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Structural basis for Robertsonian translocations in man: Association of ribosomal genes in the nucleolar fibrillar center in meiotic spermatocytes and oocytes

(acrocentric chromosomes/nucleolar organizer/rDNA/meiotic rearrangement)

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ABSTRACT **The spatial relationships of acrocentric chromosomes were studied during prophase I of meiosis in human oocytes and spermatocytes by using cytogenetic techniques, electron microscopy, and *in situ* hybridization. Ultrastructural investigations revealed an ordered arrangement of nucleolar bivalents at the zygotene and pachytene stages. The end of the bivalent corresponding to the cytological satellite was consistently attached to the nuclear envelope. The fibrillar center of the nucleolus always contained rDNA chromatin fibers emanating from the secondary constriction region. Association of ribosomal genes from two bivalents in the same fibrillar center was frequently observed. Ultrastructural studies demonstrated the close proximity of chromatids in the short arm region of the involved nonhomologous acrocentrics. A breakage/reunion model based on our data can explain the formation of all observed types of Robertsonian translocations: monocentrics and dicentrics with or without rDNA.**

Robertsonian translocations are the most frequent form of chromosome rearrangement in man, their incidence being about 1% in the general population. They result from different modes of exchange occurring within the centromeric region of two acrocentric chromosomes, thus producing a metacentric or submetacentric element (1). In man, the chromosomal segment above the centromere is divided into three parts: the proximal short arm, the secondary constriction or stalk, which is the nucleolus organizer region (NOR), and the distal cytological satellite. The five pairs of acrocentric chromosomes share multiple copies of rRNA genes variably dispersed among the NORs (2, 3). Nonhomologous chromosomes are involved in 90% of Robertsonian translocations, and the most frequently observed are the 13;14 and the 14;21 translocations (4). Most translocations between heterologs are dicentric and devoid of Ag-positive NOR (4–8).

The formation of Robertsonian translocations has been ascribed to chromatid exchange after a break between acrocentric chromosomes associated with the same nucleolus, during spermatogonial and oogonial mitosis (9). It has also been suggested that Robertsonian rearrangements result from an orderly, nonrandom process during meiotic pairing and exchange (10). Indeed, nonhomologous acrocentric chromosomes are often associated with the same nucleolus at meiotic prophase I in human spermatocytes and oocytes (11–14). The nonrandom involvement of chromosomes in translocations can also be explained by an accidental meiotic recombination between partially homologous sites on nonhomologs. If the pairing is "end-to-end," crossing-over results in two recombinants, one acentric and the other dicentric (15). The two centromeres are so close to one another that they may act as a single centromere (16).

Electron microscope studies provide more precise information about the relationship between the nucleolus and the acrocentric chromosomes. A connecting region of the nucleolus was reported in close association with the nucleolar chromomere of acrocentrics (17). Recent progress in studies of nucleolar ultrastructure has led to the conclusion that this connecting region consists of the fibrillar center which is the interphasic counterpart of the NOR (18–22). Electron microscope investigations on the human oocyte at pachytene stage of meiosis show that the ribosomal genes belonging to several chromosomes are gathered in the same nucleolar fibrillar center, where they are embedded in an argyrophilic protein. This pattern may be a factor influencing nondisjunction and translocation (22, 23).

The present study was undertaken to examine whether the structural arrangement of ribosomal genes in the human meiotic oocyte can explain the formation of the different types of Robertsonian translocations. Moreover, because there is no reason to preclude the formation of these rearrangements during spermatogenesis, we started a light and electron microscope study of human spermatogonia and spermatocytes. We investigated whether the structural pattern of ribosomal genes is similar to that observed in the oocyte and may also provide a cytological basis for Robertsonian rearrangements.

MATERIALS AND METHODS

Materials. Ovarian material was recovered from eight fetuses, ages 16 to 24 weeks. The testicular samples originated from seven normally fertile patients who had undergone a testicular operation under general anesthesia.

Electron Microscopy. Testicular biopsy specimens were fixed with 2.5% glutaraldehyde/0.1 M cacodylate, pH 7.3, for 2 hr and then postfixed with 2% osmium tetroxide in the same buffer for $2^1/_2$ hr. Ovaries were cut into 1-mm^3 pieces and fixed with 3% glutaraldehyde/0.1 M phosphate, pH 7.2/0.05 M sucrose at 4°C followed by 2% osmium tetroxide in the same buffer. After embedding in Epon, thin sections were stained with uranyl acetate and lead citrate.

Silver Staining of the NOR. *Light microscope silver staining.* After isolation and spreading (24), germ cells were stained according to the technique of Goodpasture and Bloom (25).

Electron microscope silver staining. Silver impregnation was performed by the technique of Hernandez-Verdun *et al.* (26).

***In Situ* Hybridization.** 28S rRNA was isolated by sucrose gradient from human thyroid polysomal RNA free of poly(A)$^+$

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Abbreviation: NOR, nucleolus organizer region.

RNA after chromatography on oligo(dT)-cellulose (T_3, Collaborative Research, Waltham, MA). 28S [^{3}H]cDNA (9 to 1.1 × 10^7 dpm/μg) was synthesized as described by Brahic *et al.* (27) with calf thymus DNA random primer purified by chromatography on DEAE-cellulose and [^{3}H]dCTP (50–80 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq; Amersham). Chromosome spreads were prepared for *in situ* hybridization according to Gall and Pardue (28), except that the DNA denaturation step was performed in 95% formamide containing 0.1 strength standard saline citrate (0.1× NaCl/Cit; NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 2 hr at 72°C. 28S cDNA was hybridized to meiotic chromosomes in 50% formamide in 3× NaCl/Cit for 24 hr at 42°C in a sealed moist chamber. Two nanograms of cDNA in 10 μl mixture covered by an 18 × 18 acid-washed coverslip was used per slide. Radioautographs were prepared as described (28).

RESULTS

Oogonia and oocytes

A detailed description has already been reported (22, 23). Here we outline the salient features that could be relevant to the mechanisms of translocation.

(*i*) The oogonium displays a reticulated nucleolus with several fibrillar centers.

(*ii*) At leptotene, the silver-positive NORs are located in the peripheral region of the nucleoli. The NORs correspond to fibrillar centers in the electron microscope. The mean number of active NORs is 7 per nucleus.

(*iii*) At pachytene, the mean number of silver-positive NORs is reduced to 2.5 per nucleus. The nonrandomness of the arrangement of nucleolar bivalents is clearly demonstrated by electron microscope studies. The telomere corresponding to the cytological satellite is consistently attached to the nuclear envelope. The nucleolus displays a unique fibrillar center which is segregated from the other nucleolar components. Chromatin fibers emanating from the secondary constriction region of the bivalent penetrate the fibrillar center. A majority of nucleoli are associated with one bivalent, but in a large number of oocytes (40% in our material) the nucleolar fibrillar center is connected with chromatin fibers emanating from two bivalents (Fig. 1). In some oocytes, three bivalents are associated with the same fibrillar center.

Spermatogonia and spermatocytes

Spermatogonia. The nucleus of A dark and A pale spermatogonia contained 3–5 nucleoli. In most nuclei, the nucleoli were of the reticulated (nucleolonemal) type, but the compact type was also observed. Each nucleolus displayed only one fibrillar center, consistently associated with the nuclear envelope. The fibrillar center was partially surrounded by strands of dense fibrillar component which extended as fibrillogranular strands into the nucleoplasm (Fig. 2). The nucleoli of the B spermatogonia were enlarged and had a more central location inside the nucleus. The developing nucleolonema displayed several fibrillar centers that no longer showed any connection with the nuclear envelope.

Leptotene Stage. Two types of nucleoli were observed: (*a*) residual nucleoli showing only the granular component and apparently without any chromosomal connection; and (*b*) newly formed nucleoli always facing the secondary constriction region of an acrocentric chromosome. The telomere of this chromosome was consistently attached to the nuclear envelope. These nucleoli were composed of a fibrillar center surrounded by a layer of dense fibrils. The fibrillar center was penetrated by chromatin fibers emanating from the chromosomal secondary constriction region (Fig. 3).

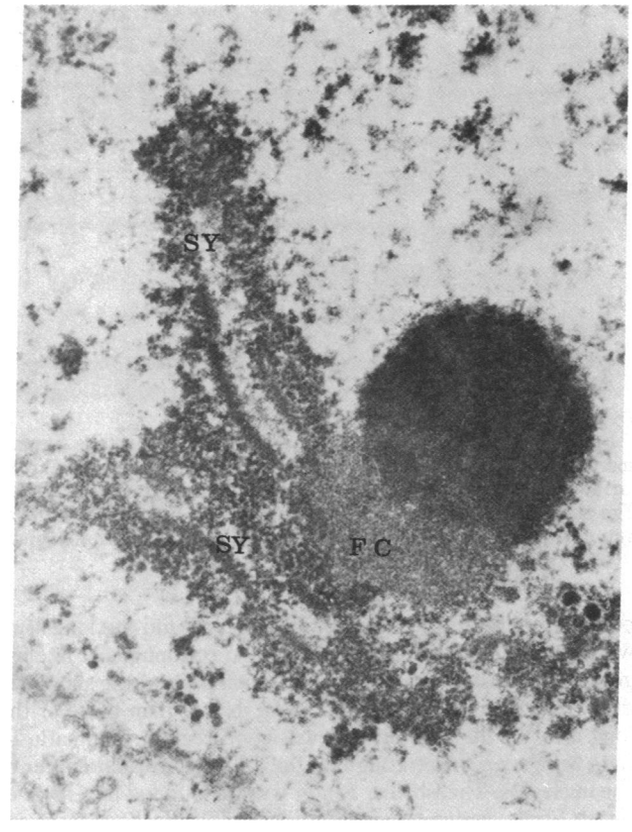

FIG. 1. Pachytene oocyte from an 18-week human fetus. The segregated fibrillar center (FC) of the nucleolus is connected with intermingled chromatin fibers which emanate from two acrocentric bivalents. SY, synaptonemal complex. (×28,000.)

Zygotene Stage. The chromosomal pairs were present as bivalents showing a synaptonemal complex. The spatial arrangement of the nucleolar bivalents was strictly nonrandomly ordered as observed in the oocyte. The telomere corresponding to the cytological satellite was attached to the nuclear envelope, anchored by a thickening of the lateral element of the synaptonemal complex. The secondary constriction region was located 0.3 μm from the nuclear envelope. It was followed by the

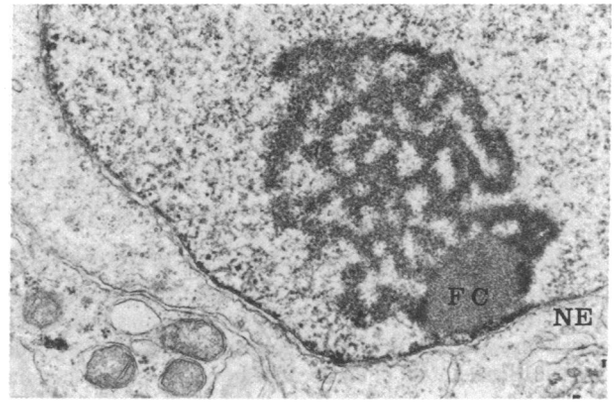

FIG. 2. Nucleolus of an A pale human spermatogonium. The unique fibrillar center (FC) is associated with the nuclear envelope (NE). (×18,000.)

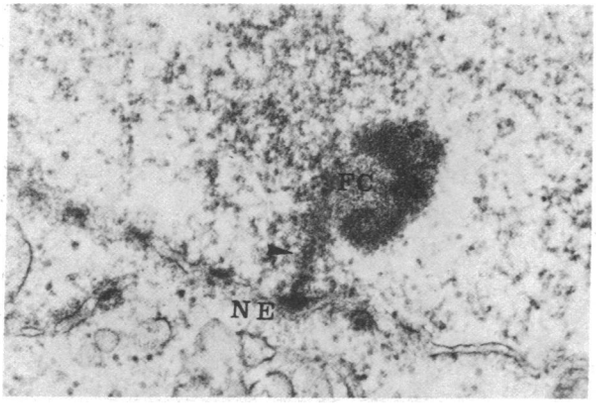

FIG. 3. Newly formed nucleolus from a leptotene-stage human spermatocyte. The nucleolar chromosome displays a single axial core (arrowhead). The telomere is attached to the nuclear envelope (NE). The nucleolar fibrillar center (FC) is penetrated by chromatin fibers emanating from the chromosomal secondary constriction region. (×22,000.)

proximal short arm, the centromeric region, and the long arm, with a synaptonemal complex becoming continuous at late zygotene. The distal (satellite) and proximal parts of the short arm displayed chromatin fibers that were denser than those of the long arm. In the secondary constriction region, chromatin fibers were seen penetrating into the adjacent fibrillar center of the nucleolus. The fibrillar center was surrounded by a layer of dense fibrils continuous with a developing fibrillogranular reticulum (Fig. 4*A*).

Among 20 nucleoli studied, 4 showed two fibrillar centers, each connected with a distinct bivalent (Fig. 4*B*). In a few nucleoli, fusion of the two fibrillar centers was observed although the connections with the bivalents were still visible (Fig. 4*C*).

Pachytene Stage. At mid-pachytene, the whole short arm—from the centromere region to the telomeric attachment site on the nuclear envelope—consisted of dense chromatin surrounding the synaptonemal complex. At late pachytene, the short arm chromatin was heavily condensed in a "basal knob" (29). The fibrillar center, segregated from the granular part of the nucleolus, was still connected with the short arm.

In 20% of the spermatocytes, two synaptonemal complexes were observed inside the basal knob, proving that two acrocentric bivalents cooperated in forming one nucleolus. The central part of the basal knob resulted from tight association of the chromatin fibers from the two bivalents, without any visible limit between them (Fig. 5*A*). The unique fibrillar center was associated with the medial part of the basal knob, at the same distance from the two synaptonemal complexes, as found in previous observations (17).

The fibrillar center was intensely argyrophilic when stained with the ultrastructural silver technique, confirming that it was equivalent to the chromosomal NOR (Fig. 5*B*). In the light microscope, the corresponding nucleolar area was also stained with silver; moreover, the connections with identifiable acrocentric bivalents could be demonstrated (Fig. 5*C*).

In situ hybridization performed with 28S [^{3}H]cDNA demonstrated that the fibrillar center contained the ribosomal genes at pachytene (Fig. 5*D*). Radioautographic studies showed the silver grains strictly limited to a spherule which corresponded to the fibrillar center, being located between the subterminal region of an acrocentric bivalent and the larger spherical part of the nucleolus. The number of grains was directly proportional to the number of bivalents associated with the fibrillar center.

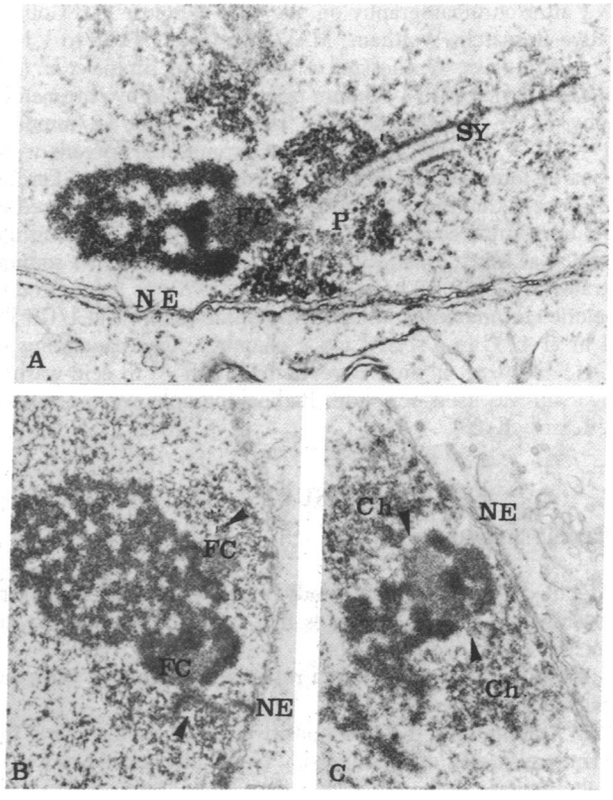

FIG. 4. Zygotene stage of human spermatocyte. (*A*) Part of nucleus showing the relationships among nucleolus, nucleolar bivalent, and nuclear envelope. The telomere of the cytological satellite is attached to the nuclear envelope (NE). The nucleolar fibrillar center (FC) is tightly connected with the secondary constriction region. Beyond that area, the bivalent shows the dense chromatin of the proximal short arm (P) in continuity with that of the centromeric region. In the long arm, the synaptonemal complex (SY) is surrounded by diffuse chromatin. (×20,000.) (*B*) Nucleolus displays two fibrillar centers (FC), each connected with the chromatin of a distinct bivalent (arrowheads). (×13,000.) (*C*) Fusion of two nucleolar fibrillar centers. The connections with the chromatin (CH) of two bivalents are visible (arrowheads). NE, nuclear envelope. (×17,500.)

DISCUSSION

Oogonia and type A spermatogonia differ in their pattern of nucleolar organization. In oogonia, the nucleolus contained a large number of fibrillar centers. In these cells, each nucleolus organizer probably passes through several fibrillar centers, as suggested in mouse oocyte (20) and in the human fibroblast (30). On the contrary, in spermatogonia of type A only one fibrillar center was observed in each nucleolus, suggesting that several NORs were associated within the same structure. In the interphase nucleus of these spermatogonia, it is obvious that the short arm of two or several acrocentrics can be located in close proximity. Therefore, at least in spermatogonia, the possibility of breakage followed by incorrect repair resulting in a translocation chromosome cannot be excluded, as suggested earlier (9). In that case, the clone of spermatozoa derived from one affected spermatogonium should contain the chromosomal rearrangement.

Ultrastructural studies revealed a strictly ordered arrangement of nucleolar bivalents in zygotene and pachytene spermatocytes and oocytes and demonstrated the proximity of chromatids in the short arm region of nonhomologous acrocentrics. The telomere corresponding to the satellite was consistently attached to the nuclear envelope. The nucleolar fibrillar center was always penetrated by rDNA-containing chromatin fibers

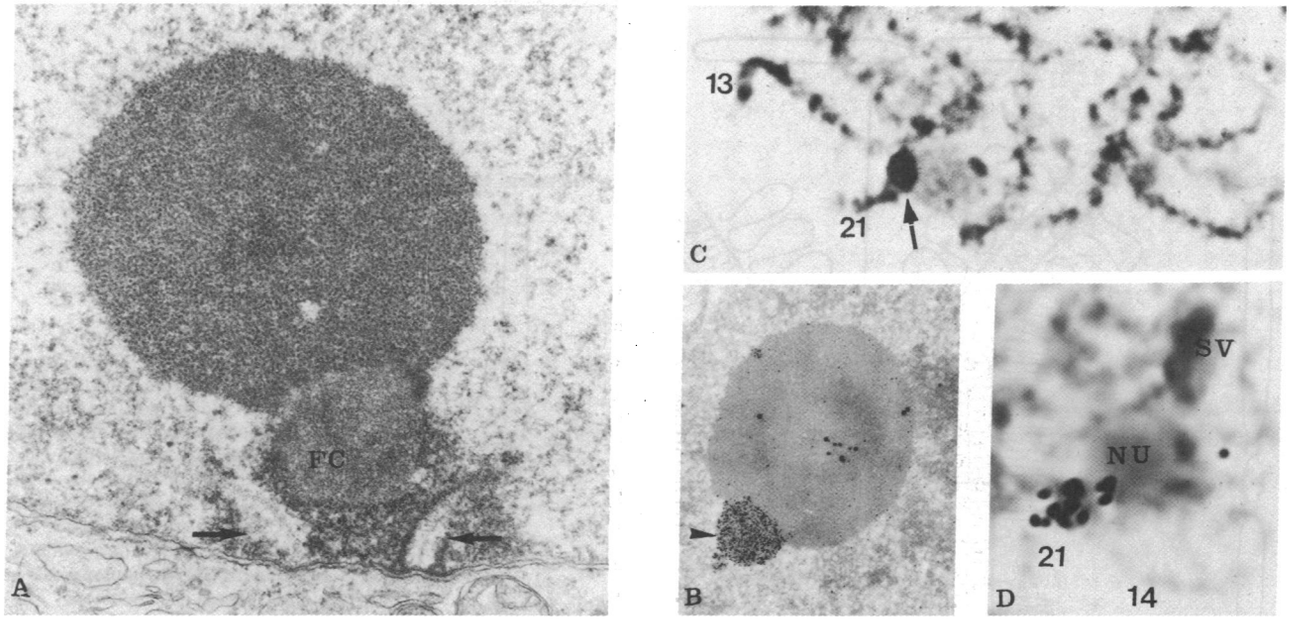

FIG. 5. Pachytene stage of human spermatocyte. (*A*) At late pachytene, the nucleolar components are segregated. The fibrillar center (FC) is connected with the medial part of a basal knob formed by aggregation of the dense chromatin constituting the short arm of two bivalents. Two synaptonemal complexes (arrows) are visible. (×27,000.) (*B*) Nucleolus stained with the ultrastructural NOR-silver technique. The segregated fibrillar center (arrowhead) is selectively argyrophilic. (×19,500.) (*C*) Detail of mid-pachytene in the light microscope. The silver-NOR stained region of the nucleolus corresponding to the fibrillar center (arrow) is connected with two acrocentric bivalents identified as 13 and 21 on the basis of their chromomere pattern. (×2,500.) (*D*) Radioautograph after *in situ* hybridization with 28S [^{3}H]cDNA. The silver grains are located in the nucleolar area corresponding to the fibrillar center. Two bivalents, identified as 14 and 21, are connected with the labeled area. NU, nucleolus; SV, sex vesicle. (×2,500.)

which emanated from the secondary constriction region. Association of ribosomal genes from two bivalents was observed in about 40% of oocytes and 20% of spermatocytes. Our observations are in agreement with tridimensional reconstructions of pachytene nuclei in human spermatocytes (31) showing that several bivalents can be associated with the same nucleolus.

When two nucleolar bivalents are located close to one another, tight contact of chromatids is formed by overlapped and even intermingled lateral loops. A breakage/reunion event between nonhomologous chromatids may result in the different observed types of Robertsonian translocation (Fig. 6). (*a*) If the abnormal recombination occurred between the centromere and the nucleolar fibrillar center, the translocation chromosome would be dicentric without NOR, a condition usually observed. (*b*) Breakage/reunion occurring inside the fibrillar center—i.e., in the NOR region—can be expected to generate a dicentric chromosome with partial preservation of the NOR. (*c*) The break points can occur on the short arm of one and the long arm of the other bivalent involved, each centromere being at a different distance from the nuclear envelope; the resulting translocation chromosome would be monocentric and devoid of rDNA. Because this latter rearrangement requires close proximity of the short arm chromatin from one bivalent and of the paracentromeric long arm chromatin from the other one, short arm polymorphism is probably a necessary condition for its occurrence. This is far from uncommon because variations in length or size can involve the satellite, the secondary constriction, and the proximal short arm. The proposed model is advantageous in that it may explain all observed types of Robertsonian translocation between nonhomologs.

It has been shown that, in humans, nucleolus organizers on nonhomologous chromosomes normally can be the sites of genetic exchanges. An analysis of the distribution of rDNA polymorphisms is consistent with a model involving unequal crossing-over between NORs (32). There is no contradiction between this information and our hypothesis for dicentric chromosome formation. Sequences such as palindromes might stimulate chromatid exchanges (33) and lead to rDNA polymorphism (34). The only difference in obtaining either rDNA polymorphism (32) or dicentric chromosomes formation (present report) is the orientation of the polarity of the DNA strands involved in the palindromic sequence. Preservation of the same polarity leads to rDNA polymorphism whereas an opposite polarity leads to dicentric formation (34). According to our ultrastructural findings on the spatial relationships between nonhomologous acrocentric bivalents, this opposite polarity can result from an inverted loop formation. The convoluted aspect of the chromatin fiber in the short arm region of associated bivalents provides the cytological basis of such loops. The necessary occurrence of an inverted loop to obtain a dicentric chromosome makes this event more uncommon than the unequal crossing-over resulting in rDNA polymorphism.

Accidental recombination between partially homologous sites on nonhomologs, with end-to-end pairing during meiosis, has been proposed as an explanation of dicentric Robertsonian translocations (15). It has been argued that, if this proposal were correct, a NOR would normally occur at Robertsonian translocation points, a condition that is seldom observed (6). Furthermore, our electron microscopic observations showed that the pattern of pairing between nonhomologs was consistently side by side, excluding the end-to-end hypothesis. The objections that have been raised (6) are overcome through the model we propose, if it can be demonstrated that the breakage/reunion nearly always occurs inside the proximal short arm, between the centromere and the NOR.

The chromosomal site of the breakage can be identified by studying, via *in situ* hybridization, the distribution of satellite DNA III in Robertsonian translocations. In the acrocentric chromosomes, this satellite DNA is located in the short arm (35,

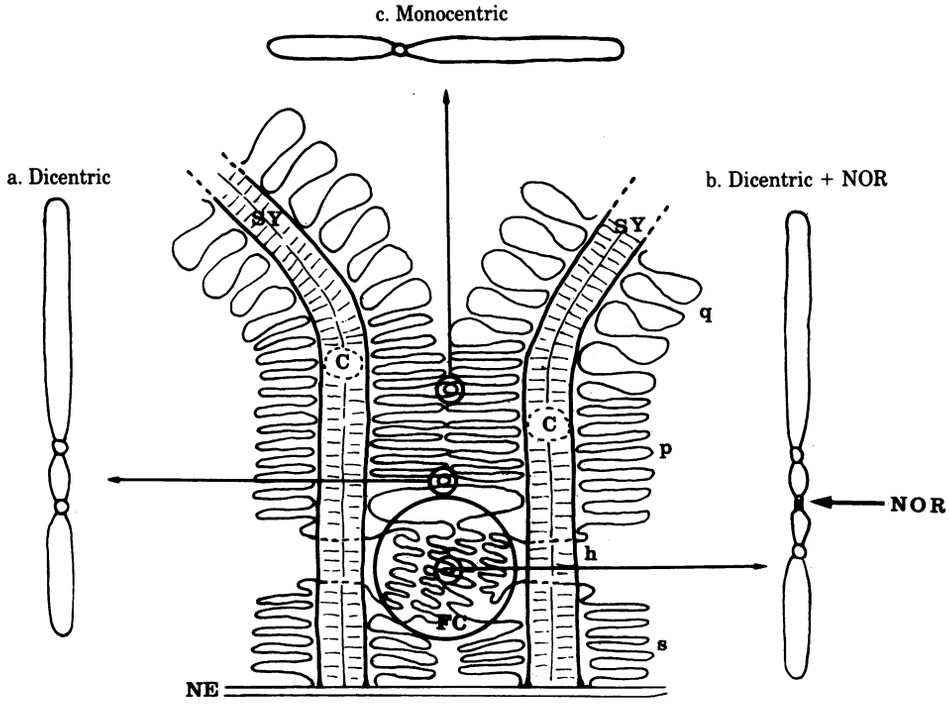

FIG. 6. Diagram showing the formation of Robertsonian translocations at pachytene stage after a breakage/reunion involving nonhomologous chromatids. (*a*) Dicentric without NOR; (*b*) dicentric with NOR; (*c*) monocentric. Only two chromatids are represented in each nucleolar bivalent. C, centromere; SY, synaptonemal complex; NE, nuclear envelope; FC, fibrillar center of the nucleolus; p, proximal part of the short arm; q, long arm; h, secondary constriction region; s, satellite.

36). In dicentric Robertsonian translocations, satellite DNA can be detected in association with each centromere, but its quantity is almost always less than that associated with the normal homolog's centromere. Because in most cases neither rDNA nor silver-positive NORs can be detected (4), the break points must almost always be within the region containing satellite DNA—i.e., between the centromere and the NOR (8). In monocentric translocations, no satellite DNA can be found in the chromosome arm that has lost its centromere. Our model is consistent with these findings.

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