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Sp1 and Sp3 regulate basal transcription of the human APOBEC3G gene

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ABSTRACT

APOBEC3G (A3G), a member of the recently discovered family of human cytidine deaminases, is expressed in peripheral blood lymphocytes and has been shown to be active against HIV-1 and other retroviruses. To gain new insights into the transcriptional regulation of this restriction factor, we cloned and characterized the promoter region of A3G. Transcriptional start sites were identified by 5′-rapid amplification of cDNA ends analysis. Luciferase reporter assays demonstrated that a 1025 bp A3G promoter sequence (from −959 to +66 relative to the major transcriptional start site) displayed constitutive promoter activity. In T cells, the A3G promoter was not inducible by mitogenic stimulation, interferon treatment or expression of HIV-1 proteins. Using a series of 5′ deletion promoter constructs in luciferase reporter assays, we identified a 180 bp region that was sufficient for full promoter activity. Transcriptional activity of this A3G core promoter was dependent on a GC-box (located at position −87/−78 relative to the major transcriptional start site) and was abolished after mutation of this DNA element. Electrophoretic mobility shift assays and chromatin immunoprecipitation assays demonstrated that the identified GC-box represented a binding site for the ubiquitous transcription factors specificity protein (Sp) 1 and Sp3.

INTRODUCTION

The recently discovered APOBEC3 family of cytidine deaminases is considered to play an important role in antiviral intrinsic immunity (1,2). In primates, the seven paralogs APOBEC3A, B, C, DE, F, G, H (A3A-H) have been described (3), and they appear to fulfill individual functions. Human APOBEC3G (A3G), the most prominent member of the APOBEC3 family has been identified as the cellular restriction factor that is responsible for inhibition of *virion infectivity factor* (Vif)-deleted human immunodeficiency virus-1 (HIV-1) replication in non-permissive cells (4). A3G is packaged into HIV-1Δ*vif* particles and causes C-to-U deaminations on the single-stranded viral DNA during reverse transcription (5–8). This leads to degradation of the uracile-containing DNA by cellular repair mechanisms or to hypermutation of the viral genome (5,6). As a result, only a marginal fraction of the A3G-containing HIV-1 particles is able to complete the replication cycle. In addition to the inhibition of HIV-1, A3G restricts replication of other lentiviruses, gammaretroviruses, deltaretroviruses, spumaviruses, long-terminal-repeat (LTR)-retrotransposons, orthohepadnaviruses and avihepadnaviruses (9–21). Interestingly, deamination seems not to be the only A3G-mediated antiviral mechanism; in the case of hepatitis B virus (HBV) and human T cell leukemia virus type 1 (HTLV-1), A3G was shown to restrict virus replication by deamination-independent mechanisms (12,13,19,22–25). Another member of the APOBEC3 family, APOBEC3F (A3F), appears to have similar activities like A3G (26,27). A3F is also packaged into HIV-1Δ*vif* particles and induces similar C-to-U deaminations, although the proteins differ in their target sequences specificity (26,28). Furthermore, A3F proteins were detected in many tissues that express A3G and are able to form heteromultimers with A3G (26,29,30). Both proteins localize to mRNA processing (P) bodies, cytoplasmic compartments involved in the degradation and storage of non-translating mRNAs (30,31).

A3G has been shown to be expressed in T cells, a relevant cell target for HIV-1 *in vivo*, but little is known about its regulation (4,29,32). There is a report describing that mitogenic stimulation of T cells upregulates A3G mRNA levels, but this was not analyzed on the transcriptional level (33). Since the A3G promoter has not been systematically analyzed so far, our aim was to clone the A3G promoter and characterize its regulation in T cells. In our study, we observed that A3G uses multiple

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transcriptional start sites (TSS). By generating a series of 5′ deletions of the A3G promoter, we identified a 180 bp region that mediated basal transcription. In T cells, transcriptional activity of this core promoter was not inducible by mitogenic stimulation or interferon treatment, but was dependent on a GC-box which was recognized by Sp (specificity protein) 1 and Sp3 transcription factors.

MATERIALS AND METHODS

Cell culture

The human T cell lines A3.01 and PM1 (NIBSC, UK) and the human myeloid cell line U937 (NIBSC, UK) were grown in complete RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin–streptomycin. The human hepatic cell lines HepG2 and Huh7 (kindly provided by Dr Thomas Pietschmann, Department of Molecular Virology, University of Heidelberg) as well as HeLa cells were maintained in Dulbecco's high glucose modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin–streptomycin. Cells lines were incubated at 37°C with 100% humidity in 5–7% CO_2 and passaged using standard cell culture techniques.

Plasmids

For cloning of an APOBEC3G promoter-driven reporter plasmid, genomic DNA was prepared from the T cell line PM1 using the DNeasy Kit (Qiagen). The DNA sequence ranging from positions −959 to +66 relative to the identified transcription start was amplified via PCR using the primers 3Gprom1025 (5′-TGTGAACGCGT TGCTGCAGGCCATCTGGATGTATATG-3′) and 3Gpromreverse (5′-ACAGCAGATCTAGGGACCTCT GATAAAGACAGG-3′). PCR reactions were performed with *Pwo* DNA Polymerase (Roche) using the following cycle conditions: one cycle 94°C for 2 min; 30 cycles 94°C for 30 s, 58°C for 60 s, 72°C for 60 s; one cycle 72°C for 7 min. The amplicon was ligated into the promoterless luciferase reporter plasmid pGL3-Basic (Promega) via MluI and BglII restriction sites, which were introduced by the primers. The resulting construct contained 1025 bp of the A3G promoter and was designated pGL3-APOprom1025. Reporter plasmids containing shorter fragments of the APOBEC3G promoter were constructed using pGL3-APOprom1025 as template and the following forward primers: for plasmid pGL3-APOprom502 (containing sequence −436/+66): 3Gprom502 (5′-TGTGAACGCGTTCCATAACATGG GGACAAGA-3′); for plasmid pGL3-APOprom225 (containing sequence −159/+66): 3Gprom225 (5′-TGTG AACGCGTCGAGGGCAGGATCCGGGAGT-3′); for plasmid pGL3-APOprom180 (containing sequence −114/ +66): 3Gprom180 (5′-TGTGAACGCGTTCTTGATGG TGGAGAGGAGG-3′); for plasmid pGL3-APOprom150 (containing sequence −84/+66): 3Gprom150 (5′-TGTGA ACGCGTGCGGGACCACCAGGGGAGGGGCTT-3′); for plasmid pGL3-APOprom120 (containing sequence −54/+66): 3Gprom120 (5′-TGTGAACGCGTTGC TGGCTCAGCCTGGTGTG-3′); for plasmid pGL3-APOprom60 (containing sequence +7/+66): 3Gprom60 (5′-TGTGAACGCGTCCCTTTGCAATTGCCTTG-3′); each in combination with the reverse primer 3Gpromreverse (described above). PCR reactions were performed with *Pfu* Ultra Hotstart (Stratagene) using the following cycle conditions: one cycle 94°C for 2 min; 30 cycles 94°C for 45 s, 58°C for 45 s, 72°C for 60 s; one cycle 72°C for 7 min. As for pGL3-APOprom1025, MluI and BglII restriction sites were introduced via the primers and PCR products were ligated into pGL3-Basic (Promega) via these restriction sites. pGL3-APOprom180mut carries two point mutations (bold) and was generated using the primer 3GProm180mut (5′-TGTGAACGCGTTCT TGATGGTGGAGAGGAGGCTCCAGCTG**TT**CGGG ACCACCAG-3′) in combination with primer 3Gpromreverse. This PCR was performed with an annealing temperature of 65°C. pGL3promE1 (containing nucleotides −114/−85) and pGL3promE2 (containing nucleotides −92/−63) were constructed by annealing the following single-stranded oligonucleotides: 114_85Plus (5′- CGCGTTCTTGATGGTGGAGAGGAGGCTCCA GCTGGA-3′) and 114_85Minus (5′- GATCTCCA GCTGGAGCCTCCTCTCCACCATCAAGAA-3′) or 92-63Plus (5′-CGCGTCCAGCTGGGCGGGACCACC AGGGGAGGGGCA-3′) and 92_63Minus (5′-GATCT GCCCCTCCCCTGGTGGTCCCGCCCAGCTGGA-3′). After annealing, the double-stranded oligonucleotides which contained the respective 30 bp of the APOBEC3G promoter and sticky ends compatible with MluI and BglII restriction sites were ligated into the pGL3-Promoter (Promega) vector. The sequences of all constructed plasmids were verified by sequence analysis. Nucleotide −219 of the cloned APOBEC3G promoter differs from the sequence in the database (GenBank™ accession number DQ147772). An A-to-C substitution is present at this position. Numbering is relative to the major transcriptional start site we identified.

The reporter plasmids pGL3-Control and phRG-TK were purchased from Promega. pGL2-CVX contains two repeats of the IFN-responsive GAS (gamma activated sequence) elements (GATCTGGATTTAGAGTAATA TGAAACTGAAAGTACTTCG) of the guanylate-binding protein (GBP) gene in front of a CMV minimal promoter and was kindly provided by Ute Pägelow and Mario Köster from the Helmholtz-Zentrum für Infektionsforschung. Plasmid pNL4-3 (NIBSC, UK) contains the full-length HIV-1_{NL4-3} genome and has been described previously (34). pcDNA3.1Vif was generously provided by Nathaniel R. Landau from the Salk Institute, La Jolla. It was generated by amplifying the Vif gene from pNL4-3 and ligating it into the pcDNA3.1 vector via BamHI and XhoI restriction sites. A 3′-WPRE element was included into the XhoI site. pBS-kRSPA-$Tat_{HIV-1(NL4-3)}$ was constructed by amplifying the two exons of Tat via PCR reaction using the molecular clone pNL4-3 as template and the following primer sets: exon1, 5ÜXho1HIV-Tat1Plus (5′-GCATGCTCGAGAT GGAGCCAGTAGATCCTAG-3′) and HIV-Tat1Minus (5′-TGCTTTGATAGAGAAGCTTGATG-3′); exon2,

15FHIV-Tat2Plus (5′-TTCTCTATCAAAGCAACCCAC CTCCCAATCCCG-3′) and 5ÜSpe1HIV-Tat2Minus (5′-GACGTACTAGTCTATTCCTTCGGGCCTGTC-3′). XhoI and SpeI restriction sites were introduced via the primers. The sense-primer of exon2 starts with a 15-mer which is homologous to the 3′ end of exon1 and necessary for fusion of both exons. PCRs were performed with Expand High Fidelity PCR System (Roche) using the following conditions: one cycle 94°C for 3 min; 35 cycles 94°C for 45 s, 55°C for 45 s, 68°C for 45 s; one cycle 68°C for 7 min. For fusion of both exons, the following PCR conditions were applied: one cycle 94°C for 3 min; 35 cycles 94°C for 45 s, 58°C for 45 s, 68°C for 60 s. After 10 cycles without primers, the sense-primer of exon1 and the antisense-primer of exon2 were added for the remaining cycles. The resulting amplicon was ligated into the pBS-kRSPA vector (35) via XhoI and SpeI restriction sites.

5′-Rapid amplification of cDNA ends analysis (RACE)

Total RNA was isolated from A3.01 T cells using RNeasy mini kit (Qiagen). The transcriptional start sites of A3G were identified using the 5′/3′ RACE Kit, 2nd Generation (Roche) according to the manufacturer's instructions. The following primers were used: RACE-APO3G1 (5′-TATCCCTTGTACACTTTGT-3′) for cDNA synthesis, RACE-APO3G2 (5′-CATACTCCTGGTCACGAT-3′) for the first PCR and RACE-APO3Gnest (5′-GAATACACCTGGCCTCGAA-3′) for the nested PCR. Reaction products were analyzed by agarose gel electrophoresis, purified using QIAquick gel extraction kit (Qiagen), T/A-cloned into vector pCR4-TOPO (Invitrogen) and sequenced.

Luciferase assay

For transient transfection of A3.01 and U937 cells, DMRIE-C transfection reagent (Life Technologies) was used (36). Cells were seeded in 6-well tissue culture plates (5×10^5 cells per well) in 1.5 ml Opti-MEM (Life Technologies) containing 0.5 μg *firefly* luciferase reporter plasmid and 3.5 μl DMRIE-C. After 4–5 h of incubation, 1.5 ml complete RPMI medium were added. HepG2 and Huh7 cell lines were transfected using LipofectAMINE Plus as recommended by the manufacturer (Life Technologies). Briefly, exponential growing cells (1.5×10^6) were transfected with 5 μl LipofectAMINE, 6 μl PLUS reagent and the required amount of plasmid DNA in a final volume of 1 ml Opti-MEM. Following 4 h of incubation, cells were washed in PBS and 3 ml of complete DMEM medium were added.

For cotransfection of reporter plasmids and siRNA into HeLa cells, HiPerfect transfection reagent (Qiagen) was used according to the manufacturer's protocol for cotransfection of adherent cells with siRNA and plasmid DNA.

Two days after transfection of the respective cell lines, cells were harvested in 100 μl (suspension cells) or 300 μl (adherent cells) of Passive Lysis Buffer (Promega) and luciferase assay was performed using the Dual Luciferase Assay System (Promega) according to the manufacturer's instructions. As an internal control, 50 ng (adherent cells) or 100 ng (suspension cells) of ph-RG-TK plasmid (Promega), which constitutively expresses *renilla* luciferase was cotransfected in every sample and *firefly* luciferase activities were normalized to *renilla* luciferase activities. Mean values (±SD) of a representative experiment performed in triplicate are shown in the figures. For stimulation of cells, final concentrations of 20 ng/ml TPA (Sigma) or 30 ng/ml IFN-α or 30 ng/ml IFN-γ (Tebu-Bio) were applied approximately 15 h before harvesting for luciferase assay.

Electrophoretic mobility shift assay (EMSA)

For preparation of nuclear extracts, 5×10^6 A3.01 T cells were washed in cold PBS and resuspended in 500 μl buffer A (10 mM HEPES pH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). After incubation for 15 min on ice, swollen cells were pressed 10 times through a syringe with a 26G needle and centrifuged at 5000 r.p.m. for 5 min. Pellets contained the nuclei and were washed in buffer A for two times and resuspended in 50 μl buffer C (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA 1 mM DTT, 1 mM PMSF). After shaking for 30 min at 4°C and centrifugation for 10 min at 13 000 r.p.m., supernatants were used as nuclear extracts.

EMSA probes were generated by annealing the following complementary oligonucleotides: APO-Sp1/3, 5′- CCA GCTGGGCGGGACCACCAGGGGAGGGGC-3′ and 5′-GCCCCTCCCCTGGTGGTCCCGCCCAGCTGG-3′; APO-Sp1/3mut, 5′- CCAGCTG**TT**CGGGACCACCAG GGGAGGGGC-3′ and 5′- GCCCCTCCCCTGGTGG TCCCG**AA**CAGCTGG-3′ according to standard procedures. Nucleotides differing from the original promoter sequence are shown in bold type. A commercially available Sp1 probe (sc-2502, referred to as Sp1cons) was purchased from Santa Cruz Biotechnology. The double-stranded oligonucleotides were 5′ end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and purified by using Nick G50 columns (Amersham).

For EMSA, 5 μg of nuclear proteins were preincubated on ice with 2 μg of poly(dI-dC) (Roche) as an unspecific competitor and 1 μg of bovine serum albumin in band shift buffer (50 mM Tris, 150 mM KCl, 5 mM EDTA, 2.5 mM dithiothreitol, 20% Ficoll) for 15 min. ^{32}P-labeled oligonucleotides (50 000 c.p.m.) were added in a total volume of 20 μl, incubated on ice for 20 min and loaded onto 5% native polyacrylamide gels in 0.5×Tris-borate-EDTA buffer. Upon fractionation, gels were dried and exposed for autoradiography. For competition experiments, 1- or 30-fold molar excess of the unlabeled APO-Sp1/3 or APO-Sp1/3mut oligonucleotides was added to the preincubation mixture. For supershift experiments, 2 μg Sp1 antibody (sc-59x, Santa Cruz Biotechnology) or Sp3 antibody (sc-644x, Santa Cruz Biotechnology) were added to the preincubation mixture and preincubation time was extended to 30 min.

Chromatin immunoprecipitation (ChIP) assay

A3.01 cells were treated with RPMI culture medium containing 1% formaldehyde for 10 min. at 37°C. Cells were washed twice with ice-cold PBS and incubated for 10 min. on ice after resuspension in SDS lysis buffer (ChIP Assay Kit, Upstate). After centrifugation, pellets were resuspended in MNase reaction buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 4% NP-40). DNA digestion was performed using 50 U Micrococcal Nuclease (Fermentas) and 1×10^7 cells per tube in a volume of 1.5 ml. After 2 min, reaction was stopped by adding 30 µl 200 mM EGTA. Further steps were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate) according to the manufacturer's instructions. 1×10^7 cells and 2 µg antibody (Sp1(Pep2) sc-59, Sp3(D-20) sc-644 or actin(H-196) sc-7210, Santa Cruz Biotechnology) were used for each immunoprecipitation. All buffers were freshly supplied with protease inhibitors. After phenol/chloroform extraction and ethanol precipitation, DNA was resolved in 16 µl H_2O. Immunoprecipitated DNA was detected by nested PCR using 4 µl of the resolved DNA in the first PCR, and 1 µl for the nested PCR. For amplification of the A3G promoter, the following primers were used: ChIP3Gplus 5′-ccacggtggcctccgagggtga-3′ and ChIP3G-minus: 5′-ctctccaccatcaagacagac-3′ (1. PCR); ChIP3G2-plus: 5′-tactctccctccctgtcccca-3′ and ChIP3G nested minus: 5′-aggctgatgcctccgcag-3′ (nested PCR). Taq polymerase (Qiagen) was used together with the following cycle conditions: one cycle 94°C for 2 min; 30 cycles 94°C for 30 s, 60°C for 60 s, 72°C for 2 min; one cycle 72°C for 10 min. As a negative control, a region in the A3G gene was targeted using the primers ChIP3Gneg_plus: 5′-taagtaccacccagagatgag-3′ and ChIP3Gneg_minus: 5′-catgatcttcatggtggcacg-3′ for both PCR steps. PCR conditions were the same as for the A3G promoter sequence, with the exception that annealing temperature was decreased to 55°C.

RNA interference and western blot analysis

Sp1 and Sp3 translation was silenced in HeLa cells using the siRNA duplexes Hs_SP1_1_HP and Hs_SP3_1_HP (Qiagen). A nonspecific siRNA (Qiagen) was used as control. HeLa cells were transfected with 150 or 300 ng siRNA per 6-well, using the HiPerfect transfection reagent (Qiagen) according to the manufacturer's protocol for reverse transfection of adherent cells in 6-well plates.

Forty-eight hours after transfection, HeLa cells were harvested for detection of Sp1 and Sp3 proteins. Cells were washed in PBS, lysed in RIPA (25 mM Tris pH 8.0, 137 mM NaCl, 1% Glycerol, 0.5% sodium deoxycholate, 1% NP-40, 2 mM EDTA pH 8, 0.1% SDS and protease inhibitors) and lysates were cleared by centrifugation. After boiling with Laemmli's buffer, samples were subjected to SDS–polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane. Sp1 and Sp3 proteins were detected using α-Sp1(Pep2) antibody (sc-59, Santa Cruz) or α-Sp3(D-20) antibody (sc-644, Santa Cruz) followed by incubation with α-rabbit-HRP (Amersham Biosciences). For detection of tubulin, α-tubulin (B5-1-2, Sigma) and α-mouse-HRP (Amersham Biosciences) antibodies were used. Signals were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

RESULTS

Characterization of the transcriptional start sites of APOBEC3G by 5′-RACE

To identify the transcriptional start sites (TSS) of APOBEC3G (A3G) in A3.01 T cells, we performed 5′-rapid amplification of cDNA ends analysis (RACE) with A3G-specific primers (see Figure 1). Agarose gel electrophoresis resolved the nested PCR products into three bands of different electrophoretic mobility with a dominant middle band (Figure 2). For each band, the DNA was cloned and sequence analysis of six or seven individual transformants was performed. We observed that the transcriptional start sites of the A3G gene were located between 58 and 361 nt upstream of the ATG start codon (Figure 1). Although TSS were variable and most sites were only detected once among the 19 clones analyzed, one TSS was identified in six individual clones. This TSS was located 66 nt upstream of the start of the published A3G mRNA sequence (GenBank™ accession number NM021822) and we defined this position as the major transcriptional start site of the A3G gene.

The core promoter of A3G is located within the region −114/+66 relative to the TSS

For characterization of the A3G promoter, we cloned the 1025 bp located at position −959/+66 relative to the identified transcription start into the promoterless pGL3-Basic luciferase reporter plasmid and designated the plasmid pGL3-Basic-APOprom1025. Similarly, pGL3-APOprom502 (containing sequence −436/+66), pGL3-APOprom225 (containing sequence −159/+66) and further 5′ deletion reporter constructs containing 180, 150, 120 or 60 bp upstream of position +65 were generated (Figure 3A). In order to analyze transcriptional activity, the luciferase reporter plasmids were transiently transfected into A3.01 T cells. Luciferase assays revealed a ~20-fold increased transcriptional activity of the 1025 bp sequence as compared to the empty vector, indicating that we had identified an active A3G promoter sequence (Figure 3B). This transcription rate was not significantly altered by the 5′ deletions leading to the 502, 225 and 180 bp fragments (Figure 3B). In contrast, a drop in luciferase activity was observed in the case of the 150 bp fragment. This construct only retained 28% of the transcriptional activity of the 180 bp promoter in A3.01 T cells and activity of the 120 bp fragment was further reduced. Comparable reductions relative to the activity of 180 bp fragment were observed in the myeloid cell line U937 and the hepatic cell lines HepG2 and Huh7 (Figure 3C), indicating that the core promoter of A3G is located within the region −114/+66 relative to the TSS.

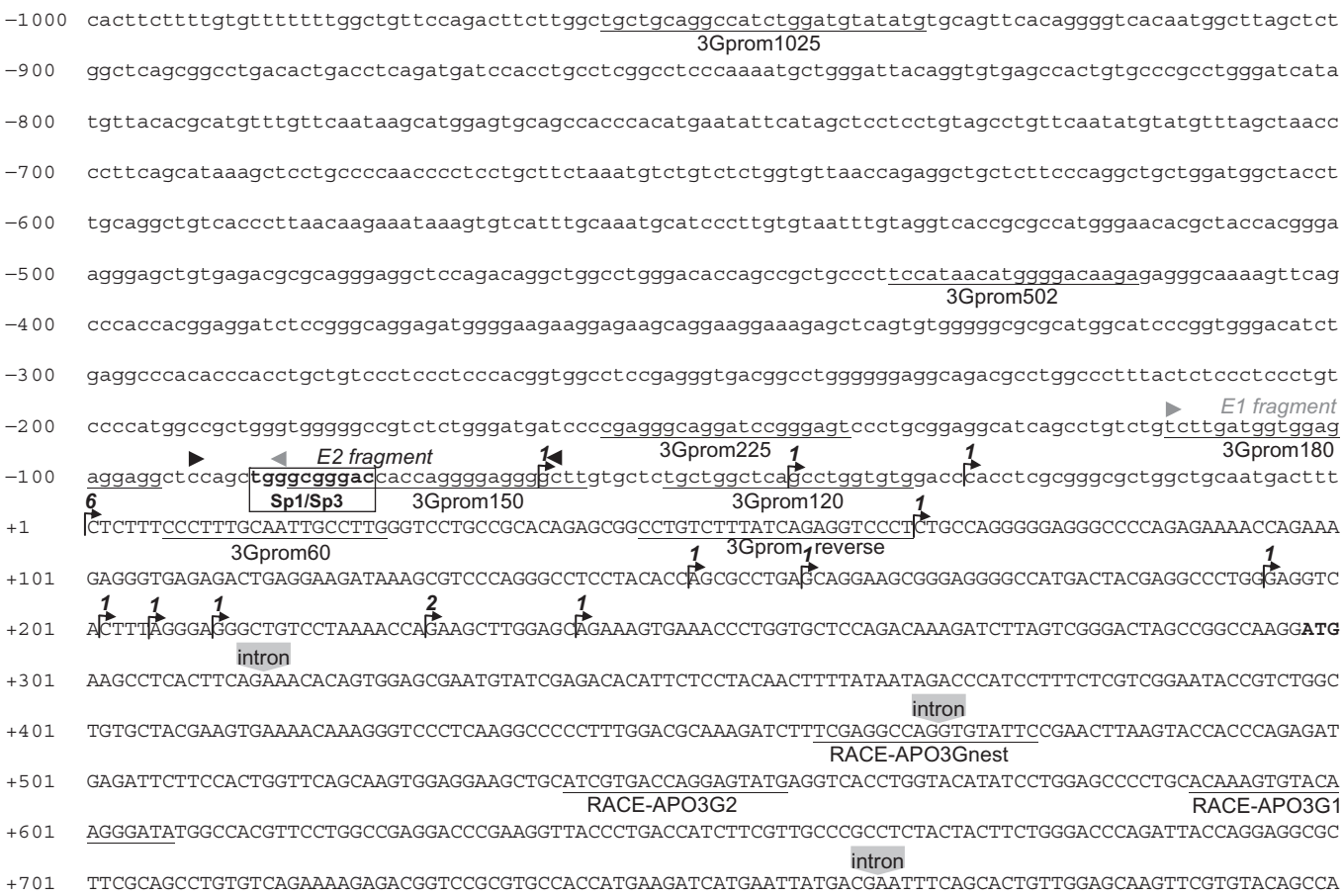


Figure 1. Sequence of the A3G promoter and the downstream region. The first 1000 bp upstream of the major TSS are shown in lower case, the first 800 bp of the transcribed sequence are shown in upper case. Introns are removed, but their positions are indicated. Arrows refer to transcriptional start sites and their observed frequency is given by the numbers above. The primer binding sites for 5′-RACE analysis and cloning of the luciferase reporter constructs are underlined and the names of the primers are annotated. Gray and black arrowheads define the regions designated E1 and E2 which were cloned into vector pGL3-Promoter and used as EMSA probes. The ATG start codon and the identified Sp1/Sp3 transcription factor binding site are shown in bold.

The A3G promoter is not inducible in T cells

According to the current knowledge, APOBEC3G plays a role in the innate defence against pathogens like HIV-1. The latter has evolved a mechanism to counteract A3G activity by expressing the regulatory protein Vif (4). Vif induces proteasomal degradation of A3G and additionally inhibits A3G activity by further mechanisms (8,37–43). To investigate whether the overexpression of HIV-1 proteins also influences A3G promoter activity, we either expressed HIV-1 Vif or the HIV-1 Tat protein, the latter has been shown to activate different viral and cellular promoters (44,45). In addition, increasing amounts of the plasmid pNL4-3, containing the full-length genome of HIV-1, were transfected. These constructs or the empty vector pcDNA3.1 were cotransfected with the 1025 bp construct into A3.01 T cells. Luciferase assays showed that the transcriptional activity of the A3G promoter was not significantly altered in the presence of Vif, Tat or HIV-1_{NL4-3} (Figure 4A), suggesting that HIV-1 is not modulating A3G expression on the transcriptional level.

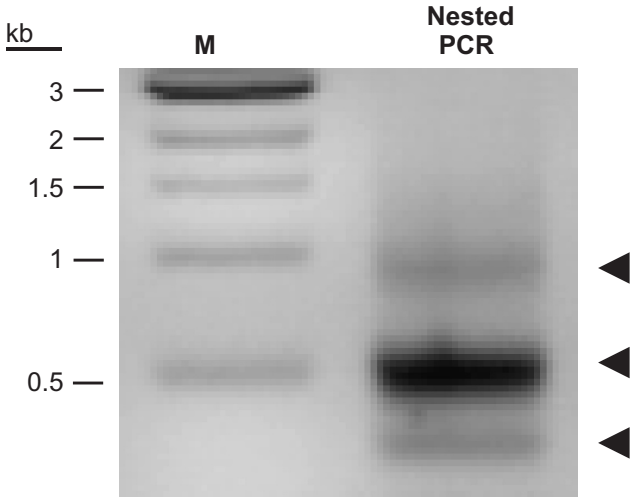


Figure 2. 5′-RACE analysis of the A3G cDNA. Agarose gel electrophoresis of size marker and A3G 5′-RACE products after nested PCR with primer RACE-APO3Gnest (see Figure 1 for primer details). Arrowheads indicate the three resulting DNA bands which were cloned and sequenced.

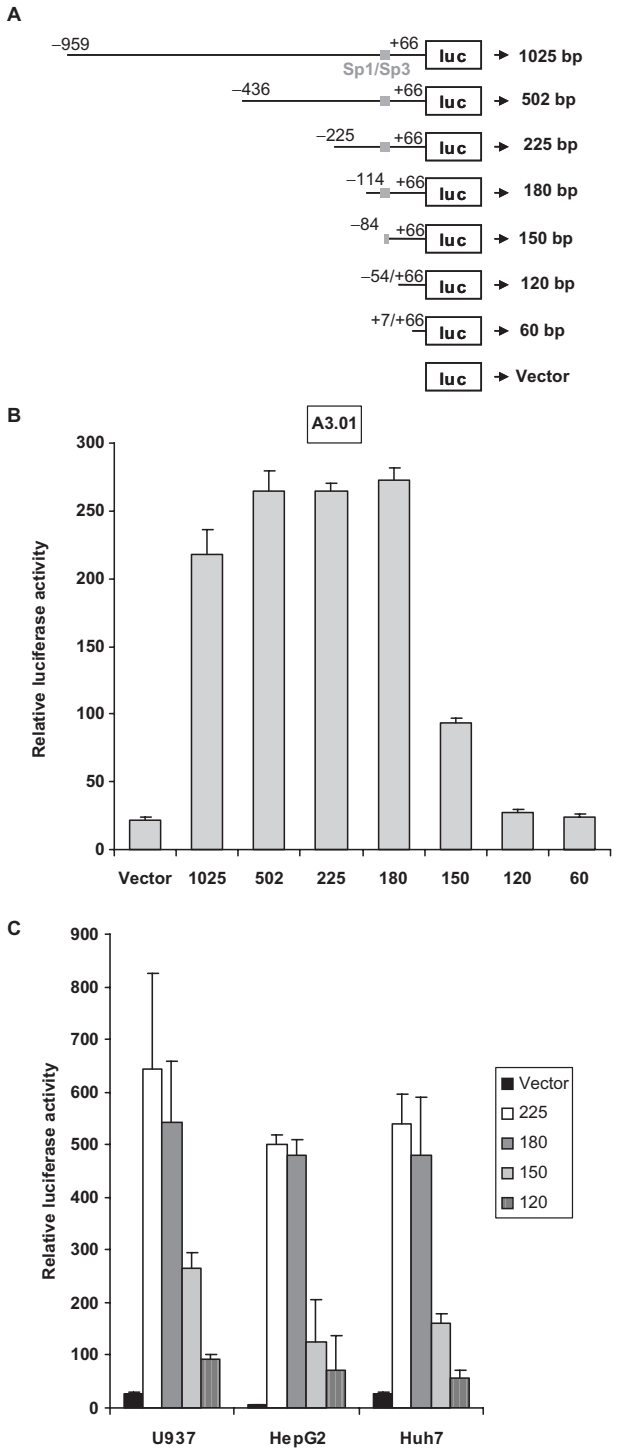
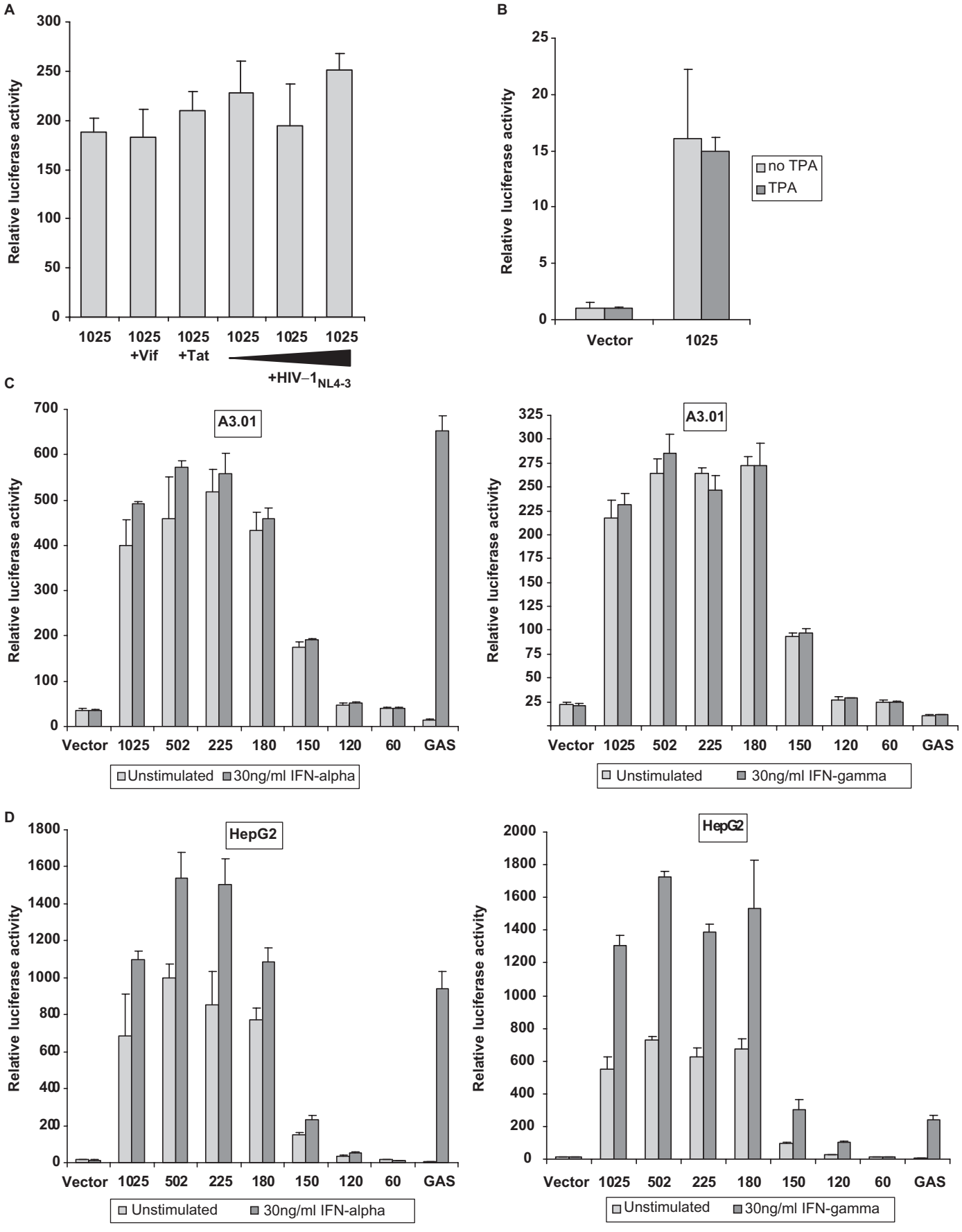


Figure 3. Luciferase activities of A3G promoter constructs in different cell lines. (**A**) A3G promoter 5′ deletion constructs of different sizes were cloned into pGL3-Basic luciferase reporter plasmids. Numbering is relative to the major TSS. A putative Sp1/Sp3 consensus site (gray square) is depicted. (**B**) A3.01 T cells were transiently transfected with the A3G promoter deletion constructs. Numbers on the *x*-axis refer to the length of the A3G promoter fragments in bp. (**C**) A3G promoter plasmids were transfected into U937, HepG2 and Huh7 cell lines. Numbers in the legends refer to the length of the A3G promoter fragments in bp. After 48 h, cells were harvested for luciferase assay. *Firefly* luciferase activities were normalized to coexpressed *renilla* luciferase activities. Mean values (±SD) of a representative experiment performed in triplicate are shown.

It has been shown that the amount of A3G mRNA in T cells is increased in response to mitogenic stimulation with phorbol ester (46–48). In addition, interferons have been described to upregulate A3G expression in hepatocytes and macrophages (46,48). To investigate whether these stimuli interfere with A3G promoter activity in T cells, we transfected A3.01 T cells with the 1025 bp promoter or the empty vector pGL3-Basic (vector) and treated the cells with phorbol ester (TPA). The luciferase assay showed that the ~15-fold increased transcriptional activity of the 1025 bp promoter relative to the empty vector was not further enhanced by TPA treatment (Figure 4B), although the functional activity of TPA was confirmed by induction of the SV40 promoter-containing reporter plasmid pGL3-Control (data not shown). Similarly, treatment of A3.01 T cells transfected with the A3G promoter deletion constructs with IFN-α or IFN-γ showed no effect (Figure 4C). Interestingly, a control plasmid (pGL2-CVX) containing two IFN-responsive GAS (gamma activated sequence) elements upstream of the luciferase reporter gene was only induced by IFN-α in these cells (Figure 4C). Since two reports describe A3G upregulation by interferons in hepatocytes (47,48), we additionally performed the experiment in the hepatic cell line HepG2. In line with these publications, we observed an induction of the A3G promoter by approximately 2-fold after IFN-α or IFN-γ stimulation (Figure 4D) with IFN-γ being slightly more potent. For both interferon types, induction of A3G promoter activity was observed for all deletion constructs except for the 60 bp fragment, indicating that the responsible region is located within the 60 nt present in the 120 bp, but not in the 60 bp fragment.

A GC-box located at position −87/−78 of the A3G promoter is important for transcriptional activity

The reporter studies shown in Figure 3 had demonstrated a drop in luciferase activity after deletion of the 30 nt at the 5′ end of the 180 bp core promoter. We therefore inspected the 30 bp sequence deleted in the 150 bp fragment and identified a GC-box at position −87/−78 (see Figure 1). The sequence TGGGCGGGAC, which is interrupted in the 150 bp fragment, represents a variant of the (G/T)GGGCGG(G/A)(G/A)(C/T) consensus motif recognized by Sp1 and Sp3 transcription factors. To analyze whether this putative Sp1/Sp3-binding site mediates transcriptional activity of the 180 bp core promoter, we introduced two point mutations which changed the sequence from TGGGCGGGAC to TG**TT**CGGGAC (mutations shown in bold). This resulted in a 71% reduction of the transcriptional activity compared to the unmodified 180 bp promoter (Figure 5A) and this value was only marginally higher than the luciferase activity of the 150 bp fragment, indicating that the identified motif is essential for basal activity of the A3G core promoter. To further examine the transcriptional potency of the 30 nt present in the 180 bp promoter, we cloned the region −114/−85 (containing all nucleotides which are deleted in the 150 bp fragment, designated E1) or the region −92/−63 (containing the putative Sp1/Sp3 motif, designated E2) into the vector pGL3-Promoter


A
Relative luciferase activity
300
250
200
150
100
50
0
1025
1025 +Vif
1025 +Tat
1025
1025
1025
+HIV−1NL4-3
B
Relative luciferase activity
25
20
15
10
5
0
no TPA
TPA
Vector
1025
C
A3.01
Relative luciferase activity
700
600
500
400
300
200
100
0
Vector
1025
502
225
180
150
120
60
GAS
Unstimulated
30ng/ml IFN-alpha
A3.01
Relative luciferase activity
325
300
275
250
225
200
175
150
125
100
75
50
25
Vector
1025
502
225
180
150
120
60
GAS
Unstimulated
30ng/ml IFN-gamma
D
HepG2
Relative luciferase activity
1800
1600
1400
1200
1000
800
600
400
200
0
Vector
1025
502
225
180
150
120
60
GAS
Unstimulated
30ng/ml IFN-alpha
HepG2
Relative luciferase activity
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Vector
1025
502
225
180
150
120
60
GAS
Unstimulated
30ng/ml IFN-gamma

(see Figure 1). This vector contains a luciferase reporter gene under the control of an SV40 promoter without enhancer sequences, and putative transcriptionally active sequences can be cloned upstream of the SV40 promoter. Luciferase assays showed that the E1 element increased SV40 promoter activity only by ~2-fold (Figure 5B). In contrast, the 30 nt of E2 enhanced the transcriptional activity of the SV40 promoter by ~4.3-fold, indicating that the intact GC-box present in the E2 element was responsible for the strongly enhanced transcriptional activity of the SV40 promoter.

Sp1 and Sp3 transcription factors bind to the GC-box at position −87/−78 of the A3G promoter

To investigate whether the GC-box represents a binding site for the transcription factors Sp1 and Sp3, we performed EMSA analyses with nuclear extracts isolated from A3.01 T cells. As probes, we radioactively labeled the unmodified E2 sequence (nucleotides −92/−63 of the A3G promoter, probe designated APO-Sp1/3) or the same region carrying the two point mutations described above (probe designated APO-Sp1/3mut). As control, we used a commercially available Sp1 consensus oligonucleotide (Sp1cons), which is known to be recognized by Sp1 transcription factors. Four DNA–protein complexes were observed in the presence of the APO-Sp1/3 probe (Figure 6A). The upper two complexes were specific since they disappeared in the presence of a 30-fold molar excess of unlabeled APO-Sp1/3 probe (Figure 6A, lane 7). Further confirmation of the specificity of these DNA–protein complexes was demonstrated by the inability of the unlabeled APO-Sp1/3mut probe to abolish binding (Figure 6A, lanes 12 and 13). As expected, the two specific complexes were also present in the case of the Sp1cons control probe (Figure 6A, lanes 2 and 3). In contrast, none of these specific complexes was observed when the mutated probe was used (Figure 6A, lanes 10 and 11), confirming that protein binding was dependent on the identified GC-box. In order to characterize the complexes, we performed supershift experiments using Sp1- and Sp3-specific antibodies. The upper of the complexes that appeared in combination with the Sp1cons or APO-Sp1/3 probes, shifted in the presence of the Sp1 antibody (Figure 6B, lanes 3 and 7), whereas the lower complex shifted in the presence of the Sp3 antibody (Figure 6B, lanes 4 and 8). Taken together, the EMSA

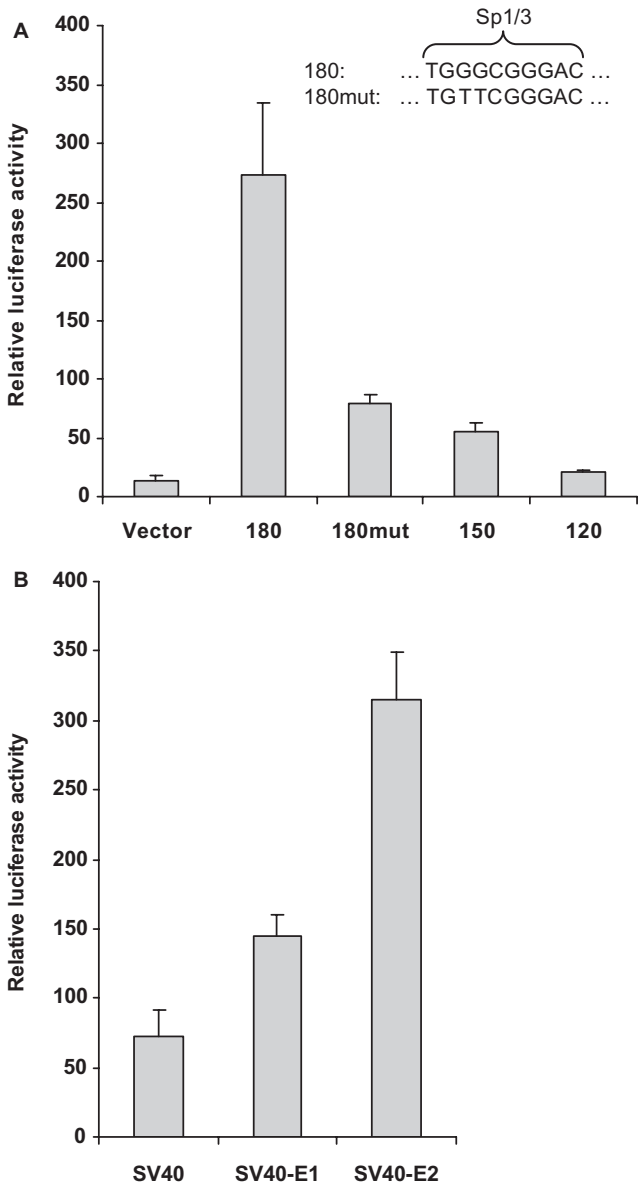


Figure 5. A GC-box mediates transcriptional activity of the 180 bp core promoter. (**A**) A3.01 T cells were transfected with reporter plasmid pGL3-Basic containing 180, 150 or 120 bp of the A3G promoter. The two G-to-T substitutions introduced into the GC-box of the 180 bp fragment (180mut) are specified. After 48 h, cells were harvested for luciferase assay. *Firefly* luciferase activities were normalized to coexpressed *renilla* luciferase activities. Mean values (±SD) of a representative experiment performed in triplicate are shown. (**B**) pGL3-Promoter reporter plasmids containing the regions E1 or E2 (see Figure 1) upstream of the SV40 promoter were transfected into A3.01 T cells. *Firefly* luciferase activities after 48 h were normalized to coexpressed *renilla* luciferase activities. Mean values (±SD) of a representative experiment performed in triplicate are shown.

Figure 4. A3G promoter activities after coexpression of HIV-1 proteins or treatment with TPA or interferons. (**A**) A3.01 T cells were cotransfected with pGL3-Basic reporter plasmid containing the 1025 bp A3G promoter and 1 μg of Vif expression plasmid, 1 μg Tat expression plasmid or increasing amounts of HIV-1_{NL4-3} (0.1, 0.5 and 1 μg). After 48 h, cells were harvested for luciferase assay. *Firefly* luciferase activities were normalized to coexpressed *renilla* luciferase activities. (**B**) A3.01 T cells were transiently transfected with pGL3-Basic reporter plasmid containing the 1025 bp A3G promoter or with empty vector. Fifteen hour before harvesting for luciferase assay, a subset of the cell culture was stimulated with 20 ng/ml TPA. Forty-eight hour after transfection, luciferase assay was performed. *Firefly* luciferase activities were normalized to coexpressed *renilla* luciferase activities and the values for the empty vectors (untreated and TPA-stimulated) were set as 1. (**C**) A3.01 T cells were transiently transfected with the A3G promoter constructs or with the interferon-responsive reporter plasmid pGL2-CVX (GAS). Fifteen hour before harvesting for luciferase assay, a subset of the cell culture was stimulated with 30 ng/ml IFN-α or IFN-γ. Forty-eight hour after transfection, luciferase assay was performed. *Firefly* luciferase activities were normalized to coexpressed *renilla* luciferase activities. (**D**) HepG2 cells were used for transfection. The experiment was performed as described in (C). Mean values (±SD) of representative experiments performed in triplicate are shown.

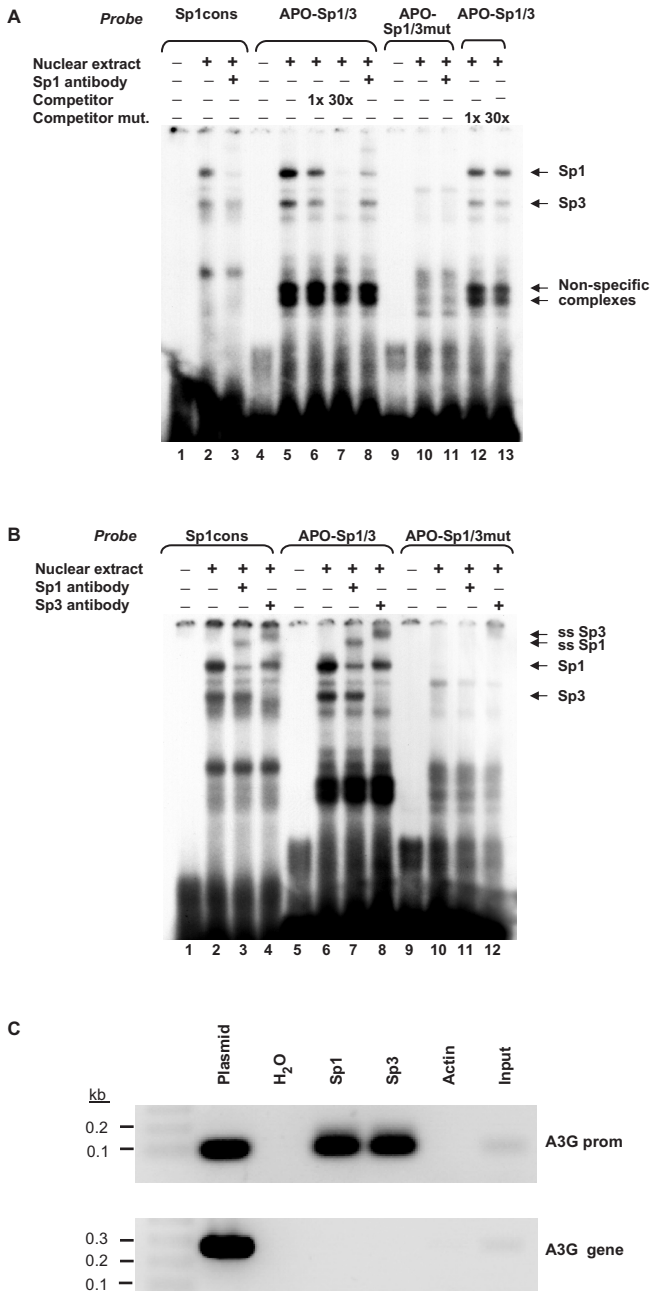


Figure 6. Sp1 and Sp3 bind to the GC-box present in the A3G promoter. (**A**) Nuclear extracts of A3.01 T cells were incubated with a ^{32}P-labeled commercial Sp1 oligonucleotide probe (Sp1cons) or labeled probes homologous to the unmodified or mutated E2 region (see Figure 1) of the A3G promoter (APO-Sp1/3 and APO-Sp1/3mut). Protein–DNA complexes were separated by polyacrylamide electrophoresis and detected by autoradiography. EMSA was performed with a 1- or 30-fold molar excess of unlabeled APO-Sp1/3 probe (competitor, lanes 6 and 7) or APO-Sp1/3mut probe (competitor mut., lanes 12 and 13). (**B**) Sp1- and Sp3-specific antibodies were added to the EMSA reactions resulting in a supershift (ss) of the respective antibody–protein–oligo complexes (lanes 3, 4, 7, 8, 11, 12). (**C**) ChIP assay was performed with DNA from A3.01 T cells. Immunoprecipitation was performed with antibodies against Sp1, Sp3 or actin. PCR primer pairs specific for the A3G promoter (upper panel) or the A3G gene (lower panel) were used. As positive controls, the sheared and cross-linked DNA before the immunoprecipitation step (input) or a plasmid carrying the target sequence (plasmid) was used as template.

demonstrated that the GC-box located on the A3G core promoter serves as a binding site for Sp1 and Sp3.

To show binding of Sp1 and Sp3 factors also in the context of the endogenous A3G promoter, a chromatin immunoprecipitation (ChIP) assay was performed. Sp1 and Sp3 antibodies, but not an actin antibody, immunoprecipitated the A3G promoter in A3.01 T cells (Figure 6C, upper panel). In contrast, no PCR signal was received with a primer pair recognizing a region in the A3G gene ~4000 bp downstream of the Sp1/Sp3–binding site (Figure 6C, lower panel). Only the positive controls showed a DNA band, with an A3G expression plasmid or the sheared and cross-linked input DNA used as template. This demonstrates the binding of Sp1 and Sp3 transcription factors to the endogenous A3G promoter.

Silencing of Sp1 and Sp3 reduces A3G promoter activity

To confirm the role of Sp1 and Sp3 in regulation of A3G promoter activity, we silenced their translation via RNA interference. Functionality of the siRNAs directed against Sp1 or Sp3 was confirmed by western blot analysis: protein levels of Sp1 as well as the long and short isoforms of Sp3 were strongly reduced in the presence of 150 or 300 ng specific siRNA (Figure 7A). An unspecific control siRNA had no influence (Figure 7A). We then cotransfected the luciferase reporter plasmid containing the 180 bp A3G promoter together with 100 ng siRNA using an optimized protocol for the cotransfection of plasmid plus siRNA. This resulted in a 31–43% reduction of luciferase activity in the presence of Sp1- or Sp3-specific siRNA compared to the control siRNA. In contrast, no influence on transcriptional activity of the 150 bp fragment, which does not contain the Sp1/Sp3-binding motif, was observed. Thus, both Sp1 and Sp3 factors are mediating transcriptional activity of the A3G promoter.

DISCUSSION

Shortly after the discovery of APOBEC3G, it became clear that this human gene plays an important role in antiretroviral defense (4). Originally identified as a restriction factor of HIV-1 infection, expression of APOBEC3G was found in human peripheral blood lymphocytes and macrophages, which represent the main target cells for HIV-1 (32). In addition, the protein was detected in lung, liver, spleen, testis and ovary, but little is known about its transcriptional regulation (29,32,47). To address this question, we cloned the human APOBEC3G promoter and analyzed its regulation in T cells. Applying 5′-RACE, we identified multiple start sites for transcription of the A3G gene. This observation is consistent with the fact that the gene appears to lack canonical CCAAT and TATA boxes (32), a condition often associated with multiple transcriptional start sites (49–51). However, one single start site was detected in 6 of 19 clones and was designated as +1. This TSS is located 66 bp upstream of the start of the published mRNA sequence (GenBank™ NM021822).

We cloned a 1025 bp promoter, which ranges from position −959/+66 relative to the identified TSS.

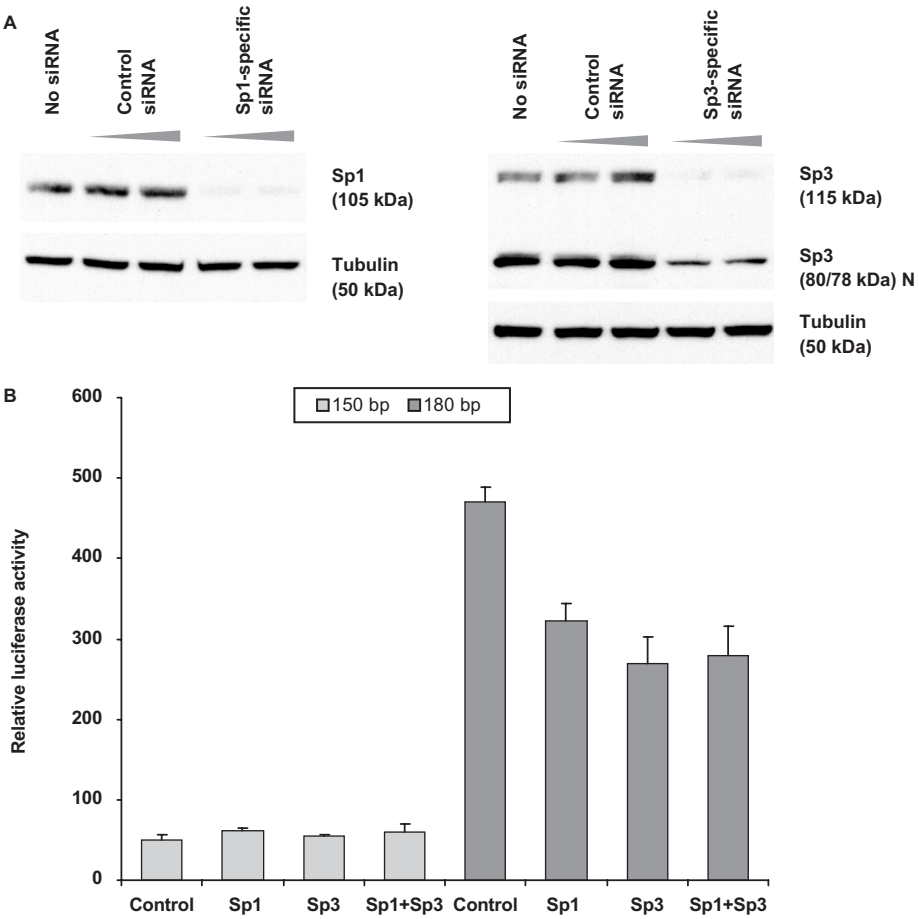


Figure 7. Silencing of Sp1 and Sp3 reduces A3G promoter activity. (**A**) HeLa cells were transfected with 150 and 300 ng of unspecific, Sp1-specific or Sp3-specific siRNA. After 48 h, cells were harvested and Sp1 and Sp3 proteins were detected by western blot analysis. As loading control, protein levels of tubulin are shown. (**B**) HeLa cells were cotransfected with reporter plasmid pGL3-Basic containing 180 or 150 bp of the A3G promoter and siRNA. Hundred nanogram of unspecific siRNA (control), Sp1-specific siRNA (Sp1), Sp3-specific siRNA (Sp3) or a mixture of 50 ng Sp1-specific plus 50 ng Sp3-specific siRNA (Sp1+Sp3) were used. *Firefly* luciferase activities after 48 h were normalized to coexpressed *renilla* luciferase activities. Mean values (±SD) of a representative experiment performed in triplicate are shown.

Luciferase reporter assays showed that this promoter was transcriptionally active in A3.01 T cells. Treatment with the phorbol ester TPA did not further enhance transcriptional activity, although upregulation of A3G mRNA levels in T cells by TPA has been described (33). Rose *et al.* used actinomycin D to block further transcription and did not find evidence for enhanced mRNA stability. Therefore, the authors suggested that an enhanced transcription rate could be responsible for the increased amount of A3G mRNA after TPA treatment. However, the promoter analysis we performed indicates a different mechanism that is independent of transcriptional regulation. In addition, we observed that the A3G promoter was not inducible by IFN-α or IFN-γ in A3.01 T cells, whereas in the hepatic cell line HepG2, a moderate induction was measured. It has been described previously that A3G gene expression is upregulated by interferons in hepatocytes and macrophages (46–48,52). In this context, two interferon-responsive elements have been identified at the positions −6/+9 and +1/+15 relative to the TSS (48). Consistent with this data, we observed an interferon induction in hepatocytes for the fragments ranging between 1025 and 120 bp length. The activity of the 60 bp fragment that does not contain these motifs was not affected. Thus, the induction we observed was most likely mediated by the described interferon-responsive elements. However, according to our results, these motifs can enhance transcription in hepatic cells, but not in T cells. This is surprising, since IFN-α-inducible signaling cascades are present in A3.01 T cells: we showed that the control plasmid harboring interferon-responsive GAS elements was markedly induced by IFN-α treatment. In contrast, the GAS element was not responsive to IFN-γ treatment in these cells, suggesting that our T cell line was not able to mediate IFN-γ-induced signals. So far, enhanced A3G promoter activity by interferons is clearly described in hepatocytes and macrophages, whereas the situation in T cells is still unclear. A recent study found no influence of IFN-α or IFN-γ on A3G expression in resting primary blood lymphocytes (52). Another publication describes an enhanced expression of A3G after IFN-α treatment in resting primary CD4 T cells, but not in

activated T cells (53). This observation is not contradictory to our results since T cell lines are mitotically active and therefore rather in an activated than in a resting state. In conclusion, the current data suggests that regulation of the A3G promoter activity by interferons is dependent on the cell type and possibly also from the cellular activation status.

We additionally analyzed the influence of HIV-1 proteins on A3G promoter activity. The HIV-1 Vif protein is neutralizing the antiviral function of A3G by a multitude of ways. Targeting the A3G protein for proteasomal degradation is considered to be the main mode of action (37–41), but it becomes more and more clear that other mechanisms are also involved. There is evidence that Vif also promotes exclusion of A3G from the virus particles and an influence on the A3G translation process is discussed (8,42,43). The question whether Vif alters transcription controlled by the A3G promoter has not been analyzed so far. Our analysis indicates that transcription from the A3G promoter is unaffected by Vif or other HIV-1 proteins. Taken together, in T cell lines, the A3G promoter appears constitutively active.

By generating a series of 5′ deletions, we showed that the core promoter is located within the region −114/+66 relative to the TSS. This 180 bp promoter was transcriptionally active in the lymphoid, myeloid and hepatic cell lines we tested, indicating that ubiquitous transcription factors are involved in regulation of A3G gene transcription. A GC-box with the sequence TGGGCGGGAC was identified at position −87/−78 of the A3G promoter. This GC-box is essential for basal promoter activity, since changing the motif to TG**TT**CGGGAC by introducing two point mutations strongly reduced A3G promoter activity. The transcriptional potency of the GC-box was further demonstrated by cloning it upstream of an SV40 promoter. This resulted in a 4-fold enhanced transcription rate. The hypothesis that a general transcription factor is involved in regulation of A3G transcription was confirmed by EMSA. Supershift analysis showed that the transcription factors Sp1 and Sp3 which are ubiquitously expressed in mammalian cells, bind specifically to the identified motif. In addition, the binding of Sp1 and Sp3 to the endogenous A3G promoter was demonstrated by a chromatin immunoprecipitation assay. This observation is consistent with the results of the 5′-RACE, since Sp1 is known to play an important role for RNA polymerase II to bind to the transcription initiation site in TATA-boxless promoters and is associated with multiple transcription initiation sites (54–56). We also tested whether overexpression of Sp1 or Sp3 proteins had an influence on A3G promoter activity. Luciferase assays revealed that A3G promoter activities remained unchanged (data not shown), most probably due to the high basal expression level of the endogenous Sp1 and/or Sp3 proteins. The Sp family of transcription factors is involved in transcriptional regulation of many housekeeping, tissue-specific, viral and inducible genes (57). Whereas Sp1 typically acts as an activator, Sp3 can serve as a repressor or activator (57). However, in the context of the A3G promoter, both transcription factors serve as activators, as shown by siRNA-mediated silencing of the single factors. Although siRNAs directed against Sp1 or Sp3 significantly reduced transcription controlled by the 180 bp promoter, transcriptional activity was not reduced to the level of the 150 bp fragment. This can be explained by the remaining low amounts of Sp1/Sp3 proteins which can be seen in the western blot analysis. Alternatively, other transcription factors could be involved in A3G regulation.

Our results also provide information about the regulation of APOBEC3F (A3F), another member of the human APOBEC3 family. A3F is expressed in many human tissues that also express A3G and both proteins have been shown to form heteromultimers, which are most likely generated through binding to an RNA intermediate (26,29,30). A3F only differs in 16 nt from the 560 nt upstream of the TSS of A3G (GenBank™ DQ146365 and DQ147772). The 180-bp core promoter region, which we identified for A3G has 100% identity with the A3F sequence. Therefore, Sp1/Sp3 transcription factors are most likely also mediating basal transcription of the A3F gene.

Our study revealed that the human A3G gene is controlled by a promoter with multiple transcriptional start sites. In A3.01 T cells, the A3G promoter appears constitutively active and is not inducible by TPA, type I or II interferons or by HIV-1 proteins. The core promoter is located within a region of 180 bp at position −114/+66 relative to the TSS. A GC-box is crucial to the function of the core promoter and represents a binding site for Sp1 and Sp3 transcription factors. Our results can serve as a basis for future studies aimed at understanding how A3G and A3F expression is controlled in different tissues and how these restriction factors can be used to develop novel therapeutic strategies against HIV-1 infection.

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Conflict of interest statement. None declared.

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