

# Switching On Gene Therapy

Using Gene Profiles to Design Drugs

C. Allen Black

**By designing an RNA molecule to carry a "switch," therapeutic genes can be introduced into cells conditionally. An example of such a molecule, termed Nucline, responds only to specific cellular environments and can be based on gene profiles from DNA arrays or other proprietary genomics information. With this method, protein from the gene product does not need to be synthesized, and because any gene can be introduced or used as a switch, a Nucline-based pharmacogenomic drug can be developed rapidly.**

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**T**he explosion of genomics information has enabled tremendous steps forward in developing rationally designed drugs. However, converting this information into pharmaceutical agents has been difficult (1) and, as a result, new drug production often has become bottlenecked at the drug discovery and development phase. Robotics and advanced drug screening techniques have decreased drug development time significantly, but it still can take up to seven years to identify and develop promising drug candidates (2).

Gene-based drugs — such as antisense, siRNA and replicating vectors — offer one solution to the bottleneck because they can be synthesized rapidly based upon genomic information. These drugs can be directed to specific cells, organs or diseases. However, the gene-based drugs developed so far have many undesirable qualities, too: antisense often fails to induce a pharmacological effect; replicating vectors can induce leukemia

and deadly immunological responses; and siRNA gene silencing effects can be inherited (3–6). Consequently, gene-based therapies have failed to develop into the large markets that small-molecule drugs have.

To address such issues with gene-based drugs, Sunpillar LLC (Pittsburgh, Pennsylvania, USA) has developed a technology that closes in on creating a useful pharmacogenomic drug by eliminating many of the deficits in gene-based drugs while maintaining desirable qualities, such as rapid rational production and cellular specificity. Nucline™ is a system for introducing therapeutic RNA coding for a gene into a cell, much like traditional gene therapy. However, with Nucline, the gene is expressed transiently and only when specific genes are present in the cell (7). Using a novel design, previously mutually exclusive properties in gene-based drug design are combined — new gene introduction with conditional but transient expression. Nucline also can perform Boolean logic operations inside the cell, much like a computer, which gives it a limited artificial intelligence that allows the drug to sense the cellular environment before deciding to release the gene.

## The Nucline Theory

**"Sensing" genes in a cell.** Naturally occurring mRNA molecules code for proteins. When natural mRNA is inserted into a cell, the ribosomes translate the coded protein. Nucline is similar to natural mRNA because it can be translated into a protein

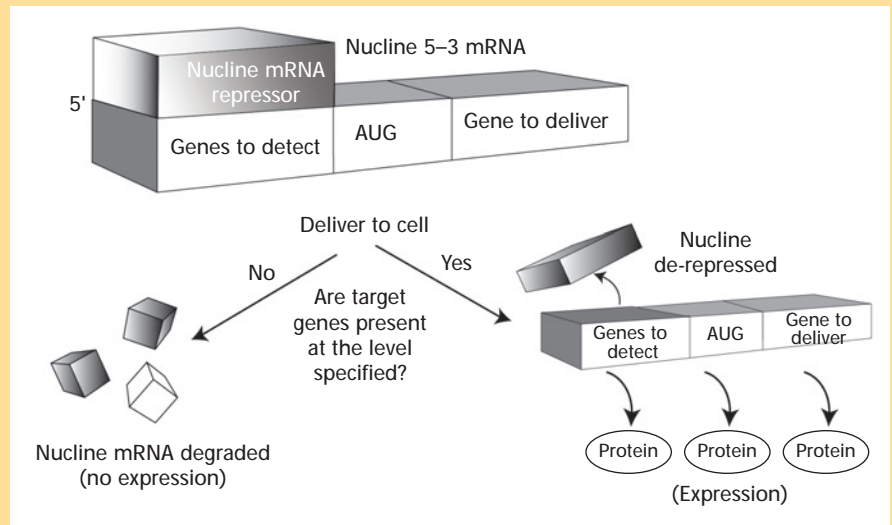
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the same way. However, unlike natural mRNA, Nucline RNA has a regulatory “switch” that prevents the molecule from being translated unless specific genes are present in the cell to switch on translation.

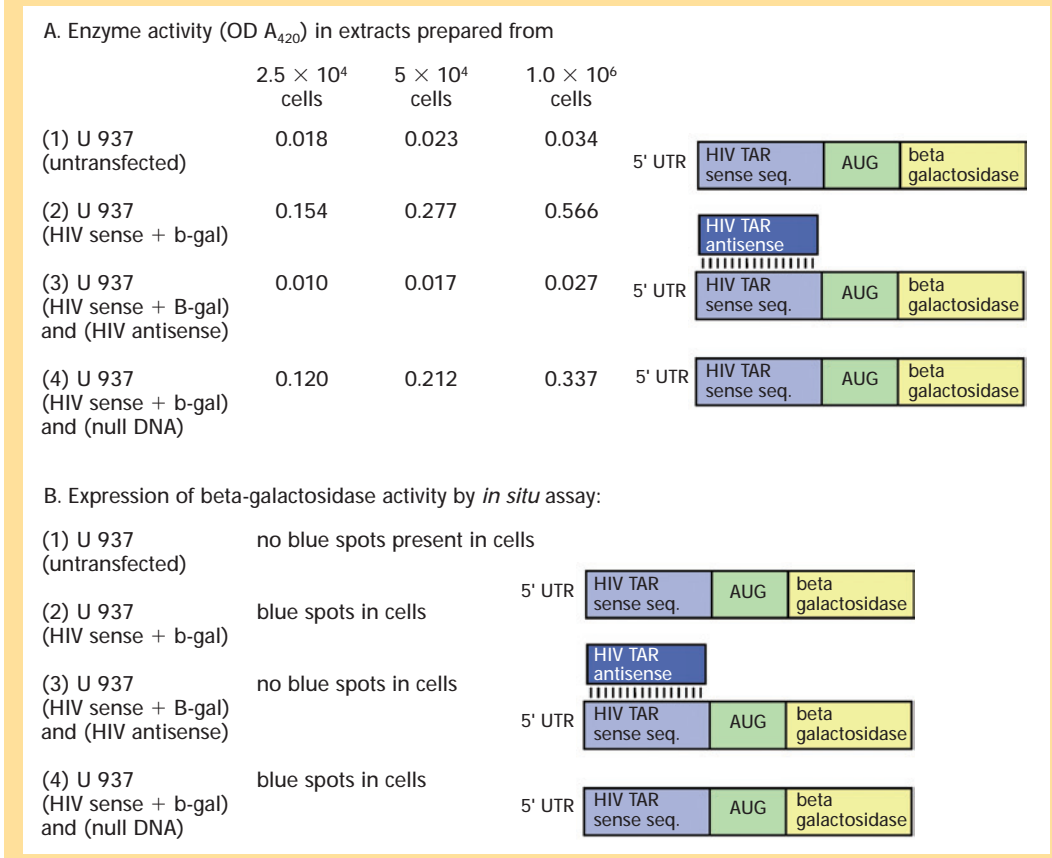
The on–off switch design for Nucline RNA exploits the principle that secondary structure in an mRNA molecule upstream of the start codon prevents translation (8). Essentially, the more stable the secondary structure upstream of the start codon the harder it is for a ribosome to bind the mRNA molecule and penetrate the secondary structure to reach the start codon and begin translation. By introducing an artificially created secondary structure in front of the start codon, Nucline RNA cannot be translated until the secondary structure is removed.

The artificial upstream secondary structure is created by inserting a short sense nucleotide sequence in the 5' untranslated region in front of the start codon on the RNA molecule. A short, complementary antisense sequence then is ligated to this sequence. The antisense oligonucleotide provides enough upstream secondary structure to completely prevent the ribosome from translating the RNA molecule (Figure 1).

Figure 2 shows an example of the effect of upstream secondary structure on *in vitro* translation (9). HIV produces a structural RNA, termed trans-activating RNA (TAR), that assists the virus in replication. Here, a eukaryotic vector carrying a 70 base-pair segment from the TAR RNA sense sequence was placed upstream of the beta-galactosidase gene and stably transfected into U937 cells. Additionally, some of the U937 cells were transfected a second time with a vector that transcribes an antisense oligo complementary to the TAR sequence, so that both the oligo and the effector gene were present in the same cell. In the singly transfected cells, beta-galactosidase activity was detected both by colorimetric assay and *in situ* counting of the blue



**Figure 1.** A schematic representation of Nucline operating in a cell. If target mRNA is present in the cell, the antisense sequence is competed off the Nucline RNA and the effector gene is translated by the cell’s ribosomes. If the target mRNA is not present, then Nucline RNA is not expressed, and is degraded.



**Figure 2.** In both U937 cell extracts (A) and *in situ* U937 cells (B), the activity of the beta-galactosidase RNA is unaffected by an unligated upstream HIV TAR RNA sequence in the 5' untranslated region (2). In the presence of HIV TAR antisense (3) but not null sequence (4), the TAR region is bound, and translation ceases because the ribosome cannot penetrate the secondary structure. The presence of a sense–antisense switch upstream of the start codon completely prevents expression of the therapeutic gene until it can be competed off by the presence of wild-type RNA.

**Table I. A summary of some of the significant similarities and differences between Nucline and SMaRT therapeutic RNA technologies**

	<b>Nucline</b>	<b>SMaRT</b>
Conditional translation of an introduced RNA?	Yes	Yes
Low dosage ratio of one molecule of drug to one cell?	Yes	Yes
Detects mutations?	Yes	Yes, but only in pre-RNAs, not structural or regulatory RNA
Requires a delivery system?	Yes, into the cytoplasm	Yes, into the cytoplasm but needs additional nuclear transport
Expresses normal proteins?	Yes	No, chimeras only
Sensitive to all forms of RNA?	Yes	No, sensitive to spliced pre-mRNA, not structural or regulatory RNA
Expression in any species?	Yes	No, the spliceosome only is present in eukaryotic organisms
Detects overexpression of non-mutated RNA?	Yes	No
Detects gene combinations and profiles?	Yes	No

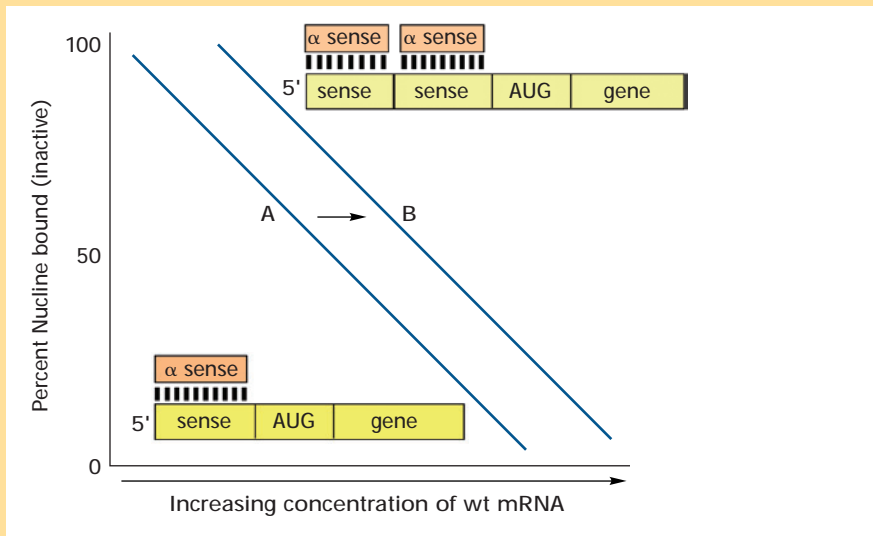
cells. The 5' HIV sense sequence upstream of the beta-galactosidase gene had no effect on protein translation. However, in the doubly transfected cells, the HIV TAR antisense oligo bound the RNA and completely prevented beta-galactosidase translation. These data demonstrate that upstream secondary structure in the form of a sense-antisense switch prevents translation.

Nucline RNA can be translated only when the antisense sequence is removed. If wild-type mRNA complementary to the antisense molecule is present in the cell, the oligonucleotide will be competed off the Nucline RNA through homologous hybridization. Essentially, when the two sense sequences are present in the cell (one in the form of the Nucline molecule and one in the form of wild-type RNA), 50% of the antisense oligos will reversibly bind wild-type RNA and 50% will reversibly bind Nucline at equilibrium. As the amount of wild-type RNA increases, more antisense oligos will bind wild-type RNA instead of Nucline. Once the antisense molecule is removed from Nucline, the secondary structure no longer exists, and the ribosome can freely translate the protein coded in the Nucline RNA. The hybridization reaction can be accelerated even by creating base pair

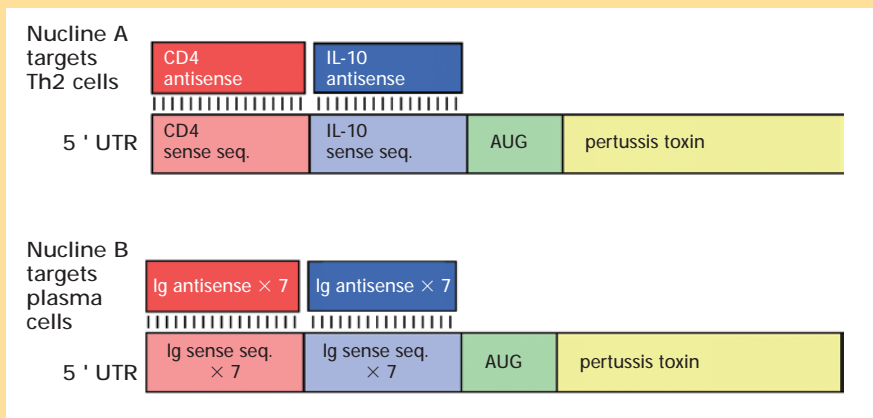
mismatches between the Nucline molecule and the antisense molecule. Over time, the freed Nucline RNA molecule is degraded, and expression ceases.

### **Manufacturing Process**

Nucline is manufactured using traditional, commonly available molecular biology techniques. A 10–100 base-pair sense sequence DNA oligo (the “switch”) is inserted in a transcription plasmid in the untranslated region immediately prior to the Adenine Uracil Guanine (AUG) start codon. Any effector gene to be translated then can be placed immediately after the AUG start codon; this includes toxins, biological response modifiers and oncogenes, etc. Using T7 polymerase, RNA transcripts containing the sense sequence linked to the effector gene are run off the plasmid and then purified using standard procedures. Finally, commercially synthesized antisense oligos are bound to the Nucline RNA. Once the antisense molecule is bound to the sense sequence upstream of the start codon, the Nucline RNA is purified by size exclusion. This raw Nucline molecule can be used in *in vitro* translation reactions or inserted into commercially available delivery systems. Nucline



**Figure 3.** A linearized binding curve for Nucline in the presence of increasing concentrations of target wild-type mRNA is shown. Curve A demonstrates the binding kinetics when only one sense–antisense repressor switch is present in Nucline. Curve B demonstrates that by increasing the number of sense–antisense sequences upstream of the start codon, more wild-type mRNA is needed to compete off the repressors. Thus, Nucline can be made to be sensitive to both sequence and expression levels of wild-type mRNA.



**Figure 4.** A schematic diagram demonstrates a potential treatment for multiple myeloma, a rare form of B-cell myeloma. Nucline A targets Th2 cells inducing B-cell proliferation by releasing pertussis toxin only in cells where CD4 and IL-10 are co-expressed. Nucline B targets hypergammaglobulinemic plasma B cells that produce 14 or more copies of specific immunoglobulin. The Nucline B construct contains 14 sense–antisense switches, but for purposes of illustration only two are shown, along with the multiplier of seven.

RNA can be introduced into cells via ballistic penetration, lipofection and electroporation.

## Advantages

**Therapeutic mRNA.** Nucline is a member of the therapeutic RNA class of drugs. Therapeutic mRNA is exogenously introduced RNA, with the simplest form being transfection with an mRNA molecule. However, more sophisticated therapeutic RNAs

have been developed. One form of therapeutic RNA technology currently in use is spliceosome-mediated RNA trans-splicing gene therapy (SMaRT™ Technology; Intronn Inc., Gaithersburg, Maryland, USA) (10). SMaRT Technology takes advantage of the fact that most eukaryotic RNA is spliced from the pre-RNA form into mRNA. By introducing an artificial splice site in pre-RNA coding for a therapeutic gene of interest, the spliceosome

joins the normal RNA to the therapeutic RNA, creating a functional mRNA molecule. Essentially, the splicing machinery in the nucleus is fooled into trans-splicing a portion of the endogenous gene onto the therapeutic gene. The resulting RNA chimera can be translated by the ribosome after it is exported back out of the nucleus. Intronn has demonstrated successfully the use of targeted therapeutic RNA to both selectively mark cancer cells and swap mutated cystic fibrosis genes for normal alleles (11–12).

Nucline RNA conditionally expresses a therapeutic protein of interest, as does SMaRT. However, Nucline differs significantly in design, which leads to greater advantages and versatility, as detailed in Table I. Instead of reacting with pre-mRNA and fooling the spliceosome in the nucleus, Nucline reacts with mRNA in the cytoplasm and fools the ribosome. Moreover, where SMaRT technology produces chimeric proteins, Nucline produces normal proteins. Nucline also can react with non-spliced RNA, such as structural RNAs and prokaryotic RNA, not just pre-RNA. Most importantly, Nucline is sensitive not only to gene sequences but also to mRNA concentration. Thus, Nucline can detect when a gene is overexpressed (as in cancer mutations of transcription promoter elements) as well as when a target gene is present (as in viral infection or protein mutation). SMaRT is limited to detecting the presence of a gene, not the gene's expression level in the cell.

### Sensing the mRNA copy number in the cell.

When sense wild-type RNA is present, the antisense oligo is competed off of the Nucline RNA in a concentration dependent manner. The threshold amount of wild-type RNA required to compete off the antisense oligo from the Nucline RNA can be adjusted by adding together multiple redundant switch sense sequences. When large numbers of repeats of the sense–antisense switch are present, more wild-type RNA subsequently is needed to compete off the antisense oligos (Figure 3). Therefore, by responding not only to specific nucleotide sequences but also the amount of wild-type RNA in a cell, Nucline can be used to target cells that overexpress wild-type mRNAs, as well as cells expressing mutations in the mRNA nucleotide sequence. To date, no other drug has been

capable of sensing gene expression levels in a cell.

**Sensing different kinds of RNAs present in the cell.** Nucline is sensitive to combinations of different wild-type mRNAs present in the cell. Instead of putting one sense sequence switch upstream of the codon, the researcher can insert multiple different switch sequences into Nucline, each switch coding for a different mRNA sequence. For example, if Nucline contains both a sense sequence for CD4 and a sense sequence for IL-10, then Nucline is only de-repressed in Th2 cells (expressing CD-4 and IL-10), not in Th1 cells (expressing CD-4 but not IL-10) or CD8 cells (expressing neither CD-4 nor IL-10). Because Nucline can be programmed to become active only when unique profiles of mRNAs are present in a cell, a company can specifically target entire gene profiles using genomics data. No current drug is capable of responding to specific combinations of gene expression in addition to overexpression.

**Performing logic operations to sense the cellular environment.** The ability to combine switches allows researchers to use Boolean logic to sense the cellular environment. Differing switches on the same Nucline molecule create an “and” statement for releasing the gene; differing switches on separate Nucline molecules delivered at the same time create “or” statements and multiple redundant switches on the same Nucline molecule create “greater than” statements.

For example, as shown in Figure 4, the combination of Nucline A that targets Th2 cells and Nucline B that targets B cells overexpressing immunoglobulin could be used to treat multiple myeloma, a rare B-cell lymphoma that results in hypergammaglobulinemia. The Boolean logic statement of the drug design is this: if a cell expresses the CD4 “and” IL-10, release pertussis toxin, “or” if a cell expresses “greater than” 14 copies of immunoglobulin RNA, release pertussis toxin. This design would kill both the hypergammaglobulinemic B cells and Th2 cells inducing proliferation while leaving all other cells unharmed.

## Disadvantages

Nucline does have some disadvantages compared to small-molecule drugs, due to

its relatively large size. It requires a delivery system because it cannot pass through the cell membrane. Also, RNase rapidly degrades RNA, so Nucline has a relatively short half-life, both *in vivo* and on the shelf. Finally, Nucline cannot directly target proteins, and in some diseases, such as prion diseases, it might be more effective to target protein function than gene function.

## Conclusion

Nucline is an important new tool for drug development. Like antisense, siRNA and SMaRT, it can respond to individual gene sequences. Like traditional gene therapy, it can introduce new genes into a cell. Like all gene-based approaches, it can be synthesized rationally and rapidly. But unlike any other technology, Nucline can be deployed conditionally based on the type and number of wild-type RNA present in a cell.

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