Nonsense-mediated mRNA decay affects nonsense transcript levels and governs response of cystic fibrosis patients to gentamicin

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Aminoglycosides can readthrough premature termination codons (PTCs), permitting translation of full-length proteins. Previously we have found variable efficiency of readthrough in response to the aminoglycoside gentamicin among cystic fibrosis (CF) patients, all carrying the W1282X nonsense mutation. Here we demonstrate that there are patients in whom the level of *CF transmembrane conductance regulator* (*CFTR*) nonsense transcripts is markedly reduced, while in others it is significantly higher. Response to gentamicin was found only in patients with the higher level. We further investigated the possibility that the nonsense-mediated mRNA decay (NMD) might vary among cells and hence governs the level of nonsense transcripts available for readthrough. Our results demonstrate differences in NMD efficiency of *CFTR* transcripts carrying the W1282X mutation among different epithelial cell lines derived from the same tissue. Variability was also found for 5 physiologic NMD substrates, *RPL3*, *SC35 1.6 kb*, *SC35 1.7 kb*, *ASNS*, and *CARS*. Importantly, our results demonstrate the existence of cells in which NMD of all transcripts was efficient and others in which the NMD was less efficient. Downregulation of NMD in cells carrying the W1282X mutation increased the level of *CFTR* nonsense transcripts and enhanced the *CFTR* chloride channel activity in response to gentamicin. Together our results suggest that the efficiency of NMD might vary and hence have an important role in governing the response to treatments aiming to promote readthrough of PTCs in many genetic diseases.

Introduction

Many inherited diseases result from nonsense mutations leading to premature termination codons (PTCs) (1). One therapeutic approach for patients carrying in-frame nonsense mutations is aimed at promoting readthrough of the PTCs to enable the expression of full-length, functional proteins (2, 3). Studies in tissue culture cells as well as in mouse models have shown that aminoglycosides can readthrough disease-causing PTCs and partially restore the expression and/or the function of these proteins (4–19). Further clinical trials in Duchenne muscular dystrophy (DMD) (20, 21), Becker muscular dystrophy (20), and cystic fibrosis (CF) (22, 23) patients have shown that aminoglycosides can promote in vivo readthrough of nonsense mutations and lead to expression of full-length proteins and/or correction of the protein function. However, in several studies, no expression of full-length functional proteins was actually observed, suggesting no response to the aminoglycoside treatment (15, 16, 21–25). This variability

in response was proposed to be associated with the identity of the PTC and its sequence context (26–28), as well as with the chemical composition of the aminoglycoside, the duration of treatment, and the method of application.

We have recently administered gentamicin to the nasal epithelium of CF patients, all of whom carried the same W1282X nonsense mutation (29). This mutation is associated with a severe form of the CF disease (30). In many of the patients, improvement of the typical CF electrophysiological abnormalities was found and expression of full-length *CF transmembrane conductance regulator* (*CFTR*) proteins was demonstrated following the treatment. In other patients, however, no correction of the electrophysiological abnormalities was found, indicating no response to gentamicin. The reasons for this variable response remained unknown. Since the templates for readthrough treatment are the transcripts carrying a PTC, we hypothesized that the level of these transcripts is a limiting factor in the response.

PTC-bearing transcripts are detected and degraded by the nonsense-mediated mRNA decay (NMD) pathway, a posttranscriptional translation–dependent surveillance mechanism that prevents the synthesis of proteins carrying PTCs (31, 32). NMD has been shown to degrade transcripts carrying disease-causing nonsense or frameshift mutations, as well as a variety of physiologic transcripts. Among these are transcripts with upstream open reading frame (uORF), transcripts containing introns in the
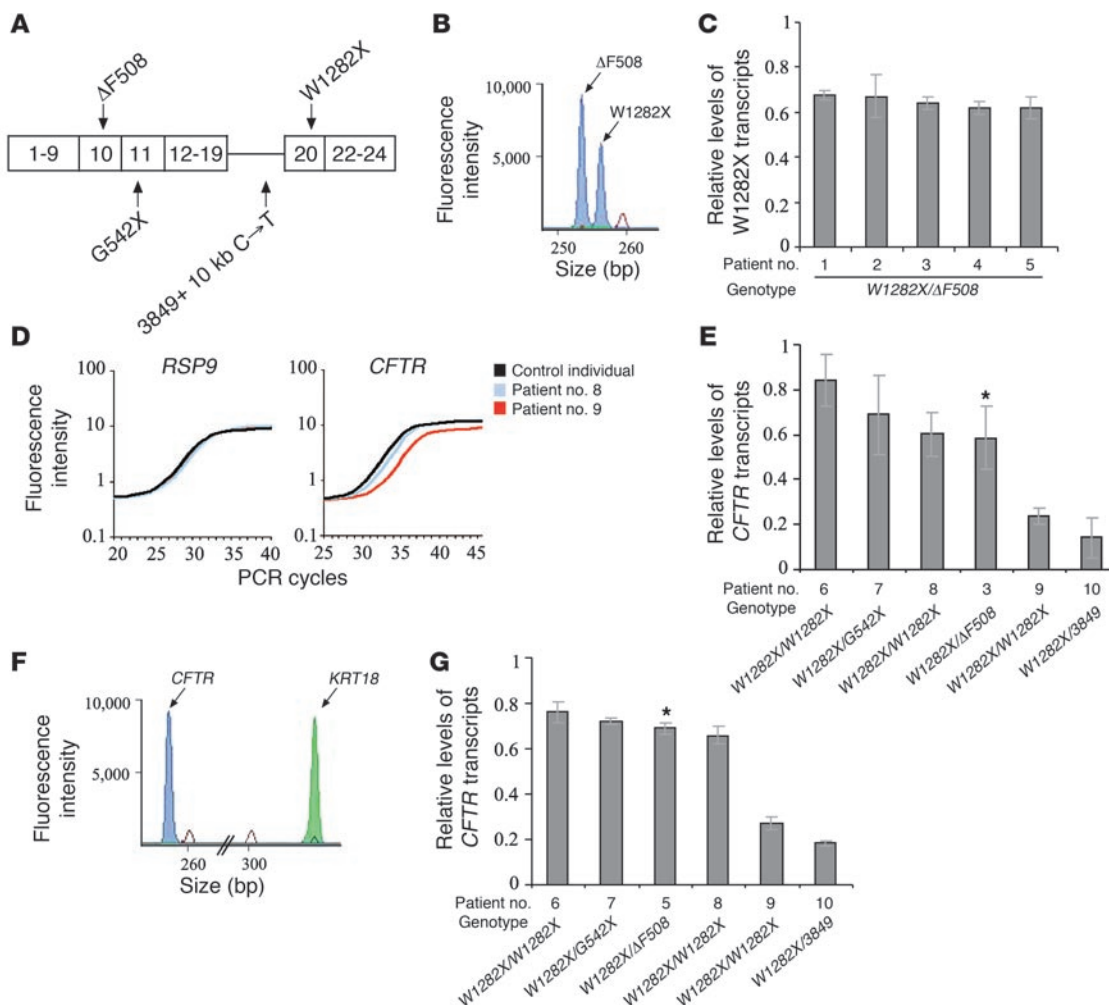
Figure 1

Levels of *CFTR* nonsense transcripts in nasal epithelium of CF patients. (**A**) A scheme of localization of the *CFTR* mutations carried by the studied patients. The numbers within the boxes mark the *CFTR* exons. The connecting horizontal line marks intron 19. (**B**) An example of GeneScan analysis of RT-PCR products from the ΔF508 region (patient 3). The size marker appears as a red peak (260 bp). (**C**) A summary of the W1282X transcript levels in the *W1282X/ΔF508* patients. These levels were compared to the level of ΔF508 transcripts. (**D**) An example of real-time PCR of the *CFTR* and the *RPS9* genes, of RNA from 2 patients (nos. 8 and 9) homozygous for W1282X and a control individual with normal *CFTR* alleles. (**E**) A summary of the *CFTR* transcript levels, in the patients carrying 2 nonsense mutations. The levels were normalized to the level of *RPS9* and compared to the normalized level obtained from a control individual with normal *CFTR* alleles. The asterisk marks the *CFTR* transcript level in patient 3, who was also analyzed as described in **C**, for comparison of the results of the GeneScan and real-time PCR analyses. (**F**) An example of GeneScan analysis of RT-PCR products of the *CFTR* and the *KRT18*, in a patient homozygous for the W1282X mutation (patient 6). The size marker appears as red peaks (260 bp and 300 bp). (**G**) A summary of the *CFTR* transcript levels in the patients carrying 2 nonsense mutations. These levels were normalized to the level of *KRT18* and compared to the normalized level obtained from a control individual with normal *CFTR* alleles. The asterisk marks the *CFTR* transcript level in patient 5, who was also analyzed as described in **C**, for comparison of the GeneScan results. The levels in **C**, **E**, and **G**, are shown as mean ± SEM.

3′ untranslated region (UTR), and transcripts derived from alternative splicing (33–37).

Here we have investigated the possibility that NMD efficiency might vary among different cells and hence affects the level of PTC-bearing transcripts available for readthrough treatment. We demonstrate that the same PTC can elicit NMD with variable efficiency among cells derived from nasal epithelium. Variability in the NMD efficiency was found for *CFTR* transcripts carrying the W1282X mutation as well as for several physiologic NMD substrates, *ribosomal protein L3* (*RPL3*), *splicing component 35 kDa* (*SC35*) *1.6 kb*, *SC35 1.7 kb*, *asparagine synthetase* (*ASNS*), and *cysteinyl-tRNA*

synthetase (*CARS*). We further showed that downregulation of NMD increased the level of *CFTR* nonsense transcripts and led to enhanced *CFTR* chloride channel activity in response to gentamicin. Together, our results suggest that the efficiency of NMD may vary among cell lines and hence affect the level of transcripts carrying PTCs, and govern the response to readthrough treatment.

Results

Different levels of nonsense CFTR mRNA in samples from patients. In order to investigate the possibility that the response of patients to readthrough treatment is associated with the level of mRNA tran-

Table 1
Study patients and response to gentamicin treatment

Patient number	Genotype	Basal NPD		Chloride transport		Response
		Before treatment	After gentamicin	Before treatment	After gentamicin	
1	*W1282X/ΔF508*	−38	−18	−1	−6	+
2	*W1282X/ΔF508*	−41	−41	−3	−5	+
3	*W1282X/ΔF508*	−43	−30	−1	−4	+
4	*W1282X/ΔF508*	−40	−30	4	−4	+
5	*W1282X/ΔF508*	−40	−30	−2	−2	+/−[A]
6	*W1282X/W1282X*	−47	−32	0	−9	+
7	*W1282X/G542X*	−43	−45	−2	−11	+
8	*W1282X/W1282X*	−40	−35	11	−9	+
9	*W1282X/W1282X*	−32	−33	2	4	−
10	*W1282X/3849+10 kb C→T*	−65	−56	3	−3	−

All values are expressed as mV. The normal range for basal potential difference in our laboratory is ≥−30 mV, and the normal range of chloride transport is ≤−4 mV (40). [A]In all previous clinical trials in CF patients, aminoglycosides (22, 23, 29) or other molecules (41–44) influenced only the chloride transport abnormality. In this patient, there was no change in chloride transport, while normalization of basal potential difference was observed. This is unusual, and hence the response of this patient remained inconclusive (+/−).

scripts carrying the W1282X mutation, we obtained RNA samples from nasal epithelial cells from patients who had participated in our double-blind, placebo-controlled, crossover trial (29). The level of nonsense *CFTR* mRNA was analyzed in pretreatment samples from these patients. Five of the patients were heterozygous for the W1282X and the ΔF508 mutations (Figure 1A). The ΔF508 mutation is a 3-bp deletion, which is not expected to be affected by NMD, hence the level of ΔF508 transcripts is similar to that of wild-type (38). Thus, the level of mRNA transcribed from W1282X alleles could be distinguished and compared to those transcribed from the ΔF508 allele. The results showed that the level of the W1282X mRNA among these patients was reduced to between 62% ± 0.05% and 68% ± 0.02% of that transcribed from the ΔF508 allele (Figure 1, B and C). The other 5 patients carried 2 alleles with *CFTR* nonsense mutations, of which at least 1 was the W1282X (Figure 1A). The *CFTR* mRNA levels in these patients were normalized to those transcribed from a control gene, the *ribosomal protein S9* (*RPS9*), which we found to be expressed at the same level in all tested individuals and whose expression was not affected by the gentamicin treatment. The level of the normalized *CFTR* nonsense mRNA in each patient was compared to the normalized level obtained from a control individual with normal *CFTR* alleles. The results revealed that in 3 patients (nos. 6, 7, and 8), the level of *CFTR* nonsense mRNA was between 60% ± 0.10% and 84% ± 0.12% of that found in a control individual with normal *CFTR* alleles, similar to the level found in the *W1282X/ΔF508* patients (Figure 1, D and E). In contrast, in 2 patients (nos. 9 and 10), the level was considerably lower, 24% ± 0.04% and 14% ± 0.09%, respectively. We further analyzed 1 patient heterozygous for the W1282X and the ΔF508 mutations (patient 3) by both GeneScan and real-time PCR analyses for comparisons of the results obtained by the different mRNA quantification methods. As shown in Figure 1, C and E, similar levels were observed. Furthermore, 2 of the patients homozygous for W1282X (nos. 8 and 9) are sisters whose parents are first-degree cousins. Although they carry identical *CFTR* alleles, there was a large difference in their levels of W1282X transcripts. Patient 8 had 60% ± 0.10% of the level found in a control individual, while patient 9 had 24% ± 0.04%. Consistent results were obtained in RNA samples derived from these patients at various time points (Supplemental Figure 1; supplemental material avail-

able online with this article; doi:10.1172/JCI28523DS1). In order to exclude the possibility that the observed variability resulted from variation in the representation of epithelial cells in the samples, we further normalized the level of *CFTR* transcripts in these patients to that of *keratin 18* (*KRT18*), a marker of ciliated and secretory epithelial cells (39), which we found not affected by the gentamicin treatment. The level of the normalized *CFTR* nonsense transcripts in each patient was compared to the normalized level obtained for a control individual with normal *CFTR* alleles. The results revealed normalized levels similar to those obtained with *RPS9* (Figure 1, F and G). It is important to note that no change in the level of nonsense transcripts was found in any of the patients following the placebo or the gentamicin treatment (Supplemental Figure 2A). Together, the results indicate that our patients can be categorized into 2 groups, one in which the level of *CFTR* nonsense transcripts is markedly reduced and the other in which the level is significantly higher.

In order to investigate the role of transcription efficiency in regulating the level of *CFTR* mRNA, we analyzed the level of *CFTR* pre-mRNA in 4 patients (nos. 2, 5, 8, and 10). The results showed that the relative *CFTR* pre-mRNA levels were not significantly different (*P* = 0.189) among the patients (Supplemental Figure 3). Hence, the markedly reduced level of nonsense *CFTR* mRNA in patient 10 (shown in Figure 1) was not the result of lower *CFTR* transcription efficiency.

The level of CFTR nonsense transcripts is associated with the response of patients to gentamicin treatment. We further analyzed whether the relative level of *CFTR* nonsense transcripts is associated with the response of the patients to gentamicin treatment. As shown in Table 1, normalization of the abnormal chloride transport (≤−4 mV) was found in 7 of the 8 patients with the higher level of *CFTR* nonsense transcripts. In some of these patients (nos. 1, 3, and 4), the normalization of chloride transport was associated with improvement toward normalization of the basal nasal potential difference (NPD) (≥−30 mV) (40). The reason for this improvement in a subset of the patients is unclear. In one patient, patient 5, there was no change in chloride transport, while normalization of basal NPD was observed. It is important to note that in all previous clinical trials in CF patients, aminoglycosides (22, 23, 29) or other molecules (41–44) affected only the chloride transport
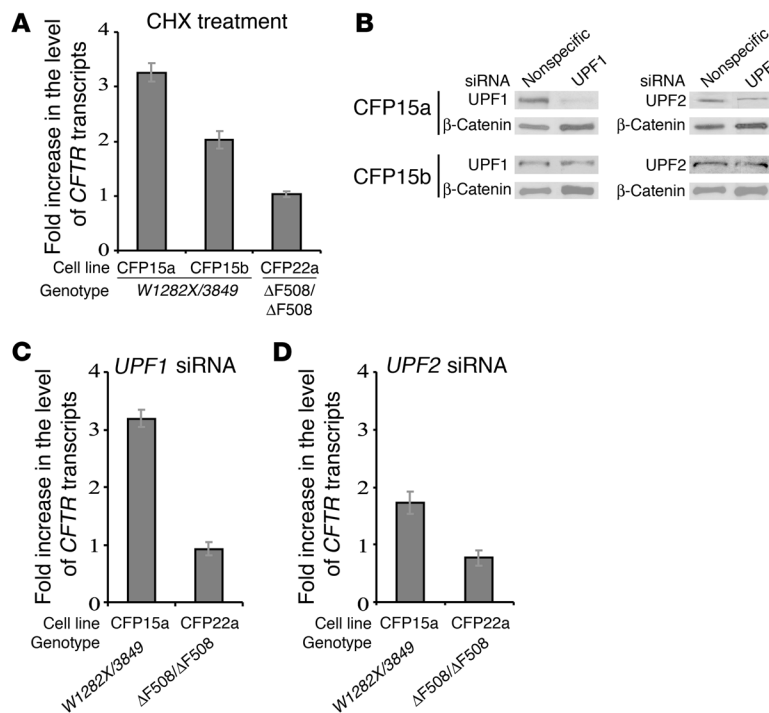
Figure 2
Effect of NMD inhibition on the level of endogenous *CFTR* transcripts. (**A**) Effect of indirect NMD inhibition by CHX on the level of *CFTR* transcripts. The level was measured by real-time PCR and normalized to that of *RPS9* and the ratio between these normalized levels following treatment was calculated and compared to the ratio in untreated cells. (**B**) Western blot analysis in CFP15a (upper panels) and CFP15b (lower panel) cells following sequence-specific downregulation of UPF1 and UPF2. (**C** and **D**) Effect of direct NMD inhibition by siRNA directed against *UPF1* (**C**) and *UPF2* (**D**) on the level of *CFTR* transcript. The level was measured and normalized as described in **A**. The increase in the level of *CFTR* transcripts is shown as mean ± SEM.

abnormality; hence, the response of patient 5 remained inconclusive. No response was found in the patients with markedly reduced transcript levels (patients 9 and 10). Together, the results suggest that the level of nonsense transcripts might be a limiting factor in readthrough of PTCs in response to gentamicin treatment, although the variability in *CFTR* transcript level cannot entirely explain the variability in the response.

Variable NMD efficiency of endogenous CFTR transcripts carrying the W1282X mutation. Previous studies showed that the level of W1282X transcripts is markedly reduced in epithelial samples derived from CF patients and in a bronchial epithelial cell line, IB3-1, heterozygous for the W1282X and the ΔF508 mutations (5, 45). These results raised the possibility that transcripts carrying the W1282X mutation are subject to NMD. PTCs that are located more than 50–55 nucleotides upstream of the final exon-exon junction can induce NMD (46). Since the last *CFTR* exon-exon junction is located more than 55 nucleotides downstream to the W1282X mutation, transcripts carrying the W1282X mutation have the potential to be subject to NMD. In order to investigate this possibility, we analyzed nasal epithelial cell lines (CFP15a and CFP15b) derived from polyps of 2 unrelated CF patients, both heterozygous for the W1282X and the 3849+10 kb C→T mutations. The latter is a change from C to T that generates a splicing donor site that can lead to inclusion of an 84-bp cryptic exon harboring a PTC (47). We examined the level of *CFTR* nonsense transcripts following treatment with cycloheximide (CHX), a known indirect inhibitor of NMD (48). The *CFTR* transcript level was normalized to that of *RPS9*, and the ratio between these normalized levels following CHX treatment was calculated and compared to the ratio in untreated cells. Following CHX treatment in CFP15a cells, a 3.3 ± 0.16–fold increase in the *CFTR* transcript level relative to untreated cells was found, while in CFP15b the increase was only 2.0 ± 0.16–fold (Figure 2A). These results suggest that endogenous *CFTR* W1282X transcripts might be subject to NMD with variable

efficiencies. As a control, we administered CHX to another nasal epithelial cell line (CFP22a), which is homozygous for the ΔF508 mutation. No change in the *CFTR* transcript level was found in these cells (Figure 2A). This indicated that the inhibition of NMD by CHX is specific to transcripts carrying PTCs.

To further study the mechanism of this variability, we tested the effect of siRNA duplexes on *UPF1* and *UPF2* (*UPF1* and *-2 regulator of nonsesnse transcripts homolog (yeast)*; also known as *RENT1* and *RENT2*, respectively). These factors are essential for the NMD pathway (49). As a control, we analyzed the effect of nonspecific siRNA duplexes. Western blot analysis in CFP15a cells showed a sequence-specific downregulation of both *UPF1* and *UPF2*. However, the downregulation of *UPF1* was more efficient (Figure 2B, upper panels). Western blot analysis in CFP15b cells revealed poor downregulation of both factors (Figure 2B, lower panels). Hence, in further experiments, only CFP15a cells were analyzed. The level of *CFTR* transcripts increased by 3.2 ± 0.16–fold in CFP15a cells in which *UPF1* was downregulated relative to untreated cells (Figure 2C). Downregulation of *UPF2*, which was less efficient than that of *UPF1*, also increased the transcript level, but to a lesser extent, by 1.7 ± 0.20–fold (Figure 2D). These results are consistent with the Western blot, which showed less efficient downregulation of *UPF2* than of *UPF1* (Figure 2B, upper panels). As a control, we treated the CFP22a cells with the *UPF1* and *UPF2* siRNA. As expected, no change in the *CFTR* transcript level was found (Figure 2, C and D). Together, the results suggest that NMD efficiency for transcripts carrying the W1282X mutation is different among different cell lines even derived from the same tissue.

Variable NMD efficiency of physiologic NMD substrates. We further investigated the possibility that the variability in NMD efficiency might be a general phenomenon and is not specific to *CFTR* transcripts carrying PTCs. For this we analyzed in the 3 epithelial cell lines (CFP15a, CFP15b, and CFP22a) the effect of CHX treatment on the level of physiologic NMD substrates. These included an alternatively spliced PTC-bearing transcript *RPL3* (50); transcripts with introns in their 3′ UTR (the *SC35* splicing isoforms *SC35 1.6 kb* and *SC35 1.7 kb*) (51, 52); a transcript with a uORF (*ASNS*) (33); and another bona fide NMD substrate with an unknown NMD-inducing feature, *CARS* (33). For all 5 transcripts analyzed, the results show an increase in the level following CHX treatment, which widely varied among the cells (Figure 3, A–E). In the CFP15b cells, the increase was significantly lower
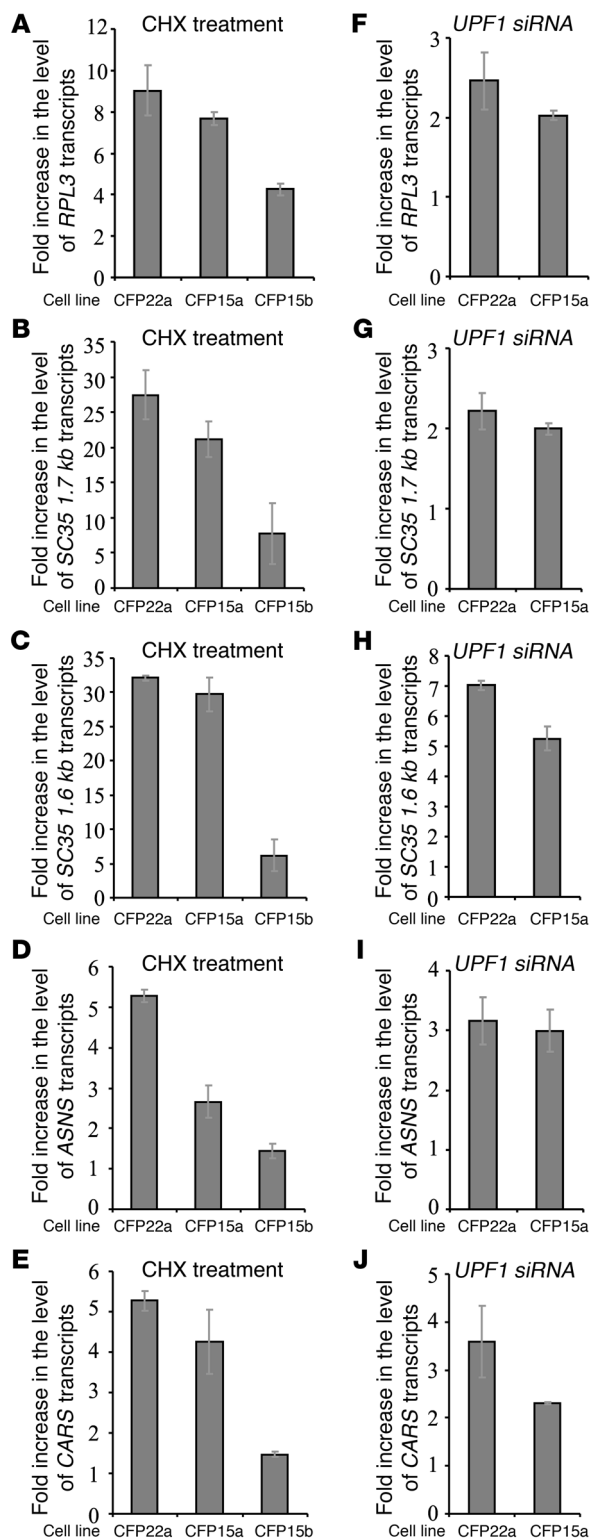
Figure 3
Effect of NMD inhibition on the transcript level of physiologic NMD substrates in nasal epithelial cell lines. The level of each analyzed transcript was measured by real-time PCR and normalized to that of *GAPDH* and the ratio between these normalized levels following treatment was calculated and compared to the ratio in untreated cells. (**A**–**E**) Effect of indirect NMD inhibition by CHX on the level of *RPL3* (**A**), *SC35 1.7 kb* (**B**), *SC35 1.6 kb* (**C**), *ASNS* (**D**), and *CARS* (**E**) transcripts. (**F**–**J**) Effect of direct NMD inhibition by *UPF1* downregulation on the level of *RPL3* (**F**), *SC35 1.7 kb* (**G**), *SC35 1.6 kb* (**H**), *ASNS* (**I**), and *CARS* (**J**) transcripts. The increase in the level of *CFTR* transcripts is shown as mean ± SEM.

resulted from the incomplete NMD inhibition following *UPF1* downregulation. Variability in NMD efficiency following *UPF1* downregulation was also found among other epithelial cell lines (unpublished observations). Together, the results following CHX treatment and *UPF1* downregulation indicated a wide variability in NMD efficiency among different cell lines, even in cell lines derived from the same tissue.

We further analyzed the relative level of all 5 physiologic NMD substrates in RNA samples from nasal epithelial cells derived from 3 patients: 2, 8, and 10. The results showed that the relative levels of all analyzed transcripts were lower in patient 10 than in patients 2 and 8 (Figure 4). This is consistent with the levels of the nonsense *CFTR* mRNA, which were lower in patient 10 than in patients 2 and 8.

NMD governs the CFTR function following gentamicin treatment. Previous studies in *Saccharomyces cerevisiae* found that suppressor transfer RNA (which promotes readthrough) led to sufficient levels of functional proteins expressed from a nonsense allele only after inhibition of NMD (53–55). Here we aimed to investigate the potential of NMD inhibition to modulate the response to gentamicin in epithelial cells derived from CF patients. Since the *CFTR* gene encodes a cAMP-activated chloride channel, we analyzed the effect of NMD inhibition on the *CFTR* function by measuring cAMP-evoked chloride efflux. The results showed that the *CFTR* channels in CFP15a cells are inactive or absent (Figure 5A). Following treatment with 50–200 μg/ml gentamicin, concentration-dependent chloride efflux was detected (Figure 5A). This indicates that gentamicin can induce productive readthrough of the PTCs in these cells. Chloride channel activity was improved in gentamicin-treated cells in which *UPF1* or *UPF2* was downregulated compared with cells treated with gentamicin alone (Figure 5, C–E). The downregulation of *UPF1* resulted in a higher activation of the *CFTR* channels relative to *UPF2*, consistent with the different efficiency of the downregulation of these factors (Figure 2B, upper panels). Interestingly, *UPF1* downregulation in cells treated with 50 μg/ml gentamicin resulted in chloride efflux comparable to that achieved with 100 μg/ml gentamicin alone (Figure 5, C and D). Moreover, *UPF1* downregulation in cells treated with 100 μg/ml resulted in an even higher activation compared with that achieved with 200 μg/ml gentamicin alone (Figure 5, D and E). To a lesser extent, this effect was also observed following *UPF2* downregulation. In order to evaluate the extent of functional improvement following NMD inhibition in CFP15a, we examined the *CFTR* function in human epithelial cells T84, which carry normal *CFTR* alleles (Figure 5B). The results show that the *CFTR* function is similar in T84 cells and CFP15a cells following *UPF1* downregulation together with treatment with 200 μg/ml gentamicin (Figure 5, compare B and E).

than that observed in the other cells. The level of these physiologic NMD substrates was further analyzed in CFP15a and CFP22a cells following *UPF1* downregulation (Figure 3, F–J). As shown in Figure 3, the fold increase following CHX treatment was higher than that observed following *UPF1* downregulation (Figure 3, compare A–E with F–J). We conjecture that this effect probably
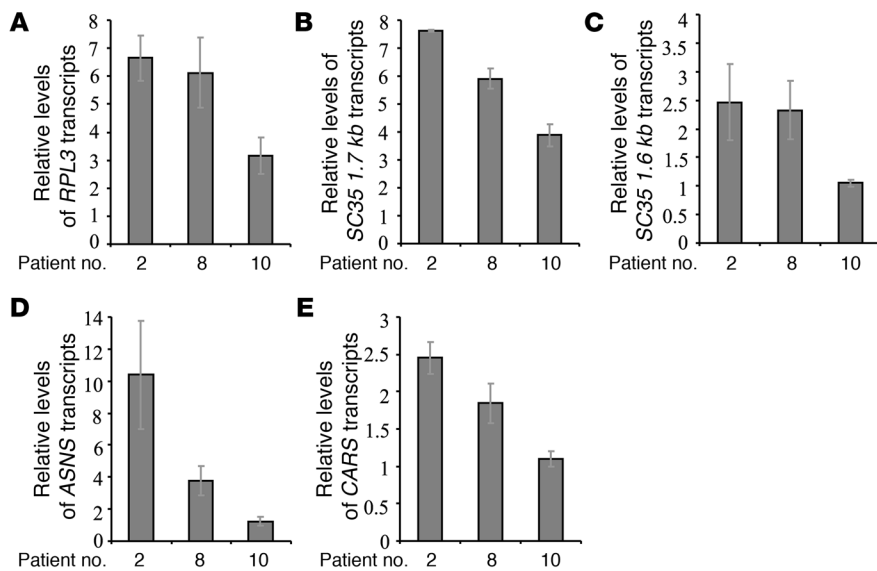
Figure 4
Relative levels of physiologic NMD substrates in RNA samples derived from CF patients. The level of each analyzed transcript — *RPL3* (**A**), *SC35 1.7 kb* (**B**), *SC35 1.6 kb* (**C**), *ASNS* (**D**), and *CARS* (**E**) — was measured by real-time PCR and normalized to the level of *GAPDH*. The relative levels are shown as mean ± SEM.

We extended the *CFTR* functional analysis to another epithelial cell line, IB3-1. Previous studies have shown that the level of the W1282X transcripts in these cells is markedly reduced in comparison to the level of transcripts derived from the ΔF508 allele (5). As shown in Figure 5G, the *CFTR* channels in IB3-1 cells were inactive. No significant activation was observed following treatment of the cells with 50 μg/ml, 100 μg/ml, or even 200 μg/ml gentamicin (Figure 5G). Following downregulation of *UPF1* together with treatment with 50 μg/ml gentamicin, no significant improvement in chloride efflux was achieved (Figure 5H). Only following downregulation of *UPF1* together with treatment with 100 μg/ml or 200 μg/ml gentamicin was an impressive *CFTR* activation observed (Figure 5, I and J). It is important to note that this *CFTR* activation was found despite a nonefficient transfection (~20%) with *UPF1* siRNA (data not shown). These results indicate that the *CFTR* activation in IB3-1 cells by gentamicin was highly dependent on NMD downregulation.

We also analyzed the *CFTR* function in the homozygous ΔF508 CFP22a cells. No *CFTR* chloride efflux was found in CFP22a cells following gentamicin treatment, as expected from cells carrying *CFTR* mutations with no potential for readthrough (Figure 5K). Furthermore, we found no improvement following downregulation of *UPF1* or *UPF2* (Figure 5K). This result is consistent with the inability of NMD inhibition to increase the ΔF508 transcript level (Figure 2, C and D). These results support the general concept that NMD affects the level of nonsense transcripts and governs the response to gentamicin.

Discussion

Our study presents what we believe to be new insights into the molecular basis of the response to treatments that promote readthrough of PTCs by showing a potential role for the NMD mechanism in governing the response. Previous studies emphasized the contribution of *cis*-elements, such as the stop codon and the first nucleotide that follows, to the response to aminoglycoside treatment. Our results indicate that factors other than the PTC locus itself, such as the level of nonsense transcripts, might limit the readthrough efficiency. In cases of efficient NMD, the level of nonsense transcripts is markedly reduced and hence is insufficient to generate enough functional proteins even when gentamicin is

provided. By contrast, in cases of less-efficient NMD, the transcript level is higher and readthrough might be effective. It was previously suggested that no clinical benefit would be derived from aminoglycoside treatment in CF patients with severely reduced levels of nonsense transcripts (56). Our results, although performed on a very small number of patients, support this hypothesis and show no correction of the *CFTR* function in patients with markedly lower levels of nonsense transcripts (Figure 1 and Table 1). The results further show that by increasing the level of *CFTR* nonsense transcripts, a significant improvement in the response to gentamicin can be achieved. This leads to increased activation of the *CFTR* channel compared with the activation with gentamicin alone (Figure 5). It follows that in the same cells, increase in the level of nonsense transcripts can allow the use of lower gentamicin concentrations to achieve chloride efflux activation similar to that achieved with gentamicin alone. Although the gentamicin concentrations used in this study were much higher than the clinical doses, the results provide a proof of concept that the level of PTC-bearing transcripts affects the response to readthrough treatment.

Here we show, for a variety of transcripts, that NMD efficiency might vary considerably among cells derived from the same cell type (epithelium) and even from the same tissue (nasal epithelium) (Figures 2 and 3). The results also clearly show that there are cell lines, such as CFP15b, in which NMD is relatively less efficient. It is important to note that in all cell lines, different transcripts are subject to different extents of NMD (Figure 3). Previously it was shown that in patients carrying PTCs in collagen X, the nonsense transcripts were subject to efficient NMD in cartilage cells, while in noncartilage cells, no NMD was observed (57). Recently, in 2 unrelated fetuses with Roberts syndrome, both homozygous for a PTC in the ESCO2 gene, different tissues showed different NMD efficiency (58). These studies suggest that the NMD efficiency of transcripts carrying PTCs might vary among different tissues. These differences probably result from the interaction between elements in transcripts that are subject to NMD and factors in the NMD machinery. Together, the results suggest that variability in NMD efficiency might be a more generalized phenomenon; however, further studies are needed.

Our results point to the possible role of NMD efficiency in regulation of normal cellular functions. Variable NMD might lead to
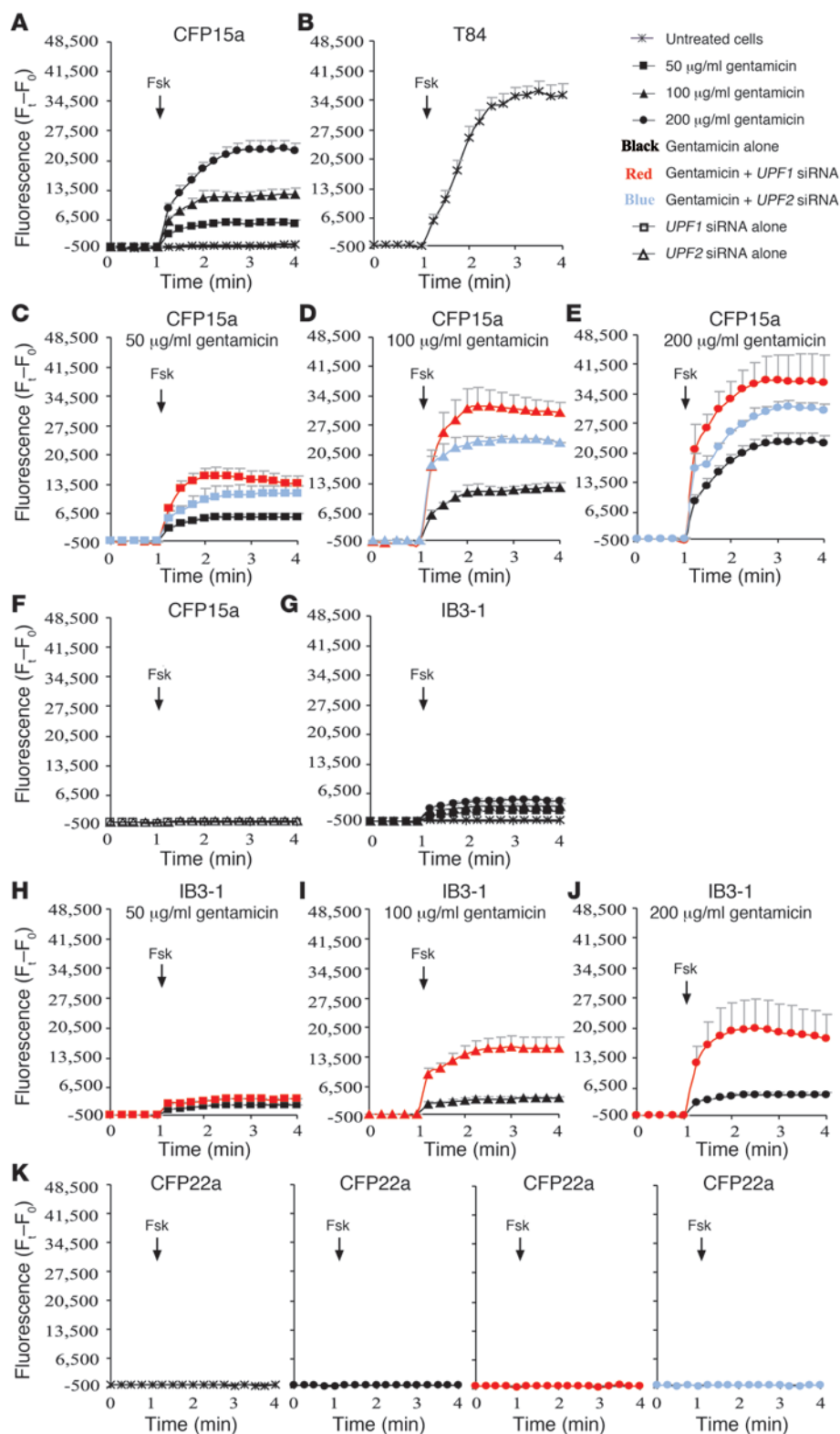
Figure 5
Effect of NMD downregulation on the *CFTR* chloride efflux following gentamicin treatment. (**A**) CFP15a cells treated with 50–200 μg/ml gentamicin. (**B**) Chloride efflux in T84 cells. (**C**–**E**) CFP15a cells treated with siRNA directed against *UPF1* and *UPF2* following gentamicin treatment with 50 μg/ml (**C**), 100 μg/ml (**D**), and 200 μg/ml (**E**). (**F**) CFP15a cells treated with siRNA directed against *UPF1* or *UPF2* alone. (**G**) IB3-1 cells treated with 50–200 μg/ml gentamicin. (**H**–**J**) IB3-1 cells treated with siRNA directed against *UPF1* following gentamicin treatment with 50 μg/ml (**H**), 100 μg/ml (**I**), and 200 μg/ml (**J**). (**K**) CFP22a cells treated with 200 μg/ml gentamicin alone and with siRNA directed against *UPF1* and *UPF2*. Fsk, forskolin. The arrow indicates the time of forskolin administered. The increase in chloride efflux is shown as mean ± SEM. F_t, fluorescence intensity at the reading time; F_0, fluorescence intensity at the beginning of the experiment.

or deleterious gain-of-function effect. For example, in β-thalassemia, the truncated proteins derived from transcripts carrying nonsense mutations cause toxic precipitation of insoluble globin chains, which might affect the phenotype of heterozygous patients (60).

It was previously suggested that readthrough-promoting reagents may not cause appreciable stabilization of nonsense transcripts due to low efficiency of readthrough or initiation of alternative surveillance mechanisms (61). In our study, we observed no increase in the level of *CFTR* nonsense transcripts following gentamicin treatment in patients and cell lines (Supplemental Figure 2). However, gentamicin concentration–dependent correction of the *CFTR* function was observed in the cells. It is possible that this resulted from low readthrough levels, which were sufficient for functional restoration (62). Similarly, another study in 2 cell lines, one compound heterozygous for nonsense alleles in the HEXB gene and the other monoallelic for a nonsense allele in TP53 gene, found no change in the level of nonsense transcripts following treatment with aminoglycosides (63). However, in IB3-1

differences in disease severity in the case of truncated proteins with partial function. This may explain the phenotypic variability among DMD and BMD patients carrying the same nonsense mutation in the dystrophin gene (59). Variable NMD efficiency might have an effect in cases where the truncated protein has a dominant-negative

cells, transcript stabilization was observed following gentamicin treatment (5). Hence, further investigation of the potential for gentamicin to stabilize transcripts in vitro and in vivo is required.

The small number of primary cells scraped from the nasal epithelium of CF patients who participated in the clinical trial did not

enable us to perform experiments aimed to directly investigate the role of NMD in response to gentamicin. However, the variability in the level of a variety of PTC-bearing transcripts suggests that the NMD mechanism might have contributed to the response of these patients to gentamicin treatment. It is worth noting that the role of posttranscriptional mechanisms in regulating the response to gentamicin cannot be excluded, except for alternative splicing of *CFTR* exon 20, which we found not be upregulated by the W1282X mutation (Supplemental Results and Supplemental Figure 4).

Finally, our results are important for the development of new therapeutic approaches aiming to promote readthrough of PTCs in many human genetic diseases. Such approaches should consider the possible effect of low levels of PTC-bearing transcripts on the response, although high transcript levels might not necessarily predict a response. It is interesting to note that we and others are currently investigating the effect of a newly developed small molecule, PTC124, on CF patients carrying the W1282X mutation (64). The role of NMD in regulating the response to this treatment is being studied in light of the results presented here.

Methods

Patients. Five of the patients were heterozygous for the W1282X and the ΔF508 mutations (patients 1–5); 3 were homozygous for the W1282X mutation (patients 6, 8, and 9); 1 was heterozygous for the W1282X and G542X mutations (patient 7); and 1 was heterozygous for W1282X and 3849+10 kb C→T mutations (patient 10), which can lead to inclusion of an 84-bp cryptic exon harboring a PTC. The clinical data of the patients and the protocol of the double blind placebo-controlled crossover trial were previously described (29).

Basal potential difference and chloride transport measured by NPD. NPD was measured in the nasal epithelium of patients before treatment and after placebo and gentamicin treatment, as described in Wilschanski et al. (29). In brief, measurements were performed between a fluid-filled recording bridge on the nasal mucosa and a reference bridge. In this way, consistent baseline measurements of potential difference were obtained. Change in the voltage response, which indicates nasal chloride permeability, was measured following generation of a large chloride chemical gradient across the apical membrane.

Cell cultures. Nasal epithelial cell lines were established from nasal polyps of CF patients using E6/E7 genes of the human papilloma virus 18 and hTERT for CFP22a, E6/E7 genes alone for CFP15a, and LT-hTERT for CFP15b (65, 66). These cells were grown in bronchial epithelial cell basal medium (Cambrex). T84 cells, which were derived from lung metastases from colorectal carcinoma, were grown in DMEM-F12 supplemented with 10% FCS. IB3-1 cells, which were derived from a primary culture of bronchial epithelia isolated from a CF patient (67), were grown in DMEM supplemented with 10% FCS.

RNA analysis. Scraped nasal epithelial cells were obtained from the patients before treatment and after treatment with placebo or gentamicin. Total RNA was extracted using the RNeasy Extraction kit (QIAGEN) or Tri-Reagent-LS (Molecular Research Center). Reactions without RNA and RT were used as controls. The level of the nonsense *CFTR* mRNA in samples of *W1282X/ΔF508* patients was analyzed by RT-PCR with primers in exons 10 and 11, flanking the ΔF508 mutation. The ΔF508 mutation is a 3-bp deletion which is not expected to be affected by NMD, hence the level of ΔF508 transcripts is similar to that of wild type (38). Thus, the level of mRNA transcribed from W1282X alleles could be distinguished and compared to those transcribed from the ΔF508 allele. Sequences for the primer pair used were as follows: forward GATTATGGGAGAACTGGAGC, reverse TTCTT-GCTCGTTGACCTCCA. The forward primer was fluorescently labeled with 6-FAM. The PCR products of the ΔF508 transcripts and the W1282X

transcripts were 254 and 257 bp, respectively. The conditions were determined by serial tertiary dilutions and by the number of cycles. Each PCR product (1 μl) was mixed with 0.3 μl of a 500-TAMRA–labeled commercial size standard (Applied Biosystems) and run on an ABI PRISM 377 system (Applied Biosystems). The analysis was performed using GeneScan software (version 3.1; Applied Biosystems). The level of the W1282X transcripts was determined as the peak area of the signal of the W1282X PCR product divided by the peak area of the signal of the ΔF508 PCR product.

For the RNA samples of the other 5 patients and of CFP15a, CFP15b, and CFP22a cell lines, we performed real-time PCR in the LightCycler (software version 3.5) using a FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The *CFTR* mRNA levels were normalized to those of mRNA transcribed from a control gene, *RPS9*. For each pair of primers, a standard curve was derived, and annealing temperature was optimized in order to exclude PCR artifacts. Moreover, we included in the analysis only experiments in which the standard curves were as expected, to ensure accurate quantification. The level of the normalized *CFTR* nonsense mRNA in each patient was compared with the normalized level obtained for a control individual with normal *CFTR* alleles. For CFP15a, CFP15b, and CFP22a cells, the level of the normalized *CFTR* transcripts was compared to the normalized level of untreated cells. Sequences of the primer pairs used were as follows: *CFTR* mRNA: forward, GAGGGTAAAATTAAGCACAGT, reverse, TGCTCGTTGACCTCCA; *RPS9*: forward AGACCCTTCGAGA-AATCTCGTCTCG, reverse TGGGTCCTTCTCATCAAGCGTCAGC.

For the RNA samples of the patients carrying 2 nonsense mutations, we also normalized the *CFTR* level to the level of *KRT18*, a gene expressed specifically at ciliated and secretory epithelial cells. Quantification analyses were performed using 2 methods: real-time PCR and GeneScan. Real-time PCR reactions were performed as with the *RPS9* (see above). For the GeneScan analysis, the *CFTR* and *KRT18* transcripts were amplified in a multiplex PCR. Two different fluorescently labeled forward primers were used: 6-FAM for *CFTR* forward primer and HEX for *KRT18* forward primer. *CFTR* primer pair used here was the same as that used for the reactions with samples from the *W1282X/ΔF508* patients. Sequences of the *KRT18* primer pair were: forward AGTCTGTGGAGAACGACATCC, reverse TGGT-GCTCTCCTCAATCTGC. The PCR products of the *CFTR* transcripts were 257 bp, and those of *KRT18* were 311 bp. Similar transcript levels were obtained using either of these quantification methods.

Analysis of the level of *CFTR* pre-mRNA was performed using the real-time PCR system, using primers in intron 5 of the gene. Two-tailed Student's *t* test was used to analyze the differences in *CFTR* pre-mRNA levels between patients 5 and 10. *P* values less than 0.05 were considered to be significant. The sequences of the primer pair were: *CFTR* pre-mRNA: forward CTCAGCTCTAGCTTCCC, reverse GCTCAGGTATCATATCTGGC. All RT-PCR experiments were repeated at least 3 times, for both patient and cell line samples.

For quantification of physiologic NMD substrates, *RPL3*, *SC35 1.6 kb*, *SC35 1.7 kb*, *ASNS*, and *CARS*, we also performed real-time PCR. The levels of each transcript were normalized to the levels of *GAPDH* transcripts. In the samples from cell lines, the normalized level of the transcripts following NMD inhibition was compared with the normalized level of untreated cells. Sequences of the primer pairs used were as follows: *RPL3*: forward GGCATTGTGGGCTACGTG, reverse CTTCAGGAGCAGAGCAGA; *SC35 1.6 kb*: forward CGGTGTCCTCTTAAGAAAATGATGTA, reverse CTGCTACACAACTGCGCCTTTT; *SC35 1.7 kb*: forward GGCGTGTATT-GGAGCAGATGTA, reverse: the same as for *SC35 1.6 kb*; *ASNS*: forward TCAGGTTGATGATGCAATG, reverse CAGCTGACTTGTAGTGGGTC; *CARS*: forward AAATTAAATGAGACCACGGA, reverse TGACATCACAGC-CAAGTGTA; *GAPDH*: forward TGAGCTTGACAAAGTGGTCG, reverse GGCTCTCCAGAACATCATCC. In order to analyze the splicing pattern

of the W1282X region, we performed RT-PCR reactions with primers in exons 18 and 21: forward GTAAACTCCAGCATAGATGTGG, reverse CCACTGTTCATAGGGATCCAA. The expected PCR product from normal splicing in this region was 492 bp.

Gentamicin treatment and NMD inhibition. Cells were treated with 50–200 µg/ml gentamicin sulphate (Biological Industries) for 18–24 hours. We indirectly inhibited NMD by treating the cells with 200 µg/ml CHX (Sigma-Aldrich) for 5 hours. siRNA oligonucleotides for silencing *UPF1* and *UPF2* expression (Dharmacon) were those described by Mendell et al. (49). siRNA oligonucleotide for luciferase (Dharmacon) was described by Gehring et al. (68). Nonspecific control oligonucleotide (Dharmacon) contained 52% GC content, similar to the GC content in both specific oligonucleotides. 10^5 cells were plated for RNA analysis into 6-well plates and for protein analysis into 100-mm plates, 24 hours before transfection. 10^4 cells were plated for functional analysis into 96-well plates, 72 hours before transfection. Two hundred nanomolar of each RNAi oligonucleotide was transfected using Oligofectamine (Invitrogen). Cells were further grown for 24–48 hours. Gentamicin was added for 18–24 hours before the cells were harvested for RNA or protein analysis or for measurements of *CFTR* function. Experiments were repeated at least 3 times.

Western blot analysis. Total proteins (20–40 µg) were extracted from the different cell lines and were subjected to SDS-PAGE as previously described (68). These proteins were probed with antibodies against *UPF1*, *UPF2*, and β-catenin.

CFTR functional analysis. CFTR function was measured by chloride (Cl⁻) efflux, using *N*-(6-methoxyquinolyl) acetoethyl ester (MQAE), a Cl⁻-sensitive fluorescence indicator (Invitrogen). Cells were loaded overnight with MQAE. The rate of Cl⁻ efflux was measured in response to exchange of extracellular Cl⁻ with nitrate (NO₃⁻), an anion that passes through the *CFTR* but unlike Cl⁻ does not quench the indicator's fluorescence. Activation of the *CFTR* chloride channel was stimulated by the cAMP agonist forskolin. The fluorescence measurements were performed using the FLUOstar Galaxy fluorescence reader (BMG LABTECH). For evaluation of *CFTR* activation, the difference between fluorescence intensity at the reading time (F_t) and that at the beginning of the experiment (F_0) was calculated.

Acknowledgments

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