

RAPID COMMUNICATIONS

RAPID ISOLATION OF METAPHASE CHROMOSOMES CONTAINING HIGH MOLECULAR WEIGHT DNA

ALAN B. BLUMENTHAL, JEFFREY D. DIEDEN, LEON N. KAPP, and JOHN W. SEDAT. From the Laboratory of Radiobiology and the Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

ABSTRACT

Metaphase chromosomes with high molecular weight DNA were isolated from Chinese hamster ovary (CHO) cells in a neutral buffer containing polyamines and chelators. The individual, unfixed chromosomes retained their centromeric and secondary constrictions, distinct sister chromatids, and complex banding patterns. The DNA from these chromosomes was 100-fold larger (2×10^8 daltons) than DNA from chromosomes isolated by other procedures. These characteristics indicate preservation during isolation of considerable native structure. In contrast to chromosomes produced by other methods, these chromosomes were stable in storage and did not aggregate, thus providing useful material for studies of the structure and biochemistry of individual chromosomes.

KEY WORDS chromosomes · DNA · Chinese hamster cells · metaphase

Several procedures have been described for isolation of metaphase chromosomes (4–7, 10, 13). Most use acidic isolation buffers (8) or a neutral buffer in the presence of 2-methyl-2,4-pentanediol plus divalent cations (15) to stabilize chromosome morphology. These isolation conditions lead to the degradation of chromosomal DNA (16) either by deperination at low pH or by nuclease activity at neutral pH in the presence of divalent cations. High molecular weight DNA has been obtained from metaphase chromosomes by raising the pH of the isolation buffer to 9.6–10.5 (14, 16). This high pH, however, may have undesirable effects on the recovery of acidic proteins from metaphase chromosomes. In this report, we present a procedure for rapidly obtaining mammalian metaphase chromosomes, at neutral pH, that contain high molecular weight DNA. We employed the buffer system described by Wallace et al. (12), which uses polyamines rather than divalent cations to stabilize chromosome structure and heavy metal chelators to prevent nuclease activity. This buffer

has been used successfully in isolating *Drosophila* polytene chromosomes and diploid nuclei without changing their native structure (9).

MATERIALS AND METHODS

Cell Lines

Chinese hamster ovary (CHO) cells were grown in McCoy's 5A medium supplemented with 15% calf serum, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer, 50 μ g/ml of streptomycin, 50 U/ml of penicillin, and 50 μ g/ml of chlorotetracycline, in either Falcon plastic T-flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) or glass roller bottles. Cultures were labeled for 12–15 h with 0.2 μ Ci/ml of [14 C]thymidine (sp act, 10 Ci/mmol) or 0.1 μ Ci/ml of [3 H]thymidine (50 Ci/mmol) before harvesting of mitotic cells.

When the cell cultures in T-flasks were ~80% confluent (doubling time was ~12 h), loosely attached cells were removed by gentle shaking, and fresh medium containing colcemid (2×10^{-7} M) was added. Cultures were then incubated for 3–4 h at 37°C, and mitotic cells were harvested by gentle shaking. Cells in roller bottles were grown to ~50% confluence. Loosely attached cells or cells incubated with colcemid, as described above, were removed by rotating the bottles at 180 rpm for 3–5

min. The mitotic index of the cells obtained from T-flasks and roller bottles was ~95%.

Chromosome Isolation

Mitotic cells were centrifuged at room temperature for 5 min at 1,000 rpm (950 *g*) in a model PR-1 International centrifuge (International Equipment Co., Needham Heights, Mass.). The resulting pellet was resuspended in ~5 ml of buffer A (15 mM Tris-HCl, 0.2 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA, 80 mM KCl, 20 mM NaCl, and 14 mM β -mercaptoethanol), pH 7.2 (12) and centrifuged at 1,500 rpm for 2 min. This was repeated twice.

Two procedures were then used to prepare suspensions of metaphase chromosomes. In procedure 1, the cell pellet was drained carefully and $\sim 2 \times 10^7$ cells were resuspended in 1 ml of buffer A plus 0.34 M sucrose, 0.1% digitonin, and 0.5 M 2-methyl-2,4-pentanediol. A 10-fold concentrated buffer A solution (pH 7.4) was used to prepare this solution. The final pH was 7.0. Digitonin was added to the solution just before use and dissolved as completely as possible by warming to 37°C. The solution was equilibrated to room temperature (22–23°C) and then added to the cell pellet. The cell pellet was resuspended in buffer A by mixing on a Vortex mixer (Scientific Industries, Inc., Bohemia, N. Y.) for 5–20 s. Chromosomes were then released by gently drawing the cell suspension up and expelling it through a 25-gauge needle ($\frac{5}{8}$ inch long) attached to a 3-ml plastic syringe. This was repeated three to four times. Foaming of the solution was avoided.

For procedure 2, the drained cell pellet was resuspended in ~20 times its volume of cold (0°C) buffer A (pH 7.2 at 25°C) plus 0.1% digitonin. 50–100 μ l of cell suspension was placed in an untreated 6 \times 50 mm glass tube or 1 ml of suspension in a 15-ml conical plastic centrifuge tube (No. 25310; Corning Glass Works, Science Products Div., Corning, N. Y.). The tube was then held on a Vortex mixer set at high speed. The mixing time was divided into 1-min intervals, and the tube was cooled on ice for 2–5 min between these intervals; total mixing time was 3–6 min. The Vortex mixer was used to release metaphase chromosomes by creating a thin layer of cell suspension on the wall of the tube through which the cells were moving at maximum velocity. Foaming of the solution was avoided.

Chromosome release was monitored by placing a drop of the suspension on a microscope slide, which had a dried droplet of ethidium bromide ($\sim 0.5 \mu$ g) on the surface, or by including 10 μ g/ml of Hoechst 33258 in the isolation buffer. The chromosomes and/or cells were then observed with a fluorescent microscope. Suspensions of free chromosomes were stored at 4°C for further analysis. No changes in chromosome morphology were observed after 2–6 wk of storage.

Chromosomes were also isolated by a modification of the method of Wray and Stubblefield (15). The mitotic cell pellet, prepared as described above, was resuspended in 2 ml of a trypsin solution (0.25% trypsin

[Grand Island Biological Co., Grand Island, N. Y.] dissolved in 0.8% NaCl, 0.04% KCl, 0.01% phenol red, 0.1% dextrose, and 0.038% Na₂HCO₃; all solutions wt/vol) and incubated at 37°C for 4 min. 2 ml of McCoy's 5A medium plus serum was added to stop the trypsin reaction. The cells were centrifuged at 950 *g* for 4 min, resuspended in McCoy's 5A medium without serum, and cooled on ice for 20 min. The cells were again centrifuged at 950 *g* for 4 min and then resuspended in isolation buffer (1 M 2-methyl-2,4-pentanediol, 0.5 mM CaCl₂, and 0.1 mM piperazine-N,N'-bis[2-ethane sulfonic acid] monosodium monohydrate), pH 6.5, and again centrifuged as above. The pellet was resuspended in chromosome isolation buffer and incubated at 37°C for 10 min. Release of chromosomes was accomplished by passing the cell suspension three to four times through a 25-gauge needle, as described above.

Alkaline Sucrose Gradient Analysis

Chromosome suspensions (0.5 ml) were layered on 0.5 ml of a lysis solution (0.5 M NaOH, 0.02 M EDTA, 0.1% NP-40) over a 5–20% alkaline sucrose gradient containing 0.1 M NaOH, 0.9 M NaCl, and 0.01 M EDTA. Lysis of chromosomes and release of free DNA were allowed to proceed by storing the gradients for 30–60 min at room temperature in the dark. The gradients were centrifuged in a Beckman SW27.1 rotor at 20°C for various times and speeds.

After centrifugation, the bottom of each centrifuge tube was punctured and the gradients were collected into 20 fractions of equal volume. 200 μ g of calf thymus DNA was added to each fraction and the DNA was precipitated by adding 1 ml of 6 M HCl and 6% sodium pyrophosphate to each fraction and cooling to 0°C. The precipitates were collected on Whatman GF/C filters, washed with 4% perchloric acid, washed consecutively with 70, 95, and 100% ethanol, and dried for scintillation counting.

Sedimentation coefficients of the DNA were calculated from calibration standards (phage T4 and λ DNAs) previously run under identical conditions in this laboratory (1). Number average molecular weights were estimated from sedimentation coefficients by the equation of Studier (11).

Chemicals

EGTA, digitonin (B grade), spermidine (A grade), and spermine were obtained from Calbiochem, La Jolla, Calif. EDTA and Tris (base) were obtained from Sigma Chemical Co. (St. Louis, Mo.). 2-methyl-2,4-pentanediol was obtained from J. T. Baker Chemical Co. (Phillipsburg, N. J.), and NP-40 from Particle Data Inc. (Elmhurst, Ill.).

RESULTS

Chromosome Isolation

The shearing procedures and buffer used re-

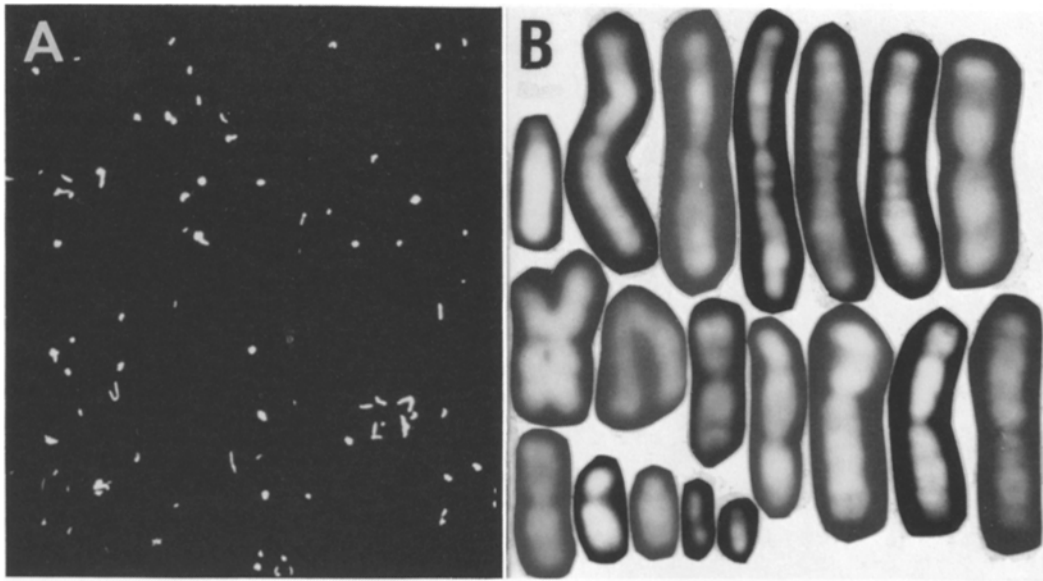


FIGURE 1 Metaphase chromosomes released from CHO cells. Chromosomes were prepared as described in procedure 2 of Materials and Methods and stained with the fluorescent dye Hoescht 33258. (A) A typical field of chromosomes observed at low power ($\times 40$ objective) showing primarily separate chromosomes (total magnification about 450). (B) Individual chromosomes, from a field as seen on the left, photographed with a $\times 100$ objective (total magnification about 4,400).

sulted primarily in single chromosomes with normal morphology (Fig. 1A). The chromosomes had the same size distribution as those in a standard Giemsa's-stained CHO karyotype (results not shown). Isolated chromosomes retained their centromeric and secondary constrictions and distinct sister chromatids (Fig. 1B). Because these chromosomes were not subjected to extremes of pH or denaturing conditions, their substructure (e.g., banding) was preserved. The chromosomes retained this morphology and did not aggregate during storage at 4°C for 2–6 wk. Digitonin was necessary to release chromosomes from the cells. 0.5 M 2-methyl-2,4-pentanediol facilitated breakage of cells (procedure 1) and release of chromosomes. In the procedure using 2-methyl-2,4-pentanediol, a pH of 6.9–7.0 was critical for preserving chromosome morphology. Triton X-100 (0.1%) also released chromosomes from the cells, but these chromosomes were easily distorted by the shear forces of isolation.

DNA from Isolated Chromosomes

The size of DNA from ^{14}C -labeled chromosomes isolated in buffer A was analyzed on alkaline sucrose velocity gradients and compared to the size of DNA from chromosomes isolated in

the Wray and Stubblefield buffer. DNA from chromosomes isolated in buffer A sedimented rapidly compared to DNA from chromosomes prepared in the Wray and Stubblefield buffer (Fig. 2A). About 56% of the DNA sedimented slowly (fractions 10–20) after isolation in the Wray and Stubblefield buffer, compared to 13% after isolation in buffer A (Fig. 2A). The number average molecular weight of the DNA isolated in the Wray and Stubblefield buffer was $(2.2 \pm 0.3) \times 10^6$ (mean \pm SD). The average molecular weight of the DNA from chromosomes isolated in buffer A (Fig. 2B) was $(2.4 \pm 1.0) \times 10^6$.

DISCUSSION

Stable, individual chromosomes with normal morphology can be isolated from mammalian (CHO) cells in a modified buffer A, originally devised by Wallace et al. (12).¹ This buffer employs the polyamines, spermine and spermidine, to stabilize chromosomes and chelators (EDTA and EGTA)

¹ The isolation procedure described here is also applicable to chromosome isolation from human cells (E-11 human diploid fibroblasts; J. D. Dieden and L. N. Kapp, unpublished observations) and *Drosophila* cells (J. W. Sedat, unpublished observations) in culture.

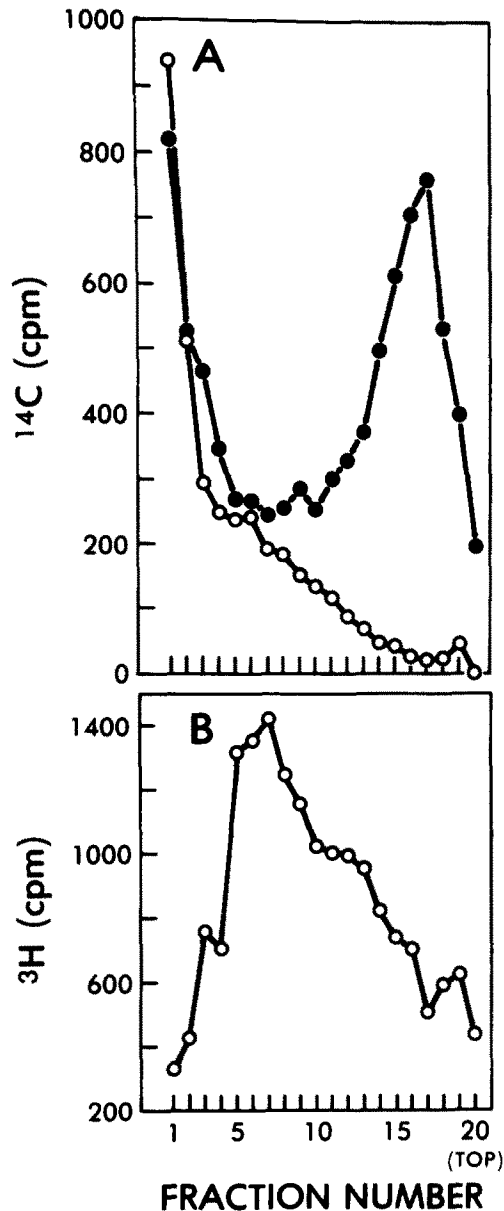


FIGURE 2 Sedimentation of DNA in isolated chromosomes. Chromosomes were prepared by procedure 1, described in Materials and Methods. The chromosome suspensions were then layered on top of a lysis solution on a 5–20% alkaline sucrose gradient and centrifuged. (A) DNA from chromosomes isolated in buffer A (○) or as described by Wray and Stubblefield (●) (15) and centrifuged in parallel gradients for 18 h at 18,000 rpm. (B) DNA from chromosomes isolated in buffer A, centrifuged for a shorter time (4 h) at 20,000 rpm to determine the molecular size distribution.

to prevent nuclease action. Buffer A has been shown to preserve *in vivo* chromosome structure, as observed by fluorescence and polarization microscopy or by high voltage, electron transmission microscopy (9). Preliminary results indicate that CHO chromosomes isolated in buffer A can be fractionated into size classes, as described by Mendelsohn et al. (8) and can be purified in gradients of metrizamide in buffer A.

DNA from chromosomes isolated in buffer A is at least 100-fold larger than that obtained from chromosomes isolated in the pH 6.5 buffer of Wray and Stubblefield. This DNA sediments rapidly in alkaline sucrose velocity gradients, indicating a molecular weight equivalent to that obtained from interphase eukaryotic cells lysed directly in alkali ($2\text{--}5 \times 10^8$ daltons) (2). Large DNA has also been obtained from chromosomes by Wray et al. using a pH 10.5 isolation buffer (16). However, the advantages of the procedure presented in this work are that high molecular weight DNA can be obtained from chromosomes that are isolated at physiological pH and maintain many of their *in vivo* structural characteristics. Also, chromosomes in buffer A are stable in storage for long periods and do not aggregate.

Chromosomes isolated in buffer A will be useful in studies of morphology and for the analyses of intact DNA and associated proteins. The stability of these chromosomes and their lack of aggregation should allow separation of individual chromosomes by flow microfluorimetry (3). They can then be used for studies of the control of DNA replication in individual chromosomes and for investigations of the DNA sequences and proteins specific to individual chromosomes.

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