Antisense drugs are short (10 to 25 bases) oligomers that mimic DNA or RNA and inhibit gene expression in a sequence-dependent manner. PMOs of various lengths (7 to 20 bases) were tested for inhibition of luciferase expression in *Escherichia coli*. Shorter PMOs generally inhibited luciferase greater than longer PMOs. Conversely, in bacterial cell-free protein synthesis reactions, longer PMOs inhibited equally or more than shorter PMOs. Overlapping, isometric (10-base) PMOs complementary to the region around the start codon of luciferase inhibited to different extents in bacterial cell-free protein expression reactions. Including the anti-start codon in PMOs was not required for maximal inhibition. PMOs targeted to 5' untranslated or 3' coding regions within luciferase mRNA did not inhibit, except for one PMO targeted to the ribosome-binding site. Inhibition of luciferase expression correlated negatively with the predicted secondary structure of mRNA regions targeted by PMO but did not correlate with C+G content of targeted regions. The effects of PMO length and position were corroborated by using PMOs (6 to 20 bases) targeted to *acpP*, a gene required for viability. Because inhibition by PMOs of ~11 bases was unexpected based on previous results in eukaryotes, we tested an 11-base PMO in HeLa cells and reticulocyte cell-free protein synthesis reactions. The 11-base PMO significantly inhibited luciferase expression in HeLa cells, although less than did a 20-base PMO. In reticulocyte cell-free reactions, there was a trend toward more inhibition with longer PMOs. These studies indicate that strategies for designing PMOs are substantially different for prokaryotic than eukaryotic targets.

**MATERIALS AND METHODS**

PMOs. PMOs were synthesized and purified at AVI BioPharma, Inc. (Corvallis, Ore.), as previously described (11), dissolved in water, filtered through a 0.2-μm-pore-size membrane (HT Tuffryn; Gelman Sciences, Inc., Ann Arbor, Mich.), and stored at 4°C. Sequences of PMOs used in the present study are shown in Table 1. The concentration of PMO was determined spectrophotometrically by measuring the absorbance at 260 nm and calculating the molarity by using an extinction coefficient calculated as described previously (4). The base sequences of nonsense PMOs were chosen randomly from a pool of bases that approximated the relative abundance of bases in the corresponding PMOs that were targeted to either *myc-luc* or *acpP*.

**Bacteria and growth conditions.** *E. coli* AS19 (26) and SM101 (9), which have defects in lipopolysaccharide synthesis that result in outer membrane permeability to high-molecular-weight solutes, were grown aerobically in Luria-Bertani broth at 37 and 30°C, respectively. Transformants that expressed pSE380myc-luc were grown in Luria-Bertani medium plus 100 μg of ampicillin/ml.

**Reporter gene.** Standard molecular biology procedures (1) were used for all constructions. All constructs were sequenced. Two reporter systems (pT7myc-luc and pSE380myc-luc) for antisense inhibition were previously constructed, as described previously (11), by fusing 30 bp of the 5' end of human c-myc to all but...
### TABLE 1. PMO characteristics

| PMO     | Target        | Sequence (5’→3’)a | Length (no. of bases) | %G+C | 2° scoreb |
|---------|---------------|-------------------|-----------------------|------|-----------|
| 328     | myc-luc       | ACG TTG A         | 7                     | 43   | 0         |
| 327     | myc-luc       | ACG TTG AG        | 8                     | 50   | 0         |
| 326     | myc-luc       | ACG TTG AGG       | 9                     | 56   | 0         |
| 208     | myc-luc       | ACG TTG AGG G     | 10                    | 60   | 0         |
| 340     | myc-luc       | ACG TTG AGG GGG   | 11                    | 64   | 0         |
| 298     | myc-luc       | ACG TTG AGG GGC   | 12                    | 67   | 0         |
| 250     | myc-luc       | ACG TTG AGG GGC A | 13                    | 62   | 0         |
| 249     | myc-luc       | ACG TTG AGG GGC AT | 14                 | 57   | 0         |
| 248     | myc-luc       | ACG TTG AGG GGC ATC | 15                 | 60   | 0         |
| 247     | myc-luc       | ACG TTG AGG GGC ATC G | 16         | 62   | 0.0625   |
| 246     | myc-luc       | ACG TTG AGG GGC ATC GT | 17                 | 59   | 0.1176   |
| 245     | myc-luc       | ACG TTG AGG GGC ATC GTC | 18               | 61   | 0.1667   |
| 126     | myc-luc       | ACG TTG AGG GGC ATC GTC GC | 20           | 65   | 0.2000   |
| 239     | myc-luc       | G TTG AGG GGC ATC GTC GC | 18           | 67   | 0.2222   |
| 240     | myc-luc       | TTG AGG GGC ATC GTC GC | 17           | 65   | 0.2353   |
| 241     | myc-luc       | TG AGG GGC ATC GTC GC | 16           | 69   | 0.2500   |
| 242     | myc-luc       | G AGG GGC ATC GTC GC | 15           | 73   | 0.2667   |
| 243     | myc-luc       | AGG GGC ATC GTC GC | 14           | 71   | 0.2857   |
| 244     | myc-luc       | GG GGC ATC GTC GC | 13           | 77   | 0.3077   |
| 329     | myc-luc       | GG GGC ATC GTC GC | 12           | 75   | 0.3333   |
| 330     | myc-luc       | GG GGC ATC GTC GC | 11           | 73   | 0.3636   |
| 331     | myc-luc       | GC ATC GTC GC | 10           | 70   | 0.4000   |
| 332     | myc-luc       | GC ATC GTC GC | 9            | 67   | 0.4444   |
| 333     | myc-luc       | ATC GTC GC | 8            | 62   | NC        |
| 334     | myc-luc       | ATC GTC GC | 7            | 71   | NC        |
| 341     | myc-luc 5’ end transcript | GGA AAC CGT TGT GGT TCT CC | 20       | 60   | 0.7500   |
| 342     | myc-luc 5’ end transcript | AC CGT TGT GGT TCT CC | 16       | 62   | 0.6875   |
| 343     | myc-luc 5’ end transcript | GT TGT GGT TCT CC | 13       | 69   | 0.6154   |
| 344     | myc-luc 5’ end transcript | GT GGT TCT CC | 10       | 70   | 0.8000   |
| 345     | myc-luc RBS and 3’ of RBS | CGT CGC GGG ATT CCT TCT | 18 | 61 | 0.3889 |
| 346     | myc-luc 5’ of RBS | AAA GGT AAA CAA AAT TAT | 18 | 11 | 0.1667 |
| 347     | myc-luc RBS and 5’ of RBS | TCC TCC TTA AAG TTA AAC | 18 | 28 | 0.3333 |
| 356     | myc-luc       | GTG TGA GGG G | 10           | 70   | 0        |
| 357     | myc-luc       | GT TGA GGG GC | 10           | 70   | 0        |
| 358     | myc-luc       | T TGA GGG GCA | 10           | 60   | 0        |
| 359     | myc-luc       | TGA GGG GCA T | 10           | 60   | 0        |
| 360     | myc-luc       | GA GGG GCA TC | 10           | 70   | 0        |
| 361     | myc-luc       | A GGG GCA TCG | 10           | 70   | 0.1000   |
| 362     | myc-luc       | GGG GCA TCG T | 10           | 70   | 0.2000   |
| 363     | myc-luc       | GG GCA TCG TC | 10           | 70   | 0.3000   |
| 364     | myc-luc       | G GCA TCG TCG | 10           | 70   | 0.4000   |
| 214     | luc, unpaired loop | AAT AGG GTT GG | 11           | 45   | 0        |
| 215     | luc, base-paired stem | TTT GCA ACC CC | 11           | 55   | 0.9091   |
| 143     | Nonsense control for myc | ATC CTC CCA ACT TCG ACA TA | 20 | 45 | NC |
| 371     | Nonsense control for myc | TGC CGA CGG CGT CTA TA | 20 | 60 | NC |
| 373     | Nonsense control for myc | TCC ACT TGC C | 10 | 60 | NC |
| 62-1    | acpP           | TTC TTC GAT GGT CAT AC | 20         | 40   | NC        |
| 62-2    | acpP           | TC TTC GAT GGT CAT A | 18         | 39   | NC        |
| 62-3    | acpP           | C TTC GAT GGT CAT | 16         | 44   | NC        |
| 62-4    | acpP           | TC GAT GGT CAT | 14         | 43   | NC        |
| 169     | acpP           | C TTC GAT GGT G | 11         | 45   | NC        |
| 379     | acpP           | TTC GAT GGT G | 10         | 40   | NC        |
| 380     | acpP           | TTC GAT GGT | 9          | 33   | NC        |
| 381     | acpP           | TTC GAT GGT | 8          | 38   | NC        |
| 382     | acpP           | TC GAT AG | 7          | 43   | NC        |
| 383     | acpP           | C GAT AG | 6          | 50   | NC        |
| 62-5    | Nonsense control | TTG TCC TGA ATA TCA CTT CG | 20 | 40 | NC |
| 62-7    | Nonsense control | G TTC TGA ATG TCA CTT | 16 | 38 | NC |
| 62-8    | Nonsense control | TCG TGA GTA TCA CT | 14 | 43 | NC |
| 170     | Nonsense control | TCT CAG ATG GT | 11 | 45 | NC |
| 384     | Nonsense control | AAT CGG A | 7 | 43 | NC |

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72. a The initiation anticodon is shown in boldface.
72. b That is, the fraction of bases complementary to base-paired, folded mRNA target. NC, not calculated.
the start codon of the gene for firefly luciferase (luc from pGL-2; Promega Corp., Madison, Wis.). The constructs were separately transformed into E. coli SM101 and AS19.

The acpP-luc reporter (pCNacpP-luc) was made by ligating a Sall-NotI restriction fragment of luc with the Sall-NotI fragment of pCNacpP (Promega Corp.), removing the adenine from the start codon by site-directed mutagenesis, and then directionally cloning a synthetic fragment of acpP (bp –17 to +23, inclusive, where +1 is adenine of the start codon) between the Nhel-Sall sites. pCNmyc-luc was made in the same way, except that the myc sequence from –14 through +16 (inclusive; numbering adenine of the start codon as +1) instead of acpP was cloned into the Nhel-Sall sites. Luciferase enzyme activity was measured in bacteria as described previously (11).

**Cell-free protein synthesis.** Bacterial cell-free protein synthesis reactions were performed by mixing reactants on ice according to the manufacturer’s instruction (Promega Corp.). Reactions were programmed with pT7myc-luc plasmid in a coupled transcription-translation reaction or mRNA synthesized in a cell-free RNA synthesis reaction (MEGAscript T7 High Yield Transcription Kit; Ambion, Inc., Austin, Tex.) programmed with pT7myc-luc. All acpP-luc reactions were programmed with pCNacpP-luc. Where indicated, cell-free reactions were composed with rabbit reticulocyte lysate as described by the manufacturer (Promega Corp.). PMO was added to a final concentration of either 100 or 200 nM as indicated in the figure legends. After 1 h at 37°C, the reactions were cooled on ice and luciferase was measured as described previously (11).

**Mammalian tissue culture.** HeLa cells were transfected in T75 tissue culture flasks (Nalge Nunc, Inc., Rochester, N.Y.) with a luciferase reporter plasmid (pCMyc-luc) by using Lipofectamine reagent (Gibco-BRL, Grand Island, N.Y.) according to the manufacturer, for 5 h in serum-free media (oN一夜, Gibco, Inc., Carlsbad, Calif.) before re-addition of recovery medium (HyQ mega Corp.). PMO was added to a final concentration of either 100 or 200 nM of luciferase, optical density at 600 nm, and CFU/milliliter.

### RESULTS

#### Various PMO lengths

The efficacy of PMO inhibition of target gene expression was tested as a function of the length of PMO. Two series of PMOs (Table 1) were synthesized by sequentially truncating subunits from either the 3' or 5' end of a 20-base PMO that is complementary to the region around the start codon of a luciferase reporter gene (myc-luc). myc is a eukaryotic gene without a similar sequence in E. coli and contributes the nontranslated 5' end and start codon of the reporter transcript. Previous results have shown that the 20-base myc PMO inhibits expression of this reporter gene (11). Each of the various-length (7- to 20-base) PMOs was added to growing cultures of E. coli SM101(pSE380myc-luc). After 8 h in culture, luciferase was measured. Compared to a culture without PMO, 3' truncated PMOs inhibited luciferase from 16 to 95% and 5' truncated PMOs inhibited luciferase from 6 to 89% (Fig. 1). There is a trend toward more inhibition with shorter PMOs, with a maximum at about nine bases. The seven- and eight-base, 3' truncated PMOs inhibited less than the same length 5' truncated PMOs. Control PMOs 371 and 62-8 with nonsense sequences inhibited 0 to 1%. The same pattern of inhibition was found when luciferase was measured at earlier time points (data not shown).

#### Cell-free protein synthesis

PMOs from the same two series were added individually to bacterial cell-free protein synthesis reactions programmed to express myc-luciferase. These experiments were designed to eliminate the effects of entry of PMOs into the cell and to test the PMOs at 37°C instead of the permissive growth temperature (30°C) of the conditional mutant SM101. PMOs truncated at the 3' end from 10 to 20 bases in length inhibited about the same (Fig. 2). Inhibition decreased sharply with 3' truncated PMOs less than nine bases in length. PMOs truncated at the 5' end from 12 to 20 bases in length inhibited luciferase about the same (Fig. 2). A sharp decrease in inhibition occurred with 5' truncated PMOs less than 12 bases in length. Nonsense sequence controls did not inhibit significantly.

#### Various PMO positions

A series of isometric (10-base) PMOs, which varied by 1 base at each end (Table 1) and was targeted to the region around the AUG start codon of myc-luc, was added to bacterial cell-free reactions programmed to synthesize myc-luc. All PMOs inhibited luciferase expression (Fig. 3). A trend toward more inhibition was apparent as the target position moved downstream of the start codon. There was no
correlation between inhibition and inclusion of the anti-start codon within the PMO sequence.

Another series of PMOs with various lengths was targeted to various positions within the transcript of the myc-luc, including the extreme 5' end of the transcript (PMOs 341 to 344), the ribosome-binding site (PMOs 345 and 347), the region upstream of the ribosome-binding site (PMO 346), the region around and immediately downstream of the start codon (PMO 126), and the 3' coding region of luciferase (PMOs 214 and 215). Each PMO or a nonsense PMO (371 or 373) was added (200 nM) to a bacterial cell-free protein synthesis reaction programmed to synthesize luciferase. After 1 h at 37°C, luciferase light production was measured. The results were that PMO 126 inhibited luciferase 79%, which was significantly \( (P < 0.001, n = 4) \) more inhibition than that caused by either of the nonsense PMOs 371 or 373 (6 or 4% inhibition, respectively). PMOs 345 and 347 that overlapped the ribosome-binding site inhibited luciferase 24 and 16%, respectively, which for PMO 345 was barely different \( (P = 0.016, n = 3) \) but for PMO 347 was not significantly different \( (P = 0.092, n = 6) \) than the nonsense control PMO 371. Inhibition by each of the other PMOs in this series ranged from 9 to 14% but was not significantly different \( (P > 0.1, n = 3 \text{ or } 5) \) than either of the nonsense controls.

Statistical analysis of all PMOs targeted to myc-luc, or only the 10-base isometric series indicated no correlation \( (r = 0.098, P = 0.54) \) and \( r = 0.46, P = 0.19 \), respectively) between inhibition in the cell-free reactions and percent G+C content. However, an analysis of 37 myc PMOs (Fig. 4), excluding those shorter than nine bases (327, 328, 333, and 334) and those in the 3' coding region of Luc (214 and 215), revealed a significant negative correlation \( (r = -0.85, P < 0.001) \) between inhibition of reporter expression and 2° score of the PMO (Table 1). An analysis of all 10-base PMOs targeted to myc-luc also showed a significant negative correlation \( (r = -0.91, P < 0.001) \) between inhibition and 2° score.

PMOs targeted to acpP. The effect of PMO was tested on an endogenous bacterial gene, acpP, which is essential for viability (34) and has been used previously to inhibit bacterial growth (11, 13). Ten PMOs, which varied in length (6 to 20 bases) and were complementary to the region around the start codon of acpP (Table 1), were added to cultures of AS19, and growth at 37°C was monitored by determining the optical density and viable cell counts. Growth curves were normal for all cultures except the one with the 11-base PMO, which grew significantly \( (P < 0.001) \) slower than the others (Fig. 5A). Viable cells were significantly \( (P < 0.001) \) reduced at 8 h in cultures grown with a PMO of 10, 11, or 14 bases (Fig. 5B). No reduction in CFU was apparent in cultures treated with a PMO of <10 or more
than 14 bases in length. Cultures without PMO or with one of the variable-length, nonsense base sequence PMOs did not inhibit growth.

**Cell-free inhibition of acpP.** PMOs of various lengths (from 6 to 20 bases) and targeted to acpP (PMOs 62-1, 62-2, 62-3, 62-4, 169, 379, 380, 381, 382, or 383) were added to bacterial, cell-free protein synthesis reactions programmed to express an acpP-luc reporter. The results were that PMOs 11 to 20 bases in length significantly inhibited luciferase expression to about the same extent (ranged from 35 to 50% inhibition). PMOs shorter than 11 bases in length inhibited luciferase expression less than 10% (range, 6 to 9%), which was not significantly different than nonsense sequence controls included PMOs 384, 62-8, and 62-5. Error bars indicate the SD (n = 3).

**PMO effects in HeLa cells.** Previous work in eukaryotic systems suggests that PMOs 13 to 14 subunits in length are ineffective (30). We treated HeLa cell cultures that expressed myc-luc with 10 μM myc PMO of two lengths (11 and 20 bases [PMOs 340 and 126]). Luciferase was measured at 7 and 24 h after treatment. The results were that at 7 h the 11- and 20-base PMOs inhibited luciferase expression 48% (standard deviation [SD] = 21, n = 2) and 58% (SD = 15, n = 2), respectively. At 24 h, the longer PMO inhibited luciferase 64% (SD = 6.7, n = 2), whereas the shorter PMO inhibited 25% (SD = 7.8, n = 2). A culture treated with the nonsense PMO 143 inhibited luciferase nonspecifically 16% (SD = 18, n = 2) and 2% (SD = 27, n = 2) at 7 and 24 h, respectively.

**PMO effects in reticulocyte lysate.** The 3′ truncated series of PMOs targeting myc-luc were tested for inhibition of luciferase in a cell-free protein translation reaction made with eukaryotic (rabbit reticulocyte) components. The 20-base PMO inhibited significantly more than the shorter PMO (Fig. 6). There was a sharp decrease in inhibition between the 20-base and the 18-base PMO. There was a trend of inhibition that generally favored the longer PMO.

### DISCUSSION

Rationales and rules for designing and positioning antisense oligomers within a target mRNA are based on experimental data, theoretical calculations, and bioinformatic screening in eukaryotic systems (8, 18, 15, 28, 32, 33). For PMO and PNA, which inhibit by blocking ribosome function (15), important design considerations include (i) oligomer length and base composition, which affect melting temperature; (ii) target secondary structure, which can interfere with oligomer hybridization; and (iii) position relative to the start codon, which may play a role in blocking ribosome function. Nevertheless, laborious methods of screening remain the rule for designing antisense drugs and identifying target positions (7, 27), particularly in bacteria, which is a relatively new application of antisense drug therapy (13, 21; B. L. Geller, J. Deere, and P. Iversen, submitted for publication).

Some rules for designing antisense antibacterial agents have...
recently begun to emerge. For PNA, a length of 10 to 12 bases is optimal (7); however, optimization of PNA length did not correlate with affinity for target (7). Other factors, such as transfer across the outer membrane of \textit{E. coli}, may have accounted for much of the improved efficacy of short PNA. It was not apparent that PMOs as short as 10 to 12 bases would be effective, given the uncertainty of the underlying factors that determined the optimal length of PNA.

We have found that PMOs 9 to 12 bases in length effectively inhibit bacterial gene expression, both in pure culture and in a bacterial cell-free protein expression system. At a reduced temperature of 30°C, PMOs as short as seven bases also caused significant inhibition (Fig. 1), probably because the melting temperature is between 30 and 37°C. The sieving effect of the outer membrane and/or the (unknown) mechanism of transport across the plasma membrane probably accounts for the reduction in efficacy of the longer PMOs (Fig. 1). Indeed, longer PMOs inhibited significantly better in cell-free expression systems than in culture. These results differ from PMO inhibition of eukaryotic gene expression, which is not significant with PMOs less than \(\sim\)16 bases in length (30). Although the basis for this difference is unknown, we speculate that differences in eukaryotic and prokaryotic ribosome structure, mechanism of subunit assembly on the mRNA, and/or uptake may be important.

Hybridization position of the PMO was also a factor. Recent evidence shows that peptide-PNA inhibited \(\beta\)-lactamase expression only when targeted to either the Shine-Dalgarno sequence (ribosome binding site) or the region around the start codon but not to anywhere else along the entire length of the mRNA (7). Our results show that the region around the start codon is an effective target site. Interestingly, it was not necessary to include the anti-start codon itself within the sequence of the PMO, as exemplified by the inhibition with PMOs 326, 208, or 340 (Fig. 2). In fact, the most effective PMOs that we tested (356, 208, and 169) were targeted slightly downstream of the start codon.

Our results also suggest that the ribosome binding site was a less effective target for PMO inhibition than the region around the start codon, at least for \textit{myc-luc}. The marginal inhibition of \textit{myc-luc} expression by PMOs targeted to the ribosome binding site may suggest that these PMOs poorly block binding of the 30S subunit. This result is different than that shown for peptide-PNA inhibition of expression of \(\beta\)-lactamase or \textit{acpP} (7). Perhaps differences in secondary structures of the three targets, differences in association constants of PNA and PMO, or contributions from the peptide used to facilitate entry of PNA would account for this difference in efficacy at the ribosome binding site.

Other characteristics of PMO were analyzed to detect a correlation with inhibition of \textit{myc-luc}. The G+C content of PMOs did not correlate with inhibition; however, a significant correlation between inhibition and theoretical secondary structure of the targeted region suggests that base pairing within the targeted region may reduce efficacy of the PMO (Fig. 4).

PMOs targeted to sequences far downstream of the AUG start codon did not inhibit expression. This result is consistent with a previous report that showed PNA lack efficacy at downstream sites (7). Each of our downstream PMOs targeted a different predicted secondary structure. One PMO (PMO 215) was targeted to a region predicted to form a stem-like structure with 10 of its 11 bases paired with a contiguous stretch of complementary bases further downstream. The other downstream PMO (PMO 214) was targeted to a region predicted to form a single-stranded region with all 11 of its bases unpaired. Target secondary structure does not appear to be a factor in the lack of efficacy of PMOs targeted to sequences well downstream of the start codon. However, this interpretation relies upon the limitations of secondary structure predictions based on one algorithm.

The efficacy of short (9- to 12-base) PMOs is somewhat unexpected for two reasons. First, in some reports, PMOs shorter than \(\sim\)16 bases are ineffective in eukaryotic systems (30), whereas in others, PMOs as short as 12 bases cause significant inhibition (15). Second, PMOs have a lower binding affinity than equivalent PNAs and would lose efficiency at a longer length than the equivalent PNA as the length decreased and the \(T_m\) fell.

The unexpected efficacy of short PMOs toward bacterial targets prompted us to test them in eukaryotic systems. Although the 20-base PMO was more effective in HeLa cells, the 11-base PMO inhibited target expression. This is consistent with the previous results (15) and shows that a PMO as short as 11 bases can cause a significant target-specific inhibition in eukaryotic cells. However, the 11-base PMO was only marginally effective in the cell-free reticuloocyte expression system, whereas the 20 base PMO caused a level of inhibition that was comparable to that found in HeLa cells. This suggests that physiological factors not present in the cell-free reactions favored the shorter PMO in HeLa cells. We speculate that cellular uptake and transport to the cytoplasm likely is the factor that favored the shorter PMO.

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