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Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor

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ABSTRACT

Chemically synthesised 21–23 bp double-stranded short interfering RNAs (siRNA) can induce sequence-specific post-transcriptional gene silencing, in a process termed RNA interference (RNAi). In the present study, several siRNAs synthesised against different sites on the same target mRNA (human Tissue Factor) demonstrated striking differences in silencing efficiency. Only a few of the siRNAs resulted in a significant reduction in expression, suggesting that accessible siRNA target sites may be rare in some human mRNAs. Blocking of the 3′-OH with FITC did not reduce the effect on target mRNA. Mutations in the siRNAs relative to target mRNA sequence gradually reduced, but did not abolish mRNA depletion. Inactive siRNAs competed reversibly with active siRNAs in a sequence-independent manner. Several lines of evidence suggest the existence of a near equilibrium kinetic balance between mRNA production and siRNA-mediated mRNA depletion. The silencing effect was transient, with the level of mRNA recovering fully within 4–5 days, suggesting absence of a propagative system for RNAi in humans. Finally, we observed 3′ mRNA cleavage fragments resulting from the action of the most effective siRNAs. The depletion rate-dependent appearance of these fragments argues for the existence of a two-step mRNA degradation mechanism.

INTRODUCTION

Studies on silencing of transgenes in plants and RNA antisense mechanisms in *Caenorhabditis elegans* have recently led to a general opportunity for suppression of gene expression by double-stranded RNA (dsRNA) through a process termed RNA interference (RNAi) (1,2). In *C.elegans* and *Drosophila*, dsRNA has already become an established tool for functional genomics (3–7) and dsRNA has been used for genomic screens of whole chromosomes in *C.elegans* (8–10). Genetic screens in different species, along with sequence similarity studies and biochemical evidence, have partly elucidated the RNAi mechanism. The dsRNA is processed to 21–25 nt short interfering RNA (siRNA) with 2 nt 3′-overhangs by the RNase III-like protein Dicer in the initiating step of RNAi (11). These cleavage products are subsequently utilised by the RNA-induced silencing complex (RISC) in the recognition and cleavage of the corresponding mRNA (12,13). In *Drosophila* embryo lysates one constituent of this complex is the PAZ/PIWI protein AGO-2 (14). Unwinding of the siRNAs to single-stranded RNA in the active RISC appears to be a prerequisite for cleavage (15) and suggests the participation of a helicase in the RNAi process. Recently, a RNA-dependent RNA polymerase (RdRP) activity has been proposed to be involved in production of secondary siRNAs in both *Drosophila* and *C.elegans* (16,17). The process involves extension of a mRNA-primed antisense strand from the primary siRNA and cleavage of the secondary dsRNA by Dicer. For extensive reviews of siRNAs and RNAi developments see Tuschl (18), Sharp (19), Zamore (20) and Nishikura (21).

In mammals, long dsRNAs result in non-specific suppression of gene expression at the translational level, mediated in part through activation of the protein kinase PKR (22). This problem was circumvented with the recent discovery that chemically synthesised siRNAs avoid this non-specific response (23). In two separate studies (23,24), siRNAs have been employed in human cells to target non-human transgene transcripts like GFP, EGFP, CAT, firefly luciferase and sea pansy (*Renilla*) luciferase. siRNA was also used against endogenous human proteins like lamin A/C, lamin B1, nuclear mitotic apparatus protein (NuMA) and vimentin (23). Interestingly, *Renilla* luciferase and vimentin protein were not efficiently targeted in some human cells. In a subsequent report (25), siRNA was used against a human homologue of the RNAi component Dicer to demonstrate its involvement in the maturation of let-7 stRNA, which belongs to a group of short RNAs involved in temporal control of expression through interference with translation (26,27). Very recently, siRNA was also used to knock down expression of HtrA2 (28). Concomitant abrogation of the apoptotic response to UV exposure demonstrated the potential of siRNA as a tool for functional genomics in human cells.

Careful review of the data implies that certain siRNA species have limited efficiency. Sites on mRNA targets are differentially accessible to ribozymes and oligodeoxynucleotides (29). We therefore wanted to test whether a position effect might be a significant factor for the observed siRNA limitations. siRNAs were designed to target a wide range of sites within the mRNA of human Tissue Factor (TF). TF is the most potent trigger of blood coagulation (30) and the demonstration of

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specific depletion of TF expression by RNAi would be of importance. In this study we report a target position dependence of siRNA efficacy in human cells. In a time-consuming reaction, a 10-fold depletion of TF mRNA and 5–10-fold depletion of TF protein and procoagulant activity were achieved with the most effective siRNA species. The appearance of mRNA cleavage fragments suggests a two-step model for the siRNA-mediated cleavage and degradation process.

MATERIALS AND METHODS

siRNA preparation

Twenty-one nucleotide RNAs were chemically synthesised using phosphoramidites (Pharmacia and ABI). Deprotected and desilylated synthetic oligoribonucleotides were purified by reverse phase HPLC. Ribonucleotides were annealed at 10 µM in 500 µl of 10 mM Tris–HCl, pH 7.5, by boiling and gradual cooling in a water bath. Successful annealing was confirmed by non-denaturing polyacrylamide gel electrophoresis. siRNA species were designed targeting sites within human protein serine kinase H1 (PSKH1) (accession no. AJ272212) and human TF (accession no. M16553) mRNAs.

Cell culture

HeLa, Cos-1 and 293 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% foetal calf serum (Gibco BRL). The human keratinocyte cell line HaCaT was cultured in serum-free keratinocyte medium (Gibco BRL) supplemented with 2.5 ng/ml epidermal growth factor and 25 µg/ml bovine pituitary extract. All cell lines were regularly passaged at sub-confluence and plated 1 or 2 days before transfection. Lipofectamine-mediated transient co-transfections were performed in triplicate in 12-well plates with 0.40 µg/ml plasmid (0.38 µg/ml reporter and 20 ng/ml control) and typically 30 nM siRNA (0.43 µg/ml) essentially as described (29). Luciferase acitivity levels were measured in 25 µl of cell lysate 24 h after transfection using the Dual Luciferase assay (Promega). Serial transfections were performed by transfecting initially with 100 nM siRNA, followed by transfection with reporter and internal control plasmids 24 h before harvest time points. For northern analyses and coagulation assays, HaCaT cells in 6-well plates were transfected with 100 nM siRNA in serum-free medium. For endogenous targets, Lipofectamine 2000™ was used for higher transfection efficiency. Poly(A) mRNA was isolated 24 h after transfection using Dynabeads oligo$(dT)_{25}$ (Dynal). Isolated mRNA was fractionated for 16–18 h on 1.3% agarose/formaldehyde (0.8 M) gels and blotted onto nylon membranes (MagnaCharge; Micron Separations Inc.). Membranes were hybridised with random primed TF (position 61–1217 in cDNA) and GAPDH (1.2 kb) cDNA probes in PerfectHyb hybridisation buffer (Sigma) as recommended by the manufacturer.

TF activity and antigen

For TF activity measurements HaCaT cell monolayers were washed three times with ice-cold barbital-buffered saline, pH 7.4 (BBS, 3 mM sodium barbital, 140 mM NaCl) and scraped into BBS. Immediately after harvesting and homogenisation the activity was measured in a one-stage clotting assay using normal citrated platelet-poor plasma mixed from two donors and 10 mM $CaCl_2$. The activity was related to a standard (31,32). One unit (U) of TF corresponds to 1.5 ng TF as determined in the TF ELISA (31,33). The activity was normalised to the protein content in the cell homogenates, as measured by the Bio-Rad Bradford or DC assays. TF antigen was quantified using the Imubind Tissue Factor ELISA kit (American Diagnostica, Greenwich, CT). The samples were left to thaw at 37°C and homogenised. An aliquot of each homogenate (100 µl) was diluted in phosphate-buffered saline containing 1% BSA and 0.1% Triton X-100. This sample was then added to the ELISA well and the procedure from the manufacturer followed. The antigen levels were normalised to the total protein content in the cell homogenates.

RESULTS

The efficiency of siRNAs is dependent on mRNA target position

We synthesised four siRNAs against each of two mRNAs of interest (Fig. 1A), human TF (30) and human PSKH1 (34), the sites chosen on each gene being the two most accessible (hTF372i, hTF478i, PSK314i and PSK546i) and the two least accessible (hTF167i, hTF562i, PSK566i and PSK739i) to ribozyme-mediated inhibition (29; M.Amarzguioui, T.Holen, E.Babaie and H.Prydz, unpublished data). The initial analysis of TF siRNA efficacy was performed in HeLa cells transiently co-transfected with siRNA (Fig. 1A) and a luciferase fusion construct, TF–LUC (Fig. 1B), using the Dual Luciferase system. The siRNAs had potent and specific inhibitory effects in the co-transfection assays. The best candidates, hTF167i and hTF372i, gave only 10–15% residual luciferase activity in HeLa cells (Fig. 1C). This level of inhibition is similar to results from previous studies using luciferase reporter plasmid systems (23,24). A possible positional effect was found, as hTF562i showed only an intermediate effect and hTF478i had very low activity. This pattern of activity was also found in Cos-1, 293 and HaCaT cells (Fig. 1C), with siRNAs from different synthetic batches and at various transfection concentrations. The siRNAs caused the same degree of inhibition over a concentration range of 1–100 nM in the co-transfection assay (see below).

To further investigate the positional effect, we synthesised several series of siRNAs against new target sites. Of the siRNAs against TF in this second series, the siRNA targeting the translation initiation site (hTF77i) and the 3′-end of the coding region (hTF929i) were both essentially inactive (Fig. 1C). Of the two others, targeting heavily base paired regions as predicted by MFold (35,36), one (hTF256i) demonstrated intermediate activity while the other (hTF459i) had low activity. Again, siRNAs targeting different positions on mRNA thus differed in activity.

We decided to explore the accessibility of the region surrounding the target site of our best siRNA, hTF167i, at a higher resolution. A third series of siRNAs (hTF158i, hTF161i, hTF164i, hTF170i, hTF173i and hTF176i) was synthesised, targeting sites shifted at both sides of hTF167 in increments of 3 nt. Each of these shared 18 of 21 nt with its neighbours (Fig. 2A). Surprisingly enough, we found that despite the minimal sequence and position differences between these siRNAs, they displayed a wide range of activities (Fig. 2B).

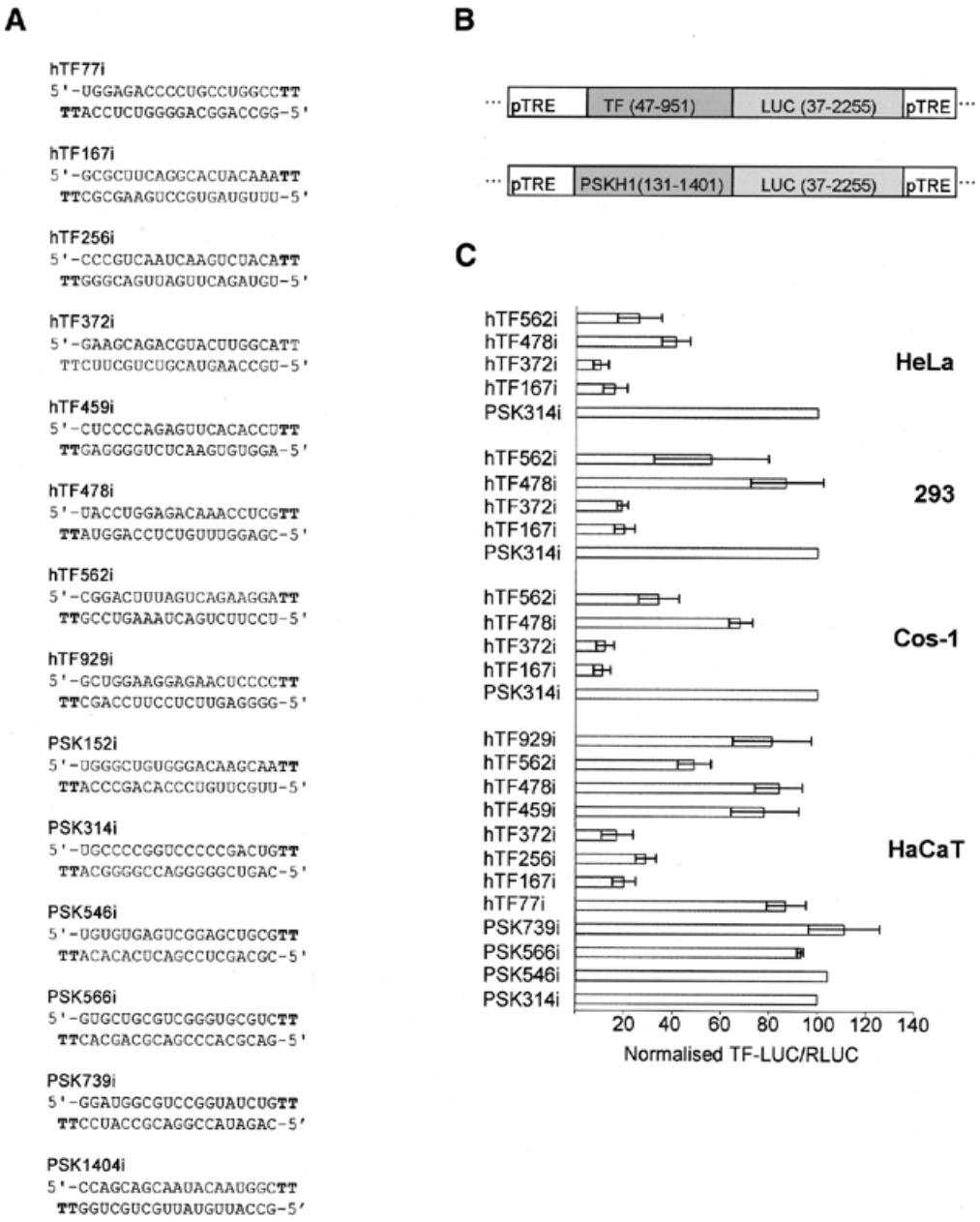

Figure 1. siRNAs, reporter constructs and RNA interference in transgene expression. (**A**) The sense (top) and antisense (bottom) strands of siRNA species, each targeting a site within mRNA for human TF (accession no. M16553) or human protein serine kinase H1 (PSKH1) (accession no. AJ272212), are shown. siRNAs were synthesised with 2 nt deoxythymidine 3′-overhangs and were numbered according to the position of the first nucleotide of the sense strand (numbering as in the GenBank entries). (**B**) Luciferase reporter constructs. The coding regions of PSKH1 and TF were cloned in-frame with the firefly luciferase (LUC) gene, into the cloning vector pTRE (accession no. U89931), producing the fusion constructs PSKH1–LUC (accession no. AF416988) and TF–LUC (accession no. AF416989). Numbering of the fusion constructs refers to that of the respective GenBank entries for PSKH1 and TF and to the pGL3-Enhancer plasmid (Promega) for LUC. TF–LUC was generated from PSKH1–LUC by PCR. The plasmid pcDNA3-Rluc (accession no. AF416990), encoding *Renilla* luciferase (RLUC), was used as an internal control (not shown). All plasmids were sequenced enough to confirm correct cloning. (**C**) RNA interference by siRNAs in reporter gene co-transfection assays. Cells were co-transfected with siRNA (30 nM) and a mixture of reporter (TF–LUC) and internal control (RLUC) plasmids. Ratios of LUC to RLUC expression were normalised to levels in cells transfected with a representative irrelevant siRNA, PSK314i.

There was a gradual change away from the full activity of hTF167i that was more pronounced for the upstream siRNAs. The two siRNAs hTF158i and hTF161i were shifted only 9 and 6 nt away from hTF167i, respectively, yet their activity was severely diminished. These results suggested that a local protein factor(s) on the mRNA caused the positional effect. However, we cannot exclude that other factors, such as sequence-dependent mRNA product release or differential efficiency of 5′ siRNA phosphorylation (15), may influence the efficacy of the siRNAs.

siRNAs cause position-dependent depletion of endogenous mRNA

Although fast and convenient, the co-transfection assay involves the use of forced expression of reporter genes, which causes difficulties of interpretation. A more direct approach is

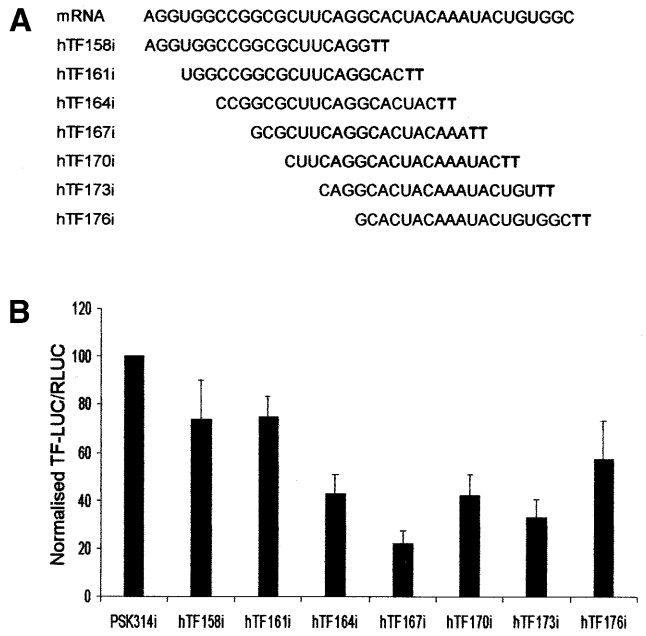

Figure 2. Investigation of siRNA position dependence at codon level resolution. (**A**) Sequences (sense strand only) of the new series of siRNAs, targeting sites surrounding hTF167. Deoxynucleotide overhangs are indicated in bold. The siRNAs were shifted in increments of 3 nt to either side of hTF167i. (**B**) Efficacy of the siRNAs in standard co-transfection assays in HaCaT cells. Different synthetic batches of the hTF167i siRNA showed similar efficacy. Results are averages of at least three independent experiments, each in triplicate.

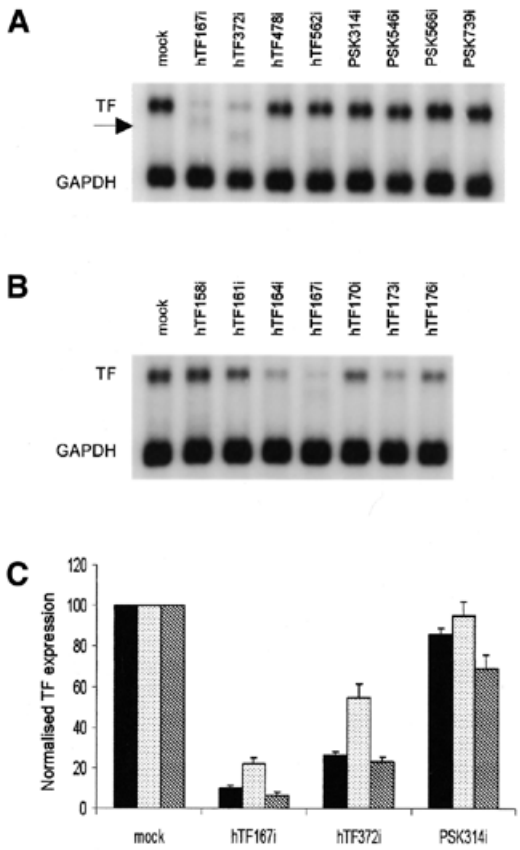

Figure 3. siRNA-mediated reduction in endogenous TF expression. (**A** and **B**) Northern analysis of TF mRNA after transfection of HaCaT cells with siRNA (100 nM). GAPDH was used as a control. The arrow indicates cleavage fragments resulting from siRNA action. (**C**) Effect of siRNAs on steady-state mRNA levels (filled bars), procoagulant activity (dotted bars) and TF protein (antigen) expression (hatched bars). For measurement of procoagulant activity and antigen, cells were harvested 48 h after siRNA transfection to accommodate the 7–8 h half-life of TF protein (31). Data are from a representative experiment in triplicate.

to measure the effect of transfected siRNA on expression of endogenous TF mRNA, which is expressed constitutively in the human keratinocyte cell line HaCaT. The two best TF siRNAs in the co-transfection assay, hTF167i and hTF372i, demonstrated strong activity. Normalised TF mRNA expression was reduced to 10 ± 1.2 and 26 ± 2.6%, respectively (Fig. 3A). Experimental reproducibility was generally higher for the northern analysis than for the co-transfection assay. The third best siRNA in co-transfection assays, hTF256i, also resulted in significant depletion of TF mRNA levels (57% residual expression; data not shown). The remaining TF siRNAs of the first two series (hTF77i, hTF459i, hTF478i, hTF562i and hTF929i), along with the control PSKH1 siRNAs, did not show any significant activity as measured by northern assay (Fig. 3A).

Interestingly, cleavage products, whose sizes were consistent with primary cleavage at the target sequences, were clearly visible below the depleted main band, though *in vitro* cleavage assays of mRNA based on RNA interference have so far not succeeded in mammalian systems (37). Our observations provide convincing evidence that siRNAs deplete the steady-state mRNA level by cleaving the mRNA. The demonstration of a cleavage product has another interesting implication. Uncapped mRNA should be quickly degraded (38,39) following cleavage and similar cleavage fragments have to our knowledge not been reported with ribozymes, DNA enzymes or RNase H-competent antisense oligos. The fact that we can observe the cleavage product implies stabilisation of a mRNA intermediate following cleavage. We defer further discussion of this matter to a later section.

There was a good, but not perfect, correlation between siRNA efficacy in the two assays. siRNAs with only intermediate activity in co-transfection assays were unable to deplete endogenous mRNA levels. Differences in activity between active siRNAs on the endogenous target were more pronounced than on transgene expression, although the activity rank order was generally conserved between the two assay systems. This observation was found to also be true for the series of siRNAs close to hTF167i. The previously demonstrated sharp boundary between active and inactive siRNAs was seen on endogenous TF mRNA (Fig. 3B), only more pronounced. For the series hTF158i–hTF167i activity increased from no mRNA depletion to 90% depletion over an interval of only 9 nt. Thus, the positional effect is demonstrated using two different versions of the same mRNA, with the results on the endogenous TF mRNA being clearer.

The TF cDNA shows an abrupt change in GC content in the region 152–198, falling from 84 to 15% for a 19 bp window. Further analysis of results from other siRNAs and their target sequences showed a slight bias of doubtful biological significance towards a correlation of higher activity and lower GC content (23,25,28; data not shown). A total of six PSKH1 siRNAs from two synthesis series had moderate to no activity

in co-transfection assays and limited effects in northern assays (data not shown). As the expression levels of PSKH1–LUC and endogenous PSKH1 mRNA regularly appeared to be lower than the corresponding hTF mRNAs (data not shown), an overabundance of target mRNA in this case was not a likely explanation for the lower silencing efficiency. We have, however, observed a correlation between the expression level and degree of silencing of the TF–LUC reporter gene. Retransfection of siRNA-transfected cells (3 days after initial transfection) with increasing amounts of the reporter gene plasmid resulted in reduced silencing with higher plasmid concentrations (data not shown). The generally lower activity of the PSKH1 siRNAs is consistent with the previously mentioned GC content bias, as the PSKH1 mRNA has a very high overall GC content (60%). No relationship between MFold-predicted mRNA structures and the potency of the siRNA was found, as siRNAs targeting sites of similar predicted secondary structures (hTF167i versus hTF478i, hTF383i versus hTF562i and hTF256i versus hTF459i) demonstrated very different activities. There was also no correlation with previous results obtained with ribozymes (29; M.Amarzguioui, T.Holen, E.Babaie and H.Prydz, unpublished data).

Inhibition of TF function by siRNAs

The effect of the siRNAs on TF protein levels and activity was assessed. Clotting, triggered by TF, was measured 48 h after transfection of HaCaT cells with selected siRNAs. The two best siRNAs, hTF167i and hTF372i, reduced the procoagulant activity of cell extracts 5- and 2-fold relative to mock-transfected cells, while the irrelevant control siRNA PSK314i had no effect on procoagulant activity (Fig. 3C). A TF-specific siRNA that was inactive in other assays (hTF478i) likewise had no effect on procoagulant activity (data not shown). Thus, a positional dependence of siRNA activity was also demonstrated in this assay. The difference in activity of the two best siRNAs also correlated well with their demonstrated efficacy in depleting mRNA. When TF antigen was measured in an ELISA assay, the reduction in antigen was essentially proportional to the reduction in mRNA (Fig. 3C) for both hTF167i and hTF372i.

We have demonstrated siRNA positional dependence in four different TF mRNA-dependent test systems. The low activity of the majority of the siRNAs may be due to non-accessibility of mRNA for cleavage, caused by higher order RNA structures or protein coverage, as suggested in a previous report (23). Prediction of secondary structures of mRNA by MFold did not provide an explanation for the position dependence (data not shown). The GC content of a region might affect its general accessibility to siRNAs indirectly through the effect on protein coverage or mRNA unwinding. It might be significant that our three best siRNAs all lie within one region of generally low GC content, with the best (hTF167i) at the edge of a deep valley of low GC content. At present, however, the factors determining the differences in siRNA efficiency remain unclear.

Competitive effects of siRNAs

The use of a single species of siRNA to target a mRNA is not a biologically relevant situation, as cells would normally encounter multiple siRNAs produced from long dsRNAs by the RNase III-like Dicer (11–14). We asked whether the complexes involved in cleaving the mRNA might have evolved to utilise multiple siRNA species, for instance through a mechanism of allosteric stimulation of RISC, and thus not work as efficiently with single siRNAs. Specifically, we wanted to know whether combinations of siRNAs would result in increased activity through synergism or addition. Testing this concept by co-transfecting various combinations of two or three siRNAs, no addition or synergism could be discerned. On the contrary, co-transfecting with an active and inactive siRNA revealed a sequence-independent competitive effect, reducing the activity of the most effective siRNA. When mixing saturating concentrations of active siRNA (hTF167i) with inactive control siRNA (PSK314i) at different ratios, a shifted dose-dependence curve was obtained (Fig. 4A). In the absence of competitor, the siRNA effect of 10 nM hTF167i was the same as that of 30 nM, yet when combining 10 nM hTF167i with 20 nM PSK314i the activity was significantly diminished. At lower ratios of active to competitor siRNA the competitive effect was progressively stronger. Investigating the generality of this competition effect beyond this siRNA pair (hTF167i versus PSK314i), we demonstrated the ability of PSK314i to interfere with the function of hTF256i and hTF372i (Fig. 4B) and that other irrelevant siRNAs (PSK546i and PSK739i) competed equally well (Fig. 4C). Furthermore, the competitive effect was also demonstrated on endogenous TF mRNA, antigen and procoagulant activity (Fig. 4D). The order of siRNA transfection did not affect hTF167i activity in competition experiments, suggesting that the initial binding of siRNAs is reversible (data not shown).

The time course of siRNA silencing

Northern analysis of cells harvested 4, 8, 24 and 48 h after the start of transfection showed maximum siRNA silencing after 24 h (Fig. 5A). There seemed to be a difference in the apparent depletion rate, as hTF167i reduced the mRNA level more than hTF173i at each time point. Similar observations were made for modified versions of hTF167i with attenuated final mRNA depletion (see below). We noted that siRNA-induced target degradation was incomplete, as a level of ~10% of the target mRNA remained even with the most effective siRNAs. This may be due to the presence of a fraction of mRNA in a protected compartment, e.g. in spliceosomes, in other nuclear locations or in non-transfected/unsilenced cells. In view of the above data and data from competition experiments, however, part of the non-degraded fraction may be explained by a kinetically determined balance between transcription and degradation, the latter being a time-consuming process (Fig. 5A).

Uptake of siRNA

To investigate the uptake of siRNAs following transfection, a FITC-tagged version of the most effective siRNA, hTF167i–FITC, was synthesised. After uptake of hTF167i–FITC by HaCaT cells, a characteristic spotty distribution was seen (Fig. 5B), as in earlier transfection experiments with FITC-tagged oligos and ribozymes. The spots are assumed to be cationic lipid micelles (containing hTF167i–FITC) that have fused with the cells. We were unable to find cells that did not display such spots and thus assume that siRNA transfection of cells is close to 100%. siRNA uptake reached its maximum and levelled off after transfection for 2–4 h. There was no evidence for uptake of siRNA in the absence of transfection agent. Cells incubated in the

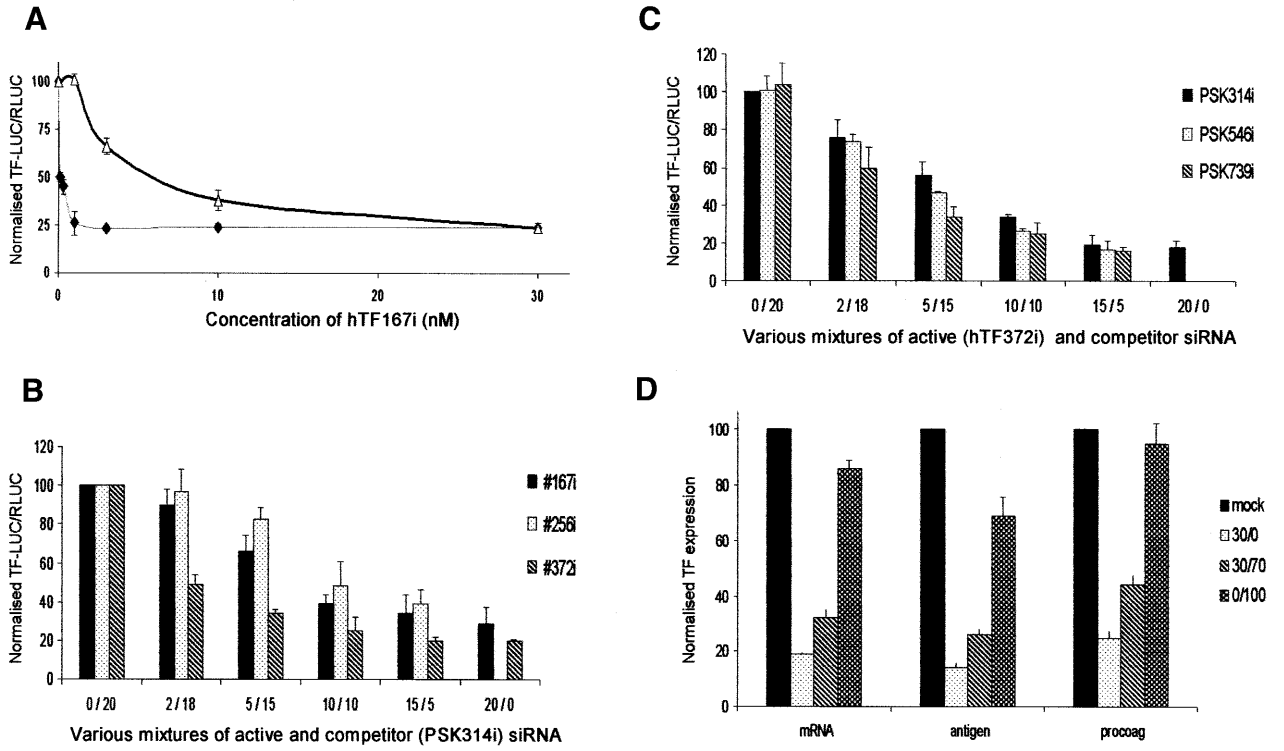

Figure 4. RNA interference by active siRNA is inhibited by the presence of inactive siRNAs. Ordinates show normalised reporter gene activity as a percent of control. (**A**) Dose dependence of active siRNA (hTF167i) in the absence (closed diamonds) and presence (open triangles) of irrelevant siRNA (PSK314i). At each concentration of hTF167i, normalised LUC/RLUC was standardised to levels in cells co-transfected with PSK314i to rule out the possibility of non-specific dose-dependent effects of siRNAs. In samples with mixtures of hTF167i and PSK314i, the total concentration of siRNA was kept constant at 30 nM by adding PSK314i to the indicated concentrations of hTF167i. (**B**) Competitive inhibition by PSK314i of various active siRNAs. (**C**) Competitive inhibition of hTF372i-mediated RNAi by different irrelevant siRNAs. Increasing concentrations of active siRNA (0, 2, 5, 10 and 20 nM) were mixed with competitor siRNA (20, 18, 15, 10 and 0 nM) to a final concentration of 20 nM. (**D**) Competitive inhibition of RNAi on endogenous TF expression by PSK314i. HaCaT cells were transfected with 30 nM hTF167i in the absence (30/0) or presence (30/70) of 70 nM PSK314i as competitor. Inactive siRNA at 100 nM (0/100) was included as a control. Complexations with Lipofectamine 2000™ were performed in parallel for northern (cells harvested after 24 h), procoagulant and antigen assays (cells harvested after 48 h). Activities and expression levels were standardised to mock-transfected cells.

presence of siRNA without liposomes did not exhibit any inhibition following transfection with reporter plasmid the next day (data not shown). Furthermore, when co-culturing siRNA-transfected cells with cells transfected with reporter plasmids, no transfer of siRNA silencing between cells was detected (data not shown), contrary to earlier reports of medium-mediated silencing in *Drosophila* S2 cells (40). We found no evidence of transfer of siRNA between cells or via the medium, thus the siRNAs seem to be taken up by cells exclusively via lipid micelles. The escape of siRNA from these lipid micelles might contribute to the slow action of siRNAs.

Persistence of siRNA silencing

Experiments in plants (41,42) and nematodes (1,43) have suggested the existence of a system whereby certain siRNA genes are involved in the heritability of RNAi-induced phenotypes. To investigate the existence of such propagators in mammalian cell lines we measured the persistence of siRNA silencing in HaCaT cells transfected at very low cell density to allow 5–10-fold cell culture expansion over a 5 day period. In an experiment based on serial transfection of reporter constructs (Fig. 5C), there was a gradual recovery of expression between 2 and 5 days post-transfection. The time-dependence of the siRNA effect on endogenous TF mRNA was similar (Fig. 5C). The level of TF mRNA in mock-transfected control cells declined gradually during this experiment, in what appeared to be cell expansion-dependent down-regulation of expression. Interestingly, the procoagulant activity showed little indication of recovering to control levels in transfected cells (Fig. 5C). Similar observations were made with hTF372i and with a combination of hTF167i, hTF372i and hTF562i (data not shown). The cause for this TF phenotype remains unknown.

Tolerance of RNAi for mismatches and modifications

The use of 2 nt deoxythymidine 3′-overhangs in these studies prompted the question of whether such overhangs might affect the efficiency of the siRNA. We synthesised a siRNA with deoxynucleotide overhangs able to base pair with the target mRNA (hTF167i-BO, Fig. 6A). Its activity was indistinguishable from that of the siRNA with non-base pairing overhangs (Fig. 6B and C). Another version of this siRNA with ribonucleotide base pairing overhangs had identical silencing activity on endogenous mRNA expression (data not shown). The same observation was made for siRNAs targeting the sites hTF173, hTF256, hTF372, hTF478 and hTF562 (data not shown). Although these modifications in the overhangs appeared to have no effect on initial silencing activity, they might affect the persistence of siRNA silencing through their differential ability to serve as templates for a putative

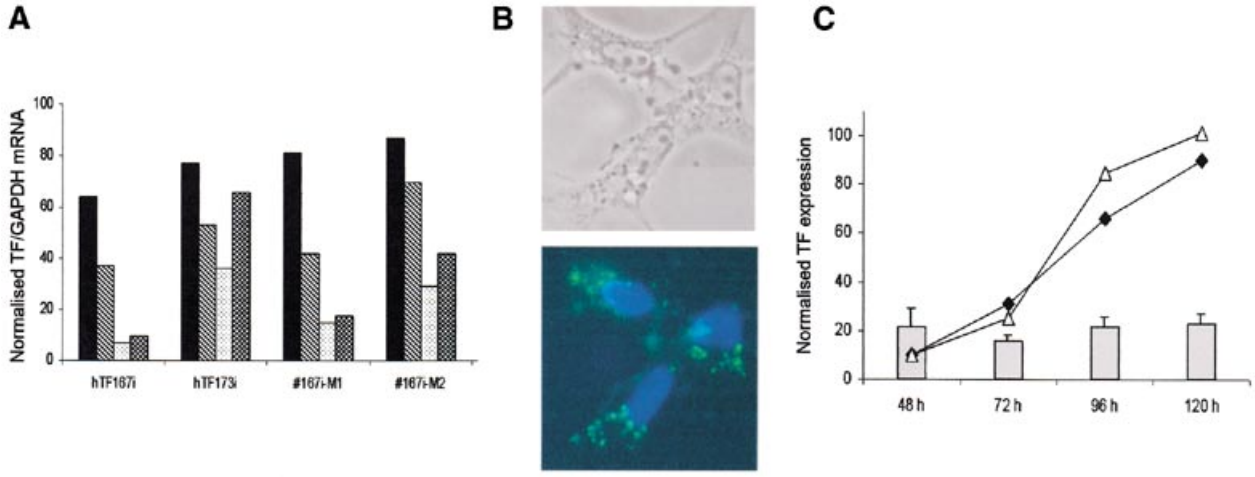

Figure 5. Time dependence of siRNA-mediated RNAi. (**A**) Time-course of mRNA silencing. Cells were transfected with 100 nM siRNA and harvested for mRNA isolation 4, 8, 24 and 48 h after initiation of transfection. Expression levels were normalised to GAPDH and standardised to mock-transfected cells at all time points. (**B**) Fluorescence and phase contrast microscopy of cells transfected with FITC-tagged hTF167i (hTF167i–FITC). (Lower) FITC signal (green) and Hoechst staining of nuclei (blue). HaCaT cells grown on coverslips were transfected for 4 h, incubated for a further 20 h, washed in PBS, fixed in methanol, mounted with DAKO mounting medium containing Hoechst reagent (2 µg/ml) and subjected to fluorescence microscopy (magnification ×40). (**C**) Persistence of siRNA silencing effect in HaCaT cells determined in a serial transfection experiment (open triangles) and by northern (closed diamonds) and procoagulant (bars) assays. In all experiments cells were harvested 48, 72, 96 and 120 h after initial transfection of 100 nM hTF167i. For the northern and procoagulation assays expression levels were standardised to mock-transfected cells as described. In the serial transfection assay cells were retransfected with the two reporter constructs 24, 48, 72 and 96 h after siRNA transfection and harvested 24 h later in each case. Normalised luciferase expression was standardised to levels in PSK314i-transfected cells.

siRNA-regenerating RdRP (16). Northern and serial transfection time-course experiments, however, revealed no significant differences in silencing between the three different versions of hTF167i, or the two versions of hTF372i, at any time up to at least 120 h (Fig. 6D and data not shown). We further tested the effect of blocking the 3′-OH position by a FITC group, thereby negating the possibility of primer extension of the siRNA antisense oligo by a putative RdRP. The activity of hTF167i–FITC was only marginally lower than the wild-type (Fig. 6B and C). The combined results indicate that some degree of modification can be introduced in the overhangs without compromising siRNA efficacy. Furthermore, the high level of activity of the 3′-OH blocked hTF167i–FITC demonstrates that initial mRNA depletion does not occur through the recently reported RdRP-dependent degradative PCR mechanism (16) in human cells. Any hypothetical human RdRP must have little effect on the persistence of siRNA-mediated silencing.

We next investigated the tolerance of the RNAi system for mismatches in the siRNA relative to the mRNA target. Low or no tolerance for mismatches would make siRNAs a valuable tool for allele-specific degradation of the aberrant mRNA in various dominant negative disorders resulting from single base pair mutations. We therefore made mutant siRNAs containing either one (hTF167i-M1) or two (hTF167i-M2) central mismatches relative to the target (Fig. 6A). The mismatches (G/G and GC/GC) were chosen to be maximally disruptive. The single mismatch reduced the effect of siRNA moderately in both co-transfection and northern assays. The double mutant, however, had almost no activity in the co-transfection assay, while still reducing mRNA levels to 30%. We interpret the gradual loss of activity in the mutants as further circumstantial evidence of a near equilibrium kinetic balance between mRNA synthesis/processing and mRNA degradation. A more direct test of the near equilibrium model was to compare the rate of mRNA depletion for ‘mutated’ and wild-type versions of hTF167i. A clear difference in depletion rate caused by active and mutant siRNAs was seen (Fig. 5A). As predicted by the model based on rates of decay and transcription, there was an apparent correlation between the rate and extent of depletion.

A similar mismatch tolerance was recently reported for siRNAs in *Drosophila* embryos (44). In another study, however, no tolerance for even a single central mutation was seen in a *Drosophila* embryo lysate assay (45). The *in vivo* system differs from the *in vitro* one in having continual mRNA production. Thus, if we assume a range of siRNA cleavage rates, all siRNAs that have so low cleavage activity that their contribution would be insignificant compared to the rate of mRNA turnover *in vivo* could possibly still significantly deplete mRNA *in vitro*. The siRNAs with high cleavage activity *in vivo* would, on the other hand, as we observed with hTF167i, lose their activity gradually when mutated.

In contrast to hTF167i-wt, hTF167i-M2 did not produce a detectable cleavage fragment. Furthermore, the appearance of a cleavage fragment with hTF167i-wt was dose dependent. It is present at saturating (30–100 nM) but not at sub-saturating (10 nM) concentrations (Fig. 6E). No cleavage fragment was seen with hTF173i, which shares 13 of 19 nt of its target sequence with hTF167i, but exhibits a reduced depletion rate compared to the latter (Fig. 5A). Combined, these observations suggest that the appearance of a cleavage product is dependent on a high enough rate of the initial cleavage step to saturate a secondary nuclease.

We annealed combinations of hTF167i wild-type and mismatched strands (Fig. 6A), producing imperfect siRNAs with 1 or 2 bp bulges. These bulges were found to have little effect on activity, with the important determinant for activity being the state of the antisense strand. Thus hTF167i-wt/M1 and hTF167i-wt/M2, containing a mutated antisense strand,

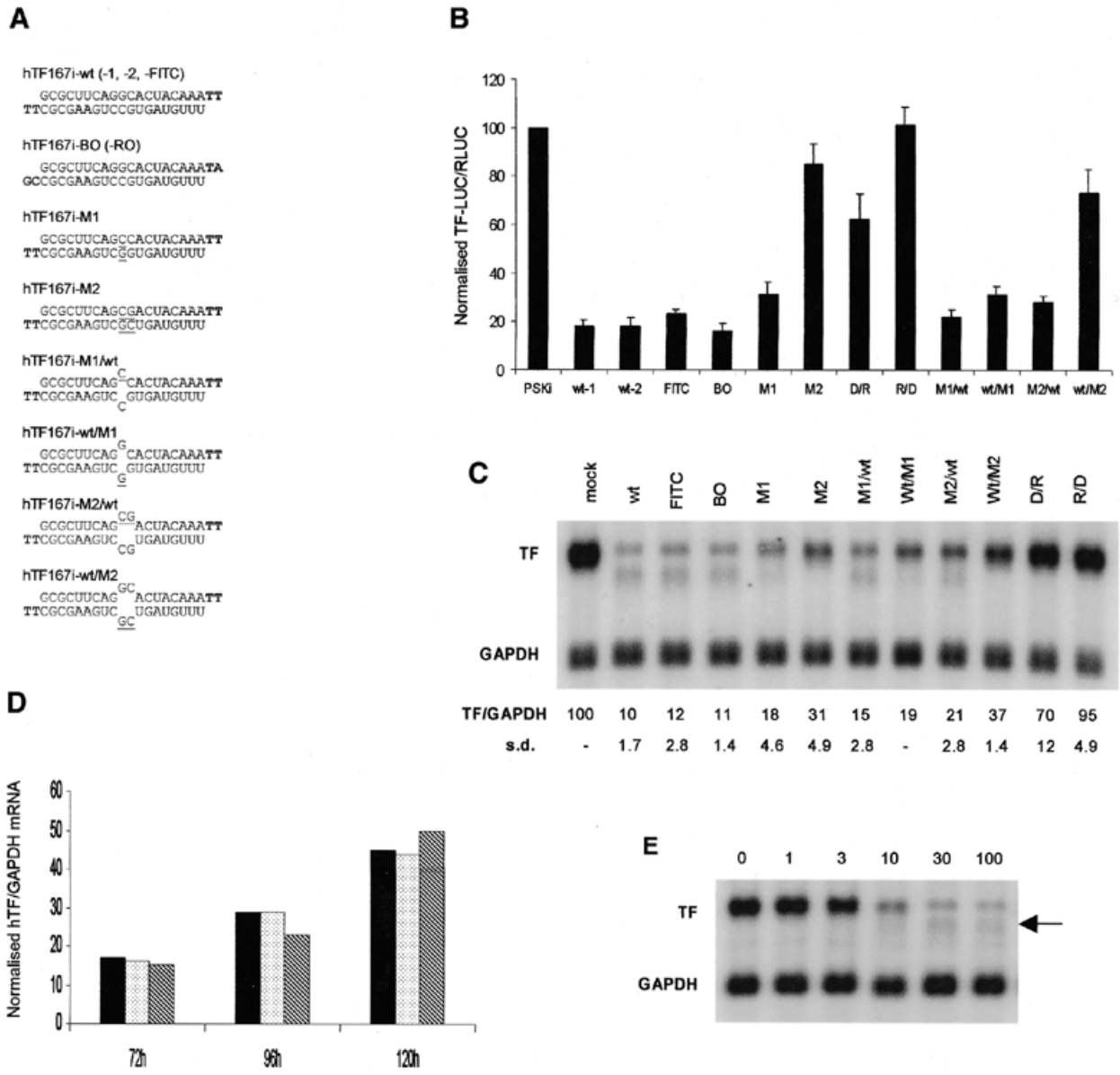

Figure 6. Tolerance of RNAi for modifications and mutations. (**A**) Sequence of modified and mutated siRNAs targeting hTF167. Two different batches of wild-type hTF167i (wt-1 and wt-2) were synthesised. Modified versions were prepared with a FITC tag on the 3′-OH of the 3′ nucleotide of the antisense strand (hTF167i–FITC) or with target mRNA base pairing overhangs consisting of either deoxyribonucleotides (hTF167i-BO) or ribonucleotides (hTF167i-RO). The siRNAs hTF167i-M1 and hTF167i-M2 have one (G→C) and two (GC→CG) central mismatches (underlined) relative to target mRNA, respectively. By combining sense and antisense strands of wild-type siRNA with partially complementary strands of the M1 and M2 mutants, imperfect siRNAs with 1 and 2 bp bulges were prepared in which the mutation relative to the wild-type siRNA was either in the sense (M1/wt and M2/wt) or antisense (wt/M1 and wt/M2) strand. (**B**) Standard co-transfections with modified/mutated siRNAs (30 nM). DNA(sense)/RNA(antisense) (D/R) and RNA/DNA (R/D) hybrid versions of hTF167i were included. (**C**) Northern analysis of RNAi by the hTF167i variants (100 nM). GAPDH-normalised expression ± SD is indicated. (**D**) Northern time-course experiment. Cells were transfected with 100 nM siRNA. Normalised mRNA levels are shown for hTF167i-wt (filled bars), hTF167i-BO (dotted bars) and hTF167i-RO (hatched bars). TF mRNA was normalised to GAPDH and standardised to levels in mock-transfected cells at each time point. (**E**) Concentration dependence (nM) of mRNA depletion by hTF167i-wt. Complexation with Lipofectamine™ was performed in one batch for all samples and complexes diluted in medium as appropriate immediately before addition to cells. The arrow indicates cleavage fragments.

behaved essentially as the non-bulged mutants and were less active than the corresponding hTF167i-M1/wt and hTF167i-M2/wt, with the modification in the sense strand (Fig. 6B and C). This strand bias has previously been seen with dsRNA in *C.elegans* (46) and emphasises that the principal role of the sense strand is to protect the antisense strand, as a single-stranded RNA of this size is degraded very rapidly *in vivo* (25). Another role has been uncovered recently whereby the identity of the sense strand is checked by gateway kinases before allowing incorporation into the RISC complex and subsequent unwinding of the siRNA (15). This is consistent with our results with DNA/RNA hybrids of hTF167i. These chimeras exhibited low or no activity in both assays (Fig. 6B and C), in agreement with recent observations in *Drosophila* (44). The strand bias was still apparent, however, with DNA being less detrimental to activity when

present in the sense (hTF167i-D/R) than in the antisense (hTF167i-R/D) strand. Finally, competition experiments demonstrated that mismatched and bulged siRNAs of low activity could compete with hTF167i as well as inactive siRNAs and competition by DNA-containing double-stranded oligos was only slightly impaired (data not shown). The process which this sequence-independent competition inhibits is not presently known.

DISCUSSION

The biological functions of RNAi seem to be control of viruses and transposons (47–50). In a study of certain aspects of the RNAi mechanism we have revealed some current limitations to the use of siRNAs as tools in functional genomics and lead compounds in drug development. We find that siRNA efficacy is highly dependent on target position. Despite this limitation, we identified one siRNA that specifically and efficiently inhibited expression of the target gene at the levels of mRNA, protein and function. The levels of TF mRNA and protein were reduced 10-fold, while TF-dependent procoagulant activity was depleted 5-fold. The effect was transient, as the mRNA levels as well as transgene expression fully recovered after 4–5 days. Our results suggest that susceptible siRNA target sites in some human genes may be rare.

We have demonstrated that inactive siRNAs compete with active siRNAs in a reversible and sequence-independent manner (Fig. 4). Mutants and other non-standard siRNAs were also able to compete. Taken together, these observations indicate lack of discrimination at one or more initial steps in the degradative process.

Even the most effective siRNAs acted relatively slowly in mRNA depletion following transfection, with the optimum effect observed after 24 h, and in no case was there total depletion of TF mRNA. Several lines of evidence suggest that this is caused by the existence of a near equilibrium kinetic balance between siRNA-mediated cleavage of mRNA and mRNA transcription and processing. These include the concentration dependence of siRNA efficacy (i.e. the increased residual mRNA or reporter gene level) in the presence of a competitor (Fig. 4), the difference in depletion rate among active siRNAs targeting different sites (Fig. 5A) and the gradual reduction in depletion rate for mutated siRNAs (Fig. 5A). We further demonstrate that RNAi to a certain degree tolerates siRNA:mRNA mismatches, with 1 and 2 bp mismatches only partially reducing the rate and extent of depletion.

The gradual recovery of gene expression within 4–5 days of transfection (Fig. 5C) constitutes evidence against the presence of a propagative system in humans maintaining the siRNA-based silencing over time. A recent report of RNAi in *C.elegans* demonstrates the need for continual siRNA production by RdRP homologues (17). Two of these RdRPs (rrf-1 and rrf-3) are intimately involved in synthetic siRNA-mediated silencing, as this pathway is abolished in a rrf-1 negative strain, while the rrf-3 negative strain actually seemed to enhance synthetic siRNA silencing. This propagative system was also proposed to operate in *Drosophila* (16), where, as in humans, RdRP homologues have not yet been identified. In both cases the mechanism seems to be production of dsRNA from mRNA by siRNA priming, with secondary siRNAs being produced by Dicer. This degradative PCR-like system was recently nominated as the mRNA degrading complex itself (16). Results from a third report (15), however, show that Dicer is ATP dependent while RISC is not. Our data with a 3′-OH blocked siRNA, which cannot be utilised as a primer by a putative RdRP, constitutes direct evidence that the proposed degradative PCR mechanism is not essential for initial mRNA depletion *in vivo* in human cells.

Finally, we observed cleavage fragments resulting from siRNA action. These cleavage fragments appeared only in the presence of a high rate of mRNA depletion. Thus, we see a cleavage fragment with wild-type hTF167i, but not with the slower hTF173i (Figs 3A and B and 5A). A double mutant of hTF167i, which results in a 3-fold increase in residual mRNA levels (Fig. 6C) and has a lower depletion rate than the wild-type (Fig. 5A), also does not produce a cleavage fragment. Even with wild-type hTF167i, the cleavage fragment disappears when the concentration of the siRNA is reduced to sub-saturating levels, resulting in less extensive cleavage of mRNA (Fig. 6E). These observations suggest that degradation is performed in at least two separate steps. We hypothesise that mRNA is cleaved in the first step by RISC. The cleaved mRNA is subsequently degraded by an exonuclease similar to that implicated in RNAi by genetic evidence in *C.elegans* (47). The accumulation of a cleavage product as seen in our northern gels would require the second step to be rate determining when RISC operates at full speed.

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