Cytological evidence for switches in polarity of chromosomal DNA

[ring chromosome formation/5'-3' phosphodiester bonds/Chinese hamster ovary (CHO) cells/harlequin staining]

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ABSTRACT From the types of ring chromosomes induced in x-irradiated Chinese hamster ovary (CHO) cells, we deduce the existence of switches in the polarity of chromosomal DNA; if there is a continuous DNA double helix along the full length of the chromosome then the polarity switches imply 3'-3' and 5'-5' phosphodiester linkages. The resolution of the method is such that we estimate that there is one polarity switch for every 10⁶ normal 3'-5' phosphodiester bonds.

In a previous communication (1) we presented data that we interpreted as indicating the presence of infrequent switches in the polarity of chromosomal DNA, i.e., the equivalent of 3'-3' opposite 5'-5' linkages. This was based on the types of ring chromosomes induced by x-irradiating Chinese hamster ovary (CHO) cells in the G₁ stage of the cell cycle. When broken chromosomes rejoin, the way in which the polynucleotide strands of DNA can rejoin is restricted according to the polarity (3'-5') of the strands (2, 3). Thus, if the two broken ends of a chromosome containing only conventional linkages rejoin, the ring that is formed can separate into two rings at the subsequent anaphase. If the fragment contains a linkage of opposite polarity, then rejoining forms a single double-sized ring. When anaphase was suppressed with Colcemid and the paired and single rings were scored in the resulting tetraploid cells, we observed that the ratio of single rings to paired rings increased from 0 to 1 with increasing ring size, indicating a length-dependent condition or event that results in the formation of single rings in the place of paired rings. This condition or event could be either a change in the polarity of the DNA or sister chromatid exchange. An estimate of the number of sister chromatid exchanges occurring during the second division (i.e., in the tetraploid cells) indicated that they could not account for the production of all the single rings observed. Since this estimate was necessarily tentative, it was imperative to devise experiments permitting direct visualization of sister chromatid exchanges. We have now performed the experiments using the fluorescence plus Giemsa or harlequin chromosome procedure (4, 5) by which it is possible to stain the two sister chromatids differentially and to visualize sister chromatid exchanges with great precision. In this way rings that depend upon sister chromatid exchanges for their conformation can be identified. The results provide new and convincing evidence for the presence of polarity reversals in the subunits of chromosomal DNA.

RATIONALE When G₁ cells are irradiated and their chromosomes are then allowed to replicate twice in the absence of cytokinesis, rings of three different origins can be identified in the resulting tetraploid metaphases (1). In addition to the paired rings and single rings formed by the orthodox rejoining of the two broken ends of a chromosome fragment with each other (Fig. 1A and C), single rings may also be formed by isocentric rejoining, i.e., reunion of complementary subunits of the DNA molecule (Fig. 1B). The essence of harlequin staining is that when two rounds of DNA replication are carried out in the presence of bromodeoxyuridine (BrdUrd), one chromatid is uniformly substituted and stains darkly with fluorescence plus Giemsa whereas its sister chromatid is bifurcally substituted and stains lightly. In such material sister chromatid exchanges can be identified clearly as exchanges between light and dark chromatids. Thus, it is possible not only to determine whether ring chromosomes are paired or single, but also to ascertain whether sister chromatid exchange is responsible for the conformations observed.

Because the centromere provides a reference point that is readily identifiable, we confine our attention to centric rings; furthermore, most rings scored were probably derived from the three large metacentric chromosomes in the CHO complement. Consider a centric fragment in which the broken ends are in regions of like polarity and rejoin in the orthodox manner to form a ring (Fig. 1A). If no sister chromatid exchange occurs, a pair of monocentric rings will be found in the tetraploid descendent cell (Fig. 1a); one chromatid of each ring will be darkly stained (Fig. 2a). A sister chromatid exchange during the first post-irradiation round of DNA replication gives rise to a single dicentric ring with a twin exchange. This ring will be a symmetrical dicentric, i.e., will have centromeres that are diametrically opposite one another. The twin exchange can be recognized because there are two label switches so that one half of each chromatid is darkly stained, and the positions of the two exchanges are trans-symmetrical with respect to the centromeres (Figs. 1a and 2a).

If the centric fragment has broken ends in regions of opposite polarity, then when the ring is formed the ends rejoin to form a continuous strand or pretzel (Fig. 1C). In the tetraploid descendent cell, a single symmetrical dicentric ring is found; this case differs from the last, however, in that one chromatid is completely labeled and one is completely unlabeled (Figs. 1e and 2e). The two chromatids of such a single ring can separate from one another, i.e., the ring is doughnut shaped rather than pretzel shaped. If a sister chromatid exchange occurs during the first post-irradiation round of DNA replication, then a pair of dicentric rings is generated. They differ from the paired rings described above as each will have the same exchange of label with respect to the centromere (twin) and be pretzel shaped, having chromatids that form one linear continuum (Figs. 1f and 2f).

Either type of radiation-induced chromosome fragment discussed above can also give rise to a single dicentric ring

Abbreviation: CHO, Chinese hamster ovary.

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* Two of these rings contained two twins.

Fig. 1. Types and origins of x-ray-induced centric rings. Rings are induced in G1 diploid cells and observed in tetraploids formed after two S periods in the presence of 10 μM BrdUrd and 0.1 μM Colcemid. Thick solid line = original thymidine-containing strand; broken line = newly synthesized BrdUrd-substituted strands; centromeres are represented by solid circles; S = DNA synthesis; SCE = sister chromatid exchange. The first row indicates the two possible types of centric fragments with respect to the polarity of their ends. The second row depicts the consequences of the orthodox mode of rejoining, A and C, and of islocus rejoining, B. The third row shows the products of replication of the three configurations of row two, both without and with sister chromatid exchange; replication of the six configurations in row 3 yields those labeled a-f in row 4. After two rounds of replication in BrdUrd one chromatid is bifilarly substituted (both strands represented as broken lines) and stains lightly, and one chromatid is unifilarly substituted (one broken line and one heavy solid line) and stains darkly. The numbers of rings observed in classes a through f are tabulated below each figure according to the number of single sister chromatid exchanges which occurred in the ultimate S phase.

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Solvable in the tetraploid descend cell as a consequence of islocus rejoining (Fig. 1B). These rings differ from single rings that originate by orthodox rejoining in that they are asymmetric; that is, the centromeres are not diametrically opposite one another (Figs. 1c and 2c), except in the rare case when the two breaks that generate the chromosome fragment are equidistant from the centromere. A sister chromatid exchange during the first round of DNA replication following the formation of this type of ring gives rise to a cis-symmetrical twin exchange, as is shown in Figs. 1d and 2d. Both the asymmetric placement of the centromeres and the presence of cis-symmetrical twin exchanges serve to distinguish these dicentric rings from those formed after breaks in regions of opposite polarity rejoin in the orthodox manner. Both these distinctions depend on the analysis of centric rings and cannot be made for acentric rings.

Sister chromatid exchanges that occur during the second post-irradiation round of DNA replication (singles) are superimposed upon the basic configurations diagrammed in Fig. 1, but do not seriously confound the distinctions among the various types of rings. An important aid in the analysis is that a single sister chromatid exchange, in addition to producing a switch in label between the chromatids, transforms a doughnut into a pretzel or vice versa.

From the foregoing, it is clear that little ambiguity is involved in assigning harlequin-stained centric ring chromosomes to the six origins depicted in Fig. 1.

MATERIALS AND METHODS

Plastic tissue culture flasks (75 cm²) were seeded with 3 × 10⁶ Chinese hamster ovary (CHO) cells in 10 ml of McCoy's 5A medium containing 15% fetal calf serum, 100 units of penicillin per ml, and 100 μg/ml of streptomycin. The cells were grown overnight in a 5% CO₂ atmosphere. The flasks were then shaken to detach dead or loosely adhering cells and the medium was replaced with 10 ml of fresh medium containing 0.1 μM Colcemid. Cells suspended by agitation of the culture were sampled each hour until a mitotic index of at least 98% was attained (6). These cells were then seeded in 25 cm² plastic flasks containing 10 ml of medium. After 1.5 hr, the mitotic cells attached to the flask, divided, and entered G1. The flasks were then irradiated with 300 rad of 300 kVp x-rays from a General Electric Maxitron (half value layer 1.65 mm Cu, 100 roentgens/min) to induce rings. The medium was then supplemented with bromodeoxyuridine (BrdUrd) to a final concentration of 10 μM and 0.2 μM Colcemid. Within 35–39 hr the cells underwent two rounds of DNA replication and became tetraploids in which each chromosome had one chromatid unifilarly substituted with
BrdUrd and one chromatid that was bifilarly substituted. These were then stained differentially with the fluorescence plus Giemsa or harlequin chromosome technique (4, 5). Tetraploid cells, most of which detach from the flasks, were collected by centrifugation of the supernatant and treated either for 3 min with 0.1 M sucrose or seven min with 0.075 M KCl to help spread the chromosomes. The cells were again concentrated by centrifugation and fixed in methanol:acetic acid (3:1). Slides were prepared by dropping a concentrated suspension of cells in fixative onto dry microslides. The slides were then stained for 15 min in 0.5 μg/ml of Hoechst 33258 (7) in 67 mM Sörensen's buffer, pH 6.8, rinsed in distilled water, and then had coverslips mounted with the same buffer. They were then placed on an x-ray viewing box containing cool white fluorescent bulbs for 45-60 min to allow the photochemical reaction to take place. The cover glass was then removed and the slides were incubated for 20 min in 1.5 M sodium chloride, 0.15 M sodium citrate (10 X SSC) at 62.5°. After further rinsing in distilled water the slides were stained in 3% Giemsa (Gurr's R66) in 67 mM Sörensen's buffer at pH 6.8. After another rinse they were dried, passed through xylene, and mounted in Gurr's DePeX.

A group of randomly chosen cells was scored completely for all chromosome aberrations for comparison with results observed in previous experiments (1). Ring-bearing cells of a second group were scored for the types of rings present. The data comprise 187 ring chromosomes observed in these two populations of cells.

RESULTS

Ring configurations are classified into six groups depending on (a) the polarity of the ring-generating fragment, (b) the type of ring-generating reunion, and (c) the presence or absence of a twin sister chromatid exchange, as shown in the first, second, and third rows, respectively, of Fig. 1. The classification of the 187 ring chromosomes observed is summarized at the bottom of the same figure; each column is subdivided in turn according to the number of single sister chromatid exchanges. The data are internally consistent in that the presence of twins is independent of the presence of singles by contingency χ², i.e., a sister chromatid exchange in the S phase immediately following x-irradiation has no influence on the probability of exchange in the subsequent S phase. Furthermore, the ratio of singles to twins is 78:34, which does not depart significantly from the 74.7:37.3 expected (2) if polarity is conserved in rejoining of sister chromatid exchanges. The raw data presented in Fig. 1 indicate that 40% [51/(51+76)] of ring-generating fragments have breaks in regions of opposite polarity.

We now inquire into the extent that this estimate may be inflated by errors in scoring. Single dicentric ring chromosomes with diametrically opposed centromeres and with one chromatid labeled and one unlabeled (Figs. 1e and 2e) are diagnostic of switches in polarity. The only source of ambiguity in scoring this class is the possible contribution of single dicentric rings from isolocus rejoining of a centric fragment in which the breaks are equidistant from the centro-
mere (Figs. 1c and 2c). We have two estimates of the possible magnitude of this error. The observation that originally led us (1) to postulate the occurrence of isolocus rejoining was the appearance, in the tetraploid descendents of cells irradiated in G1, of large numbers of single dicentric chromosomes (not rings) that were symmetric about their midpoints (i.e., mirror image dicentrics). They were as frequent as the paired asymmetric dicentrics expected from rejoining of two broken chromosomes in the orthodox manner. Symmetric dicentrics are known to result from sister union of isochromatid breaks induced in S or G2. However, we argue on three grounds that the single symmetric dicentrics observed in these experiments are the consequence of lesions induced in G1 and are not the consequence of ordinary isochromatid breaks induced in a small contaminating population of S or G2 cells: (1) single symmetric dicentrics occur together in the same cells with paired asymmetric dicentrics; (2) no other types of aberrations interpretable as chromatid aberrations are encountered in these tetraploid metaphases; and (3) the segregation of label demonstrates that paired and unpaired dicentrics alike have undergone two rounds of DNA synthesis in BrdUrd subsequent to x-irradiation. Furthermore, if single symmetric dicentrics originate in the manner postulated, then twin exchanges would appear as pairs of sister chromatid exchanges symmetrically disposed around the midpoint of the dicentric; in 190 single symmetric dicentric chromosomes we observed 48 such twins and 72 singles, in agreement with the 40:80 ratio expected (2)8.

In the present experiments 219 randomly chosen cells were scored for all types of aberrations; 206 single symmetric dicentrics (including more complex configurations containing such events) were found; the number of isolocus reunions per cell was distributed according to Poisson expectations. These observations agree very well with our earlier observations (1) as well as with the subsequent observations of Ikushima and Wolff (8). The contention of Bender et al. (9) that isolocus reunions are rare or absent is not, given the design of their experiment, in conflict with our findings. If the post irradiation mitosis is not prevented, then isolocus reunions should produce chromatid bridges at anaphase, the products of which will not appear as dicentrics in the resulting diploid metaphases; these authors did not inhibit cytokinesis, and accordingly they observed no symmetric dicentrics.

We propose to utilize our observed frequency of isolocus rejoining to estimate the number of unpaired dicentric ring chromosomes expected from isolocus rejoining in both arms of the same chromosome; we do so to assess the possibility that a significant number of rings of this origin might be misjudged as indicating switched polarity. As we can detect no more than one isolocus event per chromosome arm, we estimate the mean number of isolocus reunions per cell by using the zero term of the Poisson distribution. 82/219 cells were without single symmetric dicentrics; this leads to an estimate of 0.98 isolocus reunions per cell. We next apportion this mean among the chromosomes of the CHO karyotype according to metaphase chromosome length, and then calculate for each chromosome the probability that it will have 2, 3, etc., isolocus reunions. Then, assuming that these breaks are distributed binomially between the two arms according to their lengths, we estimate the probability that there will be at least one isolocus reunion in each arm, which is the probability that such a dicentric ring will be formed. These probabilities when summed over all chromosomes yield an expectation of 3.25 such rings in the 219 cells examined; we observed seven. Therefore, there is no reason to believe that misscored asymmetric dicentric rings are contributing to the symmetric dicentric ring class.

The second indication that misclassification of rings originating through isolocus reunion is not an important source of error comes from dicentric single rings with twin exchanges. Dicentric rings with cis-symmetric twin exchanges were classified as originating from isolocus reunion irrespective of the positions of their centromeres. Of sixteen such dicentric rings only one had centromeres that appeared to be diametrically opposite one another (e.g., the right ring in Fig. 2d), suggesting that about 1/16 of the dicentric rings arising from isolocus reunions are discernibly asymmetric. Thus the 44 asymmetric dicentric rings without twins imply the presence of about three symmetric dicentric rings from isolocus rejoining; these are included among the 49 symmetric dicentric rings, leaving 46 that originated by rejoining in an orthodox manner of fragments with breaks in regions of opposite polarity.

Two other possible scoring errors lead to underestimates of the incidence of polarity switches. First, we note from the data presented in Fig. 1 that the frequency of twins in rings that arose from orthodox rejoining of fragments with breaks in regions of opposite polarity (Fig. 1f) is low compared to the incidence of twins in the other two classes (Fig. 1b and d); we expect all three to be alike. Paired monocentric rings are, on the average, half the size of dicentric rings, and the continuity of the chromatids is more difficult to ascertain. Thus, some of the paired monocentric rings scored as doughnuts and considered to have arisen from breaks in regions of like polarity are likely to be pretzels that arose from fragments with breaks in regions of opposite polarity. Second, it is possible that some of the exchanges scored as twins in dicentric rings were in reality false twins caused by two appropriately positioned but independent singles. Other estimates (10) of this source of error suggest that less than 4% of twins arise in this fashion. False twins transfer rings from the class diagnostic of switches in polarity into the other two classes, i.e., from the class illustrated in Fig. 1e to those in Fig. 1b and d.

**DISCUSSION**

In the previous experiments (1), the relation between contour length and the ratio of single rings to paired rings allowed us to estimate the sum of sister chromatid exchanges and polarity switches per μm of metaphase chromosome length; this value was 0.09. The contribution of sister chromatid exchange to this value can be estimated from experiments in which the incidence of sister chromatid exchange was measured in diploid CHO cells stained with the harlequin chromosome method (5). This value, which was unaffected by G1 irradiation, was 0.15 sister chromatid exchange per chromosome per cell cycle or approximately 3.15 exchanges per cell; since the metaphase length of chromosomes in CHO cells is approximately 110 μm (1), there are

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8 These single symmetric dicentrics incidentally provide evidence against defined endpoints of replications of chromosomal DNA. If there were discrete termination points, then a chromosome break would produce one fragment with an initiation site subterminal to a segment without a termination site and the other with a termination site subterminal to a segment with no initiation site. Isolocus rejoining should result in replication difficulties for one or both such fragments; yet as demonstrated in our earlier study (1), every unpaired symmetric dicentric is accompanied by a normally replicated acentric fragment, a result consistent with the occurrence of defined initiation points only.
0.03 sister chromatid exchanges per \( \mu m \) of metaphase length. Subtracting this value from the 0.09 computed in the previous experiment leaves 0.06 switches in polarity per \( \mu m \) of metaphase chromosome. The observed total metaphase length of 110 \( \mu m \) corresponds to 6.6 switches in polarity per CHO chromosome complement. On the basis of measurements in other rodent species we take approximately \( 6 \times 10^6 \) base pairs as an estimate of the DNA content of the hamster complement; thus the cytological procedure employed in this work permits the resolution of one polarity switch in \( 10^6 \) phosphodiester bonds.

It is possible that the switches occur at still unidentified non-DNA links in the chromosomes. If, however, the chromosome consists of but a single DNA molecule extending from one end of a chromatid to the other (11), the switches would be 3'–3' and 5'–5' bonds. Enzymes capable of forming the 5'–5' linkages at the 5' terminus of some messenger RNAs (12) have recently been demonstrated (13). As these structures are (a) formed by post-transcriptional modification and (b) contain three phosphate groups rather than one interspersed between two ribose moieties, they do not imply the presence of 3'–3' linkages in the DNA template; they do, however, serve to demonstrate that biological systems are capable of synthesizing types of bonds similar to those suggested by our results.

We regard these polarity switches as characteristic features of the genome and not as an unusual consequence of the irradiation. In the first place, if our observations were the result of antipolar rejoining, then such rejoining would have to occur 40% of the time. Brewen and Peacock (3), however, have shown that when G2 chromatids broken with x-rays rejoin, polarity is conserved and, as mentioned above, the present results support conservation of polarity in sister chromatid exchange. In the second place, if polarity switches were unique products of irradiation and not normal chromosome constituents, then we would not expect the cell to have the capability of replicating them. The rings, however, were observed after they had replicated twice. Finally, the length dependence of unpaired ring frequency (1) is discordant with the hypothesis of antipolar rejoining of induced breaks.

Inasmuch as the switches in polarity are normal constituents of chromosomes, they must occupy defined positions in the genome. In principle their cytological positions can be determined, but in practice the methods are not yet at hand to do so. Since the majority of the rings observed were derived from the three large metacentric chromosomes, however, our observations imply that one or two of these chromosomes have a centrally located polarity switch. We dismiss the possibility that polarity switches are regular features of the centromere regions. Observations on the strand disposition of thymidine-rich sequences on opposite sides of the centromeres in metacentric chromosomes in the mouse (14) indicate that polarity is conserved across the centromere. Furthermore, in the present results we note that the estimated number of polarity switches is considerably smaller than the number of centromeres per cell. More importantly, were a polarity switch a regular feature of all centromeres, then all, rather than 40% of, centric rings might be expected to form as pretzels.

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