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The association of the nucleolus and the short arm of acrocentric chromosomes with the XY pair in human spermatocytes: Its possible role in facilitating sex-chromosome acrocentric translocations

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Summary. Sex vesicle-nucleolus association was observed in 12% of zygotene and pachytene human spermatocytes using Giemsa and NOR-silver stained preparations. The silver-positive area of the nucleolus, corresponding to the nucleolus organizer (NOR), was usually close to the XY pair. C-banding frequently showed the terminal chromomere, formed by the condensed short arm of an acrocentric bivalent, attached to the sex vesicle. When a nucleolus produced by transcription of rDNA was connected to the short arm, it seemed to be secondarily associated with the sex vesicle. Non-transcribed ribosomal genes, which did not form a nucleolus, were revealed by in situ hybridization. Autoradiographs showed the rDNA-containing short arm of acrocentric bivalents associated with the sex vesicle in 18% of spermatocytes. The difference with the frequency of nucleolus-XY pair association was partially explained by the presence of inactive ribosomal genes. Moreover, electron microscopy showed that the dimensions of the newly formed nucleoli at early zygotene did not exceed 0.5 μm; they can be missed in light microscope investigations. From early zygotene to late pachytene, close relationships were observed between the sex vesicle chromatin and that of the associated acrocentric bivalent, especially in the short arm region. These relationships might explain the frequent involvement of acrocentrics in Y-autosome and X-autosome translocations occurring during male meiosis.

Introduction

Sex chromosome/autosome translocations mostly involve acrocentric chromosomes. In Y/autosomal translocations, usually the heterochromatin of the Y long arm is translocated onto the short arm of a D or G chromosome, among which chromosome 15 is the most frequent one involved (Noël 1979; Smith et al. 1979; Davis 1981). Similarly in X/autosomal translocations, the pairs 15, 21, and 22 are affected more often than would be expected by chance (Mattei et al. 1982). Since it is generally accepted that these translocations occur during meiosis, it would be useful to investigate whether cytologic or molecular explanations could account for their preferential occurrence.

Association of the nucleolus and the XY pair forming the sex vesicle (Sachs 1954) is consistently observed in some species, for instance in mouse spermatocytes at pachytene (Ohno et al. 1957; Kierszenbaum and Tres 1974; Hofgärtner et al. 1979), although ribosomal genes are located only on autosomal sites (Henderson et al. 1976). The close contact of nucleolus and sex vesicle results from association of at least one, and often two actively transcribed NOR-carrying bivalents with the XY pair (Knibiehler et al. 1981). In man, association of nucleolus and XY pair has been occasionally observed in human spermatocytes using light microscopy (Ferguson-Smith 1964; Eberle 1966; Luciani 1970) and electron microscopy (Solari and Tres 1970a,b; Tres 1975; Holm and Rasmussen 1977), but has not been recorded in spread preparations (Moses et al. 1975). Moreover, association of an extra chromosome 21 with the sex vesicle has been observed in Down syndrome (Johannisson et al. 1983). According to Solari and Tres (1970a) the occasional association of nucleoli with the sex vesicle is not based on a functional relationship and may only reflect the tendency of heterochromatin to association.

The aim of this work is to present a revaluation of the relationships of the XY pair with the nucleolus and the short arm of acrocentrics where the ribosomal genes are located, and to discuss some of their implications in human cytogenetics. For this investigation, we performed several converging techniques, because each technique used alone is unable to provide a definitive answer to the question of whether the acrocentric chromosomes or nucleoli are randomly arranged in relation to the XY pair: (1) meiotic techniques using hypotonic pretreatments or squashing disturb the spatial distribution of nuclear structures; (2) non-transcribed ribosomal genes do not form a nucleolus. The screening of nucleoli associated with the sex vesicle leads to an underestimation of its relationships with nucleolus organizers (NORs). We used in situ hybridization to visualize both active and inactive ribosomal genes. (3) At the beginning of their formation, nucleoli are very small (about 0.5 μm) and are scarcely visible in the light microscope. Therefore, we investigated their appearance and development with the electron microscope. Moreover,

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only electron microscopy allows a precise analysis of the relationships between chromatin fibers belonging respectively to the XY pair and to acrocentrics.

Materials and methods

The testicular samples originated from eight normal fertile patients who were subjected to testicular surgical operation under general anesthesia and from six patients undergoing a testicular biopsy for infertility.

Light microscopy

Meiotic preparations were obtained from the seminiferous tubules using the technique of Luciani et al. (1974), which avoids hypotonic treatment. The germ cells were stained with Giemsa, C-banding, and according to the NOR-silver technique of Goodpasture and Bloom (1975). Eight hundred zygotene and pachytene nuclei were analyzed.

Electron microscopy

Testicular biopsies were fixed with 2.5% glutaraldehyde in 0.1*M* cacodylate buffer pH 7.3 for 2h, then post-fixed with 2% osmium tetroxide in the same buffer for 2.5h. After embedding in Epon, thin sections were stained with uranyl acetate and lead citrate, then examined in a Siemens Elmiskop 101 electron microscope at 80kV. Ultrastructural visualization of NORs was achieved by silver staining according to the method of Hernandez-Verdun et al. (1980).

In situ hybridization. rRNA (28S) was isolated using a sucrose gradient from human thyroid polysomal RNA free of poly A+ RNA after chromatography on oligo-dT cellulose (T3 Collaborative Research). 28S ^{3}H cDNA (specific activity 0.9 to 1.1 $\times 10^8$dpm/μg) was synthesized as described by Brahic et al. (1981) using calf thymus DNA random primer purified by chromatography on DEAE cellulose and ^{3}H dCTP (50 to 80Ci/mmol Amersham U.K.). Chromosome spreads were prepared for in situ hybridization according to the method of Gall and Pardue (1971), except that the DNA denaturation step was performed in 95% formamide, 0.1× standard saline citrate (NaCl/Cit) for 2h at 72°C. cDNA (28S) was hybridized to meiotic chromosomes in 50% formamide, 3xNaCl/Cit, for 24h at 42°C in a sealed moist chamber. Twenty ng of cDNA in a 20μl mixture covered by an 18×18 acid washed coverslip were used per slide. Radioautographs were prepared according to the method of Gall and Pardue (1971).

Results

In 12% of zygotene and pachytene nuclei observed in Giemsa and silver-stained preparations, at least one of the nucleoli associated with the sex vesicle (Fig. 1a). The main nucleolus and the small nucleoli were involved with nearly the same frequency.

Well-spread preparations stained with Giemsa showed the exact relationships between the short arm of acrocentrics, the nucleolus, and the sex vesicle. In the pachytene spermatocyte, the entire short arm from centromere to satellite is condensed to form a heteropycnotic terminal chromomere (Luciani et al. 1975). We noticed that it was the terminal chromomere of one of the acrocentric bivalents that was attached to the sex vesicle. The nucleolus was connected to the terminal chromomere and therefore adjacent to the sex vesicle (Fig. 1b). Occasionally, spermatocytes were observed in which the short arm of an acrocentric bivalent without any visible nucleolus associated with the sex vesicle. The condensed terminal chromomere appeared inserted into the surface of the XY pair (Fig. 1c and d). In C-banded preparations, the terminal chromomere could sometimes be observed in association with a darkly stained component of the sex vesicle that probably corresponded to the Y heterochromatin (Fig. 1e). However, it was also observed connected with the remaining chromatin of this vesicle (Fig. 1f).

When the spermatocytes were stained with silver, the axes of the X and Y chromosomes were often visualized within the sex vesicle. In a few sex vesicles, two argyrophilic bodies were seen, corresponding to the dense bodies respectively associated with the Y axis and the X axis (Solari 1980). The organizer region of the nucleolus (NOR) consistently appeared as a black round area, segregated from the other nucleolar components. The Ag-positive NOR was usually adjacent to one side of the XY pair (Fig. 2a). The location of the NOR confirmed that the XY pair-nucleolus relationships represented an association of the short arm of an acrocentric bivalent with the XY pair. In some nuclei, the sex vesicle associated nucleolus was so small that it was restricted to an Ag-stained spherule. In a few cells, two nucleoli showed relationships with the XY pair (Fig. 2b).

In situ hybridization allowed localization of ribosomal genes in acrocentric bivalents and nucleoli (Arroua et al. 1982; Stahl et al. 1983). Among 400 hybridized nuclei, ribosomal genes, indicated in radioautographs by clusters of grains, were found associated with the sex vesicle in 72 cells, i.e., in 18% of spermatocytes. Two different aspects were observed. Most often a nucleolus was associated with the sex vesicle. The labeling grains were located close to the sex vesicle in the same area as the silver-positive NOR (Fig. 2c). In well-spread nuclei the terminal chromomere of an acrocentric bivalent could be seen connected to the labeled area. In other cells, the nucleoli were far from the sex vesicle. A labeled area was consistently observed at the junction of these nucleoli with the short arm of the corresponding acrocentric bivalent. Moreover, a cluster of grains was observed on one side of the sex vesicle, in an area where one, or more seldom two, acrocentric bivalents were attached by their short arm (Fig. 2d). The absence of any visible nucleolus suggested that this cluster of grains corresponded to nontranscribed ribosomal genes.

Electron microscopy provided a partial explanation of the different association frequencies observed among in situ hybridization preparations and among Giemsa-stained slides. It also allowed a more precise analysis of the relationships between the XY pair and the short arm of acrocentrics.

The XY pair appeared during zygotene as a dense and heterogeneous mass associated with the nuclear envelope. It contained a short core corresponding to the axis of the Y and a long core corresponding to that of the X chromosome. A short synaptonemal complex attached to the nuclear envelope was formed by pairing of the two cores in a common and region corresponding to homologous segments, as described by Solari and Tres (1970a,b) and Holm and Rasmussen (1977). The packing of both autosomal and XY pair chromatin was relatively loose at mid-zygotene and increased progressively as meiotic prophase advanced. However, from mid-

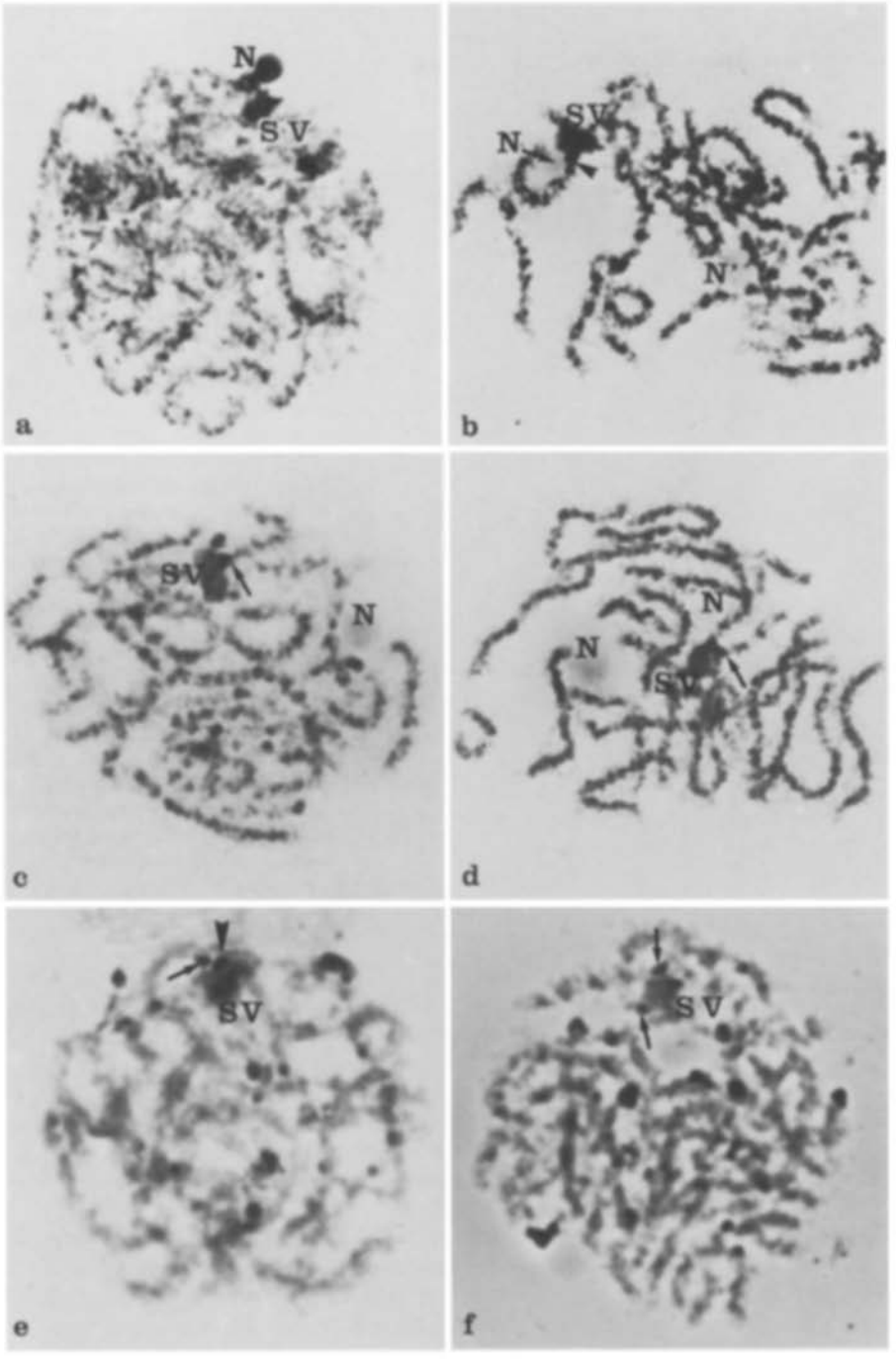


Fig. 1a–f. Pachytene stage of human spermatocytes showing the association of nucleoli (N) and/or acrocentric bivalents with the sex vesicle (SV). **a** A large nucleolus is associated with the sex vesicle. Silver staining. **b** The terminal chromomere *(arrowhead)* of an acrocentric bivalent is attached to the sex vesicle. The nucleolus (N→) connected to the terminal chromomere is secondarily associated with the XY pair. N′: main nucleolus. Giemsa staining. **c** and **d** Acrocentric bivalents devoid of nucleoli, attached to the sex vesicle by their condensed short arm *(arrow)*. Giemsa staining. **e** C-banding showing the terminal chromomere *(arrow)* of an acrocentric bivalent associated with a darkly stained area *(arrowhead)* of the sex vesicle, presumably corresponding to Y-heterochromatin. **f** C-banding. Two terminal chromomeres of acrocentric bivalents *(arrows)* are connected with lightly stained areas of the sex vesicle (× 2,000)

to late pachytene the autosomal chromatin decondensed (with the exception of the acrocentric bivalents short arms) while the sex vesicle remained densely packed. At early stages, the sex vesicle displayed dense bodies associated with the single cores but ring-like bodies were lacking, as recorded by Solari and Tres (1970a, b) and Solari (1974). The packing of chromatin and the changes of characteristic structures inside the sex vesicle, compared with the evolution of nucleoli, were useful features for establishing the sequence of substages from early zygotene to late pachytene.

At early zygotene, nucleoli developed at the secondary constriction region of nucleolar bivalents, first appearing as small fibrillar centers partially surrounded by a layer of dense fibrils. The dimensions of these structures did not exceed 0.5 μm. Thus, they were scarcely visible in the light microscope. In the electron microscope, the nascent nucleolus and the corresponding bivalent could quite often be observed in close association with the sex vesicle (Fig. 3a). The NOR-silver reaction performed at the ultrastructural level showed a nucleolar area heavily stained with silver closely associated with the sex vesicle near the nuclear envelope (Fig. 3b). This area corresponded to the fibrillar center of the nucleolus, as in most other cell types (Hernandez-Verdun et al. 1980). This localization was similar to that generally observed in the light microscope. From mid-zygotene to early pachytene, the growing nucleolus extended along the nucleoplasmic side of the sex vesicle (Fig. 3c, d) forming a large fibrillo-granular reticulum which should be easily visible in the light microscope. During

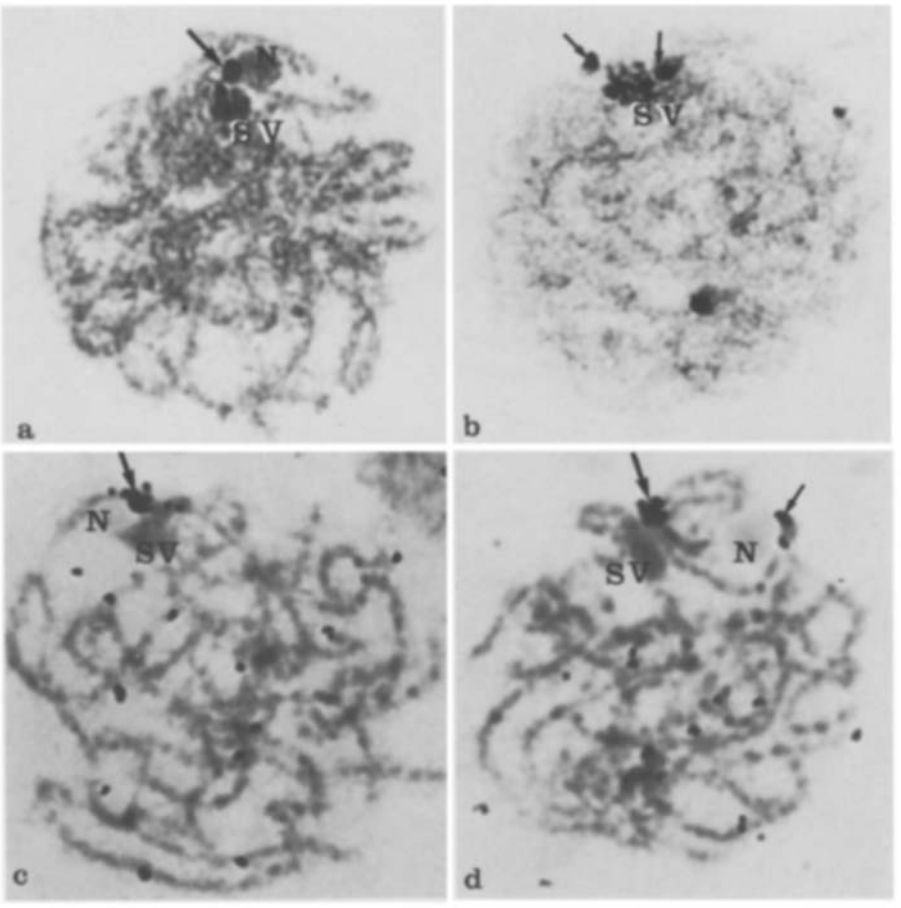


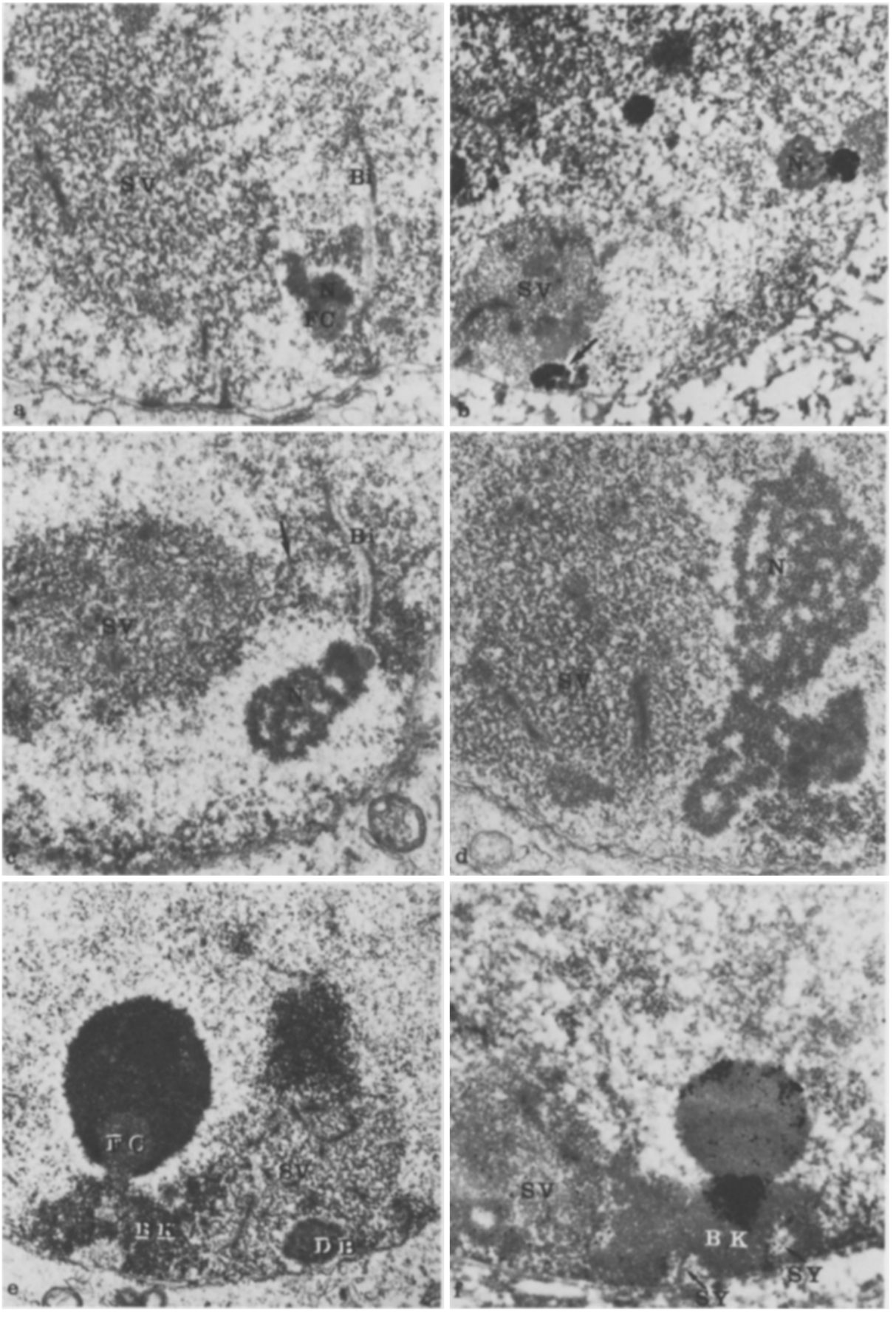
Fig. 2a–d. Relationships of silver-stained NORs (**a** and **b**) and rDNA (**c** and **d**) with the sex vesicle (SV). **a** The silver-stained NOR *(arrow)* of a large nucleolus (N) is adjacent to the sex vesicle. **b** Two small nucleoli with silver-stained NOR *(arrows)* are associated with the sex vesicle, which displays the axes of the X and Y chromosomes. **c** and **d** Autoradiographs following in situ hybridization performed with 28S ^{3}H cDNA. **c** Labeling grains *(arrow)* are seen overlying the terminal chromomere of an acrocentric bivalent and the adjacent part of the connected nucleolus (N), both associated with the sex vesicle. **d** A cluster of labeling grains *(large arrow)* is located over the sex vesicle-associated terminal chromomeres of two acrocentric bivalents devoid of nucleoli. Far from the sex vesicle, a large nucleolus (N) is connected with the labeled short arm *(small arrow)* of an acrocentric bivalent (× 2,000)

these stages the chromatin of the acrocentric bivalent came in contact over a variable length with the sex vesicle chromatin (Fig. 3c).

From mid- to late pachytene, the whole short arm of all acrocentric bivalents, from the centromere region to the telomeric attachment site on the nuclear envelope, progressively condensed to form a "basal knob" (Woollam and Ford 1964). When an acrocentric bivalent was associated with the XY pair, a close apposition was observed between the XY chromatin and the dense chromatin of the "basal knob". In most cases, simply the difference in electron density indicated the limit of each structure. Only the short arm of acrocentrics was involved in this sex vesicle connection from mid to late pachytene. During this stage, the nucleolus showed a spontaneous segregation isolating the fibrillar center from the mainly granular component. The fibrillar center was still connected with the condensed short arm (Fig. 3e). In about half of the observed nucleolus-sex vesicle associations, the main bulk of the nucleolus was not directly in contact with the sex vesicle. An interweaving of the nucleolar and sex vesicle components as described in mouse spermatocytes (Kniebiehler et al. 1981) never occurred in man. Thus, the electron microscope ultrastructural observations unequivocally demonstrated that the nucleolus-sex vesicle association was secondary to the close connection established between the short arm of an acrocentric and the XY pair.

The fibrillar center of the nucleolus was intensely argyrophilic when stained with the ultrastructural silver technique, suggesting that it was equivalent to the chromosomal NOR. The silver stained area was consistently connected with the dense chromatin of the short arm. When two acrocentric bivalents cooperated in forming a common nucleolus, the silver stained fibrillar center was located in the medial part of the "basal knob" formed by aggregation of the condensed short arms. Such an aggregate was also frequently close to the sex vesicle (Fig. 3f).

Fig. 3a–f. Electron micrographs showing the relationships of nucleoli (N) and acrocentric bivalents (Bi) with the sex vesicle (SV), from early zygotene to late pachytene in human spermatocytes. **a** Early zygotene. A newly formed nucleolus attached to an acrocentric bivalent is in close contact with the sex vesicle. The fibrillar center (FC) of the nucleolus is connected to the secondary constriction region of the bivalent (× 23,000). **b** Mid-zygotene, silver-staining. The silver-positive NOR *(arrow)* of a small nucleolus is closely associated with the sex vesicle. N: main nucleolus with a silver-stained NOR (× 14,000). **c** Late zygotene: An acrocentric bivalent with a growing nucleolus is adjacent to the sex vesicle. Close contact is observed between the chromatin of the sex vesicle and that of the nucleolar bivalent *(arrow)* (× 21,500). **d** Early pachytene: a large reticulated nucleolus is extending along the nucleoplasmic side of the sex vesicle (× 23,000). **e** Late pachytene. The condensed chromatin of the short arm of the acrocentric bivalent, forming a "basal knob" (BK), is closely apposed to the sex vesicle. The nucleolar fibrillar center (FC) is connected with the condensed short arm. DB: dense body (× 21,000). **f** Late pachytene, silver staining. The "basal knob" (BK) formed by aggregation of the condensed short arm of two acrocentric bivalents is adjacent to the sex vesicle. The nucleolar area heavily stained with silver is the fibrillar center and corresponds to the NOR. SY: synaptonemal complex (× 20,000)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Discussion

The frequency of the sex-vesicle-nucleolus association in human spermatocytes has been estimated to be 6% (Luciani 1970). In the testicular biopsies that we studied, a frequency of 12% was observed on Giemsa- and silver-stained preparations. Moreover, our investigations demonstrated that visualization of ribosomal genes increased this percentage to 18%. This difference is explained in two ways: (1) The nucleolus is lacking when the ribosomal genes are inactive (non-transcribed). However, the presence of rDNA and consequently of an acrocentric is revealed by in situ hybridization. (2) At the beginning of its elaboration, the nucleolus is so small (0.5 μm) that it is easily observable only in the electron microscope and may be missed in the light microscope.

An 18% frequency suggests that the relationship of NORs or nucleoli with the sex vesicle is not random. Assuming that every bivalent has an equal probability of associating with the XY pair, with five acrocentric bivalents, association of acrocentrics would be expected 23% of the time. This probability only indicates that any segment (i.e., short arm, centromere region, or long arm) of an acrocentric bivalent is expected to be found close to the XY pair approximately in one out of five cells. As the observed association involves only one end, always the same one, namely the short arm telomere, the chance of random association is much lower. The fact that a tight connection is established between the nucleolar telomere in the light microscope (or its equivalence in the electron microscope, the "basal knob") and the sexual pair constitutes by itself an argument in favor of a non-random relationship.

Although some similarities exist between the sex vesicle-nucleolus association in man and mouse, there are also striking differences: (1) In the mouse this relationship is almost constant, while in man it is only frequent. (2) A close association is always developed in the mouse spermatocyte between the nucleolar components and the XY pair (Ford and Woollam 1966; Solari 1974; Hofgärtner et al. 1979). The crescent-shaped nucleolus covers the nucleoplasmic side of the sex vesicle and sends invaginations into its chromatin (Solari and Tres 1967; Knibiehler et al. 1981). This close association and interweaving of the nucleolar and sex vesicle components is not observed in man. As shown by our light- and electron-microscope observations, the nucleolus is secondarily associated with the sex vesicle because it is attached to the short arm of an acrocentric bivalent. It is this short arm that is primarily connected to the chromatin of the sex vesicle with a significant frequency.

The question arises whether the short arm of acrocentrics associates with the Y or the X chromosome, or with both. Driscoll et al. (1979) observed that in human pachytene spermatocytes after Q-banding, an acrocentric bivalent was occasionally in association with the fluorescent region of the Y chromosome in the sex vesicle. C-banding confirmed that associations involving the sex vesicle usually appear to be with the Y-heterochromatin. According to Rasmussen and Holm (1978) electron micrographs of the XY pair show that a nucleolus-like structure is associated with the Y chromosome in the human pachytene spermatocyte. These observations are in accordance with those made in somatic cells, where association of Y-chromosome with the nucleolus is very frequent (Bobrow et al. 1971; Gagné et al. 1972; Therkelsen and Petersen 1971; Goldgefter et al. 1973; Iorio and Wyandt 1973). Somatic pairing of chromosomes Y and 15 has been demonstrated by Schmid et al. (1983) in lymphocyte cultures treated with 5-azacytidine, a cytosine analogue that can be incorporated into DNA where it is substituted for 5-methylcytosine. Direct contact was observed between the short arm of chromosome 15 and the long arm of the Y. The pericentromeric regions of the other acrocentric chromosomes also participated in somatic pairing, although the heterochromatin of these chromosomes is not 5-azacytidine sensitive. All these associations may be the result of the well-known attraction of constitutive heterochromatin located in the Y chromosome and in the short arm of acrocentrics. Solari and Tres (1970a) and Solari (1974) already stated that the proximity of nucleoli to the sex vesicle may only reflect the tendency of heterochromatin to association, as nucleoli are attached to heterochromatic "basal knobs". More precise information is available since Gosden et al. (1975, 1978, 1979) demonstrated that satellite DNA III is present in the short arm of all acrocentrics. The distribution is the same in meiotic male chromosomes (Seuanez et al. 1976). The 3.5-kilobase male-specific sequence, located in the long arm of the Y chromosome, is associated with human satellite III. Hybridization experiments demonstrate that a sequence, which is totally or partially homologous to the 3.5-kilobase fragment, is present in chromosome 15 (Bostock et al. 1978). A similar 3.4-kilobase sequence found in the Y chromosome DNA hybridizes to chromosomes 14 and 15. This sequence is related to human satellite III, although it shows important differences in structure (Cooke and McKay 1978). It has been recently shown that satellite III DNA derived from male tissue contains a 3.4-kilobase fragment that is located on the long arm of the Y chromosome (Gosden et al. 1984).

The presence of highly methylated DNA has been demonstrated in the short arm of chromosome 15 and in the long arm of the Y chromosome by immunofluorescence techniques after binding of antibodies specific for 5-methylcytosine (Miller et al. 1974; Schnedl et al. 1975). It is generally accepted that heterochromatin and satellite DNAs are enriched in 5-methylcytosine (review in Cooper 1983). All these data suggest that the attraction between the short arm of acrocentrics and the Y chromosome is mediated by common DNA sequences.

In our material, with C-banding the short arm of acrocentrics could be seen associated with the darkly stained Y-heterochromatin of the sex vesicle, but was also found with approximately the same frequency connected to other parts of this vesicle. Hence, our observations suggest that the short arm of acrocentrics can also be specifically associated with the chromatin of the X chromosome. Recently, sequences with repetitive segments have been identified which are located on both X and Y chromosomes (Rappold et al. 1984). Several repetitive sequence families have been mapped to the X, some of them in the centromeric region, others distributed throughout the X (review in Miller et al. 1984). Assuming that repetitive sequences play a causal role in "heterochromatic attraction" (Schmid et al. 1975), these data suggest that the X participates in attracting the short arm of acrocentrics to the sex vesicle.

A prerequisite for interchromosomal rearrangements is the proximity of the involved chromatids during the mitotic cycle or meiosis. The well-known variability of heterochromatic blocks, which most often coincide with highly repetitive DNA, shows that heterochromatin is prone to undergo intra- and interchromosomal rearrangements. De novo rearrangements of constitutive heterochromatin are well-documented in

mitotically dividing cells (review in Kurnit 1979). It has been argued that meiotic crossing-over is a rare event in constitutive heterochromatin and, according to that fact, meiosis is an unlikely source for rearrangements of heterochromatic regions (Kurnit 1979). However, it is probable that the mechanisms involved in crossing-over and interchromosomal rearrangements are not exactly the same. Meiotic recombination seems to be mediated by recombination nodules, which are specific structures located on the synaptonemal complex (Carpenter 1981; Rasmussen and Holm 1978) and are considered as organelles packaging enzymes and structural proteins that control its ordered progressing. Interchromosomal rearrangements might involve a breakage-reunion event where incorrect repair plays an essential role. Our observations do not exclude that autosome-sex chromosome translocations occur during the spermatogonial mitotic cycle. However, in demonstrating the frequent connection of the short arm of acrocentrics with the XY pair, they provide a cytologic basis for their translocation and show that its occurrence at zygotene-pachytene is a definite possibility.

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