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*DNA CIRCULARITY AND THE MECHANISM OF STRAND SELECTION IN THE GENERATION OF GENETIC MESSAGES**

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A potentially informative paradox exists in the literature of genetic transcription. Experiments[1–3] with preparations of purified DNA and transcribing enzyme (the DNA-dependent RNA polymerase, for brevity referred to as "transcriptase") have yielded RNA complementary to both strands of the DNA employed as a template. On the other hand, analyses[4–6] of transcription in the intact cell revealed that primarily only one of the two complementary DNA strands generates RNA messages. Resolution of this riddle could conceivably illuminate the mechanism operating to restrict *in vivo* transcription to one of the two strands.

As a departure for a further analysis of the difference between the *in vivo* and *in*

vitro results, the following three possibilities can be listed for the strand selection mechanism of transcription. (1) The control device functioning in the cell is not a structural component of either the DNA or the transcribing enzyme. An obvious example is an enzyme which would selectively destroy one of the two RNA complements. Others can be readily devised. (2) A structural feature of the transcribing enzyme controls selection and is rendered nonfunctional during purification. (3) The selection device is a built-in feature of the DNA which is destroyed during its isolation.

The experimental material and information currently available permits a direct test of the third alternative. It is known that, due to its inordinate length, the most likely injury suffered by DNA during isolation is fragmentation. The question immediately arises whether this is sufficient to explain the absence of strand selection exhibited in test-tube experiments. Two requirements must be satisfied for an interpretable experiment. One is a source of nonfragmented DNA. The other is a preparation of the transcriptase sufficiently free of DNAase to avoid introducing breaks in the time period of RNA synthesis.

To provide the first requisite, attention was focused on the replicating form (RF DNA) of the bacteriophage ϕX174 which infects *E. coli*. Our previous experience with RF DNA suggested that it possessed a number of obvious advantages for the purposes at hand. We have already shown[4] that messages are generated in the cell only from one of its two complements. The molecular weight of RF DNA (3.4×10^6) is small enough to minimize fragmentation during purification. A chromatographic method has been developed[3] permitting the isolation of pure RF DNA virtually free of contamination with fragments of host DNA or the single strands (hereafter called vegetative DNA) found in mature virus particles. Chromatographically purified preparations were shown[8] to be composed almost exclusively of circular DNA, thus providing another objective criterion of intactness. In addition, the possibility arose that the circularity, which had also been observed by Sinsheimer and his colleagues,[9, 10] might be related to the selective mechanism.

The necessity for a pure transcriptase was satisfied by purification to the point where no DNAase or RNAase could be detected by a sensitive assay detailed below.

It is the purpose of the present paper to describe the experiments made possible by the availability of intact DNA and nuclease-free transcriptase. The results are satisfyingly clear-cut. When intact circular DNA is employed as the template, only one strand is transcribed, as shown by both hybridization tests[13] and nearest neighbor analysis of the RNA product. However, when the DNA circles are broken, RNA complementary to both strands is found. The results clearly establish that the strand selection mechanism of genetic transcription is a feature of intact double-stranded circular DNA.

Materials and Methods.—(a) *Nucleic acid preparations:* ϕX174 RF DNA and the corresponding single-stranded DNA were isolated and purified chromatographically as described by Hayashi *et al.*[3] The columns used for RF DNA had a height to surface ratio of 1.55. All DNA preparations were tested for nuclease (§ *e*) and those employed in the experiments described showed no activity. RNA was isolated and purified according to the protocol of Hayashi and Spiegelman.[11] Purity and uniformity of RF DNA was monitored by constant specific radioactivity on repeated chromatography, banding in CsCl density gradients, and densitometer tracings of sedimentation

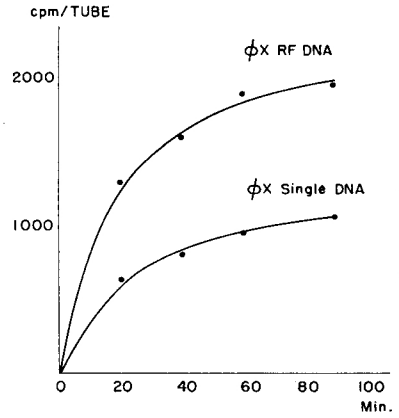

FIG. 1.—Kinetics of RNA synthesis. Each (0.25 ml) reaction mixture contained the components in the concentration described in §(*e*) of *Materials and Methods*. 150 cpm corresponds to one mμmole of GMP incorporated in RNA. RF DNA #1 and RF single-strand were used with enzyme #1. When sonicated RF was used, a curve similar to those shown was obtained but at a rate approximately $^1/_4$ that shown with intact RF–DNA as a template.

patterns using UV optics in the analytical ultracentrifuge. Such preparations are highly infectious in the protoplast test.[12]

(b) *Transcriptase purification:* The DNA-dependent RNA polymerase was isolated[2] from *E. coli* (C-122), harvested in log phase, and stored at −15°C. The last step involves an elution from a DEAE column with 0.23*M* KCl collected in fractions of about 2 ml. Each fraction was assayed for 20 min at 36.5°C for R NA polymerase activity using calf thymus DNA and the supplement detailed in (§*e*). Regions showing activity were then assayed for the presence of RNAase and DNAase. Only those fractions devoid of detectable nuclease activity were used in the experiments described. Purified enzyme was stored at 0°C. On isolation the three preparations used had the following specific activities in the units of Chamberlin and Berg,[2] #(1)-6000; # (3-1)-4000; # (4)-4,700.

(c) *Assay for DNAase and RNAase:* Each assay (1 ml) contained 90 μg of C^{14}-DNA (4,800 cpm/μg) or 20 μg of P^{32}-ribosomal RNA (3,500 cpm/μg) from *E. coli*. Incubation was carried out for 20 hr at 37°C under conditions suitable for RNA synthesis (§ *e*) except for omission of the nucleoside triphosphates. Negligible activity was assumed if less than 5% of the DNA and less than 8% of the RNA was converted to acid-soluble form. In examining the transcriptase, 100 μg of purified enzyme were tested.

(d) *GTP^{32} preparation:* GTP labeled with P^{32} in the nucleotide phosphorus was prepared according to Haruna *et al.*[14] The initial specific activity was about 3.3×10^8 cpm/μM of GTP. All counting was done in a Packard liquid scintillation spectrometer. Acid-precipitable material was washed with TCA and dried on Millipore membranes. Acid-soluble material was dried onto plastic planchets which were inserted into the vials.

(e) *RNA synthesis:* The reaction mixture (1 ml) contained 24 μg of template DNA, 40 μM of tris buffer pH 7.9, 1 μM $MnCl_2$, 4 μM $MgCl_2$, 46 μM KCl, 12 μM of β-mercaptoethanol, 40 ~ 100 μg of enzyme, 500 mμM ATP, CTP, UTP, and GTP^{32} labeled in the nucleotide phosphorus. Reaction temperature was at 36.5°C. The kinetics of RNA synthesis with circular RF DNA and mature DNA as templates are shown in Figure 1. The kinetics with disrupted circles are quite similar to those shown but at a rate corresponding to about 25% of the rate obtained with intact circles.

(f) *Analysis of nearest neighbor to guanosine:* On termination of the synthesis with GTP^{32} as the only labeled component, the contents were precipitated and washed five times with cold 3% PCA. Four mg of *E. coli* C bulk RNA were added to each tube and the contents hydrolyzed with 0.3 *N* KOH at 37°C for 15 hr. Chromatographic separation of 2′–3′ nucleotides and distribution of the radioactivity among them was carried out as described previously.[11]

(g) *Sonication of DNA:* DNA (50 γ/ml) dissolved in SSC (0.15 *M* NaCl; 0.015 *M* Na citrate) was sonicated with a Raytheon sonic oscillator at 4°C for 6 min. S_w^{20} of sonicated RF was about 8.

(h) *Hybridization:* The details of formation and detection of DNA-RNA hybrids are as described by Hayashi, Hayashi, and Spiegelman.[4, 15] Denatured DNA and RNA are mixed in 2 × SSC buffer and incubated at 42.5°C for 16 hr. The reaction mixtures are treated with pancreatic

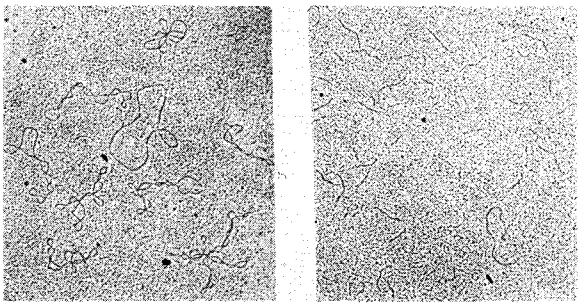

FIG. 2.—Electron microphotographs of ϕX-RF (*left*) and sonicated ϕX-RF (*right*). The method of Kleinschmidt *et al.*[24] was used. Preparations were shadowed with 12 mg of V_2O_5 at a distance of 11 cm and an angle of 6° while grids were rotating. Pictures were taken with a Siemens Elmiskop IIb at 50 kv by Barbara Chandler, Department of Zoology, University of Wisconsin.

RNAase and the resistant DNA-RNA hybrids separated on columns of methylated albumin coated on kieselguhr.

(i) *Electron microphotographs:* Electron microphotographs of intact and sonicated RF DNA were kindly taken by B. Chandler, University of Wisconsin, according to the procedure detailed elsewhere.[8]

Experimental Results.—Purity of RF DNA: As had been done previously,[3] the RF DNA was labeled with H^3-thymidine during its synthesis in the infected complex. This permits monitoring the progress of the purification by means of specific activity. Chromatography is repeated until constant specific activity of the RF DNA peak is achieved. Such preparations exhibit a homogeneous band in equilibrium centrifugation in CsCl gradients at a position corresponding to a ρ of 1.708 using *Ps. aeruginosa* N^{15}-DNA as a density marker at 1.746. Further, densitometer tracings of sedimentation profiles indicate that more than 95 per cent of the DNA sediments with an S^{20}_W of 21.0 ± 0.5, the value found originally[8] for the RF DNA. Finally, electron microphotographs show that more than 90 per cent of the material is in the form of intact circles. Figure 2 compares one of the purified RF DNA preparations used in the present study before (*left*) and after (*right*) sonic disruption.

Purification of transcriptase: In our experience, the Chamberlin-Berg procedure[2] used with *E. coli* (strain C) yielded DNA-dependent RNA polymerase remarkably free of both DNAase and RNAase as tested with radioactive substrate by the 20-hr assay described under §(*c*) above. Figure 3 shows the elution profile of enzyme (#3) and identifies two fractions used in experiments described below. The apparent splitting of the activity into two fractions is not uncommon, and its significance remains for elucidation.

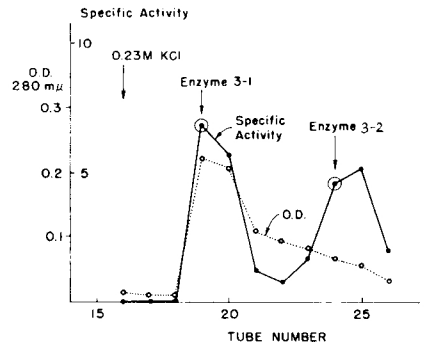

FIG. 3.—Chromatography of transcriptase. About 5 mg of fraction 3 of Chamberlin and Berg[2] was put onto a DEAE column (7 cm × 1 cm diameter), and washed with 0.16 *M* KCl in buffer B (0.002 *M* KPO_4 buffer pH 8.4, 10^{-2} *M* $MgCl_2$, 10^{-2} *M* β-mercaptoethanol, 10^{-4} *M* EDTA) until $O.D._{280}$ became less than 0.02. The enzyme was eluted with 0.23 *M* KCl in buffer B. Every 2 ml of effluent was collected. Enzyme activity was measured as described. Specific activities are expressed in arbitrary units.

TABLE 1

FREQUENCY OF NEAREST NEIGHBOR TO G IN SYNTHESIZED RNA

Theoretical					CpG	ApG	UpG	GpG
(A)	On mature strand (single)*				22	34	24	20
(B)	On complementary strand†				18	21	39	22
(C)	On both strands‡				20	28	32	20
Experimental								
No.	Template DNA	Enzyme	GMP^{32} incorp (mμM)	Incubation time (min)				
1	RF-1	1	26.8	0–20	21.1	21.4	37.4	20.1
2	RF-1	1	24.0	20–90	22.2	20.8	37.7	19.3
3	RF-1	1	52.0	0–90	20.8	20.8	38.2	20.2
4	RF-2	1	40.0	0–90	22.1	20.8	37.5	19.6
5	RF-2	3–1	20.0	0–30	21.9	21.3	36.8	20.0
6	RF-3	4	24.0	0–30	22.2	21.2	37.6	20.0
				Average	21.7	21.0	37.5	19.8
7	RF sonic-1	1	10.0	0–90	23.1	25.4	30.0	21.5
8	RF sonic-2	3–1	4.2	0–30	22.5	27.7	30.0	19.8
9	RF sonic-3	4	3.8	0–30	21.9	26.0	31.0	21.1
				Average	22.5	26.3	30.3	20.8
10	Mature-1	1	11.0	0–20	22.5	35.9	23.0	18.6
11	Mature-1	1	9.0	20–90	23.9	34.7	22.4	19.0
12	Mature-1	1	18.7	0–90	22.0	34.3	23.8	19.9
				Average	22.8	34.9	23.0	19.1
13	RF-2	3–2	3.6	0–30	21.7	25.1	33.3	19.9

* Calculated from limited replication of φX174 assuming the RNA synthesized is complementary and antiparallel.

† Limited replication data are converted into a complementary antiparallel template for the formation of an antiparallel RNA complementary to it.

‡ Calculated from extensive replication of Table 2 of Swartz *et al.*[16]

Details of RNA synthesis and nearest neighbor frequencies to G are as described in *Methods*. The mμM of GMP incorporated are those found and are not normalized to the amount of enzyme used, which varied from 40–100 μg. In experiments 2 and 11 the reaction was run for 20 min with unlabeled nucleoside triphosphates and P^{32}-GTP put in for the last 70 min. "RF-sonic" 1, 2, and 3 are sonicated aliquots of RF 1, 2, and 3. In all cases 24 μg of the corresponding DNA were used. Theoretical calculations employ the data of Swartz *et al.*[16] Numbers represent "moles-per cent."

Nearest neighbor analysis of RNA synthesized on intact and disrupted DNA circles: The elegant studies of Swartz *et al.*[16] on DNA synthesis with single-stranded DNA of φX174 provides the nearest neighbor frequencies in it, as well as its complement. From these data one can readily calculate the nearest neighbors of RNA synthesized on the original single strand, its complement, or both. Examination reveals that nearest neighbors to guanosine (G) provide the most sensitive discrimination among the three possibilities. The corresponding numbers are given as the theoretically expected values in Table 1. To decide whether intactness of circularity plays a role in strand selection, nearest neighbor frequencies to G were determined on RNA synthesized with intact circles, ruptured circles, and as an added control, on the mature single-stranded DNA. From the last, only one outcome is possible. To make certain that any differences observed could be ascribed without ambiguity to one of the two reactants, independent preparations of RF DNA and transcriptase were made and interchanged.

The frequencies of nearest neighbors to G found in the various experiments are recorded in Table 1. Experiments 1–6 involved three RF DNA preparations combined with three independent enzyme purifications. The results obtained are indistinguishable and independent of the particular pair used. Further, it will be noted that experiments 1–3 used the same pair and compared the nearest neighbors observed during different periods of the synthesis. It is clear from a comparison of expected and observed values that the best agreement has been obtained with the calculation which assumes that only one of the two strands of the RF DNA is tran-

scribed. Further, the values obtained specify that it is the strand complementary to that found in the virus which determines the base sequence of the RNA synthesized.

These conclusions are further strengthened by the results of experiments 7–9, which employed the same enzyme preparations tested in the first six experiments, but used sonicated RF DNA as templates. Here it is evident that agreement is with line C of the theoretical values, indicating that both strands of the RF DNA are functioning virtually equally in determining the base composition of the RNA synthesized. Finally, experiments 10–12 show that the enzyme has no difficulty in synthesizing RNA complementary to the mature strand if it is the only template in the reaction mixture.

Experiment 13 is reproduced since it yielded a result of possible significance. The enzyme used was #3-2, which came from the second peak of activity in Figure 3. Although free of detectable nuclease activity, it yielded a product with RF DNA suggesting that 30–40 per cent of the RNA is generated from the mature strand and the remainder from its complement. These results imply the possibility that the transcriptase in this peak is contaminated with an enzyme which can cleave the circular elements without further degradation of the double-stranded structure. Exploitation of this suggestive clue would appear to be worthy of further effort.

Hybridization tests with RNA synthesized on intact and disrupted circles of RF DNA: The analyses of nearest neighbors to G are consistent with the inference that intact circles generate complementary RNA copies from one strand, whereas with broken circles both strands of the DNA are involved. Hybridization tests can subject this conclusion to a definitive test. Figure 4 shows that RNA synthesized on either intact or disrupted circular RF DNA hybridizes to heat-denatured RF DNA. This result is expected since RF DNA contains both complements and establishes that both preparations contain hybridizable RNA. Figure 5 shows the decisive experiment in which the two types of preparations are challenged with single-stranded DNA from the virus particle. Here we see (Fig. 5*B*) that the RNA synthesized on broken circles hybridizes readily, whereas the preparation generated by the intact circles forms RNAase-resistant complexes (Fig. 5*A*) very poorly (less than 10% of 5*B*). This is precisely the outcome predicted from the nearest neighbor analysis. The RNA synthesized on circular templates should be composed principally of only one complement and, since its base composition is not complementary but similar to the mature strand, hybridization between the two should not occur. Finally, RNA generated from disrupted circles should contain both complements, one of which should be complementary to the mature strand. As a consequence, successful hybridization should occur.

Discussion.—Frequency of one- and two-strand transcription: It is of interest to compare the purity of the components used with the quantitative results obtained. Tracings of the sedimentation patterns and random field examinations of electron microphotographs both indicate that the RF DNA employed consisted of at least 90 per cent unbroken circles. This is a minimal estimate, since breakage could have occurred, for example, in preparation of grids, etc.

Comparison of the theoretical and observed ApG and UpG values listed in Table 1 provides an estimate of the relative frequency with which each strand of the DNA duplex is employed in the transcription. The data obtained with intact circular

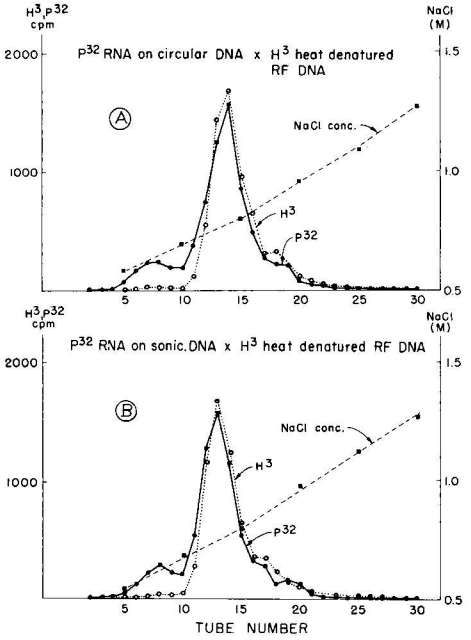

FIG. 4.—Hybridizations of RF DNA and RNA synthesized on intact (*A*) and disrupted (*B*) circles. The P^{32}-RNA was synthesized on intact (*A*) and disrupted (*B*) circles. H^3-RF DNA was sonicated, dialyzed against 0.1 × SSC, heated to 97–98°C for 10 min, and quickly cooled to 0°C. Prior sonication was necessary for complete irreversible heat denaturation. Each hybridization mixture contained 108 μg of denatured DNA and 0.1 μg of RNA. Incubation was in 0.3 *M* NaCl, 0.03 *M* Na citrate at 42.5°C for 16 hr. Subsequently, the mixture was subjected to RNAase (free of DNAase) at 30 μg/ml at 26°C for 30 min. The mixture was then loaded on a MAK column and eluted as detailed by Hayashi *et al.*[4] The H^3 identifies the DNA, and the P^{32} the RNA in the RNAase-resistant hybrid structures.

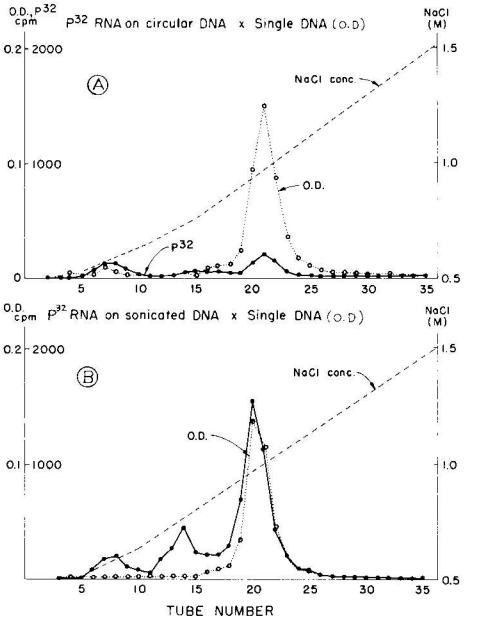

FIG. 5.—Hybridization tests of RNA synthesized on intact (*A*) and disrupted (*B*) circles with mature single-stranded DNA. The conditions of hybridization and subsequent treatment are the same as described in Fig. 4. Each hybridizing mixture contained 100 μg of mature DNA and 0.1 μg of P^{32}-RNA. The $O.D._{260}$ profile identifies the DNA, and the P^{32} the RNA in these RNAase-resistant hybrid structures.

RF DNA indicate that 90—100 per cent of the RNA synthesized has been directed by only one of the two DNA components, the complement to the mature strand. This frequency range is clearly consistent with the hybridization tests carried out with the mature strand in Figure 5*A*. Similar calculations with disrupted circles as templates indicate that both strands are transcribed with equal frequencies.

The quantitative data agree, therefore, with the estimations of purity of RF DNA for circularity. They further indicate that except for the minor fraction 3-2, the preparations employed are not contaminated with impurities which can destroy circularity. One preparation (#2) had a comparatively low specific activity (1200) and was not used in the experiments reported.

Conclusions on the nature of the strand selection mechanism: It is clear from the experiments described that we have succeeded in reconstructing in the test-tube the conditions leading to a strand selection mechanism which makes the same choice

observed[4] in genetic transcription of the intact cell. The necessary components are a nuclease-free transcriptase and intact circular double-stranded DNA. The data cannot be explained in terms of a quantitative limitation on RNA synthesis with circular DNA. In point of fact, twice as much RNA (corresponding to 3 times the weight of the template DNA) is produced in equivalent incubations with circular DNA as with ruptured material.

The data of Table 1 clearly establish that it is the intactness of the RF DNA which determines whether one or both strands are transcribed. Each of the three enzyme preparations used gave both results, the outcome being determined by the DNA included in the reaction. These results argue against a control mechanism involving a component which is separable from the DNA and the transcriptase. They are equally inconsistent with identifying the control element as either a structural or contaminating component (e.g., a nuclease which selectively destroys one RNA strand) of the transcriptase. All the data support the thesis that the strand selection mechanism is to be identified as a unique feature of circular double-stranded DNA.

DNA intactness—circularity: The experiments described do not prove that only circular DNA will retain the strand selection device. It is conceivable that any DNA molecule, open or closed, will exhibit this property, providing it has never experienced fragmentation. This possibility may soon be testable with material now available. In any event, at the present writing intact circularity has been shown to be a key controlling factor. This leads one to entertain the concept that circularity may turn out to be a general feature of strand selection in transcription. It will be recalled that the concept of a circular DNA is not a new one. It first emerged as a brilliant deduction in Jacob and Wollman's[17] formal model of their data on recombination in *E. coli*. Subsequently, genetic evidence for circularity was provided for the bacteriophages T4[18] and λ[19]. Recently, direct evidence for ring structures came from autoradiographic experiments with *E. coli*[20] and electron microphotographs of viral DNA.[8, 9, 21, 22] Finally, note should be made of Stahl's[23] ingenious ring-chain model for chromosomes, which serves to explain a number of paradoxes which grew out of fine structure genetic analysis.

In conclusion, it would not be surprising to find that circularity is not confined to bacteria and viruses. Further, it may be that a circular element will be found to constitute the unit of transcription (i.e., the transcripton).

Such circular DNA molecules are likely to contain a unique region of closure, which may also serve to start and direct the relative movement of the transcribing enzyme. The RF DNA of φX174 is an obvious choice for attempts at the chemical identification of such unique elements, if they exist.

Summary.—The experiments described were designed to resolve the following paradox; genetic transcription in the cell generates RNA strands complementary to only one of the two components of the DNA, whereas *in vitro* experiments show that both strands of the DNA are transcribed. The possibility was considered that the inconsistency could derive from the fact that test-tube experiments invariably use fragmented DNA. The experimental material consisted of a purified preparation of "replicating form" DNA of φX174 which was shown to be composed of intact double-stranded circles. The RNA synthesized on intact and disrupted circles was compared by nearest neighbor analysis and by hybridizations with the

mature single-stranded DNA. The data clearly established that intact circles generated RNA complementary to only one strand, the complement of the mature strand in the duplex. Disrupted circles produced strands complementary to both DNA components.

It appears that the strand selection mechanism which characterizes normal genetic transcription is a unique feature of circular double-stranded DNA. It can be reproduced and studied outside the cell.

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