Nonhistone Chromosomal Proteins in Synchronized HeLa Cells

(chromatin/SDS–gel electrophoresis/cell division cycle)

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ABSTRACT Chromatin was isolated from synchronized HeLa cells at different stages of the cell division cycle and fractionated into DNA, histones, and nonhistone proteins. Electrophoresis of the nonhistone proteins in sodium dodecyl sulfate–polyacrylamide gels revealed a highly reproducible pattern of 22 bands, having estimated molecular weights of 15,000–180,000, with 85% (by mass) over 40,000. The amounts of some nonhistone proteins varied during the cell cycle by as much as 50%, while others remained at a constant level. One group of nonhistone proteins (molecular weight 75,000) was greatly reduced just before the start of DNA replication (S-phase), then returned to normal levels in the mid-S phase. These results are discussed with regard to the possible role of nonhistone proteins in regulating chromosome structure and function.

Eukaryotic chromosomes contain DNA, histones, and various nonhistone proteins, many of which are acidic (1, 2). In contrast to their histone counterparts, nonhistone chromosomal proteins are electrophoretically complex, metabolically unstable, and, in some cases, tissue-specific (3–11). These characteristics have led to speculation that the nonhistone, acidic proteins may play important roles in regulating chromosome structure and function (12). We have examined these proteins in synchronized HeLa cells by high-resolution gel electrophoresis to determine whether their quantitative distribution in chromatin varies in relation to the cell division cycle.

METHODS

Cells and Synchronization. HeLa cells (S$_3$ strain) were grown in suspension culture at 2 to 4 × 10^5 cells per ml with Joklik-modified Eagle's medium (13) containing 3.5% each of calf and fetal-calf serum. Cells were synchronized by the double thymidine method (14) as detailed previously (15).

Cell Fractionation and Chromatin Isolation. All procedures were done at 4° unless noted otherwise. Cells were harvested by low-speed centrifugation, washed twice in Earle's balanced salt solution (16), and disrupted by Dounce homogenization in RSB buffer [0.01 M NaCl–1.5 mM MgCl$_2$–0.01 M Tris·HCl, (pH 7.0)]. Nuclei were sedimented at 1000 × g (3 min) and washed in 10 volumes of RSB buffer three times. The washed nuclei were adjusted to 4 × 10^7/ml of RSB buffer and disrupted by brief sonication (1 min, in 15-sec pulses, 40 W; Bronson model W185, Heat-Systems Ultrasonics, Plainview, N.Y.); breakage was 99% or more, as determined by phase-contrast microscopy. The sonicate was then layered over 30% sucrose in NaCl–Tris [0.01 M NaCl–2.5 mM Tris·HCl (pH 7.2)] and centrifuged at 4500 × g for 15 min (5000 rpm, Spinco SW27 rotor); this procedure pelleted most of the nucleoli, but only 2–6% of the chromatin (DNA). The material remaining on top of the 30% sucrose, containing 75–85% of

Abbreviation: SDS, sodium dodecyl sulfate.

the nuclear DNA, was centrifuged through 60% sucrose in NaCl–EDTA–Tris buffer [0.01 M NaCl–24 mM EDTA–2.5 mM Tris·HCl (pH 7.2)] for 100 min at 27,000 rpm (SW27 rotor). This spin was designed to leave in the supernatant heterogeneous nuclear ribonucleoprotein particles, which sediment from 40 to 250 S (ref. 17, and T. Pederson, unpublished results), and the 60S ribosomal subunits of the nucleoplasm, which contain 95% of the nuclear RNA (18). The chromatin pellet, corresponding to about 70% of the total nuclear DNA, was resuspended in NaCl–Tris and dialyzed overnight to remove sucrose.

Preparation of Histones and Nonhistone Chromosomal Proteins. The dialyzed chromatin was extracted twice in 0.4 N H$_2$SO$_4$ (30 min each, 4°); the acid-insoluble material was collected by centrifugation at 37,000 × g for 15 min. The two supernatants were then pooled (histone fraction) and made 1.0% in sodium dodecyl sulfate (SDS). The "dehistonized" chromatin was washed three times in NaCl–Tris and dissociated in 1.0% SDS containing 1.0% 2-mercaptoethanol (1 hr, 37° with intermittent stirring). This fraction (nonhistone proteins) and the histones were then dialyzed overnight against 0.1% SDS–0.1% mercaptoethanol–0.01 M phosphate buffer (pH 7.0).

Polyacrylamide Gel Electrophoresis. SDS Gels. 6 × 75-mm gels of 7.5% acrylamide [0.1% SDS–0.5 M urea–5 mM EDTA–0.1 M phosphate (pH 7.0) (19)] were overlaid with a 20-mm spacer gel of 2.5% acrylamide prepared in the same polymerizing solution, but with 0.01 M phosphate buffer (pH 6.0). Sample loads were 15 μg of histones and 30 μg of nonhistone proteins in 0.05–0.10 ml containing 0.25 M sucrose; 0.1% bromphenol blue dye was added to the samples just before loading. Electrophoresis was at 8.0 mA/gel until the dye had reached the last 50 mm of the 7.5% gel (6.5–7 hr, 21°). Approximate molecular weights were calibrated against the relative migrations (20) of bovine-serum albumin (68,000), β-galactosidase (135,000), chymotrypsinogen (25,700), cytochrome c (11,700), ovalbumin (43,000), and myoglobin (17,200).

Disc-Gel Electrophoresis in SDS. The discontinous system of Neville (21) was used, with the lower gel buffer at pH 9.18; EDTA was omitted. Electrophoresis was at 100 V for 3–3.5 hr at 21°. Both types of gels were stained with Coomassie blue (22); densitometry was at 550 nm with a Gilford model 220 spectrophotometer equipped with a linear gel transport (Gilford, Oberlin, Ohio) and strip chart recorder.

Chemical Analyses. Protein was determined by the Lowry method (23) with bovine-serum albumin as a standard. RNA was separated from DNA by the procedure of Fleck and
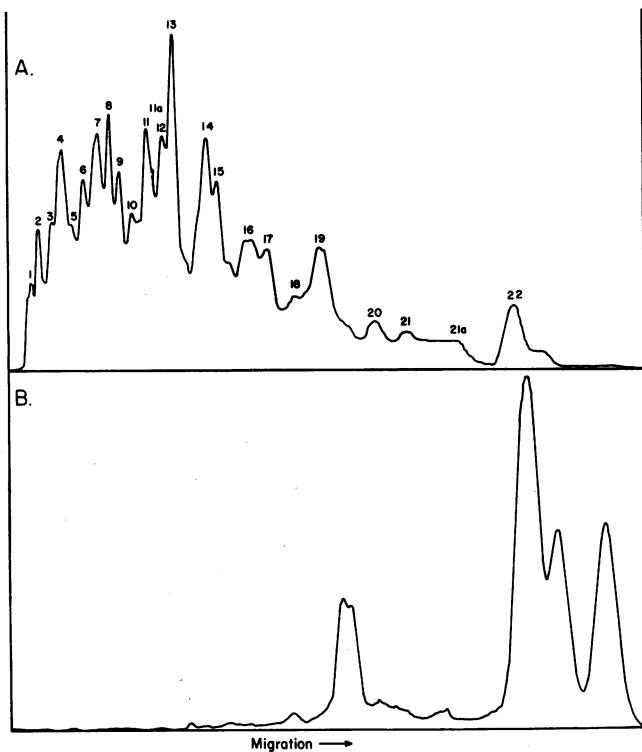
FIG. 1. SDS–polyacrylamide gel electrophoresis of HeLa chromosomal proteins. Densitometer tracings at 550 nm of Coomassie blue-stained gels shown in Fig. 2. Direction of migration, *left* to *right*. (*A*) Nonhistone chromosomal proteins. (*B*) Histones.

Munro (24) and determined by the orcinol reaction (25); DNA was measured by the diphenylamine method (25, 26).

RESULTS

Composition of HeLa Chromatin. As defined by insolubility in 0.4 N H_2SO_4, about 40% of the proteins of HeLa cell chro-
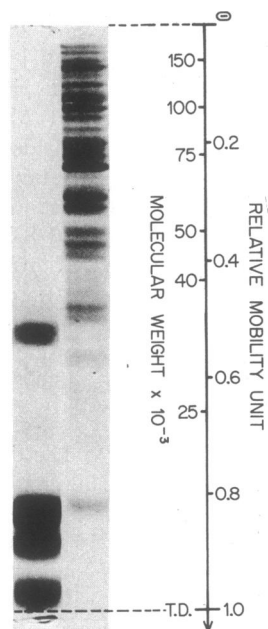

FIG. 2. SDS–polyacrylamide gel electrophoresis of HeLa chromosomal proteins. Direction of electrophoresis was *top* to *bottom*. Protein loads: 15 μg for histones (*left*) and 30 μg for non-histone chromosomal proteins (*right*). T.D. is tracking dye.

matin as isolated here are nonhistone. The relative proportions of histones (1.08 ± 0.02 μg/μg of DNA [± SE, n = 3]), nonhistones (0.70 ± 0.05), and RNA (0.055 ± 0.005) are similar to values reported for chromatin preparations from various other sources (10, 27, 28).

We were particularly concerned about nonspecific adsorption of cytoplasmic proteins to chromatin during the isolation procedure (29). Accordingly, cells were labeled for one generation with [³H]leucine and homogenized in RSB buffer; the cytoplasmic fraction was then used to homogenize an equal mass of unlabeled cells, from which chromatin was subsequently purified. From the measured specific activity of the labeled cytoplasm, and the amount of radioactivity in the final chromatin preparation (Table 1), contamination was estimated to be about 2.0% by mass (1.9 μg of cytoplasmic protein per 100 μg of chromatin).

Electrophoretic Characterization of Chromosomal Proteins. Electrophoresis of HeLa histones in SDS–polyacrylamide gels reveals four distinct bands, having approximate molecular weights of 33,000 (histone I), 14,000 (III), 11,000 (IIa and *b*), and 9,000 (IV) (Figs. 1*B* and 2). There is little other acid-soluble protein in HeLa chromatin. In contrast, when clean intact nuclei were extracted in 0.4 N H_2SO_4, electrophoresis revealed several additional polypeptides that comprised as much as 20% of the total protein entering the gel (Fig. 3). Clearly, the HeLa nucleus contains many basic proteins in addition to histones; the chromatin preparation has been significantly enriched for histones relative to these other basic nuclear proteins.

The complexity of the acidic proteins from HeLa chromatin is evident in Fig. 1*A* and 2 (right gel). There are 22 distinct bands (numbered in Fig. 1*A*) ranging from 15,000 to slightly over 180,000 daltons; most of this material is over 40,000 daltons (85% of the mass, 75% of the polypeptide chains). No aggregated material was trapped at the gel face; the complete entry of protein into the gel permitted the use of small sample loads (15–30 μg), probably a key factor in the high resolution obtained.

In view of the very high molecular weights of some of the acidic chromosomal proteins, we considered the possibility that these were undissociated DNA–protein complexes. By electrophoresing chromatin isolated from cells labeled for one generation with [¹⁴C]thymidine, no radioactivity was found in either the spacer or running gels (200,000 cpm of TCA-precipitable ¹⁴C in the chromatin sample loaded). While the possibility of SDS-insoluble RNA-protein com-
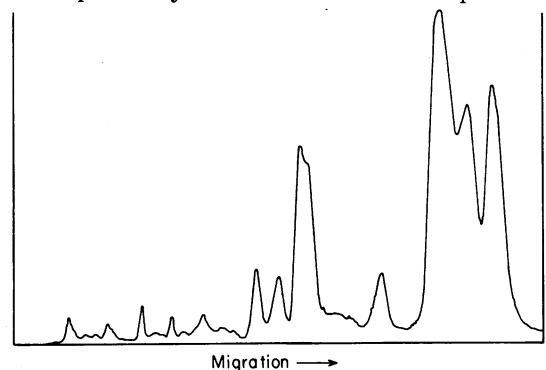

FIG. 3. Electrophoresis of total nuclear acid-soluble proteins. HeLa nuclei were extracted in 0.4 N H_2SO_4, and the acid-soluble fraction was electrophoresed as in Fig. 2. Direction of migration, *left* to *right*.

plexes entering the gel was not examined, we consider all of the Coomassie blue-positive bands to be polypeptide–SDS complexes.

The acidic protein numbered 22 (Fig. 1) migrates with an apparent molecular weight of 14,000, the same as one of the histone bands. However, data to be published elsewhere indicate that this protein has a 4.5-fold higher ratio of tryptophan to lysine than histones, indicating it is truly "nonhistone" rather than a trace of unextracted histone (52). A nonhistone protein of molecular weight 14,000 has also been observed in chromatin from rat liver and kidney and seaurchin embryos (11, 30).

It is also noteworthy that ribosomal structural proteins, which have molecular weights 10,000–55,000 (31, 32), are conspicuously absent from both the histone and acidic protein gels, indicating little contamination of the chromatin preparation by either nucleolar ribosomal precursor particles (33–35) or nucleoplasmic 60S ribosomal subunits (36).

Analysis of Nonhistone Chromosomal Proteins during the Cell Cycle. The rate of DNA synthesis and mitosis in HeLa cells synchronized by the double thymidine blockade method is illustrated in Fig. 4. Chromatin was isolated from cells harvested at the indicated times, and the nonhistone proteins were analyzed by electrophoresis in either standard SDS gels (continuous buffer) (19, 20) or in SDS-containing disc gels (21) (Fig. 5). Although the degree of resolution is similar in the two types of gels, the distribution of polypeptides differs considerably, providing separate endpoints for the cell cycle analysis. Despite the impression of cell cycle constancy in the photographs, densitometry revealed several quantitative changes, which were consistently observed in each of three separate synchronized cell experiments. Fig. 6 is a composite of densitometer tracings for the standard SDS gels shown in Fig. 5A. The arrows in the upper left panel (early G_1) indicate the polypeptides that underwent quantitative changes during the cell cycle. One of the most conspicuous shifts occurs in band 15, which is reduced by about 50% in mid-S and G_2 relative to other peaks (e.g., 14); this change is also apparent in the photograph (Fig. 5A). A relative reduction in the heights of peaks 4 and 17 is also evident in G_2. Finally, there is a striking decrease of band 11 at early S; it then reappears in mid-S. This effect is evident upon close inspection of Fig. 5A. In two other experiments, band 11 was reduced or absent in late G_1, as well as early S; thus, while the precise time of this change cannot be determined, it clearly occurs

TABLE 1. *Preparation of HeLa chromatin in the presence of [³H]leucine-labeled cytoplasm*

	cpm/mg of protein
[³H]leucine cytoplasm	260,000
Chromatin	8,520

900 ml of cells at 3×10^5/ml were suspended in medium containing half of the normal amount of leucine and were labeled for 18 hr with [³H]leucine, 0.5 μCi/ml. Cells were homogenized in RSB buffer, and the cytoplasmic fraction was used to swell and homogenize an equal mass of unlabeled cells. Aliquots of the labeled cytoplasm and final chromatin preparation were assayed for radioactivity and for protein (23). Calculation of cytoplasmic contamination was as follows: (i) 8520 cpm equals 0.03 mg of cytoplasmic protein. (ii) 1.0 mg of chromatin protein equals 1.57 mg of chromatin (because protein: DNA ratio of whole chromatin equals 1.7). (iii) Thus, contamination is 0.03 mg × 1/1.57 = 0.019 mg per mg chromatin, or 1.9% by mass.
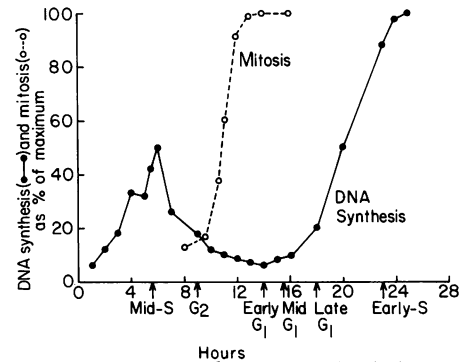

Hours

FIG. 4. Rate of DNA synthesis and mitosis in synchronized HeLa cells. 3500 ml of HeLa cells (3×10^5/ml) were exposed to 2 mM thymidine for 16 hr, resuspended in 3500 ml of fresh, warmed medium for 10 hr, then exposed to 2 mM thymidine again for 12 hr. [If the second block exceeds 12 hr, synchrony is poorer than that illustrated, since significant numbers of cells move across the G_1/S boundary before the population as a whole is released (37, 38)]. At 0 hr, the cells were resuspended in 7000 ml of fresh medium, at a density of 3×10^5 cells per ml. DNA synthesis was monitored at hourly intervals by labeling 1.0 ml of cells for 15 min with 0.5 μCi of [*methyl*-¹⁴C]thymidine, and determining the amount of incorporation into trichloroacetic acid-precipitable material. At 6 hr after release, 100 ml of the culture was removed and incubated with colchicine (0.2 μg/ml) to monitor the progression of cells into mitosis. At the times indicated by *arrows*, about 2.0 g of cells were harvested and fractionated.

at or near the G_1/S transition. Analyses of histones conducted in parallel (not shown) revealed no such quantitative changes over the cell cycle.

DISCUSSION

Depending upon one's conceptualization of the interphase nucleus, the prospect of isolating chromatin uncontaminated by nonchromosomal constituents may be viewed with either optimism or despair. The method used in the present study was designed to separate chromatin from the largest nuclear structures on the one hand (nucleoli), and from considerably smaller ribonucleoprotein particles on the other. While the
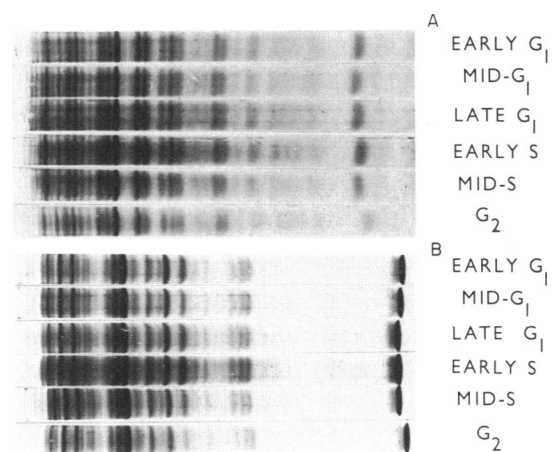

FIG. 5. Electrophoresis of nonhistone chromosomal proteins from synchronized cells. In each set of gels, successive cell cycle stages are shown going from *top* to *bottom;* direction of electrophoresis, *left* to *right.* (A) 0.1% SDS gels, sample load, 30 μg per gel. (B) Discontinuous gel electrophoresis in 0.1% SDS (21); sample load, 30 μg per gel.

absence of ribosomal structural proteins from the chromatin preparation is encouraging, as is the negligible contamination by cytoplasmic proteins, it is not possible to rule out the loss of some chromosomal components during isolation. On the other hand, chromatin prepared by more conventional methods (39) yields nonhistone proteins that display electrophoretic patterns similar to those described here (5, 11, 30, 40, 41). Therefore, we assume that the population of proteins in isolated chromatin constitutes at least an approximation of those associated with interphase chromosomes *in vivo*. We wish to also comment on our deliberate omission of mitotic stages from the cell cycle analysis of nonhistone chromosomal proteins.

Sadgopal and Bonner found that HeLa metaphase chromosomes were enriched in acid-insoluble proteins, as compared to interphase chromatin (49); this finding is compatible with the electron microscopic observation that nucleolar fragments become intimately associated with condensing chromosomes during prophase (50) and the related finding that isolated HeLa metaphase chromosomes contain substantial amounts of preformed ribosomal RNA precursors in the form of ribonucleoprotein particles (51). However, comparisons of metaphase chromosomes and interphase chromatin may be misleading, since these materials must of necessity be prepared by different techniques (49). A single method for
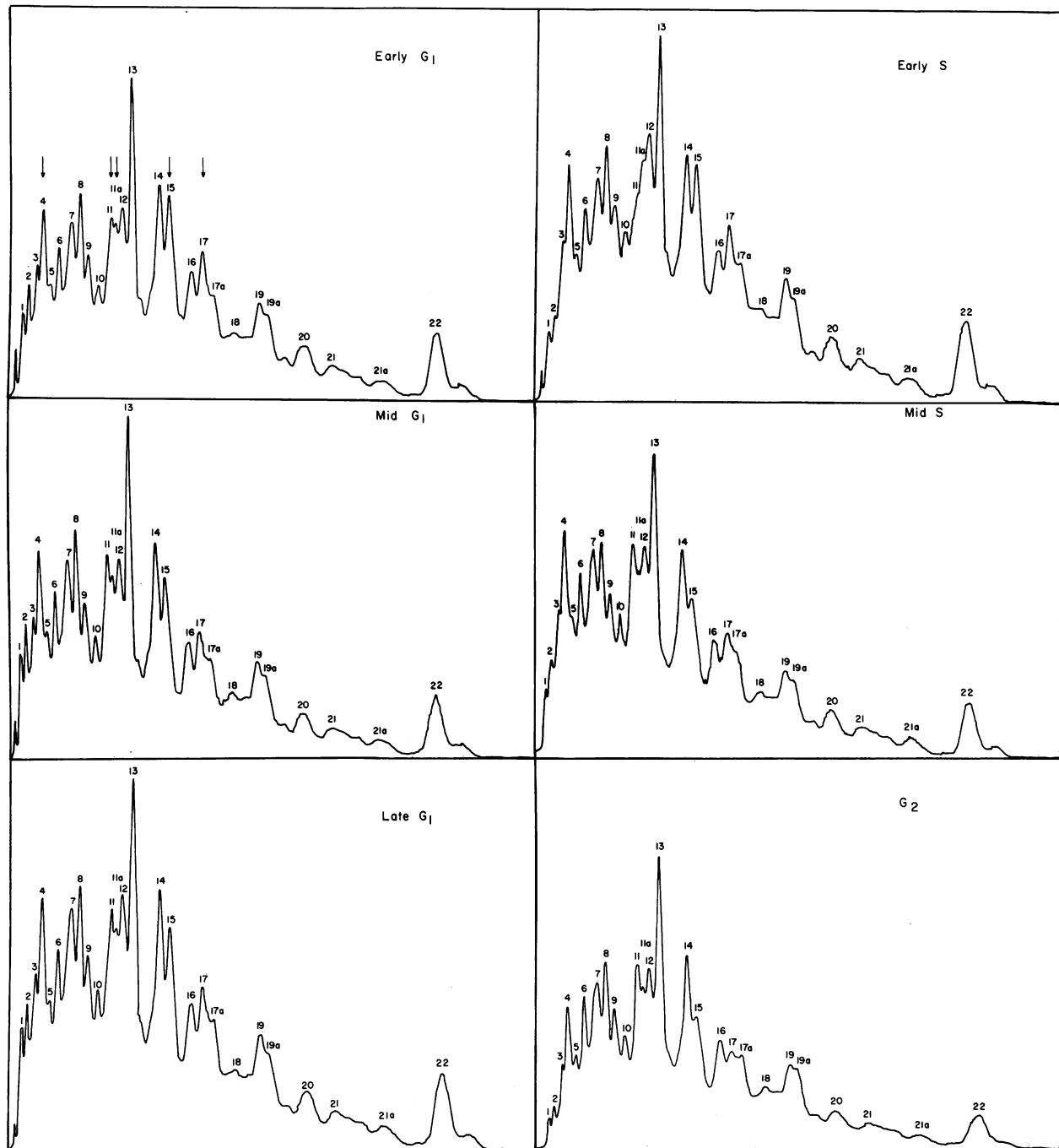

FIG. 6.   SDS–polyacrylamide gel electrophoresis of nonhistone proteins from synchronized cells. Densitometer tracings of the gels illustrated in Fig. 5A; direction of migration *left* to *right*. *Arrows* in top left panel (early G₁) indicate polypeptides that displayed quantitative changes as a function of the cell cycle.

the isolation of both materials, which properly considers the cell biology of mitosis, has not been devised.

Since nonhistone chromosomal proteins have been implicated in the regulation of transcription (12), one might view the cell cycle-dependent changes observed in this study as reflecting a program of differential gene expression that is played out as cells proceed through interphase (42). However, there is little compelling evidence that genes are transiently switched on and off during the cell cycle, particularly in higher eukaryotes. Even in the case of histone synthesis, the available data do not exclude the possibility that histone messenger RNA is synthesized throughout the cycle, but is translated only during the S phase (43); however, recent technical advances may soon permit this distinction to be made (44, 45). In the case of embryonic development, quantitative shifts in nonhistone chromosomal proteins have been observed (30), but are no more dramatic than those found in the present study with synchronized cells. Thus, even in situations where differential gene expression is probably abundant, changes in the amounts and kinds of regulatory chromosomal proteins may escape detection by present methods. This would be especially so if there were individual regulatory sites governing entire batteries of tandemly-reiterated, identical structural genes (53–55). Furthermore, it is likely that some regulatory effects of chromosomal proteins are mediated by phosphorylation (9, 12, 46), rather than by their selective deposition and removal altogether. In view of these considerations, it seems premature to attempt to relate quantitative shifts in nonhistone proteins to differential gene expression in this or any other system. What does seem clear on the basis of available data is that the overall macromolecular composition of chromatin varies throughout the G_1, S, and G_2 phases of the HeLa cell cycle. This result is compatible with recently-described structural transitions in interphase chromosomes, as resolved by chemical probes such as [³H] actinomycin (47) and DNase (48). As methods for the preparative fractionation of nonhistone proteins continue to evolve (30, 40, 41), it may become possible to assess more directly their roles in chromosome dynamics.

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