Quantitative Electron Microscopy of Intracytoplasmic Type A Particles at Kinetochores of Metaphase Chromosomes Isolated from Chinese Hamster and Murine Cell Lines

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SUMMARY

We have successfully isolated and spread individual chromosomes of CHO-K1 cells for electron microscopic karyotyping. Controlled preparation permitted a quantitative evaluation of the association between endogenous intracytoplasmic type A virus precursor particles and the centromeric region (kinetochores) of isolated chromosomes at prophase and metaphase. Our results suggest the transfer of type A particles from the cytoplasmic to the centromeric regions during early metaphase in conjunction with microtubule assembly at a time when the kinetochores are structurally mature and capable of binding microtubules. Preliminary comparable studies of the endogenous M432 virus propagated in murine cells support these findings. Our results are discussed with respect to mechanisms of intracellular movement of virus precursor particles and the interference with components of both the cytoskeleton and the mitotic apparatus.

INTRODUCTION

Intracytoplasmic type A particles are known to be precursors of retroviruses of type B and D and of certain, probably related, endogenous viruses isolated from Chinese hamster cells (Wheatley, 1974; Calafat & Hilkens, 1978; Heine et al. 1979), some murine (Callahan et al. 1976; Heine & Todaro, 1978) and certain marsupial cell lines (Hamilton et al. 1979). They persist also in other cell lines which, to our knowledge, do not release mature virus particles (Pepper & Brinkley, 1977). The reported association of these type A particles with the mitotic apparatus in some of the cell lines has attracted wider investigative interest and suggested a possible mechanism of epigenetic transmission of some of these endogenous viruses (Wheatley, 1974; Heine & Todaro, 1978). It has been shown that the type A particles congregate in the pericentriolar region of interphase cells and increase in number at the onset of mitosis (Gould & Borisy, 1977; Snyder & McIntosh, 1976), but are associated with the kinetochore region of chromosomes in metaphase-arrested cells (Gould & Borisy, 1976; Heine & Todaro, 1978). The intimate association of type A particles with these sites of microtubule nucleation (microtubule organizing centres – MTOC) has been noted by Gould & Borisy (1977) in their in vitro studies of microtubule formation, and Pepper & Brinkley (1977) could show the presence of tubulin on the surface of intracytoplasmic type A particles in Ptkt cells. The affinity for tubulin of type A particles in CHO cells was also corroborated by our previous studies with the microtubule depolymerizing agent vincristine sulphate (Heine et al. 1979). Since MTOC are determinative for the maintenance of cell
configuration (Porter et al. 1974; Miller et al. 1977; Tucker et al. 1978) the interaction of virus precursor particles with these sites could conceivably indicate a function in phenotypic transformation.

The accumulation of type A particles at centromeric regions of metaphase chromosomes suggests also the possibility that their assembly may be determined or regulated at specific chromosomal loci, perhaps associated with kinetochore activation at the onset of mitosis (Brinkley & Stubblefield, 1970). Although little is known about the localization of such postulated ‘kinetochore genes’ the nucleolus organizer regions in Ptk1 cells, located at secondary constriction sites of the X chromosome, have been morphologically correlated with them (Brinkley & Stubblefield, 1970). In any case, a localization of type A particles at the centromeric or other specific region of only certain chromosomes would be consistent with the involvement of a chromosomal genetic locus in the expression of these viruses, whereas their presence at the centromeric region of all chromosomes would favour alternative explanations for their kinetochore affinity. If, for example, the type A particles are transported to the kinetochore plates in association with the attachment of spindle microtubule fibres, an affinity for specific chromosomes would not be expected and a kinetochore association would be absent or reduced in prophase and prometaphase nuclei.

We attempted to test these hypotheses by studying the incidence of kinetochore-associated type A particles at different stages of the mitotic cycle. In this communication, therefore, we wish to report on the distribution and number of type A particles at kinetochore sites by using an adaptation of a technique of whole mount electron microscopy of isolated prometaphase- and metaphase-arrested CHO cells. Evidence will be presented that the affinity of these virus precursor particles is not selective for some chromosomal sites, but extends to the centromeric region of all metaphase chromosomes of Chinese hamster and probably also of murine cell lines, and has a preferred localization at only one sister kinetochore plate. A lesser incidence of kinetochore binding was observed in prometaphase than in metaphase chromosomes. These observations suggest that the affinity of these endogenous virus precursor particles to kinetochore regions of chromosomes is not the immediate result of control by an adjacent specific chromosomal locus, but implies the physical binding of such particles to MTOC.

**METHODS**

**Cell cultures.** CHO-K1 cells obtained from Dr G. J. Todaro, Laboratory of Viral Carcinogenesis, NCI, Bethesda, Maryland, U.S.A. were grown in Falcon plastic dishes as non-confluent monolayers in Dulbecco’s minimum essential medium (D-MEM) supplemented with 10% inactivated foetal calf serum and antibiotics. NIH/3T3 cells infected with the murine virus M432 were cultivated as described previously (Heine & Todaro, 1978).

**Colcemid treatment.** Cells were blocked in S-phase by treatment with 2 mM-thymidine for 16 to 18 h and then arrested in metaphase by the addition of 0.05 mg/ml Colcemid (Grand Island Biological Corp., Grand Island, New York, U.S.A.) in spinner medium (Eagle’s MEM for suspension cultures). Cells at the prometaphase stage were collected 30 to 45 min after addition of Colcemid. Metaphase cells were sampled after 4 to 5 h of Colcemid treatment. Cells blocked in mitosis were shaken gently from dishes, transferred to centrifuge tubes and processed further as outlined below.

**Karyotyping (light microscopy).** The G-band technique was used to identify the chromosomes (Sun et al. 1973; Dutrillaux & Lejeune, 1975). CHO cells were examined for number variation by counting chromosomes in 44 random metaphase spreads. The chromosome number had a definite mode of 20. Only a few cells contained less than 20 chromosomes; they may have been broken cells. There was no incidence of polyploidy.
Type A particles at metaphase chromosomes

Karyotyping (electron microscopy). Since banding procedures are not yet available for the characterization of chromosomes in the electron microscope we based our identification on relative size and the position of the centromere in isolated chromosomes. We employed CHO cells extensively in these studies, since the number of chromosomes per cell is low and each chromosome is of characteristic configuration. Both factors facilitated recognition of individual chromosomes in the electron microscope. Chromosomes were obtained from lysed cells by previously published procedures which were modified slightly (Gould & Borisy, 1978).

Cell lysis and electron microscopy. Metaphase-arrested cells were pelleted at low speed in a table-top centrifuge, drained and trypsinized in 1 ml of 0.25 % trypsin (in Hanks' balanced salt solution) for 1 min at 37 °C. Trypsinization was interrupted by the addition of 0.5 ml foetal calf serum. The cell suspension was pelleted at low speed; pellets were drained and suspended in 0.4 ml distilled water. The centrifugation was repeated, the resulting pellets were carefully drained to remove any residual fluid, resuspended in 1 drop of distilled water and lysed by the addition of an equal volume of 0.5 % Triton X-100 in PME buffer (20 mM-PIPES; 0.2 mM-MgCl2; 2 mM-EGTA), pH 6.9. Lysis was followed in the light microscope and free chromosomes were obtained 2 to 5 min after exposure to Triton X-100. Chromosomes were fixed by the addition of an equal volume of 2 % glutaraldehyde in 10 mM-potassium phosphate, pH 7.0. After 5 min fixation the chromosomes were transferred to ionized, carbon- and Formvar-coated grids by either one of two methods: (i) by centrifugation for 10 min of small drops of lysates on to grids followed by staining with 2 sodium phosphotungstate (PTA), or (ii) by transferring on to a grid one drop of a mixture containing equal amounts of lysate and PTA, pH 6.5, in 0.4 % sucrose. Residual fluid was removed from the grids with filter paper.

For the isolation of prometaphase cells the method was modified as follows: trypsinization was extended to 1.5 min and a 1 % solution of Triton X-100 was used for cell lysis.

RESULTS

Karyotyping of CHO cells

Karyotyping by G-banding revealed a certain similarity of our CHO-KI cell line to that described as standard 'wild-type' (WT) by Worton et al. (1977). In agreement with their results we found that each cell had a pair of normal number 1 chromosomes, one normal number 2, one number 2 with a deletion from the short arm, Z2, one normal number 3 or X chromosome, one of each number Z3, Z4 and Z7, two normal number 6, one of each number 8, Z8 and Z9, one of each number 9, 10, 11 (designated 9 to 11 in our karyograms), and one number Z10 and Z13 (Fig. 1). Missing in our cell line are one normal number 9 and chromosomes Z5 and Z12. In addition, we found regularly two marker chromosomes (Fig. 1 and 2, M), which correspond to the q and p arms of chromosome number 1. The chromosome derived from the p arm has a terminal deletion (Fig. 1, arrow).

Fig. 2 illustrates representative metaphase-arrested chromosomes isolated and spread for examination in the electron microscope as described in Methods. A comparison of Fig. 2 with Fig. 1 shows that identification of the chromosomes prepared for electron microscopic studies is possibly based on relative size and the position of the centromere.

Association of intracytoplasmic type A particles with the kinetochore region

CHO cells in early stages of mitosis

It is known that kinetochores are formed during prophase at the centromere region and that they reach maturity, i.e. they are able to interact with microtubules, only late in prometa-
phase (Brinkley & Stubblefield, 1970). Therefore, the incidence of type A particles at the
kinetochores during the early stages of mitosis was investigated to determine the nature of
their relationship to these unique chromosome structures.

After exposure of synchronized CHO cells to Colcemid for 20 to 45 min type A particles
were associated only with those chromosomes showing distinct centromeric regions and/or
kinetochores. The type A particles were located adjacent to the centromeres in all these
cases (10 out of 11 chromosomes examined) and their numbers varied greatly, ranging
between 2 and 30 particles per centromere. It is of interest to note that the particles were
located preferentially at only one of the two sister centromeres. We did not observe type A
particles near chromosomes that had not yet developed distinct centromeric regions or
kinetochores.

CHO cells arrested in metaphase

In metaphase-arrested cells, that were exposed to Colcemid for 5 h, positive identification
in the electron microscope of centromeric regions at individual chromosomes was achieved
without difficulty due to their intense staining (Fig. 2 and 3). Furthermore, kinetochores
could be identified frequently as rope-like structures at the surface of the centromeres. Type
A particles were often associated with this area. They were observed at kinetochores
(centromeres) in 78 out of 248 metaphase-arrested chromosomes (31 %). As shown in Table
1, the percentage of positive chromosomes varied among the different chromosome types and
could be as low as 10 % (chromosome 3) or as high as 67 % (Z2). Our data demonstrate

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Fig. 1. Karyotype of CHO cells, G-banding. The arrow points to the band deleted in the marker
cromosome which was derived from the p arm of chromosome 1.
clearly the ability of type A particles to associate with the kinetochore region of each type of chromosome.

Figure 3(a) illustrates four metaphase-arrested chromosomes corresponding to numbers 2, Z2, 9 and Z13. Enlargements of their centromeric region revealed the presence of type A particles adjacent to the densely stained centromeres (Fig. 3b, c, d). The close association of such particles with the kinetochores which were situated at the surface of the centromeres is shown in Fig. 4. In Fig. 4(b) the rope-like, twisted kinetochore is stretched across the centromere and at one side five type A particles are closely associated with the structure (arrow). In Fig. 4(d) the number of particles attached is much larger, about 25 to 30 and they cover much of the kinetochore. Thus, the number of type A particles at a kinetochore may vary from chromosome to chromosome. On average, there were 6 to 10 particles present at one kinetochore location, but extreme values were 1 particle (Fig. 3d) and 35 to 40 particles (Fig. 5a) per kinetochore. Fig. 5(a) also illustrates the accumulation of type A particles at only one kinetochore, whereas the other one is completely bare. Such asymmetry in distribution, with only one of the sister kinetochores decorated with type A particles, was observed rather frequently. This observation was quantified in metaphase-arrested cells with the following result: among 64 chromosomes, 49 (70%) exhibited type A particle accumulations at only one centromere, eight showed the particles at both centromeres, but with an extremely uneven distribution, and at seven chromosomes the particles were
Table 1. Distribution of kinetochore-associated type A particles with chromosomes in metaphase-arrested CHO cells (5 h Colcemid)

<table>
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<th>Chromosome number</th>
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<th>Mq</th>
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<th>Z12</th>
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<td>Chromosomes with kinetochore-associated type A particles</td>
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<td>4</td>
<td>2</td>
<td>8</td>
<td>4</td>
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Fig. 3. (a) Electron micrograph of group of chromosomes, corresponding to numbers 2, Z2, 9 and Z13 with clusters of type A particles at the centromeres. (b), (c) and (d) Enlargement of the centrometric regions to show the asymmetric accumulation of type A particles (arrows) at sister centromeres. Negatively stained with PTA.

distributed across the complete primary constriction in such a fashion as to make a correlation between particles and a specific centromere impossible (Fig. 4a). At higher magnification, the globular configuration of the particles could be recognized, as well as their hollow centre and the relatively long spikes on their surface (Fig. 5b). The diameter of the particles was measured as being approx. 75 nm and the length of the spikes was about 35 nm. [Recent measurements revealed that the previously published value for the size of murine intracytoplasmic type A particles of strain M432 (Heine & Todaro, 1978) was too high. The diam. of the murine type A particles is similar to that of the particles contained in CHO cells described here, namely approx. 75 nm.]
Type A particles at metaphase chromosomes

Fig. 4. Association of type A particles with kinetochores. (a) Chromosome Z2. (b) Enlargement of the centromeric region of (a); a kinetochore of cord-like appearance with 5 type A particles can be seen at one centromere (arrow). (c) Chromosome 1; the arrow indicates the centriole near the centromere. (d) Enlargement of the centromeric region of (c); numerous type A particles associated with the kinetochore are indicated (arrow). Negatively stained with PTA.

We observed centrioles in close proximity to the centromeres in 13 metaphase spreads (Fig. 4c, d). Nine of these centrioles were surrounded by numerous type A particles, ranging between 20 and 35 particles per individual centriole. Type A particles were not present in the immediate surroundings of the remaining four centrioles.

Metaphase-arrested NIH/3T3 cells infected with M432 virus

Due to the difficulties in karyotyping the telocentric mouse chromosomes, many of which vary little in size and thus make typing in the electron microscope nearly impossible, our results regarding the type A particle distribution at different chromosomes have to be considered as preliminary. Thus far, we have examined 73 chromosomes of different length arrested in metaphase in the presence of Colcemid for 4 h. We found 18 chromosomes (22 %) that contained type A particles in the immediate vicinity of their centromeres. The number of particles at each centromere was low, between one and five, as compared to the
Fig. 5. (a) Accumulation of numerous type A particles at only one sister kinetochore. (b) Type A particles at high magnification. Spikes on the particle surface are recognizable (arrow). Negatively stained with PTA.

Fig. 6. (a) Chromosome isolated from NIH/3T3 cells infected with murine virus of strain M432. Clusters of type A particles at sister centromeres are indicated (arrows). (b) Type A particles at higher magnification show short spikes on their surface. The arrow points to two microtubules.

The number seen at chromosomes of CHO cells (Fig. 6a). Furthermore, we observed type A particles at both kinetochores of the telocentric chromosomes in approx. the same numbers. This finding was in obvious contrast to the aforementioned asymmetric distribution of type A particles at sister kinetochores of CHO cells.

The fine structure of the murine type A particles was similar to that of the particles associated with CHO cells. As shown in Fig. 6(b), the murine particles consisted of hollow spheres, approx. 80 nm in diam., with spikes of 25 nm length extending from their surface.
Type A particles at metaphase chromosomes

DISCUSSION

The close association of intracytoplasmic type A particles, present in certain cells derived from the Chinese hamster and the mouse, with the centriolar area and the kinetochores of metaphase-arrested cells has been reported (Wheatley, 1974; Gould & Borisy, 1976, 1978; Heine & Todaro, 1978). Furthermore, a pronounced affinity of such particles for microtubules, in particular the microtubular protein tubulin, has been described (Pepper & Brinkley, 1977; Heine et al. 1979). It is apparent that the association of type A particles – of which some represent virus precursors – with cell organelles known to contribute much to both the control of cell division and the configuration of the cell, may directly interfere with normal cellular function and may lead to abnormalities in cell behaviour.

In this report, we ask whether the accumulation of type A particles at the centromeres (kinetochores) of metaphase-arrested chromosomes may be due to the activity, in individual chromosomes, of special loci situated in the immediate neighbourhood of the centromeres. This working hypothesis is supported by the view that the centromeric region is associated with a specialized gene active early during mitosis (Brinkley & Stubblefield, 1970) and that it produces a kinetochore ribonucleoprotein thought to be a gene product possibly regulating the role of the kinetochore (Rieder, 1979).

Initial attempts to test this hypothesis by studying the incidence of kinetochore-associated type A particles in somatic cell hybrids segregating hamster chromosomes [CHO-K1(HPRT−) × NIH-3T3(TK−)] were not successful because of insufficient and inconsistent segregation of the resultant hybrid clones. In this communication, however, by examining isolated chromosomes of Colcemid-arrested CHO cells in the electron microscope, we present evidence that type A particles are present at the kinetochores of at least 30% of the chromosomes and at all 18 different chromosome types. Our results permit the conclusion that the binding of type A particles to the kinetochore regions of chromosomes is not selective to some chromosomal sites, but occurs in the centromere region of all metaphase chromosomes of Chinese hamster and probably also of murine cell lines, in which these precursor particles of endogenous retroviruses are observed. Their localization is preferentially asymmetrical in CHO cells, associating with only one sister kinetochore plate. A functional asymmetry of sister kinetochores during prometaphase movement has been reported and it has been shown that, under appropriate experimental conditions in the presence of Colcemid, microtubules associate with only one of the sister kinetochores (Brinkley & Stubblefield, 1970). Microtubules were only rarely present in our preparations of isolated chromosomes. Therefore, we are not able to state which of the sister kinetochores is decorated with the type A particles.

A chromosomal association of these particles was not observed in prophase prior to the differentiation of the centromeric region and was only rarely observed in prometaphase. The frequency of kinetochore association increases, however, in mature metaphase apparently concomitant with the completion of the attachment of microtubule spindle fibres to the kinetochore plate. These observations do not support the hypothesis that type A particles are formed de novo by transcription at chromosomal loci associated with the kinetochore region, but suggest the interpretation that these particles may be transported from the centriolar region to one sister kinetochore plate in conjunction with the organization of microtubule spindle fibres. Immunological and immuno-electro microscopic studies using isolation techniques as described here may elucidate the prevalence and significance of this association of endogenous retroviruses with nucleating sites of microtubules associated with the mitotic apparatus and its possible implications for modes of epigenetic transmission.
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REFERENCES


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