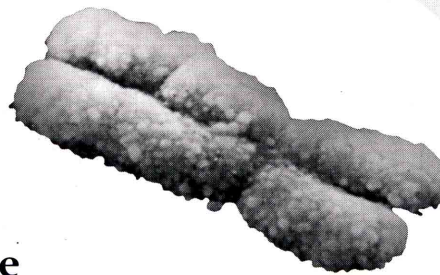


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Protein composition of human metaphase chromosomes analyzed by two-dimensional electrophoreses

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Abstract. A large amount of metaphase chromosomes were isolated from synchronized human cell lines by a polyamine procedure. All the chromosomal proteins extracted by an acetic acid extraction method were fully dissolved into the sample solutions for isoelectric focusing (IEF) or radical free and highly reduced (RFHR) two-dimensional electrophoreses (2-DEs). As a result, well-separated and highly reproducible 2-DE patterns were obtained. This could not be attained by an ordinary acetone precipitation method. The 2-DE patterns visualized using Coomassie Brilliant Blue (CBB) staining indicated that more than one hundred proteins were involved in the isolated metaphase chromosomes, although the most abundant proteins, histones, occupied a greater part of the chromosomal proteins. It

was also shown that colcemid treatment for cell cycle synchronization had little effect on the 2-DE pattern compared to that obtained without the treatment. Furthermore, no significant differences were observed in the 2-DE patterns among the chromosomal proteins prepared from two different human cell lines, BALL-1 and K562. However, 2-DE analysis of isolated metaphase chromosomes from HeLa cells apparently showed a smaller number of proteins than the BALL-1 and K562 cell lines at a neutral pI range. The present study paves the way for elucidating protein composition of human metaphase chromosomes.

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The formation of metaphase chromosomes is essential for the equal segregation of genetic information as well as the delivery of proteins to daughter cells (Sumner, 2003). Although more than a hundred years have passed since the first observation of this structure (Flemming, 1882) and a number of researchers have continued to study chromosomes (Lewis and

Laemmli, 1982; Rattner and Lin, 1985; Belmont, 2002; Swedlow and Hirano, 2003), proteomic information is quite limited to date (van Holde, 1988; Sumner, 2003). One of these situations originated from the lack of genome information, although a few chromosomal proteins, such as topoisomerase II α and condensin complex, were reported as the main components of a chromosome scaffold (Gasser et al., 1986; Maeshima and Laemmli, 2003). However, its relation to the structure of metaphase chromosomes remains controversial (Belmont, 2002; Christensen et al., 2002; Tavormina et al., 2002). The contribution of the condensin complex to chromosome condensation at mitosis was reported (Hirano and Mitchison, 1994; Ono et al., 2003). In addition, several chromosomal peripheral proteins or passenger proteins were also reported (Cooke et al., 1987; Hernandez-Verdun and Gautier, 1994). However, the importance of these proteins in the formation and maintenance of the chromosome structure, among all chromosomal proteins, was not well known.

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Recently, we reported on the preparation of large amounts of two types of human metaphase chromosomes (Sone et al., 2002). In that article, we also reported the relationship between the surface structure of two types of isolated chromosomes observed by scanning electron microscopy and examining their protein composition by SDS polyacrylamide gel electrophoresis (SDS-PAGE). It was suggested that the protein composition of metaphase chromosomes had to be analyzed to clarify the structure and function of metaphase chromosomes in combination with protein identification, using modern proteome techniques.

In the present study, we isolated a large amount of human metaphase chromosomes using the polyamine procedure, which has been frequently used in biochemical and morphological analyses (Spector et al., 1998). The chromosomal proteins were extracted by the acetic acid extraction method (Hardy et al., 1969). The separation of chromosomal proteins was performed by two-dimensional electrophoresis (2-DE), followed by the detection of proteins after Coomassie Brilliant Blue (CBB) staining. Two types of two-dimensional electrophoreses (2-DEs) – isoelectric focusing (IEF) or radical free and highly reduced (RFHR) 2-DEs – were employed for the separation of all chromosomal proteins. The effect of colcemid treatment on the protein composition for the cell-cycle synchronization was assessed by the IEF 2-DE. Furthermore, the protein compositions of three human cell lines were compared. The present article provides a basis for chromosome study based on constituent proteins.

Materials and methods

Reagents and buffers

Digitonin was purchased from Sigma-Aldrich Co. The proteins used for molecular mass references and IPG buffers 4–7 and 6–11 were purchased from Amersham Biosciences. The polyamine buffer contained 15 mM Tris-HCl (pH 7.2), 2 mM EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 0.2 mM spermine and 0.5 mM spermidine. The SDS sample buffer contained 31.25 mM Tris-HCl (pH 6.8), 25 mM DTT, 5% glycerol, 1% SDS and 0.05% BPB. The IEF sample buffer contained 7 M urea, 2 M thiourea, 65 mM DTT and 2% CHAPS. The IEF reducing buffer contained 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS and 16 mM DTT. The RFHR sample buffer contained 8 M urea and 1.5% 2-mercaptoethanol.

Cell culture and metaphase chromosome isolation

Human cell lines, BALL-1 (RCB0256) and K562 (RCB0027), were obtained from RIKEN Cell Bank (Riken, Tsukuba, Japan). The HeLa cell line was a kind gift from Prof. Yamamoto (University of Tokyo). BALL-1 and K562 cells are floating cells, while HeLa cells are adherent cells. Typically, 400 ml of 5×10^5 cells/ml BALL-1 or K562 cells were cultured in a 600 ml flask in RPMI 1640 medium (Invitrogen) containing antibiotics and 10% fetal bovine serum (Invitrogen). The cells were incubated at 37°C under a 5% CO₂-containing atmosphere. At a cell concentration of 5×10^5 cells/ml, colcemid was added at a final concentration of 0.02 µg/ml for synchronization of the cell cycle, followed by further incubation for 12 h. On an average, more than 60% of the mitotic index at the final cell concentration of 7×10^5 cells/ml was achieved by this procedure. Hereafter, unless otherwise stated, all the procedures for the purification of chromosomes were performed at 4°C. Chromosome isolation by the polyamine procedure was performed according to a previously published method (Spector et al., 1998). Cells washed with the polyamine buffer were collected by centrifugation at 440 g for 5 min. The cells were swollen under a hypotonic condition (75 mM KCl) for 30 min. After collection of the cells by centrifugation at 780 g for 10 min, cell lysis was performed with a vortex for 30 s in a polyamine buffer con-

taining 0.1% digitonin. The cell debris and nuclei were removed by two subsequent centrifugations at 190 g for 3 min, and the supernatant containing chromosomes was centrifuged at 1,750 g for 10 min. The chromosome pellet was resuspended in the polyamine buffer and centrifuged again at 1,750 g for 10 min to collect the isolated chromosomes. The isolated chromosomes were stored at –80°C in a polyamine buffer containing 70% glycerol. In this procedure, the chromosome structure is maintained by the polyamines, spermine and spermidine, instead of divalent cations such as Mg²⁺ or Ca²⁺ (Spector et al., 1998; Hudson et al., 2003). Chromosome isolation from the BALL-1 cell line without colcemid treatment was also performed using the polyamine procedure. When the chromosomes were isolated from the HeLa cells, chromosomes were isolated from the cells arrested by colcemid treatment at the mitotic phase, which were easily released from the culture dish by tapping.

Extraction of proteins from isolated chromosomes

The extraction of proteins from isolated chromosomes was performed by the acetic acid extraction method for 2-DEs (Hardy et al., 1969). Using this method, nucleic acids were removed as precipitant, while proteins were collected in a soluble form. The chromosomes were resuspended in a polyamine buffer containing 100 mM MgCl₂, followed by the addition of two volumes of glacial acetic acids. After mixing with vortex and shaking for 1 h at 4°C, the nucleic acids were removed as precipitant by centrifugation. The second extraction of the remaining pellet was then performed. The two resulting supernatants were pooled and dialyzed against 1,000 volumes of 2% acetic acid. The chromosomal proteins were lyophilized and dissolved into water containing Complete Protease Inhibitor cocktail (Roche), and the concentration of the proteins was determined using the Advanced Protein Assay Reagent (Cytoskeleton). Typically, a single extraction experiment using isolated chromosomes from 2.8×10^8 cells yields 500 µg proteins. Aliquots of the proteins were lyophilized again and stored as powder materials at –80°C.

In the case of protein extraction using acetone precipitation, twice the volume of –20°C acetone was added to the solution containing isolated chromosomes, followed by centrifugation at 15,000 g for 15 min.

Separation of chromosomal proteins by electrophoreses

One-dimensional (1-D) SDS-PAGE of the extracted proteins was performed according to a previously published method. In the case of IEF 2-DE, 500 µg of proteins extracted by the acetic acid method were solubilized in IEF sample buffer. IEF was carried out with Ettan IPGPhor (Amersham Biosciences) using Immobiline DryStrips, pH 4–7 or 6–11, at 20°C with 80,000 Vh. On completion of the focusing time, the proteins were reduced using the IEF reducing buffer and alkylated with 0.24 M iodoacetamide. The 2-DE was then carried out using 12% polyacrylamide gel. For the RFHR 2-DE of the chromosomal proteins, lyophilized proteins were solubilized in the RFHR sample buffer and incubated for 30 min at 40°C. Separation by RFHR 2-DE was performed as described previously (Wada, 1986). Generally, 1 mg of extracted proteins was applied to the RFHR 2-DE. Each protein was detected after CBB staining. Separations by SDS-PAGE and IEF 2-DE were repeated more than six times.

Detection of proteins and identification of histones

Proteins separated using 2-DEs were detected by ImageMaster 2-D Elite (Amersham Biosciences). Identification of the histones was performed by the peptide mass fingerprinting method. Briefly, the highly intense spots were excised from the gel, and in-gel digestion was performed using trypsin at 37°C. The peptides were eluted with 50% acetonitrile with 0.1% trifluoroacetic acid, and then desalted and concentrated by a ZipTip C18 (Millipore Corp.). The purified peptide solution was mixed with equal volumes of saturated cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA. The mass of the peptide was measured by a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF MS), Autoflex (Bruker Daltonics). The peptide mass fingerprints were analyzed using Mascot software (Matrix Science, www.matrixscience.com) with an NCBI database.

Results

Extraction of proteins from human metaphase chromosomes

Human metaphase chromosomes isolated using the polyamine procedure demonstrate authentic morphology upon observation by optical microscopy after Giemsa staining (Fig. 1). The surface structure of the isolated chromosomes observed by SEM showed a smooth structure covered irregularly with scaly structures (data not shown) as reported in a previous study (Sone et al., 2002). The chromosomal proteins were extracted by the acetone precipitation method, and the proteins were tested to be solubilized in the IEF buffer for 2-DE analysis. However, most proteins remained insoluble, especially the core and linker histones (Fig. 2, lane 1), and resulted in a poorly reproducible 2-DE pattern (data not shown). Then, the acetic acid extraction method, which was originally developed for the extraction of constituent proteins from ribosome (Hardy et al., 1969), was employed to extract the chromosomal proteins. However, this procedure gives rise to a complex of nucleic acids and basic proteins. Practically, no precipitant was observed after the centrifugation of the IPG buffer containing chromosomal proteins (data not shown), indicating the absence of large aggregates of the chromosomal proteins. An equal volume of SDS sample buffer was added to the supernatant, followed by its application to 1-D SDS-PAGE. The resultant pattern (Fig. 2, lane 3) was close to that obtained in the case of direct solubilization in SDS sample buffer (Fig. 2, lane 2), indicating the complete extraction and solubilization of chromosomal proteins in the IPG buffer with the present procedures. Likewise, the complete solubilization of the extracted chromosomal proteins in the RFHR sample buffer was also confirmed.

2-DEs of chromosomal proteins

The chromosomal proteins with different pI values were well separated by the IEF 2-DEs with pI 4–7 and 6–11, as shown in Fig. 3. Several well-separated intense spots were detected at around pI 6 in the IEF 2-DE pattern with pI 4–7 (Fig. 3A), while intense spots of core and linker histones were detected at the most basic region (pI 6–11) in the IEF 2-DE pattern (Fig. 3B). As indicated in Fig. 2, histones occupied a greater part of the proteins that were involved in isolated metaphase chromosomes.

As shown in Fig. 4, several basic non-histone proteins were detected in RFHR 2-DE as small and intense spots around the large and intense spots of the histones.

The numbers of spots detected by image analysis were 105 for the pI range 4–7 and 95 for the pI range 6–11. The number of spots detected in the RFHR 2-DE pattern was 125. Because protein detection was performed after CBB staining in the present analysis, a significant amount of proteins were detected. In addition, proteins of some spots in the IEF 2-DE pattern in the pI range from 4–7 presumably corresponded to the same proteins detected in the IEF 2-DE pattern in the pI range from 6–11. Therefore, it could be concluded that isolated human metaphase chromosomes contain, at least, more than one hundred proteins.

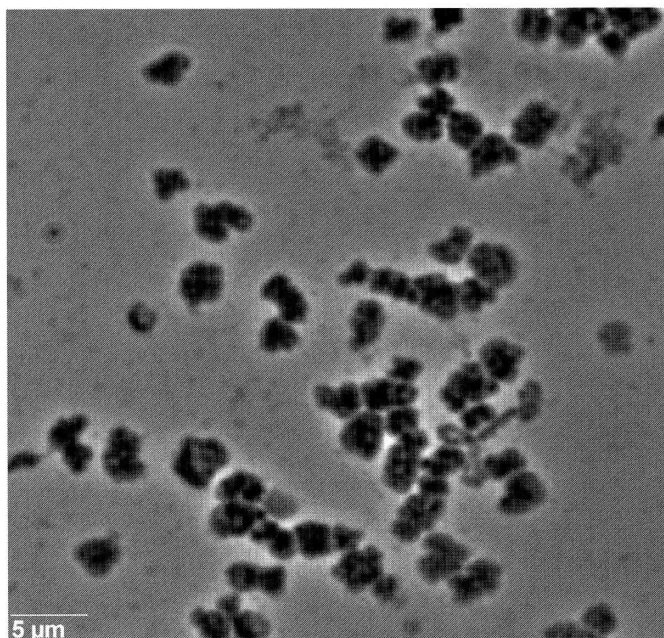


Fig. 1. Light microscopy observation of isolated human metaphase chromosomes by the polyamine procedure. Bar is 5 μ m.

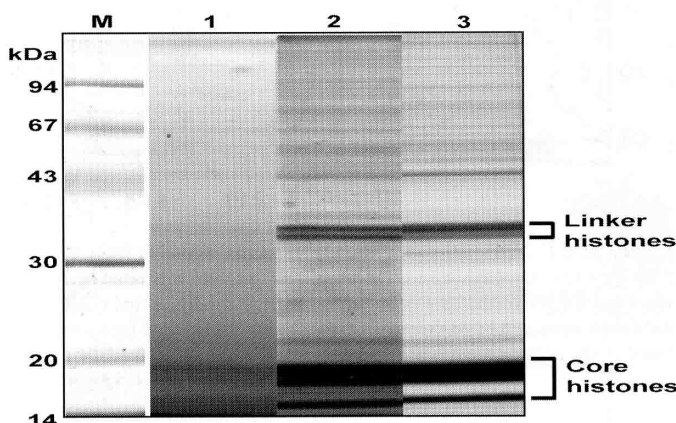


Fig. 2. Differences in solubilization of the chromosomal proteins of BALL-1 cells prepared by different protein extraction methods. (M) Molecular weight marker. (1) Chromosomal proteins were extracted by the acetone precipitation method, followed by solubilization in SDS sample buffer. (2) Chromosomal proteins directly solubilized in the SDS sample buffer. (3) Chromosomal proteins extracted by the acetic acid method (followed by solubilization in the IEF 2-DE buffer) were applied to 1-D SDS-PAGE.

Effect of synchronization by colcemid treatment on protein constituent

Figure 5A shows the IEF pattern of the proteins in the pI range from 4–7 from the isolated chromosomes without colcemid treatment. The pattern is very close to that of the proteins from the chromosomes with colcemid treatment. These

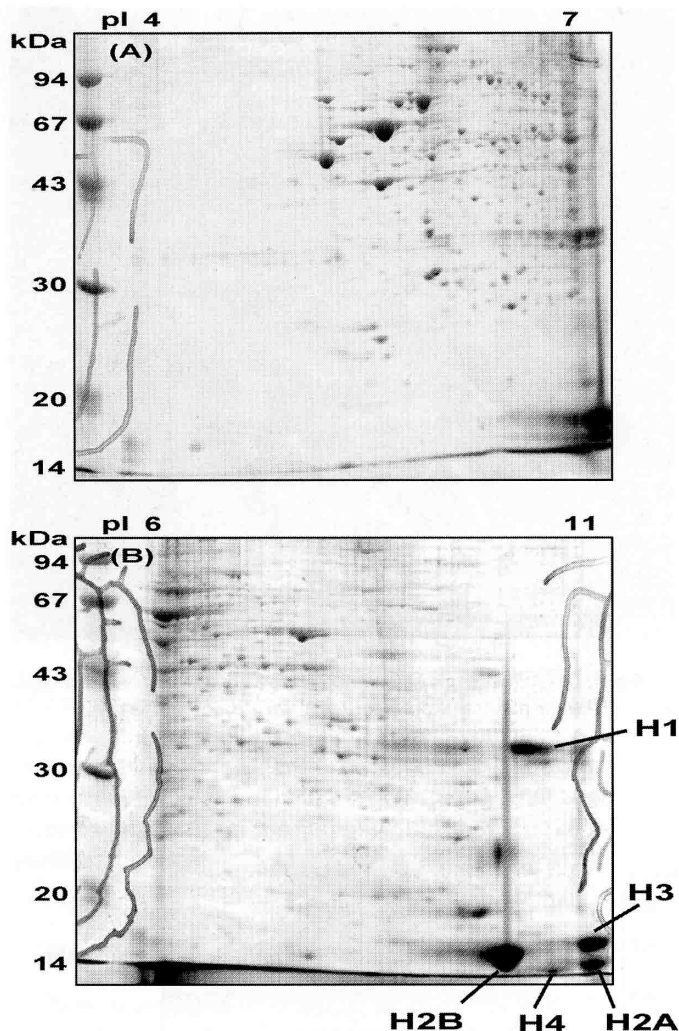


Fig. 3. IEF 2-DE patterns of the chromosomal proteins from BALL-1 cells. Proteins extracted by the acetic acid method were separated by IEF on immobilized pH gradients 4–7 (**A**) or 6–11 (**B**) in the first dimension. They were then separated by SDS-PAGE in the second dimension. Histones identified by the peptide mass fingerprinting method were indicated.

results indicate that the effects of synchronization by colcemid treatment had no significant effect on the protein composition of the metaphase chromosomes. This is reasonable since colcemid prevents the formation of the spindle apparatus responsible for cell division and does not inhibit the formation of metaphase chromosomes themselves.

Differences in protein compositions among different cell lines

Three cell lines, BALL-1, K562 and HeLa cells, were tested for the composition of chromosomal proteins. The protein composition of isolated metaphase chromosomes from the K562 cells revealed by IEF 2-DE was quite similar to that of the BALL-1 cells (Fig. 5B). On the other hand, the 2-DE pattern of the HeLa cells indicated a less number of proteins in the slightly

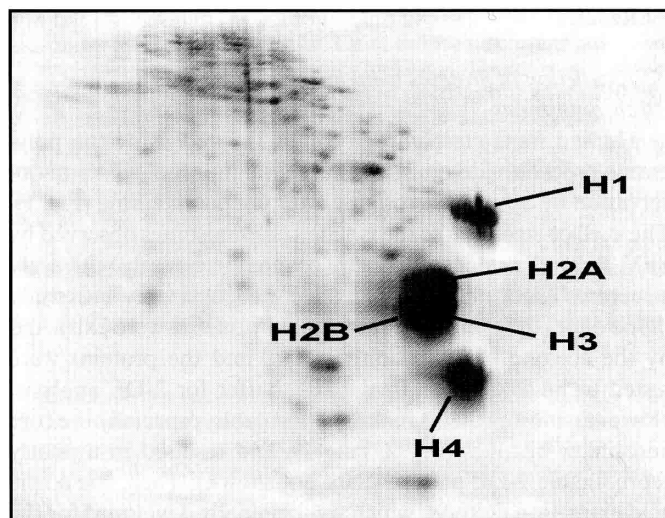


Fig. 4. RFHR 2-DE patterns of chromosomal proteins from BALL-1 cells. Proteins extracted by the acetic acid method were separated by RFHR 2-DE. Histones identified by the peptide mass fingerprinting method were indicated.

low or neutral pI region, as shown in Fig. 5C. The intense spots detected at low pI region were similar to those in the two other cell lines.

Discussion

The protein composition of metaphase chromosomes analyzed by SDS-PAGE and IEF 2-DE was previously reported (Wray et al., 1980; Adolph and Phelps, 1982; Detke and Keller, 1982). In these reports, IEF 2-DE was employed for the separation of proteins with neutral or acidic pI. On the contrary, the protein composition of metaphase chromosomes were revealed using not only SDS-PAGE and IEF 2-DE but also RFHR 2-DE in the present study. The separation of proteins with high pI value, such as histones, using IEF 2-DE is generally difficult because the formation of an immobilized pH gradient at a high pH value is limited. On the contrary, in the case of RFHR, the separation of proteins is based on the differences in the migration of each protein according to the net charge at pH 8.2, providing no practical limitation of the pI value (Wada, 1986). Actually, highly basic proteins, core and linker histones, were separated from other basic proteins (Fig. 4). As a result, the present study provides information on the overall constituent proteins including those with highly basic pI values. The number of chromosomal proteins could be estimated at least at more than two hundred based on the present 2-DE pattern. This value is comparable to that in a previous report (Adolph and Phelps, 1982), where 280 proteins were detected.

Intense spots of non-histone proteins in IEF 2-DE pattern were concentrated at the pI region from 5.5–6 and the molecular weight from 40 to 80 kDa. This pattern is similar to that observed previously (Wray et al., 1980). Some of these proteins

with large amounts might correspond to those reported in the 2-DE pattern of metaphase chromosome scaffold (Detke and Keller, 1982).

BALL-1 has a lymphocyte-like morphology and is a typical human B cell leukemia cell line (Miyoshi et al., 1977), and K562 has a lymphoblast-like morphology and is a chronic myelogenous leukemia cell line (Lozzio and Lozzio, 1975; Andersson et al., 1979). The chromosome number of the floating cells, BALL-1 and K562, ranged from 46 to 48 and from 59 to 68, respectively (RIKEN Cell Bank, <http://www.brc.riken.jp/>). The types of cell lines are similar to each other but have different chromosome numbers. As for the HeLa cells, which are epithelial-like adherent cells, the chromosome number ranges from 77 to 86. Because the chromosome structure at optical microscopic resolution is not different for all the three cell lines tested, proteins found in all the three cell lines with similar amounts are essential for chromosome structure and function. The similarity of abundant proteins of metaphase chromosomes between two species, Chinese hamster ovary and human HeLa cell lines (Wray et al., 1980), further suggests the similarity of constituent proteins of metaphase chromosomes in higher eukaryotes.

HeLa cells, which are adherent cells, become rounded during prophase and microfilament bundles are disassembled at mitosis. Further, focal adhesions, to which microfilament bundles anchor, are also disrupted, causing reduced adhesion to culture dish. Therefore, in the case of isolation of metaphase chromosomes from HeLa cells, the isolation is effectively performed from cells at mitosis, eliminating unexpected adhesion of proteins originated from cells at interphase during the chromosome isolation. Thus, proteins that were detected for only BALL-1 and K562 cells might be contaminant proteins adhered to chromosomes during the isolation processes.

The present results show that all the necessary conditions have been determined for the proteome analysis of constituent proteins of human metaphase chromosomes. It is anticipated that over a hundred chromosomal proteins will be identified in the near future for a complete understanding of the metaphase chromosomes based on protein constituent.

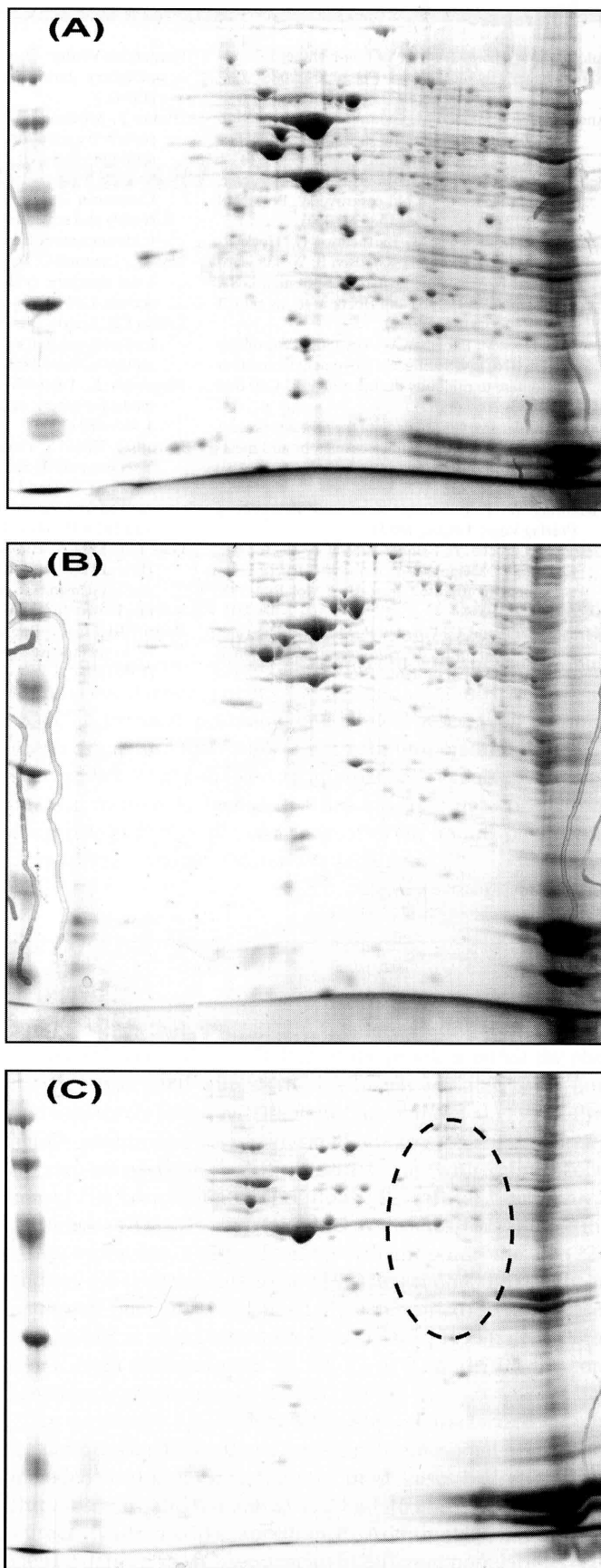


Fig. 5. IEF 2-DE patterns for isolated metaphase chromosomes prepared from (A) BALL-1 cells without colcemid treatment, (B) K562 cells and (C) HeLa cells. The HeLa cell strain contains less proteins at a slightly low or neutral pI region (dashed circle).

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