Ribonuclease-sensitive Endogenous DNA Polymerase Activity and DNA-directed DNA Polymerase in Human Tissue Culture Cell Lines

B. I. Sahai Srivastava and J. Minowada

Roswell Park Memorial Institute, Buffalo, New York 14203

SUMMARY

The 37,000 X g supernatant ("soluble") activity, the chromatin-associated DNA-directed DNA polymerase activity, and the soluble RNase-sensitive endogenous DNA polymerase activity were examined in two fibroblast cell lines (WI-38 and WI-38VA13), in thirteen lymphoid cell lines originated from normal persons or from acute lymphocytic leukemia, acute myelocytic leukemia, Burkitt lymphoma, or multiple myeloma patients, and also in cells obtained directly from patients with acute lymphocytic leukemia, acute myelocytic leukemia, multiple myeloma, or lymphosarcoma. RNase-sensitive endogenous DNA polymerase activity, which was 1/1000 to 1/10 of DNA-directed soluble DNA polymerase activity, was lowest in WI-38 cells and highest in lymphoblasts from acute lymphocytic leukemia patients. The soluble and chromatin-associated DNA-directed DNA polymerase activity of WI-38V13 cells, compared to that of WI-38 cells, and the activity of acute myelocytic leukemia, Burkitt lymphoma, and multiple myeloma cells, compared to that of normal cells, were two to ten times higher; in contrast, the activity of cells of acute lymphocytic leukemia or lymphosarcoma origin was one-third to one-half that of normal cells.

INTRODUCTION

Since the initial observations of Temin and Mizutani (31) and Baltimore (2) demonstrating the presence of RDDP in certain RNA tumor viruses, this enzyme has been found in all known oncornaviruses (21, 25), as well as in human acute leukemic lymphoblasts (10) but not in normal human lymphoid cells. Uninfected rat cells (5) and normal chicken embryos (12) contain similar endogenous RDDP activities. Since the viral enzyme could also use synthetic double-stranded RNA's and hybrids of RNA and DNA to synthesize DNA (25), several laboratories (15, 17-19, 23, 29) using these templates or natural RNA of uncertain purity (18) reported RDDP activity in a variety of cells, including normal mouse and human cells (19, 23). However, it is now apparent that the synthetic nucleic acids were not specific templates for RDDP and could not be used for demonstrating the presence of this enzyme (11, 20). The sensitivity of the reaction to RNase, its requirement for all 4-deoxyribonucleoside triphosphates, and preferably, the association of the DNA product with the RNA template should be demonstrated to show the presence of RDDP (5, 7, 12, 22). In the present study we have used these criteria for the detection of RDDP in several normal and malignant human cell lines, as well as in cells obtained directly from the patients. In addition, we have also examined soluble and chromatin-associated DDDP activity in these cells.

MATERIALS AND METHODS

One normal (RPMI 1788) and 2 malignant (RPMI 8226 and HR1K) cell lines in culture were examined at different times after change of medium for optimum enzyme activity. RPMI 1788 was established from normal human peripheral blood lymphocytes, whereas cell line RPMI 8226 originated from the peripheral blood of a patient with multiple myeloma. Cell line HR1K was derived from a Burkitt lymphoma. At the start, the cells were grown in several flat 16-oz culture bottles in Roswell Park Memorial Institute 1640 medium containing 5% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (50 units/ml). The cultures were maintained at 37° with appropriate feeding until the desired amount of cells was obtained. The cells were harvested by centrifugation (10 min at 800 X g) and resuspended in fresh medium in 2.5-liter roller bottles (Belco Glass, Inc., Vineland, N. J.) to give 4 to 5 X 10⁶ cells/ml. The bottles were rotated (2 rpm) at 37° and aliquots were removed for the determination of viable cells (by trypan blue exclusion) and immunoglobulin-producing cells (by direct immunofluorescent staining with fluorescein-labeled goat anti-human IgM or λ light chain) and enzyme assay. On the 5th day after feeding, the cultures were fed with an equal volume of fresh medium, and the cells were removed 2 days later for study. On the basis of the above study, the cells of all 13 lymphoid lines used in subsequent studies were also harvested about 24 hr after the renewal of the medium. The diploid human fibroblast cells (WI-38), grown to confluency,
were supplied by Dr. L. Hayflick, and the SV40-transformed WI-38 cells (cell line WI-38VA13), obtained from Dr. Hayflick, were grown to confluency in enriched Eagle's minimum essential medium. The mouse leukemic cell line LS178Y, supplied by Dr. H. B. Bosmann was grown as described above. The cells from acute lymphocytic leukemia, AML, MM, and LS patients were supplied by Dr. T. Ohnuma. The white blood cells from these patients were obtained by differential centrifugation. The cultured cells were harvested by centrifugation. All cells were washed 6 times with phosphate-buffered saline (pH 7.2) before enzyme extraction.

The washed cells (approximately 0.3 g, wet weight) were homogenized manually by 30 strokes in 5 ml of Buffer A (25 mM Tris-sulfate, pH 8.3; 1 mM MgSO4; 6 mM NaCl; 4 mM dithiothreitol; and 0.1 mM EDTA) in a relatively loose-fitting glass homogenizer. Glycerol was added to the homogenate to a final volume of 20%, and the contents were centrifuged at 37,000 X g for 20 min. The upper two-thirds of the 37,000 X g supernatant (soluble) was pipetted off, avoiding contamination with the chromatin-containing pellet, and used for the determination of calf thymus DDDP and RSEDP activity and protein. For the determination of the DDDP activity, various concentrations (in linear range) of the enzyme preparation (in 0.1 ml) were mixed with 20 μg (0.05 ml) of native or heat-denatured calf thymus DNA (1) and 0.15 ml of “incubation mixture” [containing (per ml) 100 μmoles Tris-HCl buffer, pH 8.3; 12 μmoles magnesium acetate; 40 μmoles dithiothreitol; 120 μmoles NaCl; 1.6 μmoles each of dATP, dCTP, and dGTP; 20 μCi TTP-methyl-3H (specific radioactivity, 7.7 mCi/μmole)]. After 1 hr of incubation at 37° (the time during which the reaction was linear), 100 μg of yeast RNA and 0.5 ml of 20% TCA were added. The precipitate was collected on presoaked (overnight in saturated sodium pyrophosphate solution) B3 membrane filters, washed with 5% TCA, dried, and counted with the use of toluene-based scintillation fluid (27).

The soluble enzyme preparations as obtained above were free of DNA; this was indicated by the lack of reaction by the diphenylamine test (4) and by the fact that all the endogenous DP reaction was sensitive to RNase. Thus, this preparation was suitable for RSEDP activity determinations. For these determinations, parallel samples of the enzyme preparation (0.1 ml) at 2 different concentrations were preincubated (37° for 10 min) either with 0.05 ml of 0.2 M NaCl or with boiled (100° for 10 min) pancreatic RNase A (10 μg in 0.2 M NaCl; Worthington Biochemical Corp. Freehold, N. J.) before incubating each with 0.15 ml of incubation mixture for 30 to 60 min. The product was precipitated and counted as indicated above. The enzyme preparation was precipitated with TCA (5%). After the pellet was washed with ethanol and ethanol:ether (1:1), it was solubilized in 0.3 N NaOH, and the protein was estimated by the procedure of Lowry et al. (14).

The crude chromatin pellet obtained as described above was repeatedly washed by gentle homogenization in 0.05 M Tris-HCl (pH 8.0) and 0.01 M β-mercaptoethanol and centrifuged (2,000 X g, 10 min). The chromatin was further purified by ultracentrifugation at 70,000 X g for 2 hr through 1.7 M sucrose (26). The pellet obtained by ultracentrifugation was washed by suspension in 0.05 M Tris-HCl (pH 8.0), and 0.01 M β-mercaptoethanol and centrifugation at 37,000 X g for 30 min. The washed chromatin pellet was dispersed in 0.01 M Tris-HCl (pH 8.0), and aliquots (0.1 ml) were used the same day for DDDP activity (without and with added calf thymus DNA) determinations as indicated above for soluble enzyme. This preparation was also used for DNA determinations (26).

RESULTS AND DISCUSSION

The data in Charts 1 and 2 illustrate the effect of the addition of culture medium on the systems studied. For 4 days after medium renewal, cell density (Chart IA) continued to increase with little change in cell viability (Chart IB). Furthermore, the level of immunoglobulin synthesis (Chart 1C) was also higher when the cells were in the exponential growth phase.

RSEDP activity (Chart 2A) did not change significantly in cell line RPMI 1788. In cell lines RPMI 8226 and HR1K, a somewhat greater variation in activity was noted, including a decline after refeeding on the 5th day.

The denatured (Chart 2B) calf thymus DDDP activity in the soluble fraction of cell line RPMI 1788 showed only insignificant changes during the course of the experiment. However, in cell lines RPMI 8226 and HR1K this activity declined on the 4th day after medium renewal but was high again 2 days after refeeding with fresh medium.

The chromatin-associated DDDP activity (data not shown) measured with and without added native calf thymus DNA showed only insignificant variation in cell lines RPMI 1788, RPMI 8226, and HR1K.

In spite of some individual variations, all these cell lines showed high DP activities when harvested about 24 hr after renewal of the medium, and thus cells incubated for this period of time were used in these studies.

The data in Table 1 show that all the endogenous DP activity was sensitive to boiled RNase; thus, no DDDP activity was observed unless DNA was added exogenously. The elimination of dATP, dCTP, and dGTP from the reaction mixture almost completely eliminated the TMP-3H incorporation, indicating that the reaction was not due to a terminal deoxynucleotidyl transferase enzyme. The data in Table 1 further show the lack of inhibition of the DP reaction by lysozyme and the sensitivity of the reaction products to DNase.
but not to boiled pancreatic RNase A. To see whether the RNase sensitivity of the endogenous DP reaction resulted from destruction of template RNA or a small RNA primer, the product of this reaction was examined. In 2 normal cell lines, examination of this product by Cs₂SO₄ equilibrium gradient analysis showed (Chart 3, A and B) the presence of a small peak (Fraction 8) coincident with the visible precipitate band of RNA in the gradient; this peak may consist of small pieces of RNA.
Chart 3. Cs$_2$SO$_4$ (A, B) and CsCl (C, D) equilibrium gradient centrifugation of RSEDP reaction products. CsCl (3.5 g) or Cs$_2$SO$_4$ (2.4 g) was dissolved in 3 ml of solution (containing products) in 5-ml nitrocellulose tubes. The tubes were then topped with 1 ml of paraffin oil and spun in a SW-50L rotor (40,000 rpm, 42 hr for CsCl; 32,000 rpm, 66 hr for Cs$_2$SO$_4$) at 20°. After centrifugation, 6 (CsCl)- or 10 (Cs$_2$SO$_4$)-drop fractions were collected by bottom puncture of the tubes. Carrier RNA (100 µg) was added to each tube and the TCA-precipitable radioactivity was collected on B$_6$ membrane filters and counted in a scintillation counter. Heated sample was heated for 10 min in a boiling water bath and then chilled. For alkali treatment, the sample was made 0.3 M with respect to NaOH, placed in a boiling water bath for 5 min, chilled, and neutralized with HCl. Densities of 1.694 and 1.71 in CsCl gradients refer to the position of marker tobacco callus and Escherichia coli native DNA, respectively. The densities of 1.44, 1.46, and 1.67 in Cs$_2$SO$_4$ gradient were calculated from refractive index readings.

of DNA attached to rather long RNA chains (22). If the reaction products were treated with alkali before centrifugation, this small peak was no longer observed (Chart 3A), indicating that the freed material now sedimented with the DNA. CsCl equilibrium gradient analysis of the product formed after 1 hr of incubation (Chart 3C) also showed a small peak (Fraction 6), which may represent an RNA-DNA hybrid. This small peak in CsCl gradient would, however, be of no significance if it were not for the presence of a hybrid peak in Cs$_2$SO$_4$ gradients as well. Most of the product formed during an incubation period of 10 to 120 min was, however, double-stranded DNA (Chart 3) with a sedimentation coefficient of 10 to 11 S in a 5 to 20% sucrose gradient (Chart 4). Thus, on the basis of the 3 criteria mentioned above, RDDP activity appears to be present in these cells. Additional work by one of us (28), the details of which are the subject of a separate report, has further shown the presence of 2 DNA polymerases in the soluble fraction from RPMI 1788, RPMI 8226, HR1K, and "fresh" acute lymphocytic leukemia cells. These polymerases were eluted from the DEAE-cellulose column at the same position from each of these cells and could use DNA-free human tRNA for the synthesis of DNA. Although the sensitivity of the endogenous reaction to RNase; its inhibition on elimination of dATP, dCTP, and dGTP from a

Chart 4. Sucrose density gradient centrifugation of the RSEDP reaction products. About 1 ml of solution containing products (obtained as given in the legend of Chart 3) was layered on 26 ml of a 5 to 20% linear sucrose gradient made in 0.01 M Tris-HCl, pH 7.6. The gradients were spun at 25,000 rpm for 16 hr in an SW25-1 rotor. Forty-drop fractions were collected by bottom puncture and the TCA-precipitable radioactivity of the fractions was determined as given in the legend of Chart 3.
the reaction mixtures and its insensitivity to lysozyme was tested for each cell type, it was not considered necessary to examine the product by Cs₂SO₄ gradient analysis in each case. As seen in Chart 5 (top), RSEDP activity was detected in each cell type examined. In general, the activity was lowest in fibroblast cells and highest in mouse leukemic and fresh acute lymphocytic leukemia cells. Several other cells of malignant origin had RSEDP activity higher than that of the normal cell lines, but in 1 normal cell line (RPMI 4170) there was also high activity. Although the RSEDP activity of the mouse leukemic cell line reported here is similar to the RDDP activity previously found in this cell line (3), the values in Chart 5 may be somewhat low, especially in cells (such as those from acute lymphocytic leukemia, AML, and LS patients) which have high endogenous RNase activity (B. I. S. Srivastava and J. Minowada, unpublished data). The presence of RSEDP activity in cells examined, including human cells of normal origin, can probably be explained on the basis of either the protovirus (30) or the oncogene (16) hypothesis. Recently, RSEDP activity has also been reported in uninfected rat cells (5), normal chicken embryos (12), acute lymphocytic leukemia cells, and phytohemagglutinin-stimulated normal human lymphocytes (8, 9).

The data in Chart 5 (bottom) further show that WI-38VA13 cells had severalfold higher soluble DDDP activity as compared to WI-38 cells. The soluble DDDP activity of cell lines of Burkitt lymphoma, AML, and MM origin was also higher compared to cell lines of normal lymphoid origin, whereas the other cells had activity in the same range as normal lymphoid cells.

Differences in the capacity of the chromatin from different cells to serve as template for its endogenous DDDP were observed (Chart 6). The chromatin studied here, like that from other sources (13, 24) was a poor template for endogenous DP. Even with the chromatin from HR1K cells, which showed relatively high activity, the addition of DNA more than doubled this activity. The high template activity of chromatin from HR1K cells in the DP reaction could not be attributed to the presence of DNase or protease in the chromatin, since these enzymes were not detected. The chromatin-associated DP activity (measured in the presence of added native calf thymus DNA) of WI-38VA13 cells was twice that of WI-38.
cells, whereas the Burkitt lymphoma cell lines (HR1K, NK9, and Raji) and MM cells (RPMI 8226) had values higher than those found in normal lymphoid cell lines (Chart 6).

This study demonstrates the presence of RSEDP activity in all cells examined, as well as the existence of quantitative differences in DDDP and RSEDP activity between cells of normal and malignant origin. This data should be evaluated, however, with regard to the possibility that some normal cell lines may have undergone changes in culture. For example, lymphoblastoid cells in culture established from normal human donors have been shown to exhibit several malignant characteristics (6, 32). On the other hand, other cell lines of malignant origin may not necessarily represent malignant cells.

ADDITIONAL
Further unpublished work of the authors with HR1K cells has shown the presence of particulate material that banded at the density of 1.16 to 1.18 g/ml in a sucrose gradient and had RSEDP activity.

REFERENCES
Ribonuclease-sensitive Endogenous DNA Polymerase Activity and DNA-directed DNA Polymerase in Human Tissue Culture Cell Lines

B. I. Sahai Srivastava and J. Minowada


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/32/11/2481

To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/32/11/2481.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.