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Lambda Phage DNA: Joining of a Chemically Synthesized Cohesive End

Abstract. *The chemically synthesized dodecamer d(pA-G-G-T-C-G-C-C-G-C-C-C) was annealed, and was covalently joined to lambda phage DNA with bacteriophage T_4 ligase. The 5′-end of the dodecamer was joined to a deoxyguanosine residue. Repair with DNA polymerase I established that the position of joining was the left-hand end of lambda DNA. This is the first time that a chemically synthesized oligonucleotide has been covalently joined to a naturally occurring DNA molecule.*

Bacteriophage lambda DNA possesses two mutually complementary single-stranded structures extending from the 5′-termini of the double helix The nucleotide sequence of these cohesive, or "sticky," ends has recently been determined by Wu and Taylor (*1*).

By means of such structures, two molecules of DNA might be joined, or one molecule might be circularized; such phenomena are observed in nature (*2*). A heterologous genetic message might be joined to appropriate phage DNA through cohesive ends, and be carried into bacterial spheroplasts. With this in mind, we recently completed the chemical synthesis of a dodecamer identical with the protruding right-hand end of lambda phage DNA (*3*). We present evidence here that this chemically synthesized deoxyribododecanucleotide (Fig. 1, box) can be physically annealed, and enzymatically attached, to the macromolecular phage DNA in specific covalent linkage.

The enzymes used in this study and the procedure for the ^{32}P labeling of the 5′-terminus of the dodecamer with polynucleotide kinase and [γ-^{32}P]-adenosine triphosphate (ATP) have been described (*4*). After labeling the 5′-terminus of the dodecamer with ^{32}P, we placed the entire reaction mixture on diethylaminoethyl (DEAE)-cellulose paper (Whatman DE-81) and developed it for 6 hours with descending 0.5*N* triethylammonium bicarbonate (TEAB) buffer (*p*H 7.6). The paper was dried and cut into 2-cm lengths. The amount of radioactivity was determined in a Packard scintillation counter with a toluene-based scintillation liquid. The labeled dodecamer moved 2 to 4 cm from the origin and was separated from excess [γ-^{32}P]ATP. Similar strips with ^{32}P-labeled dodecamer were eluted with TEAB buffer (1*M*, *p*H 7.6), and the buffer was removed by repeated evaporation.

The results of binding ^{32}P-labeled dodecamer to lambda DNA is shown in Fig. 2A. A small peak containing ^{32}P (first peak) was found to coincide with the elution position of lambda DNA. A second peak (largest) was shown by a separate experiment to coincide with the elution position of the dodecamer itself; the third peak (found only in this experiment) appeared to be a small amount of contaminating inorganic ^{32}P.

When the annealed lambda DNA and [5′-^{32}P]dodecamer were incubated with bacteriophage T_4 polynucleotide ligase, and then separated by gel chromatography, a peak containing ^{32}P was found to elute with the lambda DNA again (Fig. 2B).

A comparison of resistance to bacterial alkaline phosphatase (BAP) of the two macromolecular peaks from Fig. 2A (annealed only) and Fig. 2B (annealed and incubated with ligase) is illustrated in Fig. 3. (The phosphorus label on the original dodecamer, contained in a phosphomonoester linkage, is labile to the enzyme. Upon joining of the dodecamer to the DNA, it will become an enzyme-resistant phosphodiester.) Dephosphorylation of the first peak of Fig. 2A, and separation on DE-81 paper (Fig. 3A), produced a movement of the ^{32}P label from the origin to a position expected for authentic inorganic phosphate (P_i). Furthermore, the failure of this ^{32}P

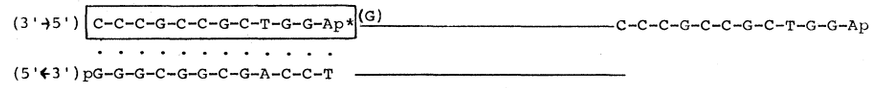

Fig. 1. Structure of the lambda DNA and its cohesive ends. The dodecamer in the box is annealed to the left-hand cohesive end.

Table 1. Joining of a synthetic dodecamer to lambda DNA with T_4 ligase. Lambda repair synthesis was carried out in a total volume of 0.3 ml, containing 28 μmole (83 m*M*) of TES (*10*) buffer (*p*H 7.0), 3.3 μmole (10 m*M*) of $MgCl_2$, 5 μmole (15 m*M*) of dithiothreitol, 27 μmole (80 m*M*) of NaCl, 5 nmole of the deoxynucleoside triphosphates indicated, and 0.5 pmole of lambda DNA (0.35 absorbance units at 260 nm). The mixture was incubated with 1.5 units of *Escherichia coli* DNA polymerase fraction 7 (*11*) for 4 hours at 5°C. The reaction was stopped by heating it for 10 minutes at 75°C. The indicated amount of [5′-^{32}P]dodecamer and 5 nmole of ATP were added, and the solution was cooled to room temperature over 1 hour. After 1 hour incubation at 0°C with 3 units of T_4 ligase, the reaction mixture was incubated for 30 minutes with 5 μg of BAP and was applied to DEAE-cellulose paper. The paper was developed with 0.5*M* potassium phosphate buffer (*p*H 6.9) and the amount of ^{32}P-labeled dodecamer that was joined to the DNA was determined by the amount of radioactivity (counts per minute) remaining at the origin. The number of counts at the origin of a control reaction mixture (without ligase) were subtracted. Joining reactions without repair were carried out with the same reaction mixture minus DNA polymerase and deoxynucleoside triphosphates.

Experiment	Repair with DNA polymerase	Extent of joining (pmole of ^{32}P)	^{32}P-labeled dodecamer (pmole)
1	No	0.06	1.6
2	No	0.21	16.0
3	Yes: dCTP, dGTP	0.20	1.6
4	Yes: dATP, dGTP	0.003	1.6

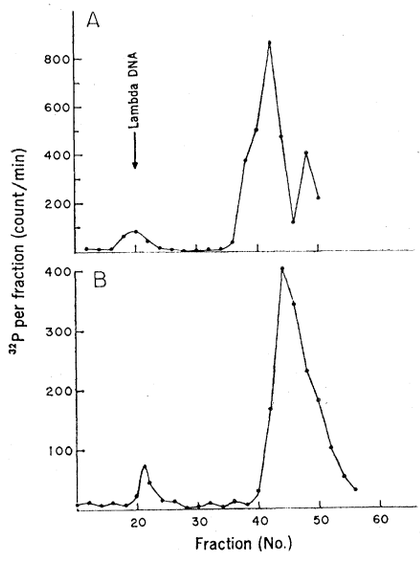

Fig. 2. Determination of [5′-^{32}P]dodecamer complex with lambda DNA. In experiment A, a reaction mixture (0.3 ml) containing 20 μmole (66 m*M*) of tris-HCl buffer (*p*H 7.6), 2 μmole (6.6 m*M*) of $MgCl_2$, 5 nmole of ATP, 1 pmole of lambda DNA (0.7 absorbance units at 260 nm), and 1.6 pmole of [5′-^{32}P]-dodecamer (≈ 18,000 count/min per pmole) was heated 5 minutes at 60°C. The mixture was annealed by cooling to room temperature over a 1-hour period. The mixture was chromatographed, at 0°C, on a column (0.9 by 43 cm) of agarose (Bio-Gel A, 0.5 m), which was developed with buffer consisting of 66 m*M* tris-HCl (*p*H 7.6) and 6.6 m*M* $MgCl_2$. Fractions (0.45 ml) were collected, and were analyzed for ^{32}P content by Čerenkov radiation (*9*). In experiment B, an identical mixture was annealed, and 3 units of T_4 polynucleotide ligase was added. The reaction was incubated for 1 hour at 0°C, and was chromatographed as in experiment A.

to adsorb to Norit (*4*) after being eluted from DE-81 paper was also characteristic of P_i. In contrast, when the first peak from Fig. 2B was treated with BAP and separated on DEAE paper, most of the radioactivity remained at the origin, thus illustrating that the label was resistant to the phosphomonoesterase.

These results indicate that a portion of the ^{32}P label in the [5′-^{32}P]dodecamer has become covalently attached to DNA after incubation with polynucleotide ligase. This fact was confirmed by a "nearest neighbor" analysis (*5*) of the material eluted as the first peak in Fig. 2B. Examination of the 3′-deoxyribonucleotides formed after digestion of the fraction of peak 1 (Fig. 2B) with micrococcal nuclease and phosphodiesterase from spleen revealed that all of the ^{32}P label was in deoxyguanosine 3′-phosphate, as required by the nucleotide sequence as determined by Wu and Taylor (*1*). As shown in Fig. 1 (left side), the expected ^{32}P transfer, with correct joining and digestion to 3′-nucleotides, would be from the 5′-deoxyadenosine residue of the dodecamer to the 3′-deoxyguanylic acid residue found on the lambda DNA.

When lambda DNA is treated with an appropriate DNA polymerase and the four usual deoxyribonucleoside 5′-triphosphates, both protruding single-stranded termini can function as templates in directing the addition of complementary nucleotides to the corresponding 3′-ends, until a perfect duplex is produced. By withholding up to three of the deoxynucleoside triphosphates, such synthesis can be limited to less extensive "repair" (*1*). Repair synthesis with dCTP and dGTP (*6*) (that is, in the absence of dATP and TTP) should cause the incorporation of nucleotides only on the right-hand end of DNA. Thus, after a repair of this type (*7*), it should still be possible to anneal, and join with ligase, the dodecamer d(pA-G-G-T-C-G-C-C-G-C-C-C) to the left-hand end of lambda DNA. The extent of joining was considerably better after repair on the right-hand end of lambda DNA (Table 1). This may be due to the fact that, after repair, annealing of the labeled dodecamer is no longer in competition with circularization and concatemer formation. In support of this hypothesis, we also found that a tenfold increase in the amount of [5′-^{32}P]dodecamer increased the extent of joining (Table 1); the results of Wu are also in agreement (*7*).

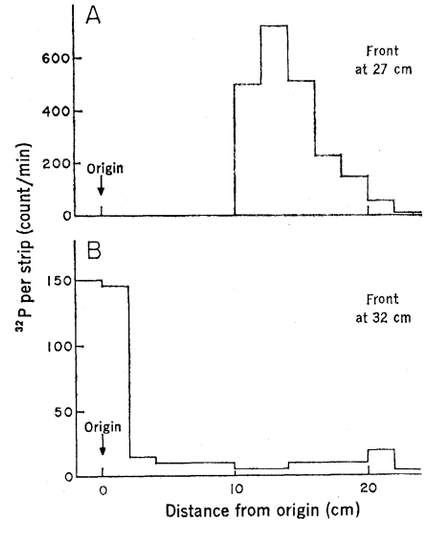

Fig. 3. Resistance to bacterial alkaline phosphatase of ^{32}P label complexed with lambda DNA. (A) A portion of the first-peak fraction seen in Fig. 2A (~ 3000 count/min) was incubated with 5 μg of BAP for 30 minutes at 60°C. The reaction mixture was then placed on DEAE-cellulose paper, and the ^{32}P radioactivity was counted, as described in the text for purification of labeled dodecamer. (B) A portion of the first-peak fraction seen in Fig. 2B (~ 400 count/min) was treated with BAP and was separated, as described above.

Further proof of specific, as opposed to random, joining is found in Table 1, experiment 4. In this case a sample of lambda DNA was first "repaired" with the two deoxynucleoside triphosphates, dATP and dGTP. Such a procedure should effect partial repair of the left-hand end of the DNA as well, and should prevent proper annealing and joining of the ^{32}P-labeled dodecamer. The joining was in fact decreased to 1/20 that of the control (Table 1). This point is also confirmed by the failure of heterologous DNA to incorporate radioactivity—substitution of calf thymus DNA for lambda DNA did not permit the joining of labeled dodecamer to macromolecule.

The results obtained from these experiments establish that the sequence d(pA-G-G-T-C-G-C-C-G-C-C-C) is specifically hydrogen-bonded to lambda DNA. These results are consistent with the sequence proposed by Wu and Taylor (*1*). However, the ligase experiment does not rule out the possibility that one nucleotide may be mismatched in such a complementary sequence without the ligase joining event being adversely affected (*8*).

To our knowledge, this is the first instance of the incorporation of a chemically synthesized DNA fragment into a naturally occurring DNA molecule.

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Gonadal Effects of Vasectomy and Vasoligation

Abstract. *During a 28-week study, vasectomy and vasoligation of immature male Wistar rats revealed that there was a significant decrease in urinary 17-ketosteroid in the vasectomized group at week 15; at week 28 there were significant decreases in the weights of the testes of the test groups, as compared to those receiving sham operations, with maximum alterations in the vasectomized rats. Small, soft discolored testes with cysts in the cauda epididymis and vas deferens regions occurred frequently in the test groups. The output of 17-ketosteroid in the urine and the findings in the testes indicate significant alterations in the morphology and function of the testes and suggest the need for caution and extensive investigations in man before recommending vasectomy as a simple, innocuous, "physiologic" means to ensure conception control.*

Valid social ends do not justify invalid, unscientific means. It has been reported that in the United States "last year 750,000 of the operations [vasectomies] were performed, and 1 million are expected in 1972" (*1*). Reports similar to this one led us to investigate what scientific data existed to justify vasectomy as demonstrably safe without sacrifice of normal physiologic patterns. The desirability of zero population growth cannot excuse the routine resort to irreversible, controversial procedures, be they drug or surgical.

Since Steinach's report in 1920 (*2*) that vasectomy caused a temporary decline in spermatogenesis of rabbits, analyses of the effects of vasectomy and vasoligation surgical procedures have been contradictory (*3–5*).

In man, other than as a contraceptive procedure, vasectomy has few proponents even though it has been proposed for alleviating senile, prostatic hypertrophy (*6*) and has been used in conjunction with prostatectomy to prevent nonspecific epididymitis and for disputed "regenerative" effects in males (*7*). Psychic disturbances have been recorded after vasectomy (*8, 9*), and unexplained thrombophlebitis and systemic disorders have been reported (*9*). Autoantibodies against spermatozoa have been found in the blood of a man with obstructed vas deferens (*10*) and in the blood of unilaterally vasectomized or vasoligated rats (*11*).

In our study, we sought to define some of the effects of vasectomy and vasoligation on testicular morphology and function. Immature albino male Wistar rats averaging 82 g were matched by weight and divided into three groups (vasectomy, group A; vasoligation, group B; and sham-operated controls, group C). The operations were performed under ether anesthesia, and the suprapubic incisions were performed under clean but not necessarily aseptic procedures (*12*). In the vasectomized rats, a minimum of 1 cm of the duct was removed. In the vasoligated rats (group B) a sterile black braided silk (Ethicon) tie was placed in the mid-region of the vas deferens. In group C (sham-operated rats) the testes and vas deferens were handled but without resection or ligation.

At week 15, the various test and control rats were placed singly in metabolism cages for collection of urine specimens for 17-ketosteroid analyses (*13*). The assay of urinary androgens has frequently been used as an index of gonadal output (*4, 14*) even though it represents the combined total of the predominant gonadal and smaller urinary 17-ketosteroid contributions of the adrenal glands (*15*).

All rats were decapitated at week 28. The sites of vasectomy, vasoligation, and other cavities of the body were examined, and the testes, seminal vesicles, adrenals, spleen, thymus, liver, and kidneys were dissected free of fat and connective tissue and weighed. Heparinized blood specimens were collected for biochemical investigations.

Urinary 17-ketosteroid values at week 15 after operation were reduced in both the vasectomized and vasoligated groups, but only group A vasectomized rats revealed statistically significant decreases compared to the controls (Table 1). Other investigators have reported no changes in the output of 17-ketosteroids in the urine of vasectomized rats (*4*).

Statistically significant reductions in the absolute weights of the testes of group A (vasectomized) and group B (vasoligated) rats were found (Table 1). In accord with the previous results, the decreases in testes weight were less in the vasoligated animals.

Gross examination of the testes at autopsy indicated that only 10 of the 31 animals in the group A rats had testes that appeared normal. In 17 animals, the pairs of testes were small, less turgid or soft, and had an abnormal purplish coloration (Fig. 1).

Table 1. Effects of vasectomy and vasoligation on urinary 17-ketosteroid output at week 15, testes weights at week 28, and white blood cell counts (WBC) at week 20 of immature Wistar rats (*N*, number of rats). Group A, vasectomy; group B, vasoligation; group C, sham control.

Group	Total urinary 17-ketosteroid		Blood count		Testes weight		Final body weight (g)
	N	μg	*N*	WBC/mm³	*N*	g	
A	30	86.85 ± 4.75	31	16876.6 ± 612.5	31	2.2014 ± 0.1829	544.7 ± 8.1
B	31	98.71 ± 3.89	31	16372.6 ± 479.6	31	2.5005 ± 0.1603	562.8 ± 8.6
C	27	106.48 ± 7.85	30	14314.2 ± 373.2	29	3.1532 ± 0.1134	540.8 ± 10.5
				Differences (%)			
A vs. C		−18.4		+17.9		−30.2	+0.7
P		.04		< .001		< .001	.77
B vs. C		−7.3		+14.4		−20.7	+4.1
P		.38		< .01		< .01	.11
A vs. B		+13.7		−3.0		+13.6	+3.3
P		.06		.52		.23	.14