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Ordered tandem arrangement of chromosomes in the sperm heads of monotreme mammals

(chromosome organization/*in situ* hybridization/platypus)

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ABSTRACT **A very old unanswered question in classical cytology is whether chromosomes are arranged randomly in sperm or whether they occupy specific positions. Even with modern methods of chromosome painting, it is difficult to resolve this question for the very condensed and almost spherical sperm head of most mammals. We have taken advantage of the unusual fibrillar sperm head of monotreme mammals (echidna and platypus) to examine the position of chromosome landmarks in a two-dimensional array. We used fluorescence and radioactive *in situ* hybridization to telomeric, rDNA, and unique sequences to show that chromosomes are arranged tandemly and in a defined order in the sperm nucleus.**

Many cytologists over the last century expressed an interest in the spatial arrangements of chromosomes within the nucleus of somatic cells and gametes. However, despite numerous early studies of plant and animal species, employing a variety of classic techniques, there was little consensus on either the general occurrence, or the possible significance of spatial order (1).

More recently, advances in fluorescent light microscopy, confocal microscopy, and *in situ* hybridization have made it possible to study the disposition of whole genomes, chromosomes, repetitive DNA sequences, and unique genes within interphase nuclei (2–4). These studies indicate that individual chromosomes generally occupy discrete domains in interphase nuclei (5–8). That the position of a particular chromosome in the nucleus may directly correlate with gene expression and phenotype is suggested by the peripheral position of the condensed inactive X chromosome in cells of female mammals, and the observation of its abnormal position in patients suffering from a severe form of epilepsy (9–11). In species hybrids and cell hybrids, at least, this spatial order has predictable consequences in the control of chromosome elimination and gene expression (12, 13).

The disposition of the chromosomes in sperm has been the subject of a number of studies over five decades. Early work concentrated on insects, which have favorably elongated sperm heads. In iceryine coccids (scale insects) the two chromosomes migrate into the spermatid in a fixed order (14). In the cave cricket, there is indirect, but clear, evidence from studies using polarized ultraviolet light that the two chromosomes are arranged end-to-end in the mature sperm head, and a strong indication that the order of the chromosomes is fixed (15). However, from an autoradiographic study of the position of the late replicating X in grasshopper spermatozoa, it was concluded that chromosomes were arranged tandemly but in random order (16). In the plant *Crepis capillaris*, the three chromosomes of the sperm pronucleus have been observed attached end-to-end (17), and end-to-end chains of the three pairs of synapsed pachytene chromosomes have also been observed to become detached from the nuclear envelope at prophase (18, 19). There is evidence from serial sectioning of eggs of the turbellarian worm *Polychoerus carmelensis* that the chromosomes are also arranged in a specific order in the egg nucleus at meiosis (20).

More recently, several attempts have been made to examine the disposition of chromosomes in mammalian sperm heads by use of fluorescence *in situ* hybridization (FISH) to mark repeated or unique sequences whose position on the metaphase chromosome was known. Zalensky and coworkers (21) showed that centromeres clustered into a dense chromocenter in decondensed human sperm but that telomeres were dispersed more peripherally. A concentration of interspersed repeat sequences at the head and of ribosomal and centromeric sequences at the equatorial region in bovine sperm suggested that the organization is not random (22). A hint of nonrandom positioning of specific centric probes is apparent in many FISH studies of aneuploidy in sperm (23), but chromosome painting reveals no significantly nonrandom relationship between two markers in decondensed human sperm, although it shows that chromosomes occupy a delineated domain in the sperm head (24). The difficulty with all these studies is that the mouse and human sperm nucleus is highly compact and nearly spherical, and there are no good morphological reference points to which the positions of gene markers can be easily compared after FISH.

Monotremes are mammals of the subclass Prototheria, which diverged from subclass Theria ("placental" and marsupial mammals) about 170 million years ago. Of the three extant monotreme species, the platypus diverged from the two echidna species 30–70 million years ago. Monotremes are unique among mammals in laying eggs, and also in possessing an elongated sperm head (25). The large, fibrillar sperm heads are very suitable for a study of chromosome arrangement in mammalian sperm.

The chromosomes of monotremes are sufficiently well known to supply useful molecular markers for such a study. All three species share a karyotype comprising six large chromosome pairs almost identical across species, and a number of small chromosomes, which cannot be individually identified (26, 27). In platypus, the diploid number is 52, and in echidna species, the diploid number is 64 in females and 63 in males. Unique among mammals, monotreme karyotypes include several unpartnered chromosomes that form a translocation chain with the sex chromosomes at male meiosis (26, 28). Prominent nucleolar organizers are present on the satellited short arm of chromosome 6 in the platypus, and at this site as well as on the satellited short arm of chromosome 3 in echidna (27). A number of genes have been mapped by *in situ* hybridization of human cDNA probes to identical sites on the large X chro-

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Abbreviations: FISH, fluorescence *in situ* hybridization; NOR, nucleolar organizing region.
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mosome and some of the large autosomes in platypus and echidna (29–31).

We used FISH and radioactive *in situ* hybridization to localize conserved telomeric sequences, ribosomal DNA, and mapped unique sequences in the fibrillar sperm heads of monotreme mammals.

MATERIALS AND METHODS

The species studied were the platypus (*Ornithorhynchus anatinus*) and the Australian echidna (*Tachyglossus aculeatus*). Platypus testis and toe web were obtained at autopsy from Barbara Evans (Melbourne University) and retained under permit RP 90–005 (Victorian Department of Conservation, Forests and Lands). An echidna was provided by the South Australian Department of National Parks and Wildlife under permit A52, W51, and one testis and a skin biopsy were removed by Ian MacDonald (Melbourne University). Cell lines were initiated from skin biopsies using a chicken plasma clot method (32). Cell lines were maintained at 32°C (the body temperature of monotremes) in DMEM (Flow Laboratories) supplemented with fetal calf serum at 10% for platypus and 26% for echidna (Flow laboratories) in an atmosphere of 10% CO_2.

Air-dried mitotic chromosome preparations were made from cultures incubated with 0.005% colchicine for 1 hr before harvest, and cells were swelled in 0.05 M potassium chloride for 20 min before fixation. To obtain meiotic preparations, testis was finely chopped in 1.5% trisodium citrate. Cells were treated with 0.05 M KCl at 37°C for 8 min and, after several changes of fixative (3:1 methanol/acetic acid), dropped onto acid-cleaned microscope slides and allowed to air dry. Slides were treated with RNase A (100 μg/ml in 2× standard saline citrate (SSC; 1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) at 37°C for 1 hr, washed in three changes of 2× SSC, and dehydrated through an ethanol series then denatured using 70% formamide and 2× SSC (pH 7.0) for 2 min, followed immediately by dehydration in first 70% then 100% ethanol at −20°C.

The probes used for *in situ* hybridization to sperm heads were telomere-specific deoxyoligomers, rDNA from *Xenopus laevis*, and unique genes whose position on monotreme mitotic chromosomes are known (Table 1). The telomere oligomers were localized by FISH. Oligomers were labeled with biotin using terminal deoxyribonucleotidyltransferase. Preparations were denatured with 70% formamide at 70°C, followed by 2–3 min of digestion with proteinase K, and hybridized overnight with 30% formamide mix (39, 40). Signal was detected by fluorescence using one round of amplification with fluorescein-labeled avidin and anti-avidin antibody (Vector Laboratories).

The rDNA and unique gene probes were localized to sperm heads by radioactive *in situ* hybridization. Probes were nick translated with ^{3}H-labeled dATP, dCTP, and dGTP [Amersham; specific activities of 84, 62, and 39 Ci/mmol (1 Ci = 37 GBq), respectively] to activities of 2×10^7 to 2×10^8 cpm/μg. Labeled probe was prepared in a mixture containing 70% formamide, 10% dextran sulfate, 2× SSC (pH 7), and a 200-fold excess of sheared salmon sperm DNA. The probe mixtures were denatured at 100°C for 10 min and then stored on ice. Probes at concentrations ranging from 0.01 to 0.1 μg/ml were hybridized for 24 hr at 37°C in 20 μl of the probe mixture, which was sealed under a siliconized coverslip. Coverslips were removed, and the slides were washed in three changes of 50% formamide in 2× SSC (pH 7) at 40°C and three changes of 2× SSC (pH 7) at room temperature, then dehydrated through an ethanol series and air dried (41). Slides were coated with tested Amersham L1 emulsion and exposed for 20–60 days before developing and staining with Giemsa. Only silver grains directly overlaying sperm heads were scored as signal, and their positions were recorded, relative to the ends of the sperm heads, on video printouts of each sperm head scored.

The proximal and distal ends of platypus sperm heads were readily distinguished by their shape, the proximal being regularly more pointed. Platypus sperm heads were divided into 10 equal-sized intervals for scoring at 21 positions and analysis of the distribution of signal. However, the difference between ends was less apparent in echidna sperm, and orientation was clear in only a minority of sperm. For the repeated sequence probes, only this subpopulation of sperm was scored, but the grain numbers were so low for unique sequences that all sperm had to be included; thus, signal could be scored only at 11 positions over 5 intervals with respect to (either) end and middle. The distribution of grains over these 10 (5) intervals was analyzed using the Z_{max} statistic (42), which tests the overall distribution for departure from nonrandomness, then identifies, in order, significantly overlabeled sites.

RESULTS

Distribution of Telomeres Along Sperm Heads. We used *in situ* hybridization with fluorescein-labeled telomeric oligonucleotides to mark the positions of the ends of monotreme chromosomes in sperm. These oligonucleotides hybridize to a highly conserved telomeric sequence in the chromosomes of all vertebrates, as well as to some centromeric, nucleolar organizing region (NOR)-associated and interstitial sites in some species (39). These probes specifically hybridized to the telomeres of platypus chromosomes, observed as a single signal on the terminus of each chromatid in monotreme mitotic and meiotic preparations (Fig. 1*a*). No interstitial, centromeric, or NOR-associated signal was detected.

The arrangement of telomere sequence in sperm was strikingly nonrandom. The label appeared to be distributed in bands, some of which could be resolved into duplicate signals, and the arrangement of these bands appeared to be similar from sperm to sperm (Fig. 1 *b* and *c*).

To determine how the chromosome ends were arranged in the sperm heads, we counted the number of fluorescent bands

Table 1. Gene probes used in this study

Gene	Symbol	Location		Probe description	Source
		Platypus	Echidna		
Ribosomal/RNA	rDNA	Element	Element	*X. laevis* cDNA 12.0-kb insert in pBR322	K. Jones, Edinburgh (33)
Phosphoglycerate kinase	PGK	Class A	Class A	*Macropus rufus* genomic DNA 1.1-kb insert in pBR322	D. W. Cooper, Sydney (34)
Glucose-6-phosphate dehydrogenase	G6PD	Xq	Xq	Human cDNA 2-kb insert in PUC18	D. Toniolo Pavia, Italy (35)
Coagulation factor VIII	F8	Xp	Xp	Human cDNA 1.5-kb insert in PGem	K. H. Choo, Melbourne (36)
Duchenne muscular dystrophy	DMD	1	1	Human cDNA 6.2-kb insert Bluescript K5	I. M. Kunkel, Boston (37)

Monotreme chomosomes are classified according to Wrigley and Graves (38). Smaller chromosomes in both species cannot be individually identified, even by G- or late replication banding, and are therefore grouped into size classes A and B.

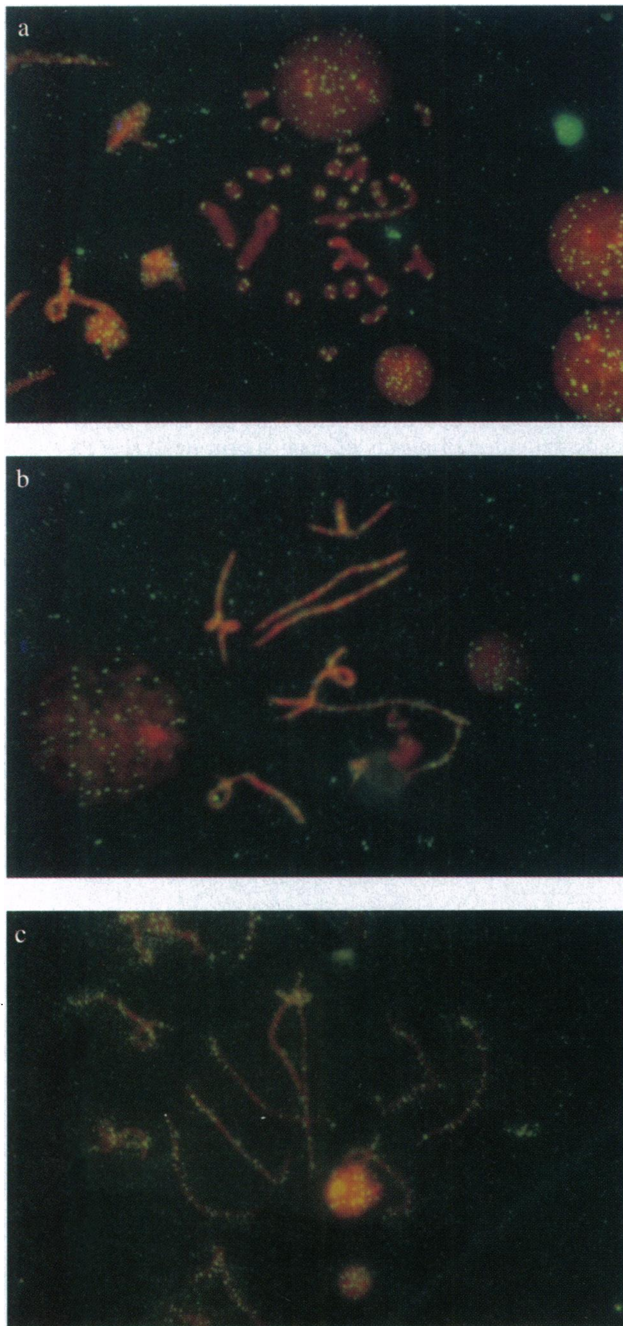

FIG. 1. Position of telomere-specific sequences on echidna meiotic chromosomes (*a*), echidna sperm heads (*b*), and platypus sperm heads (*c*).

in enlarged photographs of labeled single sperm. If chromosomes are arranged randomly or in any configuration with their ends overlapping, it should be possible to identify each telomere as signal, whereas, if chromosomes lie end-to-end, neighboring telomeres would abut and be visible as bands composed of duplicate signals. Because the platypus has a diploid number of 52, platypus sperm would be expected to possess 26 chromosomes, with a total of 52 telomeres. If these chromosomes are arranged randomly or overlapped, 52 separate fluorescent signals should be visible along the length of the sperm head, whereas if they are laid end-to-end, 27 should be apparent. As documented in Table 2, we observed 18–26 paired signals (mean = 23) along the length of platypus sperm, which is consistent with a tandem arrangement.

We obtained similar results for echidna sperm. This species has a diploid number of 63 in males, so half the sperm should contain 32 chromosomes (64 telomeres) and half 31 (62 telomeres). We counted 23–32 (mean = 27.5) fluorescent bands (Table 2), which is consistent with an end-to-end arrangement.

Locations of Heterochromatin and rDNA Along Sperm Heads. Next, we wished to test the hypothesis that chromosomes are arranged in a fixed tandem array in monotreme sperm. Therefore, we investigated the relative positions of heterochromatin and blocks of ribosomal genes along sperm heads in the two species.

In monotremes, GC-rich heterochromatin is specifically stained with Chromomycin A_3 (38). Large blocks of heterochromatin are associated with the sites of NORs on the satellited chromosomes 3 and 6 in echidna, as well as on the small, unpaired heterochromatic element that lies at the end of the translocation chain (Fig. 2*a*), which would be expected to be present in half the sperm. Half the sperm heads should therefore contain two staining sites, and the other half should contain three. Also, it should be possible to tell if these sites assume a consistent position with respect to the proximal and distal ends of the sperm. We observed sperm heads with chromomycin-positive sites at one end, both ends, and the middle with or without sites at the ends. It was hard to quantitate these because the two ends were difficult to distinguish in darkfield preparations, and staining was uneven over the preparation because of the variable presence of cytoplasm. In platypus, the heterochromatin located at the chromosome 6 NOR and on the small unpaired element was less conspicuous, and chromomycin staining material could not be reliably located in sperm heads.

To confirm the locations of the NOR regions in the sperm of both species, *in situ* hybridization was performed using a ribosomal DNA probe (Table 1). This probe showed conspicuous hybridization to three major sites (the satellited short arms on chromosomes 3 and 6 and the unpartnered element) in echidna meiotic spreads (Fig. 2*b*). Up to three labeled regions were visible on echidna sperm heads (Fig. 2*c*), at one or both ends and the middle, coinciding with the sites identified by chromomycin staining.

Because of the heavy label produced by the repeated rDNA sequences, it was possible to restrict the analysis to the minority of echidna sperm whose orientation was clear. Grain distributions were summed over 21 positions over sperm heads divided into 10 segments, producing localized peaks at the proximal, medial, and distal sites previously identified by their chromomycin staining (Fig. 3). These could be tentatively identified from the relative efficiencies with which they were labeled in mitotic and meiotic spreads, giving the order 3s > unpaired element > 6s. Taking into account the presence of the unpaired element in only half of the sperm, echidna sperm showed the heaviest labeling at the medial site (3s), the next

Table 2. Numbers of telomere signals along monotreme sperm heads

Monotreme	No. of sperm	No. of chromosomes in sperm	No. of telomeres	Expected no. of signals if tandom arrangement	Observed no. of signals (mean)	Range
Platypus	15	26	52	27	23	18–26
Echidna	19	31, 32	62, 64	32, 33	27.5	23–31

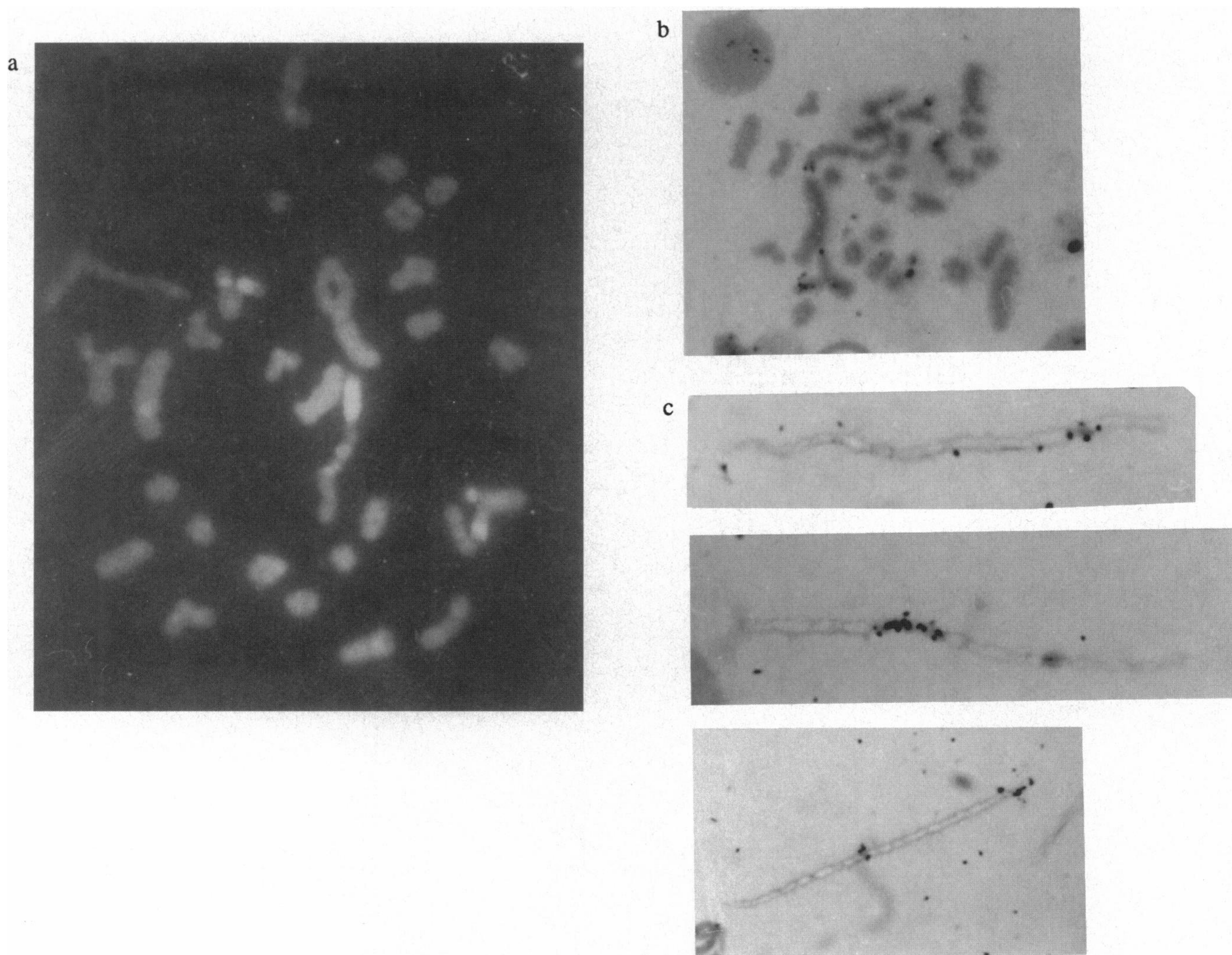

FIG. 2. Location of heterochromatin and rDNA sequences in monotreme chromosomes and sperm. (*a*) Chromomycin A_3-stained heterochromatin in echidna meiotic chromosomes. (*b*) *In situ* hybridization of ^{3}H-labeled rDNA probe to these heterochromatic regions in echidna meiotic chromosomes. (*c*) *In situ* hybridization of rDNA to echidna sperm heads showing grains located over one or both ends and/or a medial site (note that not all sperm show labeling over each site).

at the proximal site (unpartnered element) and the least at the distal end (6s).

For the platypus, two sites were labeled by rDNA in mitotic and meiotic spreads, and in sperm heads. The overall distribution showed a peak near the posterior end of the sperm and a weak signal at the anterior end. In mitotic platypus cells, the site on chromosome 6 was much more efficiently labeled than the one on the unpartnered element, and there is no signal on chromosome 3, which lacks a heterochromatic NOR. Thus, as for echidna, the proximal site represents the unpartnered element and the distal site the chromosome 6 NOR.

Locations of Unique Sequences Along Monotreme Sperm Heads. Unique gene probes, which have identified the sites of homologous monotreme genes on the X chromosomes and autosomes (Table 1), were also hybridized *in situ* to monotreme sperm heads (Fig. 3). The two X-borne genes F8 and G6PD both hybridized to sites near the posterior end of platypus sperm and to a site near one end of echidna sperm. The chromosome 1 marker DMD mapped to the anterior end of platypus sperm and near to one end in echidna. PGK, which maps to one of the class A chromosomes, assumed a medial position in the sperm heads of both species.

DISCUSSION

The numbers and distributions of telomeric signal we observed along the fibrillar sperm heads of both monotreme species studied strongly support the hypothesis that chromosomes assume a head-to-tail tandem orientation. This explains why there were only about half as many fluorescent bands as there were telomeres. Our interpretation of this distribution is shown in Fig. 4*a*.

Our observations of the position of heterochromatic blocks, and of repeated and unique genes along the sperm heads are summarized in Fig. 4*b*. The consistent position of heterochromatin and rDNA signal and the localized signals produced by unique sequences imply that the order of chromosomes in this array is at least highly nonrandom and may well be fixed. The two X chromosome probes map close together, as would be expected if the X chromosome were condensed and always occupied the same position in the sperm head. Genes known to map on other chromosomes occupy quite different positions in the sperm head. The positions of unique and repeated sequences are entirely consistent between platypus and echidna, with the exception of the relative position of the chromosome 6 NOR and the X-borne genes, which overlap to some extent. This consistency implies that the order of chromosomes in sperm has been conserved at least over the 30–70 million years since the divergence of these two species. Chromosome order must, therefore, represent an important feature of nuclear structure in monotreme sperm.

Whether this ordered tandem arrangement is a feature of sperm of all mammals or of all vertebrates (or even all animals

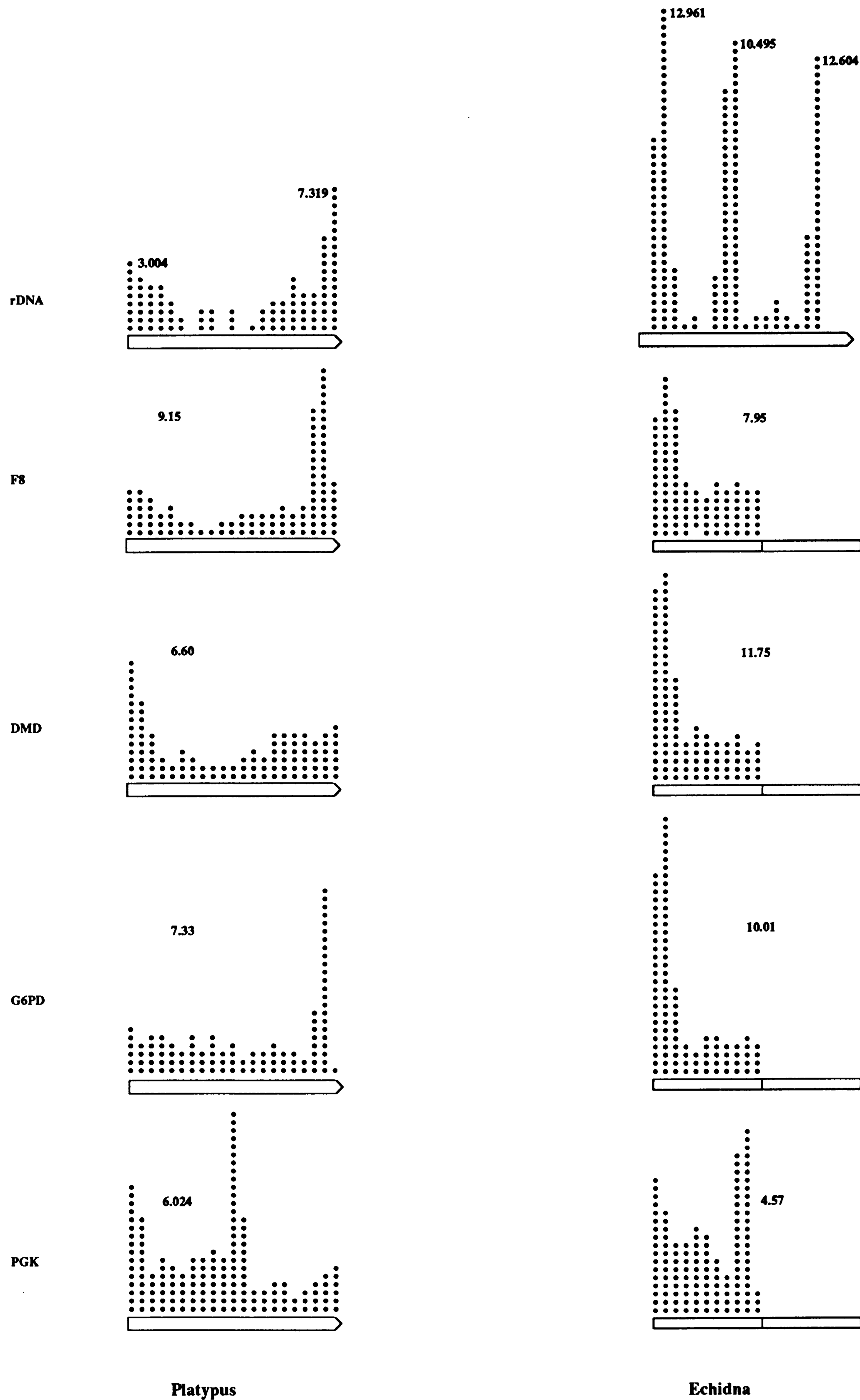

FIG. 3. Grain distributions over platypus and echidna sperm heads from *in situ* hybridization of ^{3}H-labeled probes to rDNA and four unique sequences. Grains were counted over 100 metaphases for the rDNA probe and 100 for the unique genes. Distribution was recorded over 21 equal segments of platypus and echidna sperm whose proximal (pointed) and distal ends could be distinguished; however, for the unique probes, echidna sperm whose orientation could not be distinguished had to be included; thus, scoring was performed over 11 equal segments from (either) end to middle. The numbers over the distributions represent significant Z_{max} scores for the primary (and significant secondary) peaks.

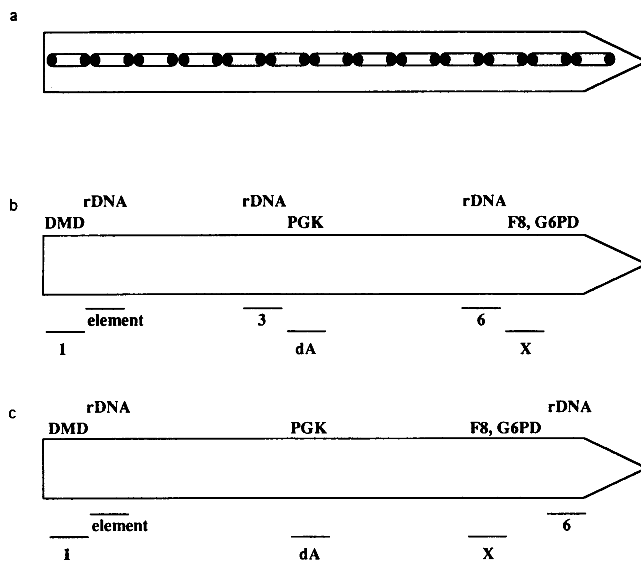

FIG. 4. Interpretation of the arrangement of chromosomes and the position of genes in monotreme sperm. (*a*) Model of the end-to-end tandem arrangement of chromosomes in sperm. The positions of specific genes within echidna sperm heads (*b*) and platypus sperm heads (*c*) suggest that chromosomes occupy a consistent order, which is conserved between species.

or all eukaryotes) remains to be determined. The relative positions of gene or chromosome probes in human and mouse sperm are not obviously nonrandom, but analysis of position within the spherical or oval sperm head remains difficult. *In situ* labeling studies of telomere, rDNA, and unique mapped genes within the fibrillar (albeit smaller) sperm heads of birds and insects would be of great value. Specific chromosome arrangement may prove to be a general feature of animal sperm, and it may be enlightening to compare the positions of homologous chromosome regions across species.

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