CMLS, Cell. Mol. Life Sci. 62 (2005) 362–376
1420-682X/05/030362-15
DOI 10.1007/s00018-004-4443-y

CMLS Cellular and Molecular Life Sciences

Research Article

Interference with MCP-1 gene expression by vector generated triple helix-forming RNA oligonucleotides

K. Kautz, M. Schwarz and H. H. Radeke*

Pharmazentrum Frankfurt, Dr.-Hans-Schleussner-Foundation, Immune Pharmacology, Clinic of the Goethe University, 60590 Frankfurt (Germany), Fax +49 69630183202, e-Mail: radeke@em.uni-frankfurt.de

Received 7 October 2004; received after revision 29 November 2004; accepted 2 December 2004

Abstract. Triple helix-forming oligonucleotides (TFOs) that specifically bind to double-stranded DNA sequences can be rationally designed, while intracellular delivery of single stranded RNA TFOs has not yet been studied in detail. In this report, we demonstrate gene and sequence-specific inhibition of MCP-1 gene expression due to interference of intracellular-generated single-stranded RNA (CU-TFO) with an overlapping SP-1/AP-1 target. Binding of synthetic 19-nucleotide (19-nt) CU-TFO to the MCP-1 promoter duplex was verified by triplex blotting. Furthermore, we confirmed binding of a 1.1-kb fusion transcript containing the 19-nt pyrimidine CU sequence to a plasmid-encoded MCP-1 promoter target duplex at pH 7.0. In tumour necrosis factor-α-stimulated HEK cells, CU-TFOs inhibited MCP-1 protein release by 76 ± 10.2% compared to intracellular-generated control oligonucleotides. Interleukin-8 as a control target gene was not affected by CU-TFO, confirming both highly specific and effective chemokine gene repression by transfectable TFO-shuttle vectors.

Key words. Triple helix-forming oligonucleotide (TFO); antigene strategy; chemokine; monocyte chemoattractant protein-1 (MCP-1); AP-1; SP-1; triplex; tumour necrosis factor; interferon-gamma.

Specific interference of gene expression has wide-ranging applications in experimental biology and is an attractive approach for the development of new therapeutics. Several gene-specific inhibitory strategies have been established in vitro but rarely tested in vivo. Synthetic DNA/RNA oligonucleotides either inhibit mRNA translation (antisense strategy) or may destroy specific mRNA molecules when delivered in the form of ribozymes or siRNA. As an alternative to these approaches, gene expression may be modulated at an earlier step, i.e. at the level of transcription, by molecules that specifically interact with the DNA double helix. Interest in oligonucleotides designed to form triple helices with double-stranded DNA is steadily increasing, partially because of their potential as artificial repressors of gene expression and as mediators of site-specific DNA cleavage. Modulation of transcription by triple helix-forming oligonucleotides (TFOs) was described in cell-free systems and in intact cells using e.g. plasmid targets transiently transfected into these live cells [1, 2], but also for endogenous genes [3]. Moreover, at a given promoter, triplex formation was shown to block the binding of various transcription factors, including SP-1, thereby inhibiting transcription initiation [4]. Triplex formation also augmented site-directed mutagenesis and promoted recombination in a site-specific manner [5–7]. Triplex-based activity in animals was demonstrated by successfully introducing site-specific genomic modifications in somatic cells of adult mice [8]. The triplex-mediated

* Corresponding author.

antigene approach has been used to selectively inhibit a wide array of clinically important genes. Examples include transcriptional inhibition of aldehyde dehydrogenase [9], interleukin (IL)-2 receptor [10], the human HER2/neu gene [11], c-myc [12, 13] and granulocyte/macrophage colony-stimulating factor [14]. The mechanisms underlying triple helix formation are reasonably well understood. TFOs specifically bind to the major groove of DNA, forming Hoogsteen- or reverse Hoogsteen-hydrogen bonds with bases in the purine-rich strand of the duplex DNA. Optimal target sequences must harbour 12–14 consecutive purines on one strand [15] since only purine bases are able to establish two Hoogsteen- or reverse Hoogsteen-type hydrogen bonds. Two principle binding motifs have been described: for the pyrimidine motif, a TFO consisting of cytosine (C) and thymine (T) binds with a parallel orientation to the purine strand of DNA via Hoogsteen bonds. Sequence specificity is mediated by binding of third-strand thymines (T) to adenine in A:T base pairs while protonated cytosines (C+) bind to guanine in G:C base pairs. For the purine-binding motif, the TFO binds antiparallel to the purine-rich strand of the DNA via reverse Hoogsteen bonds. In this case-triplex formation is mediated by binding of adenine or thymine to adenine in A:T base pairs, and guanine to guanine in G:C base pairs.

Out of a high number of possible TFO targets in the mammalian genome that we have located with a gene bank search algorithm [H. H. Radeke and B. Bruno, unpublished data], we have chosen a purine-rich 19-bp target sequence close to the ATG start site of the human chemokine, monocyte chemoattractant protein-1 (MCP-1) gene.

MCP-1, a member of the CC chemokine superfamily, functions in attracting monocytes [16], T lymphocytes [17–19], basophils [20] and B cells [21] to sites of inflammation. MCP-1 is expressed in different cell types including monocytes, T lymphocytes, endothelial cells, fibroblasts and, in the case of injury, a number of organ tissue cells, such as renal mesangial cells [22–24]. Several studies have shown that MCP-1 plays a major role in the initiation and progression of various forms of nephritis [25–31]. In addition, the expression of MCP-1 is associated with the pathogenesis of many other chronic inflammatory diseases, e.g. atherosclerosis [32] and multiple sclerosis [33]. The expression of MCP-1 is regulated by cytokines including tumour necrosis factor (TNF) [34], platelet-derived growth factor (PDGF) [35], interferon-γ (IFN-γ) [36] and IL-1β [23, 37], but stress factors and viral infections also cause MCP-1 expression [38, 39]. Several regulatory promoter regions important for MCP-1 expression have been identified. Two nuclear factor-κB-binding sites located approximately 2.6 kb from the transcription initiation site appeared to be critical elements for MCP-1 induction in response to IL-1β and TNF-α [40, 41]. Additionally, within an 150-bp segment located 5′ to the ATG translation start codon, two consensus elements for the AP-1 trans-acting factors have been described in the human MCP-1 promoter. The more proximal of these consensus AP-1 elements overlaps with a consensus site for SP-1 that appears to be important for the basal transcriptional activity of the gene [41]. Mutation of the SP-1-binding site proved to be critical for regulation by both TNF and PDGF [42]. Thus, both disease findings and gene regulatory studies at the molecular level point to a considerable proinflammatory role for MCP-1. Several therapeutic strategies including neutralizing antibodies and protein antagonists have been employed to reduce MCP-1 activity in nephritis and other inflammatory diseases [43]. These antiprotein/receptor strategies as well as inflammatory disease models in MCP-1 gene-deficient mice revealed variable and mostly limited success, which might be due in part to incomplete inhibition of MCP-1 activity [31].

The human MCP-1 promoter region contains a 19-bp TFO target site spanning the binding motif for SP-1 and partially overlapping a putative AP-1-binding site. Previously, we demonstrated that a 19-nucleotide (19-nt) purine TFO binding in an antiparallel orientation to the human MCP-1 promoter formed a sequence-specific triplex with the target DNA in vitro, blocked in vitro binding of nuclear extract proteins and recombinant SP-1 to the promoter DNA and, when added extracellularly, inhibited MCP-1 expression in HEK 293 cells by 45% [44]. In the present study, we investigated a new approach to optimise triplex oligonucleotide delivery by an intracellular shuttle vector. In this way, we sought to overcome obvious limitations of the oligonucleotide-based technology, such as insufficient delivery to nuclei and the need to apply chemically modified TFOs to ensure stability in the extracellular and intracellular environment. As described here, we achieved this goal by designing an eukaryotic expression vector transcribing a triplex-forming RNA sequence as a pyrimidine-binding motif together with the hygromycin resistance mRNA as one fusion transcript. HEK 293 cells stably transfected with this vector exhibited a marked reduction in MCP-1 expression. To confirm that triplex formation was be the true mechanism of action responsible for these significant results, we identified sequence-specific triplex formation with a synthetic 19-nt TFO. Furthermore, we showed binding of the 1.1-kb fusion transcript containing the CU-TFO to a plasmid-encoded MCP-1 promoter duplex target. Therefore, the experimental approach presented here, i.e. using targetable shuttle vectors to generate triplex-forming oligoribonucleotides inside the target cell, opens up an entirely new and promising avenue to inhibit transcription initiation of clinically relevant genes.

Materials and methods

Oligonucleotides and reagents

Non-modified oligoribonucleotides used in triplex blotting experiments were purchased from MWG (Munich, Germany) and contained the 19-nt TFO with the pyrimidine, parallel-binding CU motif (rCU-TFO 5′UCU CCC UCU CUC CCA CCU C 3′), four different controls [rGA-control (5′GAG GUG GGA GAG AGG GAG A 3′, purine], antiparallel-binding motif), rGU-control(5′GUG GUG GGU GUG UGG GUG U 3′, purine], antiparallel-binding motif), rCA-control (5′CAC ACA CCC AAC CAC ACC C 3′, scrambled sequence)] and a control with the same sequence as the pyrimidine TFO but containing one mismatch (19CU_M, 5′UCA CCC UCU CUC CCA CCU C 3′). Oligonucleotides for construction of the TVHygro vector were obtained from MWG. Oligonucleotides for PCR were purchased from Qiagen Operon (Cologne, Germany). Restriction enzymes and buffers used were from MBI Fermentas (St. Leon-Rot, Germany) if not otherwise stated.

Preparation of radio-labelled double-stranded MCP-1 promoter fragment and a plasmid containing the MCP-1 promoter duplex

The duplex DNA used for triplex blotting experiments corresponds to the region between bp –76 and bp –38 of the human MCP-1 promoter (fig. 1A–C). A modified luciferase pGL3-control vector (Promega, Madison, Wis.) containing the 39-bp target sequence between *Hind*III and *Nco*I restriction sites [unpublished data] was digested with the respective restriction enzymes *Hind*III and *Nco*I (New England Biolabs, Beverly, Mass). The resulting fragment contained the 39-bp MCP-1 promoter duplex plus 5′ overhang on each side. The fragment was electrophoretically fractionated with an 1% agarose gel, excised by a scalpel, and DNA extracted using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). The resulting 5′ overhang of the duplex DNA was end-filled and [α-^{32}P]-dCTP radiolabelled using Klenow fragment exo$^-$ (MBI Fermentas) in the presence of 0.02 mM of dGTP, dATP and dTTP and 1.85 MBq [α-^{32}P]-dCTP (Amersham Pharmacia Biotech, Freiburg, Germany). Unincorporated nucleotides were removed using the Qiaquick nucleotide removal kit (Qiagen). In the following, the radio-labelled duplex is referred to as the '39-bp MCP-1 promoter duplex'. Sequences for the duplex MCP-1_2M containing two mismatches, 5′ AGC TTT CCT GCT TGA CTC CGC CCT CTA TCC CTC GGC CCG CTT TCC 3′ and 5′ CAT GGG AAA GCG GGC CGA GGG ATA GAG GGC GGA GTC AAG CAG GAA 3′ (MWG), were annealed in equal amounts and radio-labelled as described above. The amount of radio-labelled duplex DNA used in triplex blotting experiments ranged between 3×10^{-3} and 0.1 nmol. For labelling of the plasmid pGL3 control containing the MCP-1 promoter duplex the vector was linearised by *Bgl*II restriction digestion. About 100 ng (~3.1×10^{-3} nmol) was labelled with Klenow fragment (Fermentas) according to the supplier's protocol.

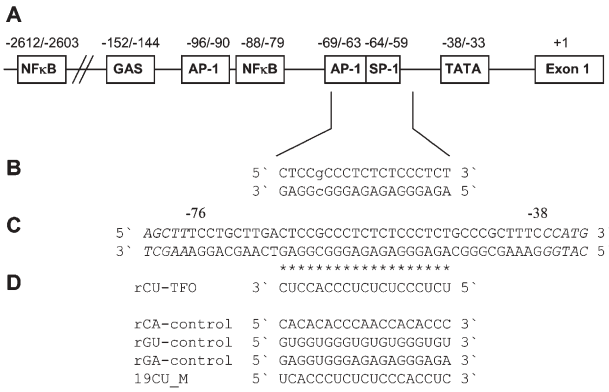


Figure 1. Structure of the human MCP-1 promoter, TFO target sequence in the promoter, sequences of the oligoribonucleotides and the 39-bp promoter fragment used in this study. The proximal promoter region of the human MCP-1 gene contains putative binding sites for the transcription factors NF-κB, AP-1 and SP-1, as well as a TATA box and a GAS (*A*). A homopurine/homopyrimidine 19-bp TFO target site was identified at bp –66 to –48, a point of mismatch is shown by small letters (*B*). A 39-bp MCP-1 promoter fragment spanning bp –76 to –38 was obtained by restriction digestion (see Materials and methods); sequences according to the restriction enzymes *Hind*III and *Nco*I which do not belong to the MCP-1 promoter are shown in italics. 5′ overhangs due to restriction digestion were end-filled and radio-labelled using Klenow fragment (*C*). Five different 19-nt oligoribonucleotides were designed: TFO as a pyrimidine oligoribonucleotide binding to the target site in a parallel manner (rCU-TFO), and four 19-nt controls including purine oligoribonucleotide (rGU-control and rGA-control), a CA oligoribonucleotide with scrambled sequence (rCA-control) and a control which was the same as rCU-TFO but with one mismatch at position 3 (19CU_M) (*D*).

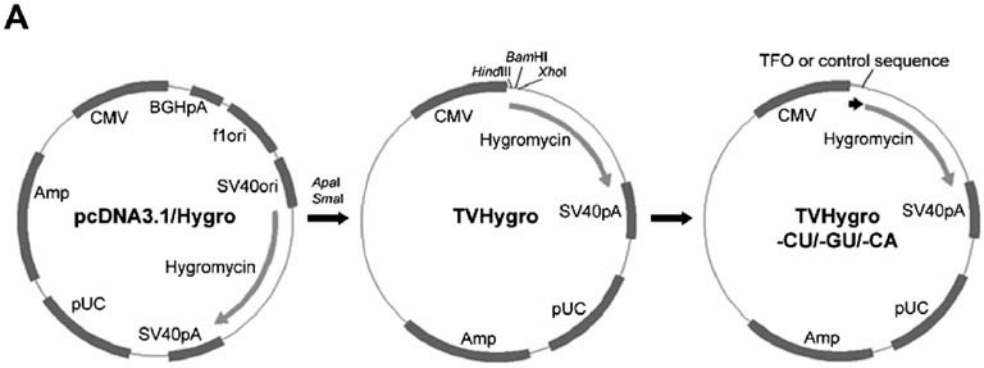
Triplex blotting

Triplex blotting is a technique described for the detection of specific RNA molecules capable of triplex formation. Triplex blots were started by electrophoretic separation of the 19-nt oligoribonucleotides (fig. 1D) by 7.0 M urea/6% polyacrylamide gels. Nucleic acids were transferred to nylon filters (Hybond-N; Amersham Pharmacia Biotech, Little Chalfont, UK) in a buffer consisting of 17 mM NaH_2PO_4, 8 mM Na_2HPO_4, followed by UV cross-linking (150 mJ/cm^2). Then, filters were pre-hybridised for 1 h at 30° or 37°C in Rapid-Hyb buffer (Amersham) adjusted to pH 5.5 or 6.7 or in a buffer containing 5×NAE (500 mM sodium acetate, 5 mM EDTA adjusted to pH 5.5 or 6.7), 5× Denhardt's solution, 1% SDS and 20 µg/ml of salmon sperm DNA. Following pre-hybridisation, blots were hybridised overnight with the radio-labelled duplex DNA containing the target sequence in the same solution at 30° or 37 °C with gentle shaking. Subsequently, filters were washed twice by shaking in 2× NAE (200 mM sodium

acetate, 2 mM EDTA pH 5.5 or 6.7) and 0.1% SDS for 20 min at 30° or 37 °C. Filters were exposed to Phospho-Imager screens (Fuji, Kanagawa, Japan), visualised using a Fuji BAS-MS Phospho-Imager (Fuji Imaging) and analysed by Phospho-Imager software (TINA; Raytest Company, Straubenhardt, Germany).

Construction of vectors TVHygro-CU, TVHygro-GU and TVHygro-CA

Transfection vectors were constructed to drive transcription of either RNA containing the 19-nt pyrimidine triplex-forming sequence targeting the MCP-1 promoter (CU-TFO) or RNA containing two different 19-nt control sequences (GU-control or CA-control). The vector TVHygro was derived from the eukaryotic expression vector pcDNA3.1/Hygro (Invitrogen, Karlsruhe, Germany) with the sequences spanning the region 1002–2079 deleted by restriction with the enzymes *Apa*I and *Sma*I. The resulting *Apa*I overhang was end-filled and the vector religated. Three different DNA sequences were cloned into the 5′ untranslated region of the hygromycin resistance gene, resulting in the vectors TVHygro-CU, TVHygro-CA and TVHygro-GU (fig. 2A). The constructs leading to TVHygro-CU (5′GTC GAC



B

TVHygro-CU

```
     5′ GTCGACAAGCTTCTCGAGTCTCCCTCTCTCCCACCTCGGATCCCGCGAT 3′
     3′ CAGCTGTTCGAAGAGCTCAGAGGGAGAGAGGGTGGAGCCTAGGGCGCTA 5′
           HindIII XhoI                        BamHI
RNA: CU-TFO          5′…UCUCCCUCUCUCCCACCUC…Hygromycin…3′
```

TVHygro-GU

```
          5′ AAGCTTCTCGAGACACCCACACACCCACCACGGATCC 3′
          3′ TTCGAAGAGCTCTGTGGGTGTGTGGGTGGTGCCTAGG 5′
             HindIII XhoI                        BamHI
RNA: GU-control       5′…GUGGUGGGUGUGUGGGUGU…Hygromycin…3′
```

TVHygro-CA

```
          5′ AAGCTTCTCGAGCACACACCCAACCACACCCGGATCC 3′
          3′ TTCGAAGAGCTCGTGTGTGGGTTGGTGTGGGCCTAGG 5′
             HindIII XhoI                        BamHI
RNA: CA-control       5′…CACACACCCAACCACACCC…Hygromycin…3′
```

Figure 2. Schematic representation of the vector construction. The vector TVHygro was derived from the eukaryotic expression vector pcDNA3.1/Hygro with the sequences spanning the region 1002–2079 deleted by restriction digestion with the enzymes *Apa*I and *Sma*I. The resulting *Apa*I overhang was end-filled and the vector religated. TFO-RNA or control-RNA coding sequences were cloned into the 5′ untranslated region of the hygromycin resistance gene. Transcription of the TFO/hygromycin and control/hygromycin fusion transcripts, was driven by the constitutive CMV promoter (*A*). Vectors were constructed to drive transcription of either a pyrimidine triplex-forming RNA sequence targeting the MCP-1 promoter (CU-TFO) or two different control sequences (CA-control and GU-control) (see fig. 1D). The three different constructs which were cloned into TVHygro are shown (*B*), leading to the vectors TVHygro-CU, TVHygro-GU and TVHygro-CA. Vector TVHygro without insert was used as a third control vector. HEK 293 cells were transfected with these vectors and single clones were isolated. Different stable cell lines were generated under continuing selection pressure (250 µg/ml hygromycin).

AAG CTT CTC GAG TCT CCC TCT CTC CCA CCT CGG ATC CCG CGA T 3′ and 3′ CAG CTG TTC GAA GAG CTC AGA GGG AGA GAG GGT GGA GCC TAG GGC GCT A 5′) and TVHygro-CA (5′ AAG CTT CTC GAG CAC ACA CCC AAC CAC ACC CGG ATC C 3′ and 3′ TTC GAA GAG CTC GTG TGT GGG TTG GTG TGG GCC TAG G 5′) were digested with the enzymes *Hind*III and *Bam*HI, and cloned into TVHygro in *Hind*III/*Bam*HI orientation. The construct leading to vector TVHygro-GU (5' AAG CTT CTC GAG ACA CCC ACA CAC CCA CCA CGG ATC C 3' and 3' TTC GAA GAG CTC TGT GGG TGT GTG GGT GGT GCC TAG G 5') was *Bam*HI and *Xho*I digested and cloned into TVHygro in *Bam*HI/*Xho*I orientation (fig. 2B). Each of the cloned sequences was transcribed in line with the hygromycin resistance mRNA under the control of the constitutive cytomegalovirus (CMV) promoter, resulting in one fusion transcript. TVHygro served as an additional control vector without insert.

Stable transfection of HEK 293 cells

HEK 293 cells (CRL 1573) were obtained from the American Type Culture Collection (Manassas, Va.). Cells for routine culture were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (BioWhittaker, Verviers, Belgium) and penicillin/streptomycin (Sigma, Deisenhofen, Germany). HEK 293 cells were transfected with the constructed vectors TVHygro-CU, TVHygro-GU, TVHygro-CA or TVHygro (no insert) using Polyfect transfection reagent (Qiagen) according to the manufacturer's instructions. Selection of TVHygro-CU, TVHygro-GU, TVHygro-CA and TVHygro transfectants was carried out in the presence of 250 µg/ml hygromycin (PAA Laboratories, Cölbe, Germany). Single transfectants were isolated and single-cell clones of each of the transfectants were expanded under continuing selection pressure.

Cell culture and cytokine treatment

For analysis of cytokine induced stimulation of chemokine-production, HEK 293 cells were plated in 24-well plates in medium as described above at a density of 9×10^4, 1.5×10^5 and 3×10^5 cells/well and grown overnight. After 18 h culture, medium was replaced with 500 µl of fresh medium per well, and cells were treated with 500 U/ml TNF-α (Bissendorf Peptides, Bissendorf, Hannover, Germany) and/or 1000 U/ml IFN-γ (Cell Concepts, Umkirch, Germany) for 24 h. Then, culture media were harvested, centrifuged for 5 min at 12,000 g to remove cell debris and stored at –20 °C until further analysis of secreted proteins by ELISA.

Isolation of nuclei, preparation of RNA and Northern blot analysis

HEK 293 cells at 90–100% confluence were washed twice and scraped into ice-cold PBS and pelleted by centrifugation at 500 g for 5 min, 4 °C. Nuclei were isolated by lysing the cells in Nonidet P-40 lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% (v/v) NP-40 for 5 min on ice. Nuclei were pelleted by centrifugation at 500 g for 5 min, 4 °C. After an additional resuspension in NP-40 lysis buffer, the nuclei were pelleted by centrifugation again and the supernatant was discarded. Nuclei were resuspended in glycerol storage buffer containing 50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA. Nuclei were frozen and stored in liquid nitrogen. RNA was isolated using the Trizol Reagent (Invitrogen) according to the manufacturer's instructions. For isolation of total RNA, cells were washed twice in ice-cold PBS and RNA was isolated according to the Trizol protocol. For Northern blot analysis, 5 or 10 µg RNA per lane were electrophoresed in a formaldehyde-agarose gel and transferred to a nylon membrane. Hybridization was performed using a radio-labelled, denatured 522-bp cDNA probe specific for the hygromycin resistance gene RNA.

Reverse transcription and PCR

First-strand synthesis was done with a ThermoScript RT-PCR System (Invitrogen) using 1–2 µg RNA. PCR was performed using Platinum Taq DNA Polymerase (Invitrogen). Thermocycler conditions: 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The following oligonucleotide primers were used at a 200 nM final concentration: upstream primer 1 = rCU 5′ TCT CCC TCT CTC CCA CCT C 3′, upstream primer 2 = rGU 5′ GTG GTG GGT GTG TGG GTG T 3′, upstream primer 3 = rCA 5′ CAC ACA CCC AAC CAC ACC C 3′ and downstream primer Hygro = 5′ TCA CGC CAT GTA GTG TAT 3′.

In vitro transcription of single-stranded 1100-nt RNA containing the 19-nt pyrimidine TFO sequence or control sequences

The vector segments encoding TFO or control sequences and an additional part of the hygromycin resistance gene were amplified by PCR using the upstream primer fw_TVHygro 5′ AGC AGA GCT CTC TGG CTA A 3′ and downstream primer rev_TVHygro 5′ TCG GCG AGT ACT TCT ACA C 3′ at 200 nm final concentration and Platinum Taq DNA Polymerase. Thermocycler conditions: 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. Products were purified using the Qiaquick PCR Purification kit. The PCR product was used as template for in vitro transcription. Product amplification included the T7 promoter sequence of the vector. In vitro transcription was performed with the T7 Ribomax Express Large Scale RNA Production System (Promega) according to the supplier's protocol. Correct sizes (1.1 kb) of the transcribed RNAs were proven by electrophoresis with 1% agarose gels.

Magnetic capture of triplex complexes containing target plasmid and single-stranded RNA transcript

Annealing of the biotinylated oligonucleotide 5′ biotin-GCG CTT CTG CGG GCG ATT TGT GTA CGC CCG 3′ (HPCL purified and delivered by MWG) to in vitro-transcribed RNA (denatured at 90 °C for 5 min) was achieved by mixing 400 pmol RNA with 400 pmol biotinylated oligonucleotide in annealing buffer (100 mM potassium acetate, 30 mM HEPES, 2 mM magnesium acetate, pH 7.4). The reaction was incubated for 1 h at room temperature. Quantitative annealing of the oligonucleotide to the single-stranded RNA was confirmed by gel electrophoresis through an 1% agarose gel. The annealing reaction was incubated with 2 µg of the vector pGL3-control (Promega) containing a 39-bp MCP-1 promoter target sequence. Incubation was performed in triplex buffer (20 mM Tris-HCl, 1.5 mM MgCl2, 140 mM KCl, 3% glycerol) for 1–24 h at room temperature to allow proper triplex formation (pH 7.0). Small aliquots of each reaction were saved for PCR analysis to confirm that equal amounts of plasmid had been used. The reaction was incubated with 100 µl of streptavidin micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 5 min at room temperature. Dilution of the beads in the binding reaction was between 1:2 and 1:10. For magnetic separation, the column (Miltenyi Biotec) was placed in the magnetic field of the VarioMACS separator (Miltenyi Biotec) and rinsed once with 250 µl of the equilibration buffer provided by the manufacturer and three times with 250 µl of triplex buffer. After that, the binding reaction was applied on top of the column matrix. The solution passed through the column by gravity and the magnetically labelled complexes were retained in the column. The column was rinsed four times with 250 µl of triplex buffer to remove non-specifically interacting plasmid. For elution, the column was removed out of the magnetic field and 250 µl of triplex buffer or H_2O was applied directly on top of the column. Elution and washing fractions were saved for RNA and PCR analysis. To evaluate the quantity of RNA which was bound to the micro beads, 40 µl of each fraction was subjected to electrophoresis through a 1% agarose gel and the relative amount of RNA in the elution fraction was quantified densitometrically.

Semi-quantitative PCR

To compare the quantity of plasmid bound to control or TFO-RNA, semi-quantitative PCR was performed using vector-specific primers. Upstream primer 5′AAGCTTT-CCTGCTTGACTCC contains the restriction site *Hind*III (AAGCTT) of the plasmid pGL3 and part of the MCP-1 promoter sequence which was cloned between *Hind*III and *Nco*I restriction sites. The sequence of the downstream primer was 5′AGACCAGTAGATCCAGAGGA, leading to a PCR product of 658 bp. The template volume applied in the PCR was calculated according to the amount of RNA present in the elution fraction. PCR was performed using Platinum Taq DNA Polymerase. Thermocycler conditions: 94 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min.

Determination of MCP-1 and IL-8 protein levels in the culture medium

Concentrations of secreted chemokines were measured by ELISA. The human MCP-1 ELISA was performed with reagents from R&D Systems (Wiesbaden, Germany); IL-8 was determined with the OptEIA human IL-8 Set from BD Bioscience Pharmingen (Heidelberg, Germany).

Statistical analysis

A series of eight independently transfected and grown HEK 293 cell lines were used for the experiments. With these lines, experiments were performed at least three times and with respect to the blots depicted, one out of a series of comparable experiments was chosen. ELISAs were always done in duplicates and repeated three or more times. Results are expressed as mean ± standard deviation (SD) and statistical significance calculated by Student's t test with significance level given in the respective figure legend.

Results

Experimental design

The human MCP-1 promoter region contains a TFO target site consisting of 19 bp, including the binding motif for SP-1 and a partially overlapping putative AP-1-binding site. To generate TFOs directly inside the cell, we reconstructed an expression vector generating single-strand RNA transcripts, which may act as TFOs and thereby inhibit MCP-1 expression (fig. 2A). As described in Materials and methods, we inserted a DNA fragment coding for a 19-nt parallel-binding CU-TFO into the 5′ untranslated region of the hygromycin resistance gene of the eukaryotic expression vector TVHygro (fig. 2B).

In vitro determination of triple helix formation by triplex blotting

Our experiments revealed that the synthetic 19-nt CU-TFO effectively and specifically initiated triplex formation with the 39-bp MCP-1 promoter duplex (fig. 1C) in vitro. The capacity of synthetic oligoribonucleotides (fig. 1D) to bind to the MCP-1 promoter was investigated by triplex blotting [45] (see Materials and methods). Here, different concentrations (0.025–1.0 nmol) of the oligoribonucleotides were electrophoretically fractionated, transferred to a nylon filter, and hybridised with the

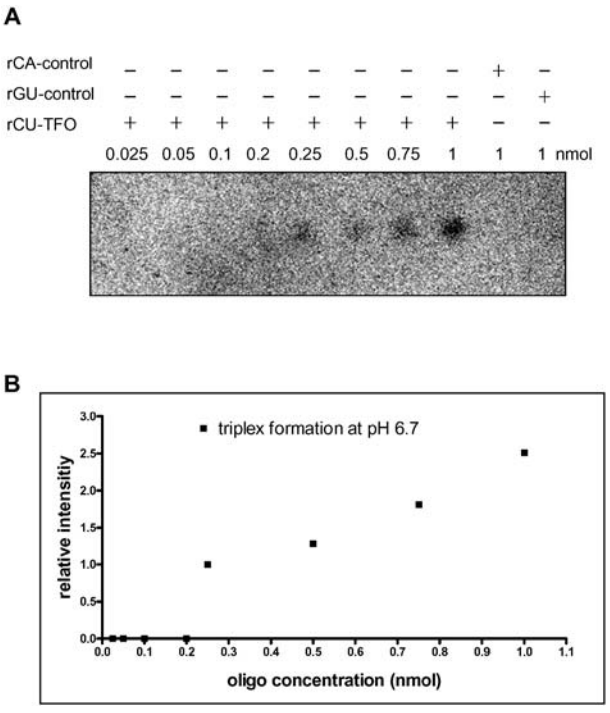


Figure 3. Participation of the pyrimidine CU-TFO in triplex formation at pH 6.7. The synthetic 19-nt oligoribonucleotides rCU-TFO, rGU-control and rCA-control (0.025–1.0 nmol) were electrophoretically fractionated through a 6% polyacrylamide gel containing 7 M urea and blotted to a nylon membrane. Hybridisation with the radioactive-labelled 39-bp MCP-1 promoter duplex (~3×10^{-3} nmol) was carried out overnight at 30 °C. Hybridisation and stringency washes were performed at pH 6.7 (*A*). Concentration-dependent binding of CU-TFO to the duplex is shown (*B*).

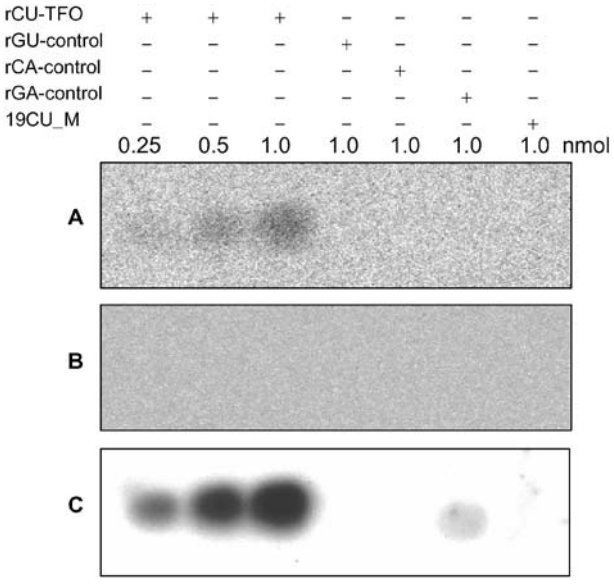


Figure 4. Specific binding of the 19-nt CU-TFO to the MCP-1 promoter duplex. Synthetic 19-nt oligoribonucleotides (0.25–1.0 nmol) were electrophoretically fractionated through a 6% polyacrylamide gel containing 7 M urea and blotted to a nylon membrane. Hybridisation with the radioactively labelled 39-bp MCP-1 promoter (~3×10^{-3} nmol) was carried out overnight at 37 °C. Hybridisation and stringency washes were performed at pH 5.5 (*A*). Hybridisation was performed as described but with a MCP-1 promoter duplex containing two mismatches at position 1 and 8 of the TFO-binding site (*B*). Blots were stripped for 1 h at 70 °C in 0.5 × NAE (50 mM sodium acetate, 0.5 mM pH 7.5) and reprobed with the linearised plasmid pGL3 control containing the 39-bp MCP-1 promoter sequence as described above (*C*).

radio-labelled 39-bp MCP-1 promoter template. Figure 3A demonstrates the predicted and specific detection of triplex-forming CU-TFO by triplex blotting with a minimum of 0.25 nmol transferred RNA. rCA-control and rGU-control were not detected by the labelled double-stranded probe even at the highest concentration (1 nmol), suggesting that these RNA species are not able to form triplex structures. Concentration-dependent binding of the CU-TFO at pH 6.7 is shown in figure 3B. The concentration of the 39-bp MCP-1 promoter was calculated to be 3×10^{-3} nmol. Under these conditions, binding of the duplex DNA to the CU-TFO could be observed at 83.3-fold molar excess of the TFO in comparison to the duplex DNA. Notably, binding of the duplex DNA to the CU-TFO was more pronounced at low pH (pH 5.5, data not shown). These results are in agreement with other investigations (see Discussion), as triplex formation involving the pyrimidine-binding motif is dependent on cytosine protonation and is therefore more likely to occur at low pH. To demonstrate binding specificity employing the pyrimidine motif, an oligoribonucleotide with a single mismatch (19CU_M) was tested. Here, a pH of 5.5 was chosen to perform experiments with the control oligonucleotide under optimal binding conditions for the pyrimidine motif. Figure 4A demonstrates that the duplex DNA (~ 3×10^{-3} nmol) did not bind to 1.0 nmol 19CU_M confirming the sequence-dependent specificity of triplex formation in this experimental setup. No triplex formation occurred using the oligo rGA-control, which represents an antiparallel, purine-binding motif. As a further control, in figure 4B, we show that a 39-bp MCP-1 duplex with two mismatches exhibited no interaction with any of the shown oligos. These results are summarized in table 1. Next, we wanted to exclude that the observed binding might be due to non-sequence-specific interactions of the CU-TFO with the duplex DNA. In principle, binding might occur through D-loop formation with partial melting of the 39-bp target DNA. Therefore, a double-stranded plasmid containing the 39-bp MCP-1 promoter duplex as a target was used for hybridisation in triplex blotting experiments, which drastically reduces the likelihood of D-loop formation. Figure 4C confirms the specific binding of the 19-nt CU-TFO to the plasmid-encoded target duplex (~ 3.1×10^{-3} nmol), whereas the control 19CU_M with one mismatch failed to bind to the target. Interestingly, and as will be discussed below, in

Table 1. Summary of the analysed oligoribonucleotides and duplexes in triplex blotting experiments.

oligo	sequence	properties	motif	triplex formation
rCU-TFO	5`UCUCCCUCUCUCCCACCUC		parallel, pyrimidine binding motif	yes
19CU_M	5`UCACCCUCUCUCCCACCUC	1 mismatch	parallel, pyrimidine binding motif	no
rGA-control	5`GAGGUGGGAGAGAGGGAGA		anti-parallel, purine binding motif	weak binding
rGU-control	5`GUGGUGGGUGUGUGGGUGU		anti-parallel, purine binding motif	no
rCA-control	5`CACACACCCAACCACACCC	scrambled	none	no

duplex	sequence	properties	triplex formation
MCP-1	5`*AGCTT*TCCTGCTTGA**CTCCGCCCTCTCTCCCTCT**GCCCGCTTTC*CCATG* 3` 3`*TCGAA*AGGACGAACT**GAGGCGGGAGAGAGGGAGA**CGGGCGAAAG*GGTAC* 5`		yes
MCP-1_2M	5`*AGCTT*TCCTGCTTGA**CTCCGCCCTCTATCCCTCG**GCCCGCTTTC*CCATG* 3` 3`*TCGAA*AGGACGAACT**GAGGCGGGAGATAGGGAGC**CGGGCGAAAG*GGTAC* 5`	2 mis-matches	no

this experimental setting (pH 5.5), we observed a weak binding of the antiparallel rGA-control to the target duplex, indicating a marginal binding affinity.

Determination of nuclear and cytosolic oligoribonucleotide transcripts

After demonstrating that the synthetic CU-TFO binds in a sequence-specific fashion to the MCP-1 promoter in cell-free setups, we verified the presence of the three different intracellular-generated RNA oligonucleotides in the stably transfected cell lines. Because, on the one hand, the interfering CU-TFO has to be present in the nuclei to exert its function and, on the other hand, the template for the hygromycin resistance protein has to reach the cytosol, we separately isolated nuclear RNA and total RNA containing the cytosolic fraction from each cell line carrying the vectors TVHygro-CU, TVHygro-GU or TVHygro-CA. Isolated RNA was reverse transcribed and subjected to PCR. Because the TFO and control sequence were transcribed in line with the hygromycin resistance gene as one fusion transcript, RT-PCR was performed using specific upstream primers for the 19-nt CU-, GU- or CA-RNA coding sequences and a downstream primer located within the hygromycin resistance gene. These primer pair combinations should result in an amplified product of 578 bp in TVHygro-CU and -CA cell lines and 522 bp in TVHygro-GU cell lines, because different restriction enzymes were used for the cloning (see Materials and methods). Figure 5A shows that the expected specific RNA sequence was present in each cell line (lane 1, 4 and 7) in the nuclei. Additionally, figure 5B confirms this finding when total RNA from the cytosolic fraction was used as PCR template. Control reactions were performed without reverse transcription to exclude DNA contamination (lane 2, 5 and 8). To confirm the exclusive presence of the CU-TFO generated in TVHygro-CU cell lines and the control RNA generated in TVHygro-CA and -GU cell lines, control reactions were performed using the primer pair for CU-TFO detection with the two control RNA templates, and the primer pair for the control RNA for the CU-TFO extract. Excluding vector cross-contamination, no PCR product was detectable in the respective samples (see lane 3, and lanes 6 and 9, fig. 5A, B). That different RNA species were synthesised in equal amounts in each cell line was shown by Northern blot analysis with a specific 522-bp antisense probe for the hygromycin resistance gene mRNA (fig. 5C).

Magnetic capture of triplex complexes

The predicted CU-TFO produced inside the cells is a fusion transcript of the 19-nt triplex-forming sequence and the messenger RNA of the hygromycin resistance gene. Therefore, we established an in vitro experiment to demonstrate that not only a synthetic 19-nt CU-TFO but also the large single-stranded RNA fusion transcript containing the CU-TFO binds to the MCP-1 promoter duplex in a sequence-specific manner. The RNA transcripts of the different vectors, TVHygro (without insert), TVHygro-CU, -CA and -GU, were generated by in vitro transcription using T7 polymerase. Transcripts with a length of about 1.1 kb contained the TFO or control sequence plus the hygromycin resistance gene mRNA. Integrity of the in vitro transcripts is shown in figure 6A. Then, the transcribed RNA was annealed to a biotinylated oligonucleotide in equimolar amounts and triplex formation was performed as described in Material and methods. Important to note is that triplex formation was carried out using the 39-bp MCP-1 target promoter duplex integrated in the pGL3 plasmid rather than using just the short duplex which served as a TFO template in figures 3, 4A and 4B. The results in figure 4C clearly

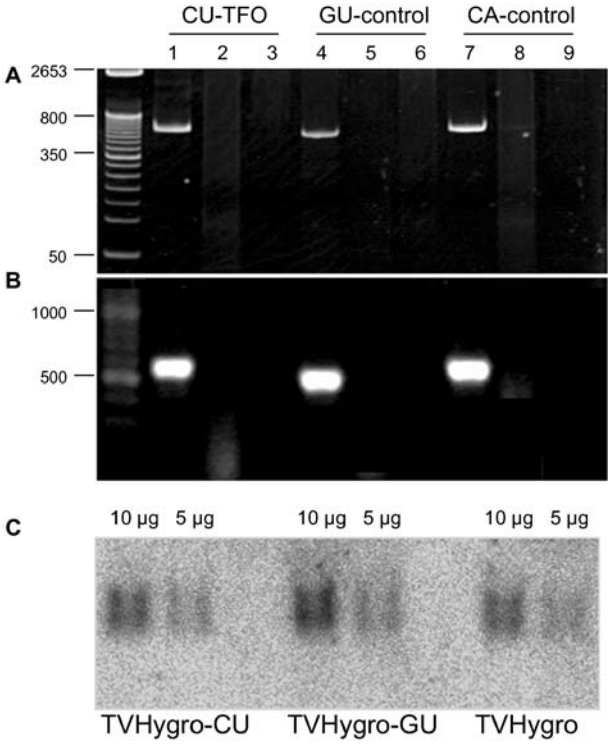


Figure 5. Presence of CU-TFO, GU-control and CA-control in the nuclei of stably transfected HEK 293 cells. RNA from cell nuclei was isolated as described in Materials and methods from each cell line, TVHygro-CU, TVHygro-GU and TVHygro-CA, and reverse transcribed. RT-PCR was performed using a specific upstream primer located in the 19-nt TFO and control RNA coding sequence, and a downstream primer located in the hygromycin resistance gene. The calculated lengths of the amplified products were 578 bp (CU-TFO and CA-control) and 522 bp (GU-control). RT-PCR products were electrophoretically fractionated through a 12.5% native polyacrylamide gel (*A*). Total RNA was isolated from each cell line and reverse transcribed. RT-PCR was carried out as described and products were electrophoretically fractionated through a 1% agarose gel (*B*). Lane 1, product CU-TFO; lane 4; product GU-control; lane 7; product CA-control; lane 2, 5 and 8; control reactions without reverse transcription to exclude DNA contamination; lane 3; control reactions with primer pair combination specific for GU-control; lane 6 and 9; control reactions with primer pair combination specific for CU-TFO. For Northern blot analysis, 5 or 10 µg RNA per lane was electrophoresed through a formaldehyde-agarose gel and transferred to a nylon membrane. Hybridisation was performed using a radio-labelled, denatured cDNA-probe specific for the hygromycin resistance gene RNA (*C*).

demonstrate that this plasmid was able to recognise and bind to 19-nt oligoribonucleotide in a sequence-specific manner via triplex formation. To mimic and estimate the likelihood of specific triplex formation under intranuclear conditions, we quantified the actual triplex-dependent enrichment of bound plasmid to the different large RNA fusion transcripts via PCR. For a specific detection, the upstream primer was set at the MCP-1 promoter target site and the downstream primer was specific for the pGL3 vector leading to a PCR product of 658 bp. The triplex complexes or control reactions were captured via the annealed and biotinylated RNA onto streptavidin-coated magnetic beads and applied to separation columns inside a magnetic field. To remove unbound RNA and plasmid, four washing steps were performed using the triplex binding buffer and in a final step, the complexes were eluted. Each fraction was saved for RNA and PCR analysis. In figure 6B, the binding of the large RNA fusion transcript to the beads is shown (lane F) and the relative amount was quantified densitometrically. To allow quantitative comparison of bound plasmid to CU-TFO versus control sequence-containing large transcripts, the PCR analysis was normalised to the amount of RNA in the elution fraction (factor 6.78). Figure 6C clearly indicates a specific triplex-dependent enrichment of the plasmid with the MCP-1 promoter target via 1.1-kb single-stranded RNA transcripts containing the specific CU-TFO rather than controls with no insert, GU or CA sequences. When summarising results of three independent experiments, the efficacy of the CU-TFO to form triplexes was three times stronger (fig. 6D).

Specific inhibition of basal and cytokine-stimulated MCP-1 gene expression

Our next aim was to analyse the effects of intracellular-generated CU-TFO versus control RNA on endogenous MCP-1 gene expression. We especially wanted to answer the question whether the TFO-based antigene strategy is limited to the target gene and effective also with different known stimuli of MCP-1 protein expression. Non-stimulated HEK 293 cells produced low basal amounts of MCP-1 in culture. HEK 293 cell lines TVHygro-CU, releasing CU-TFO, and control cell lines, releasing control RNA (GU-control, CA-control or RNA without insert derived from vector TVHygro) were plated in 24-well plates at a density of 1.5×10^5 cells/well and cultured for 24 h in the absence or presence of 500 U/ml TNF-α. To perform statistical analysis, a series of 13 independent experiments with six different stably transfected cell lines generating intranuclear CU-TFOs was carried out, and we measured a mean inhibition of 76 ± 10.2% of MCP-1 protein secretion after TNF-α stimulation as compared to measurements in two control cell lines (n=7). The basal MCP-1 protein secretion in unstimulated TVHygro-CU cell lines was reduced by 58 ± 13.1% (n=13, six cell lines) in comparison to non-stimulated control cell lines. Extending these results, inhibition of MCP-1 protein secretion was analysed in IFN-γ-stimulated cell lines to evaluate the influence of other involved transcription factors on TFO binding. One typical result is shown in figure 7. IFN-γ-induced MCP-1 expression was inhibited by 83% in CU-TFO-releasing cell lines. Combined stimulation with IFN-γ and TNF-α resulted in enhanced MCP-1 protein secretion in HEK 293 cell lines in comparison to single cytokine stimulation, suggesting an additive effect.

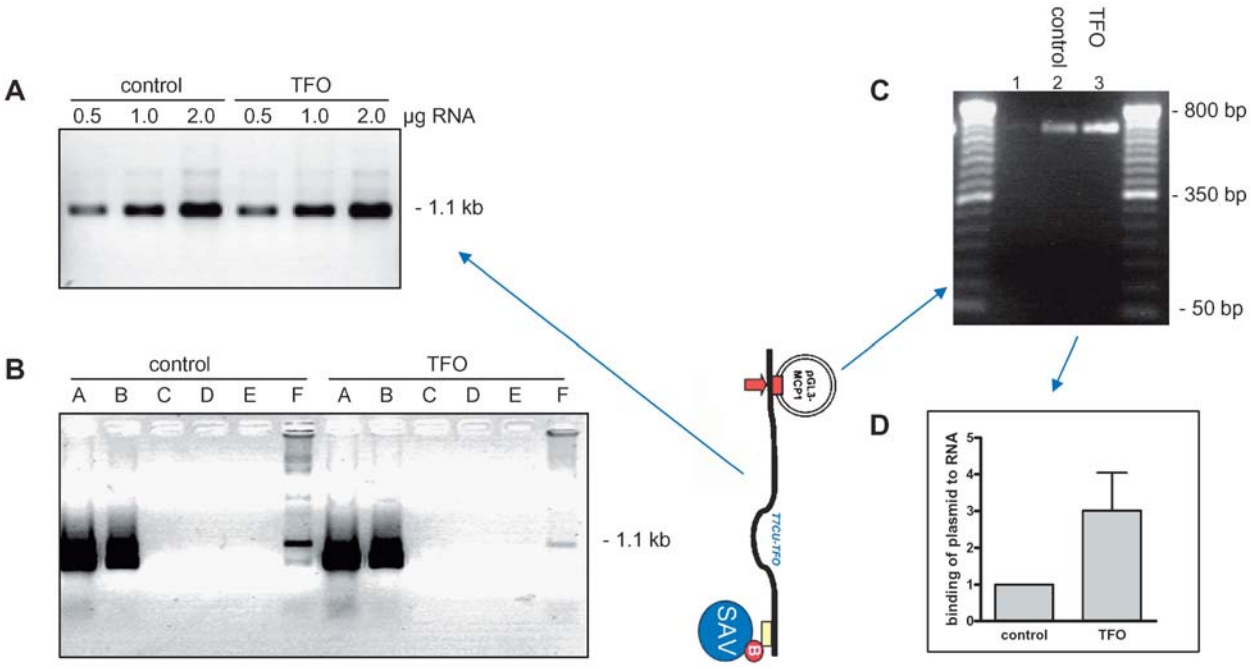


Figure 6. Binding of the in vitro-transcribed 1.1-kb CU-TFO to a plasmid-encoded 39-bp MCP-1 promoter duplex. Vector sequences including the T7 promoter and the TFO or control coding sequences were amplified by PCR and used as templates for in vitro transcription. Estimated sizes of transcribed RNA due to the transcriptional start site at the T7 promoter: 1142 bp (transcript from vector TVHygro without insert), 1149 bp (CU-TFO and CA-control) and 1111 bp (GU-control). Concentration and quality of the RNA was analysed by electrophoresis through a 1% agarose gel before each individual experiment (*A*). Biotinylated RNA transcripts were incubated with the plasmid pGL3 control containing the 39-bp MCP-1 promoter duplex to allow triplex formation. The RNA was magnetically labelled using streptavidin micro beads and the magnetic separation was performed as described in Materials and methods. All washing and elution fractions were collected and 40 µl of each fraction was subjected to electrophoresis through a 1% agarose gel. The relative amount of in vitro transcribed control or TFO bound to the beads in the elution fractions (6.78:1, respectively, see lane F) was quantified densitometrically (*B*). The amount of plasmid bound to the transcribed RNA (control or TFO) in each elution fraction (lane F) was compared by PCR (*C*). The elution fractions subjected to PCR were diluted in the same ratio as the RNA present in these fractions. 1, reaction without biotinylated RNA; 2, control; 3, TFO. Relative binding of CU-TFO to the plasmid – encoded MCP-1 promoter duplex was demonstrated in three independent experiments, using in vitro-transcribed controls without insert or with GU or CA sequences, respectively (*D*). Lane A, sample; lanes B–E, wash; lane F, elution

MCP-1 protein secretion was inhibited up to 88% in co-stimulated CU-TFO-generating cells in comparison to control cells. To further confirm that the inhibition of MCP-1 protein secretion indeed represents a gene-specific effect, HEK 293 cell lines were plated in 24-well plates at different cell densities of 9×10^4, 1.5×10^5, and 3×10^5 cells/well and cultured for 24 h in the absence or presence of 500 U/ml TNF-α. In addition to MCP-1, the release of the chemokine IL-8 was determined by ELISA. Also, in this experimental series, a marked reduction of MCP-1 protein secretion by up to 85% became apparent in the TNF-α-stimulated TVHygro-CU-transfected cell line when compared to the control vector-transfected cell line (fig. 8A). Analysing these supernatants for IL-8 protein secretion by ELISA, no inhibition of IL-8 protein secretion could be shown in CU-TFO-releasing cells, indicating that there was a sequence-specific inhibitory effect of CU-TFO only at the MCP-1 promoter site, whereas IL-8 as a suitable TNF-α-inducible control gene was not affected at all (fig. 8B).

Discussion

In this report, we demonstrated that pyrimidine triplex-forming RNA sequences generated within HEK 293 cells by specific expression vectors are effective in inhibiting the transcription of the human MCP-1 gene. As shown before, MCP-1 protein secretion could be induced by pro-inflammatory cytokines like TNF-α, IFN-γ and IL1-β in these cells [44]. A pyrimidine motif carrying TFO was chosen because triplex formation is unlikely to occur in the purine motif with RNA binding to a DNA duplex (RNA*DNA:DNA) [46, 47]. Furthermore, the formation of undesired structures which compete for triplex formation is reduced with a pyrimidine oligoribonucleotide [48]. The 19-bp target sequence is located in the promoter region of this gene and encompasses binding sites for the transcription factors SP-1 and AP-1. We showed specific binding of the 19-nt CU-TFO to the MCP-1 promoter target site in vitro at pH 6.7. Next, we have proven that the 1.1-kb TFO/hygromycin fusion transcript binds to the

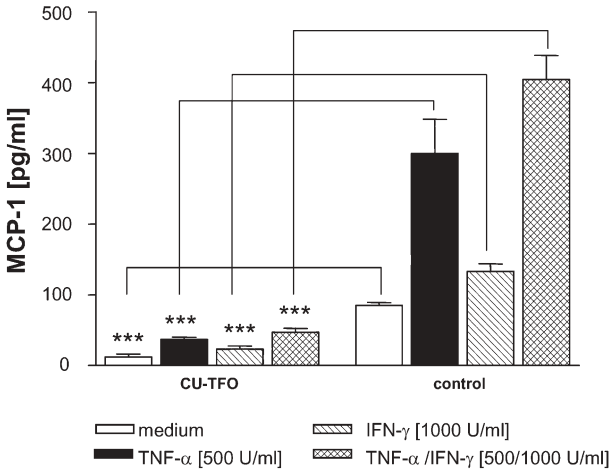


Figure 7. Inhibition of MCP-1 expression by intracellular generated CU-TFO in HEK 293 cells. Stably transfected HEK 293 cell lines generating CU-TFO or control RNA were plated in 24-well plates at a density of 3×10^5 cells/well and cultured for 24 h in the presence of 500 U/ml TNF-α, 1000 U/ml IFN-γ or TNF-α and IFN-γ together as indicated. At the end of incubation, culture supernatants were harvested and MCP-1 concentrations determined by ELISA. One experiment of three with similar results is shown. Cell culture was performed in triplicate, the error bars indicate the SD and *** a significance level of $p<0.001$ as determined by an unpaired Student t test.

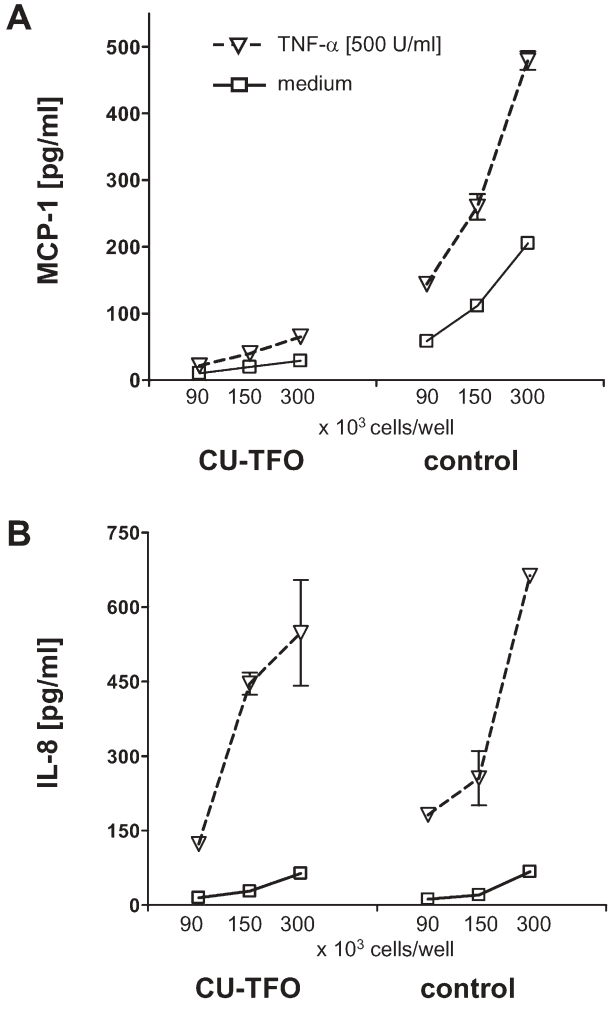


Figure 8. Sequence-specific inhibition of MCP-1 protein secretion. Stably transfected HEK 293 cells generating CU-TFO and control RNA were plated in 24-well plates at densities of 9×10^4, 1.5×10^5 and 3×10^5 cells/well and cultured for 24 h in the absence or presence of 500 U/ml TNF-α. At the end of the incubation time, culture supernatants were harvested and MCP-1 (*A*) and IL-8 concentrations (*B*) were determined by ELISA. One experiment representative of three independent experiments is shown; cell culture was performed in quadruplicate, the error bars indicate the SD.

MCP-1 promoter duplex at pH 7. We showed this by a newly developed technique measuring the relative binding of the plasmid-encoded MCP-1 promoter duplex to the TFO/hygromycin fusion transcript in comparison to control transcripts via magnetic separation of the triplex complexes. As shown, the binding of the specific TFO/hygromycin fusion transcript to the plasmid-embedded MCP-1 promoter duplex was three times more efficient than the binding of control RNA. We confirmed that TFO or control transcripts are present in the nucleus, the site of triplex formation. Finally, we demonstrated a highly effective and specific inhibition of MCP-1 protein secretion as compared to IL-8 in cytokine-stimulated cells which express the CU-TFO unlike cell lines carrying control vectors. These results proof that the triplex formation we observed in cell-free experiments was readily transferable to intact target cells by using RNA TFO-expressing vectors and led to a functional blockade of the target gene.

The potential of ribonucleotide sequences to form triplexes is supported by several in vitro studies [46, 49, 50]. We verified the binding of a 19-nt CU oligoribonucleotide to the MCP-1 promoter duplex and the formation of a triplex structure by triplex blotting. While a mismatch at position 15 (C/G inversion) was tolerated well in our triple helix motif without adverse effects on TFO binding, we observed no triplex formation using an oligoribonucleotide with CA base composition in a scrambled order (rCA-control) or using an oligoribonucleotide in the purine-binding motif (rGU-control). However, a weak binding of the GA sequence to the plasmid-encoded MCP-1 promoter target duplex occurred. Because the TFO sequence contains a symmetric sequence in the length of 11 nt (5′ ucuCCCUCUCUCCCaccuc 3′), a short double helix might form between CU-RNA and the GA-rich strand of the MCP-1 promoter instead of a triplex structure. Therefore, the binding capacity of a TFO sequence containing only one mismatch which did not interrupt the symmetric structure (5′ uca<u>a</u>CCCUCU-CUCCCaccuc 3′, mismatch underlined) was tested. Here, an antisense double helix might still be possible but triplex formation should be less favourable. Our data clearly show that no binding occurred using this kind of

control, indicating that the observed binding indeed must represent triplex formation. Another control employed consisted of a duplex DNA containing two mismatches, which did not bind to the TFO. This finding represents a strong argument that the binding of the RNA TFO shown here is not a result of unspecific duplex/oligo interaction. A few studies have been published which excluded the possibility that purine oligoribonucleotides (ORNs) form triplex structures with duplex DNA [47], but more recently Cogoi et al. [51] convincingly demonstrated triplex formation using CU, GU and, GA oligoribonucleotides. In the latter publication, these ORNs led to 38, 40 and 47% inhibition of c-Ki-ras promoter activity, respectively, when generated in HEK 293 cells intracellularly. Based on these promising results, interest is growing in developing methods to produce repressor RNA transcripts inside the target cell in vivo [51, 52]. Another strategy was discussed by Datta and Glazer [53]. They tested a vector system for the production of single-stranded DNA to serve as a TFO in mouse cells and assayed TFO-induced recombination, but no inhibition of endogenous gene expression was measured.

Rininsland et al. [54] described the specific suppression of IGF-1 receptor gene expression in rat glioblastoma cells in culture as well as in animals by using a plasmid-encoded homopurine (AG) RNA effector sequence. They showed that this RNA TFO formed a triple helix with the target DNA, whereas a homopyrimidine RNA sequence had no effect. Shevelev et al. [55] demonstrated tumour growth inhibition in an animal model employing a eukaryotic expression vector encoding a purine GA-TFO corresponding to the IGF I gene. Additionally, an antiparallel GA oligoribonucleotide binding to the rat α1 (I) procollagen promoter inhibits procollagen gene transcription [56]. Here, the authors demonstrated inhibition of gene transcription by in vitro transcription runoff experiments. Overall, while all these studies suggested that triplex formation inside the cell nuclei led to the observed inhibitory effect, no clear experimental proof has been delivered. Our data, however, clearly show that the CU sequence was capable of triplex formation with the target duplex at the human MCP-1 promoter not only with a short target duplex in triplex blots but, moreover, even when expressed within a large transcript and with the target duplex embedded in a complete plasmid.

Pyrimidine-rich triplex-forming oligoribonucleotides have a considerably higher binding affinity to the duplex DNA in comparison to their triplex-forming DNA oligonucleotide counterparts [50], but triplex formation alongside the pyrimidine-binding motif typically requires mildly acidic conditions to protonate cytosines. Several potential cytosine analogues like 8-oxoadenine derivatives exist which can overcome the need for cytosine protonation [57, 58]. On the other hand, Soukup et al. [59] selected RNA transcripts which all contained a pyrimidine recognition sequence and were able to bind to the target duplex between pH 6.5 and pH 7.4. Noonberg et al. [52] suggested that the high concentration of a triplex RNA oligonucleotide, which is generated in the nucleus in vast excess over its DNA duplex target may drive triplex binding to a critical element on a gene promoter, and block subsequent gene expression. The pH within the cell is different for various organelles, and compartments of the biosynthetic pathway are described to have a neutral pH, endosomes are slightly acidic (pH 6.2) and lysosomes range between pH 4.8 and 5.2 [60]. Llopis et al. [61] detected a pH of 6.58 for the trans-Golgi network, a major sorting site for newly synthesised molecules. In the magnetic capture assay, we could verify triplex formation at pH 7 using the 1.1-kb fusion transcript, which might be comparable to the pH in the cellular situation.

With respect to the functional antigene activity, we have successfully developed a vector-mediated synthesis of pyrimidine triplex-forming RNA species inside HEK 293 cells leading to up to 88% inhibition of TNF-α and IFN-γ co-stimulated MCP-1 protein secretion. Vector constructs used for transfection to generate stable cell lines had to cross the plasma membrane, move through the cytoplasm, and be transported into the nucleus before any transcription of their encoded genes can take place. The intracellular trafficking and cytoplasmatic transport of plasmid DNA is reviewed by Zhou et al. [62]. Here, transcription of the TFO-coding sequence was driven together with the hygromycin resistance gene as one fusion transcript from the constitutive CMV promoter. We have confirmed the presence of each of the intracellularly generated CU-, GU-, and CA-RNA sequences in the nucleus and the cytosolic fraction by RT-PCR, leading to the conclusion that the vector constructs successfully entered the nucleus. Furthermore, the transcript was sufficiently stable: the RNA was translocated into the cytosol, where it was translated into protein and enabled stably transfected HEK 293 cells to be cultured under continuous hygromycin selection pressure. The evidence of transcription and the cell culture conditions show that the vector constructs reached the nucleus where the action of TFO binding to the endogenous MCP-1 promoter target sequences takes place. No quantitative experiments were performed to evaluate the exact copy numbers or concentration of expressed transcripts, which might be possible by nuclear runoff assays.

In direct comparison with our previous data [44], this investigation convincingly shows that vector-mediated delivery of triplex-forming RNA sequences is much more efficient than adding DNA TFOs against the human MCP-1 extracellularly, which led to only 45% inhibition of gene expression. A 76–88% inhibition was reached here with an intracellular-generated CU triplex RNA sequence. To exclude the possibility that the TFO inhib-

ited MCP-1 expression by affecting TNF-α signalling, we evaluated whether the TFO interfered with the expression of IL-8, which is also induced by TNF-α. In samples identical to those with the measured reduction of MCP-1 secretion, we found no reduction of IL-8 protein secretion. Again, the control GU and CA sequences had neither an effect on IL-8 protein secretion nor on MCP-1 protein secretion. Binding of the CU-TFO to the MCP-1 RNA appears to be unlikely, because there is no matching sequence in the MCP-1 RNA for stable duplex formation with the TFO; thus an 'antisense' effect could also be excluded.

The inhibitory effect of the TFO is more pronounced in TNF-α-stimulated HEK 293 cells than in unstimulated cells (76% versus 53%, respectively), leading to the question of accessibility of chromosomal DNA targets to TFOs. Macris and Glazer [63] detected binding of TFOs to a chromosomal site even in the absence of transcription when high concentrations of the TFO were used for transfection, demonstrating that transcription at a chromosomal site increased duplex site accessibility to the TFO. Comparably, our data suggest that induction of transcription enhanced TFO binding to the MCP-1 promoter target site resulting in a stronger inhibitory effect of MCP-1 expression.

In summary, these data strongly suggest that a specific RNA TFO inhibited MCP-1 expression by a triplex-mediated blockade of transcription and, moreover, that this can be achieved by transfectable shuttle systems. Unlike siRNAs and antisense oligonucleotides which have to deal with a high number of mRNA targets, triplex-forming oligonucleotides selectively inhibit expression of specific genes directly. Released from transfectable RNA vectors they only have to reach the two alleles of the gene, which opens the perspective for a longer-lasting pharmacologic effect. Recently developed siRNA-generating vector systems are also suitable for generating RNA TFOs in vivo. Combining these improved expression vectors with our knowledge of hundreds of suitable TFO targets in the mammalian genome indicates that the intracellular TFO delivery technique may be developed to a successful gene therapeutic approach.

Acknowledgements. We would like to thank S. Spiegler and M. Herrero-San Juan for their continuous and valuable practical support, and S. Rubant for many helpful discussions. We gratefully acknowledge the opportunity to perform this study given to us by J. M. Pfeilschifter. This work was supported by grants of the Deutsche Forschungsgemeinschaft to H. H. R. (DFG-grant Ra 525/7-3) and the Dr.-Hans-Schleussner-Foundation Immune Pharmacology.

1 Faria M., Wood C. D., Perrouault L., Nelson J. S., Winter A.,White M. R. et al. (2000) Targeted inhibition of transcription elongation in cells mediated by triplex-forming oligonucleotides. Proc. Natl. Acad. Sci. USA **97:** 3862–3867

2 Intody Z., Perkins B. D., Wilson J. H. and Wensel T. G. (2000) Blocking transcription of the human rhodopsin gene by triplex-mediated DNA photocrosslinking. Nucleic Acids Res. **28:** 4283–4290

3 Giovannangeli C., Diviacco S., Labrousse V., Gryaznov S., Charneau P. and Helene C. (1997) Accessibility of nuclear DNA to triplex-forming oligonucleotides: the integrated HIV-1 provirus as a target. Proc. Natl. Acad. Sci. USA **94:** 79–84

4 Carbone G. M., McGuffie E. M., Collier A. and Catapano C. V. (2003) Selective inhibition of transcription of the Ets2 gene in prostate cancer cells by a triplex-forming oligonucleotide. Nucleic Acids Res. **31:** 833–843

5 Nagatsugi F., Sasaki S., Miller P. S. and Seidman M. M. (2003) Site-specific mutagenesis by triple helix-forming oligonucleotides containing a reactive nucleoside analog. Nucleic Acids Res. **31:** e31

6 Rogers F. A., Vasquez K. M., Egholm M. and Glazer P. M. (2002) Site-directed recombination via bifunctional PNA-DNA conjugates. Proc. Natl. Acad. Sci. USA **99:** 16695–16700

7 Vasquez K. M., Dagle J. M., Weeks D. L. and Glazer P. M. (2001) Chromosome targeting at short polypurine sites by cationic triplex-forming oligonucleotides. J. Biol. Chem. **276:** 38536–38541

8 Vasquez K. M., Narayanan L. and Glazer P. M. (2000) Specific mutations induced by triplex-forming oligonucleotides in mice. Science **290:** 530–533

9 Tu G. C., Cao Q. N. and Israel Y. (1995) Inhibition of gene expression by triple helix formation in hepatoma cells. J. Biol. Chem. **270:** 28402–28407

10 Orson F. M., Thomas D. W., McShan W. M., Kessler D. J. and Hogan M. E. (1991) Oligonucleotide inhibition of IL2R alpha mRNA transcription by promoter region collinear triplex formation in lymphocytes. Nucleic Acids Res. **19:** 3435–3441

11 Porumb H., Gousset H., Letellier R., Salle V., Briane D., Vassy J. et al. (1996) Temporary ex vivo inhibition of the expression of the human oncogene HER2 (NEU) by a triple helix-forming oligonucleotide. Cancer Res. **56:** 515–522

12 Postel E. H., Flint S. J., Kessler D. J. and Hogan M. E. (1991) Evidence that a triplex-forming oligodeoxyribonucleotide binds to the c-myc promoter in HeLa cells, thereby reducing c-myc mRNA levels. Proc. Natl. Acad. Sci. USA **88:** 8227–8231

13 Thomas T. J., Faaland C. A., Gallo M. A. and Thomas T. (1995) Suppression of c-myc oncogene expression by a polyamine-complexed triplex forming oligonucleotide in MCF-7 breast cancer cells. Nucleic Acids Res. **23:** 3594–3599

14 Kochetkova M. and Shannon M. F. (1996) DNA triplex formation selectively inhibits granulocyte-macrophage colony-stimulating factor gene expression in human T cells. J. Biol. Chem. **271:** 14438–14444

15 Cheng A. J. and Van Dyke M. W. (1994) Oligodeoxyribonucleotide length and sequence effects on intermolecular purine-purine-pyrimidine triple-helix formation. Nucleic Acids Res. **22:** 4742–4747

16 Uguccioni M., D'Apuzzo M., Loetscher M., Dewald B. and Baggiolini M. (1995) Actions of the chemotactic cytokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1 alpha and MIP-1 beta on human monocytes. Eur. J. Immunol. **25:** 64–68

17 Carr M. W., Roth S. J., Luther E., Rose S. S. and Springer T. A. (1994) Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. Proc. Natl. Acad. Sci. USA **91:** 3652–3656

18 Loetscher P., Seitz M., Clark-Lewis I., Baggiolini M. and Moser B. (1994) Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. FASEB J. **8:** 1055–1060

19 Roth S. J., Carr M. W. and Springer T. A. (1995) C-C chemokines, but not the C-X-C chemokines interleukin-8 and interferon-gamma inducible protein-10, stimulate transendo-

thelial chemotaxis of T lymphocytes. Eur. J. Immunol. **25:** 3482–3488
20 Weber M., Uguccioni M., Ochensberger B., Baggiolini M., Clark-Lewis I. and Dahinden C. A. (1995) Monocyte chemotactic protein MCP-2 activates human basophil and eosinophil leukocytes similar to MCP-3. J. Immunol. **154:** 4166–4172
21 Frade J. M., Mellado M., Real G. del, Gutierrez-Ramos J. C., Lind P. and Martinez A. (1997) Characterization of the CCR2 chemokine receptor: functional CCR2 receptor expression in B cells. J. Immunol. **159:** 5576–5584
22 Rollins B. J., Yoshimura T., Leonard E. J. and Pober J. S. (1990) Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. Am. J. Pathol. **136:** 1229–1233
23 Rovin B. H., Yoshiumura T. and Tan L. (1992) Cytokine-induced production of monocyte chemoattractant protein-1 by cultured human mesangial cells. J. Immunol. **148:** 2148–2153
24 Schwarz M., Radeke H. H., Resch K. and Uciechowski P. (1997) Lymphocyte-derived cytokines induce sequential expression of monocyte- and T cell-specific chemokines in human mesangial cells. Kidney Int. **52:** 1521–1531
25 Anders H. J., Vielhauer V., Kretzler M., Cohen C. D., Segerer S., Luckow B. et al. (2001) Chemokine and chemokine receptor expression during initiation and resolution of immune complex glomerulonephritis. J. Am. Soc. Nephrol. **12:** 919–931
26 Brown Z., Robson R. L. and Westwick J. (1996) Regulation and expression of chemokines: potential role in glomerulonephritis. J. Leukoc. Biol. **59:** 75–80
27 Hora K., Satriano J. A., Santiago A., Mori T., Stanley E. R., Shan Z. et al. (1992) Receptors for IgG complexes activate synthesis of monocyte chemoattractant peptide 1 and colony-stimulating factor 1. Proc. Natl. Acad. Sci. USA **89:** 1745–1749
28 Panzer U., Thaiss F., Zahner G., Barth P., Reszka M., Reinking R. R. et al. (2001) Monocyte chemoattractant protein-1 and osteopontin differentially regulate monocytes recruitment in experimental glomerulonephritis. Kidney Int. **59:** 1762–1769
29 Radeke H. H., Janssen-Graalfs I., Sowa E. N., Chouchakova N., Skokowa J., Loscher F. et al. (2002) Opposite regulation of type II and III receptors for immunoglobulin G in mouse glomerular mesangial cells and in the induction of anti-glomerular basement membrane (GBM) nephritis. J. Biol. Chem. **277:** 27535–27544
30 Stahl R. A., Thaiss F., Disser M., Helmchen U., Hora K. and Schlondorff D. (1993) Increased expression of monocyte chemoattractant protein-1 in anti- thymocyte antibody-induced glomerulonephritis. Kidney Int. **44:** 1036–1047
31 Tesch G. H., Schwarting, A., Kinoshita K., Lan H. Y., Rollins B. J. and Kelley V. R. (1999) Monocyte chemoattractant protein-1 promotes macrophage-mediated tubular injury, but not glomerular injury, in nephrotoxic serum nephritis. J. Clin. Invest **103:** 73–80
32 Boring L., Gosling J., Cleary M. and Charo I. F. (1998) Decreased lesion formation in CCR2–/– mice reveals a role for chemokines in the initiation of atherosclerosis. Nature **394:** 894–897
33 Mahad D. J. and Ransohoff R. M. (2003) The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). Semin. Immunol. **15:** 23–32
34 Ping D., Jones P. L. and Boss J. M. (1996) TNF regulates the in vivo occupancy of both distal and proximal regulatory regions of the MCP-1/JE gene. Immunity **4:** 455–469
35 Cochran B. H., Reffel A. C. and Stiles C. D. (1983) Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell **33:** 939–947
36 Zhou Z. H., Chaturvedi P., Han Y. L., Aras S., Li Y. S., Kolattukudy P. E. et al. (1998) IFN-gamma induction of the human monocyte chemoattractant protein (hMCP)-1 gene in astrocytoma cells: functional interaction between an IFN-gamma-activated site and a GC-rich element. J. Immunol. **160:** 3908–3916
37 Zoja C., Wang J. M., Bettoni S., Sironi M., Renzi D., Chiaffarino F. et al. (1991) Interleukin-1 beta and tumour necrosis factor-alpha induce gene expression and production of leukocyte chemotactic factors, colony-stimulating factors, and interleukin-6 in human mesangial cells. Am. J. Pathol. **138:** 991–1003
38 Faller D. V., Weng H., Graves D. T. and Choi S. Y. (1997) Moloney murine leukemia virus long terminal repeat activates monocyte chemotactic protein-1 protein expression and chemotactic activity. J. Cell Physiol **172:** 240–252
39 Glabinski A. R., Balasingam V., Tani M., Kunkel S. L., Strieter R. M., Yong V. W. et al. (1996) Chemokine monocyte chemoattractant protein-1 is expressed by astrocytes after mechanical injury to the brain. J. Immunol. **156:** 4363–4368
40 Ping D., Boekhoudt G. H., Rogers E. M. and Boss J. M. (1999) Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene. J. Immunol. **162:** 727–734
41 Ueda A., Okuda K., Ohno S., Shirai A., Igarashi T., Matsunaga K. et al. (1994) NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. J. Immunol. **153:** 2052–2063
42 Ping D., Boekhoudt G. and Boss J. M. (1999) Trans-retinoic acid blocks platelet-derived growth factor-BB-induced expression of the murine monocyte chemoattractant-1 gene by blocking the assembly of a promoter proximal Sp1 binding site. J. Biol. Chem. **274:** 31909–31916
43 Wenzel U., Schneider A., Valente A. J., Abboud H. E., Thaiss F., Helmchen U. M. et al. (1997) Monocyte chemoattractant protein-1 mediates monocyte/macrophage influx in anti-thymocyte antibody-induced glomerulonephritis. Kidney Int. **51:** 770–776
44 Marchand P., Resch K. and Radeke H. H. (2000) Selective inhibition of monocyte chemoattractant protein-1 gene expression in human embryonal kidney cells by specific triple helix-forming oligonucleotides. J. Immunol. **164:** 2070–2076
45 Noonberg S. B., Scott G. K., Hunt C. A. and Benz C. C. (1994) Detection of triplex-forming RNA oligonucleotides by triplex blotting. Biotechniques **16:** 1070–1072, 1074
46 Escude C., Francois J. C., Sun J. S., Ott G., Sprinzl M., Garestier T. et al. (1993) Stability of triple helices containing RNA and DNA strands: experimental and molecular modeling studies. Nucleic Acids Res. **21:** 5547–5553
47 Semerad C. L. and Maher L. J. III (1994) Exclusion of RNA strands from a purine motif triple helix. Nucleic Acids Res. **22:** 5321–5325
48 Lacroix L., Mergny J. L., Leroy J. L. and Helene C. (1996) Inability of RNA to form the i-motif: implications for triplex formation. Biochemistry **35:** 8715–8722
49 Han H. and Dervan P.B. (1993) Sequence-specific recognition of double helical RNA and RNA.DNA by triple helix formation. Proc. Natl. Acad. Sci. USA **90:** 3806–3810
50 Roberts R. W. and Crothers D. M. (1992) Stability and properties of double and triple helices: dramatic effects of RNA or DNA backbone composition. Science **258:** 1463–1466
51 Cogoi S., Suraci C., Del Terra E., Diviacco S., Marel G. van der, Boom J. van et al. (2000) Downregulation of c-Ki-ras promoter activity by triplex-forming oligonucleotides endogenously generated in human 293 cells. Antisense Nucleic Acids Drug Dev. **10:** 283–295
52 Noonberg S. B., Scott G. K., Garovoy M. R., Benz C. C. and Hunt C. A. (1994) In vivo generation of highly abundant sequence-specific oligonucleotides for antisense and triplex gene regulation. Nucleic Acids Res. **22:** 2830–2836
53 Datta H. J. and Glazer P. M. (2001) Intracellular generation of single-stranded DNA for chromosomal triplex formation and induced recombination. Nucleic Acids Res. **29:** 5140–5147

54 Rininsland F., Johnson T. R., Chernicky C. L., Schulze E., Burfeind P. and Ilan J. (1997) Suppression of insulin-like growth factor type I receptor by a triple-helix strategy inhibits IGF-I transcription and tumourigenic potential of rat C6 glioblastoma cells. Proc. Natl. Acad. Sci. USA **94:** 5854–5859
55 Shevelev A., Burfeind P., Schulze E., Rininsland F., Johnson T. R., Trojan J. et al. (1997) Potential triple helix-mediated inhibition of IGF-I gene expression significantly reduces tumourigenicity of glioblastoma in an animal model. Cancer Gene Ther. **4:** 105–112
56 Ririe S. S. and Guntaka R. V. (1998) An RNA oligonucleotide corresponding to the polypyrimidine region of the rat alpha 1(I) procollagen promoter forms a stable triplex and inhibits transcription. Biochem. Biophys. Res. Commun. **249:** 218–221
57 Chan P. P. and Glazer P. M. (1997) Triplex DNA: fundamentals, advances, and potential applications for gene therapy. J. Mol. Med. **75:** 267–282
58 Miller P. S., Bhan P., Cushman C. D. and Trapane T. L. (1992) Recognition of a guanine-cytosine base pair by 8-oxoadenine. Biochemistry **31:** 6788–6793
59 Soukup G. A., Ellington A. D. and Maher L. J. III (1996) Selection of RNAs that bind to duplex DNA at neutral pH. J. Mol. Biol. **259:** 216–228
60 Weisz O. A. (2003) Organelle acidification and disease. Traffic. **4:** 57–64
61 Llopis J., McCaffery J. M., Miyawaki A., Farquhar M. G. and Tsien R. Y. (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc. Natl. Acad. Sci. USA **95:** 6803–6808
62 Zhou R., Geiger R. C. and Dean D. A. (2004) Intracellular trafficking of nucleic acids. Expert Opin. Drug Deliv. **1:** 127–140
63 Macris M. A. and Glazer P. M. (2003) Transcription dependence of chromosomal gene targeting by triplex-forming oligonucleotides. J. Biol. Chem. **278:** 3357–3362