support a view that initiation of carcinogenesis reflects direct modification of DNA. Nevertheless, under cellular conditions, a polymerase could insert an occasional modified nucleotide, particularly in specific sequences that favor maintenance of base pairing or stacking.

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M1 RNA, the RNA Subunit of *Escherichia coli* Ribonuclease P, Can Undergo a pH-Sensitive Conformational Change†

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ABSTRACT: After purification from extracts of whole cells, M1 RNA, the catalytic subunit of ribonuclease P from *Escherichia coli*, apparently must undergo a change in conformation before it can function catalytically. The rate of this conformational change is dependent upon the duration of incubation at various temperatures and pH. $\Delta E^{\ddagger}$ of the transition at pH 7.5 is approximately 36 kcal/mol. The change in conformation is not sensitive to Mg^{2+} concentration between 10 and 100 mM. A decrease in A_{260} of M1 RNA in solution has been observed during the incubation period that potentiates the conformational change at 30 °C, but no direct correlation can yet be made to specific structural rearrangements.

Individual RNA molecules can assume different conformations in solution that depend on precise patterns of intramolecular or intermolecular hydrogen bonding. The ability to change their conformations with solution conditions in vitro, or with physiological states in vivo, can be important to the function of proteins and nucleic acids. RNA molecules that demonstrate this phenomenon include the leader sequences of trp operon mRNA in *Escherichia coli* (Lee & Yanofsky, 1977), tRNAs (Crothers & Cole, 1978), rRNAs (Chambliss et al., 1980; Kao & Crothers, 1980), col E1 primer RNA

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(Tomizawa, 1984; Wong & Polisky, 1985), and, possibly, snRNPs (Reveillaud et al., 1984). We have found evidence for a conformational change that, under certain conditions, controls the action of M1 RNA, the catalytic subunit of ribonuclease P from *E. coli*. We now describe the characteristics of this conformational change in M1 RNA.

Materials and Methods

M1 RNA. M1 RNA was prepared as previously described (Guerrier-Takada et al., 1983). The procedure involves phenol extraction of RNA from cell extracts, electrophoresis through polyacrylamide gels, and passage of the eluted material over a CF-11 column. Samples used for enzymatic assays or spectrophotometric studies were resuspended directly from lyophilized samples prepared by the procedure described above.

Assays for RNase P Activity. These assays were carried out as previously described by using the precursor to $tRNA_1^{Tyr}$ (pTyr)[1] from *E. coli* as substrate.

Measurements of the Lag Period in M1 RNA Activity. M1 RNA was resuspended in buffer P (50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, and 100 mM $MgCl_2$) in a polypropylene microfuge tube (capacity 1.5 mL) on ice and the mixture incubated for specified times at various temperatures in a Neslab Endocal temperature-controlled water bath. After each incubation period about 7500 cpm of substrate labeled with ^{32}P was added and incubation continued for further periods of time, fixed for each temperature. The assays were performed under conditions that corresponded to the linear portion of the curves describing the kinetics of cleavage. The results were quantitated by scanning of the autoradiographs of reaction products after electrophoresis on 10% polyacrylamide gels. Thermal equilibration of ice-cold reaction mixtures took about 30 s. This period did not affect our results at 30 °C or at any other temperature used.

Spectrophotometric Measurements. Lyophilized samples of M1 RNA were resuspended in buffer P on ice and then placed in a quartz cuvette held in a water-jacketed cuvette holder. The results of the absorbance studies were recorded by a Kipp and Zonen BD40 recorder. Recording was started immediately after the sample was placed in the cuvette. A cuvette containing buffer P alone was used as the blank standard. Measurements were made from each cuvette at 30-s intervals in a Beckman DU spectrophotometer.

Results

Kinetics of M1 RNA Action. When lyophilized M1 RNA, prepared from extracts of *E. coli*, is suspended in solution and assayed for RNase P activity, a brief lag period is observed before the reaction obeys linear kinetics (Guerrier-Takada et al., 1983). However, if the M1 RNA is subjected to a period of preincubation at 37 °C or dialysis through 7 M urea, no such lag is observed. We inferred that the lag period reflects a requirement for M1 RNA to undergo a structural transition in order to become active and that this transition involves the breaking and forming of hydrogen bonds. We have now studied the lag period further in an attempt to define more clearly its thermodynamic parameters.

M1 RNA, prepared and assayed as described under Materials and Methods, was incubated for various periods of time at 19 (room temperature), 30, 37, and 43 °C prior to the addition of substrate to the reaction mixture. Aliquots were taken from the reaction mixtures and analyzed at various times after the addition of substrate. In Figure 1, we show the data

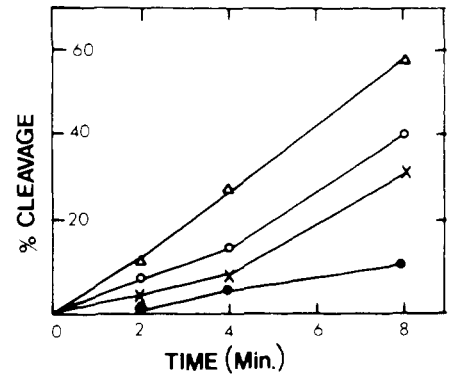

FIGURE 1: Kinetics of M1 RNA activity as a function of preincubation time. M1 RNA (11 ng/20 μL) was preincubated at 30 °C for the time indicated by the symbols described below and then assayed for activity at 30 °C as described under Materials and Methods. The abscissa represents the time after addition of substrate when aliquots were taken from the reaction mixture. Time of preincubation: (●) 5 min; (×) 10 min; (○) 20 min; (△) 30 min.

for the experiments performed at 30 °C. It is apparent that M1 RNA does not cleave pTyr with linear kinetics when the preincubation period at 30 °C is 30 min or less. The duration of the lag is clearly related to the time of preincubation. Similar results are seen, with appropriately different time scales, for the experiments performed at other temperatures (data not shown). After removal of the lag period at 30 °C, no decrease in reaction rate of M1 RNA is observed when the material is reassayed after a 30-min cooling period at 0 °C.

We assumed that a single process, such as a change in conformation from one stable state to another in which the forward rate constant is much greater than the backward rate constant, was controlling the lag period and that the preincubation time needed to eliminate the lag at a specific temperature was a valid thermodynamic parameter of the process at that temperature. Thus, a graph of the log of the time needed to eliminate the lag period (defined as the minimum time of preincubation needed to yield linear kinetics of cleavage of substrate as measured from the instant of addition of substrate to the reaction mixture) at various temperatures plotted against the inverse of the absolute temperature should yield an apparent activation energy for the type of process we describe. Such a plot is shown in Figure 2. It is linear and yields a value for $\Delta E^{\ddagger}$ of about 36 kcal/mol. We also plotted the initial velocity of the reaction (after elimination of the lag period) at various temperatures to give the activation energy of the catalytic reaction. That result, derived from the data in Figure 2, yields a value of 7 kcal/mol. The two lines in Figure 2 have slopes of different signs because we defined time, rather than its inverse, as the critical parameter in the first experiment.

In separate experiments we found that varying the magnesium ion concentration between 10 and 100 mM has no effect on elimination of the lag period, nor does the inclusion of poly(ethylene glycol) or C5 protein, the protein cofactor of RNase P, in the preincubation buffer. Furthermore, for any given temperature, if the M1 RNA sample is refrozen and thawed for reuse, it appears that about 20% of the material is in the inactive conformation once again.

Effects of pH on Elimination of the Lag Period. We studied the elimination of the lag period at 30 °C as a function of pH of the preincubation buffer [the cleavage reaction is insensitive to changes in pH between pH values of 5.5 and 9.0 (Guerrier-Takada & Altman, 1984)]. The lag was much more pronounced at pH values below 7.0 as compared to the lag observed at pH 7.5 or greater, as shown in Figure 3A. In this

[1] Abbreviations: pTyr, precursor to $tRNA_1^{Tyr}$ from *Escherichia coli*; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane.

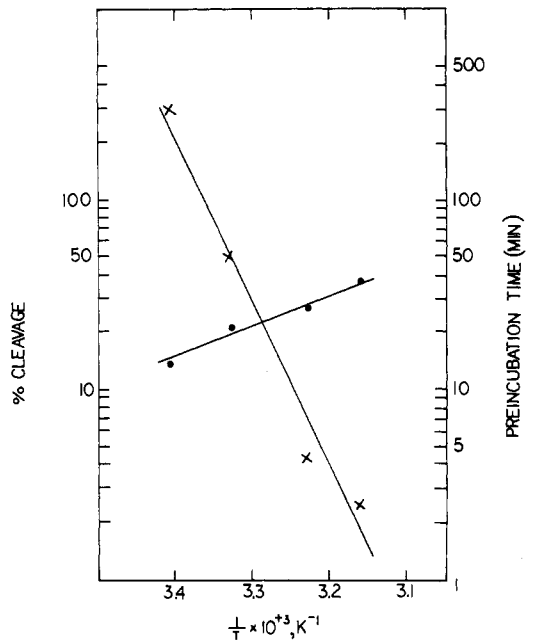

FIGURE 2: Energies of activation of reactions involving M1 RNA. (×) refers to numbers plotted on the right-hand ordinate and (●) to numbers plotted on the left-hand ordinate. The right-hand ordinate marks the log of the preincubation time required for elimination of the lag period at various temperatures. The inverse of the absolute temperature is plotted on the abscissa. The left-hand ordinate marks the initial velocity of the reaction (shown as the percent cleavage of substrate by M1 RNA, 6 min after addition of substrate to the reaction mixtures) in experiments carried out under conditions designed to eliminate the lag period completely by an appropriate preincubation time at each temperature sampled.

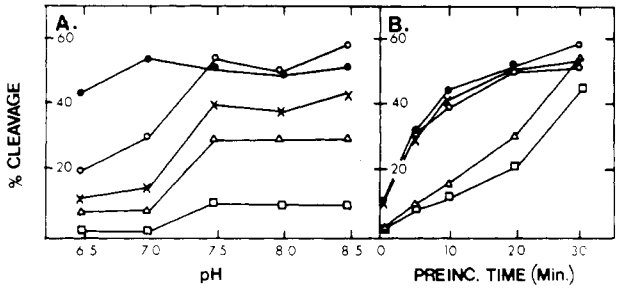

FIGURE 3: Effect of pH on removal of lag period by preincubation. M1 RNA was preincubated for various times at 30 °C and then, after addition of substrate, assayed for activity in the linear portion of the curve describing the kinetics of cleavage. The experiment was repeated with pH values of the preincubation and reaction buffer indicated in the figure. Panel A: Percent cleavage plotted against pH of preincubation buffer. Preincubation times: (□) 0 min; (△) 5 min; (×) 10 min; (○) 20 min; (●) 30 min. Panel B: Percent cleavage plotted against preincubation time for given values of pH of preincubation buffer: (□) pH 6.5; (△) 7.0; (×) pH 7.5; (○) pH 8.0; (●) pH 8.5.

figure the amount of product released at 4 min after various initial preincubation periods is plotted vs. pH of the preincubation mixture. As the preincubation time is lengthened and the lag time reduced, the dependence on pH of the length of time required to eliminate the lag period is less apparent. The data plotted in Figure 3B emphasize the effect of changing pH from 7.0, or below, to 7.5 on the removal of the lag. A similar structural transition, dependent on pH has been identified in 5S RNA by Kao and Crothers (1980).

The Lag Period and Structural Changes in M1 RNA. We examined the sensitivity to nuclease digestion of M1 RNA in solution to determine if a major structural change occurred upon removal of the lag period. The pattern of digestion by RNase T_1 (Guerrier-Takada & Altman, 1984) is very similar for M1 RNA in both configurations, indicating that no gross structural rearrangement is occurring (data not shown). However, some differences in secondary cleavage sites were observed. Apparently the general characteristics of the structure of M1 RNA in solution remain unchanged during elimination of the lag period.

We studied the structure of M1 RNA further by observing the kinetics of change in A_{260} under the conditions described for preincubation at 30 °C. When M1 RNA is suspended in buffer P and warmed to 30 °C, there is an immediate increase in A_{260} of about 10% and then a gradual decrease of about half that amount. We attribute the initial increase in A_{260} to unfolding of the molecule as it equilibrates from 0 to 30 °C. The subsequent decrease in absorbance, which has a time constant for exponential decay of about 5 min, may be the result of a single process or the net result of several processes. We note that the time constant governing the decrease in absorbance is roughly equal to that governing the rate of change of substrate cleavage at 30 °C in the absence of preincubation.

Discussion

We have shown that the lag period in the kinetics of the reaction of M1 RNA with its substrate can be analyzed as a simple kinetic process with $\Delta E^{\ddagger} \sim 36$ kcal/mol. We showed previously that the lag period can be eliminated by heating or by passage of M1 RNA through a denaturing solution, so we presume that the activation energy of the reaction could represent the *net* breakage of about 10 hydrogen bonds. During the purification of M1 RNA from cell extracts, which includes extraction by phenol, a denaturant, and ethanol precipitation, the RNA loses and apparently cannot regain the active conformation (or aggregate) during the subsequent steps in the purification procedure. Since the kinetics and thermodynamics of the necessary conformational change can be studied separately from the catalytic event, these processes can be described by the equations

$$\text{M1 RNA}^0 \overset{\Delta}{\rightleftharpoons} \text{M1 RNA} \qquad (1)$$

$$\text{M1 RNA} + \text{ptRNA} \rightleftharpoons [\text{M1 RNA*–ptRNA}] \rightarrow \text{M1 RNA} + \text{products} \qquad (2)$$

where M1 RNA^0 represents the inactive conformation, M1 RNA the potentially active state, and M1 RNA* the enzyme in the catalytically active, enzyme–substrate complex.

The structural transition in M1 RNA that occurs during elimination of the lag period has an abrupt change in pH dependence between 7.0 and 7.5. Nucleotides in solution do not have ionizable groups in this range. However, Kao and Crothers (1980) showed that 5S RNA also exhibits a conformational change that is dependent on pH with a similar abrupt discontinuity in the same pH range. They suggested that a tertiary hydrogen-bonding interaction may alter the ionization constant of a nucleotide in a cavity formed by the complex structure of an RNA in solution. Pulleyblank et al. (1985) have also postulated the existence of a protonated nucleotide, at a pH higher than that expected for a free nucleotide in solution, as an agent in the formation of nonstandard nucleic acid structures. It is well-known that the ionization constants of some free substituted groups in solution can be changed considerably when they are buried in cavities in certain protein enzyme molecules (Cotton et al., 1979; Fersht, 1977).

Differences in the pattern of nuclease digestion of M1 RNA before and after removal of the lag period are too subtle, and M1 RNA is too large (377 nucleotides) to allow a clear in-

terpretation of the structural changes that occur during the structural transition. This transition may be a prelude to formation of M1 RNA dimers, needed for catalytic activity of the enzyme (Guerrier-Takada et al., 1986), or may involve a rearrangement of parts of M1 RNA directly involved in the active site.

Further work is required to clarify the details of the conformational changes undergone by M1 RNA to generate the catalytically active species. However, our results underline the importance of RNA conformation as a means of regulating function and also highlight the capability of RNA molecules to exhibit unexpected ionization properties that are due, presumably, to the details of the folding in solution of these molecules.

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Articles

DNA Synthesis in BalB/C-3T3 *ts* 2 Cells Is Restricted by a Temperature-Sensitive Function of Late G_1 Phase†

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ABSTRACT: *ts* 2 BalB/C-3T3 mouse fibroblasts are *cdc* mutants, which arrest late in G_1, at or near the G_1/S traverse, upon full expression of the heat-sensitive lesion. The kinetics of temperature inhibition of DNA synthesis in logarithmically growing cultures reveal three stages of heat inactivation. During the first generation time equivalent, normal semiconservative, semidiscontinuous replication proceeds but is reduced as cells exit and do not reenter S phase. During a second such period, a minimal rate of normal DNA synthesis is maintained. Thereafter, as the cells move into a third aborted cell division cycle, the rate of DNA synthesis increases. However, all semiconservative synthesis is then replaced by DNA repair replication. Temperature inactivation of the *ts* 2 protein results in shutdown of nuclear DNA synthesis. In contrast, normal replication of mitochondrial DNA proceeds at control rate throughout the first stage of temperature inactivation. Synthesis of this organellar genome is quantitatively reduced as the cells move into the second phase of heat inhibition. Titration of chromatin-bound DNA with ethidium bromide revealed that wild-type cells exhibit a changing DNA topology as the temperature is raised. Temperature-inactivated *ts* 2 cells behave as though their DNA has been topologically frozen in the configuration of control cells at or near entry into S phase.

Progression of mammalian cells into DNA synthesis is dependent upon one or more biochemical events that permit or induce traverse of the G_1/S interface of the cell duplication cycle [cf. Pardee et al. (1978) and Sheinin et al. (1978b)]. Little is yet known about the nature of these processes. As one approach to this problem, we have been studying a number of mutant *dna*^{ts} mammalian cells, i.e., cells that are temperature-sensitive (*ts*) in DNA synthesis [cf. Sheinin (1980, 1984)]. These cells are higher eukaryotic analogues of what have been designated as cell division cycle, or *cdc*, mutants of yeast cells [cf. Pringle & Hartwell (1981) and Dickinson (1984)].

The present study is concerned with BalB/C-3T3 *ts* 2 mouse fibroblasts, isolated by Slater and Ozer (1976). We have now demonstrated that temperature inactivation of the *ts* 2 protein brings cells to arrest very late in G_1, at or near the G_1/S traverse (Sheinin et al., 1985b). The cells are therefore *dna*^{ts}/G_1^{ts}. Expression of the *ts* 2 defect results in inactivation

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