Antisense RNA

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I. INTRODUCTION

Following the initial discoveries of natural antisense RNAs in prokaryotes, numerous applications of antisense RNA-mediated regulation have been demonstrated in both prokaryotic and eukaryotic systems. These nontranslated RNAs, or *m*RNA-*i*nterfering *c*omplementary RNAs (micRNAs), directly repress gene expression by hybridizing to a target RNA and rendering it functionally inactive. Specificity of a micRNA for a particular transcript is conferred by extensive sequence complementarity with the "sense", or target RNA. Translation of a target mRNA is inhibited following formation of a sense-antisense RNA hybrid; in addition, the duplex molecule may be rendered susceptible to double-strand-specific cellular nucleases. Other effects of antisense RNA inhibition may include transcriptional attenuation of the mRNA as well as disruption of posttranscriptional processing events.

Artificial antisense genes have been constructed by placing the DNA fragment of a specific target gene in reverse orientation with respect to a chosen promoter. Such antisense genes have been introduced into a broad spectrum of systems, both *in vitro* and *in vivo*, enabling researchers to examine the biochemical and developmental functions of various gene products. The commercial uses of antisense RNA technology in agriculture and medicine have also become apparent as significant progress has been made in generating transgenic plants and animals in the past 5 years.

This review attempts to present the accumulated data on both natural and artificial antisense RNA regulation. While the list of reports presented here is by no means exhaustive, the reader is referred to several other review articles for additional insightful discussions on various aspects of this topic.[1-7]

II. REGULATION BY ANTISENSE RNA IN PROKARYOTIC SYSTEMS

A. Plasmid Replication and Maintenance

1. ColE1

The initiation of ColE1 DNA replication is negatively controlled at the level of primer formation by a small untranslated antisense RNA molecule (RNAI) (see also the review by Polisky).[8] The target of RNAI is the primer precursor, RNAII. Transcription of RNAII initiates at a site 555 bp upstream of the ColE1 origin,[9] and the nascent transcript forms a hybrid with the template DNA near the origin, serving as a substrate for RNase H. Cleavage of the hybridized RNAII by RNase H at the origin yields a primer for DNA replication by DNA polymerase I.[9-12] RNAI transcription begins 445 bp upstream of the origin, proceeding in the opposite direction from RNAII transcription, and terminates near the transcriptional start site of RNAII at −552 bp from the origin.[13,14] Formation of the RNAII-DNA template hybrid, and hence processing of the preprimer by RNase H, is inhibited by RNAI, which hybridizes to RNAII.[13,15] It has been proposed that binding of RNAI prevents folding of RNAII into a conformation necessary for primer formation.[13,15,16]

In vitro studies involving the substitution of G residues by I residues during transcription have shown that preprimer-DNA template hybrid formation is inhibited by alterations in the secondary structure of RNAII.[17] In addition, both the interaction of RNAII with RNAI and a deletion mutation that alters RNAII secondary structure affect transcriptional pausing as well as coupling of transcription and RNAII-template hybridization downstream, resulting in the failure of primer processing.[17] The requirement for correct folding of RNAII has also been demonstrated by analysis of point mutations that affect RNA secondary structure and prevent mature primer formation.[18]

The folded conformations of RNAI and RNAII are in turn important for their regulatory interaction. Ribonuclease sensitivity experiments have shown that RNAI has a folded structure consisting of three stems and loops[19] in concurrence with previous studies.[14,20] A stepwise pairing model has been proposed[19,21] whereby both RNAI and RNAII interact initially at their respective three loops. This transient interaction precedes formation of a more stable structure, which begins with pairing at the 5′ end of RNAI with its complementary region in RNAII and proceeds to form a full-length hybrid between the two molecules. *In vitro* binding of RNAI to RNAII, as demonstrated by change in electrophoretic mobility, is affected by various base changes that are purported to decrease loop-loop interaction without altering the folded structure.[19] Direct evidence that the binding of RNAI prevents hybridization of RNAII to template DNA has been provided by sucrose density centrifugation experiments.[18]

The interaction of RNAI and RNAII is optimally stabilized by an additional *trans*-acting regulatory element encoded ~500 bp downstream from the origin — the Rop or Rom protein.[21-24] This 63-amino-acid dimeric protein is thought to enhance the stabilization of the initial interaction between the two transcripts prior to their progressional pairing.[21,23] This enhancement results from an increase in rate constant of formation of the intermediate complex; the protein may facilitate the process by altering RNA structure to increase the potential region of interaction between the two RNA molecules, or by

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promoting the proper alignment of RNAI and RNAII for their reversible interaction.[21] Rop-mediated inhibition of primer formation requires RNAI both *in vivo* and *in vitro*.[21,23] Recent studies have provided evidence of specific interaction between Rop and the two RNAs.[25] An affinity column containing Rop immobilized to a solid support was used successfully to purify RNAI and RNAII from a RNA mixture. Based on the binding data and RNase protection experiments, it has been suggested that the protein serves as a modulator between stem structures to position the two RNAs in an optimal alignment for loop interaction.[25] X-ray crystallography of Rop has revealed its three-dimensional structure to be a dimeric molecule. Each monomer consists of two α-helices, and the entire Rop molecule has an exact twofold symmetry axis.[26] Site-directed mutagenesis experiments have shown that Rop function is affected by those mutations that alter a small number of amino acid side chains located at the extremities of the α-helix bundle on one side of the molecule.[26] It is suggested that these side chains, together with an aromatic chain of Phe-14, play an important role in RNAI-RNAII interaction.[26]

Studies involving RNAI inhibition of *in vitro* primer formation and *in vivo* characterization of mutant plasmids have indicated that RNAI also plays a key role in plasmid incompatibility.[13,15] Hybridization of RNAI to its homologous RNAII is blocked by the reversible interaction of the RNAII with the RNAI specified by a plasmid of different compatibility.

Although RNase H is absolutely required for replication of ColE1 DNA *in vitro* using purified enzymes,[9,11,18] the wild-type plasmid can be maintained in both RNase-defective (*rnh*) and RNase-DNA polymerase I-defective (*rnh polA*) bacteria.[27-30] This suggests the utilization of alternate modes of ColE1 replication contingent upon the presence or absence of these host enzymes. Studies suggest such a mechanism in *rnh polA* hosts, whereby hybridization of RNAII to the DNA template causes displacement of a single-stranded region on the non-transcribed DNA strand, followed by further unwinding by a helicase. This enables the assembly of a replisome on the extended single-stranded region, which can then initiate lagging-strand synthesis.[29,30] It has been confirmed, however, that the hybridization of RNAII to the DNA template is absolutely essential for replication to occur in all host backgrounds, and that the process is negatively regulated by RNAI.[29,30]

2. IncFII

The IncFII plasmids (which include plasmids NR1, R1, and R6) contain the information for replication control and incompatibility within a 2.5-kb region close to the origin.[31-33] As in the case of ColE1, a small untranslated antisense RNA serves both as a negative regulator in replication initiation and in incompatibility control.[34-37] Replication of FII is dependent upon the synthesis of the 33-kDa RepA (or RepA1) protein that is required for the initiation of replication at the origin.[35,38-42]

The system is efficiently regulated both at the transcriptional and translational levels.[43] *In vivo*, RepA can be produced from either of two transcripts: RNA-CX, which is transcribed from the *copB* promoter, and RNA-A, which starts from the downstream *repA* promoter.[41,44-46] Hence, RNA-A sequences are included entirely in RNA-CX. In the region upstream of the RepA coding sequence in RNA-CX, there is another coding sequence for a constitutively synthesized protein (CopB or RepA2) that represses transcription from the *repA* promoter.[47-51] RepA protein is therefore synthesized primarily from the longer RNA-CX message.

The antisense transcript (called RNAI,[34] RNA-E,[44] or CopA RNA[37]) elicits its regulatory function by binding to a complementary region within RNA-CX, thus inhibiting the translation of the RepA protein.[52] RNA-E can form two stem-loop structures;[34,53] based on the occurrence of most copy number mutations in the loop regions or in bases that affect the loop size[37,41,53-55] it has been proposed that, as in ColE1, the initial recognition between RNA-E and RNA-CX occurs via their respective loop structures. Cop T, the corresponding target region on RNA-CX messenger, occurs upstream of the RepA coding region and can form a single stem and loop.[53] *In vivo* experiments showed an inhibition of RepA expression (measured by β-galactosidase activity) when intracellular levels of RNA-E (either wild-type or hybrid transcripts) were increased.[46] Although the fate of the RNA-E/RNA-CX hybrid remains unknown, it has been proposed that binding of the two molecules alters the secondary structure downstream, rendering the RepA protein ribosome binding site inaccessible for translational initiation.[46] Alternatively, the RNA-RNA duplex may be susceptible to degradation by cellular nucleases, such as RNase III.[56]

The role of an additional constituent has been elucidated in the regulation of *repA* gene expression.[56] A previously identified 7 k reading frame,[41] which is encoded within RNA-CX, between CopB protein and RepA protein, has been expressed using lacZ fusions both *in vivo* and *in vitro*. Translational inhibition of the 7 k protein by disruption of the spacing between the ribosome binding site and the AUG start codon resulted in a tenfold decrease in *repA* expression, while production of the protein in *trans* was unable to complement the effect.[56] It has been suggested that disruption of the Cop T target stem-loop structure by ribosomes during 7 k translation prevents RNA-E/RNA-CX interaction, thus increasing *repA* expression. RNA-CX engaged in 7 k translation is therefore inaccessible to RNA-E inhibition since it cannot form the secondary structure required for recognition.[56] Based on the measured intracellular concentrations[45] of RNA-CX and RNA-E, which is produced in approximately 20-fold excess of RNA-CX,[57] it would appear that nearly all RNA-CX molecules would be sequestered by RNA-E. However, the above model potentiates an alternate pathway that balances the competition between RNA-E/RNA-CX duplex formation and accessibility of

the RepA ribosome binding site. *In vitro* experiments have demonstrated second-order kinetics of binding between RNA-E and RNA-CX, with a rate constant of $\sim 1 \times 10^6 M^{-1}s^{-1}$ at 37°C. Furthermore, the observed *in vitro* binding rate of RNA-E/RNA-CX from a copy number mutant plasmid was found to correlate well with the increased copy number phenotype observed *in vivo*.[58]

An interesting antisense RNA-mediated regulatory mechanism has been described recently for the related FII plasmid R6K.[59] Although the plasmid contains three origins (α,β,γ) of replication,[60-62] the γ origin is rarely utilized in wild-type R6K,[60] or else remains silent in certain miniplasmids.[63] One level of control is elicited by the initiator protein itself. The protein binds specifically to the seven repeated sequences (each consisting of 22 bases) at ori γ[64,65] to promote initiation of replication. The protein can also bind to an eighth repeat and two half-repeats at or near its promoter, causing autoregulation of initiator mRNA transcription.[66,67] In addition, an antisense transcript called "silencer RNA" inhibits replication by binding to an "activator" RNA, which is thought to promote initiation of replication by opening up the ori region and possibly serve as a primer for DNA synthesis.[59,63] The initiator protein enhances the inhibition of activator RNA function by specifically binding to the silencer-activator hybrid and accelerating the kinetics of the duplex formation reaction.[59] Initiator therefore acts as both a positive and a negative mediator of R6K DNA replication. While Rop protein in ColE1 augments RNAI-RNAII hybridization to a relatively small degree, the R6K initiator protein increases the rate of annealing between silencer and activator RNAs nearly 15-fold.[59]

3. IncQ

The broad host-range plasmid R1162 of the incompatibility group Q is also regulated by a small antisense RNA. This 75-base transcript is complementary to the message encoded by the *repIA* gene, one of the two contranscribed genes that constitute the *repI* region.[68-70] Although an increase in *repI* transcription is accompanied by a higher plasmid copy number,[69] the same effect is not observed for an increase in gene dosage.[68,69] The expression of the *repI* genes, which are essential for R1162 replication, is negatively controlled by the 75-base RNA. A mutation that decreases transcription of the small RNA relieves the repression of R1162 replication, resulting in an elevated copy number. The antisense transcript overlaps the ribosome binding site and first two codons of the *repA1* RNA. As in the cases of the regulatory RNAs of the ColE1 and FII plasmids, the 75-base RNA is able to form two stem-loop structures. Based on the location of the sequence complementary to the ribosome binding site at one of the loop regions, it is likely that this antisense RNA competes with the ribosomes for binding at this site.[70]

4. pT181

Regulation of copy number is established in the *Staphylococcus aureus* plasmid, pT181, by two small antisense RNAs that are complementary to the mRNAs coding for the plasmid-specific replication protein, RepC.[71-73] The two leftward transcripts, RNAIII and RNAIV, are utilized as mRNAs for RepC and contain long, untranslated 5′ leaders of 190 and 260 bases, respectively.[73,74] The translation of the RepC mRNAs can be inhibited by two countertranscripts, RNAI (80 bases) and RNAII (150 bases), which are complementary to the 5′ regions of RNAIII and RNAIV. A majority of the mutations resulting in increased plasmid copy number map in a potential loop area of RNAI, which is indicative of secondary structure interaction between RNAI and RNAIII.[73] Likewise, RNAII and RNAIV can form the same corresponding stem-loop structures in the target region. As in the preceding examples with ColE1[15,17] and the IncFII plasmids,[37,43,54] the respective single-stranded loops of the inhibitor and target RNAs may engage in the initial "nucleation" step preceding duplex formation. However, rather than a direct inhibition of translation by preventing ribosome binding, a mechanism similar to transcriptional attenuation has been suggested.

5. Plasmid Stability

In addition to the regulatory elements involved in replication, the plasmid R1 encodes two different stability functions in the *parA*$^+$ and *parB*$^+$ loci, each of which has been observed to confer a high degree of genetic stability on mini-R1 plasmids in *Escherichia coli*.[75,76] The *parB*$^+$ locus is associated with the induction of a lethal function in cells that have lost a *parB*$^+$ plasmid at cell division. The lethal factor, a 52-amino acid polypeptide, is encoded by a gene found within the locus, called the *hok* (*host k*illing) gene.[77,78] The induced overproduction of this highly toxic protein results in a rapid collapse of membrane potential together with arrest of respiration, and a drastic change in host cell morphology is observed.[78] The expression of the protein is controlled in *trans* by the product of another gene in the *parB*$^+$ locus, *sok* (*s*uppressor *o*f host *k*illing), which fully suppresses induction of the *hok* gene.[77] The *sok* regulator is a small RNA of 100 bases[79] that is transcribed in the direction opposite to that of the *hok* message. *sok* RNA has been proposed to control *hok* expression by inhibiting translation of the complementary *hok* mRNA to which it can hybridize.[77,78,80] While the *hok* mRNA is extremely stable with a half-life of 20 min or more, the *sok* RNA is rapidly degraded.[80] The differential decay of the two RNAs is the basis of the events responsible for postsegregational killing of plasmid-free cells; whereas in cells carrying plasmid, *sok* RNA prevents synthesis of the highly toxic Hok protein, in plasmid-free segregants the unstable *sok* RNAs decay, resulting in *hok* mRNA translation and, hence, rapid and selective killing of the cell.[79]

B. Transcriptional Regulation

1. crp

The autoregulation of the gene coding for cyclic AMP receptor protein (CRP) in *E. coli* occurs through the repression

of *crp* transcription by a divergent transcript called *t*ranscription *i*nhibitory *c*omplementary RNA (ticRNA).[81] Synthesis of ticRNA is activated *in vitro* by the addition of cAMP-CRP,[82] which had been previously shown to strongly inhibit *in vitro* transcription of *crp*.[83] S1 mapping experiments have identified the transcriptional initiation site of ticRNA to occur three nucleotides upstream of and on the strand opposite to that of *crp* RNA initiation. Despite the nonoverlapping nature of the ticRNA and target *crp* genes, there exists a significant extent of homology between nucleotides 2 to 15 in ticRNA and nucleotides 2 to 11 in *crp* mRNA.[82] The production of *crp* mRNA *in vitro* has been shown to be effectively repressed upon addition of a purified 92-nt divergent RNA. Furthermore, a ticRNA as small as the initial 15 nucleotides can inhibit *crp* transcription *in vitro*.[82,84] *In vivo* studies have also confirmed the cAMP-CRP-dependent repression of *crp* mRNA synthesis by ticRNA. Cells carrying a plasmid with an intact *crp* promoter gene fragment but an inactive ticRNA promoter failed to exhibit autoregulation of CRP production, while those cells carrying the control plasmid with the wild-type promoter displayed strong autoregulation.[82] S1 mapping showed that ticRNA is not produced upon adding cAMP in the cells containing the mutant promoter plasmid; the ticRNA is present in the control cells.[84] A model for ticRNA inhibition has been proposed whereby cAMP-CRP activation of the ticRNA promoter gives rise to the ticRNA that binds to the 5′ end of *crp* mRNA. The resulting hybrid structure, which resembles a rho-independent transcription terminator,[85] causes RNA polymerase pausing. The AU-rich sequences immediately following the RNA-RNA hybrid render an unfavorable instability to the *crp* mRNA-DNA hybrid region such that the *crp* transcript is released, and, hence, transcription is prematurely terminated.[81,82] The autoregulation of *crp* is the first example of control at the transcriptional level by an antisense RNA.

C. Translational Regulation

1. Bacterial Gene Expression

a. micF

The expression of the *E. coli* major outer membrane proteins, OmpC and OmpF, is regulated in response to the osmolarity of the growth medium. An increase in osmolarity is accompanied by an increase in OmpC synthesis and a concomitant decrease in OmpF synthesis, while the total amount of the two proteins remains constant.[86-88] This osmoregulation of *ompF* and *ompC*, which map at 21 and 48 min, respectively, on the chromosome,[89] is controlled by two regulatory proteins, OmpR and EnvZ, which are thought to act at a transcriptional level.[90-92]

A third regulatory molecule was identified during the characterization of *ompC*, which serves as a repressor of OmpF expression.[93,94] This regulator is a small RNA molecule transcribed from an independent promoter in the opposite orientation upstream of the *ompC* gene. A 300-bp DNA fragment including this transcriptional unit was found to encode an inhibitory function when it was introduced into *E. coli* cells on a multicopy plasmid. Upon sequencing the region, an 80-bp domain was found to contain 70% homology with the 5′ terminus (including the Shine-Dalgarno sequence and initiation codon) of *ompF* messenger. Further analysis using S1 nuclease mapping identified the transcript to be 174 bases in length. This RNA molecule, termed *micF* RNA, was hence proposed to inhibit the production of OmpF by hybridizing to the *ompF* mRNA and preventing its translation.[93,94]

micF RNA can form a stable duplex with its target;[94a] the covered section includes 44 bases of the 5′ untranslated region and 10 bases from the coding region of *ompF* messenger (of which 43 are base-paired). Both the ribosome binding site and the initiation codon of *ompF* RNA are sequestered by the micRNA.[94] Upon isolation of ^{32}P-labeled chromosomal *micF* RNA and of ^{32}P-labeled plasmid-amplified *micF* RNA, the predominant species was identified as a 4.5 S primary transcript (93 bases) with a 5′ terminal phosphorylated nucleotide at G82.[95]

It was at first suggested that the chromosomal copy of *micF* did not play a key role in osmoregulation based on experiments under steady-state conditions of a *micF* deletion strain.[96] However, a more critical study revealed that upon shifting from low to high osmolarity, the suppression of OmpF synthesis occurs more rapidly in a *micF*$^+$ strain than in a deletion strain. Furthermore, while full suppression is obtained within 15 min after the shift for the wild-type cells, the inhibition is incomplete in the *micF* deletion strain even after 90 min as measured by pulse-labeling experiments.[83] Recent results have revealed a thermoregulatory response of *micF* RNA synthesis.[97] An increase in temperature from 24 to 42°C is accompanied by an increase in *micF* RNA levels. Time course experiments have given additional evidence to the pivotal role of chromosomal *micF* in maintaining the balanced regulation of *ompF* and *ompC* expression. Although steady-state levels of *ompF* mRNA in the wild-type strain decreased dramatically within 60 min after increasing the temperature, *ompF* mRNA levels remained constant (i.e., no decrease) in a *micF* deletion strain of *E. coli*.[97]

b. isf

The transcriptional pattern of the *sulA-ompA* region of the *E. coli* chromosome (22 min) is modulated at several different levels. During normal growth the *sulA* gene (which encodes a SOS-inducible inhibitor of cell division) remains repressed, whereas the tightly linked *ompA* gene is transcribed into a stable 1250-nucleotide mRNA. Suppression of *sulA* occurs at two levels: (1) transcriptional repression by binding of the LexA protein to an operator sequence overlapping the *sulA* promoter, and (2) proteolytic attack of the SulA protein by the Lon protease.[98-101] In addition, countertranscription of *sulA* has been found to occur from a promoter located in the intergenic region between *sulA* and *ompA*. The product, a 353-nucleotide untranslated RNA (*isf* RNA), is complementary to the 3′ end of *sulA* over a region of ~250 nucleotides, and may very possibly

function as an antisense RNA to regulate *sulA* expression.[102] Following induction of the SOS response extensive transcription of *sulA* occurs, resulting in reduction of *isf* expression. In addition, transcriptional readthrough at the *sulA* terminator results in the production of a bicistronic mRNA encoding both SulA and OmpA proteins, as well as a decrease in transcriptional initiation at the *ompA* promoter due to promoter occlusion. The regulatory role of *isf* RNA is further supported by the fact that it is complementary to an essential region of *sulA* mRNA, as defined by analysis of missense mutants incapable of blocking cell division.[102] It is suggested that, as in the case of *micF* RNA, which hybridizes to *ompF* mRNA to promote a complete and efficient suppression of OmpF synthesis during the osmoregulatory response,[83,93,94] *isf* RNA hybridizes to residual *sulA* mRNA toward the end of the SOS response to allow a quicker release of the septational block.[102]

c. gvpABC OPERON

The *gvpABC* operon of the cyanobacterium *Calothrix* 7601 encodes genes involved in the formation of gas vesicles,[103,104] which provide buoyancy to the cells. The vesicle envelope is composed of a monolayer of protein molecules, which confer permeability to gas, but remain impermeable to water molecules.[105] In response to changes in environmental conditions, *Calothrix* 7601 undergoes a differentiation process, producing short filaments of small cells, called hormogonia. The formation of these hormogonia, which arise from the cellular division and fragmentation of the long filaments of vegetative cells, is accompanied by a massive production of the gas vesicles mentioned above.[105-107]

In the process of studying the regulation of the expression of the *gvp* genes, S1 nuclease mapping experiments and primer extension reactions were performed[108] to resolve the 5′- and 3′- ends of the *gvp* transcripts. Four different RNA species were found to be transcribed from the *gvpABC* operon in differentiated cells. Three of the transcripts (*gvpA*, *gvpAB*, and *gvpABC*) initiate from the same nucleotide, 56 bases upstream from the *gvpA* AUG start codon and extend for various distances in the 5′→3′ direction. The resulting transcripts are 326 bases (covering only the *gvpA* gene), 773 bases (covering the *gvpA* and *gvpB* genes), and 1408 bases (covering all three genes *gvpA*, *gvpB*, and *gvpC*) in length. The fourth transcript is a 420-base-long antisense RNA that is complementary to a portion of each of the three mRNAs of the *gvpABC* operon, including the *gvpB* AUG start codon. Transcription of the *gvp* antisense RNA is initiated within the coding region of the *gvpB* gene and ends within the upstream *gvpA* coding region. Sequence analysis indicated the absence of any potential reading frame that could give rise to a translation product.[108]

The presence of *gvp* antisense RNA in total RNAs extracted from differentiated cells, but not in total RNAs obtained from vegetative cells, is highly suggestive of its regulatory role in the expression of the *gvpABC* operon. Unlike other prokaryotic, naturally occuring antisense RNAs, the 420-base *gvp* antisense transcript is considerably larger and can hybridize along its entire length to each of the three mRNA species of the operon. Furthermore, transcription of the antisense RNA starts in the coding region of one gene and ends in that of another gene located upstream in the operon. The *gvp* antisense RNA can form duplexes with the *gvpABC* RNAs to inhibit translation by blocking ribosome binding (immediately upstream of the *gvpB* coding frame) and/or ribosome migration during translation of the mRNAs.[108]

Differentiation of hormogonia in *Calothrix* in response to environmental changes occurs without concomitant DNA replication.[106] The differentiated cells grow larger and eventually lose their gas vesicles, giving way to the regeneration of vegetative filaments.[105] In accordance with the transient nature of hormogonia production, an antisense RNA-mediated regulation of *gvpABC* expression provides an efficient and effective means of temporal control.

2. Phage Gene Expression

a. P_{aQ} RNA

Antisense RNA has been shown to participate in the regulatory events that specify the balance between lysogenic vs. lytic paths in λ bacteriophage development. The establishment and maintenance of λ lysogeny requires the products of the *cI* and *int* genes, which code for repressor and integrase, respectively.[109,110] Transcription of these genes is positively controlled by the *trans* acting cII protein that binds selectively to the −35 region of both the *cI* promoter (P_{RE} or P_E) and the *int* promoter (P_I).[111-113] A third cII-dependent transcription unit was identified in the middle of the Q regulatory gene, which gave rise to *in vitro* run-off transcripts of 220 and 320 bases in the direction opposite that of Q transcription.[114,115] *galK* gene fusions and S1 nuclease mapping indicated that the promoter for these transcripts, P_{aQ} (anti-Q), responds identically to cII activation as do P_{RE} and P_I, precisely at the same stage (10 to 12 min postinfection) of the λ infectious cycle. The shorter leftward P_{aQ} RNA, which results from termination at a potential transcription termination signal (t_{aQ}), is thought to inhibit translation of the rightward Q message by hybridization.[115] This P_{aQ}-t_{aQ} RNA-elicited repression can account for the reduction of phage late gene expression (and, hence, lytic growth) by cII, a phenomenon known as "cII-dependent inhibition".[116,117] Because the antiterminator Q protein is required for the expression of all late genes,[118] cII activation of P_{aQ} transcription, in concert with P_{RE} and P_I activation, ultimately results in favor of λ lysogeny.

b. OOP RNA

As mentioned above, the cII protein is a key factor in the coordinate regulation of the transcription units responsible for λ lysogenic development. The expression of *cII* itself is controlled at several levels, including transcription, translation, processing, and stability.[109,110] A second small antisense RNA encoded by λ has been implicated in the repression of cII

expression. This 77-base transcript, designated OOP RNA, initiates in the intercistronic region between the O and *cII* genes. While OOP RNA does not contain any open reading frames, the 3′-terminal 55 bases of the transcript are complementary to the last 17 codons of *cII* mRNA.[119-122] It has been shown *in vivo* that a multicopy plasmid carrying an OOP DNA fragment prevents *cII* expression from a derepressed prophage by ~100-fold. Inhibition was assayed using cII-dependent P_{RE}-*galK* gene fusions on multicopy plasmids.[123] In addition, overexpression of OOP sequences from an IPTG-inducible plasmid in *E. coli* gives rise to clear plaques upon infection of the cells with λ^+, indicating a developmental shift in favor of the lytic mode.[124] In contrast to the aforementioned examples of antisense RNA-mediated translational regulation, the OOP transcript is complementary to the 3′ end of its target cII message, which is indicative of a mechanism other than the direct inhibition of translation (i.e., sequestration of the ribosome binding site). It has been proposed that OOP RNA acts by forming a duplex with its target cII message, rendering it susceptible to cleavage by the host enzyme RNase III. In concordance with this theory, OOP RNA-mediated inhibition of *cII* expression is not observed in a *rnc*$^-$ *E. coli* strain.[123]

Various regulatory processes are involved in the determination of the course of λ development. While the cII protein plays a prominent role in the lysis-lysogeny decision, other genes also work in concert, both directly and indirectly, to commit the phage to one path or the other. The antisense RNAs act in a specific manner to prevent a particular target message from functioning. The inhibition of antiterminator Q expression by P_{aQ} RNA indirectly impels the phage to enter the lysogenic cycle. On the other hand, OOP RNA, which targets *cII* mRNA, functions in favor of the lytic mode. A physiological role for OOP RNA has been implicated by an observed twofold increase in *cII* expression from an induced prophage in the presence of excess anti-OOP transcript. Both cII protein and OOP RNA in this system are encoded solely by the prophage.[123] Although the above anti-OOP-induced increase in *cII* expression is not a dramatic change, OOP RNA synthesis may be augmented in those phage entering the lytic pathway — additional studies elucidating the regulation of OOP RNA transcription itself should provide further insight into the precise role of this antisense RNA.

c. sar RNA

As illustrated above for the λ phage, the development of the temperate bacteriophage P22 is also believed to be controlled in part by a small antisense RNA. This transcript, called *s*mall *a*ntisense *r*egulatory RNA (*sar*RNA), acts in collaboration with the products of the *mnt* and *arc* genes to regulate the synthesis of P22 antirepressor.[125,126] The antirepressor protein inhibits the activity of phage repressors (including P22 c2 and λcI), which shut off transcription of lytic genes during the lysogenic cycle. The antirepressor gene (*ant*) is transcribed from the strong rightward promoter P_{ant}, and is preceded by the *arc* gene. *arc* encodes a protein that binds to P_{ant}, repressing the *ant* operon during phage lytic growth. The *mnt* gene product, on the other hand, binds to and turns off P*ant* transcription during lysogeny.[127-129] The *mnt* gene is transcribed in the leftward direction from the divergent P_{mnt} promoter that overlaps P_{ant}.[130] While *ant* is transcribed late during infection as part of a large operon expressed from the upstream P_{late} promoter, the antirepressor protein is not synthesized.[131-133]

The potential role of the 68- to 69-base-long *sar*RNA as a third regulator of *ant* synthesis became apparent upon characterization of P22 mutants that were able to produce antirepressor late in infection. The mutations were mapped to the −10 region of the *sar*RNA promoter (P_{sar}) that lies in the extreme 5′ end of the *ant* gene. P_{sar} directs transcription of its RNA, which spans the entire intercistronic region between the *arc* and *ant* genes, in the direction opposite that of P_{ant} transcription.

An overproduction of antirepressor synthesis from P_{late} transcript was observed in cells infected with P_{sar} down-mutant (P_{sar}^-) phages, whereas antirepressor was not detectable in P_{sar}^+ phage-infected cells.[126] It is proposed that *sar*RNA, which is complementary to the 5′ noncoding region (including the ribosome binding site) of *ant* mRNA, hybridizes to the latter, inhibiting its translation.[125,126] Duplex formation between *sar*RNA and *ant* mRNA has been observed *in vitro*.[125] The ability of a wild-type *sar* region cloned on a plasmid to inhibit antirepressor synthesis by a P_{sar}^- phage in *trans* indicates that a direct transcriptional interference mechanism (i.e., convergent transcription) is unlikely. In fact, *in vitro* experiments have shown rather that transcription from the strong P_{ant} promoter interferes with P_{sar} transcription.[125] Therefore, effective production of *sar*RNA may not occur until the Arc protein (which is synthesized from the same operon as antirepressor) alleviates transcription from P_{ant}. In this manner Arc indirectly aids in the stimulation of *sar*RNA transcription. The importance of *sar*RNA in the establishment of lysogeny is evidenced by the clear-plaque morphology of *mnt*$^+$ *arc*$^+$ P_{sar}^- mutant infections. The precise regulation of antirepressor is thus effectuated at different levels by the actions of Mnt, Arc, and *sar*RNA, allowing the efficient establishment of P22 lysogeny.

3. Transposition and Conjugal DNA Transfer

a. Tn10

The transposable element Tn10 is also characterized by an antisense RNA regulatory system. The composite transposon contains a core region that includes the genes for tetracycline resistance, flanked by inverted repeats of the insertion sequence IS10. IS10-Right ($IS10_R$) encodes the functions necessary for Tn10 transposition and is capable of transposing independently.[134,135] The transposition frequency of a single copy Tn10 element in *E. coli* has been observed to decrease 10- to 20-fold upon introduction of a multicopy plasmid carrying $IS10_R$. This phenomenon is known as "multicopy inhibition" (MCI) and is attributed to a *trans*-acting negative factor encoded within

the outermost 180 bp of $IS10_R$.[136] An increase in the transposition is not observed due to the *cis*-acting nature of IS10 transposase.[137]

The regulatory element is a transcript produced from the pOUT promoter (RNA-OUT) of $IS10_R$. Transposition is inhibited by the hybridization of RNA-OUT to the 5′ end of transposase mRNA (RNA-IN), which is transcribed from the pIN promoter in the direction opposite that of pOUT. The two RNAs overlap and are thus complementary to each other over a 36-bp region that includes the translational start codon of transposase. Translation, but not transcription, of RNA-IN is affected by MCI, as evidenced by IS10-*lacZ* gene fusions and operon fusions. Hybridization of RNA-OUT to RNA-IN would inhibit translational initiation by preventing ribosomes from binding to the transposase messenger.[136,138] RNase III cleavage does not appear to be involved, as inhibition is unaffected in *rnc*⁻ *E. coli*.[139] In *in vitro* experiments single-base mutations resulting in increased transcription from pOUT have been observed to increase MCI, while mutations producing higher levels of pIN transcript caused a severe reduction of MCI, when introduced into MCI^+ plasmids carrying wild-type pIN-pOUT.[136]

A major 70-base-long RNA transcript (RNAout1) produced from pOUT has been identified *in vivo*.[140] The concentration of RNAout1, whose 5′ end is identical to that of the *in vitro*[138] RNA-OUT transcript, has been estimated at five to ten molecules per cell containing a single copy of Tn10.[140]

RNA-IN and RNA-OUT hybridization occurs *in vitro* with a rate constant of $\sim 3 \times 10^5$ mol/l/s.[141] While RNA-IN is not characterized by any evident secondary structures, a potential stem-and-loop structure can be assigned to RNA-OUT.[140] The pairing reaction of the two molecules is postulated to begin by the initial interaction of the loop domain of RNA-OUT with the complementary 5′ end of RNA-IN, which is followed by duplex formation in a nonrate-limiting strand-displacement reaction. As suggested by the absence of effect on the pairing reaction for mutations that result in truncated or base-substituted stems, the stem region most likely aids in the stabilization of RNA-OUT *in vivo*.[141] Despite the unaffected rate of hybrid formation, both the RNA-OUT and RNA-OUT/RNA-IN hybrid molecules are destabilized by the above alterations. Mutations in the RNA-OUT loop domain and in the 5′ end of RNA-IN, on the other hand, decrease the pairing rate dramatically. Changes that promote the formation of a closed-loop structure eliminate pairing between the mutant RNA-OUT with wild-type RNA-IN.[141]

Regulation of transposase expression by RNA-OUT is thought to be the primary means of limiting the rate of IS10 transposition per element as the number of elements in the cell increases.[141] As the frequency of transposition is directly related to the level of transposase protein,[137] translational inhibition of the transposon messenger maintains a low rate of 10^{-4} transpositions per element per generation.[138]

b. traJ

Conjugal DNA transfer by the antibiotic resistance plasmid R100 is dependent in part upon the expression of the *traYZ* operon.[142,143] *traYZ* in turn requires the plasmid-specific *traJ* gene product for its expression. This regulatory cascade culminates as the transcription of *traJ* itself is negatively controlled by the trans-acting genes *finP* and *finQ*.[144-147] It is suggested that the *finP* transcript, which is complementary to the *traJ* transcript over a 107-base region, hybridizes to the *traJ* RNA and inhibits its translation by blocking the ribosome binding site.[148] The *traJ* transcript can form two different stable secondary structures. Stem-loop IIa (bases 535 to 564)[149] forms upstream of the ribosome binding site and translational start site of *traJ*. Formation of IIa would preclude formation of stem-loop IIIa (bases 551 to 593), which includes the 3′ stem of IIa and the *traJ* ribosome binding site. *finP*, which is transcribed in the direction opposite that of *traJ*, can always form the stem-loop (IIIb) complement to IIIa.[148] The *finO* transcript is complementary to an 11-base segment of the potential IIa stem-loop region of *traJ* RNA.[150,151] It has been proposed that the *finO* transcript prevents the formation of IIa, allowing the formation of IIIa. Stem-loop IIIa can then interact with the complementary IIIb structure on *finP* RNA to initiate duplex formation,[148] in a manner similar to the mechanism described for ColE1 RNAI-RNAII pairing.[19,21] More recent studies have indicated that *finP* RNA can also bind to the 3′ ends of *traM* transcripts.[148a] The *traM* gene lies immediately 5′ to *traJ* and is transcribed in the same direction as *traJ* and the *tra* operon. The 3′ ends of the principal *traM* transcripts (705 and 562 bases long) end inside the *traJ* ORF. It thus appears that these transcripts provide additional sense RNA for binding with *finP* RNA, and the concentration of free *finP* RNA is dependent on the relative concentrations of *traJ* and *traM* transcripts. *traJ* expression is therefore regulated by the participation of both sense and antisense RNAs.[148a]

4. Plasmid Gene Expression

a. COLICIN E1 OPERON

The colicin E1 operon of the ColE1 plasmid contains a novel organization of genes that are coordinately regulated. The *cea* gene product, colicin E1 protein, forms ion channels in the bacterial membrane, leading to cell death,[152,153] while the *lys* gene product is associated with the mitomycin C-induced lethality function.[154] Both *cea* and *lys* are transcribed from a single promoter, which is induced in response to the SOS system. Read-through past the *cea* terminator results in transcription of the downstream *lys* gene. The colicin E1 immunity gene, *imm*, is located in between, and transcribed in the opposite direction to, the *cea* and *lys* genes.[155] The overlapping arrangement of the three transcripts is suggestive of an antisense RNA-regulated system of gene expression. The 3′ end of the *imm* transcript may bind to and inhibit translation of the 3′ end of *cea* RNA, which codes for the hydrophobic carboxyl-

terminal domain of colicin responsible for its activity.[152] Alternatively, the transcriptional read-through product (*cea-lys* RNA) can hybridize to the *imm* RNA, preventing its translation. In addition, the 5′ end of *imm* RNA overlaps the 5′ terminus of the *lys* transcript, including the ribosome binding site, potentiating the inhibition of either *lys* RNA or *imm* RNA translation.[155]

D. Artificially Constructed Antisense RNAs

1. Antisense RNA-Mediated Mutagenesis

Following the initial discoveries of gene regulation by naturally occurring antisense RNAs, it was speculated that artificial antisense RNAs could be synthesized by placing a DNA fragment of a specific target gene under control of a promoter, in reverse orientation.[156-158] Several such antisense RNA genes (micRNA genes) have been constructed to test the applicability of hybrid-arrested translation of specific *E. coli* mRNAs.

The mic cloning vector pJDC402 has been used to produce complementary RNAs against the mRNAs of several *E. coli* genes.[156] The plasmid contains the strong lipoprotein (*lpp*) promoter[159] and the inducible lactose promoter-operator (lac^{PO}) in tandem, followed by a unique *XbaI* site into which a DNA fragment can be inserted. The vector was also designed after the natural *micF* RNA[94] to give rise to a transcript flanked by stem-loop structures — one at the 5′ end derived from the lac^{PO} and one at the 3′ end derived from the *lpp* transcription terminator.[156]

An artificial micRNA gene targeted against *lpp* mRNA (mic[*lpp*] gene) was constructed by inserting in the opposite orientation, a DNA fragment encompassing the Shine-Dalgarno sequence and the first 29 amino acid residues from the coding region of prolipoprotein into pJDC402. The resulting mic(*lpp*)RNA is complementary to the region encompassing the ribosome binding site. Pulse-labeling experiments showed a 16-fold reduction of lipoprotein synthesis upon induction of the antisense transcript with IPTG (isopropyl-β-D-thiogalactopyranoside). The inhibition was specific for *lpp*, since the production of other outer membrane proteins is unaffected (with the exception of OmpC, whose mRNA contains a region of 80% homology near the ribosome binding site to the *lpp* mRNA). Furthermore, a duplication of the mic(*lpp*) gene in pJDC402 resulted in an even greater (31-fold) decrease in the production of lipoprotein in the presence of IPTG, signifying a clear gene dosage effect. Maximal inhibition of *lpp* expression by the induced micRNA transcript occurs within 5 min. The mic(*lpp*)RNA therefore acts after initiation of target mRNA transcription, since *lpp* mRNA has a half-life of 12 min.[160] However, Northern analysis showed that *lpp* mRNA levels are greatly diminished upon IPTG induction in cells carrying the mic(*lpp*) plasmid.[156] This specific reduction of target mRNA is analogous to the effect observed for natural *micF* inhibition. Taken together, the above results suggested that hybridization of these micRNAs to their targets not only prevents ribosome binding, but may destabilize the messages and/or render them susceptible to cleavage by ribonucleases such as RNase III.

The pJDC402 system was also used to inhibit the expression of *E. coli* outer membrane protein OmpC. Induction of a mic(*ompC*)RNA complementary to 20 bases of the leader sequence (including the ribosome binding site) and 100 bases of the coding sequence of *ompC* mRNA caused an approximately fivefold reduction in OmpC synthesis, when compared with the levels in control cells containing the vector itself. A mic(*ompC*)RNA with longer complementarity to the leader region (72 bases) was four times more effective in inhibiting the production of OmpC than the shorter mic(*ompC*)RNA, implying that 5′ leader region complementarity may be an influential factor in micRNA-mediated inhibition. These results are consistent with the greater inhibition (16-fold) observed for a mic(*lpp*) gene covering the Shine-Dalgarno region and translation initiation site as opposed to the relatively smaller effect obtained with a mic(*lpp*) plasmid complementary only to the *lpp* coding region.[156]

On the other hand, a mic(*ompA*) plasmid directed against the *ompA* coding region for amino acid residues 4 through 45 was just as (if not slightly more) effective as a mic(*ompA*) gene construct that encompassed the Shine-Dalgarno sequence and initiation codon. Furthermore, as none of the various mic(*ompA*) plasmids constructed displayed any significant inhibition of OmpA synthesis,[156] the critical determinant for susceptibility to micRNA inhibition in this particular case may lie in the high degree of secondary structure potentiated by the sequence of *ompA* mRNA.[1,161-163] Folding of the target *ompA* messenger may preclude its accessibility to hybridization with mic(*ompA*)RNA.

A polar effect of translational inhibition on the *lac* operon of *E. coli* was observed upon the induction of antisense RNAs complementary to a region of the *lacZ* (β-galactosidase) coding sequence (bases 19 to 440).[157] Cells containing the antisense *lacZ* RNA plasmid prevented IPTG-induced increase in β-galactosidase synthesis by 98%. Expression of lactose permease and thiogalactoside transacetylase, which are encoded downstream of *lacZ* on the same polycistronic message, were also inhibited to a lesser degree (80% for permease; 55% for transacetylase). Hence, an antisense RNA directed against a specific gene in an operon may affect the expression of those genes within the operon that are encoded downstream of the target. The lower inhibition observed for the genes 3′ to *lacZ* is most likely due to ribosome initiation within the polycistronic message[164] at the respective AUGs preceding the permease and transacetylase coding regions.[157] Artificially produced antisense RNAs have been shown to be most efficient when they are targeted against the 5′ noncoding region, including the ribosome binding site; however, the above study demonstrates that *lacZ* expression can be inhibited to a significant degree by antisense transcripts complementary to the coding sequences. A comparison of β-galactosidase levels in cells car-

rying plasmids with different lengths of antisense *lacZ* sequences against the coding region indicated a greater decrease in synthesis for longer antisense constructs.[158]

2. micRNA Immune System

The successful inhibition of the expression of several prokaryotic genes prompted the development of a micRNA immune system in *E. coli*.[165,166] The system is characterized by the inducible production of antisense RNAs in the bacterial cell, which are targeted to specific phage mRNAs and/or the phage genome.

E. coli cells containing micRNA plasmids directed against different regions of the coliphage SP genes were rendered resistant to phage infection to varying degrees as measured by inhibition of plaque formation.[165,166] The F-specific SP phage contains a 4.2-kb positive single-stranded RNA genome coding for maturation enzyme, coat protein, coat read-through protein, and replicase. Immunity to phage conferred by micRNA sequences targeted against the 5′ end region (including the Shine-Dalgarno sequence) of the maturation protein gene was significantly more effective than those micRNA genes directed against coat protein or replicase genes.[165,166] Further deletional analysis of the maturation protein gene region demonstrated a strong inhibitory effect (94%) on phage production in cells harboring a plasmid with only a 19-base antisense sequence encompassing the ribosome binding site (SD sequence). In addition, while the immunity conferred by the plasmid carrying antisense RNA sequences extending into the maturation gene coding region was highly specific for SP phage, the 19-base micRNA construct exerted significant inhibitory effects against both the related phages Qβ and GA.[166] Exclusion of the coding sequences presumably extends the specificity of the micRNA immune effect. Although sequence homologies in the 19-base region between phages Qβ and SP and between phages GA and SP are only ~50%, those nucleotides that are homologous include the initiation codon and three or four nucleotides of the SD sequence.[166] It may therefore be conceivable that sequestration of these two immediate regions can produce a notable micRNA immune effect. On the other hand, the most effective micRNA construct contained sequences complementary to the SD sequence of the maturation gene and the immediately preceding 13 nucleotides.[167] Inclusion of sequences complementary to the initiation codon and sequences further downstream had little effect, suggesting the dispensability of the AUG for micRNA inhibition in this particular case. Furthermore, the addition of sequences further upstream was detrimental to the potentiality of the micRNA immune effect.[167]

The studies described above suggest the feasibility of a micRNA immune system in eukaryotes against infecting viruses. Investigations currently exploring this possibility will be described in a later section.

E. RNA Secondary Structure

Although the reader may find several detailed reviews[168-172] describing various aspects of the secondary structures of RNAs, the following is a brief summary of just a few examples of secondary structure effects similar to those mediated by antisense RNAs on gene expression. In addition, the reader is referred to a recent review by Inouye[6] for a discussion on strategies and potential problems in designing artificial antisense RNAs.

1. ermSF

The inducible determinants for macrolide-lincosamide-streptogramin B (MLS) antibiotic resistance in a wide range of bacterial species are the *erm* genes.[173-178] An adenine methylase encoded by the *erm* gene confers MLS resistance by methylating adenine in 23S rRNA, which results in the reduction of affinity between the 50 S ribosomal subunit and the antibiotic.[173,174,177,178] Expression of *erm* is regulated by a posttranscriptional mechanism and is inducible by nanomolar concentrations of MLS antibiotics.[175,176]

As in the case for other *erm* determinants (e.g., *ermC*, *ermA*, *ermAM*, *ermD*, and *ermG*,[175,176,179-183] *ermSF* expression is controlled by a translational attenuation mechanism.[184,185] A 385-base 5′-terminal leader region on the *ermSF* mRNA precedes the 960-base major open reading frame encoding the methylase. This leader contains several inverted complementary repeat sequences that, by intrastrand pairing, can form alternative secondary conformations. In the inactive conformation, the SD sequence is sequestered within a paired region, preventing access by the ribosome.[186] Hence, analogous to antisense RNA inhibition, masking the ribosome binding site inhibits the translation of the methylase coding sequences. In the case of *erm* induction, the inducing antibiotic complexes with and inhibits ribosomes traversing the leader region. This causes stalling of the ribosomes as they synthesize the leader peptide, and the downstream mRNA refolds into an active conformation that exposes the methylase SD sequence. Mutational analysis by deletions in the attenuator region have confirmed this model; in addition, the tanslational attenuation mechanism concurs with the interpretation of the phenotypes of several constitutively MLS-resistant *ermSF* mutants.[186]

2. gnd

The posttranscriptional regulatory mechanism of *gnd* gene expression in *E. coli* is similar to that described above for *ermSF*.[187] The expression of *gnd* (which encodes the enzyme 6-phosphogluconate dehydrogenase [6PGD]) is dependent upon cellular growth rate, whereby 6PGD levels are repressed under slow growth conditions and induced with increasing growth rate.[188-190]

Deletion analysis of *gnd-lacZ* translational fusions has been used to define a regulatory element lying within the "internal complementary sequence" (ICS; codon 67 to codon 78), a region of the *gnd* mRNA that contains sequences highly complementary to the nucleotides surrounding the ribosome binding site (including the SD sequence). This 16-nt control element

is able to confer growth rate-dependent regulation on the *gnd-lacZ* fusions by participating in the formation of a secondary structure that sequesters the SD sequence under slow growth conditions. It has hence been described as a "*cis*-acting antisense RNA".[187] The secondary structure formed is particularly unusual in that the ICS and its target ribosome binding site are separated by a distance of greater than 200 nucleotides.[187]

The correlation between *gnd* expression and growth rate is suggested to be dependent on the uncoupling of transcription and translation, as well as on the pool of ribosomes available to compete with the ICS to initiate translation.[187] Uncoupling is achieved during conditions of slow growth (i.e., on acetate media) as a result of the transcriptional rate being greater relative to the translational rate.[191] Conversely, as growth rate increases, the rate of translation increases while the transcriptional rate remains constant; coupling is then restored.[191] In parallel, the concentration of ribosomes increases during enhanced growth so that the ribosomes can compete efficiently for the ribosome binding site, and the SD sequence is less frequently sequestered by the ICS.[187,192,193]

While the *gnd* ICS inhibitory action is similar to that of antisense RNAs, the element is thought to work only in *cis*, since the presence of an additional ICS element on a high copy number plasmid has no effect on the regulation of a wild-type chromosomal *gnd* gene. Furthermore, wild-type *gnd* provided in *trans* does not complement a growth rate-derepressed translational fusion lacking the ICS.[194]

III. REGULATION BY ANTISENSE RNA IN EUKARYOTIC SYSTEMS

A significant volume of information has accumulated on the utilization of artificial antisense RNAs to regulate the expression of both endogenous and exogenous genes in a variety of eukaryotic systems (see Tables 1 and 2). Antisense technology has been particularly suited for examining the function of specific genes in biochemical and/or developmental pathways, where conventional conditional mutants are difficult to isolate.

In vitro inhibition of mRNA translation by complementary DNAs was demonstrated in earlier experiments.[195,196] Soon thereafter, viral replication and cell transformation by Rous sarcoma virus (RSV) was shown to be inhibited in chick embryo fibroblast tissue cultures by synthetic oligodeoxynucleotides complementary to RSV terminal repeats.[197] Furthermore, direct inhibition of viral RNA translation by these oligonucleotides was demonstrated *in vitro*.[197] These experiments gave way to subsequent numerous oligonucleotide-inhibition studies, many of which have led to the development of various

Table 1
Regulation by Microinjection of Antisense RNA

Targeted gene	Host cell	Method	Complementation	Ratio[a]	Inhibition[b]	Ref.
Xenopus β-globin	*Xenopus* oocyte	Microinjection of sense and antisense RNAs	Full; 5′ end	50:1	100%[p,c] 90%[p,d]	237 237
CAT	*Xenopus* oocyte	Coinjection of sense and antisense RNAs	Full; 3′ end	10:1 10:1	90%[e] 66%[e]	221 221
Xenopus protein L1	*Xenopus* oocyte	Microinjection of antisense RNAs	Full; 3′ end just as effective	1000:1	98%[p] in 12 h 100%[p] 24 and 48 h	242 242
Drosophila Kruppel	*Drosophila* embryo	Microinjection	Full cDNA	1000:1 50:1	50% Phenocopy[k] ~4% Phenocopy[k]	238 238
Drosophila wingless	*Drosophila* embryo	Microinjection	3′ Half (1.5 kb)	—	Phenocopy[w]	239
Xenopus An1, Vg1	*Xenopus* embryo	Microinjection	Full	200 to 400:1	Unwinding activity	244 244
CAT	*Xenopus* embryo	Coinjection of sense and antisense RNAs	Full	100 to 1000:1	Unwinding activity	245 245
Mouse t-PA	Mouse oocytes	Microinjection	3′ Noncoding	4:1	~90%[e]	240

[a] Antisense RNA transcripts to sense RNA transcripts.
[b] e: % Reduction of enzyme activity.
p: % Reduction of protein steady-state level.
[c] Injection of antisense RNA followed by sense RNA.
[d] Injection of sense RNA followed by antisense RNA.
[k] Kruppel mutation.
[w] Wingless mutation.
— Not determined.

Table 2
Regulation by Artificial Antisense RNA Genes

Target gene	Host cell	Promoter	Complementarity to target	Ratio[a]	Inhibition[b]	Ref.
HSV-TK	Mouse Ltk$^+$ cells	Moust MT-1	3′ End	300:1^r	80—90%^{a}	202
HSV-TK	Mouse L cells	HSV-TK MSV-LTR	1.3 kb Coding	100:1^g	4—5×^{e}	200
5′ HSV-chicken TK fusion gene	Mouse L cells	HSV-TK	5′ Noncoding	200:1^g	100%^{e}	201
EndoB cytokeratin	F9 Embryonal carcinoma cells	HSV-TK	5′ End	5:1^r	50%^{p}	207
Mouse HPRT	COS cells	AdMLP	Intron1 + exon1 full cDNA	—	>99%^{e} >99%^{e}	208
Human *c-myc*	Human HL-60 cells	SV40	Full (−exon1)	15:1^r 10:1^g	>90%^{p}	213
Human *c-fos*	Mouse 3T3	MMTV LTR	0.19 kb 5′ end	—	Phenotypic	210
Mouse *c-fos*	Mouse 3T3	MMTV LTR	5′ Noncoding + 0.29 kb exon1	50:1^g	95%^{r}; Phenotypic	212
Rat pp60*c-src*	Rat 3T3	Mouse MT-1	Full	—	80—90%^{p}	211
SV40 large T-ag	COS1 cells	Ad-VA1	0.145 kb 5′ end	—	50—95%^{t}	203
c-*raf*-1	SQ-20B	Ad2-MLP	Full cDNA	2:1^g	>90%^{r}; Phenotypic	214
RSV *env*	R(-)Q cells	RSV LTR	*env* Coding + 5′ noncoding	12:1^g	80%^{ffu}	250
CAT	Mouse Ltk$^-$ cells	MSV LTR	Full	5:1^g	5—20×^{e}	201
Protein 4.1	*Xenopus*	MLV LTR	3′ cDNA	—	Phenotypic	220
*Drosophila hsp*26	*Drosophila* cells	hsp70	5′ Noncoding + 155a.a. coding	50—1000:1^g 2—10:1^r	90%^{p}	215
Drosophila rpA1	*Drosophila* cells	hsp70	Full cDNA	—	Phenotypic	217
Dictyostelium discoidin-1	*Dictyostelium* cells	Disc.1-a	0.32 kb Coding	50:1^g	>90%^{r,p}	218
Dictyostelium MHC	*Dictyostelium* cells	Actin-6	Full	—	250×^{p}; phenotypic	219
CAT	Carrot protoplasts	NOS CaMV 35S Carrot PAL	Full	100:1^g	>95%^{e}	204
Nopaline synthase	Tobacco plants	CaMV 35S	5′ Two thirds	10:1^r	10— 50×^{e} 8—10×^{r}	205
Chalcone synthase gene A	Petunia plants Tobacco plants	CaMV 35S	Full coding	—	Phenotypic	222
CAT	Tobacco plants	CaMV 35S	Full	1:2— 20^r	100%^{e}	206
			0.225 kb 5′ end		>60%^{e}	206
		rbcS	Full	1:10^r	None	206
Tomato PG	Tomato plants	CaMV 35S	5′ End	1:1^g 3:5^r	90%^{e} 94%^{r}	223
Tobacco RUBISCO	Tobacco plants	CaMV 35S	5′ End	1—4:3^g	67— 88%^{r} 40—63%^{r}	224
CMV coat protein	Tobacco plants	CaMV 35S	Full coding	—	s	252
PVX coat protein	Tobacco plants	CaMV 35S	Full cDNA	—	s	253
Myelin basic protein	Transgenic mice	Mouse MBP	1.2 kb cDNA	—	Phenotypic	209

[a] r: Ratio of antisense RNA transcripts: target RNA transcripts.
g: Ratio of antisense gene copy number: target gene copy number.
—: Not determined.

[b] e: % Reduction of enzyme activity.
p: % Reduction of protein steady-state level.
r: % Reduction of RNA steady-state level.
t: % Reduction of T-ag-dependent plasmid replication.
ffu: % Reduction of focus-forming units.
s: Cross-protection observed at low inoculum concentrations.
phenotypic: inhibition observed as phenotypic changes.

modifications that enhance such features as cellular uptake and resistance to nucleases. While oligonucleotide inhibition is not discussed here, the reader is referred to several articles that provide detailed reviews.[198,199]

A. Artificial Antisense Genes

1. Suppression of Exogenous and/or Transformed Genes

a. MAMMALIAN CELLS

The first study demonstrating the inhibition of specific gene expression by an artificial antisense RNA gene in a eukaryotic system utilized the herpes simplex virus (HSV) thymidine kinase (TK) gene as a target.[200] Artificial antisense genes were constructed on plasmids by the insertion of the TK structural gene, in an inverted orientation, between the HSV-TK promoter (or the murine sarcoma virus [MSV] long terminal repeat [LTR] promoter) and an SV40 polyadenylation signal. Coinjection of these antisense gene constructions with plasmid carrying the wild-type TK gene at a ratio of 100:1, respectively, resulted in a significant reduction of transient TK expression in mouse LTK$^-$ cells. TK activity was detected at four- to fivefold lower levels (compared with cells coinjected with control plasmid and wild-type TK) for cells synthesizing a 1364-base antisense RNA complementary to sense message from +51 to +1415.[200]

Additional studies that sought to determine the inhibitory function of shorter anti-HSV TK sequences gave results not unlike those observed for the prokaryotic SP phage experiments.[165,166] A plasmid containing the 5′ portion of the HSV TK gene (from −80 to +343) in reverse orientation driven by the MSV LTR promoter gave comparable levels of inhibition as those observed for antisense plasmids covering nearly the entire TK protein coding domain.[200,201] Furthermore, when a hybrid HSV-chicken TK fusion gene construct was coinjected into LTK$^-$ cells with the 5′TK antisense plasmid, complete inhibition of TK expression was observed. The chimeric gene contained the HSV-TK promoter sequences followed by 52 bp of the 5′ untranslated region fused to the chicken TK structural gene. Hence, only 52 bases of complementarity, excluding the initiator AUG codon, between the 5′TK antisense and HSV-chicken TK transcripts was sufficient for suppression of TK activity. In addition, the sequence specificity of antisense inhibition was evinced by the observation that while anti-HSV TK inhibited HSV-TK expression in LTK$^-$ cells, the expression of chicken TK, which shares no sequence homology with HSV TK, is not affected. Likewise, an anti-chicken TK construct suppressed chicken TK expression, but had no effect on HSV TK activity.[201]

Similar results as those described above were obtained when antisense vs. sense plasmids were cointroduced in LTK$^-$ cells by calcium phosphate transfection.[201] Furthermore, conditional inhibition of TK activity was demonstrated by placing the anti-HSV TK sequences under control of the MMTV LTR promoter. Upon induction with dexamethasone, a dose-dependent reduction in TK activity was observed.[201]

In another experiment, Izant and Weintraub[201] transfected the anti-HSV TK and, as a control, anti-chicken TK plasmids into L cells previously transformed to the HSV-TK$^+$ phenotype. The TK activity expressed from the chromosome was detected at sixfold lower levels in cells transfected with anti-HSV TK, compared with TK activity in control cells transformed with anti-chicken TK.[201]

In an independent study, Kim and Wold[202] designed an elegant construct to produce high steady-state levels of antisense TK transcripts in LTK$^+$ cells. Antisense TK sequences were transcriptionally fused to the dihydrofolate reductase (DHFR) protein coding region. Cells resistant to selection by progressively higher levels of methotrexate (due to overproduction of DHFR) were found to express high levels of the chimeric DHFR-antisense TK transcript. TK activity in these cells was inhibited by as much as 90%.[202]

Transient inhibition of large T-antigen gene expression by an antisense RNA transcribed by RNA polymerase III was demonstrated in COS1 cells by Jennings and Molloy.[203] In these experiments, the RNA polymerase III-dependent adenovirus VA1 gene promoter was fused to a 163-bp inverted SV40 sequence complementary to the 5′ end of the large T-antigen gene. Introduction of this construct into COS1 cells resulted in the inhibition of T-antigen-dependent replication of a cotransfected plasmid containing a SV40 replication origin. Although the suppression was transient, nearly complete inhibition (95%) of plasmid replication was observed in some experiments, indicating that expression of the chromosomal T-antigen gene could be substantially blocked by an RNA polymerase III-produced antisense transcript.[203] It has been suggested[203] that RNA polymerase III promoter-driven antisense RNA constructs may be a utile alternative to conventional RNA polymerase II promoter constructs, based on the high sequence conservation of polymerase III promoters and their activity in all cell types. Furthermore, the abundant synthesis of the normal transcription products of polymerase III promoters opens the speculation that large amounts of antisense RNAs may also be produced from these promoters.

Transient antisense inhibition of the bacterial chloramphenicol acetyltransferase (CAT) gene was achieved in LTK$^-$ cells. Upon transfection of antisense and sense CAT DNA (driven by the MSV LTR promoter) at a 5:1 ratio, there resulted a 5- to 20-fold reduction in CAT activity in comparison to cells transfected with sense CAT and antisense HSV or chicken TK plasmids.[201] From these experiments it was apparent that antisense inhibition in eukaryotic cells could be applied to an exogenously introduced prokaryotic gene.

b. PLANTS

The antisense approach is particularly suited for the characterization and/or identification of specific genes in plants and

provides a useful alternative to classic selection methods for the isolation of mutations in genes from multigene families. Antisense inhibition in the plant system was first reported by Ecker and Davis,[204] on the suppression of CAT gene expression in transiently transformed carrot protoplasts. Electroporation of antisense CAT plasmids and sense CAT plasmids into protoplasts at a ratio of 100:1, respectively, resulted in >95% reduction of CAT activity. A comparison of antisense constructs containing three different promoters (nopaline synthase [NOS], cauliflower mosaic virus [CaMV] 35 S RNA, or carrot phenylalanine ammonia-lyase [PAL]) revealed a correlation between promoter strength and degree of target gene inhibition.

Following the initial study above, antisense inhibition of transformed genes was demonstrated in transgenic plants. Introduction of a *nos* antisense gene into the genome of tobacco plants previously transformed with the wild-type *nos* gene resulted in a decrease in NOS enzyme activity (8 to 50×) as well as in *nos* steady-state mRNA levels (8 to 10×). The inhibitory activity was also present in the progeny of the transformed plants, indicating the stable inheritability of the antisense RNA-mediated effect.[205]

Transformation of transgenic tobacco plants constitutively expressing CAT activity with antisense CAT DNA showed an analogous correlation (see Reference 204) between the levels of antisense RNA synthesis and CAT inhibition. A selection strategy for plants producing high levels of antisense transcripts was applied in these experiments by designing a transcriptional gene fusion in which the inverted CAT sequences are inserted between the coding region of the hygromicin-resistance (*hyg*^{r}) gene and the 3′ end of the *nos* gene. High-level expression of *hyg* RNA, and, hence, high levels of antisense CAT RNA, was achieved by selection of regenerated plants in media containing high concentrations of hygromicin.[206] This strategy is similar to the chimeric DHFR-antisense TK transcript employed by Kim and Wold[202] in mammalian cells.

The successful antisense inhibition in transformed plants is not limited to exogenously introduced genes. The experiments described above set the precedent for the application of antisense technology in the regulation of specific endogenous plant genes to produce desirable characteristics, as well as in the development of viral immune systems. A discussion of these topics can be found in a later section.

2. Suppression of Endogenous Genes

a. MAMMALIAN CELLS

As mentioned previously, antisense RNA inhibition can be applied toward the identification and/or characterization of specific endogenous genes. Although many of the initial antisense studies in eukaryotic systems were targeted toward the inhibition of experimentally introduced genes, subsequent research has often focused on examining the suppression of various endogenous genes.

Inhibition of actin gene expression was tested in LTK$^-$ cells by introducing plasmids carrying antisense β-actin sequences fused to the MSV LTR promoter. Cell proliferation was found to diminish in LTK$^-$ cells transformed with anti-β-actin. Visual staining of BSC-1 cells injected with anti-β-actin DNA revealed marked reductions in actin microfilament cables.[201]

In their studies of the developmentally regulated endo B cytokeratin, Oshima and colleagues[207] utilized a retrovirus vector to introduce antisense endo B sequences into F9 embryonal carcinoma (EC) cells. F9 EC cells were infected by a vector containing a *neo* gene driven by the 5′-Moloney MSV LTR, followed by anti-endo B sequences (complementary to the first 625 bp of endo B cDNA) under control of the HSV TK promoter and a mutant polyoma enhancer. Although clones expressing anti-endo B RNA from the internal promoter could not be obtained, endo-B protein expression was found to be suppressed in neomycin-resistant clones expressing the colinear neomycin-antisense transcript.[207]

Reduction of endogenous hypoxanthine phosphoribosyltransferase (HPRT) activity was attained in mouse cells transfected with plasmids carrying anti-mouse HPRT sequences under the control of an adenovirus major late promoter spliced to the adenovirus tripartite leader.[208] Clonal isolates resistant to 6-thioguanine (due to inhibition of HPRT activity) were found to have reduced levels of HPRT mRNA and high levels of antisense RNA. Antisense sequences complementary to the 5′ end of the HPRT gene or sequences complementary to HPRT cDNA were equally effective in reducing HPRT expression.[208]

The first successful whole-animal model of antisense inhibition was demonstrated by Katsuki et al.[209] An antisense plasmid targeting the myelin basic protein (MBP) gene was constructed to study the effect of anti-MBP DNA on myelination in transgenic mice. The mutant shiverer mouse harbors an autosomal recessive mutation in the MBP gene that results in its MBP deficiency and hypomyelination. MBP cDNA expressed in the transgenic shiverer mouse brain had been shown to rescue the shiverer phenotype (see Reference 209 and references cited therein). A mini-gene fragment including a 1.2-kb anti-MBP cDNA sequence under the control of the mouse MBP promoter was injected into mouse zygotes that were then implanted into pseudopregnant females. Although the 5 transgenic founder mice appeared to be normal, it was found that when one of the male founders was mated to a wild-type female mouse, 10 of the 21 transgenic offspring were converted from the normal to mutant shiverer phenotype. Endogenous MBP mRNA levels were found to be decreased in these transgenic mice compared with the levels in nontransgenic littermates, and there appeared to be a correlation between reduction of MBP mRNA, of MBPs, and of myelination and mutant phenotype appearance. The presence of antisense RNA in all of the transgenic mice suggests that the decreased levels of endogenous MBP transcripts, and hence the tremor phenotype may be attributed to antisense inhibition.[209] The introduction of antisense genes into the germline of animals, as demonstrated in

the above study, facilitates the examination of the biological and developmental functions of specific cloned genes *in vivo*. Furthermore, by utilizing such features as an inducible promoter in the antisense gene construct, it may be possible to obtain transgenic animals that are homozygous for certain lethal mutations.

Antisense RNA inhibition has been found to have useful applications in studying proto-oncogene function. The technique is an effective alternative to the limited use of, for example, specific antibodies directed against proto-oncogene products. Steroid-inducible antisense c-*fos* gene constructs were stably introduced into mouse 3T3 cells to examine the function of the c-*fos* gene in growing fibroblasts.[210] Dexamethasone-induced production of anti-c-*fos* RNA complementary to the 5′ regions of the mouse or human c-*fos* gene resulted in significant reductions in colony formation and inhibited cell growth. Suppression of colony formation and cell proliferation did not occur for the induced production of sense c-*fos* transcripts in 3T3 cells. In this study, the use of the inducible MMTV promoter for antisense RNA production (see also Amini et al.[211] below) enabled the examination of a gene for which constitutive suppression may otherwise render it recalcitrant to analysis. In a subsequent independent study, dexamethasone induction of 3T3 cells transformed with multiple copies of antisense c-*fos* DNA prevented the large increase in c-*fos* mRNA and protein following platelet-derived growth factor stimulation of quiescent cells.[212] DNA replication was also greatly diminished in these cells, indicating that the expression of anti-c-*fos* RNA prevents the transition from the quiescent state into the normal cell cycle of growth and division. These observations demonstrated the requirement for the large increase in c-*fos* expression in quiescent cells following growth factor stimulation for renewed cell growth.[212]

The potential role of the c-*src* gene product in polyomavirus (Py)-mediated oncogenic transformation of rat cells was investigated by introducing antisense c-*src* gene constructs into Py-transformed FR3T3 cells.[211] The c-*src* proto-oncogene encodes a product, $pp60^{c\text{-}src}$, which possesses a protein kinase activity that is thought to play an important function in Py transformation by interacting with the Py-encoded middle tumor antigen (MTAg) (Reference 211 and references cited therein). Continued expression of MTAg is a prerequisite for Py transformation (Reference 211 and references cited therein). Production of anti-c-*src* RNA in FR3T3 cells resulted in an 80 to 90% decrease in $pp60^{c\text{-}src}$ synthesis with a concomitant reduction in $pp60^{c\text{-}src}$ protein kinase activity. Although expression of anti-c-*src* RNA did not result in reversion of transformed cell phenotype to normal FR3T3 phenotype, other parameters (i.e., focus formation, anchorage-independent growth, and tumor growth upon injection into syngenic rats) were found to be characteristic of a less transformed phenotype.[211]

In human promyelocytic leukemia HL-60 cells, high-level expression of antisense RNA from a stably integrated human anti-*myc* gene inhibited steady-state levels of *myc* protein by 90% compared with control cells.[213] As a result of antisense-mediated reduced *myc* gene expression, HL-60 cells appeared to be committed to monocytic differentiation as opposed to granulocytic differentiation. The presence of RNA duplexes in the nuclear fraction of antisense *myc* clones, and not in control clones, suggested that the complementary transcript exerts its inhibitory effect on *myc* protein synthesis by forming a stable hybrid with the target mRNA. In addition to the reduction in *myc* protein, accumulation of *myc* mRNA was diminished in clones expressing high levels of antisense transcripts. Gene fusion experiments and run-on transcription assays demonstrated that inhibition of expression in antisense transformants also occurred at a transcriptional level. The transcriptional target region that is thought to be recognized by the antisense RNA was localized to an enhancer-like 920-bp fragment of the *myc* gene promoter.[213]

The function of the endogenous c-*raf*-1 gene was investigated in radiation-resistant human laryngeal squamous carcinoma cells (SQ-20B) transfected with anti-*raf* sequences fused to the adenovirus 2 major late promoter.[214] Although the *raf* oncogene had been associated with radioresistant cancer cells (Reference 214 and references cited therein) the lack of direct evidence showing oncogene modulation of radiation sensitivity substantiates the utility of antisense inhibition in assessing *raf* function. A significant reduction in steady-state levels of endogenous c-*raf*-1 transcripts was observed in SQ-20B cells transfected with full-length antisense *raf* constructs. Reduced expression of c-*raf*-1 resulted in a decreased rate of tumor growth (compared with control cells) for anti-*raf* transfected cells that were inoculated into nude mice. Cells transfected with sense c-*raf*-1 constructs exhibited increased tumor growth after inoculation. This *raf* expression-dependent modulation in tumorigenicity indicated the direct role of the c-*raf*-1 gene product in malignant phenotype exhibition. In addition, anti-*raf* transfected cells were rendered more sensitive to radiation, implying an indirect role of *raf* with radiation response of tumor cells.[214]

b. *DROSOPHILA*

McGarry and Lindquist[215] were able to inhibit a specific member of a closely related set of genes in *Drosophila* tissue culture cells using antisense RNA. Sequences complementary to the 5′ two thirds of the message for hsp26, one of the *Drosophila* small heat-shock proteins, were placed under the control of the strong, heat-inducible *hsp70* promoter. During heat-shock, cells stably transformed with the antisense gene had a significant reduction is hsp26 synthesis (44 to 11% of wild-type level), accompanied by decreased levels of *hsp26* RNA at only severalfold higher concentrations of antisense to sense message. Expression of *hsp22*, *hsp23*, and *hsp28*, all of which contain considerable sequence homology to *hsp26*, re-

mained unaffected, demonstrating the highly specific nature of the antisense inhibition.[215] In later experiments, an antisense gene directed against *hsp70* expression suppressed production of the protein by 75% of the wild-type level during heat shock. Conversely, overexpression of other heat-shock proteins occurred, and *hsp70* synthesis persisted for a longer period during recovery suggesting a role for *hsp70* in attenuation of the response (see Lindquist as cited in Walder).[216]

The *hsp70* promoter has also been used to direct the synthesis of antisense RNA targeting the ribosomal protein rpA1 message to investigate the developmental role of *rpA1* expression in *Drosophila*.[217] In order to study inhibition *in vivo*, the antisense gene was introduced into the genome of the fly by *P*-element transformation, thus enabling heat-shock-induced production of complementary transcripts specifically at critical stages of development. Induction of antisense RNAs resulted in a severe disruption of oogenesis. The rate of egg laying was decreased by approximately 70%, and a high percentage of "small egg" female-sterile phenotype was observed. These defects were dependent upon temperature, antisense gene dosage, and level of antisense expression. Furthermore, a general decrease of mRNA levels in the ovary was found to occur with high anti-*rpA1* expression, which was suggested to be responsible for the disruption of oogenesis.[217] The anti-*rpA1*-induced conditional female-sterile phenotype observed in these experiments demonstrates the applicability of antisense inhibition of genes that are not readily amenable to mutagenesis by conventional genetic approaches, and that may exhibit altered phenotypes upon inactivation only at specific developmental stages.

c. *DICTYOSTELIUM*

Antisense genes were transfected in *Dictyostelium discoideum* cells to inhibit the expression of the three endogenous discoidin genes.[218] Production of antisense RNA complementary to discoidin *1*-α (Disc *1*-α) mRNA from the Disc *1*-α promoter resulted in a greater than 90% reduction in accumulated discoidin mRNA and protein. In this study, sense and antisense RNA hybrids are believed to form in the nucleus and be rapidly degraded, based on the detection of only low levels of endogenous discoidin message on RNA blots and the absence of detectable steady-state levels of antisense transcripts. Both the endogenous and antisense RNAs were shown to be transcribed from nuclear run-on assays. Antisense transformants exhibited a nonstreaming phenotype similar to that of discoidin-minus mutants.[218]

Knecht and Loomis[219] utilized antisense inhibition to study the function of myosin in *Dictyostelium*. Cells transformed with an antisense myosin heavy chain A (*mhc* A) gene transcribed from the *Dictyostelium* actin-6 promoter exhibited a slow-growing, abnormally large and multinucleate phenotype, indicative of an impairment in cytokinesis. MHC A protein levels were dramatically reduced by as much as 250-fold in antisense transformants. As a consequence of the lack of myosin, the sequence of developmental events following starvation was severely disrupted in anti-*mhc* A cells and, unlike control cells, did not exhibit chemotactic streaming. As in the discoidin study described above, antisense inhibition of *mhc* A complements observations of *mhc* A mutant phenotypes that may be obtained by other means, facilitating the characterization of a developmental gene.

d. *XENOPUS*

Inhibition of *Xenopus* membrane skeleton protein 4.1 expression was studied in fertilized *Xenopus* eggs microinjected with antisense protein 4.1 plasmids in order to examine the significance of protein 4.1 during embryonic development.[220] The expression of antisense protein 4.1 RNA caused a specific reduction of endogenous protein 4.1 transcripts after midblastula transition, whereas the levels of three unrelated transcripts remained unaffected. The dramatic decrease in endogenous message was concomitant with a greatly reduced accumulation of protein 4.1. The introduction of artificial partial-length sense protein 4.1 plasmids immediately after the first injection of antisense plasmids blocked the inhibitory effect, presumably by competing with endogenous wild-type message for hybridization to the antisense RNA. Thus, it is apparent that excess antisense RNA is required for effective inhibition of protein 4.1 RNA. The reduction of the membrane skeleton protein expression disrupted the normal cellular interactions in the retina between the photoreceptor outer segment and the pigment epithelium.[220] The success of the protein 4.1 inhibition may partly be ascribed to the insertion of the antisense sequences into the coding region of the CAT gene, which was previously shown to be expressed in embryos after midblastula transition under the control of the murine sarcoma virus long terminal repeat (MSV LTR).[220,221] The antisense transcript was therefore expected to appear in both the nuclear and cytoplasmic compartments as a normal messenger ribonucleoprotein as opposed to deproteinized RNA.[220]

e. PLANTS

van der Krol and colleagues[222] first reported the inhibition of an endogenous gene in plants using antisense RNA. An antisense petunia chalcone synthase (CHS) gene under the control of the CaMV 35S promoter was constructed to regulate the expression of CHS, a key enzyme in flavonoid biosynthesis. Constitutive expression of the anti-CHS gene in transgenic petunia and tobacco plants resulted in decreased amounts of CHS protein and steady-state mRNA. The reduced levels were found to correlate with phenotypic alterations of flower pigmentation. Different types of pigmentation patterns were observed among the flowers in both the transgenic petunia and tobacco plants, which did not appear to correspond necessarily to variances in antisense RNA steady-state levels. Furthermore, as the levels of anti-CHS transcripts were independent of anti-CHS gene copy numbers, it is suggested that DNA sequences

adjacent to the insertion site of the antisense gene may influence its activity both quantitatively and qualitatively. Backcrossing experiments and Southern analysis of progeny plants demonstrated the stable inheritance and co-segregation of the antisense phenotype.[222]

Expression of the endogenous, developmentally regulated polygalacturonase (PG) gene has also been inhibited in transgenic tomato plants.[223] PG, which is synthesized *de novo* from accumulated PG mRNA transcripts in ripening fruit, partially solubilizes cell wall pectin and plays a key role in fruit softening. A hybrid antisense gene consisting of an inverted 5′ 730-bp PG cDNA fragment (including a 50-bp untranslated region and initiation codon) fused to the CaMV 35 S promoter and followed by the 3′ end of the *nos* gene was introduced into tomato stem segments. Antisense PG RNA produced in transformed plants caused a reduction in PG mRNA and PG protein levels in ripe fruit. PG enzyme activity was inhibited by as much as 90% compared to ripe control fruit. As in the case of the CHS experiments, the antisense PG gene (and concomitant reduction in PG activity) is stably inherited. In addition, positional effects consequential to particular DNA insertion events is thought to be responsible for variations in steady-state antisense RNA levels that may influence antisense effects in different transformants.[223]

Rodermel et al.[224] have demonstrated the successful antisense suppression of an abundantly transcribed and expressed chloroplast enzyme, ribulose bisphosphate carboxylase (RUBISCO). The holoenzyme is composed of small subunits (SS, encoded by a nuclear multigene [*rbc*S] family) and large subunits (LS, encoded by a single chloroplast gene [*rbc*L]). In order to determine the possible effect of *rbc*S mRNA or SS protein levels on *rbc*L transcription or LS protein translation and accumulation, a *rbc*S antisense RNA expression vector was transformed into tobacco plants. Expression of anti-*rbc*S sequences in transgenic plants resulted in as much as an 88% reduction in *rbc*S mRNA levels and up to a 63% decrease in SS protein levels. Furthermore, the accumulation of LS protein (but not LS mRNA) was also depressed, and the coordinate reductions in LS and SS protein appeared to be associated with the decreased *rbc*S mRNA levels. Because pulse-labeling studies indicate equal incorporation of label into LS in transformed and control plants, and *rbc*L mRNA levels are normal, it is likely that accumulation of LS protein is regulated by translational and posttranslational factors (e.g., degradation of excess amounts of LS in the chloroplast to maintain LS and SS stoichiometry).[224] Southern hybridization experiments were performed with several progeny from a transformant with the most severely depressed levels of *rbc*S mRNA and RUBISCO holoenzyme. A negative correlation was observed between growth rate and apparent copy number of antisense gene sequences in the genomes of these plants, suggesting that the greater the antisense gene dosage and expression, the slower the plants grow. The study described above demonstrates the high potential of antisense technology in suppressing the accumulation of one of the most abundant leaf mRNA species and the most abundant soluble protein in plant cells.[224-226]

3. Antisense Ribozymes

The discovery of naturally occurring RNA molecules capable of undergoing autolytic cleavage has instigated the development of antisense RNAs possessing highly specific cleavage activities. Self-catalyzed cleavage reactions have been described for a variety of RNA molecules, including avocado sunblotch viroid RNA, several plant virus satellite RNAs, the RNA transcripts of a tandemly repeated sequence in newt, and the genomic and antigenomic RNAs of hepatitis delta virus.[227-232] The general consensus secondary structure (a "hammerhead" structure) has been described for some of these RNA enzymes, known as ribozymes,[233] whose cleavage reactions produce a 5′-hydroxyl terminus and a 2′,3′ cyclic phosphate group.[228,230-232,234] Furthermore, as the substrate requirement for cleavage is dictated only by a GUX sequence in the mRNA,[230,235] highly specific ribozymes can be designed by the inclusion of antisense RNA extensions in the 5′ and 3′ directions from the catalytic hammerhead. Hybridization of the antisense RNA "arms" of the molecule to a specific site of the target mRNA followed by cleavage by the ribozyme component would ensure highly specific and efficient inactivation of the transcript.

Haseloff and Gerlach[236] first reported the design and construction of such ribozymes targeted against three sites within the CAT mRNA sequence. The self-cleaving component from the (+) strand of satellite tobacco ringspot virus (sTobRV) RNA was designed to contain eight nucleotide-long flanking sequences complementary to three specific target sites of the CAT mRNA. The target sites were chosen to include the conserved GUC sequence immediately 5′ to the cleavage site. Each of the antisense-ribozyme transcripts (synthesized *in vitro* from T7 RNA polymerase) gave sequence-specific cleavage resulting in two RNA fragments when incubated with an 835-nt CAT transcript *in vitro*. Substrate cleavage was shown to occur in a catalytic manner, with each ribozyme participating in greater than ten cleavage events. The resulting CAT RNA fragments, whose sizes were consistent with the predicted cleavage sites, also possess 5′ terminal hydroxyl groups similar to those produced in natural autocatalytic reactions of sTobRV.[236] The above study demonstrates the powerful potential of antisense-ribozyme application both *in vitro* and *in vivo*.

B. Microinjection of Antisense RNA

The injection of *in vitro* synthesized transcripts has been utilized by researchers as an alternative method of supplying antisense RNA for the analysis of genes in cells that are not transcriptionally active. In addition, direct injection enables the introduction of a large quantity of antisense RNA for target mRNA inhibition in systems such as *Xenopus* oocytes, which

require ~50- to 100-fold excess of antisense transcript relative to mRNA[221,237] (see also below). Inhibition of target mRNA function by injected antisense transcripts is believed to occur in the cytoplasm, whereas antisense RNAs transcribed from a stably integrated gene (as described in the previous section) may render its effect in the nucleus or cytoplasm. As in the studies with antisense genes, microinjection of antisense RNA also gave rise to phenocopies of natural mutants.[238,239]

1. Suppression of Exogenously Introduced mRNA

Melton[237] first reported the inhibition of mRNA translation in *Xenopus* oocytes by microinjected antisense RNAs. Antisense β-globin RNA introduced into the cytoplasm at a 50-fold higher level than sense β-globin mRNA (which was injected 5 h later) completely blocked translation. While globin production was undetectable in these oocytes, general protein synthesis was not disrupted. Globin protein was not made when antisense β-globin RNA and globin mRNA were coinjected, presumably due to prior duplex formation. However, if β-globin message is injected first and incubated 5 h before a second injection of antisense β-globin RNA, significant reduction (>10×) of globin synthesis still occurs. Additional experiments indicated that the translational inhibition is due to antisense-sense RNA hybridization *in vivo*. The effective target region of the mRNA appeared to be localized to the 5′ end where ribosomes bind and initiate translation; hence, the antisense transcripts are capable of inhibiting initiation, but not elongation of protein synthesis.[237]

Antisense RNAs were also injected into *Xenopus* oocytes to inhibit translation of herpes TK mRNA and the bacterial CAT mRNA.[221] Injection of a mixture of CAT and TK sense mRNAs 5 to 6 h after the cytoplasmic injection of either full-length antisense CAT or full-length antisense TK RNA resulted in a specific inhibition of mRNA translation. Antisense CAT RNA inhibited CAT expression without affecting TK activity, while antisense TK RNA inhibited expression of TK but not CAT mRNA. A truncated antisense CAT RNA that left 154 bases of 5′ sense mRNA exposed (including the initiation codon and 37 amino acid coding sequences) was less effective at inhibiting CAT expression than a full-length antisense transcript. In additional experiments, CAT activity from an expression plasmid injected into the oocyte nucleus was also inhibited by antisense CAT RNA injected into the cytoplasm.[221]

2. Suppression of Endogenous mRNA

a. MAMMALIAN CELLS

Strickland and colleagues[240] utilized antisense RNA inhibition to investigate the translational activation of dormant t-PA (tissue plasminogen activator) mRNA during mouse oocyte maturation. During meiotic maturation, the stable t-PA message (which remains untranslated in the primary oocyte) acquires an extensive 3′ poly-A tail (~500 adenosine residues), is concomitantly translated, and subsequently degraded. Primary oocytes were microinjected with antisense RNAs directed against three different regions of the t-PA mRNA and assayed to t-PA activity following maturation *in vitro*. Antisense transcripts complementary to all three targets (i.e., [1] 5′ noncoding region + 115 nt coding sequence including the AUG; [2] middle coding; and [3] 3′ noncoding) inhibited t-PA expression in maturing oocytes. The 3′ antisense RNA was the most effective, inhibiting the generation of t-PA activity by as much as 90% at only a fourfold excess of antisense to sense concentration. Furthermore, injection of an antisense RNA complementary to only the 3′ terminal 103 nt inhibited translational activation as well as polyadenylation and destabilization of t-PA mRNA. RNA blot analysis revealed the possible presence of a double-strand-specific ribonuclease activity that cleaves the antisense-sense RNA duplex, yielding a stable 5′ fragment and an unstable 3′ fragment. Injections of 3′ antisense RNAs of differing lengths resulted in mRNAs shortened by the respective lengths of hybridization. In contrast, the 5′ and middle antisense transcripts did not induce mRNA cleavage in primary oocytes. Based on these experiments, the 3′ noncoding sequences appeared to be crucial for the regulated expression of t-PA mRNA.[240] More recent studies utilizing chimeric urokinase plasminogen activator reporter mRNAs fused to different portions of the t-PA 3′ noncoding region have given evidence that the AAUAAA polyadenylation signal, regulated by sequences in its 3′ untranslated region, is necessary and sufficient for t-PA mRNA translational activation.[241] The importance of the 3′ noncoding region in the posttranscriptional regulation of t-PA mRNA is further emphasized by the highest efficiency of antisense transcripts complementary to the 3′ untranslated region.

b. XENOPUS

Antisense RNAs complementary to *Xenopus* ribosomal protein L1 mRNA were microinjected into *Xenopus* oocytes in order to examine the mechanisms of ribosomal protein synthesis and assembly during oogenesis.[242] Full-length antisense RNA transcribed from a 1.3-kb cDNA fragment was injected into the cytoplasms of stage VI oocytes at ~1000-fold molar excess relative to the endogenous L1 mRNA. Introduction of this antisense transcript (which spans the entire L1 coding sequence and 3′ untranslated region, but lacks the first ten residues of the 5′ noncoding region) resulted in ~50-fold reduction in L1 synthesis within 12 h. Production of L1 protein was completely suppressed 24 and 48 h after injection, while overall protein synthesis remained unchanged. RNase protection experiments revealed that 30 to 50% of L1 mRNA is found in RNA-RNA duplex form with the antisense species, an amount that closely approximates the steady-state level of polysomal L1 mRNA in these oocytes. This indicates that the observed inhibition of L1 synthesis was attributable to, and contingent upon, hybridization of antisense transcripts to the polysomal fraction of L1 mRNA, based on the assumption that only this

fraction is accessible for duplex formation in fully grown oocytes.[242]

Microinjection of partial-length antisense RNAs resulted in repression of L1 synthesis (>50×) comparable to that observed with full-length antisense RNA.[242] An antisense transcript complementary to only the coding region adjacent to the 3′ untranslated sequences inhibited L1 production as efficiently as full-length antisense RNA, indicating that complementarity to the 5′ noncoding region is not necessary for suppression of L1 expression. Furthermore, a short 140-nt antisense RNA targeted to the 5′ untranslated region and AUG initiation codon repressed L1 synthesis only 10% as efficiently as the full-length antisense RNA. Accordingly, this RNA does not form a detectable duplex with L1 mRNA *in vivo* (but does protect a 140-nt L1 mRNA fragment from RNase digestion *in vitro*), suggesting the inaccessibility of the 5′ terminus of L1 mRNA to initiate hybrid formation in the oocyte. Alternatively, the reduced degree of antisense inhibition may be due to the inability of the 140-nt transcript to form a stable hybrid with the target L1 mRNA *in vivo*.[242]

c. *DROSOPHILA*

Injection of antisense RNAs into wild-type *Drosophila* embryos has been demonstrated to produce phenocopies of several mutant phenotypes.[238,239,243] RNA complementary to a 2.3-kb cDNA fragment of the *Kruppel* (*Kr*) gene was injected into wild-type embryos at a specific brief stage during which *Kr* is transcribed into a rare 2.5-kb poly(A)$^+$ transcript.[238] A high frequency of lethal *Kr* phenocopies was observed, and while other embryos developed *Kr* phenocopies analogous to varying degrees of *Kr* mutant phenotypes, extreme *Kr* phenocopies indicative of complete *Kr*$^+$ inhibition were not produced. The most effective tested concentration of antisense RNA, which yielded ~50% of phenocopies, was at a 1000:1 (antisense to sense) ratio. Even the weakest response (4% phenocopies) required a greater than 50-fold excess of antisense over endogenous message.[238]

The *wingless* gene of *Drosophila* has similarly been inactivated by antisense RNA, resulting in *wingless* mutant phenocopies. Antisense RNA transcribed from a *wingless* cDNA fragment produced phenocopies ranging from minimal to global *wingless* embryo phenotypes when injected into wild-type eggs.[239] Alberga and colleagues[243] utilized antisense RNA inhibition to identify the cloned *snail* (*sna*) gene. Injection of RNA complementary to a *sna* cDNA fragment resulted in weak to intermediate *sna* phenocopies in >60% of the viable embryos.[243]

3. RNA Duplex Unwinding Activity

As described earlier, microinjection of antisense RNAs has been successful in suppressing sense target mRNA expression in *Xenopus* oocytes.[221,237,242] However, in fertilized *Xenopus* eggs, injection of antisense RNA had no effect on target RNA expression, and antisense-sense RNA hybrids were found to be extremely unstable due to a RNA duplex unwinding activity.[244,245] Neither exogenously introduced CAT RNA[245] nor endogenous *Xenopus* maternal RNAs (An1 and Vg1)[244] could form stable duplexes with antisense transcripts in these experiments. Injection of RNA duplex into oocytes and eggs at different stages of embryogenesis indicated that the unwinding activity was developmentally regulated. In oocytes the activity is contained exclusively in the nucleus, and first appears in the cytoplasm upon germinal vesicle breakdown during meiotic maturation. The activity, which exists at high levels throughout early embryonic development, is again localized to the nucleus at a later stage of embryogenesis (post-MBT).[244,245] Further *in vitro* biochemical characterizations demonstrated that the activity is sensitive to proteinase K and does not exhibit a requirement for ATP or divalent cations in the crude extract. While the unwindase preferentially binds double-stranded RNA molecules, it distinguishes between intermolecular and intramolecular RNA-RNA interactions and is specific for the former. Examination of the final products of the reaction revealed that the substrate duplex is never completely unwound, but is irreversibly altered in its base-pairing properties resulting from the conversion of its adenosine residues to inosines.[246]

An unwindase activity has also been detected in a large variety of mammalian cell lines, and appears to be ubiquitously expressed in nearly all types of tissues.[247,248] The double-stranded RNA-specific activity appears to be identical to that reported in *Xenopus*,[246,249] with a 25 to 40% conversion of adenosine residues to inosine.[249] As in the case of the *Xenopus* activity, both sense and antisense strands of dsRNA are substrates for modification. The duplex is not fully unwound, but again is prevented from rehybridizing due to the base modifications. A cell cycle-dependent expression of the unwindase activity has been observed in mouse 3T3 fibroblasts.[247] Very little activity is found in cells arrested into quiescence, but the activity increases upon stimulation by fetal calf serum. In addition, continuously proliferating 3T3 cells exhibit an intermediate level of unwindase.[247]

Paradoxically, although antisense RNA molecules are unable to form stable hybrids with the target mRNAs due to an unwindase activity, this same activity produces a covalent modification of the RNA that in fact may alter its coding capacity and hence result in a nonfunctional protein. It is therefore suggested that this activity may promote antisense RNA inhibition and is unlikely to be responsible for the failure of some antisense RNA experiments.[246,249] Although a specific role for the unwinding/modifying activity has yet to be identified, it has been postulated that it may serve a regulatory function in the cell by reversing or modulating specific RNA-RNA interactions or by controlling the expression of certain RNAs in a posttranscriptional fashion. Alternatively, the activity may be

involved in the degradation of double-stranded RNA, including naturally occurring sense-antisense RNA duplexes from eukaryotic genes.[246,249]

C. micRNA Immune System Against Viral Infection

The application of antisense RNA toward the inhibition of viral infection is becoming an increasingly investigated topic. As demonstrated in the prokaryotic studies with bacteriophage SP,[165,166] a micRNA immune system in eukaryotes would enable a highly specific inactivation of viral mRNA translation and/or viral genome replication. On a practical level, the antisense RNA-mediated approach may have several distinct advantages over conventional antiviral therapies, which include testing of natural and synthetic compounds likely to interfere with viral functions. Because antisense RNAs produced in the host cell act by hybridizing exclusively to specific sequences of the viral nucleic acid, cytotoxic effects are negligible. Hence, unlike some synthetic drugs, dosage-dependent side effects would be circumvented. Furthermore, the stable transfection of tissue culture cells and the introduction of vectors carrying antisense RNA genes into germline cells enables the generation of viral resistance in plants and animals as a heritable trait. Inactivation of the target viral RNA can be enhanced by the inclusion of a ribozyme component as described above.

1. RSV

Chang and Stoltzfus[250] demonstrated antisense inhibition of RSV envelope gene (*env*) expression in quail cells. While an expression plasmid (pLC32) containing *env* cDNA sequences efficiently rescued infectious virus from R(-)Q cells containing defective RSV deleted in the *env* gene, cotransfection of pLC32 with a second plasmid (pLC25) carrying inverted *env* sequences (1725 bp of complementarity to pLC32) resulted in significantly less virus production. The extent of antisense inhibition (as measured by focus-forming unit assays) was found to be dependent on the concentration ratio of the two plasmids, with maximum inhibition (80%) occurring at five- to tenfold excesses of antisense to sense plasmid. In addition, cotransfection of pLC25 with a wild-type Prague A RSV DNA plasmid (pJD100) resulted in the inhibition of expression and replication of virus in R(-)Q cells by 70 to 80%, which persisted for as long as 8 d. Because the expression of both intron-containing (pLC32) and intronless (pJD100) plasmids were repressed by pLC25 to the same degree, it is presumed that antisense inhibition in these experiments is occurring at the level of translation rather than at the level of RNA splicing. Furthermore, antisense plasmids containing sequences complementary to the *env* 5′ noncoding region were also effective in inhibiting *env* expression.[250] It is suggested that hybridization of antisense RNA to the 5′ untranslated region of target *env* mRNA may directly block translational initiation by sequestering the AUG codon. Alternatively, sense-antisense duplex formation may disrupt secondary structure necessary for initiation of protein synthesis.[250,251]

2. CMV,PVX

Antisense RNA genes have been utilized to confer protection against several plant viruses.[252,253] A plant expression vector carrying sequences complementary to the entire coat protein (CP) coding region of cucumber mosaic virus (CMV) was introduced into tobacco plants. Transgenic plants expressing CMV CP antisense RNA driven by the CaMV 35S promoter were found to be resistant to CMV infection on inoculated and systemic leaves only at low inoculum concentrations. Although levels of detectable virus in these transgenic plants were significantly reduced compared with control plants, protection against CMV infection was overcome at high inoculum concentrations.[252] Similarly, transgenic tobacco plants expressing antisense potato virus X CP transcript from the CaMV 35S promoter were protected on inoculated and systemic leaves only at the lowest inoculum concentration tested (0.05 μg/ml).[253] It is apparent in these studies that the levels of expression of antisense transcript in these plants are not sufficient to achieve protection against virus at higher inoculum concentrations. Inhibition of viral infection by antisense-CP transcripts is not as effective as protection mediated by expression of the CP itself. Although the CaMV 35S promoter used in these experiments is a strong constitutive promoter, the effects of the antisense RNA may be overcome when viral RNA accumulates above a certain level.[252,253] Other factors, including stability of the antisense transcript and the site of integration of the antisense gene into the plant genome, may also be determinants in the effectiveness of these experiments.

D. Naturally Occurring Antisense RNAs in Eukaryotic Cells

Although RNA species that are complementary to regions of specific mRNAs have been identified in several eukaryotic systems, their functions in regulating gene expression have not been elucidated. Small, nuclear poly(A)$^-$ RNAs transcribed from the strand opposite that encoding mouse DHFR mRNA were discovered during the course of studying DHFR transcription.[254] These RNAs (ranging in size from 180 to 240 nucleotides) have heterogeneous 5′ ends that map near the DHFR promoter region and are complementary to the first 10 nucleotides of the major DHFR transcript and to a short region immediately following the DHFR mRNA translational stop codon.[254]

The occurrence of a mouse locus at which two processed poly(A)$^+$ RNA species are derived from two adjacent convergent transcription units was reported by Williams and Fried.[255] The RNAs, which are 1.2 and 3.0 kb in length, overlap by 133 bp at their 3′-untranslated ends. It is suggested that the two overlapping transcripts may be involved in some novel mechanism in regulating gene expression, possibly by the steric effects of convergent transcription or by RNA duplex formation resulting in inhibition of RNA processing and/or transport from the nucleus.[255]

Overlapping transcription units have also been identified in

the dopa decarboxylase region (*Ddc*) of *Drosophila*.[256] An 88-bp overlap exists between the genomic region encoding the 3′ terminus of *Ddc* mRNA and the 3′ terminus of an adjacent gene (of unknown function) on the opposite strand. Maximal steady-state levels of the 3′-adjacent gene transcripts occurs specifically in *Drosophila* testes tissues. In contrast, *Ddc* transcripts are present in the testes at low levels, which raises the possibility of regulatory interactions between the two RNAs, or as mentioned above, regulation by transcriptional interference.[256]

A genomic locus encoding two overlapping genes transcribed from opposite strands has been described in the rat.[257] One strand encodes gonadotropin-releasing hormone (GnRH) mRNA; the other strand gives rise to several transcripts (SH RNAs) of undefined function. Both GnRH and SH RNAs are spliced and polyadenylated, with a significant amount of shared exon sequences. The SH RNAs have identical 3′ termini but are characterized by unique 5′-sequences derived from at least two distinct promoters.[257a] While GnRH mRNA is expressed in various tissues of the central nervous system, SH transcripts were first characterized in the heart. However, the existence of SH RNAs was first discovered from isolation of a partial SH cDNA clone in a rat hypothalamic cDNA library.[257] More recent studies have shown that SH RNAs are present within at least some of the same cells that produce GnRH, and histological data indicate that the SH RNAs are localized to the nucleus.[257a] It is therefore possible that regulatory interactions may occur between GnRH and SH RNAs. The detection within GnRH neurons of a significant amount of GnRH transcripts that retain the first intron has led to the speculation that SH RNAs may regulate splicing of this intron.[257a]

In the course of examining the molecular mechanisms involved in latent infections by HSV, transcripts from the genomic region encoding the α protein ICP-0 (one of five proteins that are first to be synthesized during a productive infection) were detected in relative abundance in the nuclei of latently infected sensory neurons.[258] Northern blotting analysis using strand-specific probes revealed that these 2.6-kb-long transcripts were derived from the DNA strand opposite to that encoding ICP-0 mRNA. Further characterization of the 5′ and 3′ ends of this latency-associated transcript (LAT) using S1 nuclease and primer extension mapping revealed the complementarity between LAT and ICP-0 RNAs to be approximately 750 bases.[259,260] A possible role for this RNA in HSV pathogenesis may be to operate as a natural antisense RNA in regulating ICP-0 gene expression by reducing the pool of functional ICP-0 mRNA.[258]

Antisense RNA complementary to both the type A and type B isozymes of α-amylase mRNAs has been identified in barley seeds.[261] S1 nuclease-protection experiments revealed that the complementarity is not perfect, indicating that the antisense RNA is transcribed from a site other than the type B and type A gene coding regions. The antisense transcripts, like the α-amylase RNAs, are developmentally regulated — complementary RNAs are present in developing endosperm tissue and in mature aleurone tissue, but are absent in shoot and root tissue. A second type of antisense α-amylase RNA was detected by a less stringent assay with Northern blot analysis. Three distinct hybridizing species of 1.6, 1.4, and 1.0 kb were identified in RNA prepared from mature aleurone and/or shoot tissues. It is possible that either type of the antisense RNAs may play a role in regulating the stability and/or translation of α-amylase mRNA.[261]

A small, UG-rich RNA (102 bases) associated with MHC mRNP particles from embryonic chick muscle cells has been shown to inhibit MHC translation *in vitro*.[262] This RNA, designated translational-control RNA ($tcRNA_{102}$), specifically interacts to form complexes with MHC mRNA, increasing its RNase resistance. Upon preincubation of stoichiometric amounts of MHC mRNA and $tcRNA_{102}$, MHC synthesis is completely inhibited when the mixture is added to a reticulocyte cell-free translation system. $tcRNA_{102}$ had no effect on endogenous protein synthesis in the lysate nor on the translation of other heterologous transcripts including globin and VSV mRNAs.[262] Sequence analysis of $tcRNA_{102}$ isolated with MHC mRNPs from chicken fast muscle revealed the presence of two species of molecules, $tcRNA_{102}$(A) and $tcRNA_{102}$(B), which were identical from bases 1 to 92, but differed in their 10-base 3′-terminal sequences.[263] A significant amount of sequence homology was found to exist between the 3′ ends of both $tcRNA_{102}$(A) and (B) and the 5′ end of MHC mRNA.[264] Based on these sequence homologies, in addition to the ability of $tcRNA_{102}$ to inhibit MHC mRNA translation *in vitro* and the close association of the RNAs *in vivo*, it is suggested that $tcRNA_{102}$ may be a natural antisense RNA involved in the expression of myosin synthesis.[262-264]

In vitro studies have demonstrated a novel interaction between the RNA of CMV and its satellite RNA (sat-RNA).[265] A short complementary stretch of 33 nucleotides within the coding region of the CMV CP gene hybridizes with residues 98 to 113 and 134 to 152 of sat-RNA by forming a stable pseudoknot structure, in which the sat-RNA is twisted around the CMV RNA. Possible regulation of CMV CP synthesis by binding of the sat-RNA antisense regions has been proposed.[265]

A particularly interesting feature of the sequence and structural analysis of the tomato 7 S RNA by Sanger and colleagues[266] is the presence of five contiguous regions that can form stable hybrids with complementary sequences in the potato spindle tuber viroid (see also Symons).[267] It is suggested that pathogenesis by plant viroids may occur by hybridization between viroid sequences and 7 S RNA.[266] Such hybrid formation would interfere with 7 S RNA function in protein translocation across membranes. Furthermore, the contiguous regions are found to be conserved in several other viroids that infect tomato plants, including citrus exorcortis viroid, chrysanthemum stunt viroid, tomato apical stunt viroid, tomato plant macho viroid, and

cucumber pale fruit viroid, which presents an exciting perspective on the possibility of natural antisense pathogenesis by plant viroids.[266,267]

IV. CONCLUSIONS AND SUMMARY

The studies outlined in this review give evidence to the diversity of natural antisense RNA-mediated regulation, and demonstrate the broad applications of artificial antisense RNA inhibition in an impressive range of systems. Antisense RNA has been employed to suppress the expression of various endogenous and exogenously introduced genes in both prokaryotes and eukaryotes. The effect in each case is highly specific, as inhibition is dependent on formation of a RNA-RNA duplex between the antisense RNA and its complementary target transcript. The consequence of this hybrid formation is the inactivation of target RNA function.

While the fate of the antisense-sense hybrid has not been determined in all cases, the observed reduction in target mRNA steady-state levels that accompanies inhibition of translation product expression suggests that the duplex molecule may be rapidly degraded by cellular nucleases. Krinke and Wulff[123] have shown that OOP-RNA inhibition of λ *cII* expression appears to be dependent on RNase III cleavage in *E. coli*. In several avian cell lines, double-stranded RNA (dsRNA) and certain viruses (which contain or can form dsRNA) have been reported to induce the production of a dsRNase concomitantly with interferon induction.[268] A double-strand-specific RNase also appears to be present in mouse oocytes.[240] The unwinding/modifying activity observed in *Xenopus* eggs[244-246] as well as in mammalian cells[247,249] has also been implicated in playing a possible role in the destabilization and/or degradation of dsRNAs.

In prokaryotes regulation by natural antisense RNAs has thus far been observed to occur at three different levels: translation, transcription, and DNA replication. These regulatory RNAs can be classified into three groups (see Table 3 and Figure 1; see also review by Inouye):[6] class I, which is comprised of antisense RNAs complementary to the SD sequence and/or coding sequences of the target mRNAs; class II, which includes antisense RNAs that hybridize to noncoding sequences of the target mRNA (e.g., regions upstream of the SD sequence) or to untranslated RNAs (e.g., RNAII of ColE1); and class III antisense RNAs, which regulate transcription of the target

Table 3
Natural Antisense RNAs in Prokaryotes

Level of regulation	Antisense RNA[a]	Target	Class[b]	Function	Origin	Ref.
Translation or posttranscription	*micF* RNA (93),(174)	*ompF* mRNA	IA	OmpF synthesis	Chromosomal	93,94
	isf RNA (353)	*sulA* mRNA	IA	SOS response		102
	gvp antisense RNA (420)	*gvpABC* RNAs	IA	Differentiation		108
	oop RNA (72)	*cII* mRNA	IB	Lysis-lysogeny	Phage	121,123,124
	P_{sQ} RNA (220)	Q mRNA	IA	Lysis-lysogeny		125,126
	sar RNA (69)	*ant* mRNA	IA	Lysis-lysogeny		115
	pOUT RNA (70)	Transposase mRNA	IA	Transposition	Transposon	136
	finP RNA (150),(180)	*traJ* mRNA (traM RNAs)[c]	IA	DNA transfer		148,148a
	sok RNA (100)	*hok* mRNA	IA	Killer function		77—79
	copA RNA (90)	*repA* mRNA	II	Plasmid replication	Plasmid	34,37,44
	R1162 RNA (75)	*repI* mRNA	II	Plasmid replication		70
	pT181 RNA (88),(150)	*repC* mRNA	II[d]	Plasmid replication		73
Transcription	*tic* RNA (92)	*crp* mRNA	III	cAMP receptor protein (crp)	Chromosomal	81,82
	RNAI (108)	RNAII	II	Plasmid replication	Plasmid	13
	Silencer RNA (>143,<382)	Activator RNA	II	Plasmid replication		59,63

[a] Numbers in parentheses indicate the number of nucleotides.

[b] Class I: Contains direct complementarity to the SD sequence and/or coding regions of target mRNA resulting in direct inhibition of translation (A) and/or destabilization of the target message (B).
Class II: Bind to noncoding regions of target mRNA (such as sequences upstream of SD sequence), or to nontranslated RNAs; exerts regulatory effect indirectly.
Class III: Inhibits transcription of target gene by mechanism similar to transcriptional attenuation.

[c] See text.

[d] May be Class III; see text.

Revised from Inouye, M., *Gene*, 72, 25, 1988. With permission.

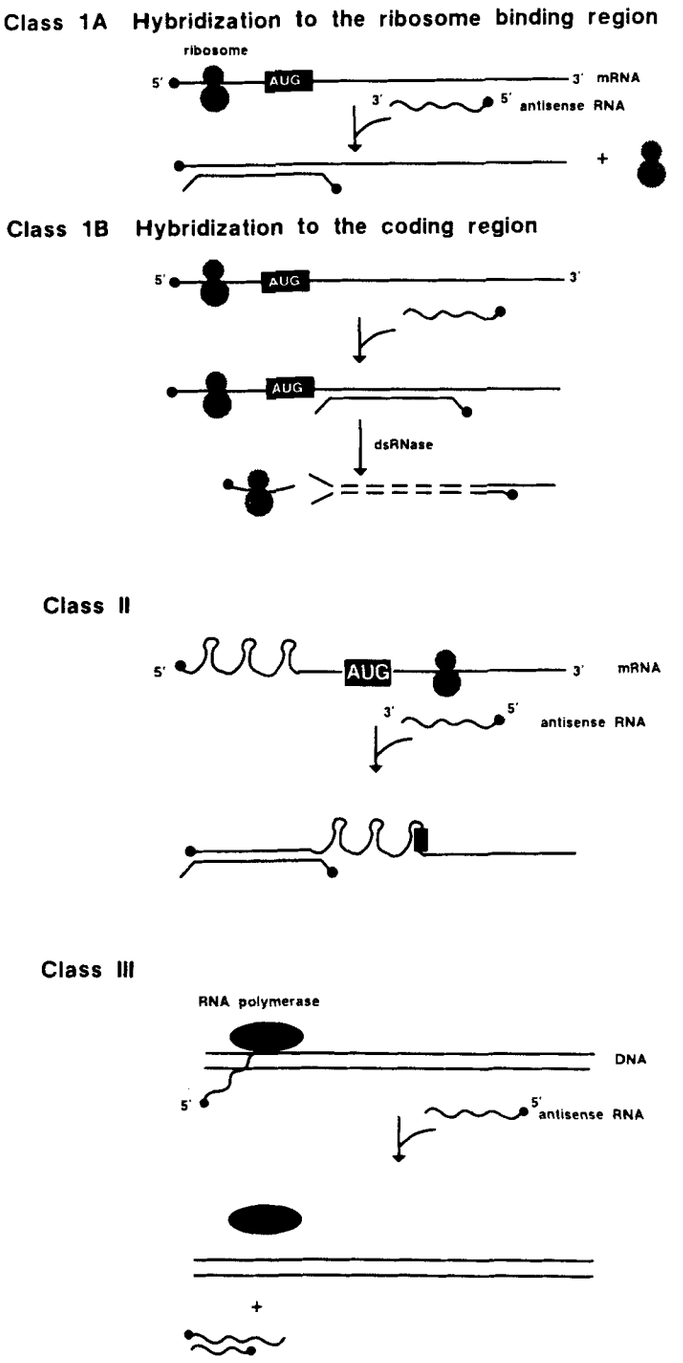

FIGURE 1. Proposed mechanisms of action for class I, II, and III prokaryotic antisense RNAs. (Adapted from Takayama, K. and Inouye, M., in *Proc. Eighth Int. Biotechnology Symposium*, Vol. 1, Durrand, G., Bobichon, L., and Forent, J., Eds., Societie Francaise de Microbiologie, 1988, 97. With permission.)

mRNA by a mechanism similar to transcriptional attenuation. While hybridization of a class I RNA to its target mRNA results in a direct inhibition of translation (class IA) and/or mRNA destabilization (class IB), binding of Class II RNAs occurs distally to their functional targets, resulting in indirect effects produced by, for example, alternative secondary structure formation that sequesters the ribosome binding site.

In experiments with artificial antisense genes in eukaryotes, antisense RNAs may exert their effects at more than one level, as in the case of antisense c-*myc* RNA, where both *myc* protein synthesis and *myc* mRNA transcription are believed to be affected by hybridization of the antisense transcript to the *myc* mRNA and to an enhancer-like region on the *myc* gene promoter, respectively.[213] On the other hand, antisense RNA may function by sequestering the target mRNA in the nucleus, preventing its transport to the cytoplasm as well as interfering with mRNA processing itself. *In vitro* experiments have demonstrated that antisense RNAs complementary to human β-globin pre-mRNA or to a chimeric globin/adenovirus E2a pre-mRNA were capable of specifically inhibiting pre-mRNA splicing.[269]

A recurring theme encountered in natural antisense RNA regulation in prokaryotic systems is that of the purported initial loop-loop interaction between the secondary structures of the antisense and sense RNA molecules. This initial recognition reaction in which the respective loop regions of the stem-loop structures transiently interact has been observed in the ColE1, IncFII, and PT181 plasmid replication systems, as well as in the regulation of conjugal DNA transfer. Recognition between RNA loop structures is not unique to antisense RNA-regulated systems; anticodon-codon base pairing of tRNAs with mRNA is a classic example of the significant nature of such interactions. Interestingly, Yavachev and Ivanov[270] have shown by computer analysis that considerable sequence homology exists between *E. coli* tRNAs and the stem-loop regions of RNAI or RNAII. A consensus sequence, the nonanucleotide AGUUGGUAG, is defined among the dihydrouridylic loops of eight tRNAs exhibiting sequence homology with loop II in RNAI. Based on these observations, the authors speculate that these two different classes of molecules may have originated from a common ancestral RNA.[270]

While the versatility of antisense RNA technology has been demonstrated by the various studies performed in many different systems, the consensus on an "optimal" antisense RNA structure for use in eukaryotes remains to be defined. Rather, it is apparent that at the present stage antisense gene constructions must be designed on an empirical basis in order to account for variables such as promoter strength and/or tissue specificity in the particular system, presence of negative and/or positive cellular factors, and stability and abundance of the antisense

vs. sense transcripts. Although in *E. coli* antisense RNAs complementary to the 5′ terminal region of the target mRNA are presumed to be most effective, in several eukaryotic systems, antisense transcripts that hybridized to other regions such as the 3′ noncoding region[240] have been shown to exert stronger inhibitory function. However, complementarity to the 5′ end of the mRNA has proven to be effective in many cases. Artificially introduced 5′-proximal secondary structures have been demonstrated to greatly affect translational efficiency of eukaryotic mRNAs, presumably by preventing stable mRNA-initiation factor complex formation.[271] In addition, antisense RNAs targeted to the 5′ terminus may suppress mRNA translation by inhibiting cap recognition.

In spite of the ambiguities encountered with different antisense constructs, there are certain parameters that can be optimized to enhance antisense RNA suppression of target gene expression (see Inouye[6] for a detailed discussion). Generally speaking, an excess of antisense over sense RNA is required for maximal inhibition. High expression of antisense RNAs can be obtained through the use of strong constitutive promoters, such as the SV40 promoter, the CaMV 35S promoter, and various retroviral LTRs. An expression vector containing the human β-actin promoter has been shown to drive high-level accumulation of γ-actin and β-tubulin antisense transcripts at levels equal to or higher than that of the highly abundant endogenous target mRNAs.[272] The use of inducible promoters such as the mouse metallothionein I promoter, murine mammary tumor virus LTR promoter, and the *Drosophila* hsp70 promoter may be desirable for the conditional inhibition of target gene expression.

Certain structural features (e.g., stem-and-loop structures) may be incorporated into the design of antisense RNAs to enhance their stability and resistance to cellular nucleases (for details see Inouye).[6] The fusion of a ribozyme component (discussed above) to antisense RNA "arms" introduces the added advantage of a catalytic constituent to enable specific and efficient target RNA inactivation.

The successful development of a micRNA immune system in higher systems would provide an attractive alternative to conventional approaches in antiviral therapy. Unlike the latter, which involve testing of natural and synthetic compounds likely to inhibit viral infection and/or replication, antisense RNA therapy circumvents dosage-dependent side effects. Furthermore, the stable transfection of tissue culture cells and the introduction of vectors carrying antisense RNA genes into germline cells enables the generation of viral resistance in plants and animals as a heritable trait.

The potential applications of antisense RNA technology in basic science and in medicine and agriculture are evident from the various studies reported to date. Whereas the identification of additional factors such as the unwinding/modifying activity introduces a novel twist to the scenario, it has at the same time elucidated previous queries as to why certain antisense-sense duplexes were particularly unstable. It is expected that as more information is accumulated the design of increasingly effective and versatile antisense RNAs will ensue.

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