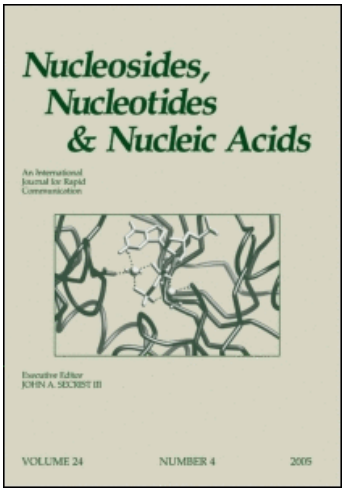

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

TARGETING OF A701G NUCLEOTIDE AT THE HUMAN ATP1A1 LOCUS USING A RNA/DNA CHIMERA

Tiziana Cervelli[a]; Grazia Lombardi[a]; Lorenzo Citti[a]; Alvaro Galli[a]; Maria Teresa Locci[b]; Giuseppe Rainaldi[b]
[a] Laboratorio di Terapia Genica e Molecolare, Istituto di Fisiologia Clinica, Pisa, Italy [b] Dipartimento di Patologia Sperimentale, Universitàdegli Studi di Pisa, Pisa, Italy

Online publication date: 12 February 2002

To cite this Article Cervelli, Tiziana , Lombardi, Grazia , Citti, Lorenzo , Galli, Alvaro , Locci, Maria Teresa and Rainaldi, Giuseppe(2002) 'TARGETING OF A701G NUCLEOTIDE AT THE HUMAN ATP1A1 LOCUS USING A RNA/DNA CHIMERA', Nucleosides, Nucleotides and Nucleic Acids, 21: 11, 775 — 784
To link to this Article: DOI: 10.1081/NCN-120016480
URL: http://dx.doi.org/10.1081/NCN-120016480

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 21, Nos. 11 & 12, pp. 775–784, 2002

TARGETING OF A701G NUCLEOTIDE AT THE HUMAN ATP1A1 LOCUS USING A RNA/DNA CHIMERA

Tiziana Cervelli,[1] Grazia Lombardi,[1] Lorenzo Citti,[1] Alvaro Galli,[1] Maria Teresa Locci,[2] and Giuseppe Rainaldi[1,*]

[1]Laboratorio di Terapia Genica e Molecolare,
Istituto di Fisiologia Clinica, Area della Ricerca del CNR,
via G. Moruzzi, 1, 56127 Pisa, Italy
[2]Dipartimento di Patologia Sperimentale,
Università degli Studi di Pisa, via Roma, 55, 56126 Pisa, Italy

ABSTRACT

The single base substitution mediated by chimeric RNA/DNA oligonucleotide is a new promising approach of gene therapy for single base mutation diseases. We exploited this approach to render HeLa cells resistant to ouabain by introducing a single base substitution in the alpha 1 subunit of the NA^+/K^+ ATPase human gene. The chimeric oligonucleotide was administered to HeLa cells by electroporation and the frequency of ouabain resistant cells determined. The results showed that the chimeric RNA/DNA oligonucleotide failed to enhance the frequency of ouabain resistant cells supporting the controversy about the conflicting results of the technique.

In mammalian cell genetics, introducing a single base change in genomic DNA could be very useful to study gene function. Moreover, targeting single base DNA to correct point mutation is an ideal approach of gene therapy for diseases due to single gene mutation.

*Corresponding author. Fax: +39 050 315-3327; E-mail: g.rainaldi@ifc.cnr.it

DOI: 10.1081/NCN-120016480 1525-7770 (Print); 1532-2335 (Online)

Recently, a strategy for site-directed repair of single base mutation has been presented[1] and extensively demonstrated.[2–4] The model hypothesizes that upon delivery into the cell of a chimeric RNA/DNA oligonucleotide containing a mismatched nucleotide enzimatic activity involved in recombination can align the two strands with the two corresponding strands of the genomic DNA. The mismatch produced by the alignment triggers the mismatch repair enzymes of the cells, which mutate the mismatched nucleotide.[5,6] The approach has been successfully applied in animal,[7–9] plant[10,11] and yeast[12] cells.

We exploited the chimeric RNA/DNA oligonucleotide approach to introduce a specific mutation in the human ATP1A1 locus coding for the α subunit (isoform 1) of the membrane Na^+/K^+ ATPase. This mutation is sufficient to confer the resistance to ouabain (OUA^R),[13] a cardiotonic glycoside that binds to α1 subunit of the enzyme thus inhibiting its activity.[14] Since the mutation is dominant, cells bearing the mutation acquire the ability to form colonies in ouabain-containing medium.[15] As several mutations of the Na^+/K^+ ATPase locus have been previously characterized in the HeLa cell line,[13] we decided to test the ability of a chimeric RNA/DNA oligonucleotide to introduce a specific mutation in these cells. The target region of ATP1A1 locus of HeLa recipient cells was amplified by PCR and RT-PCR. The results are shown in Fig. 1. The products of PCR were of different length, indicating the presence of a splicing site located nearby the target region (Fig. 1A, B). Sequence analysis (not shown) of the HeLa DNA allowed us to design the 68 bp chimeric RNA/DNA oligonucleotide ChiA760G containing 25 bp homologous to the target region (Fig. 1A). To produce the specific mutation, ChiA760G was also designed with the corresponding mismatch (Fig. 2). The gene targeting mediated by a chimeric RNA/DNA oligonucleotide is expected to induce a single base substitution. We used ChiA760G to convert the triplet GAT coding for aspartic acid into the triplet GGT coding for glycine of the alpha 1 subunit of the Na^+/K^+ ATPase human gene. Cells containing the changed aminoacid should become resistant to ouabain and, therefore, able to form OUA^R colonies (Fig. 2). ChiA760G conformation was verified by PAGE technique. A conformomer of 34 bp was the most abundantly present thus demonstrating that ChiA760G was assembled in the expected double strand structure (Fig. 3).

ChiA760G was transfected by electroporation in HeLa cells and the frequency of OUA^R colonies measured. The effects of ChiA760G on the induction of gene correction of alpha 1 subunit of the Na^+/K^+ ATPase gene are reported in Table I. The spontaneous frequency of OUA^R colonies was 8.2×10^{-6} viable cells. Following electroporation without ChiA760G, the frequency of OUA^R colonies was 7.2×10^{-6} viable cells. When ChiA760G was transfected into HeLa cells by electroporation, the frequency of OUA^R colonies was 4.4×10^{-6} viable cells. The ANOVA analysis showed that the

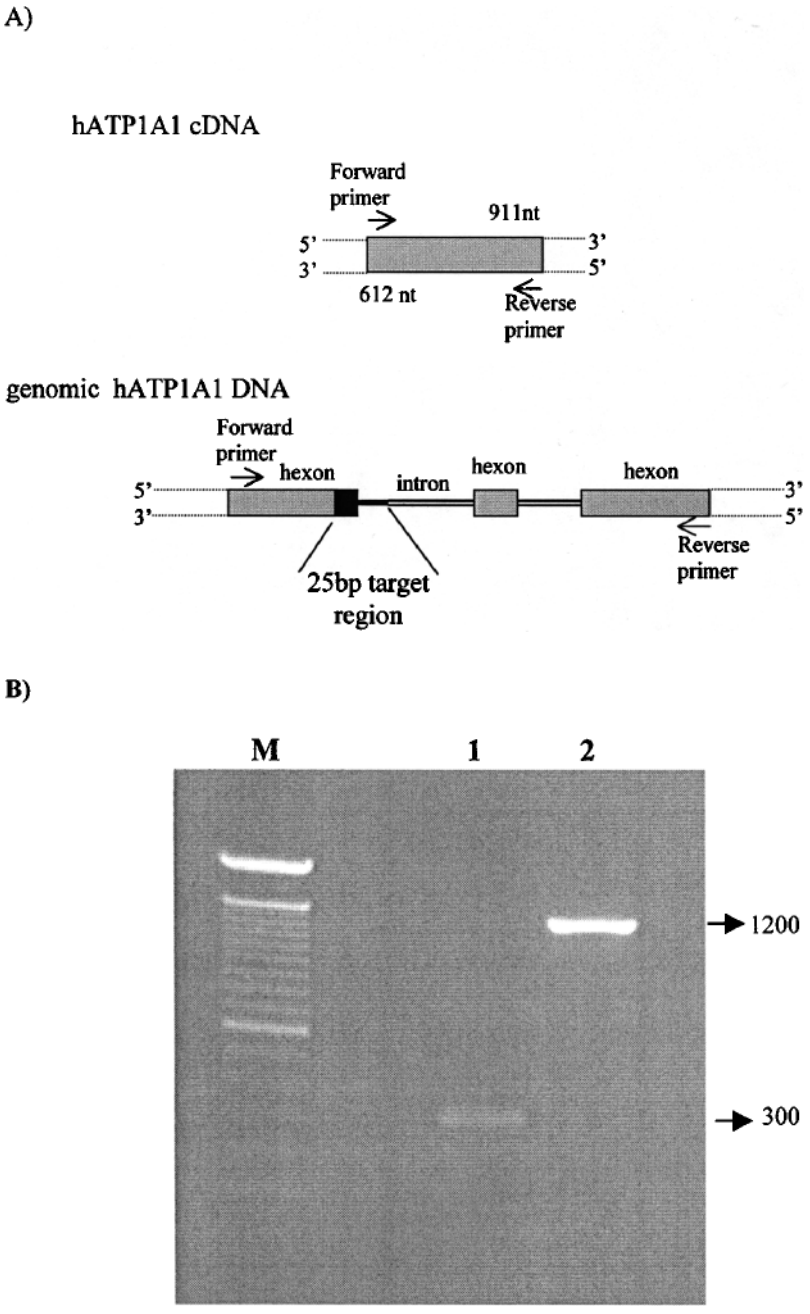

Figure 1. Characterization of the target of ChiA760G: A) structure of the hATP1A1 cDNA, the genomic hATP1A1 locus and the localization of the ChiA760G target (black box). PCR amplification was carried out using the pair of primers shown by the arrows. Genomic DNA or total RNA was extracted from HeLa cells. PCR from cDNA gave a 300 bp fragment (B, lane 1) from genomic DNA a 1,200 bp (B, lane 2). M: marker.

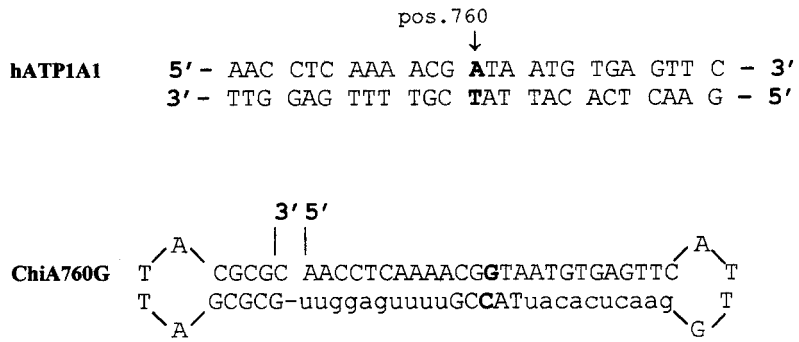

Figure 2. Schematic representation of ChiA760G. It consists of 68 nt. The upper strand of 25 nt was complementary to the target genome DNA (except for a single mismatch A → G) and was flanked by DNA residues that from poly-T hairpins at both ends and a complementary second strand. Bold letters indicates the mismatched nucleotides; small letters indicate the ribonucleotide residues; capital letters indicate DNA sequence.

frequencies were not significantly different indicating that ChiA760G failed the induction of A701G mutation in the triplet GAT.

To rule out the hypothesis that a low transfection efficiency of ChiA760G could be responsible for the lack of OUA^R induction, ChiA760G was cotransfected with a 20 bp long fluorescent single stranded oligonucleotide (FITC-ODN) and 24 h later the localization of FITC-ODN was detected by confocal laser microscopy.

The results are shown in Fig. 4. Almost 100% cells transfected with ChiA760G were fluorescent and showed high concentration of FITC-ODN inside the nucleus (Fig. 4, A). In absence of transfection, the fluorescence was distributed throughout the cytosol without specific accumulation (Fig. 4, B). Therefore, we can reasonably conclude that ChiA760G entered the cells and reached the nucleus.

The expression time we used to select for OUA resistance is routinely used in cell culture experiments.[15] Moreover, as another point mutation has been efficiently and specifically corrected by a chimeric oligonucleotide using HeLa as recipent cells,[16] we can reasonably exclude dysfunctions of recombination and/or repair processes of these cells. Therefore, the failure of OUA^R induction can be attributed to the inaccessibility of the Na^+/K^+ ATPase target locus to ChiA760G. The hypothesis that the correction efficiency may be affected by chromosomal localization of the target and, consequently, by the accessibility remains to be investigated by appropriate experiments.

The inducibility of gene correction by RNA/DNA oligonucleotides in mammalian cells is a controversial matter since the high variability of the gene correction frequencies. Gene correction is routinely assessed by PCR-RFLP analysis rather than clonal analysis. Most positive results are based

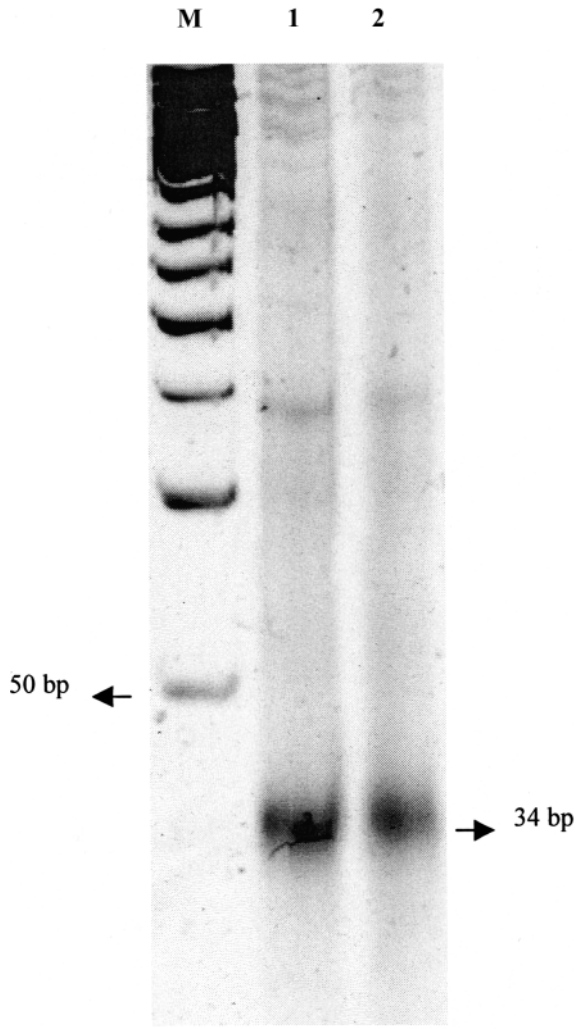

Figure 3. PAGE-migration profile of ChiA760G no submitted (lane 1) and submitted to denaturation-reannealing cycle (lane 2). M: marker.

on PCR amplification and sequencing of PCR product shortly after transfection.[1,4] False positive results can be obtained[17,18] in such way. The clonal selection is more stringent in that respect. In fact, when clonal selection was used as mutational read out, the gene correction was indeed a low frequency event.[10,11] Nevertheless, other loci have been reported to be unsensitive to the chimeric RNA/DNA oligonucleotide approach[20,21] and we do not know the causes of that. While the critical steps of the gene correction have been identified,[6,12,22,23] the molecular mechanisms at the basis of the chimera/DNA complex formation as well as of the recruitment of the proteins that should process the complex not yet.

Table I. Frequency of OUA^R Colonies in HeLa Cells Transfected by Electroporation with ChiA760G

Treatment	Viable Seeded Cells	OUA^R Colonies (n)	Frequency of OUA^R
No electroporation			
	2.7×10^6	26	9.5×10^{-6}
	2.7×10^6	25	9.1×10^{-6}
	3.6×10^6	22	6.0×10^{-6}
Electroporation			
	2.3×10^6	22	9.6×10^{-6}
	3.4×10^6	26	7.6×10^{-6}
	2.0×10^6	9	4.5×10^{-6}
Electroporation+ ChiA760G			
	4.0×10^6	17	4.2×10^{-6}
	8.9×10^6	45	5.1×10^{-6}
	1.9×10^6	6	3.8×10^{-6}

In conclusion, the gene correction by chimeric oligonucleotides is still an open aspect of gene targeting since its functioning is too much sophisticated to represent a real alternative to gene replacement approach[24] that is more flexible and simple to realize.

MATERIALS AND METHODS

A701G Mutation

The chimera approach was used to introduce the A701G mutation in the human α_1 subunit of the Na^+/K^+ ATPase. This mutation, corresponding to A631G mutation of Na^+/K^+ ATPase sheep cDNA, causes the change of the aspartate 121 to glycine and, has been reported to induce OUA^R in human cells.[13]

As only one mismatch should be present in the pairing region between DNA and chimeric oligonucleotide, the forward 5′-GGGGTTCT-CAATGTTACTGT-3′ and reverse 5′-CGGTCTCCTCCTTTTACTTC-3′ primers (spanning from nucleotide 612 to 911 of human α_1 subunit of the Na^+/K^+ ATPase cDNA) were used to amplify DNA and cDNA of HeLa cells. Genomic DNA was extracted according to the procedure reported in Miller et al.[26] whereas total RNA was isolated and purified with the ''SV and RNA isolation'' kit (Promega). RNA was retrotranscribed to cDNA by using the ''Enhanced avian RT-PCR'' kit (Sigma Chemical CO, St. Louis MS, USA). The PCR conditions were: 94°C/1 min, 58°C/1 min, 72°C/1 min for 31 cycles. PCR products were analysed by agarose gel electrophoresis and purified by using a commercial purification kit.

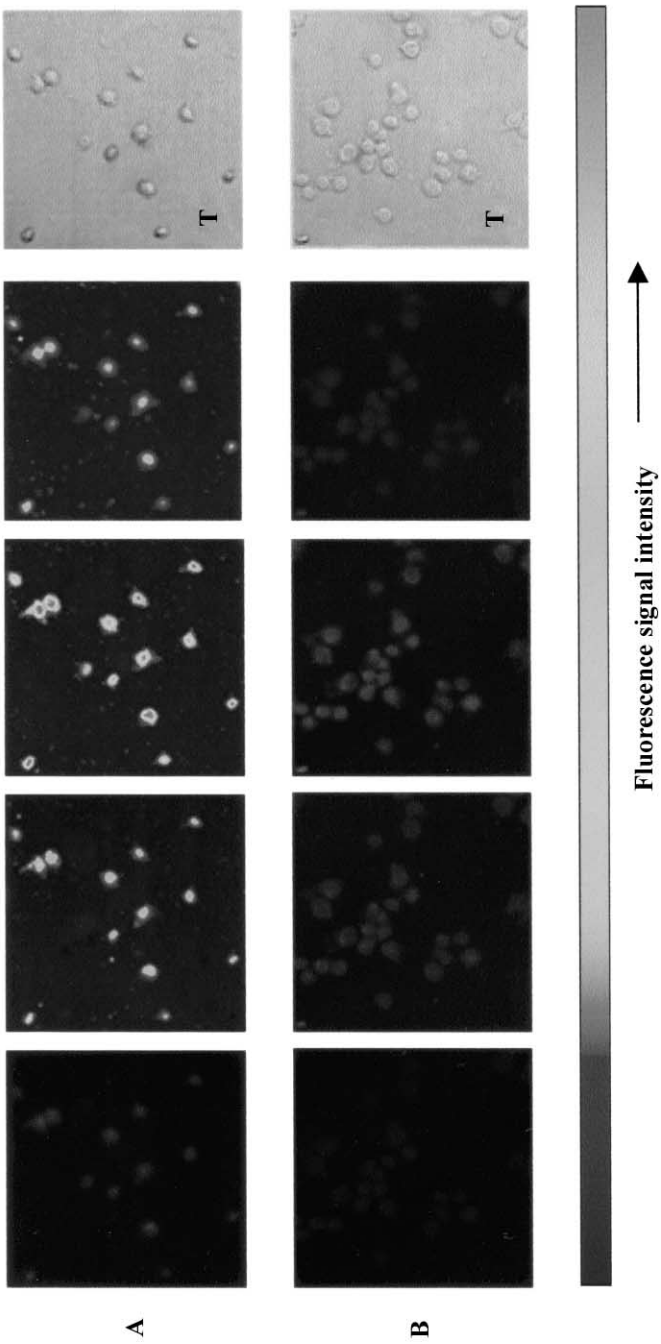

Figure 4. Localization of FITC-ODN 24 h after the electroporation in HeLa cells. The subcellular localization of FITC-ODN was determined by direct fluorescence. The fluorescence images (left to right) are obtained at increasing depths in 2-steps of 2.5 µm. T: total images of cell topographies; A: transfected HeLa cells, B: untransfected HeLa cells.

Synthesis

ChiA760G was synthesized according to the standard phosphoroamidite chemistry using the automatic synthesizer Expedite 8909 (PerSeptive Biosystem, Framingham, MA, USA). Thereafter, ChiA760G was purified by ion exchange preparative liquid chromatography, desalted and stocked at $-20°C$. Since ChiA760G was designed to assume a double strand structure, the acquisition of this conformation was tested. ChiA760G was dissolved in bidistilled water, denaturated for 10 min in boiling water and let to reanneal up to the temperature of water reached 37°C. Samples were loaded on 15% acrilamide:bis-acrilamide (29:1) gel and run at 145 V for 2 h and at 250 V for other 2 h. Afterwhich, the gel was stained with ethidium bromide and bands visualized under UV light.

Cell Line and Culture Conditions

As HeLa cells have been successfully corrected by a chimeric RNA/DNA oligonucleotide,[16] they were used as recipient of ChiA760G. Cells were cultured in Dulbecco's medium (DMEM) supplemented with 10% fetal calf serum, 10 UI/mL penicillin and 100 μg/mL streptomycin under humidified conditions in a 6% CO_2 atmosphere.

Electroporation

250 μL of DMEM without serum and antibiotics and containing 6 μg ChiA760G were used to resuspend a pellet of 3.5×10^6 cells. The suspension was transferred to 50×4 mm cuvette (Equibio) and incubated on ice for 10 min. Afterwhich the cuvette was exposed to one pulse (330 V, 1000 μF, 200 Ω) using the Electroporator II apparatus (Invitrogen) connected to a power supply (330 V, 25 mA, 25 W). The cells suspension was then cooled for 15 min in ice. Afterwards, cells were grown for 48 h to allow the occurrence of gene correction.

Selection of OUA^R Colonies

Thereafter, cells were trypsinized and seeded in 100 mm diameter dishes at cell density of 5×10^5. 2 h after seeding, 0.1 μM OUA was added to dishes.[15] OUA^R colonies, which normally appeared after 10–14 days, were stained for 90 min in 6 mL cristal violet solution (91% distilled water, 4% formaldehyde, 5% cristal violet) and counted. The frequency was calculated by dividing the total number of OUA^R colonies by the number of total viable cells. Cell viability was determined by measuring the colony forming ability.

Laser Confocal Microscopy

Following electroporation, 5×10^4 cells were seeded on a glass coverslip and incubated in complete medium for 24 h at 37°C under humidified conditions in a 6% CO_2 atmosphere. Thereafter, cells were fixed with 4% paraformaldeyde for 10 min. Slides were mounted with an antifade reagent (Vector) and observed with a fluorescent microscope connected to a Radiance plus Confocal Scanning system (Bio-Rad) equipped with Ar/He laser diodes. Fluorescent signals were detected using 488/568 emission wavelengths. Digitized images were processed with the Photoshop program (Adobe system, San Josè, LA, USA).

REFERENCES

1. Cole-Strauss, A.; Yoon, K.; Xiang, Y.; Byrne, B.C.; Rice, M.C.; Gryn, J.; Holloman, W.K.; Kmiec, E.B. Science **1996**, *273*, 1386–1389.
2. Xiang, Y.; Cole-Strauss, A.; Yoon, K.; Gryn, J.; Kmiec, E.B.J. Mol. Med. **1997**, *75*, 829–835.
3. Kren, B.T.; Cole-Strauss, A.; Kmiec, E.B.; Steer, C.J. Hepatology **1997**, *25*, 1462–1468.
4. Kren, B.T.; Bandyopadhyay, P.; Steer, C.J. Nature Med. **1998**, *4*, 285–290.
5. Kmiec, E.B. Gene Therapy **1999**, *6*, 1–3.
6. Cole-Strauss, A.; Gamper, H.; Hollomann, W.K.; Muñoz, M.; Cheng, N.; Kmiec, E.B. Nucleic Acids Res. **1999**, *27*, 1323–1330.
7. Kren, B.T.; Parashar, B.; Bandyopadhyay, P.; Chowdhury, N.R.; Chowdhury, J.R.; Steer, C.J. Proc. Natl. Acad. Sci., USA **1999**, *96*, 10,349–10,354.
8. Rando, T.A.; Disatnik, M.H.; Zhou, L. Proc. Natl. Acad. Sci., USA **2000**, *97*, 5363–5368.
9. Alexeev, V.; Igoucheva, O.; Domashenko, A.; Cotsarelis, G.; Yoon, K. Nature Biotech. **2000**, *18*, 43–47.
10. Zhu, T.; Peterson, D.J.; Tagliani, L.; St. Clair, G.; Baszczynski, C.L.; Bowen, B. Proc. Natl. Acad. Sci., USA **1999**, *96*, 8768–8773.
11. Beetham, P.R.; Kipp, P.B.; Sawycky, X.L.; Arntzen, C.J.; May, G.D. Proc. Natl. Acad. Sci., USA **1999**, *96*, 8774–8778.
12. Rice, M.C.; Bruner, M.; Czymmek, K.; Kmiec, E.B. Mol. Microb. **2001**, *40*, 857–868.
13. Croyle, M.L.; Woo, A.L.; Lingrel, J.B. Eur. J. Biochem. **1997**, *248*, 488–495.
14. Lingrel, J.B.; Kuntzweiler, T. J. Biol. Chem. **1994**, *269*, 19,659–19,662.
15. Rainaldi, G.; Colella, C.; Piras, A.; Mariani, T. Biol. Chem. Interact. **1982**, *42*, 45–51.
16. Santana, E.; Peritz, A.E.; Iyer, S.; Uitto, J.; Yoon, K.J. Invest. Dermatol. **1998**, *111*, 1172–1177.
17. Stasiak, A.; West, S.C.; Egelman, E.H. Science **1997**, *277*, 460–462.
18. Thomas, K.R.; Capecchi, M.R. Science **1997**, *275*, 1404–1405.
19. Alexeev, V.; Yoon, K. Nature Biotech. **1998**, *16*, 1343–1346.

20. Zhang, Z.; Eriksonn, M.; Falk, G.; Graff, C.; Presnell, S.C.; Read, M.S.; Nichols, T.C.; Blombäck, M.; Anvret, M. Antisense Nucl. Acid Drug Dev. **1998**, *8*, 531–536.
21. Brown, B.D.; Notley, C.R.; Hough, C.A.; Lillicrap, D. Molecular Therapy **2000**, *1*, part 2.
22. Gamper, H.B.; Cole-Strauss, A.; Metz, R.; Parekh, H.; Kumar, R.; Kmiec, E.B. Biochemistry **2000**, *39*, 5808–5816.
23. Gamper, H.B.; Parekh, H.; Rice, M.; Bruner, M.; Youkey, H.; Kmiec, E.B. Nucleic Acid Research **2000**, *28*, 4332–4339.
24. Yáñez, R.J.; Porter, A.C.G. Gene Therapy **1998**, *5*, 149–159.
25. Price, E.M.; Rice, D.A.; Lingrel, J.B. J. Biol. Chem. **1989**, *264*, 21,902–21,906.
26. Miller, S.A.; Dykes, D.D.; Polesky, H.F. Nucleic Acids Res. **1988**, *16*, 1215.

Received October 26, 2001
Accepted August 5, 2002