells or lymphocytes 48 h post-treatment is unstable, possibly because the high levels of chimeraplast and polycationethylenimine needed to induce nucleotide substitution is cytotoxic.

**K Index entrieseywords:** Alzheimer's disease; apoE genotypes; gene repair; synthetic oligonucleotides

## Introduction

Apolipoprotein E (apoE) is a circulating 34-kDa polymorphic protein that in plasma functions as an acceptor of cellular cholesterol and as a ligand to mediate hepatic clearance of lipoprotein remnant particles (Mahley and Ji, 1999; Mahley et al., 2000). In helps redistribute cholesterol between cells and acts as a ligand for several cell-surface receptors. In plasma, and in cerebrospinal fluid, about half of all apolipoproteins are apoE (Koch et al., 2001), most being synthesized by astrocytes and microglia (Strittmatter and Bova Hill, 2002). ApoE where it is a major apolipoprotein[1], fulfills several functions, all mediated via receptor-dependent pathways, including maintenance of cholesterol homeostasis and local cholesterol redistribution within tissue undergoing repair or re-modelling, developmental processes and synapse formation (Beffert et al., 1998; Beffert et al., 2004). it functions as an acceptor of cellular cholesterol and also clears lipoproteins via receptor-dependent pathways[2,3]. The two common isoforms of apoE arise from nonsynonymous coding single nucleotide polymorphisms (SNP) of the wild-type *APOE3* gene. The rarest allele ε2 (8% frequency, C→T, Arg158Cys) causes recessive hypercholesterolaemia[2], while the ε4 allele (15% frequency; C→T, Cys112Arg) produces a dominant hypercholesterolaemia and is a strong risk factor for restenosis[4] and smoking-related heart disease[5]. ApoE alleles are also strongly linked to Alzheimer's disease (AD) and several chronic neurodegenerative diseases; the ε4 allele imparts risk, while ε2 appears protective[6,7]. the rarest allele ε2 (8% frequency, C→T, Arg158Cys) appears protective (Gasparini et al., 1998; Saunders, 2000). The mechanism(s) underlying these associations is unknown, but may reflect isoform differences in intracellular apoE trafficking or in receptor-dependent cell signalling pathways[3,8,9]. (Mahley et al., 2000; Strittmatter and Bova Hill, 2002; Sacre et al., 2003)

Recently, synthetic RNA/DNA oligonucleotides have been used to repair point mutations in episomal and genomic DNA[10,11]. (Richardson et al., 2002; Rice et al., 2001). These reagents, often termed chimeraplasts, are double-stranded hairpin-capped molecules which are designed to bind specifically to the defective DNA, enabling the cell's own repair machinery to recognize and correct the faulty DNA. Several groups have reported promising results using chimeraplasts (Richardson et al.,

2002; Rice et al., 2001; Cole-Strauss et al., 1996; Kren et al., 1998; Alexeev et al., 2000; Bertoni and Rando, 2002),[10-15], including our own which has successfully converted the dysfunctional *APOE2* gene to wild-type *APOE3*, both *in vitro* and *in vivo*[16] (Tagalakis et al., 2001). Here, we apply chimeraplasty to recombinant Chinese hamster ovary (CHO) cells expressing human apoE4, and also to cultured lymphocytes from a patient homozygous for the ε4 allele, in an attempt to convert mutant *APOE4* to wild-type *APOE*3. Despite early encouraging results in short-term studies, we were unable to achieve consistent and reproducible conversions in the long term.

## Materials and Methods

### *Cell culture*

The cloned CHO cell line expressing human apoE4 that was used in these studies was produced by the method previously described for CHO-E2 cells (Tagalakis et al., 2001). These recombinant cells were cultured in Iscove's modified Dulbecco's medium with 10% dialyzed FBS (fetal bovine serum; Sigma), supplemented with 2 mM glutaMAX and 1% non-essential aminoacids (Life Technologies). Lymphocytes were isolated from the heparinized-blood of a patient homozygous for the ε4 allele and immortalized with Epstein-Barr virus (Negri et al., 1991). These transformed cells were grown as suspension cultures in RPMI 1640 medium, supplemented with 10% FBS and 2 mM L-glutamine.

### *Chimeraplasty and transfections*

The RNA/DNA oligonucleotides were synthesized commercially by MWG-Biotech (Ebersberg, Germany) to designs dictated by the target DNA (Fig. 1A). One strand of the self-associating duplex, termed the 'targeting strand (Gamper et al., 2000), had ten complementary 2'-*O*-methyl RNA residues flanking each side of the 5-base-DNA stretch. The protected RNA bases resist RNase H-mediated degradation, while four T-residues in each loop and a 5 bp GC clamp ensured that they self-associated into a double-hairpin. The other 'correcting strand' was all DNA and, except for the intended base mismatch, was complementary to the genomic DNA target. Cells were seeded into 6-well plates ($2 \times 10^5$ cells/well) 24 h prior to transfections. Chimeraplasts (400-1000 nM) were pre-incubated for 10 min with linear 22-kDa

polyethyleneimine (PEI) (ExGen 500; TCS Biologicals Ltd.), at different amine:phosphate molar ratios (5:1 to 9:1) and with the addition of 150 mM NaCl. The complexes (50 µl) were then added to each well of cells growing in 0.5 ml of serum-containing medium. After 4-6 h, monolayers were washed with phosphate-buffered saline and incubated with 2 ml of fresh medium for 48 h prior to harvesting the cells and extracting genomic DNA. In the long-term experiments, we continued to passage and culture cells for 10 days before isolating clones by limiting dilution and ring cloning.

***ApoE genotyping and DNA sequencing***

Genomic DNA (DNeasy kit; Qiagen) was extracted from cells for routine PCR-RFLP genotyping (Hixson and Vernier, 1990), amplifying a 227 bp product by 36 cycles (94 °C for 30 sec and 68 °C for 30 sec) with the primer pair, 5'-TCCAAGGAGCTGCAGGCGGCGCA-3' (sense) and 5'-ACAGAATTCGCCCCGGCCTGGTACACTGCCA-3' (antisense) and separating the *Hha*I digested fragments on 20% Tris-buffered EDTA-polyacrylamide gels (Invitrogen). Each genotype gave a specific combination of *Hha*I fragment sizes: apoE2, 91 and 83 bp; apoE3, 91 and 48 bp; and apoE4, 72 and 48 bp (Fig. 1b). A locus-specific PCR was also devised to avoid the appearance of ambiguous fragments, which often result from routine PCR-RFLP analyses involving the apoE4 genotype (Wu et al., 2000). This involved 36 cycles of PCR (94 °C for 30 sec and 68 °C for 30 sec) with the primer pair, 5'-AGGCCCGGCTGGGCGTGGA-3' (sense) and 5'-TGGGAGGCGAGACGCACCCG-3' (antisense) and was designed to amplify a region around the E4/E3 locus, whilst abolishing two *Hha*I restriction sites. The final 118 bp product contains only one *Hha*I site compared to the 4-6 present in the 227 bp product generated during routine PCR-RFLP analysis (Fig. 1B). Each genotype gave a specific combination of *Hha*I fragment sizes following digestion and separation on 20% Tris-buffered EDTA-polyacrylamide gels: apoE4, 85 and 33 bp; apoE3, 118 bp; and apoE4/E3, 118, 85 and 33 bp. The sense primer from the routine PCR-RFLP analysis, and antisense primer from the locus-specific analysis, were used for automated DNA sequencing of purified PCR products.

***'Spiking' experiments***

Two different experiments were designed to evaluate if the chimeraplast, whether

intact or degraded, could function as a primer or template in the PCR-based analyses. In the first study, increasing amounts of intact chimeraplast (3 pg-300 ng) were added directly to the PCR mix, which also contained 100 ng of CHO-E4 DNA. In the second study, CHO-K1 cells were transfected with different amounts of apoE4-to-E3 chimeraplast (600-1000 nM) and 24 h-48 h later DNA was extracted and mixed 1:1 with CHO-E4 or E4-lymphocyte DNA; 100 ng of this total DNA was used in the standard 25 µl PCR reaction. PCR-RFLP analyses were performed as outlined above.

## Results

### *Short-term conversion of APOE4 to APOE3 in recombinant CHO cells and human lymphocytes*

Subconfluent CHO-E4 cells were treated with a 68-mer apoE4-to-E3 chimeraplast (Figure 1a), at a range of concentrations and amine to phosphate (PEI:RNA/DNA oligonucleotide) molar ratios. A clear conversion was seen at each concentration tested using routine PCR-RFLP[17] (Hixson and Vernier, 1990) (Figure 1Bb), although the most efficient conversion was achieved at 800 nM and an 8:1 amine:phosphate molar ratio (Figure 2Aa). These conversions were confirmed by direct sequencing (Figures 2Bb-2Ee), while the same apoE4-to-E3 chimeraplast was used as a negative control and had no effect on CHO-E2 cells (Figure 2Aa).

The *APOE4* gene was also targeted in cultured lymphocytes from a homozygous ε4/ε4 patient for 16 h with increasing concentrations (400-1000 nM) of PEI:chimeraplast at amine:phosphate molar ratios of 5:1-7:1. A clear conversion of *APOE4* to *APOE3* was seen at 800 and 1000 nM, whereas the genotype of E2 lymphocytes, used as a negative control, was unchanged (Figure 3Aa). A 7:1 ratio of PEI to oligonucleotide and 800 nM of chimeraplast gave the highest conversion and this was confirmed by direct sequencing (Figures 3Bb-3eE). However, analyses of the conversions by PCR-RFLP for both cell types were complicated by the appearance of an additional 'unexpected' band at 83 bp. This 'unexpected' band may reflect partial digestion, and its frequent appearance has been noted previously during routinewhen genotyping of apoE4/E3 or apoE4/E4 patients (Wu et al., 2000)[18]. To circumvent this problem a new locus-specific PCR-RFLP was devised

(Figure 1cC) and used in selected studies.

***APOE4 to APOE3 gene conversion by chimeraplasty is not artefactual***

Two methods were employed to investigate whether *APOE4* to *APOE3* conversions could be attributed to PCR artefacts. Firstly, increasing amounts (1 pg-300 ng) of intact apoE4-to-E3 chimeraplast were mixed into a standard PCR-RFLP reaction containing 100 ng of DNA extracted from CHO-E4 cells. This procedure did not give rise to a PCR-generated artefact as no diagnostic apoE3 band (91 bp) appeared (Figure 4Aa). Secondly, CHO-K1 cells were transfected with varying amounts of apoE4-to-E3 chimeraplast (600-1000 nM); 48 h later DNA was extracted from the cells and mixed 1:1 with CHO-E4 or E4-lymphocyte DNA. Again, using locus-specific PCR-RFLP analysis, there was no evidence of any artefactually-produced band, this time using locus-specific PCR-RFLP analysis, implying that any intracellular degradation products do not act as PCR templates or primers (Figure 4Bb).

***Unsuccessful long-term apoE4 to apoE3 conversion in CHO-E4 cells***

CHO-E4 cells were transfected with 800 nM apoE4-to-E3 chimeraplast at an amine:phosphate ratio of 8:1 at an amine:phosphate ratio of 8:1, which gave optimal conversion (Figure 2Aa), and maintained in culture for 10 days, passaging every other day. The cells were then cloned by limiting dilution and ring isolation, expanded and their cellular DNA extracted for analysis by locus-specific PCR-RFLP and for some clones by direct sequencing. No conversion to apoE3 was detectable by PCR-RFLP analysis for any of the clones (Figure 5Aa). Direct DNA sequencing confirmed the genotypes were unchanged and provided no evidence that any of the individual clones analysed might have been converted to the apoE3 genotype (Figure 5B6b).

## Discussion

Chimeraplast-directed substitution of single bases within a gene is a powerful technology with enormous potential. Importantly, like other tissues and organs, the brain as well as liver and other organs is amenable to gene transfer[19] (Shi et al., 2001). Conversion of apoE4-expressing cells to apoE3 is an intriguing possibility; as the ε4 allele is strongly associated with AD and a variety of other neurodegenerative

disorders (Gasparini et al., 1998; Saunders, 2000)[6,7] and also , as well as predictsing poor prognosis in traumatic brain injury (Kay et al., 2003)[720,19]. Therefore, targeting the *APOE4* gene in brains of patients with AD to express apoE3 (or apoE2) could prove beneficial, and an attractive therapeutic possibility, which could be explored first in transgenic mice expressing human apoE4 (Xu et al., 1996; Gong et al., 2002) [210,212].

Here, in short-term preliminary experiments, we targeted recombinant CHO cells expressing apoE4 with an 68-mer apoE4-to-E3 chimeraplast of standard design (Tagalakis et al., 2001)[16] 68-mer. Evidence for a successful conversion of the *APOE4* gene was obtained both by PCR-RFLP analysis (91, 72 and 48 bp bands) and by direct sequencing of the PCR product, which showed an apoE4/E3 genotype for treated cells. Genomic *APOE4* was also targeted in cultured transformed lymphocytes from a homozygous ε4/ε4 patient using the same chimeraplast; clear conversion to the apoE4/E3 genotype was seen by PCR-RFLP and confirmed by sequencing. These conversions did not occur through a PCR artefact. The diagnostic band did not appear in spiking experiments when the chimeraplast was added directly to the PCR mix or when DNA extracted from transfected CHO-K1 cells, which potentially contained degraded chimeraplast fragments, was used in the PCR reaction. Therefore, we reject the possibility that intact chimeraplasts, or chimeraplast degradation products generated intracellularly, can act as primers and/or template, as suggested by some researchers (Thomas et al., 1997; Zhang et al., 1998)[232].

When CHO-E4 cells were cultured for 10 days after chimeraplasty and then cloned, no conversion was detected in over 90 clones analysed by locus-specifc PCR-RFLP. This longer term study implied that the conversion to *APOE3* was not stable, in contrast with our previous finding for *APOE2* to *APOE3* gene repairs (Tagalakis et al., 2001)[16] or the reports of other researchers targeting different genes[13-15] (Kren et al., 1998; Alexeev et al., 2000; Bertoni and Rando, 2002). One explanation for this apparent instability might be an irreversible cytotoxic action of the transfection complex on the CHO-E4 cells, perhaps making them vulnerable to apoptosis. Consistent with this possibilityInterestingly, we found that the concentration of apoE4-to-apoE3 chimeraplast needed for successful conversion was high (800 or 1000 nM; Figures 2 and 3), whereas our previous conversions with apoE2-to-apoE3 chimeraplasts were accomplished efficiently with lower amounts (200 or 400 nM)

(Tagalakis et al., 2001)[16]. Thus, a two-fold or greater increase in chimeraplast, or perhaps more significantly its carrier PEI, which has potential cytotoxicity (Fischer et al., 1999; Olsen et al., 2003)[24,43] [xx], might have contributed to the instability of the repair. An alternative, though less likely explanation, is that the switch from an apoE4 to apoE3 phenotype promoted cell death, perhaps because of a difference in intracellular trafficking of the two apoE isoforms (Strittmatter et al., 2002; DeKroon and Armati, 2003)[8,25,54]. However, it should also be noted that our cloning analysis may have missed low-level stable conversions of ≤1 %, which have previously been reported in mammalian cells using sensitive reporter gene assays (Thorpe et al., 2002a; Tran et al., 2003; Nickerson and Colledge, 2003)[26,65-28,87].

Our current failure to achieve detectable long-term correction of the dysfunctional *APOE4* gene mirrors several recent reports highlighting failure or poor reproducibility when targeting other genes (Van der Steege et al., 2001; Albuquerque-Silva et al., 2001; Taubes, 2002)[29,98-31,0]. This is perhaps not entirely unexpected as the technology is relatively new and evolving with many factors yet to be optimized (Graham et al., 2001; Yoon et al., 2002)[32,1,33,2]. One problem may be the quality of the chimeraplast itself; the reagents are long molecules which tend to self-associate during synthesis, both features that would promote formation of N-1 failure fragments and other impurities (Manzano et al., 2003)[34,43]. Indeed, the higher concentration of chimeraplast needed to convert *APOE4* to *APOE3*, compared to *APOE2* to *APOE3* conversions (Tagalakis et al., 2001)[16], may simply reflect a lower quality reagent. An additional problem, in some ways common to general gene therapy strategies, is to ensure efficient delivery to the cell nucleus (and *in vivo* to the target organ itself), avoiding reagent degradation or cytotoxic effects (Ogris and Wagner, 2002)[35,54]. Finally, more work is needed into basic mechanisms by which cells accomplish mismatch repair, an area about which we remain largely ignorant. Stimulation of repair by synchronizing cell cycles to the S-phase (Majumdar et al., 2003)[36,64] or by increasing expression of RAD51 (Thorpe et al., 2002a,b; Liu et al., 2001)[26,65,37,76,38,87] appear useful starting points for improving efficiency. Given the enormous potential of oligonucleotide-directed gene repair, it is hoped that such developments will allow progress towards the final goal — a viable treatment for disease caused by ing point mutations, including neurodegenerative disorders associated with the ε4 allele.

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## FIGURE LEGENDS

**Figure 1** Chimeraplast design and apoE genotype analysis by PCR-RFLP. (a) The sequence of the apoE4-to-E3 chimeraplast, which is internally matched[16], is shown with the DNA residues in capital letters and the 2'-*O*-methylated RNA bases in lowercase. The all-DNA 'correcting strand' is underlined with the central mutating residue double-underlined, while the DNA mutator region of the 'targeting strand' is shown in bold. (b) Routine genotyping[17] was performed by amplifying a 227 bp PCR product from exon IV and restriction isotyping with *Hha*I. Cleavage sites for *Hha*I are shown by arrows and are given for apoE2, E3 and E4 amplified sequences; codons 112 and 158 are depicted as filled boxes. (c) Locus-specific PCR-RFLP was introduced to avoid ambiguities arising from routine genotyping. E4/E3 locus-specific primers were designed to amplify a smaller fragment, whilst abolishing two *Hha*I sites during the PCR reaction. The complete sequence of the amplified 118 bp product, which encompasses the E4/E3 locus, is shown; the residues matching the PCR primers are in bold and the two C nucleotides which are modified during PCR to T are underlined. The four nucleotides in bold (**GCGC**) between the primer pair denotes the only *Hha*I site now left in the PCR product with the underlined cytosine being the nucleotide targeted by chimeraplasty.

**Figure 2** Conversion of the apoE4 cDNA in stably-transfected recombinant CHO cells to apoE3 by chimeraplasty. (a) Clear conversions of apoE4 to apoE3 cDNA (C→ T) were seen 48 h after transfecting recombinant CHO-E4 cells with the 68-mer apoE4-to-E3 chimeraplast at 800 nM at increasing amine:phosphate (PEI:RNA/DNA oligonucleotide) molar ratios (6:1-8:1), as judged by appearance of the diagnostic 91 bp band. (b-e) Sequencing chromatograms of chimeraplast-treated CHO-E4 cells. As expected, the untreated CHO-E4 cells (b) had only a C at the respective codon (112) of the apoE cDNA (arrowed), whereas partial gene conversion was evident in CHO-E4 cells treated with 800 nM of chimeraplast, since the T expected for apoE3 was additionally present (c-e). The highest conversion (e)

was seen at an amine:phosphate molar ratio of 8:1 (e), whereas lower conversion efficiencies were observed at ratios of 6:1 (c) and 7:1 (d).

**Figure 3** Converting genomic the *APOE4* gene to *APOE3* in human lymphocytes. (a) Clear conversions of the *APOE4* gene to *APOE3* (C→ T) wasere seen 48 h after transfecting EBV-transformed lymphocytes from a patient homozygous for the ε4 allele with the 68-mer apoE4-to-E3 chimeraplast using increasing oligonucleotide concentrations and different amine:phosphate molar ratios, as judged by appearance of the diagnostic 91 bp band. Ladder, 10 bp markers; E2-control, lymphocytes from an ε2/2 patient treated with the apoE4-to-E3 chimeraplast. (b-e) Sequencing chromatograms of chimeraplast-treated E4-lymphocytes. Partial gene conversion was confirmed (b**)** by direct sequencing ofas the PCR product from treated E4-lymphocytes (800 nM, 6:1 amine:phosphate molar ratio), sincewhich had the T predicted for apoE3 was present as well as the C of apoE4. The highest conversion (c) was seen at 800nM and a 7:1 amine:phosphate molar ratio, while lower conversions were noted at 1000 nM and at 6:1 (d) or 7:1 (e) amine:phosphate molar ratios.

**Figure 4** Chimeraplast-mediated conversion of E4 to E3 conversion is not artefactual. (a) Varying amounts (1 pg-300 ng) of intact apoE4-to-E3 chimeraplast were added to the routine PCR-RFLP reaction mix containing DNA extracted from untreated CHO-E4 cells. This PCR reaction did not produce artefacts as no diagnostic band (91 bp) appeared. Lad, 10 bp markers; Con, control unspiked CHO-E4 cells. (b) Cultured CHO-K1 cells were transfected with increasing amounts of apoE4-to-E3 chimeraplast (600-1000 nM) and 48 h later DNA was extracted and mixed 1:1 with DNA from untreated CHO-E4 cells or E4-lymphocytes. Analysis by locus-specific PCR-RFLP failed to reveal a diagnostic apoE3 band (118 bp), implying that chimeraplast-degradation products did not generate artefacts. Lad, 10 bp markers.

**Figure 5** Long-term conversion of the *APOE4* gene to *APOE3* was not successful in recombinant CHO-E4 cells. (a) CHO-E4 cells were transfected optimally with 800 nM apoE4-to-E3 chimeraplast at an 8:1 amine:phosphate molar ratio. The cells were maintained in culture for 10 days, passaging several times, and then 93 clones isolated and expanded by limiting dilution and ring cloning. Cellular DNA was isolated for analysis by PCR-RFLP and, in some cases, by direct sequencing; no conversion to apoE3 was detectable in any of the clones. (a) Locus-specific PCR-RFLP analysis of 8 clones picked consecutively (clones 9-16), which all show complete absence of the diagnostic 118 bp. Con, control untreated CHO-E4 cells. (b) Sequence chromatogram of the 118 bp PCR product from a representative clone (clone 15). There was no evidence for conversion of the G in apoE4 (circled) to the A expected for apoE3.

Figure 1

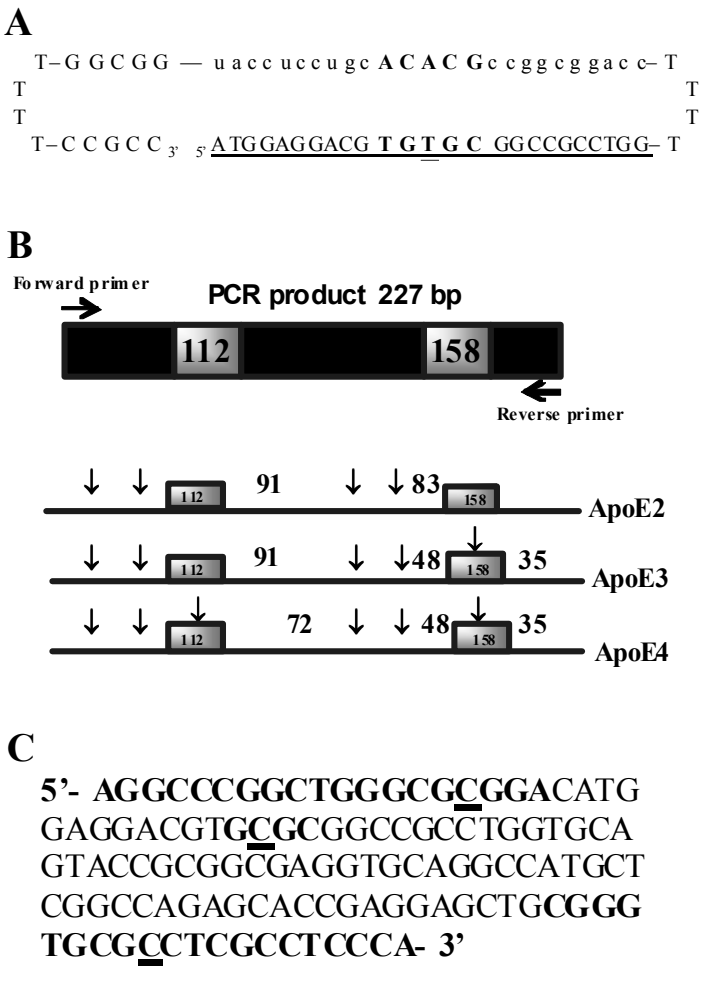


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Figure 2

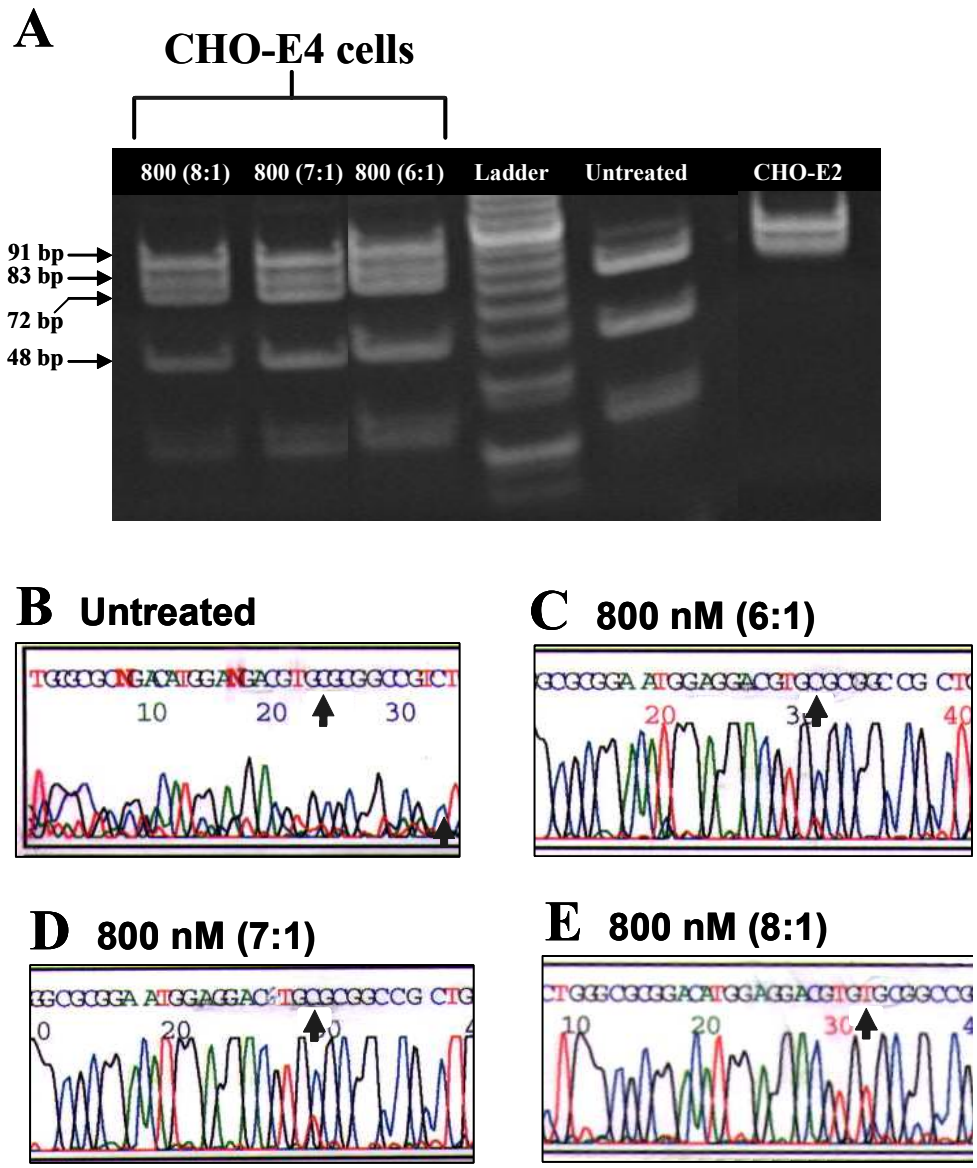

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Figure 3

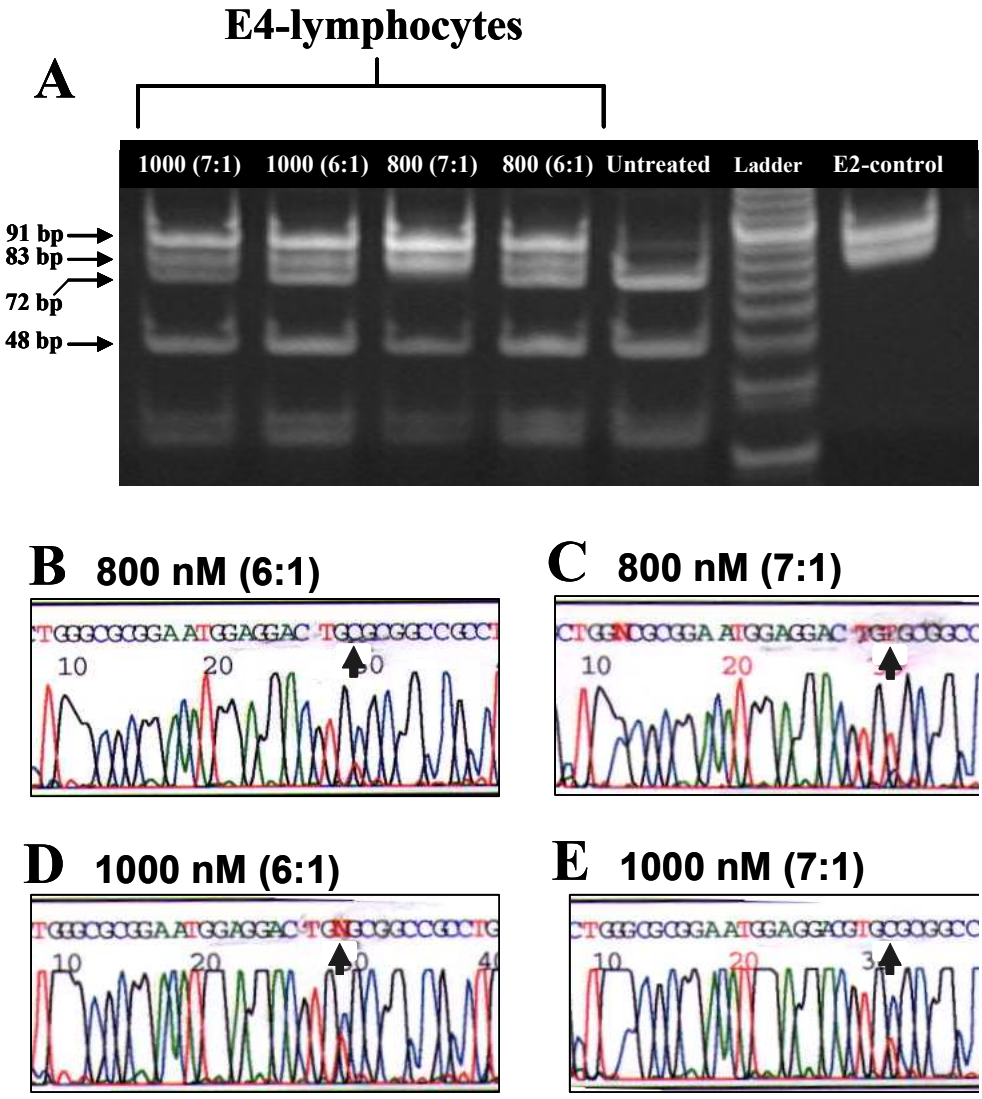
A
E4-lymphocytes
1000 (7:1)
1000 (6:1)
800 (7:1)
800 (6:1)
Untreated
Ladder
E2-control
91 bp
83 bp
72 bp
48 bp
B 800 nM (6:1)
C 800 nM (7:1)
D 1000 nM (6:1)
E 1000 nM (7:1)

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Figure 4

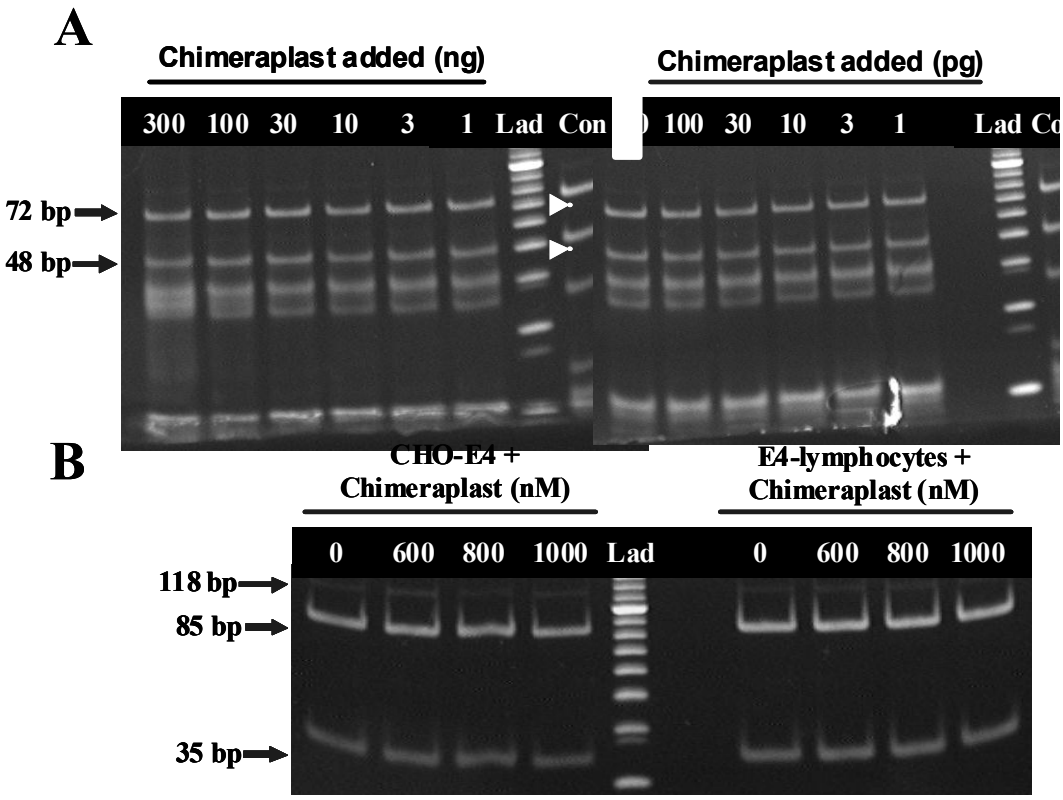
A
Chimeraplast added (ng)
Chimeraplast added (pg)
300 100 30 10 3 1 Lad Con
100 30 10 3 1 Lad
72 bp
48 bp
B
CHO-E4 +
Chimeraplast (nM)
E4-lymphocytes +
Chimeraplast (nM)
0 600 800 1000 Lad
0 600 800 1000
118 bp
85 bp
35 bp

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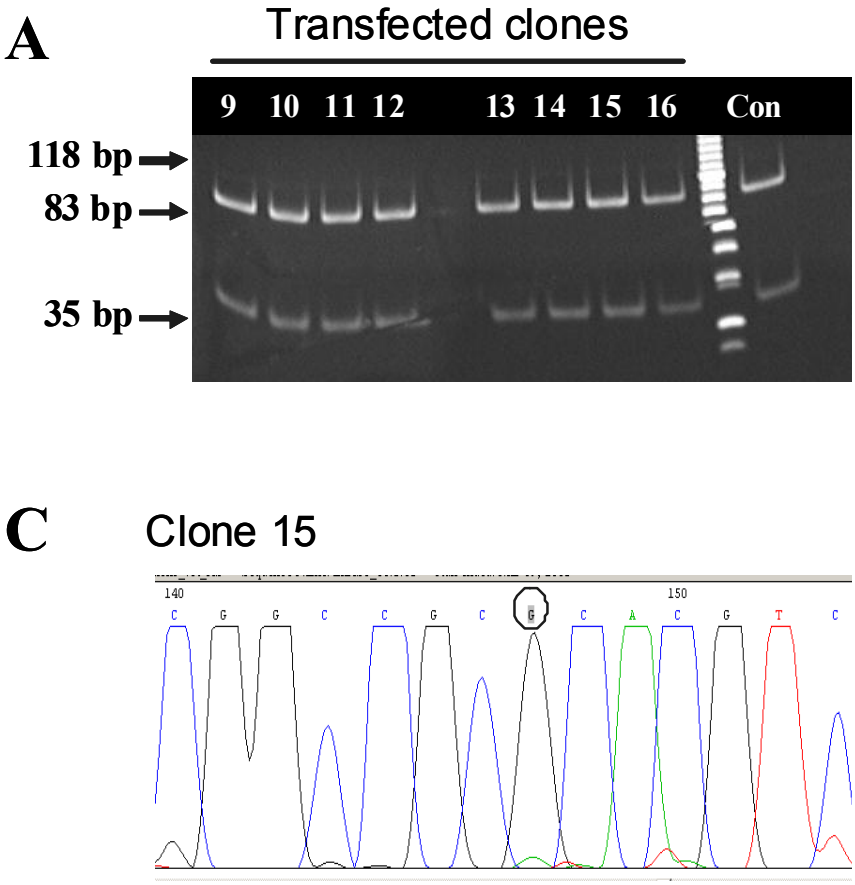
Figure 5


A
Transfected clones
9 10 11 12 13 14 15 16 Con
118 bp
83 bp
35 bp
C
Clone 15
140
150

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