Asymmetric Transcription of Deoxyribonucleic Acid by Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase of Krebs II Ascites-Tumour Cells

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Early studies of DNA transcription into RNA *in vitro* by using purified bacterial RNA polymerases (EC 2.7.7.6; RNA nucleotidyltransferase) appeared to indicate that the process was symmetrical, i.e. that both strands of the DNA molecule were used as template for the enzymic formation of RNA of complementary base sequence (Geiduschek, Moohr & Weiss, 1962). On the other hand, studies *in vivo* with bacteriophage systems suggested that only one strand of the DNA was used as template for RNA formation (asymmetric transcription) (Hayashi, Hayashi & Spiegelman, 1963; Marmur *et al.* 1963; Tocchini-Valentini *et al.* 1963). Enzymic studies (Geiduschek, Tocchini-Valentini & Sarnat, 1964; Hayashi, Hayashi & Spiegelman, 1964; Green, 1964; Luria, 1965) suggest that there might be some factor in the structure of the enzyme molecule or in the configuration of the template DNA that governs the symmetry or asymmetry of transcription.

This communication presents the results of an investigation into the synthesis of RNA *in vitro* catalysed by the DNA-dependent RNA polymerase associated with the chromatin material from Krebs II mouse ascites-tumour cells. Some of the properties of this enzyme preparation have been described by Eason & Smellie (1965). Such a preparation, it was thought, might contain the template DNA, and any RNA polymerase associated with it, in a configuration close to that occurring *in vivo*. Moreover, no attempt was made to displace histones, or other basic proteins, from the DNA to enhance the activity of the RNA polymerase.

Nuclei were isolated from the Krebs II cells after osmotic disruption of these cells (harvested 6–7 days after implantation) by the method described by Smellie, Keir & Davidson (1959). After collection by centrifugation at 600*g* for 10 min., they were washed four times with 10 mM-tris-HCl buffer (pH 7.2)–20 mM-2-mercaptoethanol (TME buffer) and then ruptured by freezing and thawing three times in 3 vol. of TME buffer. The disrupted material was centrifuged at 12000*g* for 10 min.; the supernatant fluid was discarded and the sediment was washed twice with further portions of TME buffer to remove any remaining nuclear ribosomes, or other soluble material, and finally

suspended in an equal volume of TME buffer. This preparation, which contains the bulk of the cell DNA together with the DNA-dependent RNA-polymerase activity (Eason & Smellie, 1965), was used in this study of the synthesis of RNA *in vitro*. The ³H-labelled RNA was prepared by incubating 0.83 ml. of this preparation at 37° for 30 min. with 0.16 μ mole (100 μ o) of [³H]ATP (Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A.), 0.4 μ mole each of CTP, GTP and UTP, 0.1 μ mole of tris-HCl buffer, pH 7.2, and 14 μ moles of MnCl₂ in a final volume of 1 ml. After incubation, sodium lauryl sulphate was added (final concn. 0.01%) and the suspension extracted with 80% (w/v) phenol with vigorous shaking for 10 min. The resulting emulsion was separated by centrifugation at 10000*g*, and the aqueous phase was removed and re-extracted with phenol and then with ether to remove the phenol traces. After removal of ether with a stream of N₂ the solution was treated with deoxyribonuclease (10 μ g./ml.) in the presence of MgCl₂ (5 mM) and bentonite (1 mg./ml.). After further phenol extraction as described above the resulting RNA solution was dialysed overnight against 1 l. of 15 mM-NaCl–1.5 mM-sodium citrate, pH 7, and then centrifuged at 20000*g* to remove traces of bentonite. The yield of [³H]RNA from such a preparation ranged from 175 to 500 μ g. and its specific activity ranged from 181 to 973 trichloroacetic acid-insoluble disintegrations/min./ μ g. of RNA.

When this ³H-labelled RNA was tested for sensitivity to ribonuclease A under the conditions described by Weissmann, Borst, Burdon, Billiter & Ochoa (1964) up to 6–7% of the labelled RNA was ribonuclease-resistant. Such an observation suggested that at least 92% of the product was single-stranded RNA, while the remaining resistant material might be double-stranded RNA. Further studies using the conditions of Weissmann *et al.* (1964) showed that the ribonuclease-resistance of the ³H-labelled product showed a fairly sharp thermal transition at about 105°, as measured by heating to various temperatures, cooling rapidly and testing with ribonuclease. This is a characteristic of double-stranded RNA rather than an RNA–DNA hybrid (Weissmann *et al.* 1964). It is noteworthy that the

RNA synthesized by a similar enzyme preparation from mouse-liver nuclei was only 1.5% resistant to ribonuclease and this resistance remained unchanged even after heating the RNA to 120°.

To determine whether or not the single-stranded ³H-labelled product synthesized by the Krebs II-cell DNA-dependent RNA polymerase had arisen as a result of the enzymic transcription of both DNA strands, or just one, it was subjected to conditions that might be expected to convert any single strands of RNA having complementary base sequence into a ribonuclease-resistant duplex (i.e. maintaining the RNA solution, 250 µg./ml., in 375 mM-NaCl-37.5 mM-sodium citrate, pH 7.2, for 2 hr. at 90° followed by slow cooling). This treatment did not significantly increase the original ribonuclease-resistance of the preparation and indicates that there are not two species of single-stranded RNA synthesized, having complementary base

sequence. Further, if the transcription process catalysed by the enzyme preparation is asymmetric, it should be possible to form a hybrid between the ³H-labelled enzyme product and denatured DNA of Krebs II cells, and also to prevent the formation of this hybrid by the addition of a large excess of unlabelled Krebs II-cell RNA. The assumption is made that the RNA isolated from intact Krebs II cells has originated as a result of the transcription *in vivo* from one strand of the cells' DNA. This assumption is supported by the results of hybridization and self-annealing studies with ³H-labelled Krebs II-cell RNA. Such an RNA, isolated after incubating Krebs II cells for 1 hr. at 37° in Earle's medium containing 5 µC of [³H]-uridine/ml. under the conditions described by Eason, Cline & Smellie (1963), had little resistance to ribonuclease, and, after treatment similar to that already described, which might be expected to anneal RNA strands of complementary base sequence, no significant rise in ribonuclease-resistance could be detected.

The results presented in Table 1 demonstrate that hybrid formation between denatured Krebs II-cell DNA and ³H-labelled product of Krebs II-cell RNA polymerase was considerably inhibited by the addition of a large amount of unlabelled Krebs II-cell RNA. On the other hand, addition of unrelated RNA species had much less effect.

The observation that up to 90% inhibition of hybrid formation could be achieved is significant, since if the RNA synthesized by the RNA polymerase were the result of a symmetrical transcription process then it should only be possible to achieve a 50% inhibition of hybrid formation.

The results of these studies suggest that at least 93-94% of the RNA synthesized by Krebs II-cell DNA-dependent RNA polymerase is single-stranded and arises as a result of the enzymic transcription of only one of the strands of the DNA template.

The significance of the remainder of the product cannot yet be decided. Although it has some of the properties of double-stranded RNA rather than those of an RNA-DNA hybrid, which is formed when single-stranded DNA is used as primer for bacterial RNA polymerases (Warner, Samuels, Abbott & Krakow, 1963; Sinsheimer & Lawrence 1964), the mechanism whereby it might be formed is unknown. One possibility is that, during preparation of the enzyme, the correct enzyme-template relationship is partially destroyed and a small amount of symmetrical transcription is allowed to take place.

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Table 1. *Inhibition of hybrid formation between Krebs II-cell DNA and ³H-labelled product of Krebs II-cell RNA polymerase by various RNA samples*

DNA was isolated from Krebs II cells by the method of Marmur (1961) and was denatured in water at a concentration of 50 µg./ml. by heating at 100° for 10 min. followed by rapid cooling. RNA-DNA hybridization was carried out by mixing 12 µg. of ³H-labelled enzymically synthesized RNA and 20 µg. of denatured Krebs II-cell DNA in a final volume of 1.2 ml. of 0.5 M-KCl-10 mM-tris-HCl buffer, pH 7.2, and maintaining this solution at 67° for 2 hr. and then digesting with 6 µg. of ribonuclease A (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) for 30 min. at 25°. After this treatment the RNA-DNA hybrids remaining were collected on cellulose nitrate filters by the method of Nygaard & Hall (1963); the filters were dried and then immersed in 5 ml. of scintillator fluid [0.5% of 2,5-diphenyloxazole plus 0.03% of 1,4-bis-(5-phenyloxazol-2-yl)benzene in AnalaR toluene] and assayed for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer with suitable corrections for quenching.

Origin of unlabelled RNA	Amount of unlabelled RNA added (µg.)	[³ H]RNA converted into RNA-DNA hybrid (%)
—	None	8.00
Krebs II ascites-tumour cells	161	3.40
Krebs II ascites-tumour cells	966	0.84*
Hamster cells (BHK21/C13 line)†	360	7.90
<i>Micrococcus lysodeikticus</i> cells	484	7.20

* In this type of experiment no detectable RNA-RNA hybrids were formed.

† Macpherson & Stoker (1962).

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Oxygen Consumption of Isolated Pancreatic Islets of Mice Studied with the Cartesian-Diver Micro-Gasometer

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Studies of the insulin release from the endocrine pancreas and quantitative biochemical analyses of micro-dissected pancreatic islets have significantly contributed to our present knowledge of the function and biochemistry of the pancreatic islet β -cells. However, we have so far only limited information about several important metabolic characteristics of these cells, e.g. the mechanism behind the glucose stimulation of insulin release (cf. Field, 1964). The purpose of the present communication is to demonstrate a new approach to metabolic studies of mammalian β -cells, utilizing Cartesian divers in micro-respirometric analyses of isolated and surviving pancreatic islets.

Methods. Adult female mice with the obese-hyperglycaemic syndrome from a strain originating at the Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A., were used. The animals were starved for 18 hr. before decapitation. The pancreas was then excised and suspended in Krebs-Ringer phosphate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1964), maintained at 2°. The method used for the subsequent micro-dissection of fresh islets has been described in detail (Hellerström, 1964).

The oxygen consumption of the isolated islets was

measured with the aid of Cartesian divers (Linderström-Lang, 1937, 1943; Holter, 1943) with a total volume of 8–12.5 μ l. The basic equipment and the handling of the divers mainly conformed to the directions given by Holter (1943, 1961). The silicone-coated divers were either of the 'standard' type, with a bulb between the neck and the tail, or of the cylindrical type. The total gas volume of the charged divers at equilibrium pressure was about 5 μ l., i.e. a displacement in the pressure of 1 mm. of Brodie solution at 37° corresponded to a change in the gas content of about 0.0004 μ l.

When an islet had been isolated from the surrounding tissue, it was washed briefly in fresh Krebs-Ringer phosphate buffer and sucked into a braking pipette (Holter, 1943), together with 0.5 μ l. of buffer solution. The medium with the islet was placed as a bottom drop in the bulb of a standard diver, the neck of which was sealed with 0.7 μ l. of 1% (w/v) KOH, 0.5 μ l. of paraffin oil and an appropriate amount of flotation medium. After transfer to the flotation vessel the diver was allowed to equilibrate for 10 min. before the measurements started. When the islet respiration was measured at 30°, the standard divers were filled at room temperature. In experiments performed at 37° the