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THE EFFECTS OF *ABCB1* 3'-UNTRANSLATED REGION VARIANTS ON mRNA STABILITY

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Abbreviations: P-gp, P-glycoprotein; UTR, untranslated region; ABC, ATP binding cassette; Flp293, HEK293 Flp-In; APC, allophycocyanin.

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Abstract

Genetic variation in *ABCB1*, encoding P-glycoprotein (P-gp), is a potential cause of interindividual variation in drug response. Numerous studies have focused on the effects of coding region variants on P-gp expression and function, while few noncoding region variants have been investigated. The 3'-untranslated region (UTR) regulates mRNA levels or stability via RNA-protein interactions with mRNA degradation machinery. mRNA stability is a key regulatory step controlling ABCB1 mRNA expression that ultimately impacts P-gp levels and function. We hypothesized that *ABCB1* 3'-UTR polymorphisms alter mRNA stability by disrupting RNA-protein interactions. An ethnically diverse panel of DNA samples was sequenced to identify 3'-UTR polymorphisms and determine allele frequencies. The three most common variants, along with reference ABCB1, were stably expressed in cells in order to measure mRNA half-life. The calculated half-life for ABCB1 reference in HEK293 cells was 9.4 ± 1.3 h and was similar to that estimated for the 3'-UTR variants. Endogenous ABCB1 mRNA decay was similar in lymphoblastoid cell lines carrying 3'-UTR variant and reference alleles. While the examined ABCB1 3'-UTR variants have no effect on ABCB1 mRNA stability, these data represent one of the first attempts to determine the influence of genetic variation in UTRs on ABCB1 mRNA levels.

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P-glycoprotein (P-gp) is a member of the ATP-binding cassette (ABC) transporter superfamily and its physiological role is to remove intracellular compounds via energy-dependent efflux. The distribution of P-gp in various barrier and excretory tissues can hinder drug therapy because many different types of drugs are P-gp substrates (Litman et al., 2001; Szakacs et al., 2006). Furthermore, variability in drug pharmacokinetics is widely observed for P-gp substrates, suggesting that there are interindividual differences in P-gp expression and function (Eap et al., 2002; Goh et al., 2002; Urquhart et al., 2007). The kinetics of P-gp function are dependent upon transport activity and the abundance of P-gp molecules. It is generally accepted that the structure of P-gp, encoded by the *ABCB1* gene, regulates P-gp transport function. However, the mechanisms that control membrane P-gp expression levels are expected to be distinct from those that control transport activity.

The level of ABCB1 mRNA expression is an important determinant of P-gp expression levels. Interindividual differences in ABCB1 mRNA expression levels observed in the liver (Schuetz et al., 1995), small intestine (Moriya et al., 2002; Nakamura et al., 2002; Lindell et al., 2003) and kidney (Uwai et al., 2004) may contribute to the variable drug response of P-gp substrates. Genetic variation in *ABCB1* is one possible mechanism influencing ABCB1 mRNA levels. Several studies have investigated the effects of coding region *ABCB1* polymorphisms, such as 2677G>T and 3435C>T, on mRNA expression. The 3435C>T synonymous variant is the most commonly studied with respect to mRNA levels. It was initially linked with increased mRNA expression (Moriya et al., 2002), however, data from subsequent studies are not in agreement (Siegmund et al., 2002; Uwai et al., 2004; Owen et al., 2005; Haenisch et al., 2007). An *in vitro* study measured different mRNA stabilities between the C and T alleles of 3435 (Wang et al., 2005), so it is possible that this variant causes changes at the cellular level that may extend to clinical phenotypes.

Mechanisms that alter mRNA levels can change P-gp expression and potentially P-gp transport activity. Steady-state ABCB1 mRNA levels reflect the balance between gene

transcription (*i.e.* synthesis) and mRNA stability (*i.e.* decay). Recent evidence has demonstrated that the 3'-UTR of mRNA is an important regulatory site controlling interactions with mRNA degradation machinery (Hollams et al., 2002; Tourriere et al., 2002; Mangus et al., 2003; Wilkie et al., 2003). 3'-UTR RNA-binding proteins that recognize specific mRNA sequence elements and secondary structure dictate the fate of mRNA transcripts. Polymorphisms in the 3'-UTR of *ABCB1* could disrupt RNA-protein interactions, resulting in altered mRNA stability.

The current studies were designed to test whether polymorphisms in the 3'-UTR of *ABCB1* affected mRNA stability. *ABCB1* 3'-UTR polymorphisms were identified in an ethnically diverse panel of DNA samples. Stable cell lines expressing ABCB1 reference and the three most common 3'-UTR variants were established in order to measure the rate of ABCB1 mRNA decay after cessation of transcription. The estimated mRNA half-life served as a measure of ABCB1 mRNA stability.

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Materials and Methods

Materials

Trizol, zeocin, hygromycin, the pcDNA5/FRT and pOG44 plasmids and HEK293 Flp-In (Flp293) cells were obtained from Invitrogen (Carlsbad, CA). Actinomycin D (Sigma-Aldrich, St. Louis, MO) was dissolved in 100% DMSO and stored at -20°C. Murine IgG2a MRK16 antibody was obtained from Kamiya Biomedical Co. (Seattle, WA, USA) and goat anti-mouse IgG allophycocyanin (APC) from Invitrogen. Cell culture media and reagents were purchased from the UCSF Cell Culture Facility (San Francisco, CA).

Cell Culture

Growth media for HEK293 Flp-In cells contained DME-21 supplemented with 10% FBS, 1% penicillin/streptomycin and either 100 μg/mL zeocin (parental cells) or 75 μg/mL hygromycin (ABCB1 stable cells). Human lymphoblast cell lines (GM12814, GM12815, GM 17102, GM12892, GM18506 and GM18562) were obtained from the Coriell Cell Repository and maintained in suspension with RPMI 1640 medium supplemented with 15% FBS and 1% penicillin/streptomycin. All cell lines were passaged every 2-4 days in T25 (lymphoblasts) or T75 (HEK293) flasks and maintained in 5% CO_2 at 37° C.

Identification of ABCB1 3'-UTR Variants

Genomic DNA from 247 human samples from the Coriell Institute was used to identify 3'-UTR polymorphisms in *ABCB1*. Direct sequencing of the 3'-UTR was performed with forward and reverse primers (5'-GGTGTTTCAGAATGGCAGAGTC-3' and 5'-CTGCTTAACCATTCCCACAAAA-3', respectively) using previously reported methods (Kroetz et al., 2003; Leabman et al., 2003).

ABCB1 plasmids

Full-length ABCB1 cDNA was subcloned into pcDNA5/FRT and used as a template to create 3'-UTR variant plasmids. The +193A>G variant was already present and was changed back to the reference nucleotide. Mutagenesis primers were designed for +89A>T (forward 5'-

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AACACTTACAGAATTTTGAAGAGGTATCTGT and reverse 5'-ACAGATACCCTCTTCAAAATTCTGTAAGTGTT), +146G>A (forward 5'-GTCTTCAGAGACTTCATAATTAAAGGAACAG and reverse 5'-CTGTTCCTTTAATTATGAAGTCTCTGAAGAC) and reversion of +193A>G (forward 5'-AAGTGGAGAGAAATCATAGTTTAAACTGCAT and reverse 5'-ATGCAGTTTAAACTATGATTTCTCTCCACTT). The QuikChange Site-directed Mutagenesis Kit (Stratagene) with *Pfu* turbo polymerase was used according to the manufacturer's protocol to introduce the desired variants. Direct sequencing verified the base changes.

ABCB1 Flp293 Stable Cell Lines

HEK293 Flp-In cells (Flp293) were seeded at 5 x 10^5 cells/well in 6-well plates in medium without antibiotics. One day later cells were transfected with 3.6 μg p0G44, 0.4 μg ABCB1/pcDNA5/FRT and 6 μL lipofectamine 2000 in a total well volume of 2 mL. Fresh medium was added ~5 hours after transfection. The following day cells were split into six new wells and 2-3 hours later medium containing 75 μg/mL hygromycin was added. Drug selection lasted 10-14 days and fresh hygromycin media was added every 2-3 days. Surviving cell colonies were propagated and screened for surface P-gp expression by flow cytometry. P-gp positive clones were further screened for ABCB1 mRNA expression using TaqMan quantitative real-time PCR.

ABCB1 mRNA half-life in Flp293 Stable Cell Lines and Lymphoblast Cell Lines

ABCB1 reference, +89A>T, +146G>A and +193A>G Flp293 cell lines were seeded in 12-well plates at 6 x 10^5 cells/well and allowed to grow for 24 hours. For each cell line, triplicate wells were seeded for each time point. Medium containing 7.5 μg/mL actinomycin D was added to the wells and cells were harvested over a 24 hour period. Total RNA was isolated from each sample using Trizol and quantitated using UV spectrophotometry (NanoDrop Technologies, Wilmington, DE). Equal amounts of total RNA were reverse transcribed using M-MLV reverse

transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. TaqMan primers (forward 5'-TGATCATTGAAAAAACCCCTTTG and reverse 5'-TCCAGGCTCAGTCCCTGAAG) and probe (5'-(6-FAM)-ACGGAAGGCCTAATGCCGAACACATT-(BHQ1)) for *ABCB1* were designed using Primer Express (Applied Biosystems, Foster City, CA), and were purchased from Integrated DNA Technologies (Coralville, IA). The 25 μL reaction volume contained 10X TaqMan Buffer (UCSF Genome Core, San Francisco, CA), 5.5 mM $MgCl_2$, 200 μM dNTPs (Promega, Madison, WI), 0.625 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 1 μL cDNA, 500 nM forward and reverse primers, 200 nM probe and PCR-grade H_2O. Reactions were run on an ABI Prism 7700 and cycling conditions were: 12 minutes at 95°C followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. The threshold limit was set so that it intersected all samples during the log-linear phase of amplification and the corresponding C_T values for each set of triplicates were normalized to the average t = 0 value using the equation for percent mRNA remaining: $(2^{(C_{T,\ t=0} - C_{T,\ t=x})}) * 100$, where x equals time after t = 0 in hours. The decay slope (*k*) for percent mRNA versus time was determined using logarithmic data fitting (Excel). The half-life was calculated from the equation, $t_{1/2} = \ln 2 / k$. All experiments were carried out in triplicate.

For each lymphoblastoid cell line, cells were seeded in three wells of a 6-well plate at 5 x 10^5 cells/well in 4 mL of media. After 24 hours, actinomycin D was added to each well at a final concentration of 7.5 μg/mL and 0.5 mL aliquots were removed at each time point (0, 2, 4, 8, 12 and 24 h). Cell aliquots were pelleted and Trizol was added to isolate total RNA. ABCB1 mRNA expression and ABCB1 mRNA half-life were determined as described above except that double the amount of RNA was reverse-transcribed, and double the volume of cDNA was used in each TaqMan reaction. The experiment was performed twice.

Statistical Analysis

Half-life values from three experiments for *ABCB1* reference, +89A>T, +146G>A and +193A>G Flp293 cell lines were averaged and the standard deviation was calculated. Student's *t*-test was used to determine if the mean half-lives for the variants were different than reference with an α value of 0.05.

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Results

ABCB1 3'-UTR Variants

The 3'-UTR of *ABCB1* was sequenced in 247 DNA samples from ethnically diverse populations from the Coriell Institute. Nine polymorphic sites were identified in this 380 bp region with allele frequencies ranging from 0.5 to 14%. *ABCB1* 3'-UTR variants were chosen for study based on their relatively high frequency in any one ethnic group and occurrence as a single nucleotide substitution. There are three polymorphisms that meet these criteria and for which stable cell lines were generated: +89A>T, +146G>A, and +193A>G. The +89A>T variant is found in African Americans and Caucasians, while +146G>A is only found in African Americans (Table 1). *ABCB1* +193A>T is a cosmopolitan polymorphism found in all four of the sampled ethnic populations.

Genetic Analysis of the ABCB1 3'-UTR

Little research has been done on the 3'-UTR of *ABCB1* so predictive measures were used to ascertain the potential impact of *ABCB1* +89A>T, +146G>A, and +193A>G on mRNA stability. Sequence alignments with other mammalian species using Clustal W (ver 1.83) were used to compare the 3'-UTR of human *ABCB1* with corresponding sequences from mouse, rat and rhesus monkey. The sequences ranged in length from 350 to 401 base pairs and 175 base positions were 100% identical when compared to the human sequence (Fig. 1). Using the human sequence as a reference, the sequence similarity of these four species is 46% and it increases to 90% if only human and rhesus monkey are compared. There are six 3'-UTR variant sites that are 100% identical in the three other species: +21, +89, +146, +193, +252 and +355. The other three variant sites are only conserved in rhesus monkey. The multiple species alignment also revealed contiguous sections of 100% conservation that could indicate functional importance, so the *ABCB1* 3'-UTR sequence was analyzed against a database of known 5'- and 3'-UTR sequence elements (Pesole and Liuni, 1999). An IRES element, which enables translation initiation independent of the 5' cap, was found between +282 and +380; however,

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IRES elements generally are located in the 5'-UTR (Pesole et al., 1999). No other potential RNA binding sites were identified using UTRscan (www.ba.itb.cnr.it/UTR).

mRNA Half-life of ABCB1 3'-UTR Variants in Flp293 Stable Cells

Stable cell lines expressing ABCB1 reference, +89A>T, +146G>A and +193A>G were established using the Flp-In system, which is designed to insert a single copy of a gene at the same genomic location. The host Flp293 cells originate from HEK293 cells and have endogenous ABCB1 mRNA expression. However, the ABCB1 Flp293 reference, +89A>T, +146G>A and +193A>G stable cell lines demonstrate significant overexpression of ABCB1. TaqMan real-time PCR determined ABCB1 mRNA levels increased ~80-fold and P-gp surface expression quantitated by flow cytometry showed a ~25-fold increase as compared to the host Flp293 cells. Additionally, the steady-state mRNA levels before actinomycin D exposure (t=0) for the ABCB1 3'-UTR variants differed by no more than 15% of reference (n=3; data not shown).

The mRNA stability of transcripts is commonly determined by measuring mRNA half-life. ABCB1 Flp293 reference, +89A>T, +146G>A and +193A>G stable cell lines were exposed to actinomycin D to stop whole-genome transcription. The ABCB1 mRNA levels over a 24 hour period were normalized to the t = 0 point to convert the data into percent mRNA remaining for each cell line. A plot of percent mRNA versus time shows the rate of ABCB1 mRNA decay for each cell line (Fig. 2) and provides a measurement of mRNA half-life. The average half-lives from three experiments for +89A>T, +146G>A and +193A>G were 8.3 ± 1.4, 9.6 ± 0.8 and 10.3 ± 1.2 h, respectively, and were not significantly different than reference (9.4 ± 1.3 h). As a negative control, the ABCB1 reference cell line was not exposed to actinomycin D to verify constant ABCB1 mRNA expression during the 24 h period.

mRNA Half-life of ABCB1 3'-UTR Variants in Lymphoblastoid Cells

The effect of ABCB1 3'-UTR variants on mRNA stability was also studied in human-derived lymphoblastoid cell lines in order to investigate the effect of these polymorphisms on

endogenous ABCB1 mRNA decay. Lymphoblastoid cell lines from the Coriell Institute were homozygous for either the +89A>T (GM18506), +146G>A (GM17102) or +193A>G (GM12892 and GM18562) polymorphisms. Two cell lines homozygous reference for all 3'-UTR variants were selected as controls (GM12814 and GM12815). There was sufficient ABCB1 mRNA decay over 24 h for the six lymphoblastoid cell lines, however, at each time point there was substantial variability (Fig. 3). ABCB1 mRNA levels in lymphoblastoid cell lines approached the detection limits of the assay to accurately determine mRNA half-life, but data from two experiments suggest there is no difference in mRNA decay between the ABCB1 reference and 3'-UTR variant lymphoblastoid cell lines.

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Discussion

ABCB1 mRNA degradation is an important cellular mechanism controlling P-gp expression that ultimately impacts apparent P-gp activity. The 3'-UTR of mRNA transcripts is considered the key regulatory region that interacts with mRNA degradation machinery. Protein binding sites and secondary structure in the 3'-UTR will either promote or repress mRNA degradation, resulting in changes to steady-state mRNA levels (Hollams et al., 2002; Tourriere et al., 2002; Mangus et al., 2003; Wilkie et al., 2003). Our studies established a stable expression system to test the hypothesis that *ABCB1* 3'-UTR variants alter mRNA stability. Standard methods for generating stable cell lines are unable to control for multiple plasmid insertion and transcriptional differences due to random genomic integration of the plasmid(s). The Flp-In system eliminated copy number and transcriptional activity as variables, and allowed us to attribute potential differences in ABCB1 mRNA levels to changes in mRNA decay.

The numerous efforts to associate *ABCB1* coding region SNPs with clinical phenotypes have not produced conclusive results. It is possible that unidentified SNPs in linkage disequilibrium with the commonly studied SNPs may be causative. To our knowledge, the +89A>T, +146G>A and +193A>G variants have not been investigated, and were chosen for study based on a high allele frequency in at least one ethnic group and a significant degree of evolutionary conservation at these sites. It is important for *ABCB1* pharmacogenetic research to examine non-coding polymorphisms, especially in relation to their probable mechanistic effects *in vitro*.

Regulatory proteins bind to specific elements in the 3'-UTR and interact with RNA degradation machinery by promoting or repressing degradation (Tourriere et al., 2002). A common sequence motif containing AU repeats is thought to confer rapid degradation and one study hypothesized the AU-rich regions of the c-myc and ABCB1 3'-UTRs could destabilize β-globin mRNA. In HepG2 cells, c-myc, ABCB1 and β-globin mRNA have half-lives of 30 min, 8 h and >24 h, respectively (Prokipcak et al., 1999). Chimeras containing the β-globin coding

region with either the ABCB1 3'-UTR or the c-myc 3'-UTR showed that β-globin half-life was only decreased by c-myc (Prokipcak et al., 1999). It should be noted that our measured half-life for reference ABCB1 in HEK293 cells (~9.4 h) is very similar to that seen in HepG2 cells.

The mRNA stability of ABCB1 is a regulated process dependent on multiple factors, such as stress and cell type. The human leukemia cell line K562 is a disease-state model for studying mechanisms controlling P-gp overexpression due to drug exposure. ABCB1 mRNA half-life in K562 cells is 1 h but short-term incubation with drugs, such as vinblastine and doxorubicin, increased the half-life to 10 h with no changes in transcriptional activity (Yague et al., 2003). Studies in rats have discovered that carcinogenesis in the liver can modulate the mRNA stability of Pgp1-3 (human P-gp orthologs) by dramatically increasing their normally short half-life (2 vs. 12 h) (Lee et al., 1998). Subsequent studies determined that the increased mRNA stability in rat liver tumors was associated with a decrease in Pgp mRNA fragments, indicating there was less mRNA degradation (Lee et al., 2005). Even in healthy organs free of stress, rat Pgp mRNA has tissue-specific half-lives that range from 2-12 h (Lee and Ling, 2003).

There are no literature reports discussing possible regulatory sites in the 3'-UTR of *ABCB1*. Our sequence analysis indicates that there may be functionally important segments based on high conservation with other mammalian species, however, the UTRscan database did not find any probable elements. The 3'-UTR field is rapidly growing and has yet to reach the same knowledge base as promoter research, which has identified thousands of transcription factors and DNA binding elements (Matys et al., 2003). In contrast, UTRscan currently searches for 31 known degenerative elements so it is possible there are many more yet to be identified.

The stability of mRNA may be altered by 3'-UTR polymorphisms if recognition of specific mRNA sequence and secondary structure by regulatory proteins is disrupted (Shen et al., 1999; Hollams et al., 2002; Tourriere et al., 2002). A polymorphism in the 3'-UTR of human TNFα changes binding affinity for a multi-protein complex that contains the HuR regulatory protein (Di

Marco et al., 2001). HuR binds AU-rich elements in the 3'-UTR of certain genes (Peng et al., 1998) and has been shown to stabilize mRNA containing TNFα 3'-UTR sequence motifs (Dean et al., 2001). There is one report that the 3435C>T synonymous variant decreases mRNA stability (Wang et al., 2005), but to our knowledge no pharmacogenetic research of this type has been conducted for *ABCB1* 3'-UTR variants. Thus, our mRNA half-life data represent novel findings as to the effects the +89A>T, +146G>A and +193A>G polymorphisms have on ABCB1 mRNA stability and demonstrate the utility of using stable cell lines made with Flp-In technology for these measurements. Similarly, studies in lymphoblastoid cell lines carrying 3'-UTR variants suggest that decay of endogenous ABCB1 mRNA is not affected by these specific polymorphisms. Future mRNA stability experiments should investigate other *ABCB1* 3'-UTR variants and/or haplotypes. Furthermore, the 5'-UTR may play a role in mRNA stability based on the closed-loop hypothesis (Wilkie et al., 2003). The possible interactions between both UTRs in the cytosol suggest 5'-UTR polymorphisms could modify regulatory protein interactions that propagate to the 3'-UTR and impact mRNA stability.

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Figure Legends

Figure 1**.** *ABCB1* 3'-UTR sequence alignment with selected mammalian species. Clustal W was used to align the *ABCB1* 3'-UTRs of human, rhesus monkey, rat and mouse. Variant positions are designated in the human sequence (underlined) and homology for each reference allele in all (dark highlight) or some (light highlight) species is shown. Complete homology for each base position is marked with an asterisk. UTRscan predicted an IRES element from +282 to +381 and it is shown in the human sequence with a dashed underline.

Figure 2. mRNA half-life of ABCB1 reference and 3'-UTR variants in Flp293 stable cells. The mRNA decay of ABCB1 reference (■), and three 3'-UTR variants (+89A>T (●), +146G>A (▼) and +193A>G (♦)) was measured over 24 h in the presence of 7.5 μg/mL actinomycin D. The reference cell line without actinomycin D served as a negative control (□). The percent mRNA remaining was calculated as described in the Materials and Methods section. Each point is the mean ± S.D. of three experiments.

Figure 3. ABCB1 mRNA decay in lymphoblastoid cell lines. The degradation of ABCB1 mRNA in six lymphoblastoid cell lines was measured over a 24 h period in the presence of 7.5 μg/mL actinomycin D. The ABCB1 reference GM12814 (□) and the 3'-UTR variant (GM 17102 (♦), GM18506 (●) and GM 18562 (■)) cell lines showed mRNA decay in response to Actinomycin D treatment. The percent mRNA remaining was calculated as described in the Materials and Methods section. Each point is the mean ± S.D. of three samples from one representative experiment.

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Table 1. Alleles and frequencies of *ABCB1* 3'-UTR polymorphisms

Position[a]	Alleles	NCBI SNP ID (build 126)	Golden Path Position (3/06 build)	Allele frequency (%)			
				AA[c] n=100	CA n=100	AS n=30	ME n=10
+21	T>C	rs28364275	chr7:86971474	8	0	0	0
+77	ACTT>-	rs2235052	chr7:86971418	7	0	0	5
+89	A>T	rs17064	chr7:86971406	11	6.5	0	0
+146	G>A	rs28364277	chr7:86971349	9	0	0	0
+169	G>GACAGAGA	rs28364278	chr7:86971326	9.5	0.5	0	0
+193	A>G	rs3842	chr7:86971302	13	14	15	5
+252	A>C	rs28364279	chr7:86971243	4.5	0	0	0
+316	G>A	rs28364280	chr7:86971179	3	0	0	0
+355	T>C	N/A[b]	chr7:86971140	0	0	1.7	0

[a] SNP location is relative to the TGA stop codon.

[b] SNP is not found in the NCBI database

[c] The populations studied were AA = African American, CA = Caucasian American, AS = Asian American and ME = Mexican American. The number of chromosomes resequenced for each ethnic group is shown.

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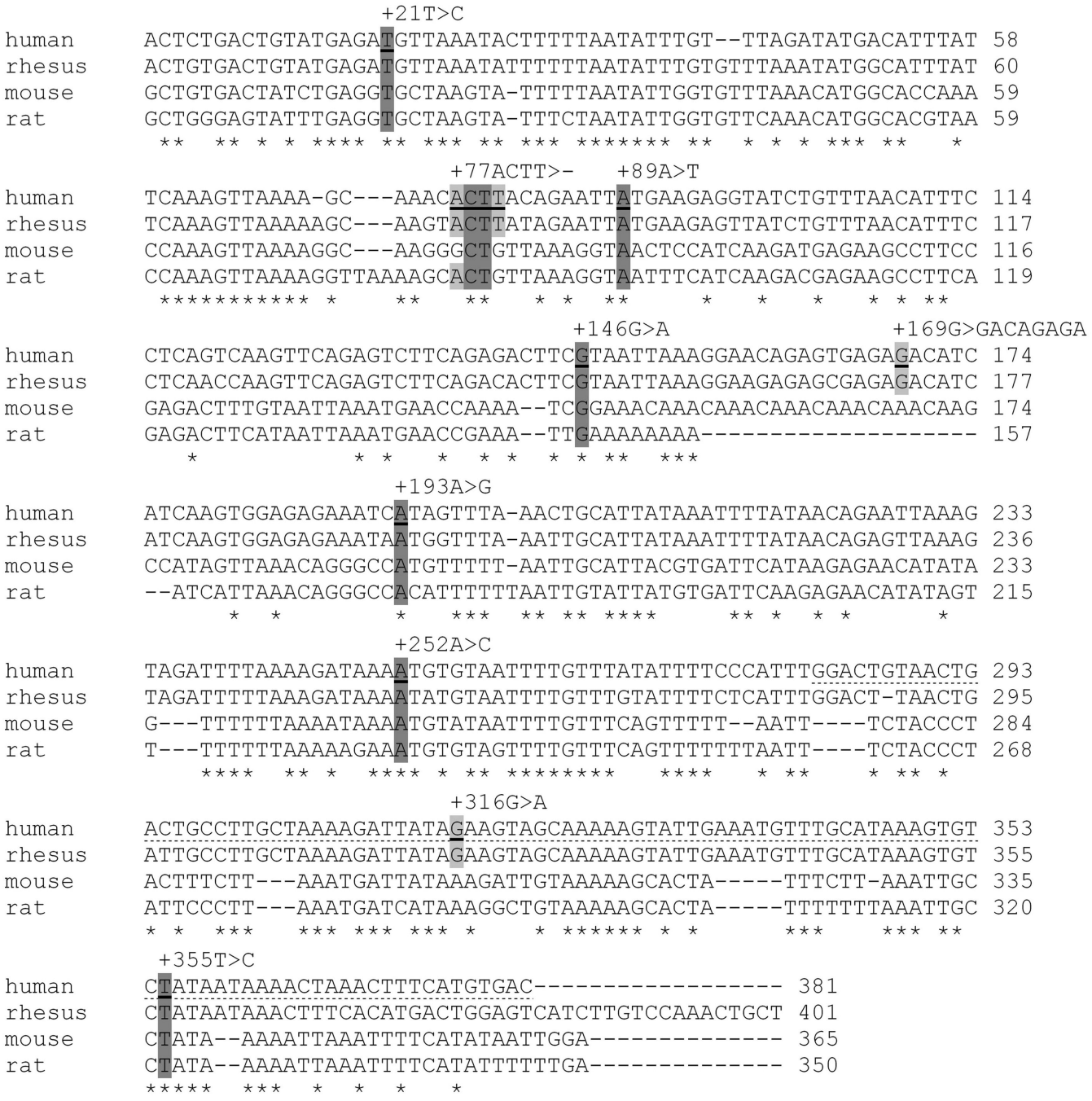

Fig. 1

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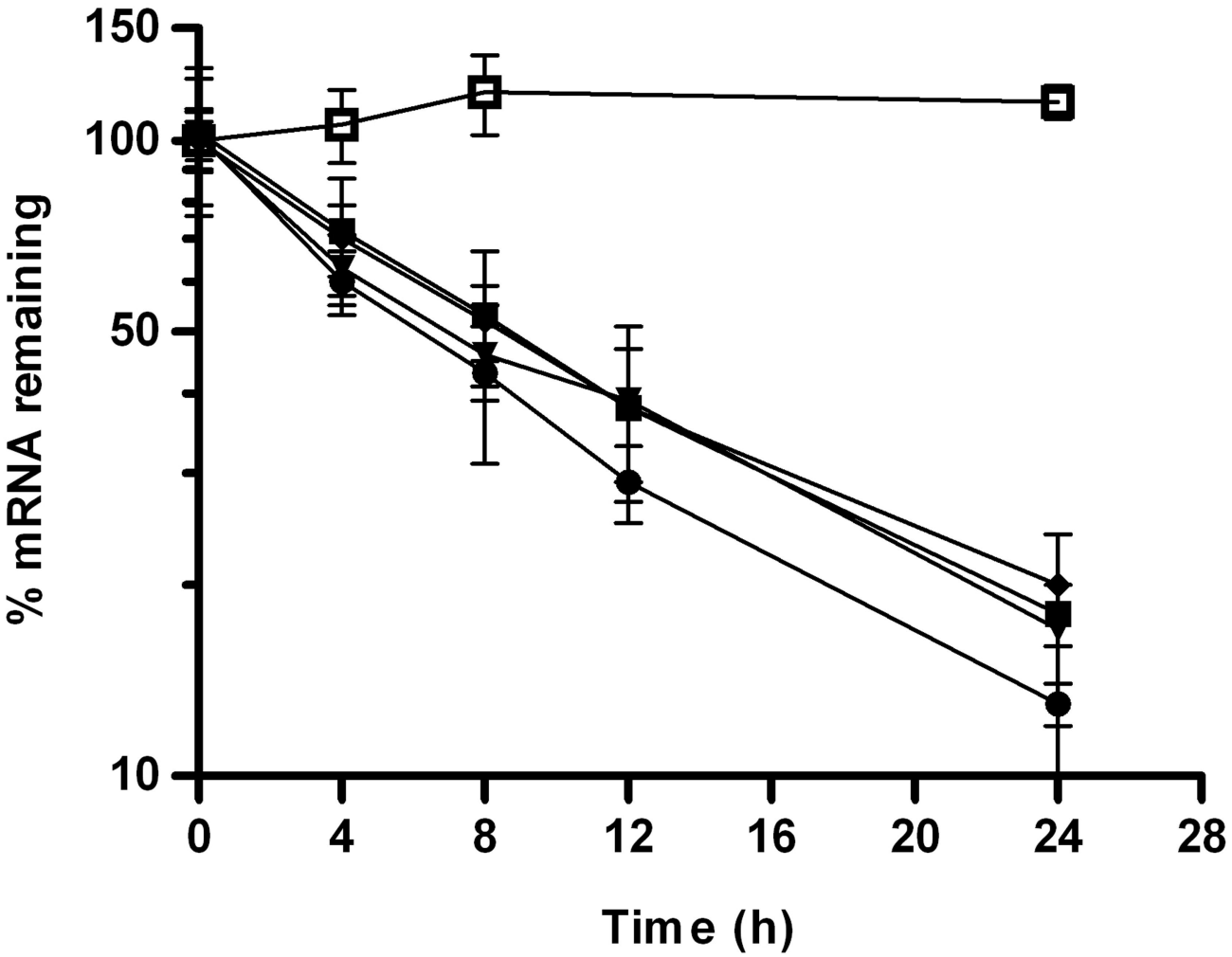

Fig. 2

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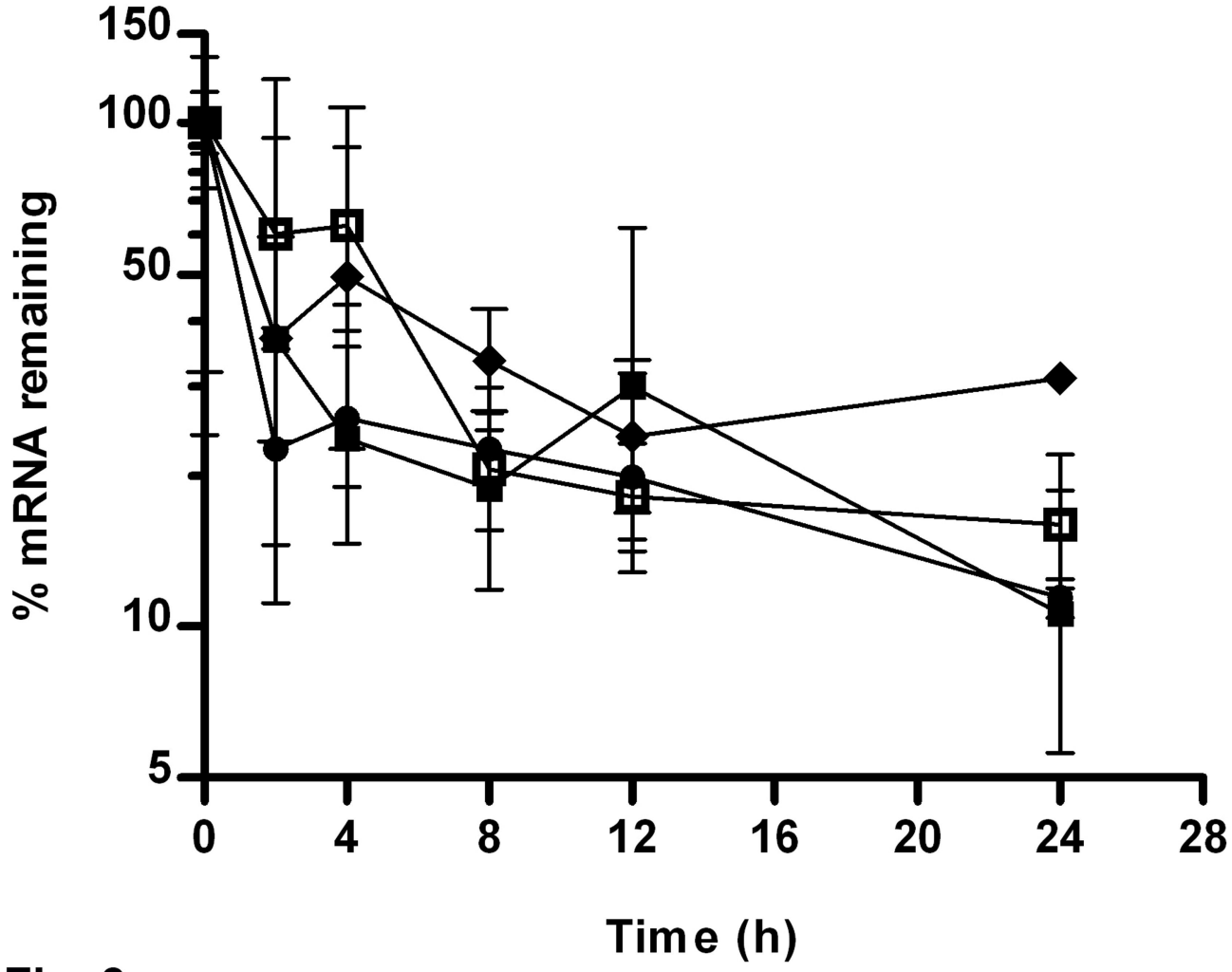

Fig. 3