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Characterization of DNA polymerase β mRNA: cell-cycle and growth response in cultured human cells

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ABSTRACT

DNA polymerase ß (ß-polymerase) is a housekeeping enzyme involved in DNA repair in vertebrate cells. We used a cDNA probe to study abundance of ß-polymerase mRNA in cultured human cells. The mRNA level in synchronized HeLa cells, representing different stages of the cell-cycle, varied only slightly. Contact inhibited fibroblasts AG-1522 contained the same level of mRNA as growing cells. The steady-state level of mRNA in fibroblasts is equivalent to 6 molecules per cell. The results indicate that the ß-polymerase transcript is "low abundance" and is neither cell-cycle nor growth phase responsive.

INTRODUCTION

Sequence comparison of ß-polymerases from human and rat indicates that the enzyme has been highly conserved (1), suggesting that it is essential for survival. ß-polymerase is found in all tissues, and the level of its enzymatic activity in a mammalian cell is relatively low and independent of cell growth and cell-cycle stage (2,3). These findings support the concept that ß-polymerase is a constitutively expressed "housekeeping" enzyme required for essential DNA metabolism events separate from replication of genomic DNA (2-5). DNA synthesis during both DNA repair and recombination are examples of two such DNA metabolism events, and the idea that ß-polymerase is involved in some types of DNA repair has been strongly supported by inhibitor studies (6-10).

ß-polymerase is a 39 KDa single-chain polypeptide (11-14) of 335 amino acids. In humans, ß-polymerase is specified by a single-copy gene spanning approximately 35 kb on the short arm of chromosome 8 (15). The gene yields a polyA$^+$ mRNA of about 1.4 kb (1,16). Information about steady-state levels of this mRNA is a

necessary initial step toward learning the overall regulation of the enzyme. In the present paper, we report the steady-state abundance of the ß-polymerase mRNA in cultured human cell systems representing different stages of the cell-cycle and different states of cellular proliferation. The results indicate that the level of ß-polymerase mRNA in dividing cells is <10 molecules per cell and is not responsive to changes in cell-cycle stage or to cell growth.

MATERIALS AND METHODS

Cell Culture and Synchronization.

HeLa cells (S3) at a concentration of 0.5 x 10^6 cells/ml were maintained in spinner culture at 37°C. Cells were fed every 48 hours with Joklik's modified Eagle's minimal essential medium plus 10% fetal calf serum and 1.25 µg/ml fungizone. Cell synchrony was achieved by a modification of the method described by Collins (17). Cells were maintained in 2 mM thymidine for 14 hr, then suspended in fresh medium, and allowed to grow in the absence of thymidine for 9 hr. Cells were exposed to a second 2 mM thymidine block for 14 hr, then released from the block, centrifuged, and resuspended in fresh medium. Under these conditions greater that 90% of the cells were at the G1S boundary, as judged by the subsequent movement of cells through the cell-cycle (17). Human fibroblasts AG-1522 were grown as described previously (18).

PolyA$^+$ RNA from Cultured Cells.

Total RNA from 10^7-10^8 cells was isolated by guanidinium thiocyanate extraction followed by pelleting RNA through a 5.7 M CsCl solution (19). PolyA$^+$ RNA was isolated from total RNA by double passage through oligo(dT)-cellulose (20). In addition, an alternative method was used. Partially purified polyA$^+$ RNA was obtained by single adsorption of total RNA to Hybond-mAP (Amersham) paper according to the protocol supplied by the manufacturer. From 1 mg total RNA loaded onto a two cm^2 piece of affinity paper, 25-35 µg polyA$^+$ RNA was obtained; this was concentrated by ethanol precipitation and used for gel electrophoresis or blot analysis.

Radioactive DNA Probes.

The cDNA probe for human β-polymerase mRNA has been described (1); cDNA probes of human β-actin and rat β-actin mRNA were obtained from L.H. Kedes, Stanford University and B. Paterson, NIH, respectively. The inserts were cut from plasmid DNA with EcoRI, isolated by electrophoresis, and nick-translated to specific activity 10^8 cpm/μg DNA with ^{32}P-labeled dNTP. ^{35}S-poly(dT) (10^6 dpm/μg) was prepared as described (21).

RNA Dot- or Slot-blots.

Samples (50 μl) containing increasing amounts of RNA were supplemented with 30 μl 20XSSC and 20 μl 37% formaldehyde, heated at 60°C for 15 min and applied to nitrocellulose BA85 (0.45 μM) membrane using a dot- or slot-blot apparatus (all from Schleicher and Schuell). Each membrane was washed once with 0.5 ml 20XSSC. Membranes were dried in air and heated at 80°C for 2 hr and then were hybridized as described below.

Northern Blot Analysis.

RNA denaturation, electrophoresis in 1.5% agarose/2.2 M formaldehyde/50% formamide gels, and electroblotting to Gene Screen membrane (New England Nuclear) were performed as described (22); hybridization was as described below.

Membrane Hybridization.

Membrane in a sealed bag (16) was prehybridized at 42°C for 4 hr and then hybridized with ^{32}P-labeled probe at the same temperature for 24-48 hr in the presence of 50% formamide (16). Membrane was washed twice in 2XSSC plus 0.17% SDS for 5 hr at room temperature, then twice in 2XSSC plus 1% SDS for 30 min at 65°C, and twice with 0.1XSSC, 0.1% SDS for 3 min at room temperature. Hybridization with ^{35}S-poly(dT) probe, 0.4 μg/ml, was as described (21). Membranes were exposed to Kodak XAR-5 film for 24-72 hr with intensifying screen at -70°C or room temperature.

RESULTS

In this study, we examined the quantity of β-polymerase mRNA in growing and quiescent fibroblasts and in HeLa cells synchronized at different stages of the cell-cycle. First, the size complexity of β-polymerase mRNA in several human cell lines

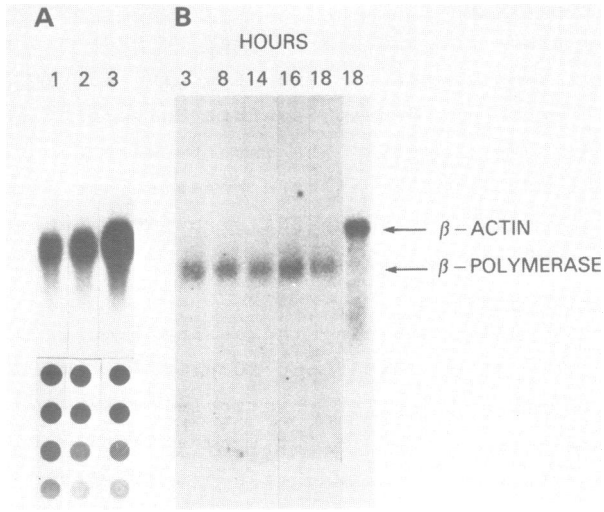

Fig. 1. Northern blot analysis of polyA$^+$ RNA from human cell lines and HeLa cells at different stages of the cell-cycle. Photographs of autoradiograms are shown. PolyA$^+$ RNA from each batch of cells was isolated, electrophoresed, electrotransferred to Gene Screen membrane and hybridized to a human ß-polymerase cDNA probe or a human ß-actin cDNA probe as described under Materials and Methods. In Panel A, 3 µg polyA$^+$ RNA was used from growing layer cultures of human fibroblast AG-1522 (lane 1), EBV-transformed human lymphocytes (lanes 2) or human teratocarcinoma NTera2D1 (lane 3). The photograph at the bottom shows dot-blot analysis of the polyA$^+$ RNAs using ^{35}S-polyd(T) as probe. In panel B, HeLa cells were released from block and collected after 3, 8, 14, 16 hr as described under Materials and Methods. Numbers at the top of lanes refer to hours after release from thymidine block.

was determined; this was evaluated prior to detailed analysis because two abundant size classes of ß-polymerase mRNA had been found in several rat tissues (16). However, as shown in Figure 1, only one size of transcript, ~1.4 kb, was found in the polyA$^+$ RNA from human cell lines, normal skin fibroblasts (AG-1522), teratocarcinoma (NTera2D1), lymphocytes transformed with EB virus, and HeLa cells (S3). Based upon these relatively simple hybridization patterns, we used dot- or slot-blot hybridization analysis to precisely quantify ß-polymerase mRNA.

Number of ß-polymerase mRNA Molecules Per Cell.

PolyA$^+$ RNA from fibroblasts AG-1522 and a standard of M13

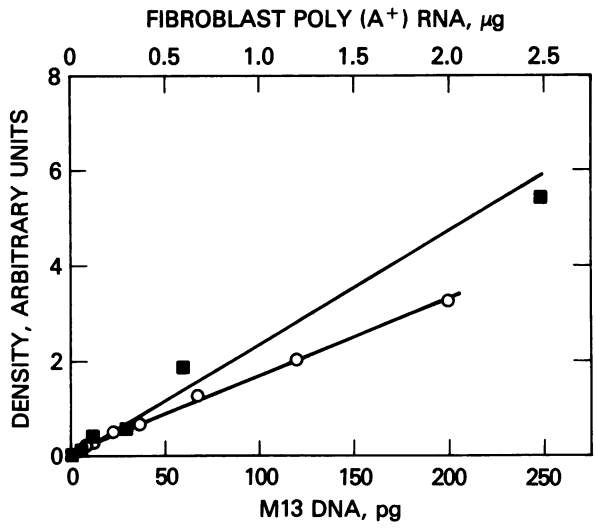

Fig. 2. Standard curve for calculation of amount of β-polymerase mRNA. Dilutions of single-stranded M13 DNA containing a β-polymerase cDNA insert (■) or polyA$^+$ RNA from growing fibroblast (0), were slot-blotted and probed with human β-polymerase cDNA. Autoradiograms were scanned by densitometer, and signals were plotted against the amount of nucleic acid applied to the slot. The linear relationships were used for calculation of β-polymerase sequence content.

DNA carrying a β-polymerase cDNA insert (1014 nt) were slot-blotted to nitrocellulose membrane and hybridized with the β-polymerase cDNA probe. Autoradiograms eventually were obtained and scanned by densitometer. Signals were plotted against the amount of nucleic acid applied, and a linear part of the dose-response curve with M13 DNA (Figure 2) was used for calculating the number of molecules of β-polymerase mRNA in the sample of polyA$^+$ RNA. Assuming equal hybridization efficiency to the cDNA insert and mRNA, we found that β-polymerase mRNA represents 0.004% of total polyA$^+$ RNA from fibroblasts AG-1522. This indicates that the average fibroblast cell contains 6 molecules of the 1.4 kb transcript. These cells are estimated to contain approximately 100,000 enzyme molecule each (18), and this protein's stability corresponds to a half-life of approximately 20 hr (25). Six molecules of the 1.4 kb mRNA would be sufficient for production of 213,000 molecules of the 335 amino acid enzyme

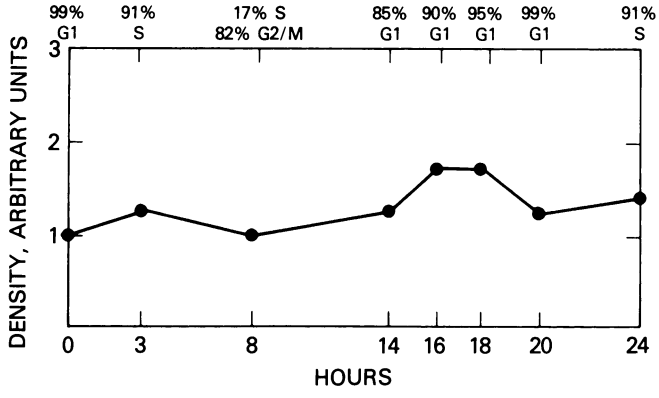

Fig. 3. Quantitative analysis of β-polymerase mRNA levels in synchronized HeLa cells at different phases of the cell-cycle. PolyA$^+$ RNA was prepared from HeLa S3 cells collected 0, 3, 8, 14, 16, 18, 20, and 24 hr after release from thymidine block, as described under Materials and Methods. The cells began to enter S at 2 hr and progressed through S until 8 hr whereupon they entered G2M. At 10 hr they began to divide and then enter G1; at 22 hr they began to re-enter S phase. RNA was dot-blotted on to Gene Screen membrane. The membrane was probed with ^{32}P-labeled human β-polymerase cDNA or ^{35}S-poly(dT). The intensity of autoradiogram signals was determined by densitometry, and the concentration of β-polymerase polyA$^+$ RNA was calculated and standardized to the concentration from cells at 0 hr. The plot shows relative β-polymerase polyA$^+$ RNA levels as a function of time after release from block (lower abscissa) or percentage of cells in phases of the cell-cycle (upper abscissa). Average values from replicate samples are shown; the range was $\pm$ 30%.

during the 22 h cell-cycle, assuming amino acid polymerization at 15 amino acids per second per ribosome (23) and 10 ribosomes per mRNA (24). Thus, the β-polymerase mRNA may be maximally utilized in order to maintain a constant cellular level of the enzyme.

Levels of β-polymerase mRNA During the Cell-Cycle.

With some genes differences in mRNA abundance are associated with progression of a cell through stages of the cell-cycle. To examine this possibility for β-polymerase, we studied mRNA abundance in HeLa S3 cells at different stages of the cell-cycle. It is known that the enzymatic activity of β-polymerase is almost constant in these cells as a function of the cell cycle (26, 27). Synchronization of cells was by double thymidine block in liquid

TABLE I
Comparison of β-Polymerase mRNA Levels in Growing and Quiescent Cells

Cell Line	Growth Phase[b]	Relative mRNA Abundance[a] β-polymerase	β-actin
Human Fibroblast (AG-1522)	Growing	1.0	1.0
	GO	1.1	0.4
Mouse Fibroblast (3T3)	Growing	1.0	1.0
	GO	0.9	0.5
Hamster Ovary (CHO)	Growing	1.0	1.0
	GO	1.0	0.5

[a]PolyA$^+$ RNA was tested by quantitative dot-blot analysis; human or rat β-actin cDNAs were used for human and rodent RNA, respectively.
[b]GO refers to quiescent cells from contact inhibited layer cultures for AG-1522 and 3T3 and to isoleucine deprived cultures of CHO cells.

culture, as described previously (17, 27). In this procedure, HeLa cells do not enter a Go state (28-30), but are arrested at the G1 boundary, and cultures then are collected at different times after release from block (0, 3, 8, 14, 16, 18, 20 and 24 hr) contain enriched populations at different stages of the cell-cycle. PolyA$^+$ RNA from these cells was dot-blotted and hybridized to the β-polymerase cDNA probe; the amount of polyA$^+$ RNA in each preparation was standardized by hybridization to ^{35}S-poly(dT). Autoradiograms eventually were scanned by densitometer, and in Figure 3, results with the β-polymerase probe are plotted as a function of time after release from thymidine block. The level of β-polymerase mRNA increased slightly in G1 and was constant throughout other stages of the cycle. Similar results were obtained when blots were probed with a human β-actin cDNA, in agreement with results by others (31, 32).

β-polymerase mRNA in Growing and Contact Inhibited Fibroblasts.

Fibroblast AG-1522 were collected both from the logarithmic phase of growth and several days after cells had reached stationary phase through contact inhibition. These quiescent fibroblasts are envisioned as resting out of the cell-cycle in a state termed GO. Throughout this growth curve the level of β-polymerase enzymatic activity is known to remain constant (18). PolyA$^+$ RNA from these cells was analyzed, and the results indicate that contact inhibited fibroblasts had the same level of β-polymerase mRNA as growing fibroblasts (Table I). Similar results were obtained with mouse 3T3 cells and with CHO cells, which were included for comparison. Probing of these polyA$^+$ RNAs with ^{35}S-labeled poly(dT) demonstrated that the preparations contained similar amounts of polyadenylated material. In the case of each cell line, we found that the β-actin mRNA level was lower in GO cells than in growing cells.

DISCUSSION

In this study, we examined the relationship between β-polymerase mRNA level and DNA replication using two approaches. We found first that the mRNA level showed no significant change during the HeLa cell-cycle, and second, that fibroblasts held in quiescence for 3 days contained the same amount of this mRNA as growing cells. Several experiments were conducted to evaluate these results: With each type of culture, the overall recovery of total RNA and polyA$^+$ RNA was about the same, indicating that differential degradation of RNA during purification probably was not involved. The same blots were probed with a β-actin cDNA, to compare the β-polymerase mRNA level with that of a well characterized,constitutively expressed mRNA (31, 32). As expected, relative β-actin mRNA levels were similar to those reported. Thus, we conclude that the β-polymerase mRNA level does not fluctuate as a function of DNA replication.

The control of the level of β-polymerase mRNA can be interpreted in light of the fact that the enzyme activity level is constant in AG-1522 cells under the growth conditions used here (18). The mRNA level corresponds to only about 6 molecules per cell. Thus, the mechanism of this "low abundance"

regulation, whether at steps in production of cytoplasmic polyA$^+$ RNA or in controlling its stability, appears to operate in the presence of a constant level of enzyme protein. It is interesting to note that several constitutively expressed eukaryotic proteins are thought to have autogeneous regulation of transcription (33), and the control of abundance of mRNA for some housekeeping genes may be at the level of RNA stability in the cytoplasm (34). In experiments not shown, we found that both growing and contact inhibited fibroblasts AG-1522 could be efficiently transfected with transient expression vectors containing the bacterial chloramphenicol acetyltransferase gene joined either to an SV-40 promoter or to the human ß-polymerase promoter (35, 36). Activity of the ß-polymerase promoter, as revealed by chloramphenicol acetyltransferase expression, was found to be the same in growing and confluent fibroblasts. This suggests that transcription at the ß-polymerase promoter may be at about the same level in these cells even though genomic DNA replication is active in one case and not in the other.

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