QUANTITATIVE CONSERVATION OF CHROMATIN-BOUND RNA POLYMERASES I AND II IN MITOSIS

Implications for Chromosome Structure

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ABSTRACT

RNA synthesis almost ceases in mitosis. It is ambiguous whether this temporal, negative control of RNA synthesis is solely because of the nature of chromosomes per se, (i.e., their condensed state), or to a physical loss of RNA polymerases along with other nuclear proteins which have been shown to pass into the cytoplasm in mitosis, or to their combined feature. Aside from such regulatory considerations, a question has also been raised as to whether RNA polymerases are constituents of metaphase chromosomes. To clarify these aspects of RNA polymerase-chromatin interaction in mitosis, the enzymes in chromosomes were quantitated and their levels compared to those in interphase nuclei and cells at various phases of the cell cycle.

The results show that the amounts of form I, form II, and probably form III enzymes bound to a genome-equivalent of chromatin stay constant during the cell cycle. Thus, the mechanism for the negative control of RNA synthesis in mitosis appears to exist in the chromosomes per se, but not to be directly related to the RNA polymerase levels.

This quantitative conservation of chromatin-bound RNA polymerases implies that they may persist as structural components of the chromosomes in mitosis.

KEY WORDS RNA polymerases ∙ mitosis ∙ chromosomes ∙ chromosomal proteins ∙ cell cycle

One of the biochemical events which distinguishes the mitotic phase from the G1, S, and G2 phases of the cell cycle is the virtual absence of nuclear transcription in mitosis (reviewed in reference 42), with the exception of some synthesis of small molecular weight RNA (24, 65). Since RNA synthesis resumes immediately after the metaphase-to-telophase transition while chromatin decondenses (42), this temporal on-off event has been explained as follows: Because of the high magnitude of chromatin packing, RNA polymerase molecules can not bind to nor transcribe through genes (reviewed in reference 43). Evidence to support this interpretation is that, like condensed heterochromatin from interphase nuclei (1, 13, 31, 58), metaphase chromosomes served as poor templates in vitro for exogenous RNA polymerases (24, 27, 56). It has also been shown that metaphase chromosomes have a low level of endogenous RNA polymerase activity (24, 37, 38, 53, 61). Another biochemical event characterizing mitosis, however, is a transfer or release into cytoplasm of nuclear proteins (2, 41, 44, 45, 54). When these facts are considered together, therefore, it is difficult to decide whether the low endogenous activity is a result of the
nature of mitotic chromatin per se or physical loss of the enzyme molecules along with other nuclear proteins from the condensed chromatin or their combined feature. To our knowledge, the possibility of such enzyme loss has not yet been examined. For an understanding of the mechanism responsible for the absence of RNA synthesis in mitotic chromosomes, therefore, it appears necessary to determine whether such loss of RNA polymerases occurs or the enzymes are present but masked in mitosis. Previously, other workers who studied HeLa cells (3) and Physarum (18) found that the total cellular RNA polymerase activity of mitotic cells approximated that of interphase cells. However, since these studies analyzed only whole cells, it remained to be ascertained whether RNA polymerases are conserved in mitotic chromosomes, and if they should be conserved, to what extent. Recently, Gariglio et al. (16) addressed this question using mitotic and growing populations of mouse Balb/C3T3 cells and found that the same level of RNA polymerases I and II existed in Sarkosyl extracts of mitotic and growing cells; the authors concluded that there is nearly as much initiated RNA polymerases in mitotic chromosomes as in growing cells. We have recently introduced a new technique which permits isolation of morphologically intact chromosomes at pH 7.5 from cultured cells, without appreciable degradation of protein and DNA and inhibition of RNA polymerases. Since any mechanical shearing of chromosomes which would lead to adventitious binding of enzymes to chromosomes was avoided in the isolation procedure, this technique enabled us to quantitate the RNA polymerases associated with chromosomes and to compare their levels to those in interphase nuclei. In agreement with the finding of Gariglio et al. (16), our results strongly indicate that nearly the same level of chromatin-bound RNA polymerase forms I and II are conserved in chromosomes and interphase nuclei.

More than 50% of chromosomal proteins are nonhistone proteins which contain many polypeptides, also found in interphase chromatin (references 14, 51, and W. Wray, a personal communication), but it is totally unknown what they represent enzymatically or structurally. Therefore, aside from regulatory considerations mentioned above, a question has been raised as to whether RNA polymerases are nonhistone protein constituents of chromosomes (14, 53, 61). The present enzyme quantitation results seem to imply that RNA polymerases of the chromatin-bound type persist as structural components of the chromosomes in mitosis.

MATERIALS AND METHODS

Chemicals

All chemicals were reagent or analytical grade. DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.; unlabeled ribonucleoside triphosphates from P-L. Biochemicals, Inc, Milwaukee, Wis.; [3H]guanosine 5'-triphosphate (15 Ci/mmol) and [3H]uridine (26 Ci/mmol) from Amersham/Searle Corp., Arlington Heights, Ill.; Ammonium sulfate (ultra pure grade) from Schwartz/Mann Div., Beckton, Dickinson & Co., Orangeburg, N. J.; Calif thymus DNA, dithiothreitol, bovine serum albumin fraction V (BSA), phenylmethylsulfonylfluoride (PMSF) and Tribase, from Sigma Chemical Co., St. Louis, Mo.; diethylaminoethyl (DEAE)-cellulose DE/81 filter discs (2.5-cm Diam) and GF/C glass fiber filters from Whatman, Inc., Clifton, N. J. and a-amanitin from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Nuclease-free pronase was from Worthington Biochemical Corp., Freehold, N. J. Beef lung heparin (1,000 U/ml) was from Upjohn Co., Agricultural Prods. MKT, Kalamazoo, Mich. High molecular weight DNA was prepared from exponentially growing DON cells (below) by the pronase-phenol extraction methods as described elsewhere (33).

Cells

A line of Chinese hamster cells, DON, has been maintained as either monolayer or suspension in RPMI 1640 medium supplemented with antibiotics (34, 35). There was no contamination with pleuropneumonia-like organisms (PPLO). For mitotic synchronization (34, 35, 57), a confluent monolayer grown at 6 x 10^6 cells/cm² was trypsinized and then the cell suspension distributed to Falcon plastic flasks (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) (75-cm² surface area) at an initial density of 6 x 10^6 cells/cm². 16 h later, Colcemid (Grand Island Biological Co. Gibco), Grand Island, N. Y.) was added at 0.06 µg/ml and 2 h later the metaphase cells selectively shaken off, as described previously (34, 35, 57). Using these metaphase cells, synchronization was initiated in a fresh medium prewarmed at 37°C in a spinner bottle at 2 x 10^6 cells/ml, and G1, S, and G2 populations were harvested at 2, 6, and 10 h later. The degree of synchronization was as described previously (34, 35, 57).

Isolation of Metaphase Chromosomes and Interphase Nuclei

Detailed procedures of chromosome isolation will be published elsewhere. Briefly, the new method used in this study preserved RNA polymerase activities and avoided the degradation of proteins and DNA. The following procedures apply for mitotic cells collected...
from 20 flasks after 2-3 h Colcemid treatment: Cells were centrifuged at 1,000 g for 3 min and resuspended in 10 ml TCMPD (10 mM Tris-HCl, pH 7.5, 2 mM CaCl₂, 2.5 mM MgCl₂, 0.5 mM PMSF, 0.5 mM dithiothreitol) at 10°C for 60 s. Cell swelling took place at this step and the cell suspension was quickly chilled at 1°C. Thereafter, all the procedures were carried out between 1°C and 4°C. Cells were lysed in the presence of the nonionic detergent Triton X-100, and pelleted as above (Fig. 1). Interphase nuclei were isolated from log phase or synchronized cultures by essentially identical procedures described by Jaehning et al. (20) and Manck (36). After centrifugation, cells, nuclei, or chromosomes were washed with 30 ml of TCMPD containing 0.5% Triton X-100, and pelleted as above (Fig. 1).

**Quantitative Solubilization of RNA Polymerases**

The method used was essentially the same as described by Jaehning et al. (20) and Mauck (36). After final centrifugation, cells, nuclei, or chromosomes were suspended in TGED (10 mM Tris-HCl, pH 8.0, 25% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol), and the suspension adjusted to 0.3 M (NH₄)₂SO₄. Enzymes were solubilized by sonication at 1°C using a Branson Model 185 Sonifier (Bronson Sonic Power Co., Danbury, Conn.) with a micro tip, at position 3 of the output control, using 3 or 4 consecutive pulses of 5 s each. After dilution with 2 vol of TGED, the insoluble materials were removed by centrifugation at 40,000 rpm for 30 min at 2°C in a Beckman-Spinco SW41 rotor (Beckman Instruments, Inc., Spino Div. Palo Alto, Calif.). The supernate was saved, diluted with 3 vol of TGED, and centrifuged again. The pellet was dispersed into TGED-0.3 M (NH₄)₂SO₄, sonicated and processed similarly. >95% of the calculated total activities based on the experiments with interphase nuclei (see Fig. 2) were reproducibly solubilized by this method.

**DEAE-Sephadex A-25**

**Column Chromatography**

Methods for enzyme fractionation on a DEAE-Sephadex column (47, 48) were essentially the same as described earlier (32, 33), except that the column size (bed volume, 2-5 ml) was scaled down. DEAE-Sephadex A-25 was washed and precycled according to the method described by Weil and Blatti (60). The column was equilibrated with TGED containing 0.05 M (NH₄)₂SO₄ and the enzyme solution applied. There was no enzyme activity in the void fractions. The enzymes were eluted with a linear (NH₄)₂SO₄ gradient, 0.05-0.55 M (48, 49). Fractions of 0.2-0.5 ml were collected with an ISCO Model 328 fraction collector.

**RNA Polymerase Assay**

Reaction mixtures contained in a final volume of 250 µl, 12.5% glycerol, 50 mM Tris-HCl, pH 8.0, 50 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MnCl₂, 5 mM MgCl₂, 0.5 mM dithiothreitol, 5 mM NaF, 0.25 mM PMSF, 0.4 mM each of ATP, CTP, UTP, and 0.04 mM [³H]GTP (117 cpmdpmol). After 10 min of incubation at 25°C, aliquots of 50 or 100 µl were placed onto DE/81 filters and immediately dried under infrared light (4). Each filter was washed five times with 5 ml of 5% Na₂HPO₄, distilled water, ethanol, and ether and the retained radioactivity determined in a toluene base-NCS (Amersham/Searle) scintillant with a counting efficiency of 45% with a Beckman liquid scintillation counter (LS-3150). When solubilized enzymes were used, the DNA isolated from DON cells was included in excess in the reaction mixture (500 µg/ml). Under these conditions, [³H]GMP incorporation was linear for up to 30 min of incubation, and dependent on the presence of DNA templates and four ribonucleoside triphosphates. 1 U of solubilized enzyme activity was defined as incorporation of 1 pmol of [³H]GMP into RNA/10 min. When nuclei or chromosomes were assayed for endogenous activity, MnCl₂ was omitted from reaction mixtures because it inhibited >60% of the endogenous transcription by precipitating chromatin. Apparent Michaelis constant (Km) of GTP for forms I and II, based on the Chinese hamster enzymes partially purified by DEAE-Sephadex, was 0.032 and 0.038 mM, respectively (Fig. 3). Since the values of enzyme activities were obtained at the approximate Km of 0.04 mM, the GTP concentration employed was also rate-limiting in our reaction. However, since the [³H]GMP incorporation was linear for up to 30 min (Fig. 4) and the maximal incorporation of [³H]GMP was at most 2% of the input radioactivity, it seems unlikely that the consumption of substrate significantly affected the RNA synthesis with the incubation times. Further, the rate of [³H]GMP incorporation was proportional to the level of enzyme concentration used (Fig. 4) and to the concentration of nuclei (Fig. 5). Accordingly, the numbers of enzyme units (Vmax) at saturating levels of GTP can be obtained by multiplying the presented values by 1.78 and 1.82 for forms I and II, respectively (as calculated from Fig. 3). In this paper, however, only the actual numbers of enzyme units obtained at 0.04 mM of GTP are presented, because without knowing Vmax, the values reflected well without significant error (~2%) the relative activity levels of forms I and II and the ratio of both enzymes.

** Sucrose Gradient Analysis of Transcripts**

As one approach to ascertain if isolated nuclei and chromosomes preserved an intact transcriptional machinery, the sizes of transcripts made by endogenous activities...
FIGURE 1  Phase contrast photographs of nuclei isolated from log phase cultures and chromosomes. Bars, 10 μ.
FIGURE 2 Solubilization of RNA polymerases by sonication. The data are the averages from two experiments. Nuclei isolated from log phase cultures were sonicated in three consecutive pulses of 5 s each (step 1). The sonicates were centrifuged at high speed (see Materials and Methods). The chromatin pellet was dispersed in the buffer, sonicated, and centrifuged as above (step 2), and this procedure repeated once again (step 3). Total RNA polymerase activity was determined in the supernates and the resuspended chromatin pellets. (a) Activity solubilized by sonication. 100% activity represents the sum of steps 1–3 activities plus the endogenous activity of the final pellet, all determined with exogenous DNA (500 μg/ml). (b) The enzyme activity in chromatin before and after sonication steps. Before sonicating the nuclei, the activity was 2.69 U/μg DNA. The activity left in the final pellet was 0.04 U/μg DNA.

FIGURE 3 Effect of GTP concentration on the rate of RNA synthesis: Lineweaver-Burk plotting (28) with varying GTP concentration. Forms I and II, partially purified through DEAE-Sephadex column, were incubated for 10 min with the standard assay mixture. DNA was present in excess (500 μg/ml). (a) Form I. (b) Form II.

FIGURE 4 Incorporation of [3H]GMP into RNA as a function of incubation time. The assay mixtures containing various concentrations of enzymes (from interphase cells) were incubated for up to 30 min. At each time point, 50 μl aliquot was removed, spotted onto DE/81 filters, and processed as described in Materials and Methods. Assays were done with (△, ○, □) 0.5 μg/ml α-amanitin.

Miscellaneous Procedures

DNA was determined by a modified diphenylamine reaction (46) and the protein by the method of Lowry et al (29). Calf thymus DNA (Sigma) and bovine serum albumin fraction V (Sigma) were used as standards. Ionic
RESULTS

Characterization of Transcriptional Machinery in Isolated Nuclei and Chromosomes

First, we examined whether or not isolated materials reflected the characteristics of RNA synthesis in the cells from which they were obtained. Table I compares the endogenous RNA polymerase activity of isolated nuclei and chromosomes as well as the rate of uridine uptake by metaphase and interphase cells. The endogenous activity was determined under conditions where the incorporation of \([\text{H}]\text{GMP}\) was proportional to the amount of input nuclei or chromosomes (Fig. 5a) and proceeded almost linearly (Fig. 5b). Only a low level of endogenous activity was found in chromosomes in comparison to the activity in nuclei (Table I). This reflected well the limited rate of RNA synthesis of metaphase cells, determined by \([\text{H}]\text{uridine}\) uptake, compared to the rate exhibited by log phase cells (Table I).

No significant increase in \([\text{H}]\text{GMP}\) incorporation over the endogenous level by nuclei and chromosomes took place even when assayed with exogenous DNA (Table I), indicating that the major endogenous activity in isolated nuclei and chromosomes represented the chromatin-bound type. Thus, it appeared that most of unbound types, if any, were washed away from the chromosomes and nuclei during the isolation. The sucrose gradient profiles of in vitro transcripts appear in Fig. 6. Interphase nuclei synthesized RNA of a wide range of molecular sizes including 45S molecules (Fig. 6a), but their transcripts formed in the presence of 0.5 \(\mu\text{g/ml}\) \(\alpha\)-amanitin exhibited two peaks, at 5S and 28S (Fig. 6a). In contrast, the chromosomes synthesized mostly small molecular weight RNA (Fig. 6b), as had previously been found for intact metaphase cells (24, 65). The virtual absence of high molecular weight RNA from the chromosomal transcripts suggested that the chromosome preparations were substantially free of interphase nuclei, preserving intact transcriptional machinery. From these gradient profiles of transcripts synthesized by known amounts of DNA in nuclei and chromosomes (Fig. 6b), it could be inferred that the major endogenous activity in isolated nuclei and chromosomes represented the chromatin-bound type.

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**Table I**

Rate of RNA Synthesis

| Source                | No. of exps | Cells | [H]uridine incorporation/min | [H]GMP incorporation/min |
|-----------------------|-------------|-------|-------------------------------|--------------------------|
| Isolated nuclei or chromosomes |             |       |                               |                          |
| Log phase cells*      | 3           | 69,100|                               | -DNA 1.33                |
|                       |             |       |                               | +DNA 1.37                |
| Metaphase cells       | 3           | 3,630 |                               | -DNA 0.069               |
|                       |             |       |                               | +DNA 0.079               |

* Log phase cells were composed of 20% G1, 50% S, 25% G2, and 5% mitotic phases, on the average.
† 5 × 10⁴ cells were incubated for 10 min at 37°C in 2 ml of RPMI 1640 medium containing 20 \(\mu\text{Ci}\) of \([\text{H}]\text{uridine}\) (26 Ci/mmol). Under this condition, \([\text{H}]\text{uridine}\) uptake was linear for up to 30 min. Incorporation was terminated by 5% trichloroacetic acid, and the acid-insoluble materials were collected on Whatman GF/C filters.
§ Nuclei or chromosomes, containing up to 25 \(\mu\text{g DNA}\) (Fig. 5) were assayed in the standard reaction mixture with or without 500 \(\mu\text{g/ml DNA}\) at 25°C for 10 min.

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6), the levels of nuclear endogenous activity that synthesized small molecular weight RNA at 5S regions appeared to persist rather constant in mitosis.

Levels of RNA Polymerases Solubilized from Interphase Nuclei and Metaphase Chromosomes

From the above data, it appeared that the endogenous RNA polymerase activities were considerably diminished in metaphase chromosomes. To answer the main question of this study as to whether RNA polymerase molecules are truly absent or are conserved within chromosomes, but their activities are merely masked, the RNA polymerase activities in isolated chromosomes were quantitated after solubilization using the method of Jaehning et al. (20), previously applied to whole cells. Inclusion of BSA was found to be necessary for quantitating the enzymes presumably by preventing their inactivation by dilution, especially when the small amounts of chromosomes were solubilized. The results appear in Table II. Interestingly, the solubilized enzyme levels per 1 μg of DNA-equivalent of interphase nuclei from log phase cells and of chromosomes were approximately the same. Thus, the enzyme levels per unit genome, as found in chromosomes, appeared to remain relatively constant throughout the cell cycle.

On the basis of the results with α-amanitin, the activity of DON cell enzymes proved to represent mostly form I. Differences in the activity level determined in the presence of 0.5 μg/ml and 200 μg/ml of α-amanitin were regarded to represent form III activities (60). Such activities in DON cells comprised <5% of the total activity, so that an accurate quantitation was difficult. Accordingly, form I and II activities are reported in the present quantitation study. The yields of forms I and II per genome equivalent from chromosomes were each approximately the same as their respective yields from the nuclei (Table II).

Levels of RNA Polymerases during the Cell Cycle

Comparison of the RNA polymerase activities between metaphase chromosomes and G1 nuclei...
was also made to further examine if the changes in the chromatin configuration, from condensed to decondensed, affected the amount of enzymes bound. At 2 h after metaphase, the chromatin decondensed and the nuclear membranes were formed completely, but without any significant increase in the bound enzyme levels (Table III). Together with the above enzyme quantitation data on chromosomes and nuclei from log phase cells, this finding strongly indicated that the amounts of chromatin bound enzymes per unit genome stayed relatively constant regardless of the change in the chromatin configuration, and also indicated that since the genome size per nucleus doubles after DNA replication, the amount of enzymes per nucleus also doubles during the cell cycle. To further learn relationships between the levels of chromatin-bound enzymes and total enzymes (bound and unbound) in a cell during the cell cycle, the enzyme activity per cell was also determined. This attempt was made using fairly pure populations of G1 and S, and less synchronized populations of G2 cells. The results appear in Table IV. The amount of enzymes per cell increased in parallel with the increase in genome size. It was also confirmed that the enzyme per unit genome in a cell was constant regardless of various genome sizes in the cell cycle. Comparison of the results in Table IV with those in Table II shows that 50% of the total enzymes in a cell represent bound types. However, this value would represent only the estimated levels of enzyme bound to chromatin, because (a) the possibility is not ruled out that the enzyme loss could result partially from the inactivation of enzyme itself during the isolation procedures and (b) no enzyme quantitation with cytoplasm was done because the enzymes in postnuclear supernates were too diluted to assay.

### Table III
**Levels of RNA Polymerases at Metaphase-G1 Phase Transition**

| Source                  | No. of exps | Enzyme activity (U/µg DNA equivalent) |
|-------------------------|-------------|---------------------------------------|
| Metaphase chromosome    | 2           | 2.65                                  |
| G1 nuclei               | 2           | 2.85                                  |

Cell synchronization was initiated from metaphase (see Materials and Methods), and 2 h later, the cultures were harvested as G1. G1 nuclei and chromosomes were prepared according to the method described in Materials and Methods.

### Table IV
**RNA Polymerase Activity Levels in the Total Cell Extracts at Various Phases in the Cell Cycle**

| Series | Cells       | No. of exps | Units enzyme activity per 10^9 cells per µg DNA |
|--------|-------------|-------------|-----------------------------------------------|
| A      | Log phase (I) | 4           | 4.70 5.16                                    |
|        | Metaphase (M) | 4           | 6.40 5.42                                    |
|        | M/I ratio    |             | 1.36 1.05                                    |
| B*     | Metaphase (M) | 3           | 6.63 5.44                                    |
|        | G1           | 2           | 3.36 5.60                                    |
|        | S            | 2           | 4.65 5.42                                    |
|        | G2           | 1           | 5.12 ND                                       |
|        | M/G1 ratio   |             | 1.97 0.97                                    |
|        | M/S ratio    |             | 1.42 1.00                                    |
|        | M/G2 ratio   |             | 1.29 ND                                       |

Metaphase cells were prepared from log phase cultures exposed to 0.06 µg/ml of Colcemid for 2 h. Synchronization was initiated, and G1, S, and G2 phase populations obtained at 2, 6, and 10 h later. 2-5 × 10^5 cells were used for quantitative solubilization in the presence of 1 mg/ml BSA.

* Three series of experiments using synchronized cultures were carried out in which two series were used for preparing M, G1, and S, and one series from M and G2. Degree of synchronization in G2 population was such that ~40% of the cells were still synthesizing DNA (34, 35).

**Fractionation of RNA Polymerases over DEAE-Sephadex A-25 Column**

On the basis of the results with α-amanitin, it appeared that at least two different enzyme forms existed in metaphase chromosomes and in interphase nuclei (Table II). To learn further basic characteristics of these enzymes, they were separated over a DEAE-Sephadex column. As shown in Fig. 5, essentially identical profiles were obtained for all the enzymes either from interphase and metaphase cells or from chromosomes and nuclei. The ionic strength at which each form eluted was as expected from established studies (reviewed in reference 49). A small peak for form III was recognized in the cell extracts, but not clearly in the isolated nuclei or chromosomes (Fig. 7), indicating the above mentioned possibility that some enzymes were inactivated during isolation procedures. However, that a small peak represents form III was confirmed by its resistance to α-amanitin (0.5 µg/ml) in the reaction mixture (Fig. 8).
The proportions of forms I and II, determined by column chromatography and α-amanitin sensitivity, are summarized in Table V. There was no significant change in the proportions of the two forms during the cell cycle (Fig. 7). The sensitivity profiles of form I and II enzymes in interphase and metaphase cells against various concentrations of α-amanitin were identical (Fig. 9).

Levels of RNA Polymerases Associated with Chromatin

As shown above (Table II), the virtual absence of significant increase in the endogenous activities of nuclei and chromosomes by added DNA appeared to indicate that there was no significant amount of unbound types in isolated nuclei and chromosomes. To rule out the possibility that the RNA polymerases were of cytoplasmic origin and became absorbed onto chromosomes during the isolation procedures, the following experiments were also carried out: When chromosomes were washed extensively with 75 mM NaCl-20 mM EDTA, pH 8.0 (adjusted with 1 N NaOH), and then with 10 mM Tris, pH 8.0, the chromosome structure was converted to a mass of chromatin, as observed under a phase-contrast microscope after staining with azur C. The analytical results obtained with this material appear in Table VI. A decreased protein/DNA ratio compared to the original material indicated that nearly 60% of the proteins were removed. However, the residual chromatin not only retained >50% of the initial activities, but also preserved a high proportion of form I enzyme (Table VI), as judged by the resistance to α-amanitin. Since comparable enzyme retention was noticed also in the chromatin prepared in the same way from interphase nuclei, it appears that the major portion of enzymes found in chromosomes represent the bound type. Since form I is readily solubilized under these conditions (6, 50), one would expect that the above washing would have selectively removed most of form I, had it simply been absorbed to chromosomes from cytoplasm.

![Figure 7](image7.png)  
**Figure 7** DEAE-Sephadex chromatography of RNA polymerase activities from nuclei, chromosomes, and cells. Enzymes are from (a) 2.0 × 10^7 log phase cells, (b) 2.1 × 10^7 metaphase cells, (c) interphase nuclei containing 450 μg DNA and (d) chromosomes containing 220 μg DNA.

![Figure 8](image8.png)  
**Figure 8** DEAE-Sephadex chromatography of RNA polymerase activities solubilized from metaphase cells. The sonicates representing 2.1 × 10^7 metaphase cells were applied over a column (0.8 cm² × 4.5 cm bed size), and eluted with a 13.0-ml linear (NH₄)₂SO₄ gradient. Fractions of 0.5 ml each were collected, and 30 μl aliquots assayed in the absence (— -- - —) or presence (— Δ —) of 0.5 μg/ml of α-amanitin.
**Activation of Endogenous RNA Polymerases by Heparin in Metaphase Chromosomes**

One crucial question to the present finding is whether RNA polymerases in metaphase chromosomes were artifactual, resulting from adventitious binding of enzymes or were due to the real existence of enzyme-gene complex. One might expect that if the latter was the case, such enzyme-gene complexes would synthesize RNA under appropriate conditions where any RNA synthesis-restricting factors are removed from chromosomes. This expectation was examined.

As an agent which removes histones and some other proteins, we chose heparin, because this polyanion, like Sarkosyl as previously demonstrated by Gariglio et al (16), has been shown to activate the initiated RNA polymerases while blocking the initiation of RNA synthesis by free enzymes (11, 17). We found that chromosomes treated with heparin at concentrations of >10 U/ml, tended to swell and lose their birefringence under phase contrast optics. To assure the heparin effect on chromosome enzymes, 100-250 U/ml of heparin was used. Results from three separate experiments are shown in Table VII. It was found that heparin could activate 10-fold the endogenous polymerase activity.

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**TABLE V**

Proportions of RNA Polymerase I and II Solubilized from Interphase Nuclei and Metaphase Chromosomes

| Source       | Determination              | No. of determinations | Enzyme activity* |
|--------------|----------------------------|-----------------------|------------------|
|              |                            |                       | Form I | Form II |
| Chromosomes  | α-amanitin sensitivity     | 10                    | 76      | 24      |
|              | DEAE-Sephadex              | 3                     | 80      | 20      |
| Nuclei       | α-amanitin sensitivity     | 11                    | 81      | 19      |
|              | DEAE-Sephadex              | 5                     | 77      | 23      |

* Form III activity is not included.

**TABLE VI**

Levels of RNA Polymerases Associated with Chromatin Prepared by Washing with 75 mM NaCl-20 mM EDTA and then 10 mM Tris-HCl, pH 8.0

| Source | Washing | Protein/DNA | No. of exp | Enzyme Activity, total |
|--------|---------|-------------|------------|------------------------|
|        |         |             |            | Form I | Form II |
|        |         |             |            | %     | %     |
| Nuclei | No      | 4.56        | 2          | 2.80   | (100)  |
|        | Yes     | 1.85        | 2          | 1.52   | (54)   |
| Chromosomes | No     | 3.70        | 2          | 2.70   | (100)  |
|        | Yes     | 1.71        | 2          | 1.68   | (62)   |

Nuclei or chromosomes were suspended in 20 ml of 75 mM NaCl, 20 mM EDTA, pH 8.0, 0.5 mM PMSF, 0.5 mM DTT using a glass-teflon homogenizer (Potter-Elvejehem type), and centrifuged. Washing was repeated twice. Pelleted materials were then swollen for 60 min in 2,000 vol of 10 mM Tris, pH 8.0-0.5 mM DTT, and centrifuged. After another washing, the formed chromatin was subjected to enzyme solubilization (see Materials and Methods). For unit of activity, see Table II.
TABLE VII

Activation of Endogenous RNA Polymerase Activities in Metaphase Chromosomes

| Exp. No. | Assay condition | [3H]GMP incorporation pmol/μg DNA/10 min |
|----------|-----------------|----------------------------------------|
| 1 Control | 0.078           |
| Plus heparin, 100 U/ml | 0.847          |
| 2 Control | 0.084           |
| Plus heparin, 100 U/ml | 0.885          |
| 3 Control | ND              |
| Plus heparin, 250 U/ml | 1.000          |
| Plus heparin, 250 U/ml and 0.5 μg/ml α-amanitin | 0.822          |

Chromosomes were isolated from log-phase cells treated with Colcemid for 2 h, suspended in TGMED (TGED plus 5 mM MgCl₂), and incubated for 10 min at 25°C with the standard reaction mixtures (Mn²⁺ was omitted) in the presence or absence of heparin. The incorporation of [3H]GMP into acid-insoluble materials was determined as described in Materials and Methods.

* Data from three separate experiments.

RNA polymerase activity of chromosomes. Since a portion of such activated activity was sensitive to 0.5 μg/ml of α-amanitin (Exp 3), it appears that both forms I and II are in the initiated state within the chromosomes.

Can Free Enzymes Bind to Genes under Chromosome Isolation Procedures?

The foregoing experiments strongly suggest the absence of free enzymes in chromosomes and nuclei, but did not rule out the possibility that during the swelling of the cells the free enzymes were bound to genes in the chromosomes. If such free enzymes were really bound to genes firmly, these enzyme-gene complexes could not utilize exogenous DNA as template (Table I) and could still be activated by heparin (Table VII). Therefore, we examined whether or not the free enzyme was bound to genes under chromosome isolation procedures. Results are shown in Table VIII. It should be noted that in the protocol the exposure of the chromatin to the enzymes was about four times longer than the exposure of the chromosomes to the disrupted cell contents in the standard isolation procedure. Under this condition, only a small portion of enzymes was found to bind to chromatin, but this was not large enough to account for a higher level of enzyme activities present in nuclei and chromosomes (Tables II and III).

DISCUSSION

Two methods have been developed to obtain relatively large quantities of metaphase chromosomes at neutral pH from several types of cultured cells (30, 61). These methods employed 10⁻³ M ZnCl₂ (30) or 1 M hexyleneglycol (61, 62) as a chromosome stabilizer and because of their near-physiological pH, circumvented the loss of acid-soluble proteins (30, 61) inherent in other methods which used low pH (5, 7, 19, 52, 55). Nevertheless, in our hands, the presence of ZnCl₂ in the isolation buffer resulted in an irreversible reduction by 60% or more of enzyme activity (unpublished data) and the hexyleneglycol treatment of metaphase cells at 37°C for 10 min., as recommended by Wray and Stubblefield (61, 62), caused an appreciable degradation of chromatin proteins (unpublished data) as well as DNA (61). The method used in the current work (see Materials and Methods), though
similar to one devised by Maio and Schildkraut (30), eliminated such drawbacks by including a protease inhibitor, PMSF, and by swelling the cells in the absence of ZnCl₂ at relatively low temperature. Details of the present method will be published elsewhere.

The present findings strongly suggest that RNA polymerases of the chromatin-bound type remain quantitatively conserved when interphase chromatin condenses into mitotic chromosomes. This conservation appears not to be artifactual, because (a) the chromosomes contained virtually no free enzymes which were able to transcribe exogenous DNA templates added in the reaction mixture (Table I) and (b) substantial amounts of enzyme activity, especially of form I, were recovered from chromosomes (Table VI) even after extensive washing with 75 mM NaCl-20 mM EDTA, pH 8.0, and then with 10 mM Tris, pH 8.0, a procedure which is known to wash off effectively form I from nuclei and nucleoli (6, 12, 50). In the presence of heparin which is well known to block the initiation of RNA synthesis, certain endogenous RNA polymerase activities were activated in the chromosomes (Table VII). Adventitious binding of proteins to nuclei or chromatin has been shown to occur at a certain ionic strength (23, 40). The existence of RNA polymerases in the chromosomes cannot be a result of such binding, because of the low ionic strength which was used to isolate them (see Materials and Methods). As a matter of fact, the extensively washed chromatin could not absorb a significant amount of RNA polymerases under conditions as such used in the present isolation procedures (Table VIII). Thus, the present study offers direct evidence to support the predictions of other workers that RNA polymerases would remain in mitotic chromosomes (18, 53, 61). These predictions came from the findings that (a) some level of endogenous RNA polymerase activity existed in chromosomes (24, 53, 61) and (b) the presence of inhibitors of protein synthesis did not block the normal resumption of RNA synthesis as cells entered G₁ from mitosis (53). These studies, however, did not include enzyme quantitation nor pay attention to the possibility that RNA polymerases might shuttle back and forth between chromatin and cytoplasm in mitosis. Of interest was the finding of Simmons et al. (53) that chromosomes dialyzed for 16 h under conditions known to preserve RNA synthetic capacities exhibited an increased level of endogenous activity; however, the enzyme level of the chromosomes was not compared quantitatively to that of the nuclei (53). Since the increased activity was partially inhibited by 0.5 μg/ml of α-amanitin (53), their results may be an indication that form II enzymes in chromosomes were somehow unmasked by dialysis. Whether this was related to the degradation of some restricting factors in chromosomes is unknown.

Previously, Gariglio et al. (16) also addressed the question of RNA polymerase levels in mitosis, using mouse Balb/C3T3 fibroblasts. Based on the analysis of Sarkosyl extracts of total interphase and mitotic cells, these authors stated: “We infer that there is nearly as much initiated RNA polymerase in mitotic cell chromosomes as found in growing cells.” It should be pointed out, however, that direct analysis of isolated chromosomes was not performed. The finding that heparin activated the endogenous RNA polymerases of chromosomes (Table VII) not only substantiated their inference but also suggested that some restricting factors were removed by these agents.

It has been shown that both unbound and bound enzymes exist in nuclei (63, 64). Generally, the unbound types leaked out of the nuclei which were isolated in solutions of low ionic strength. Since the current isolation procedures employed a hypotonic solution also (see Materials and Methods), the results permit no conclusion about the possible presence in chromosomes in situ of unbound types which would have been readily removed during the isolation.

Certain proteins bound to DNA stay constant throughout the cell cycle; for example, deoxyribonucleoproteins from interphase and metaphase cells have a constant buoyant density in CsCl and, hence, there probably is a constant ratio of nonhistone proteins to DNA throughout the cell cycle (R. Hancock, as cited in reference 14). The similarities in the polypeptide composition of nonhistone proteins from metaphase and interphase chromatin are greater than the differences (8, 9, 10, 14, 22, 26, 51). The similar protein/DNA ratio found in metaphase and interphase chromatin in the current work argues for such constancy. Thus, RNA polymerases may also belong to such proteins that bind to DNA in a constant ratio in the mitotic cell cycle.

The rate of RNA synthesis in synchronized cell cycles has well been studied in HeLa (39), CHO (15), and DON cells (25). Although some important differences in the mode of rate change after the resumption of RNA synthesis in early telophase appeared among these studies (as reviewed in reference 39), a rather general conclusion was that
the rate of total RNA synthesis increases during interphase and doubles when cells transverse from G1 through G2 phases (43, 59). This may be explained in part by the present data in that the amount of bound enzymes per cell was increased by at least 50% as cells transversed from G1 to G2. The results can be related to knowledge of the rate of change of RNA synthesis during the cell cycles.

Persistence of RNA polymerases at almost constant levels per genome throughout the cell cycle agrees with the findings of Hildebrandt and Sauer (41) who quantitated RNA polymerases in total cell extracts from synchronously dividing macroplasmodia of Physarum, and found that neither the level of enzymes per unit of protein in the extract nor the proportion of enzyme forms changed during the period from early S phase through G2 (43). Identical results were obtained when total cell extracts of HeLa cells were examined (3). Thus, the constancy of enzyme levels per genome during the cell cycle seems to be a uniform phenomenon in various kinds of cultured cells.

The present study eliminated the possibility that in DON cells RNA polymerases of chromatind-bound type represent a class of proteins which shuttle back and forth between nuclei and cytoplasm in mitosis (2, 41, 44, 45, 54), but rather indicated that they are conserved even when chromatin condenses. Therefore, a mechanism for negative control of RNA synthesis in mitosis must be present within the chromatin. Whether this mechanism is the physical condensation of chromatin itself, or whether it is this feature combined with repressor-like molecules (56) remains to be determined.

An implication of the present study is that RNA polymerases, at least forms I and II, persist as structural components of metaphase chromosomes. No quantitation was carried out for form III enzymes because of its low activity level in the DON cell line and because of the limited amount of chromosome materials available. Nevertheless, since the synthesis of low molecular weight RNA occurs continuously in mitosis both in vivo (24, 65) and, in the current work, in vitro (Fig. 6), the persistence of a form III-gene complex in chromosomes is also probable. It would be of interest to localize in situ the enzymes on metaphase chromosomes. Such an attempt has actually been done in the case of RNA polymerase II on polytene chromosomes of Drosophila melanogaster (21). Using the technique of indirect immunofluorescence, Bautz and co-workers demonstrated that form II is present almost exclusively in puffs and interband regions (21). Since RNA polymerases are concentrated in euchromatin and are scarce in heterochromatin (1, 13, 31, 58), the same technique might differentiate in situ the active from the inactive chromatin on metaphase chromosomes.

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