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Studies on antisense inhibition of translation in vitro

Anomalies and re-evaluation

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Experiments were carried out to better characterize antisense control of translation. Results in an *E. coli* system confirmed specific inhibition of poly(U) translation. At low concentrations, certain homopolymers (including poly(rA)) stimulated translation. Oligo(dA$_n$) was inhibitory at $n \leq 8$. Translation of globin mRNA in reticulocyte lysates indicated that ssDNA 15-mers targeted at β-globin mRNA inhibited both α- and β-globin production. Sequences targeted immediately downstream of the AUG were the least effective in inhibition. These and other anomalies are discussed here in relation to those of others, emphasizing caution in performing antisense experiments.

Antisense; Translational regulation; DNA oligomer; RNA oligomer

1. INTRODUCTION

The ability to control translation in a well defined manner could open the door to vast advances in the study of gene expression and in the treatment of disease. Antisense oligonucleotides act directly and indirectly through base pairing of their complementary sequence to the target. Unlike antibiotics, hormones, proteins, and the like, antisense oligomers function by well characterized interactions and are, therefore, a prime candidate for molecular design. The concept of using complementary nucleotides for targeted interactions is not a new one; but, the idea did not become popular until recent advances in DNA synthesis made oligomers readily available. It seems that Ochoa's group, in 1961, may have been the first to show that antisense molecules could be used to control protein synthesis [1]. While studying the genetic code in an *E. coli* translation system, they determined that poly A completely inhibited poly U-dependent synthesis of poly-phe. Early on, Russian biochemists suggested the use of the specific interaction of DNA with RNA to alkylate a specific sequence within that RNA [2]. In another use, antisense oligomers were made against various regions of tRNA in order to determine their function during aminoacylation [3,4]. One of the first uses of antisense DNA to inhibit translation in eukaryotes was that of Paterson and Bishop [5]. They made cDNA from chick embryo-muscle mRNA and hybridized it to the mRNA in order to determine the protein products of the abundant mRNA classes during embryogenesis. Using rabbit reticulocyte lysates, Paterson et al. [6], and Hastie and Held [7], showed that α- and β-globin translation could be specifically arrested by hybrids of plasmid DNA fragments to mRNA, hence the term Hybrid ARrest Translation, HART. In addition, adenovirus-2 DNA fragments were used in HART to determine the location within the genome, of various protein-coding regions. Although HART resulted in complete and selective inhibition of translation, this was achieved using long double-stranded-DNA fragments with hybridization to the mRNA prior to protein synthesis. The double-stranded DNA fragments used for HART required special incubation conditions in order to promote DNA:RNA hybrids without re-annealing of the DNA strands. These requirements are not suitable for in vivo experiments or for clinical treatment. Therefore, short single-stranded antisense deoxynucleotides were synthesized and used to control gene expression in vivo. Thus, Stephenson and Zamecnik showed that hybridization of antisense DNA 20-mers to Rous Sarcoma Virus mRNA inhibited its translation [8], viral replication, and cell transformation [9]. The large number of recent advances in antisense have been thoroughly covered in recent books [10,11] and review articles [12,13].

Despite the wide usage of anti-mRNA nucleotides in vitro and in vivo, their interactions have only been partially characterized, and nearly every system presents anomalies. In addition, many studies are concerned only with the immediate effect of anti-mRNA nucleotides on each biological system. In this communication, using in vitro polypeptide synthesis systems, we exam-

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ined the details of conditions under which anti-mRNA nucleotides caused inhibition of polypeptide synthesis. The experiments were performed under conditions where ongoing protein synthesis can be inhibited by antisense. These conditions are desirable for the selective inhibition of certain protein synthesis clinically as well as biologically. In the *E. coli* system, using homopolymers of ssDNA or RNA, we showed that inhibition of translation occurred within 4 min and often reached 100% at ratios of sense to antisense of much less than one. However, we observed unexpected stimulation of poly-U translation by low concentrations of poly G, poly A, and oligo $(dA)_{5-9}$, although not with poly dA. In rabbit reticulocytes, there was crossover inhibition of α-globin synthesis by anti-β-globin-mRNA oligomers. Tandem oligomers in the initiation region of the mRNA could stimulate synthesis of the alternate globin molecule. These results and those of others rule out a simple optimistic outlook on the use of antisense oligomers, and antisense experiments must be performed with full consideration for the effect of antisense molecules, including unexpected anomalies.

2. MATERIALS AND METHODS

2.1. *Hybrid arrest in the* E. coli *translation system*

The iS-30 extract of *E. coli* was prepared essentially as described [14]. The *E. coli* used were mid-log MRE600 obtained from Grain Processing Corp. (Muscatine, IA). The concentration of the iS-30 extract was 54 mg/ml. Translation using the iS-30 was carried out essentially as originally described [14]. The final reaction mixture (42.4 μl) contained: 40 mM Tris-HCl pH 7.8, 10 mM $MgAc_2$, 1.8 mM ATP, 0.15 mM GTP, 3.9 mM PEP, 120 ng/μl pyruvate kinase, 70 ng/μl leucovorin, 127 mM NH_4Cl, 18 mM KCl, 3.3 μg/μl *E. coli* tRNA (Schwarz/Mann), 6 mM β-mercaptoethanol, 2.75 nCi/μl of [^{14}C]phe (Schwarz/Mann), and 132 or 13.2 ng/μl poly U or poly A and antisense RNA or DNA (in the concentrations indicated), and 7.3 μg/μl iS-30 (as described above). Prior to iS-30 addition, the potential antisense nucleotide, poly U, and reaction mixture were incubated in a volume of (36.7 μl) for 2 min at 37°C. After the preincubation, the iS-30 was added and further incubation was carried out at 37°C for 35 min. In some experiments, no preincubation was performed, but inhibition was observed very quickly after addition of antisense nucleotide. Unless indicated, aliquots of 40 μl were taken from each reaction mixture and radioactivity was determined in hot TCA-insoluble material [15]. In some experiments, poly A and [^{14}C]lys replaced poly U and [^{14}C]phe for translation. Assay of polypeptide synthesis required sodium tungstate/TCA precipitation since poly-lys is soluble in TCA [16].

2.2. *Hybrid-arrested translation in rabbit reticulocyte lysates*

Rabbit reticulocyte lysates were prepared according to the method of Pelham and Jackson [17]. Small aliquots of the lysate (stored at −70° C) were treated to remove endogenous mRNA by incubation of each 400 μl at 20°C for 15 min with 5.3 μl, 7.6 mg/ml CPK; 4 μl, 100 mM $CaCl_2$; and 12 μl, 2 U/μl micrococcal nuclease. After incubation, 8 μl of 100 mM EGTA was added to inactivate the Ca^{2+}-dependent nuclease, and the treated lysate was kept on ice.

Translation was carried out at 30°C for 30 min in a 30 μl reaction mixture which contained: 10 μl of an energy mix, 3 μl of 10 or 50 ng/μl rabbit globin mRNA (BRL), the indicated amount of single-stranded DNA oligomer, and 10 μl of lysate. The energy mix contained 63 mM HEPES, 25 mM $CrPO_4$, 6.3 mM DTT, 0.1 mM amino acids minus methionine, 0.2 mM KAc, 2.3 mM $MgAc_2$, 150 μg/ml spermidine, and 2–3 μM [^{35}S]methionine (300 μCi). When the translation was complete, aliquots of 5 μl were removed from each reaction mixture for use in TAU-PAGE (see below) and for detection of hot TCA-insoluble radioactive material [15]. Filters for detection of protein synthesis in the rabbit reticulocyte system required slightly different treatment due to the quenching effect of the globin molecule [17]. Between the TCA precipitations and the MeOH wash, the filters were incubated at room temperature for 2 h, with decolorizer containing 60 ml, 10% TCA; 30 ml, 30% H_2O_2, and 30 ml, 88% HCOOH.

2.3. *Triton X-100 acid urea polyacrylamide electrophoresis (TAU-PAGE)*

TAU-PAGE was carried out in order to quantitate α- and β-globin synthesis during hybrid-arrested translation in the rabbit reticulocyte system. The procedure was carried out essentially as described by Rovera et al. [18]. The 1.5 mm-thick gels were formed and run using a Model 200 apparatus from Aquebogue machine shop (Aquebogue, NY). The separating gel (60 ml) contained 24 ml acrylamide/bis-acrylamide (29.8%/0.2%); 3 ml, glacial acetic acid; 21.6 g urea; 5.6 ml, 20% (w/v) Triton X-100; 360 μl, ammonium peroxydisulfate; 300 μl TEMED; and deionized water. The poured gel was overlaid with 5% acetic acid and was allowed to polymerize for at least one hour. The stacking gel (30 ml) contained: 6 ml, acrylamide/bis-acrylamide (29.8%/0.2%); 1.5 ml, glacial acetic acid; 10.8 g, urea; 2.8 ml, 20% (w/v) Triton X-100; 240 μl, ammonium peroxydisulfate; 300 μl TEMED; and deionized water. The gel was allowed to polymerize for at least 4 h.

After preparation, gels were first pre-electrophoresed in 5% acetic acid running solution, at 200 V for 1 h. Then, 25 μl of 1 M cysteamine was added to each well, and pre-electrophoresis was continued at 90 V for 45 min with fresh running solution. Sample buffer (2 ml) was stored in small aliquots and contained 0.48 g urea, 100 μl β-mercaptoethanol, 20 μl 0.2% pyronin Y, and a final concentration of 5% acetic acid. A 1 to 5 μl aliquot of the reaction mixture for globin synthesis was mixed with 20 μl of the above sample buffer and was added to wells after they were washed out with running solution. Electrophoresis was from anode to cathode at 150 V for 16 h. The gels were stained with 0.2% (w/v) Coomassie brilliant blue R in 50% MeOH:7% acetic acid [19]. Staining was carried out for at least 1 h, and was followed by destaining in repeated changes of 50% MeOH:7% acetic acid until the background color was removed. The gels were dried overnight and subjected to autoradiography directly on the film, at −70° C. The resultant autoradiograms were scanned using a Shimadzu CS-930 TLC densitometer, and the α- and β-globin bands were then quantitated using a Bioquant Digitizing Morphometry System.

2.4. *Antisense oligonucleotides used in the reticulocyte system*

The oligomers used in the rabbit reticulocyte system were synthesized by Applied Biosystems (Foster City, CA). These oligonucleotides were greater than 99% pure by reversed-phase HPLC. Six complementary DNA sequences were chosen according to the α and β globin mRNA sequence; two 15-mers each were chosen to hybridize in tandem, immediately following the initiation AUG of α- or β-globin mRNA. (α_1, n.t. 40–54 and α_2, n.t. 55–69)(β_1, n.t. 57–71 and β_2, n.t. 72–86). One 20-mer each was chosen within a coding region of low secondary structure within the α- and β-globin mRNA (α_3, n.t. 196–211; and β_3, n.t. 222–241). The sequence for these oligonucleotides, and their expected position of hybridization are listed in Table II.

3. RESULTS

3.1. *Antisense studies in iS-30 extracts of* E. coli

3.1.1. Stimulation of poly-U translation by antisense RNA and other RNA homopolymers

The initial studies on the effect of antisense were carried out by addition of RNA homopolymers to a poly-U

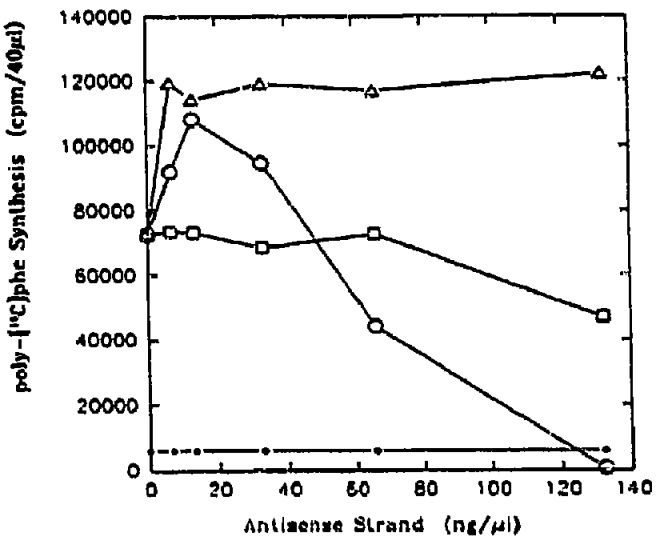


Fig. 1. RNA homopolymers as antisense inhibitors of poly-U translation in vitro. Translation of poly U in iS-30 was carried out as described in section 2. The reaction mixtures (42.4 μl) contained poly U at a final concentration of 132 ng/μl. The RNA homopolymers were added at various final concentrations as indicated in the figure. After a 2 min pre-incubation of poly U with single-stranded RNA, at 37°C, iS-30 was added, and the reaction mixtures were further incubated for 35 min. Aliquots of 40 μl were then taken from each reaction mixture and radioactivity was determined in the hot TCA-insoluble material. [^{14}C]poly-phe synthesis is indicated in cpm/40 μl. The polyribonucleic acids used were: ○–○, poly A; □–□, poly C; △–△, and poly G. In one set of reactions, •–•, poly U and poly A were added in a 1:1 ratio at various concentrations up to 132 ng/μl.

translation system in iS-30 extracts of *E. coli*. Fig. 1 indicates the extent of poly-U translation when various amounts of homopolymer were added. Translation is shown as [^{14}C]phe incorporated into hot TCA-insoluble radioactivity. While poly-U translation was completely inhibited by the addition of equal amounts of poly rA, this degree of inhibition was not seen with either poly G or poly C. When the ratio of poly U to poly A was kept at 1:1, complete inhibition of poly-U translation occurred at all concentrations used. Although these results were expected, two unexpected observations were made in this experiment. The first was that poly A, when added at low concentrations, stimulated poly-phe synthesis rather than inhibiting it. The second was that this stimulation was not limited to antisense nucleotides. Poly G stimulated poly-phe synthesis by almost twofold at every concentration used. Not every homopolymer stimulated the poly-phe synthesis, however. Thus, no such stimulatory effect was observed with poly C. It should be pointed out that in the absence of poly U, no stimulation of poly-phe was observed with these homopolymers (in confirmation of classical work by Nirenberg and Matthei [14]) (data not shown). The stimulation by poly G was further examined over a wide range of Mg^{2+} concentrations in an effort to better understand its mechanism. Table I lists the amount of hot TCA-insoluble radioactivity detected in the poly-U system at various Mg^{2+} concentrations in the presence and absence of poly G. While the poly-G stimulation was dramatic at 10 mM Mg^{2+}, it did not occur at 15 and 20 mM. It is clear that the RNA homopolymers may be used to inhibit poly-U translation in a manner consistent with base-pairing specificity; however, the finding of specific stimulation of poly-phe formation by certain unrelated nucleotides as well as antisense nucleotides indicates an unknown parameter which must be taken into account in antisense experiments.

3.1.2. Inhibition of poly-U translation by antisense DNA homopolymers — lack of stimulation

In addition to using RNA as an antisense inhibitor of poly-U translation, ssDNA was also used. Fig. 2 demonstrates the specificity and concentration effect of

Table I

Poly-G stimulation of poly-U translation at various Mg^{2+} concentrations

mM Mg^{2+} final	Poly-U translation (cpm/40 μl)		Change due to poly-G addition
	−poly G	+poly G	
10	31,135	94,783	+63,648
15	99,290	91,215	− 8,075
20	68,307	61,527	− 6,780
25	26,699	48,556	+21,857
30	11,766	24,732	+12,966

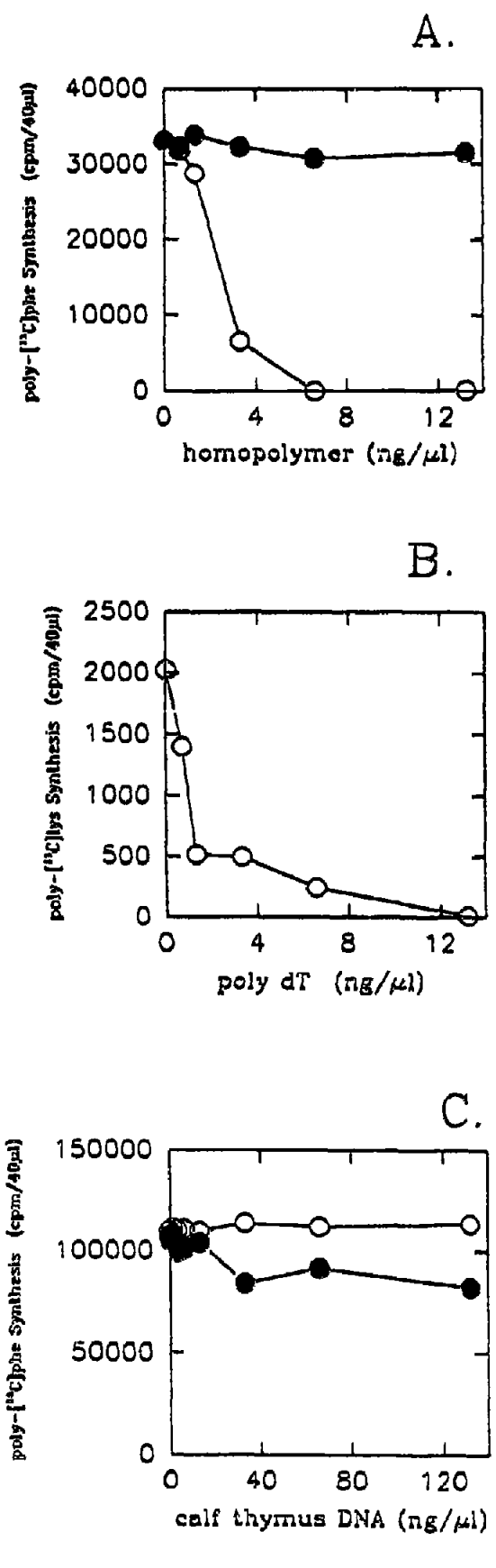
Translation of poly U in iS-30 was carried out as described in section 2. The reaction mixtures (42.4 μl) contained poly U at a final concentration of 132 ng/μl and poly G at a final concentration of 13.2 ng/μl. After a 2 min incubation at 37°C, iS-30 was added, and the reaction mixtures were incubated for an additional 35 min. Aliquits of 40 μl were taken from each reaction mixture and radioactivity was determined in the hot TCA-insoluble material. Each value is the mean of two experiments.

Table II

Sequence and of antisense oligonucleotides used for inhibition of rabbit globin mRNA translation in rabbit reticulocyte lysates

Notation	Sequence	Positions matched in globin mRNA
α_1	5′-AGC-GGG-AGA-CAG-CAC-3′	40–54
α_2	5′-GAT-GTT-GGT-CTT-GTC-3′	55–69
α_3	5′--CC-GTG-GGC-TTT-GAT-CTG-CTC-3′	196–211
β_1	5′-ACT-GGA-CAG-ATG-CAC-3′	57–71
β_2	5′-CGC-AGA-CTT-CTC-CTC-3′	72–86
β_3	5′--GC-CTT-CAC-CTT-AGG-ATT-GCT-3′	222–241

These sequences were chosen from the known sequence of rabbit α- and β-globin. The positions for hybridization of the antisense oligomers to mRNA are indicated as nucleotide position on the mRNA, with position 1 being the first A within the CAP.



ssDNA on translation. As shown in Panel A, poly dA readily inhibited poly-U translation while poly dT was completely ineffective. In contrast, poly dT was completely effective in inhibition of poly-rA translation, as shown in panel B. This inhibition occurred even when the antisense strand was present at only 1/4 the concentration of the message. Control DNA, which should not hybridize with poly U, was used and results are shown in panel C. Either native or heat-denatured calf-thymus DNA was added to the poly-U translation system. Although the double-stranded calf-thymus DNA clearly had no effect on the degree of translation, the denatured calf-thymus DNA inhibited poly-U-dependent poly-phe synthesis by about 20% at a ratio of 1:1 with poly U. We can conclude that ssDNA was more effective than RNA for inhibition of translation, and that it was specific for its antisense strand. The slight inhibition (20%) observed with single-stranded calf-thymus DNA was probably the result of its random sequence which may hybridize with the homopolymer. An important difference between these experiments and those described in Fig. 1 is the lack of stimulation by any of these homo-DNA polymers. The stimulation observed by antisense RNA homopolymers was not observed at all with antisense DNA polymers.

The expected inhibition by antisense DNA homopolymers was observed without preincubation, as shown in Fig. 3. In this experiment, a kinetic analysis of antisense DNA inhibition was performed. At every point after poly-phe synthesis had begun, poly-U translation was completely inhibited within 4 min by addition of poly dA in a 1:1 ratio with the message. Inhibition continued for at least 15 min after poly-dA addition. For DNA homopolymers in vitro, we may conclude that inhibition of protein synthesis by antisense strands was very fast and was stable for a significant length of time.

←

Fig. 2. Single-stranded DNA homopolymers as antisense inhibitors of poly-U translation in vitro. Translation in iS-30 was carried out as described in section 2. The reaction volumes in each experiment were 42.4 µl. After a 2 min pre-incubation of poly U with homopolymers, at 37°C, iS-30 was added, and the reaction mixtures were further incubated for 35 min. Aliquots of 40 µl were taken from each reaction mixture and radioactivity was determined in the hot TCA-insoluble material. The amount of poly-phe or poly-lys synthesized is indicated in cpm/40 µl. Panel A. Poly U was present at a final concentration of 13.2 ng/µl. Reactions were carried out in the presence of poly dA (○–○) or poly dT (●–●). Panel B. Poly A was translated and was present at a final concentration of 13.2 ng/µl. Reactions were carried out in the presence of various concentrations of poly dT, as indicated. Poly-lys synthesis was determined using Sodium Tungstate and TCA for precipitation as described in section 2. Panel C. Poly U was present at a final concentration of 132 ng/µl. Calf thymus DNA, either native (○–○) or denatured (●–●) was added at the indicated concentrations.

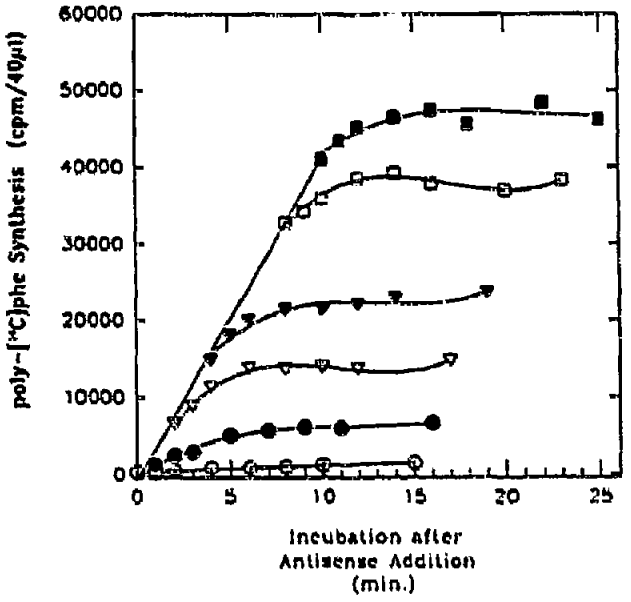


Fig. 3. Time course of inhibition of poly-phe synthesis by poly dA. Translation of poly U in iS-30 was carried out as described in section 2. The reaction mixtures (84.8 μl) contained poly U at a final concentration of 132 ng/μl. Each reaction was started by the addition of iS-30 in the absence of an antisense strand. After polyphenylalanine formation for 0, ○-○; 1, ●-●; 2, ▽-▽; 4, ▼-▼; 8, □-□; and 10 min, ■-■; poly rA (132 ng/μl) was added and the incubation was continued at 37° C. Aliquots of 10 μl were taken from the reaction mixtures at the indicated times and were treated to determine radioactivity in the hot TCA-insoluble material. Poly-phe synthesis is indicated in cpm/40 μl.

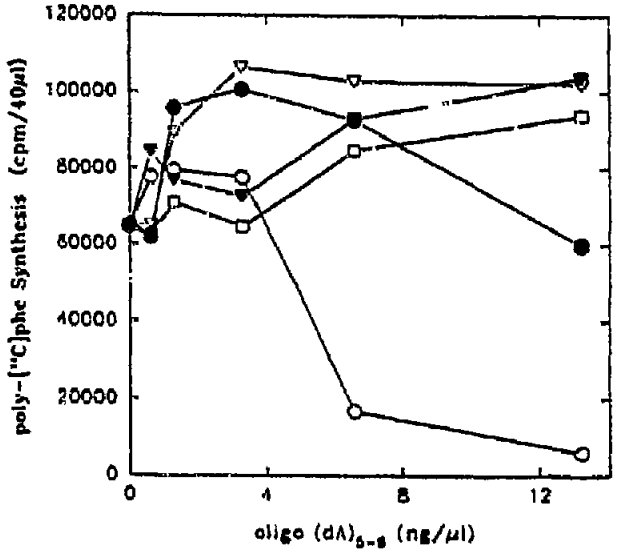


Fig. 4. Effect of $(dA)_n$ length and concentration on its inhibition of poly-U translation. Translation of poly U was carried out in iS-30 as described in section 2. The reaction mixtures (42.4 μl) contained poly U at a final concentration of 13.2 ng/μl. The single-stranded DNA oligomers were added at various final concentrations as indicated in the figure. After a 2 min incubation at 37° C, iS-30 was added, and the reaction mixtures were incubated for an additional 35 minutes. Aliquots of 40 μl were taken from each reaction mixture, and radioactivity was determined in the hot TCA-insoluble material. The extent of poly-phe synthesis is indicated in cpm/40 μl. The $(dA)_n$ oligomers used were: ○-○, dA_5; ▼-▼, dA_6; ▽-▽, dA_7; ●-●, dA_8; and □-□, dA_9.

3.1.3. Determination of the effective size of poly dA for inhibition of poly U-dependent poly phe formation — stimulation of poly-phe by antisense deoxy oligomer

The experiment shown in Fig. 4 was carried out in order to determine the shortest antisense oligomer which is effective in inhibition. These data were collected using the poly-U translation systen and antisense oligomers of dA between 5 and 9 nucleotides in length. The translation of poly U was partially inhibited by dA_8 and was effectively inhibited by dA_9. In contrast to the results with antisense DNA homopolymers, a distinct stimulation of poly-phe synthesis was observed with antisense oligo DNA. As in the case with antisense RNA polymers, the inhibition was observed only with low concentrations of added antisense oligo DNA. It is apparent that the stimulation was also dependent upon length of the antisense strand. The effectiveness of oligomer in stimulating translation increased between dA_5 and dA_7; at a length of eight, inhibition began to overcome this stimulation.

3.2. *Antisense studies in rabbit-reticulocyte lysates*

In order to determine the effect of antisense oligonucleotides on eukaryotic translation, various synthetic ssDNA oligomers were added to rabbit reticulocyte lysates using rabbit globin mRNA as an exogenous message. As shown in Table II, three types of antisense sequence were selected for both α and β-globin mRNA. Without regard to mRNA secondary structure, oligonucleotides were used which corresponded to the first 15 nucleotides immediately downstream of the AUG (α_1 and β_1), and the next 15 nucleotides downstream (α_2 and β_2). In addition, a sequence was chosen which was complementary to a similar position in the middle of the α or β-globin mRNAs (α_3 and β_3, respectively). In this latter case, the positions were chosen from regions of the mRNAs which, according to Zuker's least-energy RNA-folding programs [20], were involved in a low degree of intramolecular base pairing. Table III shows the degree of α- and β-globin production in the presence of these three types of antisense sequences. The amounts of α- and β-globin production were quantitated by densitometry of autoradiograms using TAU-PAGE gels of the final reaction mixtures. In general, it may be seen from Table III that antisense oligomers designed against α-globin mRNA specifically inhibited α-globin synthesis. The expected results of antisense theory stopped here. First, contrary to the general notion, the most effective antisense oligomer was not α_1 (near the AUG). Second, we observed as much as 24% stimulation of β-globin synthesis by α_3. The third anomaly was the inhibition of α-globin synthesis by oligomers targeted at β-globin mRNA (β_1, β_2, and β_3). Inhibition of α-globin synthesis was often equal to

that of β-globin. The fourth and most strange anomaly was the sudden appearance of specificity by β_1-β_2 while independent use of these individual oligomers resulted in non-specific inhibition. In addition, this inhibition of β-globin synthesis by β_1-β_2 was significantly reduced by the presence of α_1-α_2. These findings, general inhibition, lack of dose dependence, and even specific stimulation of protein synthesis by antisense oligonucleotides, could result in serious alteration of the expected outcome of an antisense experiment and suggest that this technique be used with great care so as to better understand any secondary effects.

4. DISCUSSION

It is generally accepted that antisense molecules have great potential for use in experimental gene regulation in both research and clinical applications. Despite widespread use of the technique and an understanding of its specificity [10,11] — Watson and Crick base pairing —it suffers from many unknowns. Our data was collected from well characterized in vitro systems under standard conditions. As described in section 3, both prokaryotic and eukaryotic representative systems were studied. We set out to characterize anti-mRNA inhibition of translation and to show its specificity. However, though the expected results were obtained in many cases, both the prokaryotic and eukaryotic systems produced results indicating that additional caution should be exercised when conducting antisense experiments and interpreting their results.

The iS-30 extract from *E. coli* is relatively simple and is almost entirely dependent upon exogenous mRNA for translation. In general, the inhibition of translation in this simple system was consistent with base-pairing specificity. However, low concentrations of the anti-mRNA (poly rA and oligo dA, but not poly dA) significantly stimulated poly-phe synthesis. This stimulation was observed with poly G also but not with poly C. The observation that a slight increase in Mg^{2+} abolished the stimulation suggests that these homopolymers caused structural changes in the ribosomes or an alteration of affinity between translational components. It is unlikely that the homopolymers are simply protecting poly U from digestion by nuclease since the stimulation was nucleotide specific (poly C did not stimulate).

Anomalies observed in antisense inhibition of translation were not limited to the prokaryotic system. The effect of antisense oligomers on translation in eukaryotes was studied in rabbit reticulocyte lysates, and at least three types of inconsistencies were observed. The first was non-specific inhibition by antisense oligomers. We observed specific inhibition of globin synthesis by α-globin antisense oligomers (α_{1-3}); however, individual β-antisense oligomers (β_{1-3}) caused inhibition of both α-and β-globin synthesis. Dash et al. obtained similar non-specific inhibition (data was not shown) and attempted to explain these results by suggesting that a 60% complementarity found between α-globin mRNA and their β-globin antisense 30-mer was enough for this inhibition [21]. However, as may be seen in Table IV, the only place of complementarity of >60% is that for α_1 at position 394 of the β-globin mRNA. In fact, there is only one obvious difference between the regions of complementarity for the α and β antisense oligomers on their opposing mRNAs. Each of the β antisense oligomers has 6 or more sequential nucleotides complementary to the 5′-nontranslated region of the α-globin mRNA. In contrast, none of the α antisense oligomers has more than 3 sequential nucleotides complementary to the β-globin 5′-nontranslated region. While there are several sequences where either the α or β antisense oligomers could hybridize within the coding region of their opposing mRNAs, we might expect that these hybrids would be easily removed by the elongating ribosome. Good-

Table III

Inhibition of rabbit-globin mRNA translation by specific oligodeoxynucleotides

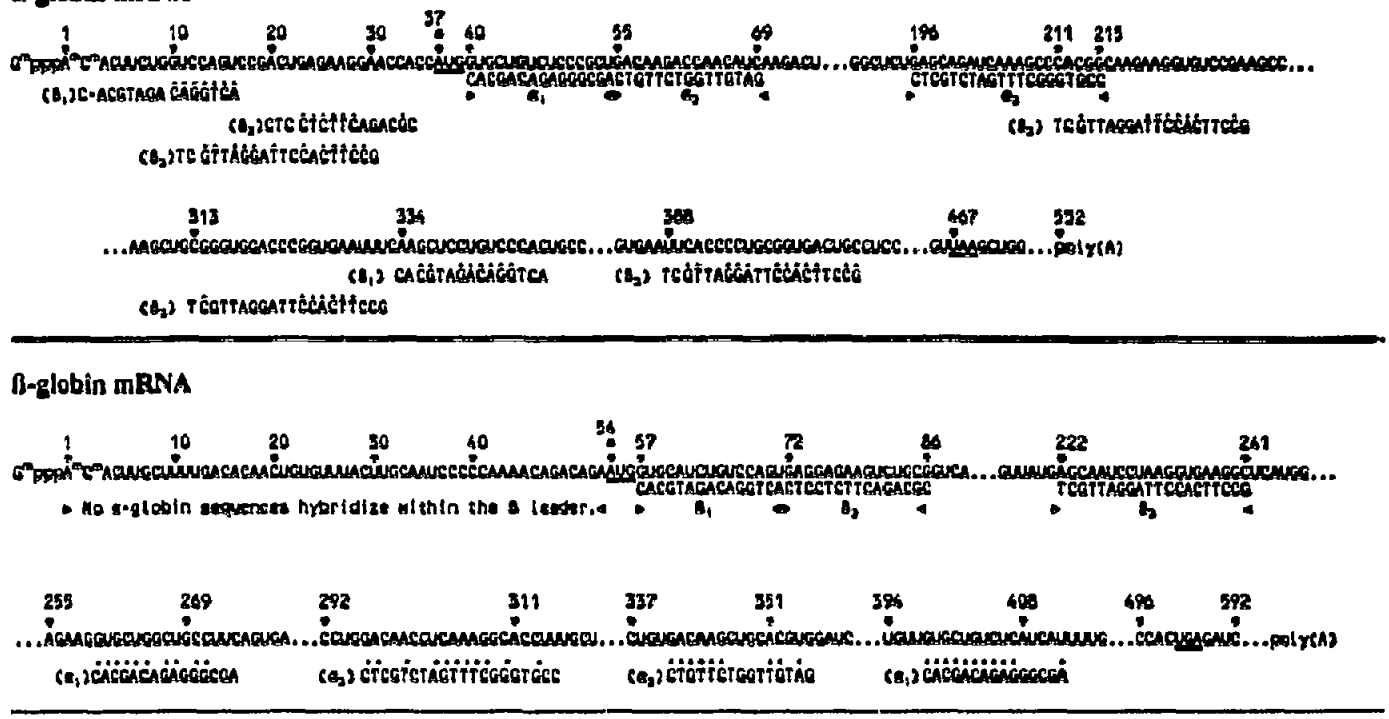
Antisense oligomer added	Amount (ng/μl)	Percent inhibition	
		α-globin	β-globin
α_1	40	15	−1*
	10	24	7*
	2	12	4*
	0.4	27	9*
α_2	40	51	−2*
	10	47	4*
	2	43	4*
α_3	40	36	−1*
	10	36	−24
	2	29	−16
β_1	40	39	38
	10	37	39
	2	52	58
β_2	40	61	67
	10	55	64
	2	68	74
β_3	40	−12	19
	10	19	39
	2	20	44
$\alpha_1\alpha_2$	40	74	−10
	10	66	−1*
	2	37	6*
	0.4	26	9*
$\beta_1\beta_2$	40	32	38
	10	20	37
	2	2*	−4*
$\alpha_1\alpha_2\beta_1\beta_2$	40	76	25
	10	79	30
	2	59	38

Hybridization of short DNA sequences complementary to rabbit α and β globin RNA was carried out as described in section 2 in a reaction containing: 75 ng of rabbit globin mRNA, the indicated amount of oligomer (see Table II). Negative values represent stimulation of synthesis.

*These values do not represent significant inhibition/stimulation since a typical standard deviation (S.D.) was about 15%. The stimulation of β-globin synthesis by $\alpha_1\alpha_2$ at 40 ng/μl had a S.D. of only 9.8.

Table IV

Complementarity between the globin mRNAs and their antisense oligomers



child et al. [22] found specific inhibition by β-globin antisense oligomers surrounding the AUG (n.t. 56–75) in contrast to our results (n.t. 57–71) and those of Dash et al. (n.t. 270–300) [21]. Goodchild et al. suggested that the use of very high concentrations caused the lack of specificity observed by Blake et al. [23]. This may be the cause of our non-specific inhibition since our maximum oligomer concentration in the case using the individual β antisense oligomers was 100 μM. In order to explain the non-specific inhibition of both globin chains by only one α- or β-globin antisense oligomer, Blake et al. had suggested that inhibition of synthesis of one chain leads to inhibition of the other by some physiological mechanism [23]. However, no molecular explanation of such a mechanism has been offered. It is possible that these anomalies may be caused by the subtleties of the three-dimensional structure of the mRNAs, therefore taking into account inter- and intra-molecular interactions [24–27]. In some cases, antisense oligomers have been shown to be completely non-specific in inhibiting mRNA translation [28]. To further complicate the situation, unlike the individual β-globin antisense oligomers, tandem oligomers immediately downstream of the initiation codon acted specifically to inhibit β-globin synthesis (Table III, $\beta_1\beta_2$). The mechanism for disappearance of α-globin inhibition by the addition of a second β-globin oligomer in tandem is not presently understood.

The second anomaly observed was that the degree of inhibition by β-globin antisense oligomers often was not dependent upon the concentration of the oligomer. While it is expected to get a dose-dependent response between the antisense oligomer and its effect, Goodchild et al. [29] and Bertrand et al. [30] indicated some erratic results with certain oligomers. This type of result seemed random and has no adequate explanation at present. The third anomaly observed in our eukaryotic system was, as discussed in the prokaryotic section, the stimulation of translation by antisense oligomers. In our system, under conditions where $\alpha_1\alpha_2$-globin antisense oligomers caused the greatest inhibition of α-globin synthesis (i.e. at 40 ng/μl), β-globin production was stimulated. Somewhat different stimulation was observed by Blake et al. who used antisense to the 5′-noncoding region of β-globin mRNA in wheat-germ extracts and rabbit reticulocyte lysates [23]. These oligodeoxynucleotides were targeted to the 5′-end of β-globin mRNA (n.t. 2–14) or to a sequence within the coding region (n.t. 59–66), indicating that stimulation can occur with antisense oligomers complementary to various positions in the RNA. Boiziau and his colleagues also observed stimulation of globin synthesis in the wheat-germ system using an antisense oligomer and its acridine derivative targeted to the coding region (n.t. 113–229)(data not shown)[31]. It is also interesting to note that Liebhaber and his group found stimulation of

mRNA binding to 80S ribosomes only when the anti-mRNA (cDNA, in these experiments) used was complementary to a sequence in the 3'-non-coding region of human β-globin mRNA (n.t. 446–551) [26]. Note that Liebhaber's work was carried out by stringent pre-hybridization of antisense to the target. Bertrand et al. found as much as 50% stimulation of rabbit α- and β-globin production by rabbit reticulocyte lysates in the presence of certain concentrations of α- or β-anomers complementary to cap sequences (n.t. 1–15) [30]. Interestingly, they did not see this effect in the wheat-germ translation system (see RNase H discussion below). With all of these examples of stimulation, there have been few ideas proposed to explain them. Although the extent of all of these stimulations is relatively slight, the effect of a 20% stimulation in the amount of an unknown protein could confuse the interpretation of results of antisense experiments.

Our results concerning the importance of target position on the effectiveness of an antisense oligomer revealed that our 20-mers targeted at the coding region of the mRNA were as effective as the 15-mers designed to bind near the initiation codon. This contrasts with the general observation that antisense inhibition of protein synthesis within the coding region is often ineffective, the antisense strand being displaced from the mRNA by the purported helix-destabilizing activity of the elongating ribosome [32,33]. Inhibition is thought to be most effective when the antisense strand binds to mRNA sequences at the 5'-cap of the mRNA, while sequences immediately adjacent to the AUG codon or within the 5'-nontranslated region were also shown to be somewhat effective targets (α, AUG thru +18; β, AUG thru +5)(β, -8 thru +12) [22,26,32]. Recent studies on the mechanism of antisense inhibition suggest that a high level RNase-H activity may be responsible for our observation that anti-mRNA targeted to regions within the coding region is an effective inhibitor [30,31]. This is the case with certain preparations of reticulocyte lysates, as well as the wheat-germ and *Xenopus* oocyte systems. When RNase-H activity is low, RNase H-mediated cleavage of the mRNA cannot compete with displacement of the DNA from the mRNA. This limits the region of effective inhibition to the 5'-cap and the 5'-nontranslated region [30]. The anomalous non-specific inhibition observed by our oligonucleotides is also related to RNAse H activity. Regions of homology as short as 15 pairs has been shown to direct RNase H-mediated cleavage [22]. However the possible presence of RNase H in the reticulocyte lysate would not explain our results since our longest match of β-antisense within α-globin mRNA is only 7 pairs. Despite the fact that RNase-H activity may explain some of the unexpected results, other unexpected anomalies described in this paper strongly suggest the utmost caution in planning and interpreting antisense experiments.

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