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An Extraordinary Temperature Dependence of the Reovirus Transcriptase*

A. M. Kapuler†

ABSTRACT: The RNA-dependent RNA polymerase (transcriptase) found in the subviral core of reovirus has an unusual temperature dependence for polyribonucleotide synthesis. The rate of RNA synthesis is 13–15-fold higher at 44° than at 34°. The temperature optimum is between 47 and 52° and Arrhenius plots are biphasic. There is a profound activation between 30 and 40° and a more normal temperature coefficient between 40 and 50°. There are no major differences in the species of RNA being synthesized at 37.5, 45, and 52°. Replacement of normal substrates by the analogs 5-bromocytidine 5′-triphosphate or 5-bromouridine 5′-triphosphate does not qualitatively alter the temperature dependence and nucleoside 5′-triphosphates (NTPases) bound with the transcriptase in the subviral core do not show the abnormal temperature effect. The kinetics of synthesis of a reaction preincubated for 2 min at 51° and shifted to 34° are no different from those of a reaction maintained exclusively at 34°. A cooperative conformational change in the template RNA which directly affects polymerase activity is considered as a potential explanation for the phenomenon.

Analysis of the life cycles of animal viruses has revealed some novel phenomena. One such unexpected event was the discovery that several diverse viruses contain template-bound polymerases as a structural component of the mature virion. Kates and McAuslan (1967) and Munyon *et al.* (1967) found a transcriptase in the core of vaccinia, a DNA virus, while Shatkin and Sipe (1968) and Borsa and Graham (1968) uncovered a RNA-dependent RNA polymerase in reovirions and Baltimore *et al.* (1970) a comparable enzyme in vesicular stomatitis virions.[1] We succeeded in isolating the reovirus transcriptase directly from virus infected L cells in subviral particles (Levin *et al.*, 1970). This enzyme, as well as other preparations from mature virions (Skehel and Joklik, 1969) or infected cells (Gomatos, 1968), synthesizes ten mRNA molecules which correspond to the ten double-stranded RNA molecules or chromosomes found within the virus particle. While characterizing the transcriptase, we found that a NTPase[2] activity capable of hydrolyzing either ribo- or 2′-deoxyribo 5′-triphosphates to their corresponding 5′-diphosphates was intimately associated with the polymerase in the subviral particle (Kapuler *et al.*, 1970). In this paper, we examine the effect of temperature on the synthesis of reovirus mRNA by the subviral particle polymerase. RNA synthesis is measurable at 30–32°, but not below, and has an optimum rate at 50–52°. Between 34 and 44°, a 13–15-fold stimulation of RNA synthesis takes place without a qualitative alteration in the species of RNA being made. A potential explanation of the phenomenon is that at physiological temperatures a conformational change in the template RNA occurs leading to a profound effect on the transcriptase activity.

Experimental Section

The Dearing strain, type 3, reovirus RNA polymerase (or transcriptase or mRNA synthetase) was isolated in the

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† Present address: Life Sciences, Microbiology Section, University of Connecticut, Storrs, Conn.

[1] This line of investigation has recently culminated with the demonstration that the virions of RNA tumor viruses contain RNA-dependent DNA polymerases (Temin and Mizutani, 1970; Baltimore, 1970), thus substantiating Temin's heretical DNA provirus hypothesis (Temin, 1964).

[2] The following unusual abbreviations are used: SVP, subviral particle; NTPase, nucleoside 5′-triphosphatase; E_a, activation energy; 5BrCTP, 5-bromocytidine 5′-triphosphate; 5BrUTP, 5-bromouridine 5′-triphosphate.

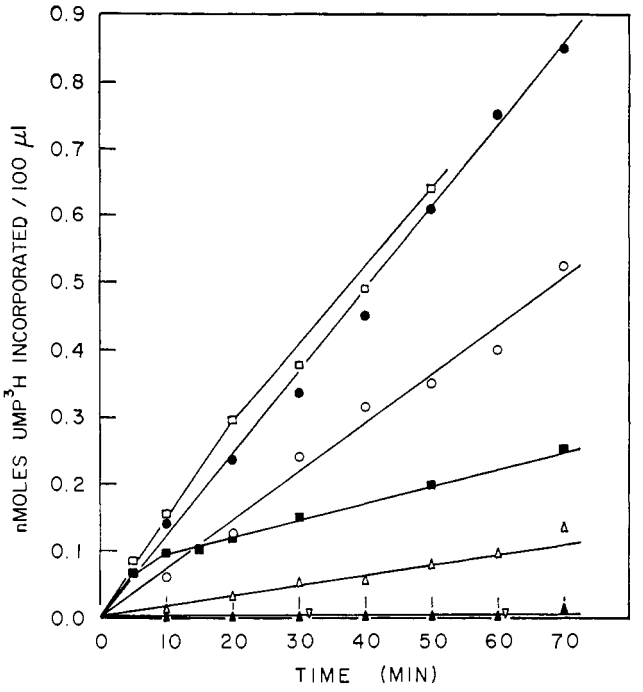

FIGURE 1: Kinetics of synthesis with reovirus transcriptase at different temperatures. Seven reactions twice normal scale were prepared under standard conditions and each one was incubated, one at a time, at a different temperature. Aliquots of 25 µl were removed as indicated in the figure and the incorporation of UTP-t (specific radioactivity 56,000 cpm/nmole) was measured: 31° (▲); 37° (△); 41° (○); 47° (●); 52° (□); 58° (■); 52°, minus GTP (▽).

laboratory of Dr. G. Acs from virus infected L cells as described previously (Kapuler *et al.*, 1970; Levin *et al.*, 1970). The subviral particle (SVP) preparations used in these experiments had specific activities of 250–300 units/mg of protein where a unit corresponds to the incorporation of 1 nmole of a radioactive precursor into acid-insoluble RNA in 30 min at 37°. RNA, insoluble in 5% trichloroacetic acid, was trapped on 27-mm Schleicher & Schuell Bac-T-Flex filters. Standard reactions were performed in 100 µl and contained 10 µmoles of Tris-HCl (pH 7.9), 30 µmoles of NH_4Cl, 750 nmoles of $Mg(OAc)_2$, 200 nmoles of ATP, CTP, UTP, and GTP, 10 nmoles of dithiothreitol, and 0.1–0.2 unit of enzyme. A constant-temperature water bath equipped with a Haake circulator and having a maximum temperature variation of ±0.1° was employed in all precision experiments. Reactions were brought to proper temperature by preincubation for 2 min prior to the addition of the enzyme. Radioactive and nonradioactive NTP were obtained from Schwarz BioResearch Inc., and P-L Biochemicals Inc., respectively. Radioactivity was measured by standard liquid scintillation procedures. 5-BrCTP was prepared from CTP by bromination in formamide; purification was effected by chromatography on Dowex 1-X8 (Cl^-). 5-BrCTP was converted into 5BrUTP by deamination with nitrous acid; 1.25 ml of glacial acetic acid and 2.1 g of sodium nitrite were added to 0.65 mmole of 5BrCTP in 10 ml of water and the solution was maintained at 4° for 24 hr. The reaction was then neutralized to pH 8 with ammonium hydroxide and the nucleotides were precipitated as Ba salts with ethanol. 5BrUTP was separated from 5BrCTP by chromatography on Dowex-1 Cl (X2) using 0–0.8 M LiCl gradient, 0.025 M in HCl. NTPases were assayed by the thin-layer chromatographic and colorimetric procedures described in a previous publication (Kapuler *et al.*, 1970).

Results

The Basic Observation: the Temperature Dependence of the Reovirus Transcriptase. The kinetics of RNA synthesis at 6 different temperatures are illustrated in Figure 1. Several distinguishing features of the reovirus polymerase reaction are apparent immediately. The rate at 37° is 22% that at 41° and 13% that at 47°. The temperature optimum is between 47 and 52° and the reaction proceeds even at 58°. At this latter temperature, the kinetics are biphasic with a rapid initial rate and a slower, linear second velocity. Below 52°, the polymerization rate is linear for at least 70 min. At 31°, the rate of the reaction is virtually nil and no different from a three nucleotide control incubated at 52°.

The temperature dependence of the polymerase reaction was measured in a series of reactions each incubated for 30 min at a given temperature between 30 and 58°. Between 30 and 40°, the rate of the reaction increased with a rapidity uncharacteristic of enzymatic reactions. The rate at 42° is six times that at 36°; between 34 and 44° it changes 13-fold. Beyond 40–42°, a more normal temperature dependence is observed (Figure 2A).

When the data are plotted as a function of reciprocal temperature the biphasic character of the rate of RNA synthesis is more pronounced (Figure 2B). Activation energies (E_a) for the two slopes correspond to 22 and 66 kcal/mole for the higher and lower temperature curves, respectively.

The polymerase found in mature virus particles has a temperature dependence comparable to that described for the subviral particle preparation. Thus, mature reovirus, isolated by well established procedures in the absence of detergents (Gomatos and Tamm, 1963) and activated with chymotrypsin (Shatkin and Sipe, 1968), had relative rates of synthesis under standard conditions at 35, 41, and 50° of 0.017, 0.670, and 1.000, respectively. However, not all subviral polymerase preparations exhibited identical temperature optima or absolute activity curves. Occasionally a preparation would show some activity (5–10% of maximal) between 28 and 30°. In these circumstances, the temperature optimum was 1–3° lower. In all cases, the apparent cooperative activation and abnormal temperature coefficient were observed.

Characterization of the Temperature Effect. (1) REVERSIBILITY OF THE REACTION. There are several possible reasons for the rapid change in the rate of RNA synthesis between 32 and 40°. To help distinguish among them, a temperature shift experiment was performed. The kinetics of RNA synthesis in a reaction incubated for 2 min at 51° followed by a shift to 34° were no different from those in a reaction maintained exclusively at 34° (Figure 3). Thus high temperature does not irreversibly activate the polymerase. The process leading to the increased rate is rapidly and completely reversible.

(2) PRODUCTS PRODUCED AT DIFFERENT TEMPERATURES. In previous studies (Levin *et al.*, 1970), we found that the products of the SVP polymerase were 10 single-stranded RNA molecules which annealed specifically to the viral double-stranded RNAs. These studies were performed at 37°; the analysis employed sucrose density gradient centrifugation and

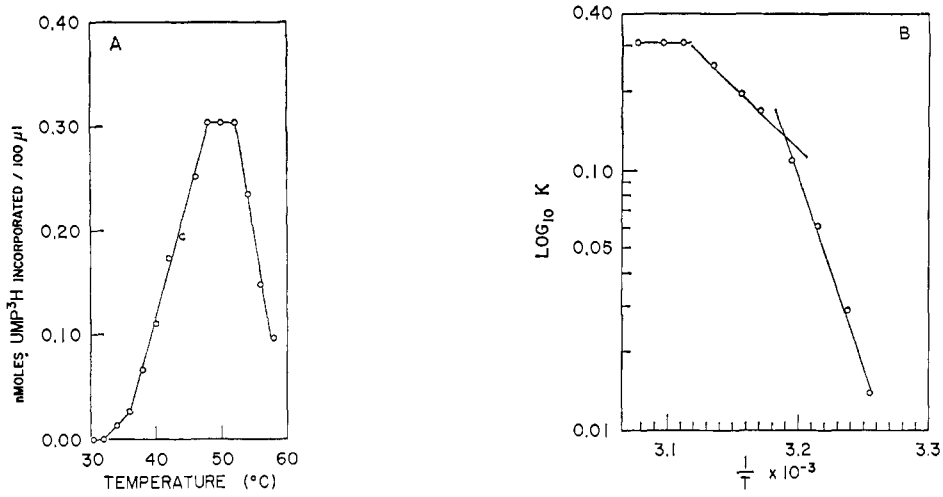

FIGURE 2: Rates of RNA synthesis as a function of temperature (A) and Arrhenius plot of the data presented (B). A series of standard reactions half normal scale were prepared; a single one was incubated for 30 min at each temperature with a constant amount of enzyme. The polymerization of UTP-*t* (specific radioactivity 112,000 cpm/nmole) was followed. The activation energies calculated from the slopes of the two lines are 66 and 22 kcal/mole. The units for *k* are nmoles of UTP-*t* incorporated/30 min; the temperature is in degrees Kelvin.

acrylamide gel electrophoresis. The products made by the transcriptase at 45 and 52° are compared with those made at 37° using the sucrose density gradient procedure. As illustrated in Figure 4, there are minor differences between the profiles of the RNA's made at the three temperatures. Further increases in temperature produce RNA profiles with diminishing amounts of large (l) (see Bellamy *et al.*, 1967; Bellamy and Joklik, 1967, for nomenclature) RNA and increasing quantities of smaller RNA molecules having discrete sizes. The products made between 37 and 52° do not change radically and hence the basis of the temperature dependence is not a qualitative alteration in the nature of the products. These data do not exclude the possibility that a few of the 10 mRNAs are synthesized differentially as a function of temperature.

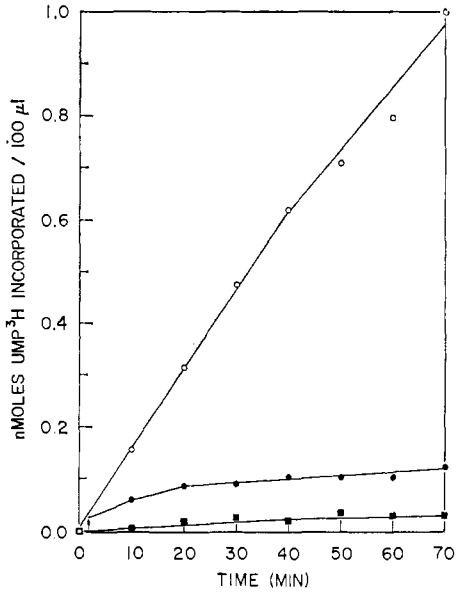

FIGURE 3: Effect of a temperature shift on the rates of RNA synthesis with the reovirus transcriptase. Three reactions twice normal scale were prepared under standard conditions. One reaction was incubated at 34°, the second at 51°, and the third at 51° for 2 min followed by a shift (denoted by the arrow) to 34° for the duration of the experiment, Aliquots of 20 µl were taken as indicated in the figure and the incorporation of UTP-*t* (specific radioactivity 56,000 cpm/nmole) was measured: 34° (■); 51 → 34° (●); 51° (○).

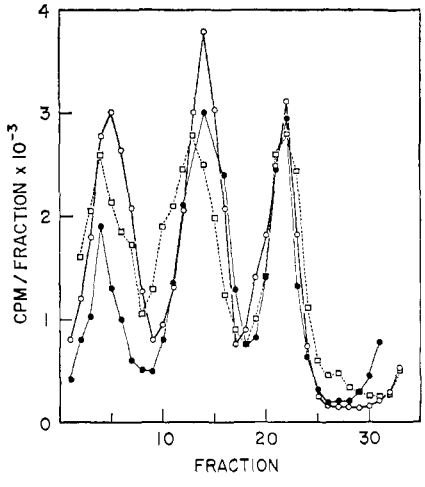

FIGURE 4: Analysis if the transcriptase products produced at different temperatures by sucrose gradient centrifugation. Three standard reactions were incubated at 37.5, 45, and 52°, respectively, for 90 min. The reaction performed at 37.5° was twice normal scale. Incorporation of UTP-*t* (specific radioactivity 144,000 cpm/nmole for 37.5° and 178,000 cpm/nmole for 45 and 52°) was measured. The reactions were ended by the addition of sodium dodecyl sulfate (to 1%) and EDTA (to 0.01 M) and then layered on performed 5–20% sucrose gradients containing 0.1 M potassium acetate pH 5.5 and 0.001 M EDTA. The gradients were centrifuged at 35°K for 11 hr in a SW41 head of a Spinco ultracentrifuge. Fractions were collected from the bottom of the tubes in the conventional way and analyzed for acid-insoluble radioactivity. The results are presented such that the peak fractions of the *s* molecules coincide: 37.5° (●); 45° (○); 52° (□).

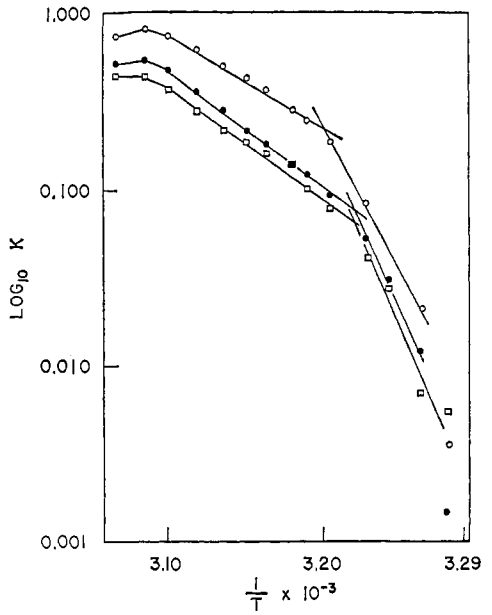

FIGURE 5: The effect of substrate analogs on the temperature coefficient of the reovirus transcriptase reaction: rates of synthesis as a function of reciprocal temperature. Three series of parallel reactions were prepared under standard conditions, each reaction half normal scale. One series contained the four natural substrates (○), the second 5BrUTP in place of UTP (●), and the third 5BrCTP in place of CTP (□). Polymerization of CTP-*t* (specific radioactivity 73,500 cpm/nmole) was measured in the first and second series and polymerization of UTP-*t* (specific radioactivity 70,000 cpm/nmole) in the third. One reaction of each series was then incubated at each temperature for 20 min. All reactions received equal amounts of polymerase. The units for *k* are nmoles of UTP-*t* incorporated/20 min. The temperature is in degrees Kelvin.

(3) EFFECT OF SUBSTRATE H-BONDING ABILITY ON THE TEMPERATURE DEPENDENCE. The reovirus transcriptase will accept the pyrimidine analogs 5-bromoCTP and 5-bromoUTP as replacements of CTP and UTP, respectively. The rates of RNA synthesis with saturating levels of these analogs are between one-third and one-half that observed with the four natural triphosphates. In both cases, the rates of RNA synthesis are linear for several hours and the products are the full complement of single-stranded RNA molecules (A. M. Kapuler, L. Altwerger, D. C. Ward, and G. Acs, manuscript in preparation for *Virology*). Since polynucleotides containing BrCMP or BrUMP residues replacing CMP and UMP have increased thermostability (Michelson *et al.*, 1967), we sought to determine whether the biphasic character of the polymerase temperature dependence could be affected selectively by replacing a normal substrate with an analog having increased H-bonding ability. The results found in Figure 5 demonstrate that H-bonding ability on the part of the substrates or the product RNA are not responsible for either the biphasic temperature dependence of polymerization or for the 32–40° activation.

(4) TEMPERATURE DEPENDENCE OF THE POLYMERASE-ASSOCIATED NTPASES. Within the reovirion is an enzyme capable of hydrolyzing nucleoside 5′-triphosphates to their 5′-diphosphates and P_i. Functionally, these NTPase activities can be separated from the polymerase based on thermostability and substrate specificity (Kapuler *et al.*, 1970). As an internal control for the 32–40° temperature activation of the polymerase, the ability of the cores to hydrolyze GTP, ATP, and CTP was measured between 30 and 50° under polymerase assay conditions. As found in Table I, these activities, in contrast to the polymerase, only triple their rates of triphosphate hydrolysis between 30 and 40°.

TABLE I: NTPase Activities at Different Temperatures.

Temp (°C)	Substrate (nmoles of P_i Released/30 min)[a] ATP	CTP	GTP
30	14.4	4.0	3.0
33	26.0	9.3	6.0
36	34.0	11.5	10.0
39	42.2	14.2	12.8
42	47.2	14.8	11.8
45	52.0	9.6	9.5
48	53.5	10.0	9.5
51	55.5	7.4	8.2

[a] The release of P_i was measured colorimetrically under standard polymerase assay conditions (see Experimental Procedures).

Discussion

There are several different mechanisms that could account for the dramatic temperature dependence of the reovirus transcriptase. Among these are possibilities suggested in part by other nucleic acid polymerizing systems.

The general interpretation of the temperature induced stimulation of enzymatic activity relies on the assessment of the empirically observed E_a as ΔH^* using absolute reaction rate theory (Stearn, 1949; Johnson *et al.*, 1954). Enzymes with Q_{10}'s between 1.5 and 4 can be understood within the context of this theory and the change in the rate of synthesis by the reotranscriptase between 40 and 50° is explicable within these terms. Below this temperature, the enzyme has a Q_{10} of 13–15 and an activation energy in excess of 66 kcal/mole, properties inconsistent with well-established mechanisms. The magnitude of temperature stimulation between 30 and 40° is suggestive of a cooperative phenomenon such as, for example, a helix–coil transition in either a polypeptide or a polynucleotide. The temperature shift experiment establishes that high temperature does not irreversibly activate the polymerase; thus the temperature effect is rapidly reversible and simple denaturation of a protein inhibitor with increasing temperature does not account for our observation. The kinetics further substantiate this conclusion in that a temperature- and time-dependent inactivation of an inhibitor would give exponential rather than linear rates of synthesis. These data also eliminate the notion that initiation is rate limiting at low temperature. Alternative to a thermal inactivation process is a cooperative temperature induced conformational change either in the template RNA, in the tertiary structure of the transcriptase, or in the organization of the subviral core. The core RNA is inaccessible to pancreatic

ribonuclease A and micrococcal nuclease (deoxyribonuclease II) and thus far it has not been possible to reconstitute active polymerase from isolated single or double stranded RNA and core proteins (J. Wachsman, H. Klett, G. Acs, and A. M. Kapuler, unpublished results). No data attest to the conformation of the template RNA within the core and it may not be a simple double helix. If it were at least in part single stranded, then a temperature dependent conformational change could alter its template efficacity. In the QB replicase system, double stranded RNA does not prime (Feix *et al.*, 1968) and Weissmann and his collaborators (Weissmann *et al.*, 1968) have suggested that during RNA synthesis, the template and its complementary product do not collapse into a classical duplex structure. Furthermore, there is a large change in the rate of the replicase reaction between 27 and 37° (S. Spiegelman, personal communication) thus establishing a formal if not mechanistic similarity to the reovirus system. Also comparable to the reovirus polymerase, the Arrhenius plot of the temperature dependence of minus strand synthesis by the fowl plaque virus RNA polymerase is biphasic. The low temperature portion of the curve has an E_a of 36 kcal/mole and a corresponding Q_{10} of 4.5 (Scholtissek and Rott, 1969). It is conceivable then that the polymerases from QB virus, fowl plaque virus, and reovirus have in common a temperature dependent conformational transition in their templates which profoundly affects their activity. This predicts that incorporation of base analogs into the reovirus template RNA should shift the 32–40° stimulation to a higher temperature if the analogs are those which increase the thermostability of a helix. Conversely, analogs such as inosine in the template should shift the activation process to a lower temperature.

Finally, as an alternate interpretation, there may be a functional integration between the synthesis of RNA and its export from the particle, a phenomenon recently discovered by Kates and Beeson (1970) for the *Vaccinia* virion transcriptase. Inhibition of transport would then interfere with synthesis. A thermally induced cooperative change in the permeability of the particle would then accelerate the rate of RNA synthesis and could account for the temperature dependence.

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