SUPPLEMENTAL METHODS AND FIGURES

Upstream Stimulatory Factor is Required for Human Angiotensinogen Expression and Differential Regulation by the A-20C Polymorphism

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Supplemental Experimental Procedures

Mutant Vectors, Adfections, and Transcriptional Reporter Assays: Mutant vectors 4U and 8U were generated from haplotype vectors 4 and 8, respectively, using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) with the oligo 5'-CCTCAGCTATAAATAGGGCggatgGACCCGGCCAGGGGAAGAAGC-3' and its reverse complement sequence (lower case letters denote mutated nucleotides in the USF consensus sequence). HepG2 adfections utilized calcium phosphate precipitates to deliver AGT reporter vectors, Renilla luciferase vector (Promega), and adenoviruses to cultured cells [1]. Viral particles (MOI=200), 5 μg AGT reporter plasmid, and a 1:500 stoichiometric dilution of Renilla vector were added to 1 mL serum-free medium. 25 μL of 1 mol/L $CaCl_2$ was added, mixed and incubated for 20 min. Media was removed from a well of a 6-well dish and replaced with the adfection mixture for 30 min at 37°C. The mixture was removed, the cells were rinsed once with medium, and then cell culture medium was added for 24 hours at 37°C prior to using the Dual-Luciferase Reporter Assay System (Promega) as previously described [2]. Co-transfection/transduction experiments in HK-2 and CCF cells first required a 2-day (HK-2, MOI=200) or 3-day (CCF, MOI=500) exposure to virus in 1 mL of normal cell medium (with only 1% FBS) followed by transfection and use of the Dual-Luciferase Reporter Assay System (Promega).

Gel Shift, Competition Gel Shift, and Supershift Assays: Nuclear extracts were prepared and quantified from HepG2, HK-2, and CCF cells as previously described [3]. Double-stranded DNA oligos were made and, when applicable, radiolabeled. The sense strand of each oligo (other than

the mismatched oligo and the ΔUSF oligo) was 5'-GATCTAGGGCXTCGTGACCCGGCCYGGGGAAGAA-3' where X=A or C for the -20 SNP and Y=A or G for the -6 SNP. The sense strand of the mismatched oligo was 5'-GATCTAGTTAGGATTGACCCGTAACTTTGAAGAA-3' and the sense strand of the ΔUSF oligo was 5'-GATCTAGGGCGGATGGACCCGGCCAGGGGAAGAA-3'. Gel shifts and supershifts were carried out as previously described [3] except that they used 5% polyacrylamide gels, 7 μg of nuclear extract per well, and 1 μg poly[d(A-T)] per well (Roche Diagnostics). Supershifts included 1 μg antibody against USF1 (sc-229X, Santa Cruz Biotechnology), USF2 (sc-861X, Santa Cruz Biotechnology), FRA1 (sc-605X, Santa Cruz Biotechnology), or β-actin (ab8227, Abcam).

Primers: Primers for quantification of hUSF1 and hUSF2 were 5'-TTGTCTGAAGAACTGCAGGGACTT-3' for FLAG-USF1 and 5'-GCAGGAGACCTTCAAAGAGGCC-3' for FLAG-USF2 whereas a common reverse primer in the FLAG tag was used for both 5'-CTACTTGTCATCGTCGTCCTTGTAATC-3'.

The effectiveness of the AdshUSF1 and AdshUSF2 adenoviruses was tested using the primers: 5'-TCAGCATAATGAAGTGGAGCGTCG-3' and 5'-CAAGCTTTGGATAGAATCCCACCT-3' for hUSF1, 5'-GCACAACGAAGTGGAGCGGAG-3' and 5'-GCAACTCCCGGATGTAATCGCAG-3' for hUSF2, 5'-TCTATGACCCAGGCAGTGATCCA-3' and 5'-AACAGCTGTTGTACTCCCAGATGT-3' for mUSF1, and 5'-CAGCACAATGAAGTGGAACGGAGA-3' and 5'-ATGTAATCGCAAGCCTTGGACAG-3' for mUSF2.

Primers used for detection of the AGT reporter vectors in plasmid immunoprecipitation experiments are 5'-TGGTCTGGCCAAGTGATGTA-3' and 5'-AACAGTACCGGAATGCCAAG-3' with 32 cycles of PCR. Primers and probes used for the custom TaqMan SNP real-time PCR detection assay were as follows: 5'-AGCCTGGGAACAGCTCCAT-3' forward primer, 5'-ACAGTACCGGAATGCCAAGCT-3' reverse primer, 6FAM-AGGGCATCGTGACC-MGBNFQ -20A probe, VIC-CTATAAATAGGGCCTCGTGA–MGBNFQ -20C probe (Applied Biosystems).

Primers used for detection of hAGT in CCF cells were 5'-TGGTCTGGCCAAGTGATGTA-3' and 5'-CTCCTCCCGGCCTTTTCCTCCTA-3' (236 bp amplicon). Primers used for detection f hAGT in transgenic mouse tissues were 5'-TGGTCTGGCCAAGTGATGTA-3' and 5'-GGCTTACCTTCTGCTGTAGT-3' (152 bp amplicon).

Real-time PCR reagents for assessing the efficiency of AdshUSF1/2 administration in vivo included the following TaqMan primers and probes obtained from Applied Biosystems: Mm01290210_g1 (mUSF1), Mm00495959_m1 (mUSF2), Mm00599662_m1 (mAGT), Hs01586213_m1 (hAGT), 4352664 (mβ-ACT), and 4352662 (mGAPDH).

References

(1) Fasbender A, Lee JH, Walters RW, Moninger TO, Zabner J, Welsh MJ. Incorporation of adenovirus in calcium phosphate precipitates enhances gene transfer to airway epithelia in vitro and in vivo. *J Clin Invest.* 1998;102:184-193.

(2) Dickson ME, Zimmerman MBRK, Sigmund CD. The -20 and -217 promoter variants dominate differential angiotensinogen haplotype regulation in angiotensinogen-expressing cells. *Hypertension.* 2007;49:631-639.

(3) Shi Q, Black TA, Gross KW, Sigmund CD. Species-Specific Differences in Positive and Negative Regulatory Elements in the Renin Gene Enhancer. *Circ Res.* 1999;85:479-488.

Supplemental Figures and Legends

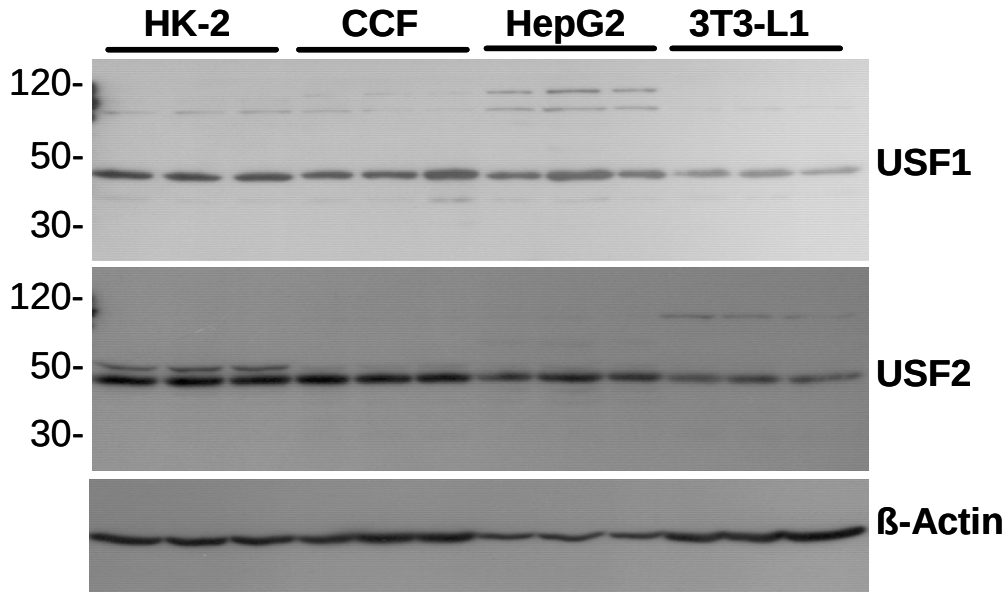

Figure S1. *USF1 and USF2 Expression.*

Western analyses for USF1 and USF2 were performed using 40 μg of total cellular protein extracts from HK-2, CCF, HepG2, and differentiated 3T3-L1 cells. Three independent protein extracts were tested from each cell line. Size markers are as indicated (kD). Membranes were stripped and re-probed for β-actin.

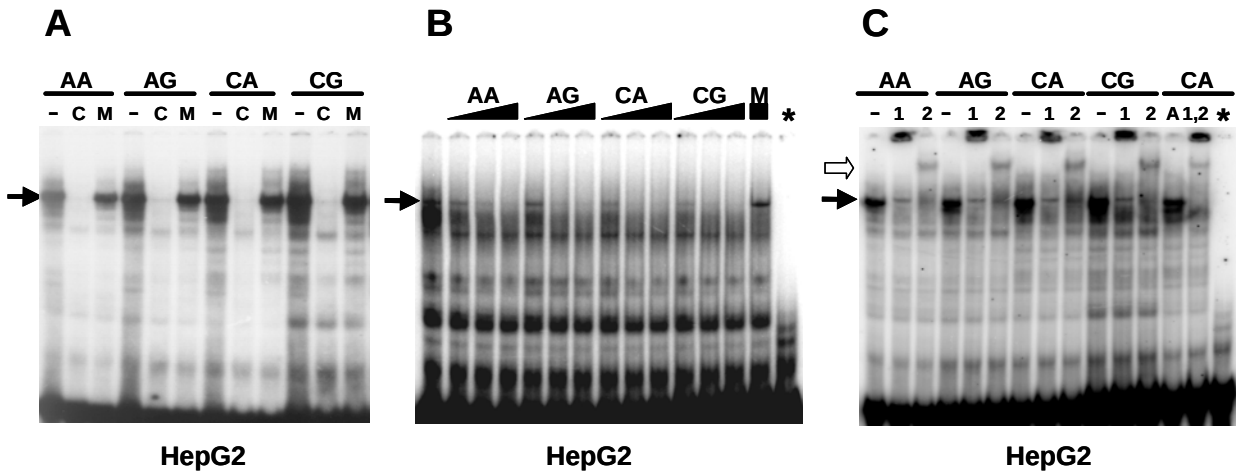

Figure S2. *Electrophoretic Gel Mobility Shift Assays*.

EMSA analyses utilizing nuclear extracts prepared from HepG2 cells and the oligonucleotides as indicated (AA, AG, CA and CG, detailed in Figure 1B). A is an EMSA with competition using mismatched cold competitor (50-fold molar excess)(M) or using cold competitor (labeled as C) derived from the same sequence as the radiolabeled probe. B is an EMSA using the CA radiolabeled probe with increasing amounts (10-, 25-, or 50-fold molar excess) of the indicated cold competitor or mismatched competitor (M). C is an EMSA using the indicated probes and antibody against USF1 (1), USF2 (2), both (1,2) or β-Actin (A). * indicates free probe using radiolabeled CA oligo without nuclear extract. Arrow, specific gel shift band; Open arrowhead, supershift bands.

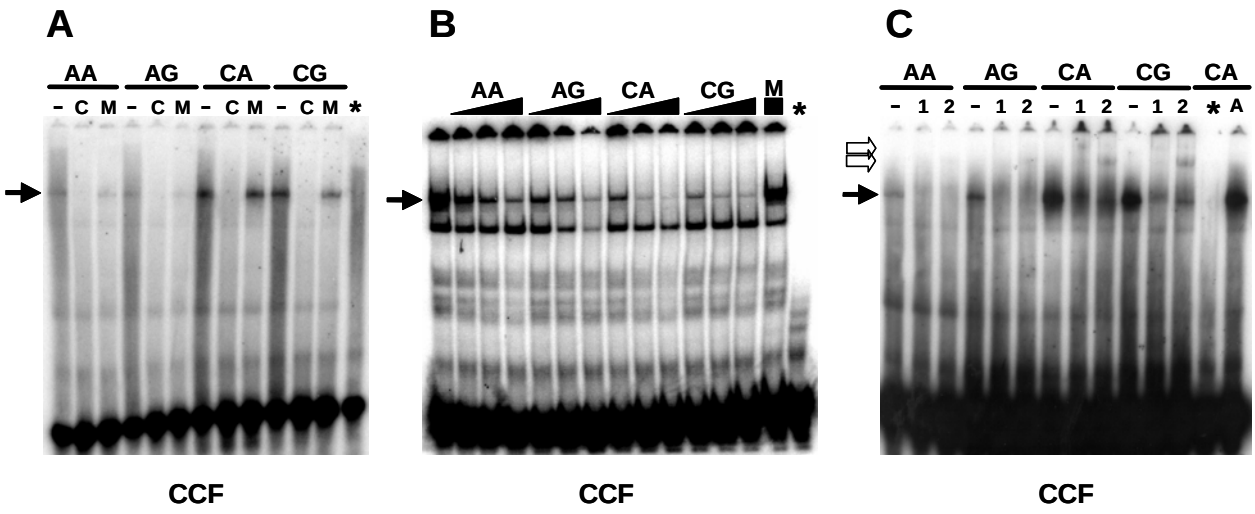

Figure S3. *Electrophoretic Gel Mobility Shift Assays*.

EMSA analyses utilizing nuclear extracts prepared from CCF cells and the oligonucleotides as indicated (AA, AG, CA and CG, detailed in Figure 1B). A is an EMSA with competition using mismatched cold competitor (50-fold molar excess)(M) or using cold competitor (labeled as C) derived from the same sequence as the radiolabeled probe. B is an EMSA using the CA radiolabeled probe with increasing amounts (10-, 25-, or 50-fold molar excess) of the indicated cold competitor or mismatched competitor (M). C is an EMSA using the indicated probes and antibody against USF1 (1), USF2 (2), or β-Actin (A). * indicates free probe using radiolabeled CA oligo without nuclear extract. Arrow, specific gel shift band; Open arrowhead, supershift bands.

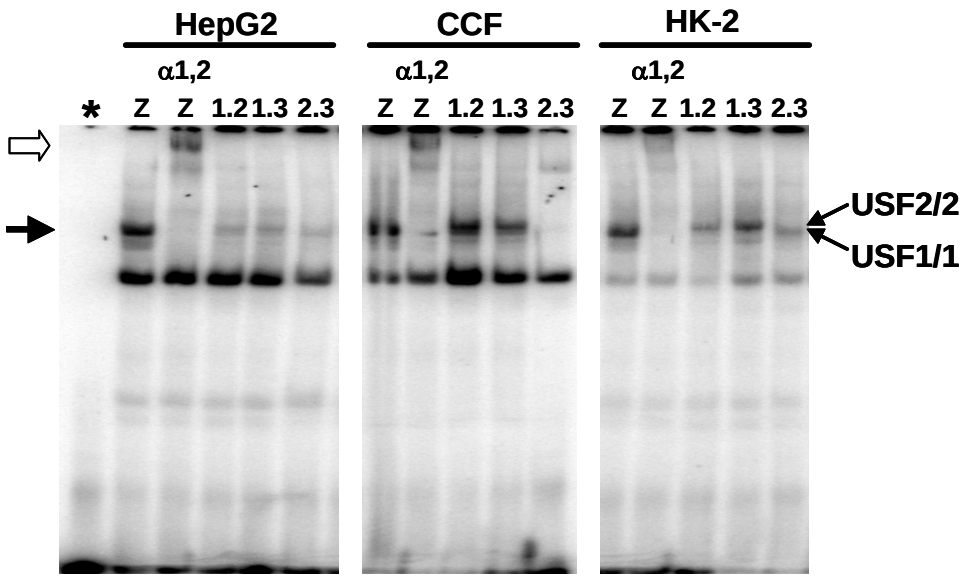

Figure S4. *Electrophoretic Gel Mobility Shift Assays on AdshRNA Treated Cells*.

EMSA analyses utilizing nuclear extracts prepared from the indicated cell type using the CA probe. Z indicates AdLacZ treated cells whereas 1.2, 1.3 and 2.3 indicate the AdshRNA vectors targeting USF1 or USF2. α1,2 is antibodies against USF1 and USF2 in supershift. * indicates free probe using radiolabeled CA oligo without nuclear extract. Arrow, specific gel shift band; Open arrowhead, supershift bands. The position of the USF1/1 and USF2/2 homodimers is indicated.