# A novel application of antisense phosphate-methylated DNA for stimulation of protein production rates

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# A novel application of antisense phosphate-methylated DNA for stimulation of protein production rates

by Saskia A.M. Biezen, Sylvia A.M. Vanhommerig, Marcel H.P. van Genderen and Henk M. Buck

*Laboratory of Organic Chemistry, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, the Netherlands*

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SUMMARY

A specific antisense phosphate-methylated 22-mer, targeted at the repressor binding site in the lac operon of *E. coli*, is able to stimulate the $\beta$-galactosidase production rate by 32% at a concentration of 1 $\mu$M. Although transcription is reduced due to hybridization of the antisense oligomer with the DNA coding strand, the translation is enhanced since the 5'-end of the mRNA is protected from enzymatic breakdown by the hybridized phosphate-methylated 22-mer. These results show that phosphate-methylated DNA oligomers can be used to induce overproduction of a selected protein in a completely novel way. It is suggested that this new regulatory mechanism may also occur under natural conditions with antisense RNA molecules.

## INTRODUCTION

Recently, we introduced phosphate-methylated DNA, which hybridizes strongly to natural DNA and RNA due to the absence of phosphate-phosphate charge repulsions between the strands in the duplex (Koole et al., 1986; Koole et al., 1987; Koole et al., 1989; van Genderen et al., 1989). It has been shown that complexation of a phosphate-methylated oligomer can specifically block the replication of single-stranded DNA *in vitro* (Moody et al., 1989a), and interferes with the transcription of double-stranded DNA in *Escherichia coli* (Moody et al., 1989b) and *Salmonella typhimurium* (Moody et al., 1990). In the latter cases, the phosphate-methylated oligomers were sense, i.e., complementary to the template strand in the DNA duplex only. We have now investigated the effect of an antisense phosphate-methylated 22-mer on the ex-

pression of the β-galactosidase gene in *E. coli*. The antisense oligomer can hybridize both to the coding DNA strand, and to the messenger RNA (mRNA) which is synthesized during transcription. Therefore, it must be expected that the translation process from mRNA to protein is now also influenced. We will demonstrate, that by selecting a hybridization target on the 5′-terminus of the mRNA, the messenger is stabilized and the protein production rate is enhanced.

## RESULTS AND DISCUSSION

The lac operon (see fig. 1) was used as a well-defined model system for testing the effect of the phosphate-methylated 22-mer d(AAT.TGT.TAT.CCG.CTC.-ACA.ATT.C) on gene expression. The synthesis of the phosphate-methylated DNA was performed according to Moody et al. (1989a). The target site in the DNA duplex is the binding site of the lac repressor protein, which prevents the action of the RNA polymerase enzyme, and hence transcription. On the mRNA, the target does not overlap the Shine-Dalgarno sequence (essential for ribosome binding; Shine and Dalgarno, 1974) or the structural genes, so translation will not be affected. However, the degradation of mRNA proceeds in the 5′→3′ direction (King and Schlessinger, 1987), and the presence of the phosphate-methylated 22-mer on the 5′-end of the mRNA is expected to stabilize the messenger, since nuclease enzymes cannot hydrolyze the hybrid of RNA with neutral DNA.

The biological experiments on β-galactosidase production were performed as reported before (Moody et al., 1989b). Transcription is started at t = 0 by addition of the artificial inducer isopropyl-β-D-thiogalactoside to cells, in which the lac operon is repressed due to the absence of the natural inducer lactose. Repeating the experiments with and without phosphate-methylated 22-mer showed that the β-galactosidase production rate can be determined with an accuracy of 8%. For the control experiments, a linear relation was found for the β-galactosidase synthesis versus time, yielding a standard production rate. In the presence of the antisense phosphate-methylated oligomer, the production

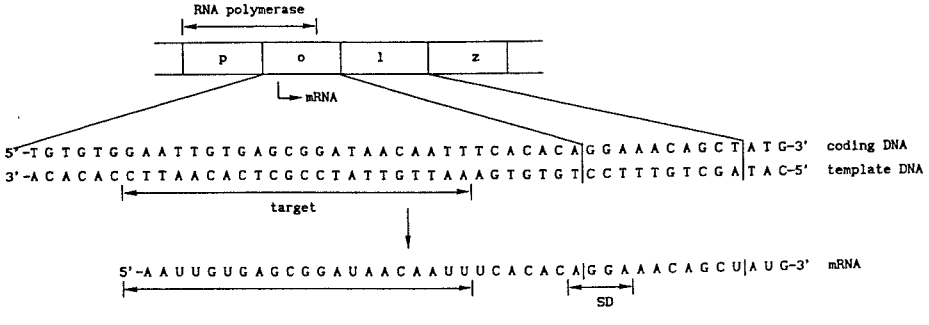


Fig. 1. Schematic representation of the lac operon, with the promoter (p), operator (o), leader sequence (l), and the structural gene for β-galactosidase (z). The target area is indicated in the DNA duplex and in the mRNA. Ribosome binding occurs around the Shine-Dalgarno sequence (SD), and translation starts at the AUG triplet.

curves were found to be reproducible within the experimental error. A typical experiment with a concentration of 1 $\mu$M antisense phosphate-methylated 22-mer is shown in fig. 2. Initially, the $\beta$-galactosidase production rate (i.e., the slope of the curve) is less than in the control experiment, as was earlier also found for the sense phosphate-methylated 22-mer (Moody et al., 1989b). Since the transcription is started at $t = 0$, almost no mRNA is present then, and transcriptional blocking must occur via hybridization of the antisense phosphate-methylated 22-mer with the coding DNA strand. Later, hybridization can also occur with the newly synthesized mRNA, which will influence the translation rate. Therefore, a situation arises in which changes in both transcription and translation affect the $\beta$-galactosidase production rate. As is seen in fig. 2, a steady-state is reached after ca. 60 min, since then a constant synthesis rate is found. This steady-state rate is consistently observed to be 32% higher than the standard production rate under identical circumstances.

Since the addition of random phosphate-methylated oligomers does not affect the $\beta$-galactosidase production (Moody et al., 1989b), it can be concluded that a sequence-specific mechanism must occur to induce the overproduction. This indicates that hybridization with the mRNA occurs, which can lead to a longer lifetime of the messenger and increased translation to protein. Since the phosphate-methylated oligomer is neutral, and therefore not recognized by proteins, an effect on e.g. ribosome binding may be excluded. Therefore, the results may be rationalized via a more efficient translation of the mRNA, since hybridization on the DNA level can only inhibit protein production. We analyz-

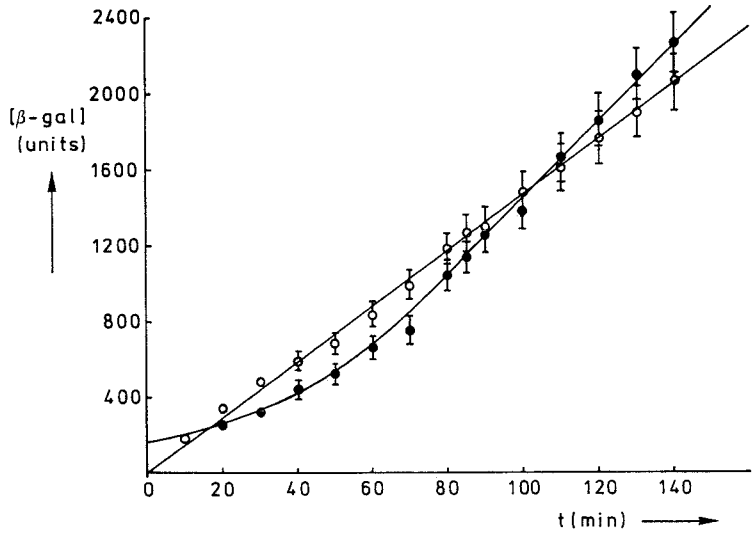


Fig. 2. Production of $\beta$-galactosidase by *E. coli* cells in the presence (●) and absence (○) of the antisense phosphate-methylated 22-mer at 1 $\mu$M concentration. The amount of $\beta$-galactosidase ([$\beta$-gal]) is expressed in units, as defined before (Moody et al., 1989b). The curve through the filled data points before $t = 60$ min is a best-fit exponential function to describe the pre-steady-state behaviour (see text). Error bars are indicated for all data points.

ed the mRNA concentration as a function of time, due to the synthesis from the DNA template ($k_1$), and breakdown by ribonucleases ($k_2$). Assuming constant enzyme and nucleoside triphosphate concentrations, it holds that:

$$\frac{d[mRNA]}{dt} = k_1[DNA] - k_2[mRNA]$$

or:

$$[mRNA] = \frac{k_1}{k_2}[DNA](1 - e^{-k_2 t})$$

The production rate of $\beta$-galactosidase via translation of mRNA ($k_3$) is now given by (with constant enzyme and amino-acid concentrations):

$$\frac{d[\beta\text{-gal}]}{dt} = k_3[mRNA] = k_3\frac{k_1}{k_2}[DNA](1 - e^{-k_2 t}) = H(1 - e^{-k_2 t})$$

where H is the steady-state production rate. Before t = 60 min, the pre-steady-state behaviour indeed fits an exponential curve that approaches the steady-state straight line. In the presence of 1 $\mu$M of antisense phosphate-methylated oligomer, H is 32% higher than the slope found in the control experiment. Apparently, the value of $k_2$ (breakdown by nucleases) is more strongly decreased than $k_1$ (transcription) by the phosphate-methylated DNA. (The higher value of $k_2$ in the control experiment is also obvious from the absence of an initial exponential curve: the steady-state is reached very quickly.) This may be due to the fact that hybridization to single-stranded mRNA is more efficient than to double-stranded DNA, so nucleases acting on the mRNA are more strongly inhibited than RNA polymerase in transcription of the DNA.

At concentrations of 5 and 10 $\mu$M antisense phosphate-methylated 22-mer, it is seen that the balance between lower transcription rate and more effective translation is different, viz. 12% stimulation and 6% inhibition of $\beta$-galactosidase production rate, respectively. Here, the transcriptional blocking dominates the increased translation rate.

## CONCLUDING REMARKS

From the present results it can be seen that $\beta$-galactosidase production rate is stimulated by 1 $\mu$M of the antisense phosphate-methylated oligomer, aimed at the 5'-end of the mRNA. This procedure is a novel approach for stimulation of protein synthesis on the various levels of gene expression. Up to now, hybridization-arrest techniques have only been used for the blocking of transcription and translation. The ability of antisense phosphate-methylated DNA to either block or enhance protein production allows numerous applications for biomedical and biotechnological purposes. Various diseases are characterized by a deficiency in the production of a protein (e.g., Factor VIII in hemophilia, phenylalanine hydroxylase in phenylketonuria, or insulin in diabetes mellitus type 2), which may be enhanced with antisense phosphate-methylated DNA. In-

creasing the production of a specified protein in industrial micro-organisms offers possibilities for more efficient biotechnological processes.

In addition, the present findings may have implications for the genetics of micro-organisms. In several bacteria, it is known that natural antisense RNA molecules are produced which can interfere with the translation of mRNAs via hybridization-arrest. We now offer the hypothesis that these antisense RNAs can also be used by the cells to stimulate the production of a required protein, which constitutes a new regulatory mechanism.

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