apoptotic bodies and the secreted bacteriotoxic substances may impede intra- and extracellular bacteria. This would explain the pivotal role observed for the CD95/CD95 ligand system in host defense against infection with *P. aeruginosa*.

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15. Lungs were fixed for 2 days and embedded in paraffin; 6-μm sections of lung were cut, removed from paraffin, and digested with proteinase K for 2 min, and endogenous peroxidase was blocked with H_2O_2. Sections were stained with terminal deoxynucleotidyl transferase in the presence of biotinylated deoxyuridine triphosphate and developed with the ABC complex and 3-amino-9-ethylcarbazole. Counterstaining was done in hematoxylin.
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Specific Mutations Induced by Triplex-Forming Oligonucleotides in Mice

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Triplex-forming oligonucleotides (TFOs) recognize and bind to specific duplex DNA sequences and have been used extensively to modify gene function in cells. Although germ line mutations can be incorporated by means of embryonic stem cell technology, little progress has been made toward introducing mutations in somatic cells of living organisms. Here we demonstrate that TFOs can induce mutations at specific genomic sites in somatic cells of adult mice. Mutation detection was facilitated by the use of transgenic mice bearing chromosomal copies of the *supF* and *cII* reporter genes. Mice treated with a *supF*-targeted TFO displayed about fivefold greater mutation frequencies in the *supF* gene compared with mice treated with a scrambled sequence control oligomer. No mutagenesis was detected in the control gene (*cII*) with either oligonucleotide. These results demonstrate that site-specific, TFO-directed genome modification can be accomplished in intact animals.

By inducing site-specific mutations in the genome, heritable changes can be achieved in gene function and expression. Triplex formation, through recognition of a specific region of duplex DNA by a single-stranded TFO, offers an attractive strategy to modify a mammalian genome. TFOs have so far been used successfully to modify genes and gene function in cells in vitro (*1*). With a view toward a therapeutic application, we have investigated the potential of a triplex approach to target specific mutations in somatic cells of mice in vivo. Transgenic mice (C57BL/6 mice containing multiple copies of a chromosomally integrated λsupFG1 vector, designated 3340) were previously generated (*2*) containing a 30–base pair (bp) triplex-target site within the *supFG1* mutation–reporter gene. We previously demonstrated TFO-targeted mutations in the *supFG1* gene in vitro on intracellular plasmid targets and on a chromosomal locus in a fibroblast cell line established from these mice (*3*–*5*). The results revealed 10- to 100-fold induction of site-specific mutations in cells treated with the specific TFO. Although psoralen conjugation to the TFO, coupled with UVA (ultraviolet, long wave) irradiation, generally increased the frequency of mutations, targeted mutagenesis was seen even without psoralen photoproduct generation, suggesting a substantial triplex-mediated process of mutagenesis. On the basis of these results, the oligonucleotides in this work were not conjugated to psoralen, and no other DNA-damaging agent (or mutagen) was required to induce mutagenesis. This affords an advantage

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in that UVA activation is not required, and there is no apparent toxicity to the animals.

Mice were given daily intraperitoneal (i.p.) injections with 1 mg day^{-1} of either AG30 or SCR30 for five consecutive days. AG30 is the specific TFO designed to bind to the polypurine site in the *supFG1* gene, whereas SCR30 is a control oligonucleotide with the same base composition as AG30 but with a scrambled sequence that differs at 12 positions (*6*). Using gel mobility shift assays and deoxyribonuclease I (DNase I) footprinting, we found that AG30 binds with high affinity (equilibrium dissociation constant $\sim 10^{-9}$ M) and specificity to the target site, whereas SCR30, having mismatches in the triplex binding code, does not. Ten days after injection, mouse tissues were collected for mutation analysis (*7*). The combined mutation frequencies in a variety of tissues from AG30-treated mice were increased by about fivefold compared with tissues from SCR30-treated mice (Table 1). The mean mutation frequencies in liver, skin, kidney, colon, small intestine, and lung from AG30-treated mice were significantly higher than those from SCR30-treated mice (*P* values calculated by the Student's *t* test are listed in Table 1). These data demonstrate an oligonucleotide-specific induction of mutagenesis in mice. Additionally, these data indicate efficient tissue uptake and distribution of oligonucleotides in mice after i.p. injections.

In a previous study, the tissue uptake and distribution after i.p. administration of G-rich, propylamine end-capped, phosphodiester oligonucleotides (similar to AG30 and SCR30) was examined (*8*). This study demonstrated substantial uptake in a number of tissues except for the brain. Because oligonucleotides do not efficiently cross the blood-brain barrier after i.p. injection, mutagenesis in brain tissue was measured as an internal control. Consistent with this, mutagenesis was not induced above background levels in brain tissues analyzed from mice treated with either AG30 or SCR30 (Fig. 1A). In contrast, all other tissues tested from AG30-treated animals showed an average fivefold increased induction in mutagenesis compared with tissues from SCR30-treated animals (Fig. 1A).

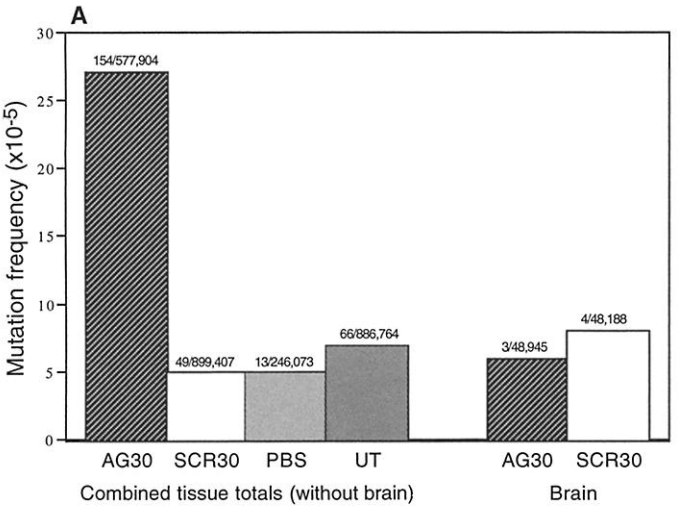
The levels of mutagenesis obtained from mice injected with SCR30 were similar to those observed in mice injected with phosphate-buffered saline (PBS) only (Fig. 1A), showing that SCR30, even at a dose of 1 mg day^{-1} for 5 days, does not have any detectable mutagenic effect in the animals. The values for both the SCR30- and PBS-injected animals, furthermore, were essentially the same as those observed in untreated animals in the same 3340 lineage (*9*) (Fig. 1A). These results not only provide additional evidence that AG30-mediated mutation induction is occurring through a sequence-specific, triplex-mediated mechanism, but also indicate that nonspecific oligonucleotides are not generally mutagenic in animals.

To further confirm that the induced mutagenesis obtained from AG30 treatment resulted from a triple-helix–dependent event and not from some hypothetical, nonspecific effect of AG30 on DNA metabolism, we tested the effect of AG30 and SCR30 in the Mutamouse mutagenesis model (*10*). We chose to use the lambda *cII* gene as a mutation reporter, because this locus has been well studied (*11*) and does not contain the AG30 triplex target site. The *cII* control gene showed no induction of mutagenesis in animals treated with either AG30 or SCR30 compared with background levels (Table 2). These results rule out any nonspecific mutagenic effect of AG30 and are consistent with a gene-specific, triplex-mediated effect of AG30 in inducing an increased level of mutagenesis in the *supFG1* gene of 3340 mice.

Mutant plaques were isolated from a va-

Table 1. Targeted mutagenesis of the *supFG1* gene in 3340 mice by TFOs. Mice were injected i.p. with 1 mg of TFO per day for 5 days ($\sim$50 mg kg^{-1} day^{-1}). AG30 is the specific TFO, SCR30 is the control TFO. Values of mutation frequency for skin, liver, and kidney are reported as the mean ± SD. For colon, small intestine, and lung, values are reported as the mean ± the difference between the two values. *P* values were calculated by the Student's *t* test.

TFO	Tissue	No. of mice	Mutants/total plaques	Mutation frequency ($\times 10^{-5}$)	*P* value
AG30	Skin	5	32/144,768	21 ± 10	
SCR30	Skin	5	8/213,944	4 ± 1	≤0.006
AG30	Liver	5	38/170,685	22 ± 7	
SCR30	Liver	5	13/296,783	4 ± 2	≤0.0004
AG30	Kidney	5	34/110,937	27 ± 13	
SCR30	Kidney	5	12/185,818	6 ± 2	≤0.007
AG30	Colon	2	29/76,930	38 ± 4	
SCR30	Colon	2	8/73,275	11 ± 0	
AG30	Intestine	2	8/18,214	46 ± 13	
SCR30	Intestine	2	4/46,397	9 ± 5	
AG30	Lung	2	13/56,370	23 ± 4	
SCR30	Lung	2	4/83,190	5 ± 1	



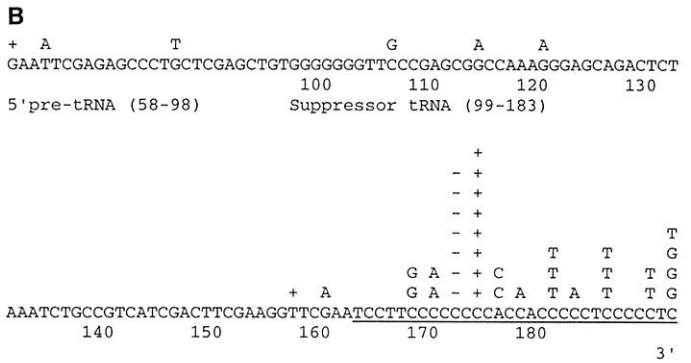


Fig. 1. (**A**) Targeted mutagenesis of the *supFG1* gene in somatic tissues of 3340 mice. The combined totals from all tissues tested, except brain, are displayed in the first set, with brain only in the second set. The number of mutants/total plaques is listed above each bar. UT, untreated mice. (**B**) Mutations in the *supFG1* gene in tissues from mice treated with AG30. The 30-bp triplex target site is underlined. The *supFG1* gene sequences were determined as described (*5*). Base changes are indicated above the sequence. (+) and (−) represent single-base insertions or deletions.

riety of tissues from AG30-treated animals, and the *supFG1* genes were analyzed by DNA sequencing. The sequences of *supFG1* mutations from a number of AG30-treated mouse tissues are listed in Fig. 1B. The results are consistent with a TFO-directed effect with the majority (85%) of the mutations concentrated within the 30-bp triplex target site. Nearly 40% of the mutations are single-base insertions or deletions in a stretch of eight contiguous Gs. It is possible that these mutations resulted from slippage errors during TFO-induced repair, because this site has been shown previously to be prone to slippage events (*2*, *5*).

Indeed, the genome sites conducive to high-affinity triple-helix formation (consisting of polypurine sequences with segments of mononucleotide repeats) are precisely the kinds of sequences that are hot spots for insertion and deletion mutagenesis in a template dislocation and slippage mechanism, as proposed by Streisinger (*12*). As such, the 30-bp polypurine triplex target site in *supFG1* is an "at-risk motif" (*13*) that is a hot spot for both spontaneous and induced mutagenesis. Even in control animals, the 30-bp, G-rich polypurine site is a hot spot for mutations, with about 50% of spontaneous mutations occurring in that site, and even more in the absence of DNA mismatch repair (*2*). However, specific TFO treatment increases the proportion of mutations at this site and also increases the absolute mutation frequency at this site more than fivefold, in a highly sequence-specific manner. Hence, the hypermutability of G-rich (or A-rich) sites is a property that actually enhances the utility of TFOs that preferentially bind to such sites in efforts directed at somatic gene knockout.

In a previous study of directed mutagenesis by psoralen-conjugated TFOs within the *hprt* gene in Chinese hamster ovary cells (*14*), mostly large deletions rather than single-base insertions or deletions were seen. However, in that study, the polypurine target site was situated within an intron, and so the type of slippage mutations we observed would have had no effect on the coding region and so would not have been detectable. In another study where psoralen-conjugated TFOs were used to target mutations in yeast, only single-base substitution mutations within a specific ochre codon were seen, rather than slippage events (*15*). However, the yeast study made use of an assay requiring specific reversion of the ochre stop codon, instead of a forward mutation assay, and so neither slippage mutations nor base substitutions elsewhere in the target site would have been observable.

We have investigated the potential of a triplex-based strategy to target mutations to a specific gene in the somatic tissues of mice. The results demonstrate substantial induction of mutagenesis in a variety of tissues with a TFO (AG3O) designed to bind to the chromosomal triplex target site in transgenic animals. We detected no induction of mutagenesis with either AG30 or SCR30 in the *supFG1* gene in brain tissue, or in the *cII* control gene lacking the AG30 triplex target site. Additionally, no induced mutagenesis was detected in the target gene in any of the tissues tested with the control oligonucleotide, SCR30. These findings demonstrate the successful application of a site-specific, DNA-binding reagent to specifically induce genome modifications on a chromosomal target in intact animals.

Although the mechanism of the triplex-directed mutagenesis is not fully established, evidence suggests that the triplex structure can invoke the nucleotide excision repair machinery, either directly or by transcription-coupled repair (*16*). This possibility is consistent with results of previous studies that demonstrate a lack of triplex-induced mutagenesis or recombination in nucleotide excision repair–deficient cells (*4*, *17*). The single-base insertions and deletions that were detected in the triplex target site in mice may therefore be a result of template misalignment in association with TFO-induced repair of this "at risk" site (*13*).

The ability to induce mutations in living animals by means of TFOs affords a powerful strategy to modify the genome. In addition, recent developments in oligonucleotide technology have advanced the potential of gene medicines for other approaches, such as specifically binding to RNA [antisense and ribozymes (*18*, *19*)], DNA [antigene TFOs, peptide nucleic acids, polyamides, and RNA/DNA chimeras (*1*, *20*–*22*)], and protein [aptamer oligomers (*23*)] targets. Our data showing a lack of mutagenesis by the scrambled sequence control (SCR30) are promising for these other nucleic acid–based strategies because nonspecific oligonucleotides do not appear to be mutagenic.

In addition, for gene-targeting technologies to provide a therapeutic benefit, oligonucleotides must be shown to be capable of binding specifically to chromosomal targets in intact animals. The in vivo distribution of oligonucleotides has been studied with a variety of modified oligonucleotides in mice (*8*, *24*, *25*). These studies established that small DNA molecules can be administered by i.p. or intravenous injections and gain access to tissues (outside the central nervous system) and to cell nuclei. The work presented here extends these studies by demonstrating that chromosomal DNA throughout the somatic tissues of an animal can be targeted by nucleic acids. This finding has important implications for the ability to use small molecules to modify gene structure and function in animals.

Although the overall efficiency of mutagenesis demonstrated with AG30 is low, our data provide the initial evidence that sequence-specific, DNA-modifying reagents may prove useful in intact animals. These observations should provide motivation to further enhance triplex technology to improve the efficiency of gene targeting, thereby allowing the targeted modification of genes in whole animals.

Table 2. Lack of TFO-induced mutagenesis of the *cII* gene in Mutamice. Mice were injected i.p. with 1 mg of oligonucleotide (either AG30 or SCR30) per day for 5 days (~50 mg kg^{-1} day^{-1}) or with PBS.

TFO	Tissue	No. of mice	Mutants/total plaques	Mutation frequency ($\times 10^{-5}$)
AG30	Skin	2	14/337,500	4
SCR30	Skin	2	19/344,500	6
None (PBS)	Skin	2	16/271,000	6

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7. Transgenic mice (3340) were generated with about 15 copies of the λsupFG1 shuttle vector, containing the *supFG1* tRNA suppressor gene, integrated on chromosome 7 (*2*). Mice (8 weeks of age) were injected (i.p.) with oligonucleotides for 5 days, and 10 days after the last injection, mice were killed and tissues collected. Genomic DNA was isolated from mouse tissues, and λ packaging extracts were used to excise and package the vector DNA into viable phage particles for analysis in a lacZ(am) strain of *Escherichia coli* to detect mutations that occurred in the mouse (*2*). In the presence of a wild-type *supFG1* gene, the amber mutation in the β-galactosidase gene is suppressed and plaques are blue in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal, whereas the mutant plaques are white.
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Learning-Induced LTP in Neocortex

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The hypothesis that learning occurs through long-term potentiation (LTP)– and long-term depression (LTD)–like mechanisms is widely held but unproven. This hypothesis makes three assumptions: Synapses are modifiable, they modify with learning, and they strengthen through an LTP-like mechanism. We previously established the ability for synaptic modification and a synaptic strengthening with motor skill learning in horizontal connections of the rat motor cortex (MI). Here we investigated whether learning strengthened these connections through LTP. We demonstrated that synapses in the trained MI were near the ceiling of their modification range, compared with the untrained MI, but the range of synaptic modification was not affected by learning. In the trained MI, LTP was markedly reduced and LTD was enhanced. These results are consistent with the use of LTP to strengthen synapses during learning.

Most cortical excitatory synaptic connections appear to be capable of persistent bidirectional modification. The ability for LTP or LTD to modify individual synapses has made LTP or LTD the most widely held candidate mechanism for learning. Experimental evidence supports this view but has not demonstrated that synaptic modifications that occur during learning use LTP or LTD (*1*). Saturation of synaptic efficacy before learning, using LTP-inducing stimuli in vivo, interferes with hippocampally mediated spatial learning (*2*–*5*). This result suggests that saturating synapses impairs learning but does not demonstrate that this same modification mechanism is used when natural learning occurs. In addition, it has been shown that synapses likely to be activated during learning change their efficacy after learning occurs. In the amygdala (*6*, *7*) and the motor cortex (*8*), candidate pathways capable of LTP are stronger after learning, but it has not been tested explicitly whether LTP strengthened these pathways.

We have previously demonstrated that layer II/III horizontal connections in rat primary motor cortex (MI) are capable of LTP and LTD (*9*, *10*) and are strengthened with forelimb motor skill learning (*8*). Strengthening is present in MI opposite to the trained forelimb ("trained MI") but not in the hindlimb area of MI or in the same rats' ipsilateral "untrained MI" (*8*). If LTP was used to achieve synaptic enhancement during learning and this process is saturable, then subsequent attempts at electrically induced LTP after learning should produce less LTP. Critical measures necessary to test this prediction are the identification of baseline synaptic strength, measured as field potential amplitude (*11*), and the upper and lower limits of modification, termed the "synaptic modification range," in trained and untrained pathways.

We defined the synaptic modification range using repeated LTP or LTD induction until saturation was reached. We used this synaptic modification range model to test whether LTP is a mechanism engaged in learning and to examine whether learning affects this range. Theoretically, the range could remain unchanged, shift, or expand as a result of learning. We know that learning increases the strength of horizontal connections because there is an absolute change in field potential amplitude in these pathways after learning (*8*). An unchanged synaptic modification range predicts less LTP but more LTD in trained pathways compared with controls. Thus, synapses closer to the ceiling of their modification range cannot express much additional LTP, and this would be consistent with the use of LTP in learning.

Rats were trained for five successive days to reach with their preferred forelimb into a box and retrieve small food pellets (*12*). Grasp attempts began during the first session, and success rate improved during the first three training days, when it became asymptotic (Fig. 1A). After training, field potentials evoked across layer II/III horizontal connections in the MI forelimb region were recorded simultaneously from both hemispheres (*13*) in slice preparations (*14*). Field potential amplitudes were 1.59 ± 0.10 times larger (N = 32) in the trained MI compared with the control, untrained MI forelimb region. There was no interhemisphere difference in paired control animals and in the hindlimb MI of trained animals (Fig. 1B) (*15*). Stimulation intensities producing half maximal response amplitudes were not significantly different in the trained and untrained MI (22.0 ± 1.24 μA and 21.1 ± 1.22 μA, respectively; N = 21; P = 0.44), indicating that the differences in field potential amplitude could not be explained by the use of different stimulating intensities.

After 5 days of training, repeated theta burst stimulation (TBS) (*16*) produced less LTP in the trained MI than in the opposite, untrained MI. In a striking example shown in Fig. 2A (top), no LTP could be produced in the trained MI horizontal connections despite repeated induction attempts. Simultaneous recordings in the untrained MI of the same slice resulted in normal amounts of LTP, with complete saturation at 163% of baseline (Fig. 2A, bottom). We examined whether these apparently saturated synapses in the trained MI retained the capability to undergo potentiation by first bringing them to lower strength using low-frequency stimulation (LFS) (*17*) (66% of baseline, Fig. 2A, top). Subsequent TBS potentiated these synapses, demonstrating their capacity for LTP (124% of renormalized baseline). These data suggest that the large field potential amplitude that appears in MI horizontal connections after learning reflects a population of strengthened synapses that retains the mechanism for LTP.

Both the amount of LTP and the number of attempts to reach saturation were lower in the trained MI. Considered as a group, LTP in animals trained for 5 days was saturated at 114.5 ± 3.6% of baseline in the trained MI. By comparison, the untrained MI was saturated at 152.1 ± 9.9% of baseline (N = 11, P < 0.001), comparable to levels seen in control rats (Fig. 2B). In 3 of these 11 cases, no LTP could be induced in the trained MI (Fig. 2A, top). In these cases, LTP was nev-

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