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Creation of a Vector for Regulated Expression of Antisense RNA in Escherichia Coli

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CREATION OF A VECTOR FOR REGULATED EXPRESSION OF ANTISENSE RNA IN ESCHERICHIA COLI

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Master of Science

by

Wen-Jun Wu

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Creation of a Vector for Regulated Expression of Antisense RNA in *Escherichia coli*

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I dedicate this thesis to my loving wife and my understanding parents. Without their spiritual and financial support, this research would have never been possible.

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CREATION OF A VECTOR FOR REGULATED EXPRESSION OF ANTISENSE RNA IN ESCHERICHIA COLI

Wen-Jun Wu **January, 1992** **46 Pages**

Directed by: Dr.Claire Rinehart, Dr.Valgene Dunham, and Dr.Frank Toman

Department of Biology Western Kentucky University

A plasmid (pGEM-SD) was constructed in which the Shine-Dalgarno sequence had been deleted from pGEM-7zf(+) plasmid to generate a vector for regulated expression of antisense RNA. The binding of antisense RNA to mRNA provides a potent mechanism by which specific transcripts can be translationally inactivated. Although part of the *lac* operator sequence was deleted in pGEM-SD plasmid, it was proven that mRNA still can be induced under the control of *lac* promoter. Recombinant plasmids were generated by ligating bacterial genomic DNA into pGEM-SD plasmid, but the orientation of the inserted gene with respect to the *lac* promoter has not been determined.

These preliminary results show that gene expression can be turned on and off via the pGEM-SD vector. Future experiments will screen antisense recombinant clones for those which would inhibit specific gene products by plating on different selective indicator media both with and without the inducer IPTG. This will allow investigation of the biological functions associated with genomic DNA sequences by means of gene mapping with antisense RNA.

Introduction and Literature Review

Gene expression in both prokaryotes and eukaryotes is controlled by the products of regulatory genes. According to a great number of studies in the past 20 years, the products of such genes were determined to be proteins termed activators (Rosenberg and Court, 1979) or repressors (Miller and Reznikoff, 1980). During the past decade, naturally occurring regulatory genes have been discovered whose RNA transcripts directly control gene expression. These newly discovered RNA repressors are highly specific inhibitors of gene expression. The regulatory RNA contains a sequence that is complementary to the target RNA, and binding of the two RNAs occurs by base pairing. The term "antisense RNA" has been coined to designate this regulatory RNA. The genes that direct the synthesis of antisense RNA are designated antisense genes (Green *et al.*, 1986).

Discoveries that antisense RNA can inhibit gene expression in natural systems led to the development of strategies to artifically regulate genes using antisense RNA. With relatively simple manipulations, antisense RNA complementary to a chosen mRNA can be synthesized *in vitro* or *in vivo* and may be used to inhibit the expression of the respective target gene. The function of endogenous target genes has now been suppressed both in prokaryotes and eukaryotes by artifical antisense genes (Simons and Kleckner, 1988; Van der Krol *et al.*, 1988). The ability to deactivate specific genes holds great promise for medicine. For example, it may someday be possible to control viral diseases with antisense RNA molecules that seek and inhibit viral gene products inside human cells.

Base-pairing specificity is the key to antisense RNA's inhibition of genes (Figure 1). In the DNA duplex of a gene, there are hydrogen bonds between opposing pairs of adenine (A) and thymine (T) bases and between guanine (G) and cytosine (C) bases. The matched sense and antisense strands of DNA complement each other. During the transcription of DNA into RNA, the antisense DNA strand acts as a template for assembling a complementary (sense) messenger RNA molecule (in which U-for uracil-substitutes for T). A single-stranded messenger RNA is translated into a protein on the cellular ribosomes. Messenger RNA is the only transcription product of most duplex DNAs; however, some genes are regulated by the additional transcription of an antisense RNA from the sense DNA strand of the same cistron. If both antisense RNA and mRNA are made, then the antisense RNA and the messenger RNA will bind with each other. A variety of factors may then prevent the translation of protein: the RNA duplex may be rejected by the ribosomes; it may be degraded by enzymes; it may never leave the nucleus; or the A bases may be modified chemically to become inosine bases, thereby scrambling the genetic code on the messenger RNA (Weintraub, 1990).

Functional regulation of specific target RNAs by antisense RNA has been shown or proposed to operate in various prokaryotic systems (Green *et al.*, 1986; Simons and Kleckner, 1988). In most of the systems listed in Table 1, antisense RNAs and their target mRNAs are transcribed from the complementary strands of the same region of DNA. However, for the two *Escherichia coli* chromosomal genes, each antisense RNA is transcribed from a region away from the target gene (Andersen *et al.*, 1989). On the other hand, the *ant* mRNA of phage P1 and P7 and its antisense RNA (c4 repressor) are transcribed from the same promoter (Citron and

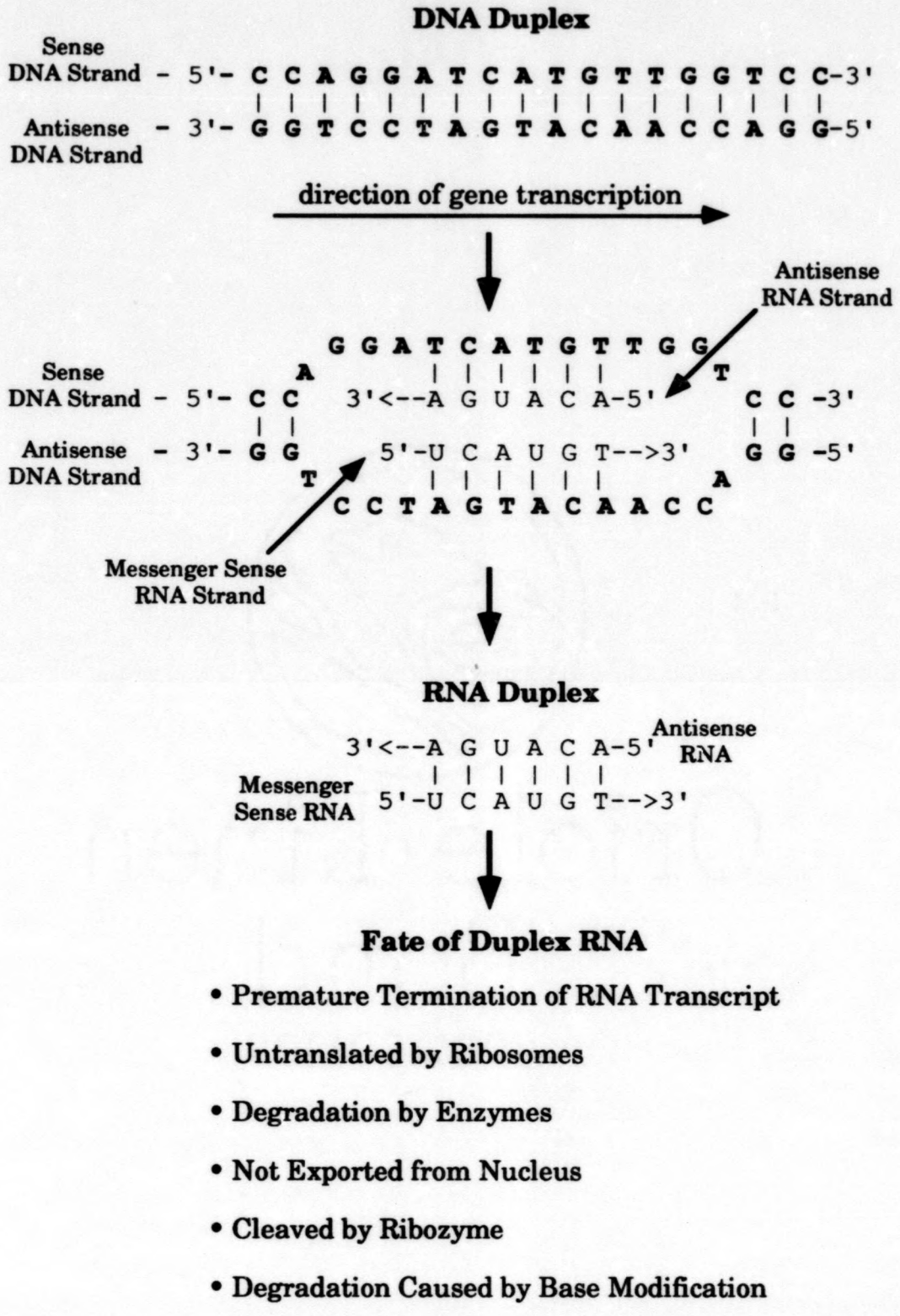

Figure 1. Base-pairing specificity is the key to antisense RNA's inhibition of genes.

Table 1. Naturally occurring antisense RNAs *

	Antisense RNA	Target RNA	Function Controlled	Level of Control **
Plasmid				
ColE1	RNA I	RNA II	Replication	Primer formation
IncF	*cop*A RNA	*rep*A mRNA	Replication	mRNA stability
IncI	*inc* RNA	*rep*Z mRNA	Replication	Translation
ColE2	*cop* RNA	*rep* mRNA	Replication	Uncertain
R1162	*ct* RNA	*rep*1 mRNA	Replication	(Translation)
R6K	silencer	activator	Replication	Uncertain
pT181	RNA I	*rep*C mRNA	Replication	Transcription termination
IncF	*fin*P RNA	*tra*J mRNA	Conjugation	(Translation)
IncFII	*sok* RNA	*hok* mRNA	Host killing	(Translation)
Phage				
Lambda	*aQ* RNA	*Q* mRNA	Lysis/lysogeny	Uncertain
Lambda	*oop* RNA	*c*II mRNA	Lysis/lysogeny	mRNA stability
p22	*sar* RNA	*ant* mRNA	Lysis/lysogeny	(Translation)
P1 , P7	*c*4 repressor	*ant* mRNA	Lysis/lysogeny	(Translation)
Transposon				
IS10	RNA-OUT	*tnp* mRNA	Transposition	Translation
Bacterial				
E.coli	*mic*F RNA	*omp*F mRNA	OmpF synthesis	Translation
E.coli	*tic* RNA	*crp* mRNA	Crp synthesis	Transcription termination

* : Taken in part from Eguchi *et al.*, 1991.

** : Speculated level of control is given in parentheses.

Schuster, 1990).

Several different mechanisms have been suggested for the inhibition of gene expression by antisense RNA (Weintraub, 1990; Table 2). Type I inhibition of translation, by blocking the ribosome binding site and/or the initiation codon, has been suggested since ribosomes only bind efficiently to single stranded RNA and not to duplex RNA (Stormo, 1986). Examples of type I inhibitions are found in the regulation of β-galactosidase synthesis by antisense RNA (Ellison *et al.*, 1985), regulation of IS10 transposition (Ma and Simons, 1990), *E.coli* OmpF synthesis (Andersen *et al.*, 1989), reduced creatine kinase activity in KC transformants (Ch'ng *et al.*, 1989), and microinjection with antisense RNA in frog's eggs (Melton, 1985). Type II inhibition functions by binding the antisense RNA to a target mRNA thus increasing its susceptibility to digestion by RNase III or other RNases as seen for *rep* mRNA of IncFII plasmid R1 (Blomberg *et al.*, 1990), GUS mRNA in transgenic plants (Cannon *et al.*, 1990), ribosomal protein L1 synthesis in *Xenopus* oocytes (Wormington, 1986), and antisense oligonucleotides acting in *Xenopus* embryos (Dagle *et al.*, 1991). Type III inhibition is based on evidence found by Kim and Wold (1985) that an RNA duplex formed in the nucleus of cultured mammalian cells was not transported to the cytoplasm. Additionally, Crowley *et al.* (1985) found that in *Dictyostelium* the RNA duplex formed in the nucleus was highly unstable and rapidly degraded. Also, Yokoyama and Imamoto (1987) suggested that decreased MYC RNA transcription in the antisense clones is regulated by the formation of an RNA duplex in the nucleus. Such base-paired structures may preclude processing and export events required for mRNA function and expression. Type IV inhibition functions through the binding of an antisense RNA to a target RNA resulting in the formation of a

Table 2. Summary of mechanisms for the inhibition of gene expression by antisense RNA

Type	Mechanism	Selected references
I	Inhibition of translation	Ellison *et al.* (1985) Ma and Simons (1990) Andersen *et al.* (1989) Ch'ng *et al.* (1989) Melton (1985)
II	Increase RNases susceptibility	Blomberg *et al.* (1990) Cannon *et al.* (1990) Wormington (1986) Dagle *et al.* (1991)
III	Inhibition of message transports to cytoplasm	Kim and Wold (1985) Crowley *et al.* (1985) Yokoyama and Imamoto (1987)
IV	Premature transcription termination	Novick *et al.* (1989) Okamoto and Freundlich (1986)
V	Base modification leading to degradation	Bass and Weintraub (1988) Kimelman and Kirschner (1989)
VI	Ribozyme activity	Cech (1986) Haseloff and Gerlach (1988)

transcription terminator or terminator-like structure which induces premature transcriptional termination, such as in the case of *rep*C transcription of plasmid pT 181 (Novick *et al.*, 1989) or for *crp* transcription of *E.coli* (Okamoto and Freundlich, 1986). Type V inhibition functions through base modification. For example, an RNA complementary to a part of the mRNA for the basic fibroblast growth factor in *Xenopus* oocyte has been shown to direct the double-stranded specific covalent modification (adenosine to inosine) of the complementary region of the mRNA (Bass and Weintraub, 1988), resulting in the abrupt degradation of the mRNA during maturation of the oocyte (Kimelman and Kirschner, 1989). Type VI inhibition uses ribozyme activity as illustrated by Cech (1986). He has shown that certain naturally occurring antisense RNA can cleave at a point where there is a specific triplet of nucleotide bases, G-U-C, in the target. Haseloff and Gerlach (1988) have also constructed several antisense chloramphenicol acetyl transferase (CAT) templates capable of specific catalytic cleavage of the CAT mRNA.

Most naturally occurring antisense RNAs have the potential to form one or more stem-loop structures with themselves (Simons and Klecker, 1988). Many of the loops are the most stably favorable size, six to seven nucleotides (Uhlenbeck *et al.*, 1973). Inspection of most systems suggests that many of the stems are extremely stable. The stems of antisense RNA not only allow formation of appropriate loops, they also determine the stability/instability of the antisense molecules (Simons and Kleckner, 1988). Genetic analysis using mutants of several different plasmids have suggested that the specificity of initial recognition between the antisense RNA and target RNA is determined by the nucleotide sequence of the loop portion of one major stem-loop structure of each antisense RNA (Figure 2).

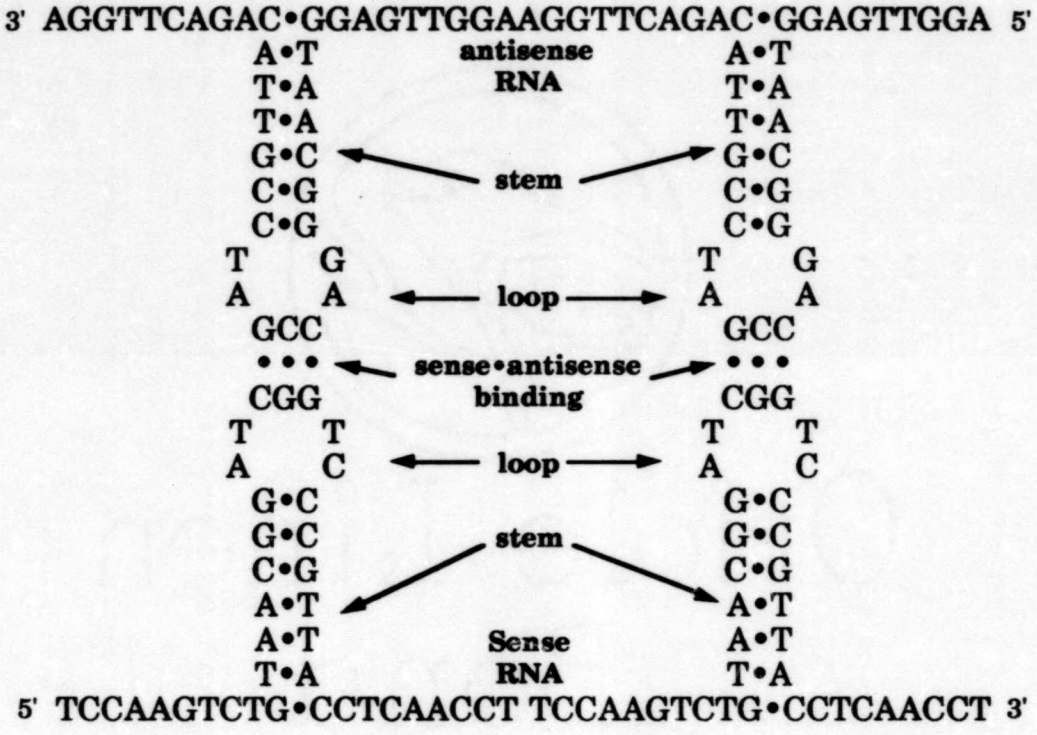

Figure 2. The initial recognition between the antisense RNA and target RNA.

Moreover, some antisense RNAs from closely related plasmid families share an identical sequence in the loop portions of the major stem-loop structures. Because these shared sequences are complementary, the initial interaction of the antisense RNAs and the target RNAs of these plasmids probably occurs between the identical combination of complementary loop nucleotide sequences, suggesting an evolutionary significance (Eguchi *et al.*, 1991).

Although gene expression can be regulated by artificial antisense RNA, it did not work effectively (Simons and Kleckner, 1988). Artificial antisense constructs can be effective, but only when antisense RNA transcription is driven by a very strong promoter and the antisense construct is present on a high copy number plasmid (Coleman *et al.*, 1985; Hirashima *et al.*, 1986). Such constructs generally lack the stem-loop features of natural antisense systems that stabilize the antisense RNA, permit rapid nucleation of stable binding, and/or provide for nucleation near an RNA end.

Regulation of gene expression by antisense RNA was first recognized as a naturally occurring mechanism in prokaryotes. Plasmid replication and copy number seem to be controlled by complementary RNA transcriptions in the *E.coli* ColE1 plasmid (Tomizawa *et al.*, 1981). Similarly, *E.coli* plasmid IncFII (Brady *et al.*, 1983) and *Staphylococcus aureus* plasmids (Kumar and Novick, 1985) appear to exhibit control by a complementary RNA mechanism. Translation of the *E.coli* Tn10 transposase mRNA can be inhibited by an RNA complementary to its 5'-end (Simons and Kleckner, 1983). The expression of *Omp*F in *E.coli* is regulated by an antisense RNA complementary to the ribosome binding site and initiation codon (Mizuno *et al.*, 1984).

By construction of antisense RNA targeted to form a duplex with the ribosome binding site and initiation codon of lipoprotein, OmpA and OmpC protein, Coleman *et al.* (1984) demonstrated inhibition of the synthesis of these proteins in *E.coli*. In addition, Coleman *et al.* (1985) used antisense RNA to block effectively the proliferation of the ssRNA bacteriophage SP in *E.coli*. Antisense RNA has also inhibited the replication of Rous sarcoma virus in cultured cells (Chang and Stoltzfus, 1987). Noteworthily, Sczakiel and Pawlita (1991) showed that HIV-1 replication is reduced in human T-cell lines constitutively expressing HIV-1 antisense RNA targeted against the 5' leader-gag region. In mammalian cells, coinjection of the thymidine kinase (TK) gene and a plasmid with the TK gene inserted in reverse orientation resulted in a decrease in TK expression in TK^- mouse L cells (Izant and Weintraub, 1984). In addition, Kim and Wold (1985) produced cell lines that transiently express antisense TK RNA by transfection of plasmid containing the dihydrofolate reductase gene immediately followed by an antisense TK fragment. Upon transfection of these plasmids into TK^+ mouse L cells, a significant decrease in thymidine kinase activity was observed. Wormington (1986) reported the inhibition of synthesis of an endogenous protein in frog oocytes by microinjection of antiribosomal protein L1 RNA. Subsequently, Qian *et al.* (1988) showed that antisense *rp*A1 expression severely disrupted oogenesis and produced a "small egg" female-sterile phenotype. Smith *et al.* (1988) indicated that polygalacturonase (PG) antisense RNA generated in transgenic tomato plants caused a reduction in PG enzyme activity when the fruit ripened. In the meantime, Sandler *et al.* (1988) reported the successful suppression of nopaline synthase enzymatic activity in the leaves of tobacco plants via the overproduction of RNAs complementary to the nopaline synthase mRNA.

Recently, Cannon *et al.* (1990) have shown leaf-specific inhibition of GUS gene expression in transgenic plants by using an antisense RNA with a 41-base homology spanning the translation start codon of the gene.

Regulation of gene expression is generally controlled at the level of translation in most systems by means of antisense RNA. Surprisingly, Yokoyama and Imamoto (1987) showed that a human antisense MYC gene stably introduced into the human promyeloleukemia cell line HL-60 can inhibit not only MYC protein synthesis but also transcription of the endogenous MYC gene, implying that antisense RNA can regulate transcription of the MYC gene. The decreased transcription of MYC appears to commit HL-60 cells to monocytic differentiation without the help of a chemical inducer. In addition, Cornelissen and Vandewiele (1989) demonstrated that expression of antisense bialaphos resistance (bar) gene reduces the bar mRNA steady state level and ratio of phosphinotricin acetyl transferase synthesis per bar messenger. Available evidence in the literature suggests that the maximal inhibition of protein synthesis occurred when a functional ribosome binding site at the 5'-end of the mRNA is targeted by antisense RNA. Nevertheless, recent observations by Ch'ng *et al.* (1989) demonstrated that antisense RNA complementary to 3' coding and noncoding sequences of creatine kinase B (CK-B) potently inhibits translation, and only one or two antisense transcripts is sufficient to block the expression of one CK-B mRNA.

Numerous reports indicated that antisense RNA hybridized to the Shine-Dalgarno (SD) sequence and the translation initiation site of the target mRNA inhibited protein synthesis more efficiently than one that is not bound to these regions. Those findings prompted the construction of an inducible vector devoid of the vector SD region so that antisense SD

sequence(s) carried by the vector would not bind to inherent undeleted vector SD sequence and circularize. Also, it was intended to prevent antisense RNA binding with ribosomes at the vector SD region and attenuating its binding affinity for target mRNA. Therefore, this constructed vector could be used to regulate the expression of any specific gene in *E.coli*. Construction of antisense recombinant vectors, by inserting the bacterial genomic DNA fragment in the opposite orientation under the control of an inducible promoter, would allow the expression of antisense SD and translation initiation sequences specific to target mRNA. The inducibility of such constructs will allow selective inhibition of any *E.coli* gene including potentially lethal genes. Future experiments will use this vector system to investigate the biological functions associated with various DNA sequences and will allow gene mapping of bacterial genomic DNA with antisense RNA probes.

Materials and Methods

Bacterial Strains, Plasmid, and Media

Escherichia coli ATCC 25922 was used for genomic bacteria in this study. *Escherichia coli* JM109 was used for all DNA-mediated transformation of bacteria. Plasmid pGEM-7zf(+), shown in Figure 3, was used as plasmid cloning vector, and contains part of the *lac*Z gene and all of the ampicillin resistance gene. *E.coli* ATCC 25922 was obtained from DIFCO. *E.coli* JM109 and plasmid pGEM-7zf(+) were obtained from Promega. Cultures of *E.coli* ATCC 25922 were grown overnight at 37°C with vigorous shaking in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Cultures of *E.coli* JM109 harboring pGEM-7zf(+) were grown overnight at 37°C with vigorous shaking in LBA medium (LB medium containing 100 μg/mL of ampicillin). For experiments requiring induction of the *lac* operon with isopropyl-β-D-thiogalactopyranoside (IPTG), 10-mL aliquots of LBA medium were inoculated with 10 μL of the appropriate overnight cultures and 35 μL of IPTG was added (70 μg/mL, final concentration) prior to growth overnight in a 37°C shaker.

Reagents and Solutions

Recipes for all the reagents and solutions referred to in the materials and methods are found in the Appendix.

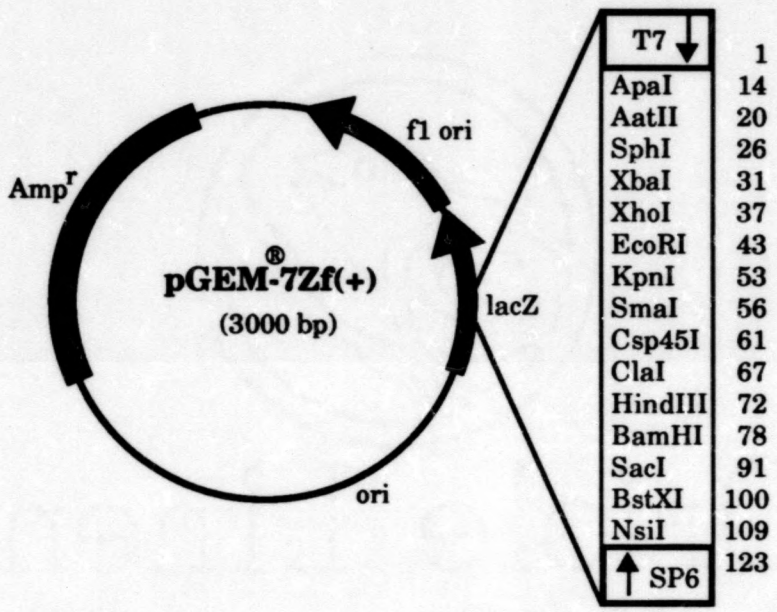

Figure 3. pGEM-7zf(+) vector map. The right column shows the directions of T7 and SP6 transcription start, and multiple cloning sites.

Preparation of Competent Cells and Transformation

The acid salt method was used for the preparation of transformation competent cells (Alexander, 1987). Approximately 30 ng of plasmid DNA was incubated with 200 μL of competent cells on ice for 30 minutes. The mixture was then heat shocked, which allowed the plasmid DNA to efficiently enter the cells, and was diluted with 800 μL of LB medium. The cells were grown in nonselective LB medium to allow synthesis of plasmid encoded antibiotic resistance proteins for one hour, then plated on LBA medium to allow identification of plasmid-containing colonies (Ausubel *et al.*, 1991).

Isolation and Purification of Plasmid DNA and Bacterial DNA

Large quantities of plasmid DNA used in this work were prepared by large scale plasmid preparations (Ausubel *et al.*, 1991). Transformed bacterial cells were grown in 250 mL of LBA medium overnight. Plasmid DNAs were released from JM109 host cells by the alkaline lysis method (Birnboim, 1983), precipitated in 1/4 volume of 10M ammonium acetate and 2 volumes of 100% ethanol, and purified by polyethylene glycol (Lis, 1980). RNA was removed from the preparation by treatment with boiled pancreatic RNase (Ausubel *et al.*, 1991), followed by an extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) to remove proteins. DNA was precipitated from the aqueous phase with 2 1/2 volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate, pH 5.5.

Large amounts of bacterial genomic DNA was prepared by scaling up mini-prep method (Ausubel *et al.*, 1991). Pelleted bacterial cells from 10 mL saturated LB cultures were lysed with sodium dodecyl sulfate (SDS) (0.5%, final concentration) and proteins were removed by digestion with

proteinase K (100 μg/mL, final concentration) at 37°C for one hour. Cell wall debris, polysaccharides, and remaining proteins were removed by selective precipitation with hexadecyltrimethyl ammonium bromide (CTAB; 1%, final concentration) and high-molecular-weight DNA was recovered from the resulting supernatant by isopropanol precipitation (Ausubel *et al.*, 1991). Quantitation of the plasmid DNA was performed by UV spectrum analysis scanning from 200nm to 320nm (Maniatis *et al.*, 1982). Plasmid DNA was characterized by restriction digestion (total reaction volumes were 20 μL; Maniatis *et al.*, 1982) and agarose gel electrophoresis.

Agarose Gel Electrophoresis

Agarose gels were prepared according to Maniatis *et al.* (1982). The gel (total volumes were 50 mL) was mixed with 2.5 μL of ethidium bromide (10 mg/mL) then run at 100 volts for about 1-1.5 hours in 1X TBE electrophoresis buffer (Appendix). DNA was visualized under UV light and a polaroid picture was taken with an orange filter (Maniatis *et al.*, 1982).

Unidirectional Deletion With Exonuclease III

Exonuclease III (Exo III) unidirectional deletion was done according to Promega Protocols and Application Guide. Before Exo III digestion, about 12 μg of plasmid DNA was dissolved in 60 μL of 1X ExoIII buffer (Appendix). Meanwhile, 7.5 μL of S1 mix (Appendix) were added to each of 25 small tubes and left on ice. When the DNA was warmed to 37°C in a water bath, 500 units of ExoIII (Promega) were added. Aliquots of 2.5 μL samples were removed immediately at 20 second intervals into the tubes

containing the S1 mix on ice. After all the samples had been taken, the tubes were moved to room temperature for 30 minutes. Once the S1 nuclease digestion was complete, 1μL of S1 stop buffer (Appendix) was added and heated at 70°C for 10 minutes to inactivate the S1 nuclease (Promega). Once S1 nuclease was inactivated, samples from each time point were transferred to 37°C and 1 μL of Klenow mix (Appendix) was added to each. These samples were incubated for 3 minutes and then mixed with 1 μL of the dNTP mix (Appendix), and kept at 37°C for 10 minutes. The samples were moved to room temperature, 40 μL Ligase mix (Appendix) were added and the samples were incubated at 14°C overnight. Once the ligation reaction was complete, 10 μL of the ligation mixture were added to competent cells for transformation as described above.

DNA Sequence Analysis

DNA sequencing procedures were according to the "Step-By-Step Protocols For DNA Sequencing With Sequenase Version 2.0" (United States Biochemical Corporation) with some modifications. This DNA sequencing method is based on the dideoxynucleotide method of Sanger *et al.* (1977).

In order to prime efficiently, double-stranded plasmids were converted to a single stranded form prior to sequencing. This is accomplished by alkali denaturation of supercoiled plasmid DNA (Lim and Pene, 1988).

The annealing mixture (10 μL) containing 7 μL of single-stranded DNA (approx. 1 μg), 2 μL of Sequenase reaction buffer (Appendix), and 1 μL of primer (an equal molar ratio of the template; Appendix) was heated at 65°C for 2 minutes, placed in a beaker of 65°C water and allowed to cool slowly to room temperature over a period of 30 minutes. Once the

annealing was complete, the tubes were placed on ice and the following components were added: 1 μL of 0.1 M dithiothreitol (DTT), 2 μL of 1X Labeling mix (Appendix), 0.5 μL of α-S^{35} dATP (10μCi/μL; 600 Ci/mmol), 1 μL of Mn buffer (Appendix), and 2 μL of diluted Sequenase Version 2.0 (1 part Sequenase Version 2.0 (Appendix) to 7 parts Sequenase dilution buffer (Appendix). These tubes were mixed thoroughly and incubated for 5-10 minutes at room temperature. Once the labeling reaction was complete, 3.5 μl of labeling reaction mixture were transferred to each of the four tubes labeled A, C, G, T containing 2.5 μL of appropriate termination mix (Appendix). When these tubes had been incubated at 37°C for 30 minutes, 4 μL of termination stop solution (Appendix) were added to all tubes.

Gel mix (50 mL) was made up with the following components: 7.5 mL of 38% acrylamide/2% bis-acrylamide, 25 g of urea, 5 mL of 10X TBE buffer, and 17.5 mL of water. After the urea was dissolved, 30 μL of 100% N,N,N',N'-tetramethylethylenediamine (TEMED) and 130 μL of 10% ammonium persulfate were added to polymerize the gel. The gel mix was poured into an ultra-thin gel assembly (0.3mm) and allowed to polymerize. The gel was pre-run for 30 minutes at 35 watts. Samples were heated to 80°C for 2 minutes and 2-3 μL were loaded per lane. The samples were resolved on the gel at 35 watts for two hours. The gel was soaked in 10% glacial acetic acid/10% methanol for 30 minutes, and dried at 80°C under vacuum for one hour. The gel was exposed to X-ray film (Kodak) with Du Pont Hi-Plus Intensifying Screen at -70°C for one week.

Preparation of Bacterial RNA

RNA was isolated from *E.coli* JM109 cells, and extracted by the procedures described by Ausubel *et al.* (1991). Bacterial cells were

harvested from a 10-mL culture by centrifugation for 10 minutes at 12,000 x g, followed by resuspension in protoplasting buffer (Appendix). Cell walls were removed by lysozyme (400 μg/mL, final concentration). The resulting protoplasts were lysed with SDS (1.5%, final concentration). Diethylpyrocarbonate (DEPC), a potent inactivator of ribonuclease, was added to the lysate (2.9%, final concentration). One half volume of saturated NaCl was then added to coprecipitate the SDS, protein, and chromosomal DNA. Insoluble material was removed by microcentrifugation at 12,000 x g for 10 minutes. The supernatant fluid contained predominantly RNA, which was recovered by ethanol precipitation (Ausubel *et al.*, 1991).

Preparation of the Nick-Translated Probe

The procedure for labeling DNA by nick translation was described by Bethesda Research Laboratories in their nick translation kit. In a 1.5 mL microcentrifuge tube, in which 7 μL of α-S^{35}-dATP had been dried, the following components were added: 1 μL of Solution A1 (Appendix), 1 μL of plasmid DNA (0.2 μg), 1 μL of Solution C (Appendix), and 2 μL of sterile water. After mixing, the sample was incubated at 13°C for one hour followed by the addition of 1 μL of Solution D (Appendix), 2 μL of E.coli tRNA (1μg/μL), and 92 μL of TE buffer (Appendix). The labeled plasmid DNA was then extracted with 100 μL of phenol/chloroform/isoamyl alcohol (25:24:1). In order to determine the amount of incorporated radioactivity, the labeled DNA was separated from the unincorporated radioactive precursors by spun-column chromatography as described by Maniatis *et al.* (1982). The amount of radioactivity incorporated into the labeled plasmid DNA was determined from counting an aliquot in a Packard Tri-Carb 1500 liquid

scintillation analyzer (Maniatis et al., 1982).

Northern Blot Analysis

RNA was denatured essentially as described by McMaster and Carmichael (1977). Briefly, RNA (up to 20 μg/3.7 μL reaction mixture) was incubated in 1 M glyoxal/50% dimethylsulfoxide (DMSO)/10mM sodium phosphate buffer, pH 7.0, at 50°C for one hour. When the incubation was complete, the RNA samples were cooled on ice and 4 μL glyoxal loading buffer (Appendix) were added prior to electrophoresis on a 1.2% agarose gel in 10mM sodium phosphate buffer, pH 7.0. Glyoxalated RNA was transferred immediately from the agarose gel to a nitrocellulose filter by the capillary blot method using 20X SSC solution (Appendix), essentially as described by Thomas (1980). After 15-24 hours, the nitrocellulose filter was dried under a lamp and baked in an oven for 2 hours at 80°C.

Prehybridization solution (Appendix) was as described by Wahl *et al.* (1979) with the exception that dextran sulfate was not used. Although dextran sulfate can increase the rate of hybridization (Wahl *et al.*, 1979), it is unnecessary for most purposes. The RNA blots were prehybridized for 8-20 hours at 40°C. Probe that had been boiled for 5-10 minutes was added to a final activity of 500,000 CPM/mL of prehybridization solution (Ausubel *et al.*, 1991) and hybridized for 20 hours at 40°C. The filters were washed as follows : two times in 1X SSC plus 0.1% SDS at room temperature (15 minutes each) followed by two washes in 0.25X SSC plus 0.1% SDS at room temperature (15 minutes each). After these washes, high stringency washes were performed with 2 changes of 0.1X SSC plus 0.1% SDS for 15 minutes at 50°C. After washing, the filter was dried and exposed to film as described above.

Results

Constructing a Vector

A vector for the expression of antisense RNA should minimally contain an inducible promoter, an antibiotic resistance marker, a multiple cloning site, an origin of replication, and preferably replicate to a high copy number in the target cell. pGEM-7zf(+) is such a plasmid vector with several additional features including an f1 origin of replication for making single stranded DNA, SP6 and T7 promoters diametrically opposed and flanking the multiple cloning site, and a blue/white selection system using the *lac*Z gene to determine if the plasmids are recombinants or not. It is this last feature, the IPTG inducible *lac*Z gene, into which antisense DNA genes will be inserted, that requires modification. Since this gene is not only transcribed but the mRNA is also translated, any antisense genes contained in the transcripts will find that ribosomes are bound to the RNA which may potentially attenuate the functionality of the antisense RNA domain. Also, if the antisense RNA contains an antisense SD region, this region could potentially bind to the SD region found in the *lac*Z gene portion of the transctipt. Such binding would prevent both translation of the *lac*Z transcript RNA and binding of the antisense RNA domain to it's target mRNA. Therefore, it was decided to remove the SD sequence from the pGEM-7zf(+) plasmid and sacrifice the blue/white colony selection features in order to insure that recombinant antisense RNA transcripts would not be interfered with by vector sequences. Figure 4 shows the positions of the *lac*Z start codon (160-162), SD sequence (168-173), *lac* operator (180-200), and *lac* promoter (207-236) in pGEM-7zf(+) plasmid. These positions were

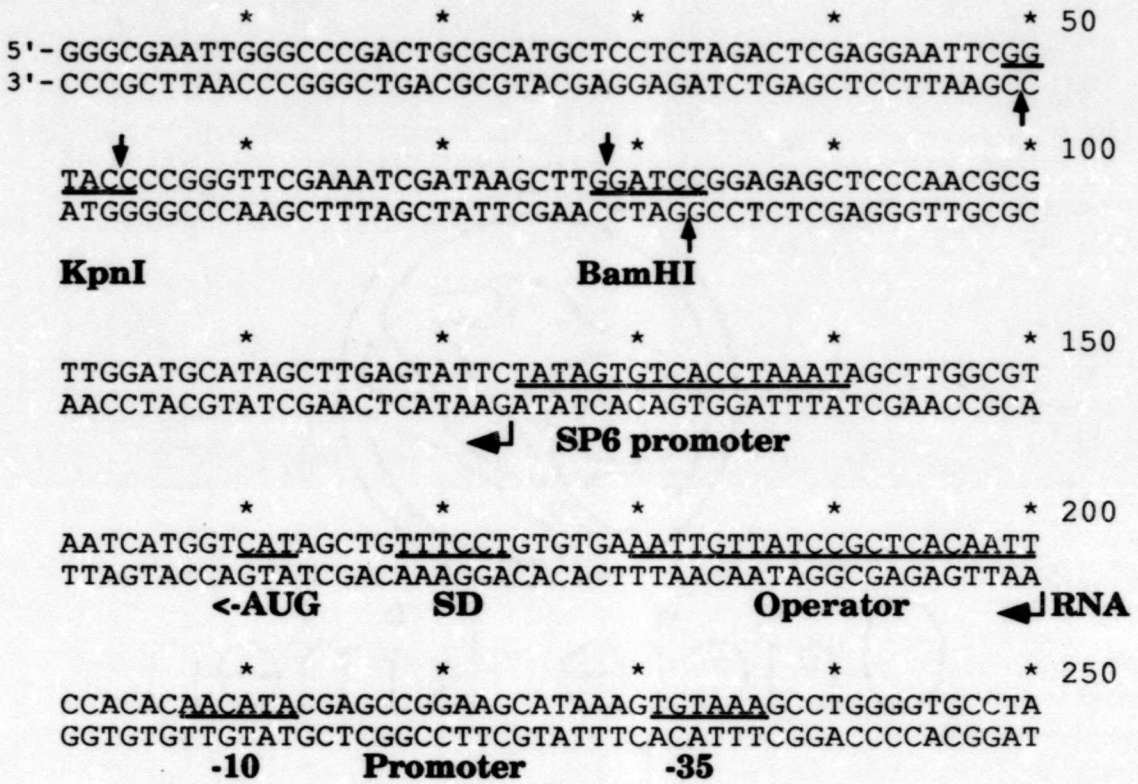

Figure 4. Partial nucleotide sequence of pGEM-7zf(+) plasmid. This DNA sequence shows *lacZ* start codon (160-162), Shine Dalgarno sequence (168-173), *lac* operator (180-200), and *lac* promoter (207-236).

located with computer analysis using MacVector (IBI).

There are two useful features of Exo III: (1) processive digestion at a very uniform rate and (2) failure to initiate digestion at DNA ends with four-base 3'-protrusions (Henikoff, 1984). About 12 μg of closed circular pGEM-7zf(+) plasmid DNA was subjected to digestion with Kpn I, which leaves a four-base 3'-protrusion, and BamH I, which leaves a 5' protrusion. Since these restriction enzymes cut at only one site along the whole DNA sequence, they created a template for Exo III which would give unidirectional digestion. When ExoIII was added to the restricted DNA, digestion proceeded from the 5'-protruding end left by the BamH I enzyme. Aliquots were removed at 20 second intervals and the reactions stopped. Subsequent S1 nuclease digestion trimmed protruding single-stranded ends of DNA. Successive addition of Klenow DNA polymerase for blunt ends formation and T4 DNA ligase to circularize the restricted DNA, was followed by transformation and plating. To screen for clones containing deletions of the SD sequence, miniprep DNA was digested with Puv II because of the location of two sites flanking the region to be deleted (base 323 and 2890 of the unmodified pGEM-7zf(+) plasmid). Two restriction fragments would be generated, 433 and 2567 base-pair (bp) from the Puv II digestion of pGEM-7zf(+) plasmid. From Figure 4 it can be seen that a deletion from the BamH I site (at base 78) to a site between the SD and operator sequences would remove 95 to 101 bp. Such a deletion would generate small Puv II fragments between 307 and 313 bp. Figure 5 shows the size ranges of the small Puv II restriction fragments generated from the digestion of a number of deleted clones. Lanes 7 to 1 show a decreasing range of size in the small Puv II restriction fragment compared with the undeleted control (lane 9). Lane 4 shows a small restriction fragment of

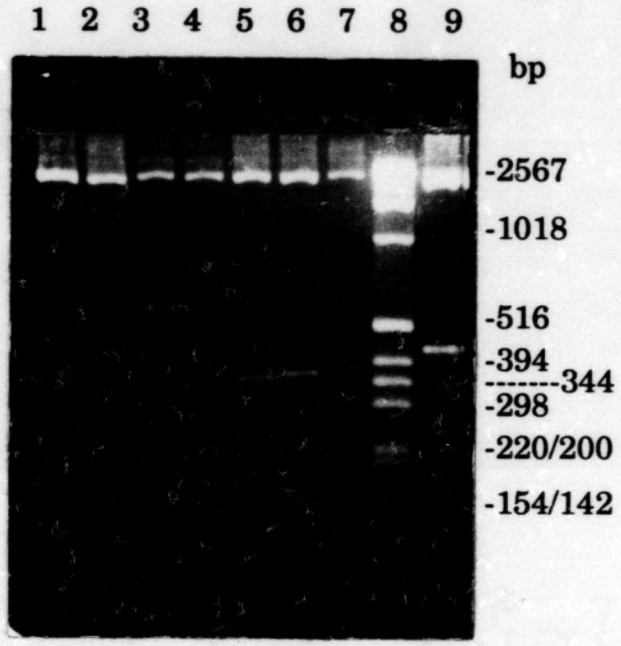

Figure 5. Electrophoresis of Puv II cut DNA isolated from pGEM-7zf(+) clones that were generated by Exo III treatment. Lane 7-1, plasmid DNAs from different time point clones (from 20 seconds to 2 minutes and 20 seconds). Lane 8: 1 Kbp DNA ladder (BRL). Lane 9: original pGEM-7zf(+) DNA. Electrophoresis was on a 0.6% agarose gel.

around 298 bp, that was used for subsequent DNA sequencing.

DNA Sequence Analysis

In order to confirm the size of the deletion, DNA was sequenced by the chain termination method. Heat denatured sequencing reactions were run on a 6% polyacrylamide gel containing 8 M urea. Figure 6 is an autoradiograph of this gel and demonstrates that the deletion ranges from bases 50 to 188. Actually, the 4-base 3' overhang, generated by cutting with the Kpn I restriction enzyme at base 53, was deleted during the S1 nuclease treatment so that base 49 but not 53 is the border base in this deletion. In this clone not only has the SD region been deleted but also part of the *lac* operator sequences. This plasmid was subsequently called pGEM-SD and Figure 7 shows the nucleotide sequence at the new operator/promoter region.

Northern Blot Analysis

To determine if RNA was being transcribed from non-recombinant pGEM-SD plasmid, bacterial cells containing pGEM-SD plasmids were grown with and without IPTG. RNA samples were isolated from these cells and separated on a denaturing agarose gel, using glyoxal/DMSO as the denaturant. The separated RNA was then transferred onto a nitrocellulose filter. High specific activity (10^8 CPM/μg) DNA probes were generated from the pGEM-SD plasmid by nick translation. The size and amount of a specific RNA species was determined by hybridizing the RNA on the filter to the labeled DNA probe. Figure 8 shows the results of the hybridization. Lane 2 shows that an extra mRNA band (5.2 Kbp) was induced by IPTG when compared with the uninduced RNA in lane 1. The

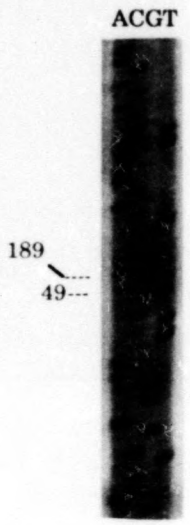

Figure 6. Autoradiograph of a DNA sequencing gel. This plasmid DNA was isolated from pGEM-7zf(+) clone as shown in lane 4 of Figure 5. This sequencing gel shows that bases from positions 50 to 188 were deleted. Electrophoresis was on a 6% acrylamide/bisacrylamide (19 : 1) gel containing 8 M urea. The running buffer was 89 mM Tris borate (pH 8.0) and 2 mM EDTA.

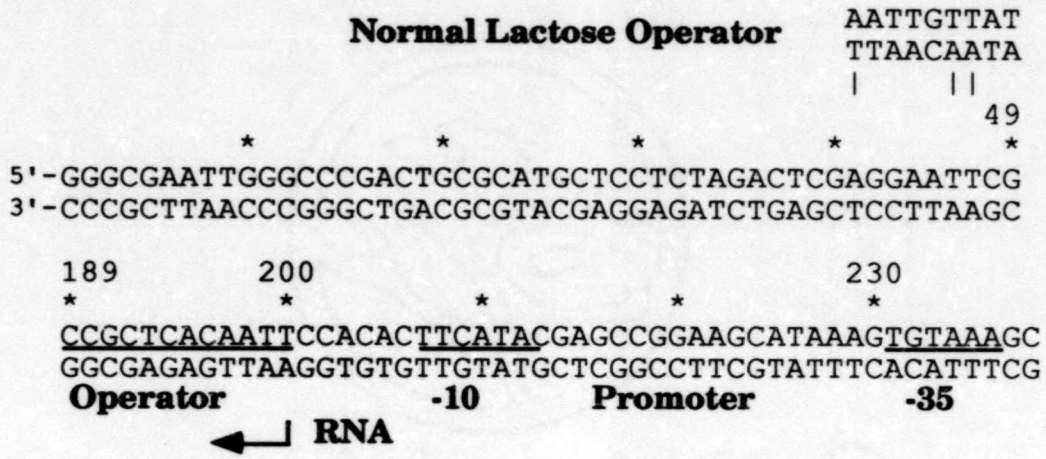

Figure 7. Partial nucleotide sequence of pGEM-SD plasmid. This DNA sequence shows the lactose transcription start, part of *lac* operator sequence (189-200), and *lac* promoter sequence (207-236). Part of normal *lac* operator sequence (180-188) of pGEM-7zf(+) plasmid is shown on top row for comparison.

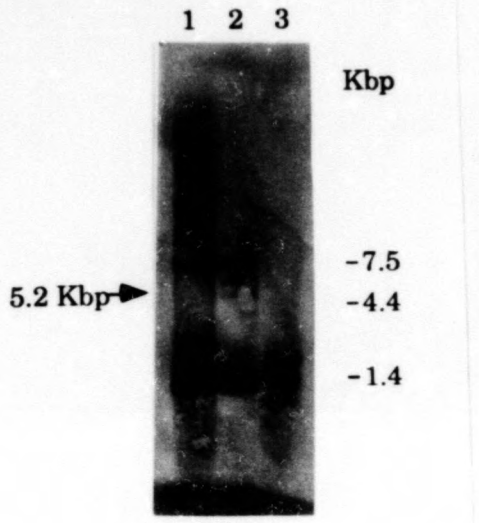

Figure 8. Northern blots of bacterial mRNA. The full-length nucleotides (2862 bp) of the pGEM-SD plasmid was nick-translated with α-S^{35}-labeled dATP and used as the probe. RNA was isolated from pGEM-SD plasmid-containing *E.coli* JM109 cultures without IPTG (Lane 1), RNA isolated from pGEM-SD plasmid-containing *E.coli* JM109 cultures treated with IPTG (Lane 2), and 0.24-9.5 Kbp RNA ladder (BRL) (Lane 3) were hybridized with the α-S^{35}-labeled nick-translation probe. Arrow indicates the 5.2 Kbp mRNA that was induced with IPTG. The size of RNA was determined by staining the gel with ethidium bromide.

size of the induced mRNA corresponds to the length of an RNA transcribed from *lac* promoter to ampicillin resistant gene terminator (1 2/3 round of pGEM-SD plasmid).

Construction of Recombinant Plasmids

Recombinant plasmids were made to determine if the inserted bacterial genomic DNA could be expressed as RNA from the pGEM-SD plasmid vector. Since longer antisense RNA inhibits translation more effectively, wild type strain of *E.coli* ATCC was cut with the 6 base-cutter enzyme (Sal I). The average 4.0 Kbp Sal I fragment from the bacterial genomic DNA can be visualized in Figure 9. Lane 1 shows the cut DNA resulted in a smear centered at 4.0 Kbp while lane 2 uncut DNA shows a larger smear region. pGEM-SD plasmid was cut to completion with Xho I as shown by the 3 Kbp band in lane 3 of Figure 9. The 4.0 Kbp Sal I fragment was ligated into the Xho I site of the pGEM-SD plasmid and because Sal I ends and Xho I ends are compatible sticky ends, the inserted DNA can be cloned in either of the two possible orientations with respect to the vector sequences. Recombinant ligated plasmids were transformed into *E.coli* JM109 and selected by screening for recombinant fragment sizes generated from EcoR I digestion of miniprep DNA. The restriction fragments were analyzed by electrophoresis using a 0.7% agarose gel (Figure 10). Two visible bands are shown (lane 3), indicating that the pGEM-SD recombinant clone contains an inserted bacterial DNA of approximately 4 Kbp. This clone could be used for northern hybridization analysis.

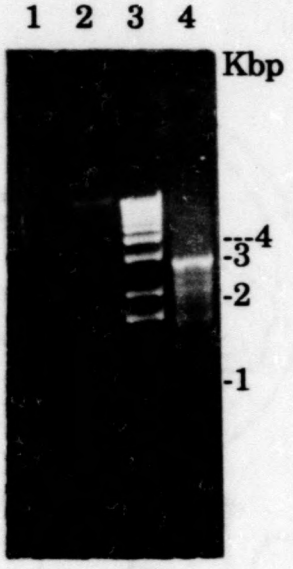

Figure 9. Electrophoresis of cut bacterial genomic and pGEM-SD DNA on a 0.7% agarose gel. Lane 1: 1 μg of bacterial genomic DNA cut with Sal I. Lane 2: 1 μg of uncut bacterial genomic DNA. Lane 3: 1 Kbp DNA ladder. Lane 4: 0.3 μg of pGEM-SD plasmid DNA cut with Xho I.

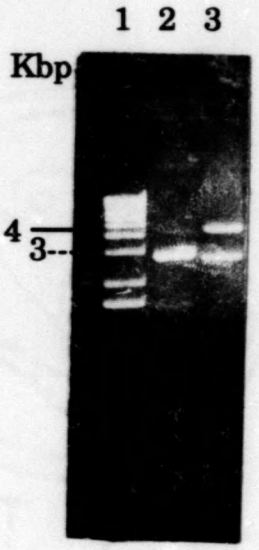

Figure 10. Agarose gel electrophoresis of vector and recombinant plasmid miniprep DNA. DNA samples were cut with EcoR I and electrophoresed on a 0.7% agarose gel. Lane 1: 1 Kbp DNA ladder. Lane 2: pGEM-SD plasmid DNA. Lane 3: pGEM-SD plasmid DNA containing a fragment of *E.coli* genomic DNA.

Discussion

The aim of the current study was to construct a potent vector devoid of a SD sequence and to show that gene expression can be regulated via this vector. It has been suggested that a SD sequence plays a crucial role in the selection of the translation initiation starts in prokaryotes and the control of translational efficiency (Shine and Dalgarno, 1974). When a SD sequence is absent from mRNA, it will prevent ribosome binding and inactivate translation of mRNA. Since the pGEM-7zf(+) vector contained a SD sequence, it was necessary to delete this region to prevent antisense RNA with a SD region from folding back onto the vector SD region. The present results demonstrate that the constructed pGEM-SD plasmid was devoid of the SD sequence and that RNA was inducible with IPTG. These results are similar to the conclusion that *mic*RNAs can be induced under control of the *lac* promoter (Coleman *et al.*, 1984). Not only *lac* promoter but also λP_L promoter has been used for regulation of gene expression. λP_L promoter is controlled by the temperature-sensitive CI repressor protein. Thus, at 30°C, no anti-mRNA would be made, but at 42°C anti-mRNA would be synthesized extensively. Pestka *et al.* (1984) have shown that antisense-β-galactosidase RNA can be induced under control of the λP_L promoter. The *lac* promoter was chosen over λP_L promoter in this study because when the temperature raises to 42°C part of gene products would be turned off under control of the λP_L promoter.

The pGEM-SD plasmid is missing not only the SD sequence but also part of the *lac* operator sequence (refer to Figures 4 and 7). Although part of the *lac* operator sequence (nucleotides 180-188) was deleted in pGEM-SD

plasmid, Figure 8 shows that mRNA still can be induced. In Figure 7, comparing the *lac* operator of pGEM-SD plasmid to normal *lac* operator of pGEM-7zf(+) plasmid, bases at positions 41, 46 and 47 of pGEM-SD plasmid are identical to bases at positions 180, 185 and 186 of the deleted operator region. Methylation and cross-linking studies have shown that the residues at positions corresponding to positions 41, 46, and 47 in the pGEM-SD plasmid are closely associated to the operator (Miller and Reznikoff, 1980) and conservation of these residues in the pGEM-SD plasmid undoubtedly contributes to it's continued inducibility.

It was shown in Figure 10 that the pGEM-SD vector can carry inserted bacterial DNA of approximately 4 Kbp. More studies will be required to show that the mRNAs are inducable with IPTG from the recombinant plasmid. In order to get longer antisense RNA to inhibit translation more efficiently, *E.coli* ATCC strain was cut with Sal I resulting in average 4.0 Kbp restriction fragments. The length of the antisense RNA is an important variable to be considered for gene regulation. The longer *mic* (*omp*C) RNA was 4-fold more effective at inhibiting OmpC production than the shorter *mic* (*omp*C) RNA (Coleman *et al.*, 1984). Also Ellison *et al.* (1985) suggested that longer antisense RNA transcripts were more effective at blocking β-galactosidase synthesis than shorter transcripts.

Recombinant plasmids that have been created could contain some inserted genes in either the sense or antisense orientation relative to the *lac* promoter. Since recombinant plasmids have been generated already the next step will be to screen recombinant clones for those which would inhibit specific gene products. This screening can be done by plating these clones on different selective indicator media both with and without the inducer

IPTG and observing phenotypic differences. About 200 recombinant clones have been plated on different selective indicator media (Motility Indole Lysine Medium, Motility Indole Ornithine Medium, Lysine Decarboxylase Agar, Violet Red Bile Agar, Methyl Red and Voges-Proskanev Agar, EMB Agar, and m Endo Agar LES) with and without IPTG, so far no phenotypic difference has been observed. Since a bacterial haploid genome contains approximately 4.2×10^6 base pairs, a particular 4000-bp fragment of interest only comprises one part in 10^3 of a preparation of genomic DNA. In generating a useful recombinant DNA library from genomic DNA, a huge population of clones is necessary to ensure that the library contains at least one version of every sequence of interest. The likehood that a sequence of interest is present in such a random library can be estimated by simple statistics based on the Poisson distribution (Clarke and Carbon, 1976). Specifically, the number of independent clones, N, that must be screened to isolate a particular sequence with probability P is given by

$$N = \ln(1-P) / \ln[1-(I/G)]$$

Where I is the size of the average cloned fragment, in base pairs, and G is the size of the target genome, in base pairs. In this study, approximately 4750 clones would need to be screened to have a 99% chance of isolating a desired sequence. Since this cloning method allowed fragments to be inserted in two possible orientations relative to the *lac* promoter, only half the clones will be in the antisense orientation. Therefore, the number of clones needed to be screened will be doubled to find one particular antisense fragment. Since in this study only 200 clones were screened, it was not surprising that antisense clones were not found specific for any of the metabolic pathways monitored. The number of clones to be screened could be reduced by more than half if cDNA would be directionally cloned into the

pGEM-SD vector in the antisense orientation.

Like pGEM-SD plasmid, plasmid pBD6 was constructed to generate anti-*lacZ* mRNA lacking a synthetic ribosome binding site adjacent to the λ P_L promoter and the *lacZ* ribosome binding site in reverse orientation (Daugherty *et al.*, 1989). Surprisingly, there still was a significant amounts of β-galactosidase was synthesized. Some aspects need to take into consideration to accout for this phenomenon. Several studies have reported that the abundance of antisense transcripts targeted to 5' noncoding sequences, the initiation codon, and coding sequences must exceed the abundance of the mRNA by 1-2 orders of magnitude to effectively block expression of the targeted gene (Green *et al.*, 1986). Also, the degree of complementarity between the antisense RNA and target mRNA is considerable for gene expression by antisense RNA (Ellison *et al.*, 1985). Therefore, these suggestions may partially explain why, in 200 screened recombinant clones, none were found specific for any of the metabolic pathways monitored. Further experiments are required to quantitate the concentration of antisense RNA versus mRNA targets in bacterial cells. The degree of complementarity between the antisense RNA and target mRNA is less considerable for gene expression in this study. Because E.coli ATCC and JM109 strains are the same bacterial cells just different species, they would contain the homologous gene sequences.

In this preliminary work, a potential antisense vector was constructed and it was shown that gene expression can be turned on and off at will. Hopefully, this vector system can provide a way to selectively turn off the expression of any *E.coli* gene including potentially lethal genes. This induced lethality may be a more effective tool in the study of essential genes than conventional temperature-sensitive mutations. Further experiments

are required to reach the long-term goal of gene mapping with antisense RNA.

Appendix

Agarose Gel Electrophoresis

10X TBE buffer

0.89 M Tris
0.89 M Boric acid
20mM EDTA, pH 8.0

Unidirectional Deletion With Exonuclease III

10X ExoIII buffer

660mM Tris-HCl, pH 8.0
6.6mM $MgCl_2$

S1 mix (for 25 time points)

172 µL deionized water
27 µL 7.4X S1 buffer
60 units S1 nuclease

7.4X S1 buffer

0.3 M Potassium acetate, pH 4.6
2.5 M NaCl
10mM $ZnSO_4$
50% Glycerol

S1 stop buffer

0.3 M Tris base
0.05 M EDTA

Klenow mix

30 µL 1X Klenow buffer
5 units Klenow DNA polymerase

1X Klenow buffer

20mM Tris-HCl, pH 8.0
100mM $MgCl_2$

dNTP mix

0.125 mM each of dATP, dCTP, dGTP, and dTTP

Ligase mix

790 µL deionized water
100 µL 10X ligase buffer
100 µL 50% polyethylene glycol (PEG)
10 µL 100mM dithiothreitol (DTT)
5 units T4 DNA ligase

10X Ligase buffer

500mM Tris-HCl, pH 7.6
100mM $MgCl_2$
10mM ATP

DNA Sequencing Analysis

Sequenase reaction buffer

200mM Tris-HCl, pH 7.5
100mM $MgCl_2$
250mM NaCl

Primer (-40) 0.5 pmole/µL

5'-GTTTTCCCAGTCACGAC-3'

5X Labeling mix

7.5µM each of dGTP, dCTP, and dTTP

Mn buffer

0.15 M Sodium isocitrate
0.1 M $MnCl_2$

Sequenase Version 2.0 (13 units/µL)

Clones of T7 DNA polymerase have been modified by USB.

Sequenase dilution buffer

10mM Tris-HCl, pH 7.5
5mM DTT
0.5 mg/mL BSA

ddA Termination mix

80μM each of dGTP, dATP, dCTP, and dTTP
8μM ddATP, 50mM NaCl

ddC Termination mix

80μM each of dGTP, dATP, dCTP, and dTTP
8μM ddCTP, 50mM NaCl

ddG Termination mix

80μM each of dGTP, dATP, dCTP, and dTTP
8μM ddGTP, 50mM NaCl

ddT Termination mix

80μM each of dGTP, dATP, dCTP, and dTTP
8μM ddTTP, 50mM NaCl

Termination stop solution

95% Formamide
20mM EDTA
0.05% Bromophenol blue
0.05% Xylene cyanol

Preparation of Bacterial RNA

Protoplasting buffer

15 mM Tris-HCl, pH 8.0
0.45 M Sucrose
8mM EDTA

Preparation of the Nick-Translated Probe

Solution A1

500mM Tris-HCl, pH 7.8
50mM $MgCl_2$
100mM 2-mercaptoethanol
100 μg/mL Nuclease-free bovine serum albumin (BSA)
0.2mM each of dCTP, dGTP, dTTP

Solution C

0.4 unit/μL DNA polymerase I
40 pg/μL DNase I
50mM Tris-HCl, pH 7.5
5mM Mg-acetate
1mM 2-mercaptoethanol
0.1mM Phenylmethyl-sulfonyl fluoride (PMSF)
50% Glycerol
100 ug/mL Nuclease-free BSA

Solution D : stop buffer

300mM Na_2EDTA, pH 8.0

TE buffer

10mM Tris-HCl, pH 8.0
1mM EDTA, pH 8.0

Northern Blot Analysis

Glyoxal loading buffer

50% Glycerol
10mM Sodium phosphate, pH 7.0
0.4% Bromophenol blue

20X SSC

3 M NaCl
0.3 M Trisodium citrate, pH 7.0

Prehybridization solution

25mM KPO_4, pH 7.4
5X SSC
5X Denhardt's solution
50 μg/mL Herring sperm DNA
50% Formamide

100X Denhardt's solution

2% Ficoll 400
2% Polyvinylpyrrolidone
2% BSA

Literature Cited

Alexander, D.C. 1987. An efficient vector-primer cDNA cloning system. *Meth. Enzymol.* **154**, 41-46, Academic Press, New York.

Andersen, J., Forst, S.A., Zhao, K., Inouye, M., and Delihas, N. 1989. The function of *micF* RNA. *J. Biol. Chem.* **264**, 17961-17970.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. 1991. *Current Protocols In Molecular Biology,* Vol. **1**. John Wiley and Sons: New York.

Bass, B.L., and Weintraub, H. 1988. An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell* **55**, 1089-1098.

Bethesda Research Laboratories procedure for labeling DNA by nick translation. 1990. Bethesda Research Laboratories Life Technologies, Inc. Gaithersburg, MD.

Birnboim, H.C. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. *Meth. Enzymol.* **100**, 243-255, Academic Press, New York.

Blomberg, P., Wagner, E.G.H., and Norstrom, K. 1990. Control of replication plasmid R1: The duplex between the antisense RNA, CopA, and its target, CopT is processed specifically *in vivo* and *in vitro* by RNase-III. *EMBO J.* **9**, 2331-2340.

Brady, G., Frey, J., Danbara, H., and Timmis, K.N. 1983. Replication control mutations of plasmid R6-5 and their effects on interactions of the RNA-I control element with its target. *J. Bacteriol.* **154**, 429-436.

Cannon, M., Platz, J., O'Leary, M., Sookdeo, C., and Cannon, F. 1990. Organ-specific modulation of gene expression in transgenic plants using antisense RNA. *Plant Mol. Biol.* **15**, 39-47.

Cech, T.R. 1986. RNA as an enzyme. *Sci. Am.* **255, November**, 64-75.

Chang, L., and Stoltzfus, C.M. 1987. Inhibition of Rous sarcoma virus replication by antisense RNA. *J. Virol.* **61**, 921-924.

Ch'ng, J.L., Mulligan, R.C., Schimmel, P., and Holmes, E.W. 1989. Antisense RNA complementary to 3' coding and noncoding sequences of creatine kinase is a potent inhibitor of translation *in vitro*. *Proc. Natl. Acad. Sci USA* **86**, 10006-10010.

Citron, M., and Schuster, H. 1990. The c4 repressors of bacteriophages P1 and P7 are antisense RNAs. *Cell* **62**, 591-598.

Clarke, L., and Carbon, J. 1976. A colony bank containing synthetic *col*E1 hybrids representative of the entire *E.coli* genome. *Cell* **9**, 91-99.

Coleman, J., Green, P.J., Inouye, M. 1984. The use of RNAs complementary to specific mRNAs to regualte the expression of individual bacterial genes. *Cell* **37**, 429-436.

Coleman, J., Hirashima, A., Inokuchi, Y., Green, P.J., and Inouye, M. 1985. A novel immune system against bacteriophage infection using complementary RNA (micRNA). *Nature* **315**, 601-603.

Cornelissen, M., and Vandewiele, M. 1989. Both RNA level and translation efficiency are reduced by antisense RNA in transgenic tobacco. *Nucleic Acids Res.* **17**, 833-843.

Crowley, T.E., Nellen, W., Gomer, R.H., and Firtel, R.A. 1985. Phenocopy of discoidin I-minus mutants by antisense transformation in *Dictyostelium*. *Cell* **43**, 633-641.

Dagle, J.M., Weeks, D.L., and Walder, J.A. 1991. Pathways of degradation and mechanism of action of antisense oligonucleotides in *Xenopus laevis* embryos. *Antisense Research and Development* **1**, 11-20.

Daugherty, B.L., Hotta, K., Kumar, C., Ahn, Y.H., Zhu, J., and Pestka, S. 1989. Antisense RNA: Effect of ribosome binding sites, target location, size, and concentration on the translation of specific mRNA molecules. *Gene Anal. Techn.* **6**, 1-16.

Eguchi, Y., Itoh, T., and Tomizawa, J.I. 1991. Antisense RNA. *Ann. Rev. Biochem.* **60**, 631-652.

Ellison, M.J., Kelleher III, R.J., and Rich, A. 1985. Thermal regulation of β-galactosidase synthesis using antisense RNA directed against the coding portion of the mRNA. *J. Biol. Chem.* **260**, 9085-9087.

Green, P.J., Pines, O., and Inouye, M. 1986. The role of antisense RNA in gene regulation. *Ann. Rev. Biochem.* **55**, 569-597.

Haseloff, J., and Gerlach, W.L. 1988. Simple RNA enzyme with new and highly specific endoribonuclease activities. *Nature* **334**, 585-591.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351-359.

Hirashima, A., Sawaki, S., Inokuchi, Y., and Inouye, M. 1986. Engineering of the mRNA-interfering complementary RNA immune system against viral infection. *Proc. Natl. Acad. Sci. USA* **83**, 7726-7730.

Izant, J.G., and Weintraub, H. 1984. Inhibition of thymidine kinase gene expression by antisense RNA: A molecular approach to genetic analysis. *Cell* **36**, 1007-1015.

Kim, S.K., and Wold, B.J. 1985. Stable reduction of thymidine kinase activity in cells expressing high-levels of antisense RNA. *Cell* **42**, 129-138.

Kimelman, D., and Kirschner, M.W. 1989. An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in *Xenopus* oocytes. *Cell* **59**, 687-696.

Kumar, C.C., and Novick, R.P. 1985. Plasmid pT181 replication is regulated by two countertranscripts. *Proc. Natl. Acad. Sci. USA* **82**, 638-642.

Lim, H.M., and Pene, J.J. 1988. Optimal conditions for supercoil DNA sequencing with the *Escherichia coli* DNA polymerase I large fragment. *Gene Anal. Tech.* **5**, 32-39.

Lis, J.T. 1980. Fractionation of DNA fragments by polyethylene glycerol induced precipitation. *Meth. Enzymol.* **65**, 347-353, Academic Press, New York.

Ma, C., and Simons, R.W. 1990. The IS10 antisense RNA blocks ribosome binding at the transposase translation initiation site. *EMBO J.* **9**, 1267-1274.

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York.

McMaster, G.K., and Carmichael, G.G. 1977. Analysis of single-stranded and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**, 4835-4838.

Melton, D.A. 1985. Injected anti-sense RNAs specifically block messenger RNA translation *in vivo*. *Proc. Natl. Acad. Sci. USA* **82**, 144-148.

Miller, J.H., and Reznikoff, W.S. 1980. In "The Operon". Cold Spring Harbor, New York.

Mizuno, T., Chou, M.Y., and Inouye, M. 1984. A unique mechanism regulating gene expression: Translational inhibition by a complementary RNA transcript (micRNA). *Proc. Natl. Acad. Sci. USA* **81**, 1966-1970.

Novick, R.P., Iordanescu, S., Projan, S.J., Kornblum, J., and Edelman, I. 1989. pT181 plasmid replication is regulated by a countertranscript-driven transcriptional attenuator. *Cell* **59**, 395-404.

Okamoto, K., and Freundlich, M. 1986. Mechanism for the autogenous control of the *crp* operon: Transcriptional inhibition by a divergent RNA transcript. *Proc. Natl. Acad. Sci. USA* **83**, 5000-5004.

Pestka, S., Daugherty. B.L., Jung, V., Hotta, K., and Pestka, R.K. 1984. Anti-mRNA: Specific inhibition of translation of single mRNA molecules. *Proc. Natl. Acad. Sci. USA* **81**, 7525-7528.

Promega Protocols and Applications Guide. 1991. (D.E. Titus, Ed.) pp. 90-98. Promega Corporation. Madison, WI.

Qian, S., Hongo, S., and Jacobs-Lorena, M. 1988. Antisense ribosomal protein gene expression specifically disrupts oogenesis in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **85**, 9601-9605.

Rosenberg, M., and Court, D. 1979. Regulatory sequences involved in promotion and termination of RNA transcription. *Ann. Rev. Genet.* **13**, 319-353.

Sandler, S.J., Stayton, M., Townsend, J.A., Ralston, M.L., Bedbrook, J.R., and Dunsmuir, P. 1988. Inhibition of gene expression in transformed plants by antisense RNA. *Plant Mol. Biol.* **11**, 301-310.

Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Sczakiel, G., and Pawlita, M. 1991. Inhibition of human immunodeficiency virus type 1 replication in human T cells stably expressing antisense RNA. *J. Virol.* **65**, 468-472.

Shine, J., and Dalgarno, L. 1974. The 3'-terminal sequence of *E.coli* 16 S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. *Proc. Natl. Acad. Sci. USA* **71**, 1342-1346.

Simons, R.W., and Kleckner, N. 1983. Translational control of IS10 transposition. *Cell* **34**, 683-691.

Simons, R.W., and Kleckner, N. 1988. Biological regulation by antisense RNA in prokaryotes. *Ann. Rev. Genet.* **22**, 567-600.

Smith, C.J.S., Watson, C.F., Ray, J., Bird, C.R., Morris, P.C., Schuch, W., and Grierson, D. 1988. Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* **334**, 724-726.

Stormo, G.D. 1986. Translation Initiation. In"Maximizing Gene Expression" (W. Reznikoff, L. Gold, Eds) pp. 195-224. Boston: Butterworths.

Thomas, P.S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* **77**, 5201-5205.

Tomizawa, J., Itoh, T., Selzer, G., and Som, T. 1981. Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc. Natl. Acad. Sci. USA* **78**, 1421-1425.

Uhlenbeck, O.C., Borer, P.N., Dengler, B., and Tinoco, I. 1973. Stability of RNA hairpin loops: A_6-C_m-U_6. *J. Mol. Biol.* **73**, 483-496.

United State Biochemical Corporation Protocols for Sequenase Version 2.0. 1990. Step-By-Step Protocols For DNA Sequencing With Sequenase Version 2.0. United Stated Biochemical Corporation. Cleveland, Ohio.

Van der Krol, A.R., Mol, J.N.M., Stuitje, A.R. 1988. Modulation of eukaryotic gene expression by complementary RNA or DNA sequences. *Biotechniques* **6**, 958-976.

Wahl, G.M., Stern, M., and Stark, G.R. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. USA* **76**, 3683-3687.

Weintraub, H.M. 1990. Antisense RNA and DNA. *Sci. Am.* **262, January**, 40-46.

Wormington, W.M. 1986. Stable repression of ribosomal protein L1 synthesis in *Xenopus* oocytes by microinjection of antisense RNA. *Proc. Natl. Acad. Sci. USA* **83**, 8639-8643.

Yokoyama, K., and Imamoto, F. 1987. Transcriptional control of the endogenous MYC protooncogene by antisense RNA. *Proc. Natl. Acad. Sci. USA* **84**, 7363-7367.