THE CHEMICAL SYNTHESIS OF DEOXYRIBO-OLIGONUCLEOTIDES COMPLEMENTARY TO A PORTION OF THE LYSOZYME GENE OF PHAGE T4 AND THEIR HYBRIDIZATION TO PHAGE SPECIFIC RNA AND PHAGE DNA

M.T. DOEL* and M. SMITH**

Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver 8, B.C., Canada

Received 4 May 1973

1. Introduction

We are approaching the problem of isolating specific mRNA* molecules by the use of a synthetic deoxyribo-oligonucleotide, complementary to a portion of the mRNA, covalently attached to a solid support. This will be used to isolate the mRNA by hydrogen bonding specifically to the complementary region.

Considerable evidence derived from model studies using synthetic deoxyribo-oligonucleotides attached to cellulose suggests that this approach is feasible with short oligonucleotides within the range of chemical synthesis [1, 2]. Purely statistical considerations predict that an oligonucleotide, 9 or 10 residues long, should be unique in a molecule the size of the T4 genome ($\sim 2 \times 10^5$ base pairs) [1, 3]. Also supporting this idea are the observations of Wu that deoxyribo-oligonucleotides as short as a nonanucleotide can be specifically hybridized to phage genomes, e.g., the cohesive ends of phage λ [4] or phage 186 [5]. However, efficient and specific binding to a very long single stranded molecule may be complicated by the presence of self-complementary sequences and the larger number of potential sites and may require the use of longer oligonucleotides. It has been reported that in hybridization of short complementary oligonucleotides to single strands of ϕ80 psu^+_{III} DNA a heptadecanucleotide could be specifically bound to the r-strand of the DNA. It has been suggested that the base sequence of the oligonucleotide plays a large part in determining the shortest oligonucleotide which will bind effectively [6]. We have focused our efforts on the mRNA for lysozyme from T4 since the work of Streisinger et al. [7, 8] on the amino acid sequences of lysozymes from a variety of frame-shift mutants has defined a tract of about 20 nucleotides in this mRNA.

We have synthesized a series of oligonucleotides up to a dodecanucleotide complementary to a part of this region and have examined their hybridization to T4 specific RNA and to single strands of T4 DNA. The results suggest that the dodecanucleotide can bind specifically to its complementary sequence on a long single stranded nuclei acid and should therefore be useful in isolation of lysozyme mRNA.

2. Materials and methods

2.1. *Chemicals*

Deoxyribonucleoside 5′-phosphates were obtained from Raylo Chemicals, Edmonton, Alberta. All other chemicals were reagent grade and were purified extensively before use. γ^{32}P-ATP (specific activity > 20/mmol) was obtained from New England Nuclear Corp. T4 polynucleotide kinase was the gift of Dr. R.C. Miller, other enzymes were from P.L. Biochemicals. Phage stocks were obtained from Dr. B.D. Hall and RNA

* Medical Research Council of Canada Postdoctoral Fellow.
** Medical Research Associate of the Medical Research Council of Canada.
† The abbreviations used are those suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (European J. Biochem. (1970) 15, 203.

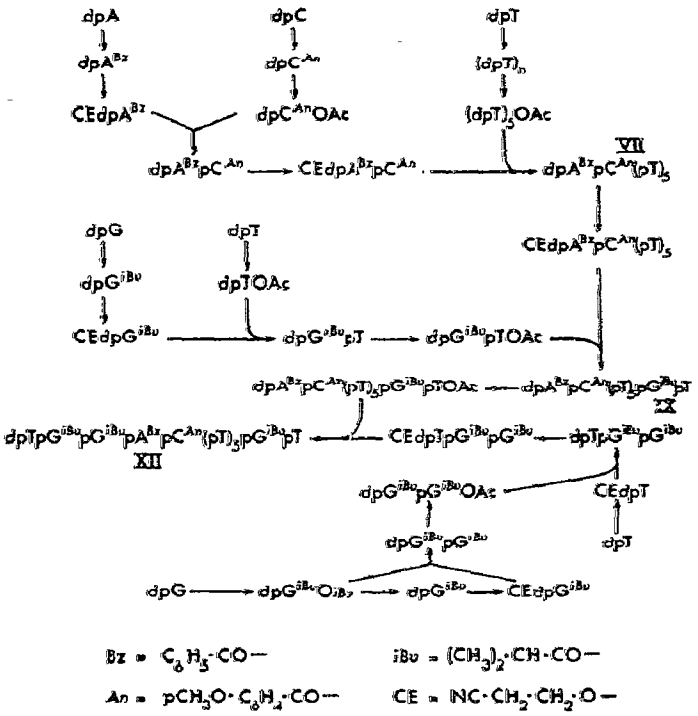

Fig. 1. Schematic representation of the chemical synthesis of some oligonucleotides complementary to T4 lysozyme mRNA.

prepared from *E. coli* B infected with T4 acq or the lysozyme deletion mutant G223 was the gift of Dr. E.T. Young, both of the University of Washington, Seattle.

2.2. *Synthesis of a dodecanucleotide*

The dodecanucleotide dpT—G—G—A—C—T—T—T—T—G—T (XII) and the heptanucleotide dpA—C—T—T—T—T—T (VII) and nonanucleotide dpA—C—T—T—T—T—T—G—T (IX) were synthesized by the methods of Khorana et al. These procedures have been extensively reported [9]. A similar oligonucleotide corresponding, instead of complementary, to this region of the lysozyme gene of T4 has been synthesized using slightly different methods by Narang et al. [10]. The strategy of the synthesis is shown in fig. 1.

2.3. *Labelling of oligonucleotides with ^{32}P*

The terminal 5′-phosphate group was removed from the oligonucleotides by *E. coli* alkaline phosphatase and the 5′ termini rephosphorylated with γ^{32}P-ATP and T4 polynucleotide kinase using the method of Richardson [11]. Oligonucleotides VII, XI and XII in fig. 1 were labelled in this way.

2.4. *Preparation and strand separation of phage DNA*

High titre phage stocks, grown on *E. coli* B in M9 mineral salt medium supplemented with tryptophan, were purified by sedimentation through 5—20% sucrose gradients. The phage DNA strands were separated by the (U, G) binding technique [12].

2.5. *Hybridization of synthetic oligonucleotides to DNA and RNA in solution*

The RNA used in these experiments was isolated from *E. coli* B infected for 20 min with T4 or the lysozyme deletion mutant G223, it was not further fractionated. Typical hybridization solutions contained 100 μg of RNA and 1 pmole of labelled oligonucleotide in 0.5 ml of 70 mM sodium chloride, 70 mM potassium phosphate pH 7.0. The mixture was heated to 65°C for 5 min then cooled to 37°C and incubated overnight. The sample was applied to a column of Sephadex G-50 (27 cm × 0.9 cm) and eluted at 25°C with the buffer. 1 ml Fractions were collected and added to 5 ml of water and the ^{32}P measured by its Cerenkov radiation [13].

Hybridization to T4 phage DNA strands was performed in 0.5 ml of 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0 (SSC). Varying amounts of labelled oligonucleotide were hybridized to 5 μg aliquots of DNA for 16 hr at temperatures between 0°C and 45°C before being separated on columns of Sephadex G-100 (27 cm × 0.9 cm). 1 ml Fractions were collected, added to 5 ml of water and the ^{32}P measured by its Cerenkov radiation.

2.6. *Hybridization of labelled oligonucleotides to DNA immobilized on nitrocellulose filters*

DNA strands were immobilized on Millipore HAWP (5 μg DNA per 25 mm disc). Hybridization was carried out by the method of Denhardt [14]. The hybridized filters were finally dried and the ^{32}P measured in a PPO—POPOP—toluene scintillation fluid.

3. Results and discussion

The results from the hybridization of oligonucleo-

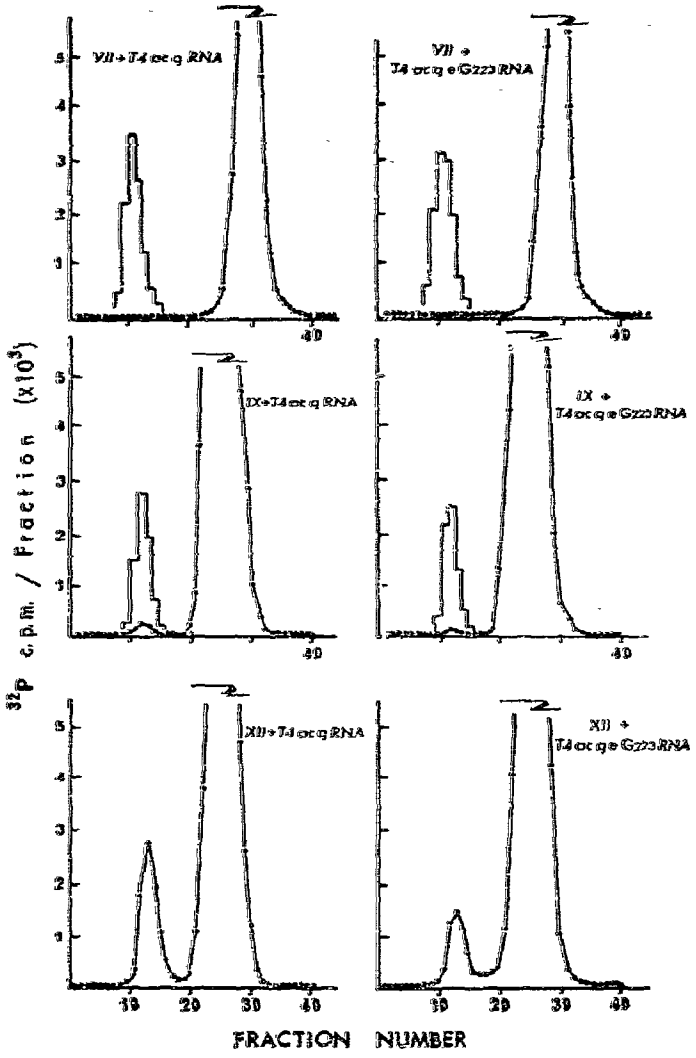

Fig. 2. Hybridization in solution of ^{32}P-labelled oligonucleotides to RNA preparations from T4 infected *E. coli*. Conditions were as described under Materials and methods.

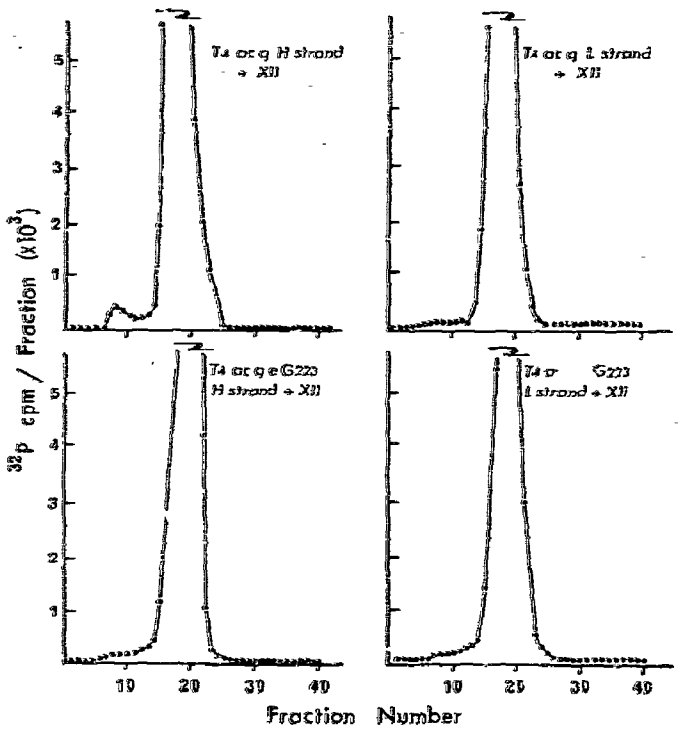

Fig. 3. Hybridization of ^{32}P-labelled dodecanucleotide to single DNA strands in solution. In the experiments shown 5 μg of DNA and 4.0 pmol of oligonucleotide were hybridized at 45°C for 16 hr.

tides to RNA preparations, as shown in fig. 2, display an interesting gradation of effects. As expected no hybridization at all was detected with the heptanucleotide VII under the conditions employed. Under the same conditions the nonanucleotide IX showed some binding both to RNA from *E. coli* infected with wild type T4 and to RNA from *E. coli* infected with the T4 mutant G223 lacking almost the entire lysozyme gene. This suggests that some site outside the lysozyme gene is binding the oligonucleotide. In a control experiment (not shown) RNA from uninfected *E. coli* bound an insignificant amount of radioactivity. The dodecanucleotide, XII, showed considerable binding to both RNA preparations but showed a marked preference for the wild type RNA. This RNA seemed to bind several times more oligonucleotide than would be expected (about 5.8 pmol/μg total RNA) on the basis of estimates of the amount of lysozyme mRNA present in the preparation (about 1 pmol/mg total RNA). However, uncertainties in this kind of estimation make it difficult to quantitate these data accurately.

The use of purified intact DNA strands enables a more accurate idea of the amount of binding to be obtained. Typical results from DNA hybridizations are shown in fig. 3. Two major conclusions can be drawn, firstly that the oligonucleotide binds preferentially to the H strand as defined by Guha and Szybalski [12]. This is as expected since lysozyme mRNA is known to be transcribed from the L strand [15]; thus the

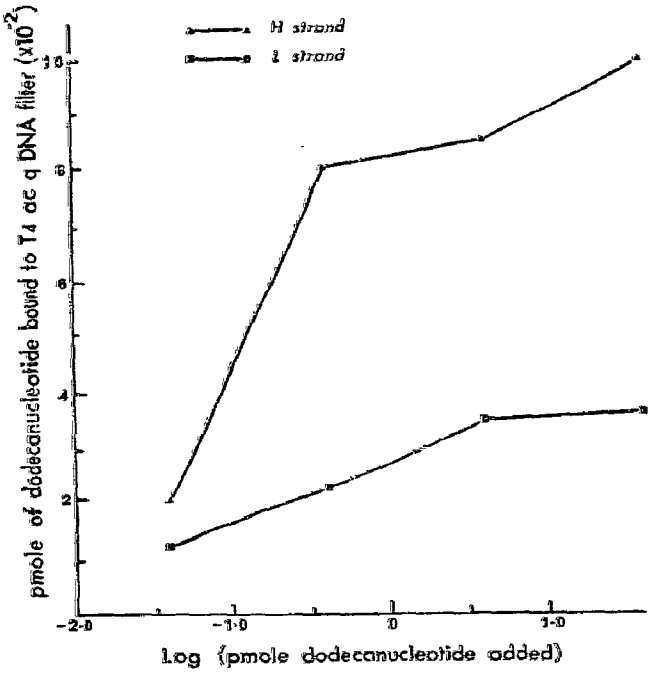

Fig. 4. Hybridization of ^{32}P-labelled dodecanucleotide to DNA strands from wild type T4 acq on nitrocellulose filters. Each filter carried 0.04 pmol of DNA strand.

sequence of the oligonucleotide is the same as the L strand and should complement the H strand. Secondly, both DNA strands of the deletion mutant G223 are equivalent in that they bond only small amounts of the oligonucleotide suggesting that the site of binding is within the lysozyme gene.

The amount of oligonucleotide bound to a DNA strand when the binding site is saturated can be determined by solution hybridization using increasing amounts of oligonucleotide. This is more conveniently done, however, by use of the filter hybridization technique. In fig. 4 are shown the results of a saturation experiment in which filters carrying separated strands of wild type T4 DNA were challenged with increasing amounts of labelled dodecanucleotide. As in the case of the solution hybridizations there is a marked preference for binding to the H strand. This binding seems to level out at about 0.09 pmol of nucleotide bound to 0.04 pmol of DNA. This is more than twice the theoretical value for steichiometric binding, however, if the L strand figures are used as a blank and subtracted, a value close to the predicted one is obtained. Denhardt's method utilizes 3 × SSC as the solvent for hybridization, it would be interesting to repeat these saturation experiments at lower salt concentrations to see the effects of this on the degree of binding at saturation. Experiments are in progress with DNA from other lysozyme mutants having slightly altered base sequence in the area of the synthetic dodecanucleotide. It is hoped that these will define more exactly the specificity of the hybridization.

From these results it seem sthat a specific interaction can be obtained between the synthetic dodecanucleotide and T4 lysozyme mRNA or T4 DNA strands. Therefore it is reasonable to suggest that using this oligonucleotide on an insoluble support [1, 2] a method could be developed for the isolation of T4 lysozyme mRNA.

Acknowledgements

We are grateful to Drs. B.D. Hall, R.C. Miller and E.T. Young for many helpful and stimulating discussions. This research was supported by a grant from the Medical Research Council of Canada.

References

[1] Astell, C.R. and Smith, M. (1972) Biochemistry 11, 4114.
[2] Astell, C.R., Smith, M. and Doel, M.T., manuscript in preparation.
[3] Thomas, Jr., C.A. (1966) in Progress in Nucleic Acid Research (Davidson, J.N. and Cohn, W.E., eds.), Vol. 5, p. 315, Academic Press, New York.
[4] Wu, R. (1972) Nature New Biol. 236, 198.
[5] Padmanabhan, R., Padmanabhan, R. and Wu, R. (1972) Biochem. Biophys. Res. Commun. 48, 1295.
[6] Besmer, P., Miller, R.C., Caruthers, M.H., Kumar, A., Minamoto, K., Sande, J.H. vande and Sidavara, N. (1972) J. Mol. Biol. 72, 503.
[7] Terzaghi, E., Okada, Y., Streisinger, G., Emrich, J., Inouye, M. and Tsugita, A. (1966) Proc. Natl. Acad. Sci. U.S. 56, 500.
[8] Okada, Y., Streisinger, G., Owen, J., Newton, J., Tsugita, A. and Inouye, M. (1972) Nature 236, 338.
[9] Khorana, H.G. et al. (1972) J. Mol. Biol. 72, 219–426.
[10] Narang, S.A. Itakura, K., Bahl, C.P. and Wigfield, Y.Y. (1972) Biochem. Biophys. Res. Commun. 49, 445.
[11] Richardson, C.C. (1965) Proc. Natl. Acad. Sci. U.S. 54, 158.
[12] Guha, A. and Szybalski, W. (1968) Virology 34, 608.
[13] Clausen, T. (1968) Anal. Biochem. 22, 70.
[14] Denhardt, D.T. (1966) Biochem. Biophys. Res. Commun. 23, 641.
[15] Kasai, T., Bautz, G.K.F., Guha, A. and Szybalski, W. (1968) J. Mol. Biol. 34, 709.