Gene Therapy

Chimeraplasty

Many inherited diseases that are potential candidates for gene therapy are the result of single base pair mutations. The ideal gene therapy would correct these genetic defects by site-specific repair. During the past five years, a new strategy has emerged for making such corrections, based on a series of DNA repair experiments using hybrid DNA-RNA oligonucleotides called chimeraplasts. These are structured as double-stranded hairpins in which one strand contains two blocks of RNA residues flanking a stretch of DNA and the second strand is made up entirely of complementary DNA.

Compared with other structures, a chimeraplast shows increased stability, better resistance to nucleases, and improved localization to and stable complex formation with genomic target sites. The mechanism of conversion has been studied in mammalian cell-free extracts and in bacterial systems, and it is believed to be a mismatch repair process, distinct from homologous recombination.

A chimeraplast is designed to be complementary to a target sequence except for a single nucleotide mismatch. Its binding to genomic DNA is apparently enhanced by the stretches of RNA, while the all-DNA strand of the chimeraplast activates endogenous repair mechanisms that alter the sequence of the targeted gene. A four-stranded intermediate configuration or double D loop structure ensures that both strands of the target DNA are converted. A major advantage of targeted nucleotide conversion by chimeraplasty is that regulation of gene expression remains under the control of the endogenous promoter at its native site.

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Development of chimeraplasty in the early 1990's showed that transcriptional activity is not required, nor does it appear to significantly influence the rate of conversion. Most mutated genes appear to be amenable to correction, and many genes have been succesfully targeted in mammalian, bacterial, and plant cells. There is no evidence of untargeted genomic changes or any increase in recombination frequencies.

The technique of chimeraplasty can either replace or exchange, insert or delete, DNA bases using the same heteroduplex hairpin structure. The process is independent of cell replication, although large numbers of chimeraplasts must be delivered to the nucleus. Interestingly, under conditions in which RNA-DNA hybrids are active in promoting nucleotide exchange, corresponding all-DNA duplexes are essentially inactive despite significant uptake by the nucleus.

The potential impact of the technology was thoroughly realized in a 1996 paper from the laboratory of Eric B. Kmiec at Thomas Jefferson University, which reported chimeraplast-mediated correction of the sickle cell mutation in the human β-globin gene in a lymphoblastoid cell line.

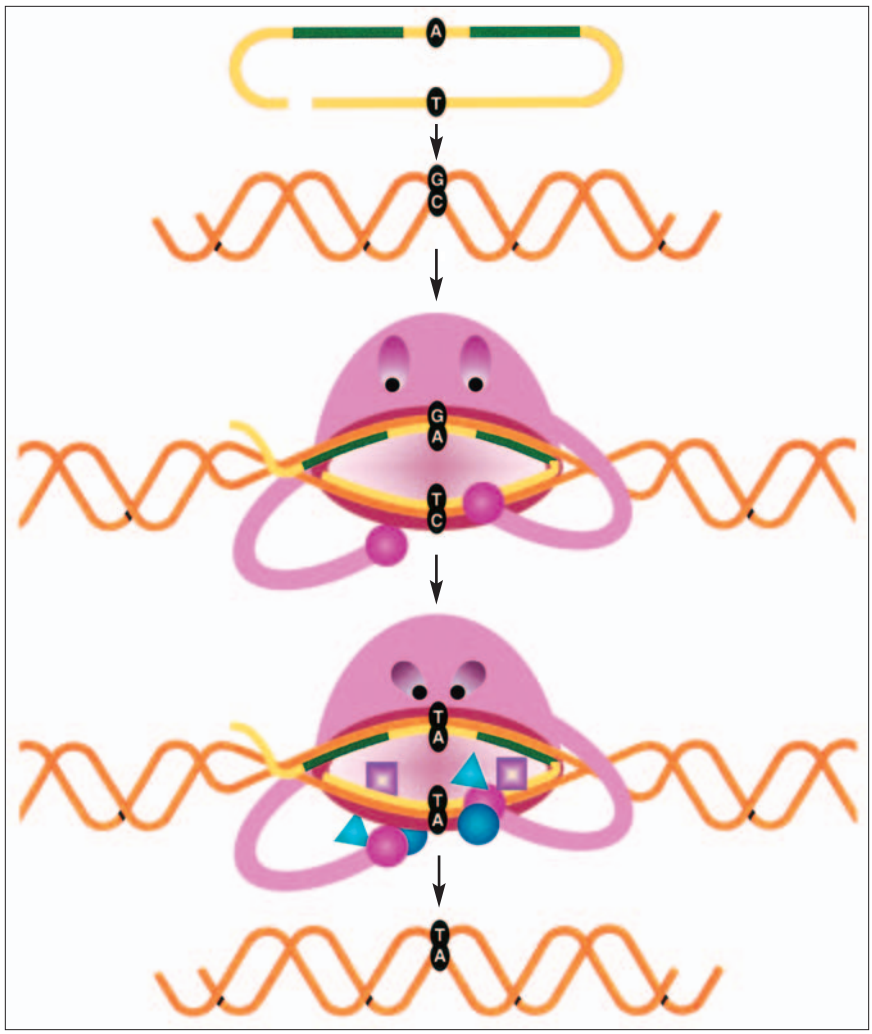
Preliminary studies emphasized the need for efficient delivery of chimeric oligonucleotides. Because nucleic acids have been successfully delivered to the liver via the galactose-specific asialoglycoprotein receptor, we made use of that receptor to study chimeraplasty in liver cells. For one delivery system, we covalently attached lactose, a disaccharide of glucose and galactose, to polyethyleneimine, which acts as a polycationic compacting agent for chimeraplasts. To try another approach, we incorporated galactocerebroside into anionic liposomes. Chimeric oligonucleotides were reproducibly delivered to hepatocytes in large numbers by these delivery systems, both in cell culture and in vivo.

Chimeraplasts were designed to introduce site-specific missense mutations into genomic DNA in cultured human hepatoma cells and in nonreplicating isolated rat hepatocytes. Subsequently, in a series of in vivo experiments, we successfully introduced mutations into the factor IX gene in a significant proportion of rat hepatocytes.

The missense mutation inactivated factor IX, and the conversion was site-specific, dose-dependent, and independent of the transcriptional activity of the target strand. We observed genomic DNA conversion frequencies as high as 60%, and reverse transcriptase PCR as well as genomic Southern hybridization analysis confirmed the changes in the factor IX gene pool in the liver.

As a final proof of principle, we determined that factor IX coagulant activity was reduced by 40%, resulting in a prolonged activated partial thromboplastin time. Thus, the observed changes in gene sequence were associated with altered phenotypic expression of the mutated factor IX protein, which remained unchanged in the rats for almost two years.

To determine whether the mutation was stable after cell replication, rats were subjected to 70% partial hepatectomy several weeks after chimeraplast treatment. In this model of liver regeneration, the remaining hepatocytes replicate in synchronous waves and



A chimeraplast is a double-stranded nucleic acid structure with a double hairpin configuration, in which stretches of RNA (*green*) flank a short DNA region that includes a single base pair mismatched to the mutated one in the target genomic DNA (*orange*). After the chimeraplast pairs to its target, endogenous DNA repair systems (*pink*) recognize the mismatch and activate factors that permanently convert the genomic sequence to the correct sequence designed into the chimeraplast.

restore the mass of the liver within two weeks.

Factor IX activity and nucleotide conversion were determined periodically through 18 months, and the observation was that the regenerated liver had in fact inherited the genomic change. The studies confirmed that site-directed nucleotide conversion by chimeraplasty was both permanent and replicatively stable.

Mismatch repair pathways endogenous to hepatocytes appear to be sufficiently active for this technology to be applied to liver-related disorders resulting from single base pair mutations. An example is Crigler-Najjar syndrome type I, in which there is no activity of uridine diphosphate glucuronosyl (UDPG) transferase, the microsomal enzyme that catalyzes conjugation of free bilirubin with glucuronic acid.

We used chimeraplasty to insert a guanosine residue into the mutant UDPG transferase gene of Gunn rats to correct the single-nucleotide deletion that causes the elevated serum bilirubin levels. The Gunn strain is well characterized and is an accurate animal model for the human syndrome. We successfully corrected the genetic lesion in the liver and established enzyme activity to the extent that serum bilirubin levels were reduced by more than 50% from control levels. Genotypic and phenotypic changes were stable for more than 18 months, and no untoward effects were observed.

Better understanding of the cellular repair processes that mediate the observed changes in DNA sequence demonstrated by this technology, coupled with further improvements in delivery systems, may provide an important advance in gene therapy for tissue types other than the liver. Chimeraplasty has now been used in plants, for example, and has resulted in heritable changes in the genomes of tobacco and maize.

In a recent report, both topical application and intradermal injection of chimeraplasts to albino mice resulted in dark pigmentation of hairs in localized areas as a result of restored tyrosinase activity. Chimeraplasty has also corrected a carbonic anhydrase gene mutation in kidney, both in vitro and in vivo. A recent success in restoring dystrophin expression in animal models may lead to a long-awaited cure for muscular dystrophy.

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