TEMPERATURE-SENSITIVE RNA POLYMERASE OF A MAMMALIAN CELL MUTANT

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SUMMARY

In our attempts to characterize a temperature-sensitive mutant (ts-2) of monkey kidney cell line BSC-i, we have provided evidence to show that the mutant was defective in the synthesis or processing of rRNA (45, 28, and 18 s) molecules at 39.5 °C and there was indication that the nucleolar RNA polymerase (I) or some factor associated with its functional activity was thermolabile at the restricted temperature of 39.5 °C. The enzyme in the wild type cell line was, however, stable at this temperature. Though conclusive evidence to implicate RNA polymerase (I) was not obtained in these experiments, temperature-sensitivity tests with crude enzyme extracts of the RNA polymerase in ts-2 showed that it contained a temperature-sensitive factor which was either degraded rapidly or failed to be eluted out of the DEAE-Sephadex column. This temperature-sensitive factor was not affected by α-amanitin.

INTRODUCTION

We have already reported (Naha, 1970, 1971) partial characterization of a temperature-sensitive mutant (ts-2) of an African green monkey kidney cell line (BSC-i) which did not support growth of Simian virus (SV 40) at the restricted temperature of 39.5 °C; the parent cell line was, however, amenable to SV 40 at both 33 °C and 39.5 °C. Characterization of this mutant cell line has been continued in the hope that it may shed light on the early events of host/virus interactions during the growth of a tumour virus. The mutant cell line was defective in [3H]thymidine incorporation at the restricted temperature (39.5 °C), but there was a continuous uptake of precursors labelled with [3H]uridine under the same conditions (Naha, 1970). Autoradiographic studies suggested the site of the defect to be in the nucleolus (Naha, 1971). There was very little incorporation of [3H]thymidine into the nucleolus at the restricted temperature, compared to the parental cell line, whereas [3H]uridine-labelled precursors of RNA appeared mostly in the nucleus and relatively little in the cytoplasm under the same conditions. It was suggested that at the restricted temperature most of the [3H]uridine-labelled material failed to reach the cytoplasm, possibly due to the defective utilization or turnover of uridine-labelled precursors.

Additional evidence of nucleolar defect in the mutant cell line at the restricted temperature was obtained from phase-microscopic studies (Naha, 1971); the optical density of the nucleoli of the cells grown at 39.5 °C was significantly lower than of those at the permissive temperature (33 °C). We therefore studied the fate of the [3H]-uridine-labelled precursors with regard to ribosomal RNA (rRNA) synthesis in the mutant and the wild type cell lines, and the rate of incorporation of uridine triphosphate (UTP) by RNA polymerase extracts.
MATERIALS AND METHODS

Cell cultures

The parental cell line (BSC-1) and the mutant ts-2 were maintained in culture in L-15 medium, supplemented with 10% foetal calf serum at 33 °C as reported previously (Naha, 1970).

Extraction of cellular RNA

Monolayer cell cultures were trypsinized (Naha, 1970) and seeded in 60-ml (20-oz.) bottles at a density of 1-3 x 10⁶ per ml in L-15 medium and incubated overnight (18 h) at 39.5 °C, the temperature non-permissive for the mutant cell line. Cells were then exposed to [G-3H]-uridine at 50 µCi/ml for 1 h at 39.5 °C ('pulse'). Chase experiments were performed with replicate culture bottles by replacing radioactive with non-radioactive medium and incubating for a further 2 h at 33 °C. Cells were then trypsinized, chilled at -2 °C, and washed with phosphate-buffered saline (PBS). Total cellular RNA was extracted with hot phenol (Scherrer & Darnell, 1962) by the modified method of Penman (Penman, 1966; Wagner, Katz & Penman, 1967), and spun in 5-20% sucrose gradient for 16 h at 20000 rev/min in an SW 27 rotor at a temperature of 5 °C.

Isolation and fractionation of DNA-dependent RNA polymerases

The following adaptation of the method of Roeder & Rutter (1969) was used. Nuclei were suspended in 10 M sucrose, 25 mM MgCl₂, 5 mM Dithiothreitol (DTT), 50 mM Tris-HCl, pH 7.4. To this suspension 0.08 ml per ml of 4.0 M (NH₄)₂SO₄ (adjusted to pH 7.9 with ammonia) was added with vigorous mixing. The viscous mixture was permitted to stand in ice for 10 min, then sonicated with 10-s bursts from a Branson sonicator with a power setting of 3, separated by 30-s cooling periods, for a total sonication time of 60 s. The suspension was immediately diluted with 2 vol. of 0.05 M Tris-HCl, pH 7.9; 5 mM MgCl₂; 0.1 mM EDTA; 0.5 mM DTT and 25% (w/v) glycerol. The resulting opalescent suspension was centrifuged at 105000 g (max) for 1 h.

The clear faint yellow suspension was carefully aspirated and 0.42 g of (NH₄)₂SO₄ per ml of solution was added to it during 30 min with constant stirring at 4 °C. Following addition of (NH₄)₂SO₄ stirring was continued for an additional 30 min. The milky suspension was centrifuged at 105000 g (max) for 90 min, the tubes were drained, and the grey-tan pellet suspended in a minimal amount of 50 mM Tris-HCl (pH 7.9), 50 mM MgCl₂, 0.5 mM DTT, 0.1 mM EDTA, 25% glycerol. This suspension was dialysed against 1000 vol. of the same buffer containing 0.05 M (NH₄)₂SO₄ for 12 h. The dialysate was centrifuged at 105000 g for 60 min and the pellet discarded. The supernatant containing the enzyme (fraction 4) was immediately applied to a DEAE-Sephadex A-25 column (0.9 x 30 cm), washed with 10 ml of 0.05 M (NH₄)₂SO₄ in the above Tris-glycerol buffer, and the enzymes eluted with a linear gradient of 0-1 to 0.5 M (NH₄)₂SO₄ in the same buffer; 0.7-ml fractions were collected.

The peaks of enzyme activity were pooled and either frozen immediately in liquid nitrogen or dialysed briefly against 0.05 M Tris-HCl, pH 7.9, 0.5 mM MgCl₂, 0.1 mM DTT, 25% glycerol and then frozen. In addition, conductivity and protein content were determined on the column fractions.

Preparation of DEAE-Sephadex

The resin was swollen for 72 h in distilled water, mixed gently with 0.5 M NH₄OH for 30 min, washed with water until the pH of the eluent was 8.0, then suspended in 0.5 M H₂SO₄ for 30 min and washed with water to pH 7.0. The gel was then washed twice with 3 vol. of 0.5 M (NH₄)₂SO₄, three times with 0.05 M (NH₄)₂SO₄ and twice with 0.05 M (NH₄)₂SO₄ in Tris-HCl, pH 7.8, 5 mM MgCl₂, 0.1 mM Na₂EDTA, 0.5 mM DTT and 25% glycerol. The column was washed with 50 bed volumes of the last (NH₄)₂SO₄ solution prior to use.
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Nuclear RNA polymerase assay

Nuclei (Freedman, 1965) were suspended in 50 mM Tris-HCl, pH 7.9, 25 mM KCl and the protein content of the suspension adjusted to 1 mg/ml (Lowry, Rosebrough, Farr & Randall, 1951). The incubation mixture contained: 0.25 mg nuclear protein per ml; 0.05 mM MgCl₂; 0.10 mM (NH₄)₂SO₄; 0.8 mM ATP, GTP and CTP; 7 μM [³H]uridine triphosphate (³H-UTP) (specific activity 1 mCi/μmol); 50 mM KCl; 25 mM β-mercaptoethanol; 75 mM Tris-HCl, pH 7.9 (37 °C), and 5% glycerol, in a total volume of 0.08 ml. Incorporation was carried out for 20 min at 37 °C, 0.05-ml samples were pipetted on to Whatman no. 1, 2.4-cm disks and immediately dropped into ice-cold 10% trichloroacetic acid (TCA) containing 5% (w/v) sodium pyrophosphate. After 20 min the disks were washed 3 times in 5% TCA plus 5% sodium pyrophosphate followed by 95% ethanol, absolute ethanol, ethanol:ether (1:1), and ether, then dried. The dried disks were placed in 10 ml toluene containing 0.1% 2,5-diphenyloxazole and 0.05% 1,4-bis-4-methyl-5-phenyloxazolyl 1-benzene. Radioactivity was measured in a Beckman L-200 liquid scintillation spectrophotometer with an efficiency for ³H in this disk system of about 7%.

DNA-dependent RNA polymerase assay

The activity of the enzymes from the column eluent, and of the pooled enzymes, was measured in an 0.08-ml incubation mixture containing: 0.8 mM ATP, GTP and CTP; 7 μM ³H-UTP (specific activity 1 mCi/μmol); 77 mM KCl; 0.02 mM Na₂ EDTA; 0.14 mM DTT; 2 mM MnCl₂; 0.04 M (NH₄)₂SO₄; 75 mM Tris-HCl, pH 8.3 (37 °C); glycerol 7%; and denatured calf thymus DNA, 0.1 mg DNA/ml. The optimum salt concentration was similar to that described previously (Roeder & Rutter, 1969); each enzyme was assayed at the ionic strength for optimum incorporation. Incubation was carried out at 37 °C for 30 min and an 0.05-ml sample was pipetted on to a Whatman no. 1 disk and processed as described above.

Reagents

Analytical reagents were used throughout. ATP, GTP and CTP as the respective Na salts and Dithiotreitol were purchased from Sigma Chem. Co., ³H-UTP as the ammonium salt was a product of the Radiochemical Centre, Amersham, Bucks, England, and had a specific activity of 1 mCi/μmol. α-Amanitin was kindly supplied by Professor T. Wieland of Heidelberg, Germany.

RESULTS

Synthesis of rRNA molecules

The sedimentation profiles of 45, 28 and 18 s rRNA radioactive molecules in total cellular RNA of the mutant (ts-2) and parent lines suggested (Fig. 1) that the mutant was blocked in the synthesis of large rRNA molecules (45, 28 and 18 s) at the restricted (39.5 °C) temperature; the parental cell line, however, formed significant amounts of these larger rRNA molecules during 1 h of incubation at 39.5 °C. Whether structurally similar 4-5 s molecules were synthesized in the mutant under these conditions could not be determined from these experiments, because the sedimentation profiles of these peaks were identical for the mutant and the parental lines. The effect of the mutation appeared more dramatically in chase experiments when replicate cultures were transferred to non-radioactive growth medium at the permissive temperature of 33 °C. During 2 h of incubation at 33 °C, 28, 18 and 4-5 s rRNA molecules were synthesized in the mutant cell line; the amounts of ³H]uridine incorporated were twice those of the parental cell line, as indicated by the radioactivity profiles. It appeared
that after the temperature shift from 39.5 to 33 °C new rRNA was made from pre-
cursor materials already synthesized in large amounts by cells before the temperature
shift. The defect responsible might be either in (i) phosphorylation of the nucleotides
or (ii) nucleolar RNA polymerase (I) activity. Preliminary results indicated that
nucleotide triphosphates, diphosphates and monophosphates were synthesized by the
mutant cell line at the restricted temperature before the temperature shift. We then
looked for temperature sensitivity in the RNA polymerases of the mutant cell line.

It should be mentioned here that shorter pulses (10–30 min) of the radioactive
labelling failed to show the presence of high-molecular-weight rRNA in the mutant
cell line; the profile looked more or less identical to that for 1-h labelling at the restricted
temperature. This implies that the small amount of rRNA present after 1 h incubation
at 39.5 °C represented molecules in the process of being synthesized when the cells
were seeded, or it might be attributed to 'leakiness' of the mutant cell line.

**Fractionation of RNA polymerase**

Two difficulties were encountered during the isolation and fractionation of RNA
polymerases of the mutant cell line: (i) poor recovery of enzymes from the gross
amount of cells used, and (ii) instability of the nucleolar polymerase (I) fraction. In
the case of parental cell line, however, both recovery and stability were greater. Never-
theless, preliminary results provided good indication (Fig. 2) that there were probably
3 different species of RNA polymerase present in the monkey kidney cell line, with
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Fig. 2. DEAE-Sephadex column chromatography of RNA polymerase from ts-2 mutant cell line. Of the material collected at arrowed peaks the first was insensitive, the second sensitive to α-amanitin. Details in text.

similar mobilities to those reported by Roeder & Rutter (1969) for sea-urchin embryos. The first peak contained such small amounts of material that no further tests could be performed on it. The second and the third peaks were nucleolar polymerase (I) and nucleoplasmic polymerase (II), as evidenced by their insensitivity and sensitivity, respectively, to α-amanitin (Kedinger et al. 1970).

Temperature-sensitivity tests on the 2 RNA polymerase species (I and II) from ts-2, isolated from the DEAE-Sephadex columns, gave unexpected results. Although the activity of the crude extracts was 50–60 % lower at 39 °C than at 33 °C (see following section), this was not the case with either of the separated enzyme peaks. Even if the first peak was temperature-sensitive it was not large enough to account for 50–60 % of the activity. Possible explanations are that a temperature-sensitive subunit (or a factor like sigma) was not eluted out of the Sephadex column or that it was degraded during the process of extraction. An attempt is now being made to improve on the stability in the extraction procedure. Another possibility could be that temperature sensitivity affected one of the subspecies of polymerase 1a and 1b (Chesterton & Butterworth, 1971), if such distinct species does exist in BSC-1 cell lines.

Temperature sensitivity in the crude enzyme extracts (fraction 4)

Fraction 4 constituted the centrifugal dialysate before being applied to the Sephadex column. The crude enzyme extracts from the parental and mutant cell lines, tested for the rate of 3H-UTP incorporation (Fig. 3) in absence of α-amanitin, indicated that incorporation by the wild type enzyme was not affected by temperature between 33 and 39 °C (optimal efficiency at 37 °C), whereas the mutant enzyme functioned about 50–60 % less efficiently at 39 °C, and 30–35 % less efficiently at 37 °C, when compared to the permissive temperature 33 °C, at the end of 20 min of incubation.

In the presence of α-amanitin (0.05 µg/ml) the same amount (0.05 ml) of wild type crude enzyme extract had undergone about 40–60 % reduction in efficiency in
Fig. 3. Effects of temperature and presence or absence of α-amanitin on the rate of $^3$H-UTP incorporation by crude enzyme extracts (fraction 4) of RNA polymerases of the wild type (BSC-1) and the mutant (ts-2) cell lines. A, without amanitin; B, with amanitin, 0.05 μg/ml. ——, wild type; ——, ts-2. ▲, ×, and ●, 33, 37 and 39 °C, respectively.

$^3$H-UTP incorporation, but the remaining activity did not show any significant difference over the temperature range. In the case of the mutant enzyme, on the other hand, the effect of temperature was significant; there was a reduction of 70-80% at 37 and 39 °C, after α-amanitin treatment, compared to the rate of incorporation at 33 °C. This indicated that a temperature-sensitive fraction did exist in the crude enzyme fraction 4. Experiments are now in progress to minimize the time required for the entire extraction procedure, so as to avoid any possible denaturation during storage where ordinarily maximum loss of activity has been observed.

DISCUSSION

Regulatory mechanisms of mammalian cells are poorly understood. In order to explore possibilities of studying such mechanisms temperature-sensitive mutants of mammalian cells were isolated. This is probably the first time an attempt has been made to characterize biochemically conditional lethal mutants in mammalian cells. In the mutant described in this paper, it appeared that a mutation in the nucleolus (chromatin) blocked the synthesis of rRNA molecules at the restricted temperature, but, once the temperature-sensitive block was removed by shifting culture bottles to a permissive temperature, rRNA synthesis proceeded normally. Since rRNA was synthesized and processed in the nucleolus, the nucleolar RNA polymerase (I) was thought to be implicated. Though direct evidence for this is awaited, there is enough circumstantial evidence to suggest that a factor present in the crude enzyme extracts of the mutant cell line was temperature sensitive at 39.5 °C and that in the presence of α-amanitin, temperature sensitivity increased from 50 to 80% against the controls.
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at 33 °C. Better recovery of the mutant enzyme and increased stability of the enzyme fractions are now being attempted.

The functions of the different species of RNA polymerases are not yet clearly understood. In this case, it would be interesting to know the sequential effects of inhibition of RNA polymerase (I) and rRNA at the restricted temperature on the cellular regulatory processes of the mutant cell line. Preliminary results (manuscript in preparation) indicated that ribonucleoproteins, histones, and possibly messenger RNA were not synthesized.

A preliminary report of this work was presented at the First International Conference on Cell Differentiation held at Nice in September 1971.

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REFERENCES


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