Indirect immunofluorescence staining with human anti-kinetochore antibodies was used to study the position of centromeres during vertebrate spermiogenesis. Many species of Amphibia have a low chromosome number and very large spermatids and spermatozoa. The number of kinetochore dots correlates exactly with the haploid chromosome number. This implies that kinetochore duplication occurs in the interval between meiosis I and meiosis II. The nonhomologous centromeres are arranged in tandem during the entire course of spermiogenesis and in mature spermatozoa. A higher order centromere arrangement was found in spermogenic cells of Anura and Urodela. In mammals, immunofluorescence analysis is complicated by the extreme condensation of chromatin during spermiogenesis and the high chromosome numbers. Nevertheless, centromere–centromere associations were observed in mammalian round spermatids and sporadically in testicular spermatozoa. This indicates that pair-wise association of centromeres is a universal principle of centromere arrangement at the postmeiotic stage.


to whom reprint requests should be addressed at Department of Human Genetics, University of Würzburg, Koellikerstraße 2, D-8700 Würzburg, Federal Republic of Germany.

157

Copyright © 1990 by Academic Press, Inc.

All rights of reproduction in any form reserved.


Paired Arrangement of Nonhomologous Centromeres during Vertebrate Spermiogenesis

T. HAAF, 1 H. GRUNENBERG, AND M. SCHMID

Department of Human Genetics, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany

INTRODUCTION

Autoimmune sera from CREST-syndrome scleroderma patients bind to kinetochores of mitotic chromosomes and to prekinetochores of interphase nuclei in a highly specific manner [1]. Indirect immunofluorescence staining with naturally occurring antibodies provides a valuable cytochemical method to render centromeres/kinetochores visible throughout the cell cycle [2] and during meiosis [3, 4]. After detergent treatment, it is possible to identify kinetochore dots in mature mammalian spermatozoa [5]. This proves that the kinetochore antigen is retained during spermiogenesis and passed into the zygote at fertilization.

In mammalian spermatid nuclei, however, the number of kinetochore dots observed is always considerably less than the haploid chromosome number. The number of fluorescent dots shows a binomial distribution with a mean value of half the expected number. Based on this observation, del Mazo et al. [6] postulated a different centromere organization in meiotic and postmeiotic cells. Another hypothesis has been proposed by Brinkley et al. [3], which states that nonhomologous centromeres may be fused into pairs.

In the present study, centromeres/kinetochores were immunolocalized in spermatids and spermatozoa of widely divergent vertebrates. Amphibian species with very large spermatid nuclei and low chromosome numbers were particularly suited for such investigations. During spermiogenesis, the centromeres of nonhomologous chromosomes showed a strong tendency to arrange themselves in tandem.

MATERIALS AND METHODS

Immediately after each animal was sacrificed, the testes were dissected out. When larger animals were used, an approximately pean-sized biopsy of a testicle was sufficient. Testis material from human males was obtained by orchiectomy for therapeutic reasons. The tunica albuginea of each testis was removed and the seminiferous tubules were collected in balanced phosphate buffer (PBS, pH 7.3). The seminiferous tubules were minced as finely as possible in a petri dish with a pair of small scissors. Using Pasteur pipets the cells were then vigorously resuspended in 10 ml of PBS. The cell suspension was allowed to settle in a centrifuge tube; after about 15 min, the supernatant was removed with a pipet and used as a source of spermatogenic cells. All postmeiotic stages were present in the supernatant in adequate numbers.

The cells were washed in PBS and centrifuged at 1400 rpm for 8 min. The pellet was resuspended in a hypotonic mixture consisting of 10 mM Hepes (pH 7.3), 30 mM glycerol, 1.0 mM CaCl2, and 0.8 mM MgCl2 [7] and incubated at 4°C for 10 min. Hypotonic treatment was designed to engorge the cells without rupturing the cell membrane. The cell density in the hypotonic solution was adjusted to 106 cells/ml. Aliquots of 0.5 ml of the hypotonic solution were centrifuged (Cytospin 2, Shandon) onto clean glass slides at 800 rpm for 4 min. After cyt centrifugation the slides were fixed in −20°C methanol for 30 min and then immersed in ice-cold acetone for a few seconds. Following three 10-min washes in PBS, the preparations were ready for immunofluorescence staining.

Spermiogenic cells of various mammalian species (humans, primate, hamster, harvest mouse, and mouse) and Amphibia (Bufo asper, Necturus maculosus, and Triturus alpestris) were used as substrate for kinetochore staining. The preparations were incubated at 37°C with kinetochore-specific antibodies in a humidified incubator for 30 min.
Sera containing high titres of anti-kinetochore antibodies were obtained from CREST-syndrome scleroderma patients [8]. The autoimmune sera were diluted 1:100 with PBS for indirect immunofluorescence staining. Following three additional washes in PBS, the slides were incubated for 30 min at 37°C with fluorescein (FITC)-conjugated anti-human IgG (Dianova), diluted 1:20 with PBS. The slides were washed three more times with PBS and then counterstained for 1 min with Hoechst 33258 (50 μg/ml in PBS) or ethidium bromide (1 μg/ml). The slides were again rinsed in PBS and finally mounted in Mowiol 4-88 (Calbiochem).

RESULTS

Indirect immunofluorescence was performed on spermiogenic cells of different mammalian and amphibian vertebrates. Centromeres/kinetochores were selectively stained by human autoantibodies from scleroderma CREST patients. The two-dimensional centromere arrangement observed in flattened cells was used to draw conclusions about the topological relationship among centromeres in the living spermatid nucleus. Although hypotonic treatment and cytocentrifugation, by necessity, go along with a partial destruction of the natural chromosome arrangement, it was possible to demonstrate highly nonrandom centromere patterns during vertebrate spermiogenesis. The immunocytochemical techniques themselves cannot produce the impression of an ordered arrangement if such order does not exist.

In preparations with cell suspensions, many histological characteristics, e.g., location of a cell within a tissue, are lost. In seminiferous tubule cells, however, the spermatids and spermatozoa can be unequivocally identified on the basis of their size and shape. Early spermatids, whose nuclei are completely spherical, originate from the telophase of the second meiotic division. The diameter of spermatid nuclei is distinctly smaller than that of diploid cells. With increasing maturity and volume reduction, their chromatin condenses uniformly within the nucleus. During the late spermatid stages, the cells undergo an elongation phase in which the nuclei assume a characteristic shape (e.g., sickle-shaped in mice or rod-like in toads). Spermatozoa probably represent the most differentiated cells of the body; their heads contain the tightly packaged haploid genome together with a tiny amount of cytoplasm.

Toads of the genus Bufo (Anura) are endowed with 22 submetacentric to metacentric chromosomes. All bufonids have large spermatids identifiable in every stage of differentiation and elongated mature sperm nuclei. Figure 1 demonstrates immunofluorescence staining of centromeres/kinetochores in both round spermatids and testicular spermatozoa of Bufo asper. In order to determine the accurate number of anti-kinetochore antibody-binding sites per haploid nucleus, it was often necessary to
focus the microscope in more than one optical plane. The majority of nuclei displayed 11 discrete kinetochore dots (Figs. 1a, 1c, and 1d). This means that each chromosome (chromatid) of the haploid nucleus is provided with a functioning centromere/kinetochore that serves as an attachment site for the spindle apparatus. Immunofluorescence staining permitted the identification of aneuploid spermatids and spermatozoa (Fig. 1b). From 100 postmeiotic cells analyzed, only 88 showed an euploid centromere number; seven cells contained 10 centromeres ($n-1$), whereas five cells contained 12 centromeres ($n+1$). Based on these observations, the rate of aneuploid germ cells is expected to be approximately 10%, which is comparable to that of mammals. Chromosomally unbalanced gametes do not appear to be subject to a strong selective process.

The spermiogenic cells of *B. asper* showed very distinct centrometric associations. The paired centromeres did not come into direct contact, but maintained a characteristic distance from each other which corresponded to the size of the two kinetochore dots themselves. Round spermatids usually contained three pairs of centromeres (Figs. 1a and 1b). In the elongated spermatids and testicular spermatozoa, four to five fluorescent double dots could be observed (Figs. 1c and 1d). This suggests that at least 8 of the 11 nonhomologous centromeres in haploid cells are arranged in pairs. The centromere pairs were distributed evenly along the entire length of the rod-like nucleus. During the elongation of the spermatid nucleus, however, the centromeres became preferentially situated near the nuclear membrane.

Centromere arrangement during spermiogenesis of salamanders and newts (*Urodela*) was of particular interest. The centromeres were always found in the basal half of the round spermatid (Fig. 2), which is direct evidence for a higher order organization within the haploid nucleus. It is presumed that after meiosis II centromeres maintain their anaphasic position throughout the entire process of spermiogenesis; polarization is thought to result from the traction on the centromeres exerted by the spindle fibers [9]. However, another conspicuous feature of spermiogenesis in *Urodela* is not satisfactorily explained by this model, which consists of the pair-wise arrangement of nonhomologous centromeres in the basal part of the spermatid nucleus. Figure 2 illustrates this phenomenon with representative nuclei of *N. maculosus* and *T. alpestris*. Experimental evidence suggests that at least half of the centromeres are associated into pairs. Such a specific centromere pattern cannot be due to meiotic pairing at prophase I or to chromosome positioning at anaphase II. Since nonhomologous pairing of centromeres usually increases during spermatid development, centromeric association must be considered as a dynamic process germane to spermiogenesis.

![FIG. 2. Centromere arrangement during spermiogenesis of salamanders and newts. (a) Late spermatid nucleus of *Necturus maculosus* ($n=19$). (b) Early spermatid nucleus of *Triturus alpestris* ($n=12$). The upper panels demonstrate anti-kinetochore immunofluorescence; the lower panels show chromatin staining with Hoechst 33258. The constitutively heterochromatic regions are characterized by their bright Hoechst 33258 fluorescence. Note the conspicuous polarization of all centromeres in the basal part of the spermatid nucleus. The nonhomologous centromeres are usually arranged in pairs. Bar represents 10 µm.](image)

Mammalian spermatids contain varying numbers of kinetochore dots (Fig. 3). However, even in the very early round spermatids, the number of fluorescent dots observed was often lower than expected for the haploid set of chromosomes. As a rule, the number of antibody-binding sites further decreased during spermatid differentiation, whereas the size of the individual fluorescent dots remained unchanged. In addition, small and highly condensed spermatids are insufficiently flattened by cytocentrifugation, so that centromeres were often visible in many different optical planes. Therefore, the analysis of centromere topology in this material posed considerable technical difficulties. Figure 3 presents the centromere patterns during spermiogenesis of different mammals including human and mouse. In all species tested, at least some centromeres occurred in pairs. The phenomenon of centromeric association was generally less pronounced than that described for the large spermatids of Amphibia. Nevertheless, it was possible to demonstrate pair associations of centromeres even in the testicular spermatozoa of mammals (Fig. 3e). The centromeres did not occupy any notable preferential position within the nuclei of mammalian spermatids or spermatozoa.

**DISCUSSION**

Indirect immunofluorescence using anti-kinetochore antibodies permits the localization of centromeres dur-
argue against the hypothesis that all centromeres are clustered together. It is more likely that the kinetochore regions of the highly condensed chromosomes in late spermatids are partially masked and therefore escape immunocytochemical detection. In addition, clustering of centromeres reported by Brinkley et al. [3] is found only in a subset of late spermatids. In the present study, we demonstrated up to 14 individual fluorescent dots in the late spermatid nuclei and testicular spermatozoa of mouse. Anti-kinetochore antibodies failed to label the centromere regions in mature mammalian spermatozoa; the kinetochore antigens specifically recognized by CREST autoantibodies are completely blocked by condensed chromatin [3]. Decoding the spermatozoa by detergent renders the appropriate epitopes accessible for the antibody molecules, but the internal order of the nucleus is largely destroyed by such treatments. Mammalian spermiogenic cells are therefore a difficult starting material for the investigation of centromere topology. Immunofluorescence staining certainly does not permit the unambiguous localization of all centromere regions during mammalian spermiogenesis.

In the large spermatids and spermatozoa of most amphibian species, however, the positions of all centromeres are easily detected. In contrast to mammals, the paired arrangement of centromeres was most obvious in amphibian spermatozoa. Nonhomologous centromere association thus appears to act as an important organizing principle which produces a certain degree of order within the spermatozoon nucleus. Centromeric association does not depend on the presence of centromeric heterochromatin. In Bufo, for example, major amounts of the constitutive heterochromatin are located in telomeric and interstitial chromosome sites [10]. The exact mechanism underlying centromere–centromere association during the various stages of spermiogenesis remains unclear. Specific centromeric DNA sequences that are responsible for centromeric organization as well as specific proteins surrounding the kinetochore complex could mediate such pairings.

Centromeric association is not confined to spermiogenic cells. In a recent study, Schmid et al. [11] reported a supernumerary microchromosome as a constitutional change in a human karyotype; this additional element showed a strong tendency for association with the centromere of any of the normal chromosomes. In the human tumor line MDA-MB 231, the centromere-containing chromosome fragments from pulverized cells consistently exhibited somatic associations [2]. Moreover, a pair-wise arrangement of centromeres was also observed in interphase nuclei of rat kangaroo and Indian muntjak cells [12].

Apart from centromeric association, the large spermatids of nonmammalian vertebrates display additional higher order centromere arrangements. In Urodela, for example, all centromeres are localized in the basal part

FIG. 3. Centromere arrangement during mammalian spermiogenesis. Nuclei were stained with anti-kinetochore antiserum and counterstained with ethidium bromide. Note the double-fluorescent dots representing tandem associations between nonhomologous centromeres. (a) Early and late spermatid stages from human (n = 23). (b) Middle spermatid stages of the primate Callithrix geoffrey (n = 23). (c) Early and late spermatid stages of Djungarian hamster (n = 14). (d) Early spermatid stages of harvest mouse (n = 34). (e) Middle spermatids and testicular spermatozoa of mouse (n = 20). Bar represents 10 μm.
of the nuclei during entire spermiogenesis. This polarization of the centromeres is an impressive example for the relationship between chromosomal arrangement and cellular differentiation. The pattern observed by centromere immunofluorescence is compatible with the earlier results obtained by C-banding techniques [9]. In the elongated spermatozoa of Bufo, the centromeres are preferentially clustered around the nuclear membrane. Only mammalian spermatids showed a relatively random topological distribution of both paired and unpaired centromeres. Obviously, this observation does not preclude the possibility that individual (noncentromeric) chromosome regions maintain a specific position in the spermatid nucleus, since the analysis of chromosome topology during mammalian spermiogenesis is complicated by technical factors.

Using immunocytochemical methods, we showed the intranuclear distribution of centromeres to be highly nonrandom in spermatids and spermatozoa of vertebrates. The paired arrangement of nonhomologous centromeres represents a biological phenomenon common to all vertebrate species. In contrast, the higher order centromere arrangement can vary considerably from one species to another. These two types of nonrandom centromere arrangement are integral parts of the spermiogenic process, and, possibly, anticipate specific functional or structural properties of postmeiotic cells.

This study was supported by the Deutsche Forschungsgemeinschaft (Ha 1374/3-1).

REFERENCES


Received October 13, 1989