Replication status of the fragile X chromosome and its implications for reproductive performance in the Indian mole rat (*Nesokia indica*)

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Summary. In all fertile females the fragile X chromosome was almost always late replicating (inactive) in an average 82% of cells whereas in infertile females, it was early replicating (active) in about the same percentage of cells. These observations strongly suggest a correlation between the replication (activity) status of the fragile X chromosome and reproductive performance.

Keywords: fragile X chromosome; replication status; reproduction; Indian mole rat

Introduction

The Indian mole rat (*Nesokia indica*) is a fossorial rodent (adult weight approximately 250 g) and has a diploid chromosome complement of 42. The X chromosome which is metacentric (denoted as X^m) is the largest in the complement (composite type, 17.34% of total chromosome length (TCL)) and has a euchromatic short arm and a heterochromatic long arm (10.9% TCL). The totally heterochromatic Y chromosome, which is also metacentric (Y^m), is the second largest in the complement (11.33% TCL).

Mole rats of this species are characterized by an extensive polymorphism of the sex chromosomal heterochromatin. The four polymorphic X-chromosome types arise due to deletion of heterochromatin at four specific sites and are designated as X^{d1} , X^{d2} , X^{d3} and X^a (Rao *et al.*, 1983; Thelma *et al.*, 1988). These sites also correspond to 4 folate-sensitive fragile sites which have been termed fraXq1, fraXq2, fraXq3 and fraXc (Tewari *et al.*, 1987). These sites have been observed in every female mole rat examined so far. In all fertile females with X^mX^m constitution one or more fragile sites are expressed on only one of the two X chromosomes in about 30–40% of cells (heterozygous expression). However, in infertile females with a similar X^mX^m constitution, these sites are not only expressed in a much higher frequency (around 70%) but, in about 4–10% of cells, both of the X chromosomes express these sites (double heterozygous/homozygous expression). The single active X chromosome in fertile males (X^mY^m) never expresses fragile sites but in infertile males 4–13% of cells express these sites on the X chromosome (hemizygous expression) (Tewari *et al.*, 1987). These observations prompted study of whether there was a correlation between the replication status of the fragile X chromosome and reproductive performance.

Materials and Methods

Animals. The study used 14 female mole rats (10 from the wild and 4 laboratory bred) with either an entire composite type X chromosome (X^m) or with polymorphic X chromosome types (X^{d1} , X^{d2}). The females were 4–8 months of age; 9 individuals were proved fertile while 5 were unable to reproduce even after prolonged periods of mating with fertile males (Thelma *et al.*, 1988).

Fig. 1. Chromosomes of the Indian mole rat. **(a)** Diagrammatic representation of the composite X-chromosome showing deletion sites on one chromatid and fragile sites on the other chromatid. The stippled portion represents constitutive heterochromatin. **(b)** Cut-outs of the X chromosome showing a fragile site ($\rightarrow$) $fra X_{q1}$ as a non-staining region, $fra X_{q2}$ as a chromatid break, $fra X_{q3}$ as a chromosome deletion, and $fra X_c$ as a chromosome break near the centromere. **(c)** and **(d)** Partial metaphases from females (fertile and infertile respectively) showing a late replicating X chromosome with a fragile site ($fra X_{q1}$ $\rightarrow$) (c) and an early replicating X chromosome with a fragile site ($fra X_{q2}$ $\rightarrow$) (d).

Cultures. Heparinized blood withdrawn from the retino-orbital sinus of mole rats anaesthetized lightly with ether was cultured in folic acid-deficient McCoy's medium 5a (Centron Labs, Bombay, India) for detection of fragile sites. Simultaneously, control cultures were grown in RPMI medium for all the individuals. All cultures of 5 ml each were supplemented with 10 μ g phytohaemagglutinin/ml (HA17, Wellcome Diagnostics, Dartford, UK) and 5% bovine calf serum. Cultures were incubated for 52 h at 37°C. To identify the late replicating X chromosome by differential labelling, 5-bromo 2-deoxyuridine (BrdU; 10 μ g/ml) was added 6.5 h before harvest. Colchicine (0.03 μ g/ml) was

Table 1. Replication status of fragile X chromosomes in fertile and infertile Indian mole rat females

Individuals*	Genotype	Total no. of cells scored (no. with fraX)	No. of cells with:		G value†
			Late replicating fragile X	Early replicating fragile X	
Fertile					
1 PW	X ^m X ^m	95 (39)	31	5	20.89
2 PW	X ^m X ^m	75 (20)	7	1	5.05
3 PW	X ^m X ^m	95 (29)	14	1	13.44
4 PW	X ^m X ^m	42 (13)	8	4	1.35
5 PW	X ^m X ^m	77 (21)	11	3	4.85
6 F1	X ^m X ^m	91 (46)	14	5	4.43
7 F1	X ^m X ^m	141 (36)	8	2	3.85
8 F2	X ^m X ^m	102 (21)	5	1	2.91
9 F2	X ^m X ^m	59 (14)	6	1	3.96
Pooled			104	23	60.73
G-test of heterogeneity (G _H)					-4.888‡
Infertile					
1 PW	X ^m X ^m	40 (35)§	6	20	17.22
2 PW	X ^m X ^m	92 (77)§	5	15	5.23
3 PW	X ^m X ^m	67 (19)§	3	14	7.22
4 PW	X ^m X ^{dl} 1/X ^m X ^{dl} 2	206 (137)§	21	116	72.54
5 PW	X ^m X ^{dl} 1/X ^m X ^{dl} 2/ X ^{dl} 1 X ^{dl} 2	96 (50)§	11	39	16.62
Pooled			46	204	118.83
G-test of heterogeneity (G _H)					-2.213‡

Comparable numbers of metaphases scored in control cultures showed no fragile sites.

*PW = parental generation, wild population; F1 = first generation; F2 = second generation.

†All values except ‡ are significant ($P < 0.05$).

§3–8% of these cells expressed fragile sites on both the X chromosomes (homozygous expression) in this category.

added 4 h before harvest. Chromosomes were prepared by a standard air-drying technique followed by fluorescence plus Giemsa staining (Perry & Wolff, 1974).

Results

Fragile sites were not observed in any control cultures grown from the 14 individuals. However, in cultures grown in folate-deficient media fragile site(s) on the X chromosome(s) (fraXq1, fraXq2, fraXq3 and fraXc) were observed for all the animals. These sites were characterized by non-staining region(s), chromatid break(s) and/or chromosome break(s)/deletion(s) (Fig. 1b). Of the 4 sites the most frequently expressed were fraXq1 and fraXq2 which were evident as chromosome breaks or deletions. The resultant acentric fragments were, however, rarely observed in the metaphases scored. Apparently, they are either eliminated from the cells or form micronuclei (unpublished). The triradials characteristic of the fragile sites in man were not seen in mole rats.

Since all 4 fragile sites observed were only due to folate deficiency, all cells expressing one or more of these sites on any one of their two X chromosomes were considered for the analysis of fragile X chromosome. Of these only those in which the short arm of the two X chromosomes were differentially labelled with BrdU were included for the evaluation of early and late replicating fragile X chromosomes. Analyses of the replication status (early/late) of the fragile X chromosome revealed that, in a majority of the cells of fertile females, the fragile X chromosome was late replicating (Fig. 1c) whereas in infertile females it was early replicating (Fig. 1d; Table 1). A G-test of significance (Sokal & Rohlf, 1969) for each of these categories substantiated the non-

random expression. The pooled G-test of heterogeneity within each category was not significant (Table 1).

Discussion

A non-random association between the functional status of the fragile X chromosome and fertility status of the individual is apparent from this study. It is well known that in somatic cells of all mammalian females one of the two X chromosomes is inactivated and therefore only one X chromosome remains functional. When expressed on the inactive X chromosome, fragile sites seem to be inconsequential, as shown by females with proven fertility. However, when fragility is expressed on the active X chromosome in a female it seems to be invariably coupled with reproductive inability. This probably suggests that some sequences located at these specific sites (corresponding to fragile sites) on the X chromosome influence the orchestrated events ultimately resulting in reproductive ability. We suggested that, when these specific sequences on the active X chromosome become non-functional due to one or more new mutations, these sequences express fragility (like the inactive X chromosome sequences in fertile category but at a comparatively higher degree) and such females become infertile.

Such an association of these specific sequences with reproductive (in) ability derives additional support from the observation that the fragile sites on the single active X chromosome in males (X^mY^m) are found only in the infertile individuals but *not* in the fertile males (Tewari *et al.*, 1987). In addition, males with deleted X chromosome heterochromatin ($X^{d1}2Y^m$; $X^mY^m/X^{d1}3Y^m/X^aY^m$, mosaic constitution of animal caught from wild), in which there has been a loss of such specific sequences, are also sterile and show both meiotic and structural abnormalities (Juyal *et al.*, 1989). Structural anomalies in the gonads and infertility have also been reported for men with fragile X chromosome (Johannisson *et al.*, 1987).

Therefore, considering that some human carrier females of the fragile X chromosome tend not to reproduce (Sherman *et al.*, 1985), it may be of interest to assess the functional status of the fragile X chromosome in such cases.

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