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Inactivation of mammalian X-chromosome during spermatogenesis: Temporal expression of genes in the laboratory mouse

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Abstract. At zygotene/pachytene stage of meiosis in mammalian testis, the X—Y heterobivalent is sequesterd into a heterochromatinized body whose genetic inactivity is shown by lack of uridine incorporation. For the genic level evaluation of the X-inactivation, activities of three X-linked genes were assayed in testicular cell types in the laboratory mouse. While hypoxanthine phosphoribosyl transferase is functional at least up to pachytene stage of primary spermatocytes, glucose-6-phosphate dehydrogenase appears to be active only in the Sertoli cells. No transcript of the muscle specific muscular dystrophin gene was obtained from its regular 5' promoter. Thus, inactivation of X-linked genes in testis occurs at different stages during spermatogenesis, independently of heterochrornatinizati on of the XY-body. We propose that Xist transcript, the putative regulator of X-inactivation in female soma, is also the regulator in testis. However, due to its extremely low level in testis the transcript may regulate by "spreading" in a gradient and affect the genes in a temporal order. Thus, besides other factors, physical proximity of the genes to Xist may determine the stage of their inactivation.

Keywords. X-inactivation; pseudoautosomal; Xist; HPRT; G6PD; mdx.

1. Introduction

An evolutionary advantage of the sex chromosomal monosomy in mammalian male lies in averting recombination between their sex determining regions, the prerequisite for maintaining identity of the sexes. Cytological and molecular studies show that notwithstanding substantial diversification, the X and Y share a small region of homology, the pseudoautosomal region (PAR) through which they pair and recombine, and ensure proper segregation during meiosis (Burgoyne 1982; Polani 1982; see Rappold 1993). Disparity in the number of Xs in the sexes is compensated by inactivation of one X in female. The inactive X-chromosome in the female somatic cells is cytologically identified as a heterochromatic body in interphase (Barr and Bertram 1949) that does not incorporate uridine (Comings 1966). Curiously, heterochromatinization and the lack of uridine uptake (Monesi 1965) is also a characteristic of the male X chromosome during meiotic division in the testis. At about the zygotene stage a heterochromatic XY body (also called sex vesicle) is formed (Solari 1974), indicating occurrence of X-chromosome inactivation (XCI) also in testis. It has been proposed that the XCI in mammalian female may have evolved from the more primitive event in the male germ cells (Cooper 1971; Lifschytz and Lindsley 1972; Brown and Chandra 1973).

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However, while much study has been done on the XCI in female somatic cells, investigations in the testis have been few and sporadic. The few reports on the XCI in male have, however, indicated similarities with the female. For instance, one of the pseudoautosomal genes in mouse, steroid sulphatase, which escapes inactivation in female also remains active in all the testis cell types in the mouse (Raman and Das 1991). But the most impressive similarity is found in the expression of the putative XCI regulating gene, Xist, which is active only in the inactive X in the female and whose expression during development coincides with the time of inactivation of the X-chromosome (Borsani *et al* 1991; Brockdorf *et al* 1991; Brown *et al* 1991). The Xist transcript has also been located in the Sertoli and premeiotic germ cells in the testis of man and mouse (Salido *et al* 1992; Brown *et al* 1992; Brockdorf *et al* 1992; Kay *et al* 1993). Yet some of the features of female XCI, like (i) how much of the X is inactivated, (ii) at what stage in spermatogenesis is it inactivated and (iii) whether it occurs in *en bloc* or segmentally in different regions remain to be satisfactorily tested in the testis.

In this paper, we present results on the pattern of inactivation of three X-linked genes, hypoxanthine phosphoribosyl transferase (HPRT), glucose-6-phosphate dehydrogenate (G6PD) and muscular dystrophin (mdx) in different testis cell types to elucidate XCI in mammals.

2. Materials and methods

All the experiments were carried out on albino mice of the Parkes strain, bred and reared in our laboratory.

2.1 *Separation of testicular cell types*

The procedure for the separation of different testicular cell types was essentially that of Meistrich (1977). Briefly, the single cell suspension of testis was separated in a 2–4% BSA gradient either under unit gravity by loading the cells on a STAPUT column (Johns Scientific, Canada) for 4-6 h, or by centrifugation in a density gradient centrifuge (Cell Separator ZS; Edmund Buhier, Germany) at 400 rpm for 20 min. Several fractions of the gradient (each of 25 ml in STAPUT and 5 ml in centrifuge) were collected and after visual inspection under microscope, were grouped into three major cell types; gonials, pachytenes and spermatids. Throughout the processing, the cells were maintained at 4°C. Separation in the cell separator worked much better than the column with respect to the time taken, purity of the fractions (~ 85% pure for a given cell type) and requirement of the initial material. Somatic tissues from the same mice were used for comparable studies. Cell extracts from liver, whole testis and separated cell fractions were prepared in 0·9% NaCI containing 0·2 M phenyl methyl sulphonyl fluoride (PMSF). The extracts were centrifuged at 12 K for 30 min at 4°C and the supernatant was collected as cytosolic fraction for enzyme assay.

2.2 *Autoradiographic evaluation of the enzyme HPRT (EC 2.4.2.8)*

A cell suspension from the whole testis (approx. 10^7/ml) was incubated with 20 µCi

of [^{3}H]hypoxanthine (sp. act. 45 Ci/mM; Amersham), the substrate for HPRT, for 3 h (37°C). The cells were fixed in acetic acid-methanol and spread on glass slides which were coated with Kodak NTB2 emulsion for autoradiography and developed in D19b after 10 to 15 days of exposure in dark.

2.3 *Assay of the enzymes G6PD* (*EC 1.1,1.49*) *and lactate dehydrogenase* (*EC 1.1.1.27*)

The quantitative estimation of G6PD enzyme activity was done spectrophotometrically in liver, testis and different testicular cell types by the standard method of Brinster (1966). The qualitative evaluation was done electrophoretically in 7·5% polyacrylamide gel (60 mA for 3 h at 4°C). The enzyme band was resolved with the substrate-specific staining (Selander *et al* 1971) for 30 min at 37°C. Lactate dehydrogenase (LDH) activity was detected on the same samples in conditions similar to those for G6PD. The enzyme bands were visualized by the substrate-specific staining (lithium lactate, NAD, NBT, PMS) for 30 min at 37°C.

2.4 *RNA extraction, Northern hybridization*

Total RNA from whole testis and liver was prepared by the single-step acid guanidium thiocyanate-phenol-chioroform extraction method of Chomczynski and Sacchi (1987). Northern (50 µg of RNA per lane) and dot blot (20 µg/sample) hybridizations were done as per standard protocols (Maniatis *et al* 1982). For Northern analysis, RNA was run on l% agarose gel in 6·7% formaldehyde. Integrity and relative amounts of RNA were checked by ethidium bromide staining of 18 and 28S rRNA on the same gel. Following hybridization (50% formamide, 6 × SSC, 0·5% SDS, 5 × Denhardt's, 500 µg/ml sheared and denatured yeast tRNA) for 12 h at 42°C, the hybridizations were carried out for 36 h at 42°C. The hybridization buffer additionally contained 5% Dextran sulphate and 1×10^7 cpm of the randomly primed ^{32}P-labelled DNA probe. The blots were washed for 30 min with 1 × SSC, 0·l% SDS (68°C). For autoradiography, the blots were exposed to X-ray film, using intensifying screens, for two days.

2.5 *In situ hybridization*

Following the protocol of Graham *et al* (1989) hybridization was performed on 6 µm thick sections of adult testes, fixed in paraformaldehyde and embedded in paraffin. The dewaxed and serially rehydrated sections were treated respectively with proteinase K (1 µg/ml) and acetic anhydride. They were prehybridized (50°C for 2 h) in the mixture having 50% formamide, 0·3 M NaCI, 20 mM Tris (pH 8·0), 5 mM EDTA, 10 mM DTT, 10% Dextran sulphate, 1 × Denhardt's and 0·5 µg/ml yeast tRNA. After washing off the salts in 2 × SSC and water, respectively, the sections were hybridized with the denatured ^{35}S-labelled DNA probes ($l \times 10^4$ cpm/slide) for 48 h at 50°C in a humidified environment. Later, the slides were washed respectively in 5 × SSC (1 h, 48°C) and 2 × SSC in 50% formamide (50°C, 15–30 min). The air dried slides were coated with Kodak NTB2 emulsion for autoradiography, developed after 48 h in D19b developer and stained with 5% Giemsa.

3. Results

3.1 *HPRT*

Activity of the enzyme was recorded in the autoradiograms of individual cells incubated with its substrate, [^{3}H]hypoxanthine. It was taken to be active only in the labelled cells. Whereas, most of the Sertoli and spermatogonial interphases, and early meiotic prophase (leptotene, zygotene and pachytene) cells were labelled, 30% of pachytenes and cells beyond this stage (meiotic metaphases, round and elongating spermatids) were unlabelled (table l, figure 1).

Table 1. [^{3} H]hypoxanthine incorporation in testicular cells of mouse.

Cell types	No. cells observed	Labelled (%)
Pachytene	201	70·1
Interphase	388	54·6
Spermatids	41	0·0
Sperm heads	289	0·0

In order to assess the steady state level of its transcripts, RNA from the whole testis of mice of different ages was Northern hybridized with pHPT5, a cDNA probe of HPRT (Konecki *et al* 1982; 1·3 kb Pst I insert in pBR322). Northerns were done on the RNA from the 6 day (have only Sertoli and gonials), 16 day (besides Sertol is and gonials, the first flush of meiotic prophase cells), 21 day (emergence of spermatids; enriched in pachytenes) and the adult testis. A band at ~ l·6 kb was seen in all the samples, that of the day 6 being very weak (figure 2a). A visual estimate indicated that intensity of the signal increased from day 6 to 21, but in the adult it was weaker than the day 21.

The cellular localization of the HPRT transcripts was done on histological sections of adult testis by RNA *in situ* hybridization (RISH). While LDH-X (Tanaka and Fujimoto 1986) was used as the positive control, the slides were hybridized with a vector plasmid pGEM-3Z for negative control. In contrast to some sporadic grains in the sections hybridized with plasmid, the sections hybridized with pHPT5 showed a high density of grains in each section in all the cell types (figure 2B). The LDH-X RISH also displayed strong hybridization in all the cell types (figure 2B). Since both the controls behaved as expected, the signal with pHPT5 is taken as genuine.

3.2 *G6PD*

The enzyme activity, analysed on polyacrylamide gels and spectrophotometrically, was not detectable in the enriched fractions of pachytenes, spermatogonials, and spermatids, but there was a discernible activity in the lysates of the whole testis and liver both as a band in the gel (figure 3) and by the shift in absorbance. Occurrence of two bands in the testis sample was due to differential degrees of

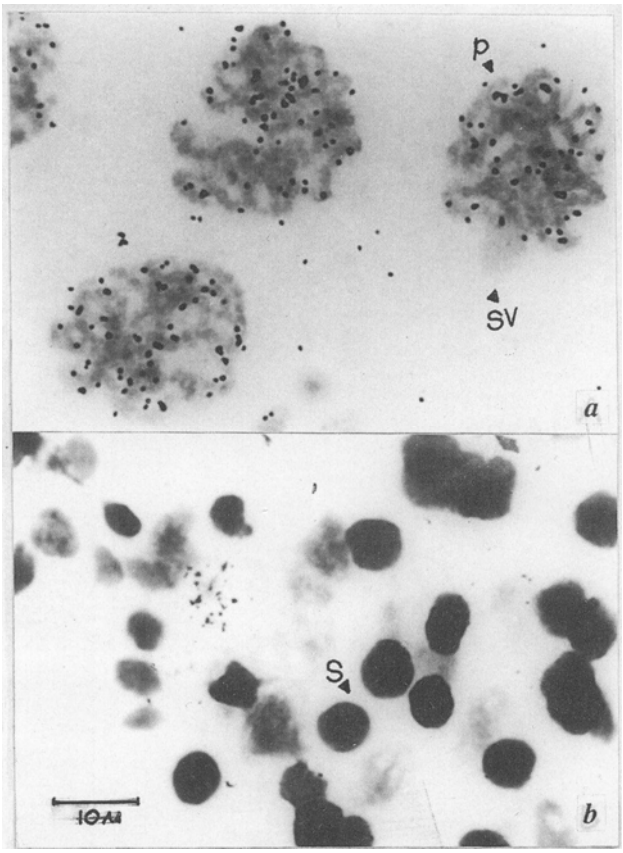

Figure 1. Cellular autoracliographs showing incorporation of [^{3}H]hypoxanthine in testicular cell types (**a**) Pachytene and **(b)** spermalids. All the pachytenes (p) are labelled while spermatids (**s**) show no grains. The XY body is also unlabelled in pachytenes except a sporadic grain (arrow), confirming near global inactivity of the sex chromosomes.

oxidation of the enzyme, as earlier shown in rabbit and human (Ninfali and Palma 1990). That the observed lack of the activity in individual cell types was not because of the degradation of proteins during the process of separation, was confirmed by showing the activity of another oxidoreductase enzyme, LDH, on the same cell lysates (data not shown). The enzyme activity was further compared between adult testis and the 6 day-old mice which consist of only the Sertoli and spermatogonial cells. The level in the neonats (0·36 IU/mg protein) was 1·6 times of the adult (0·21 IU/mg protein).

For the transcript analysis, whole testes RNA (20 µg/sample) from 6, 16, 21 day and adult mice was dot hybridized with a human G6PD probe, pGD-T-5B (2·6 kb *Eco*RI cDNA insert in pUCl9; Martini *et al* 1986). Densitometric scanning (540 nm) of the autoradiograms revealed the signal to be most prominent in the 6 day old mice (figure 4) in which enzyme activity was also higher than adults.

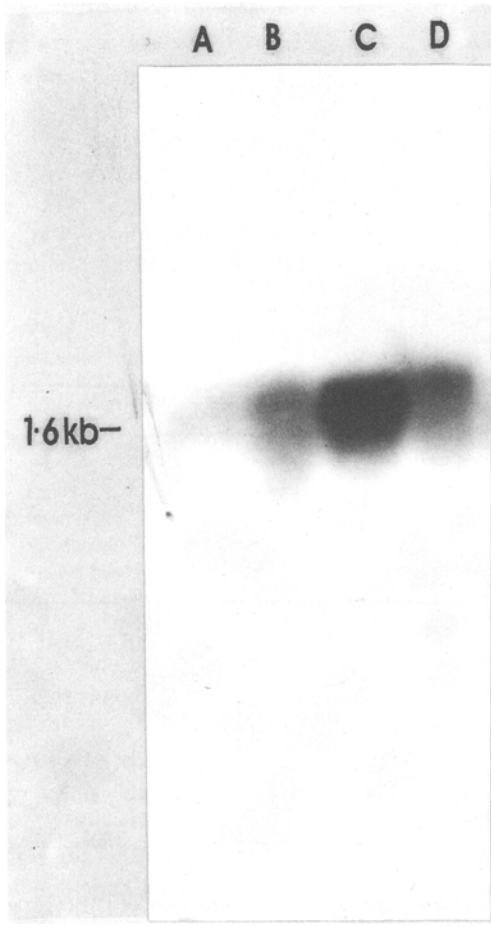

Figure 2A. pHPT5 hybridized northern blot, showing the presence of ~ 1·6 Kb mRNA transcript of HPRT in 6 (Lane A), 16 (B) and 21 day (C), and adult (D) in testicular total cellular RNA of the laboratory mouse.

3.3 *mdx*

Although muscle specific, transcripts of mdx are seen in a variety of tissues. Various transcripts utilizing different promoters (regular 5' upstream promoter or some downstream within the gene) have been encountered in heart, brain, liver and kidney (Hoffman *et al* 1987; Lederfein *et al* 1992). The probe used by us, belonged to the 5' cDNA fragment of the gene which expresses most abundantly in heart (Roffman *et al* 1987). We studied its expression in Northerns as well as RISR but failed to get any signal by either method. Parallely used LDH-X probes for the Northerns (on the same blots used for mdx) and RISH gave expected signals, suggesting that the transcript, at least from the 5' promoter of the gene, was not synthesized in testis.

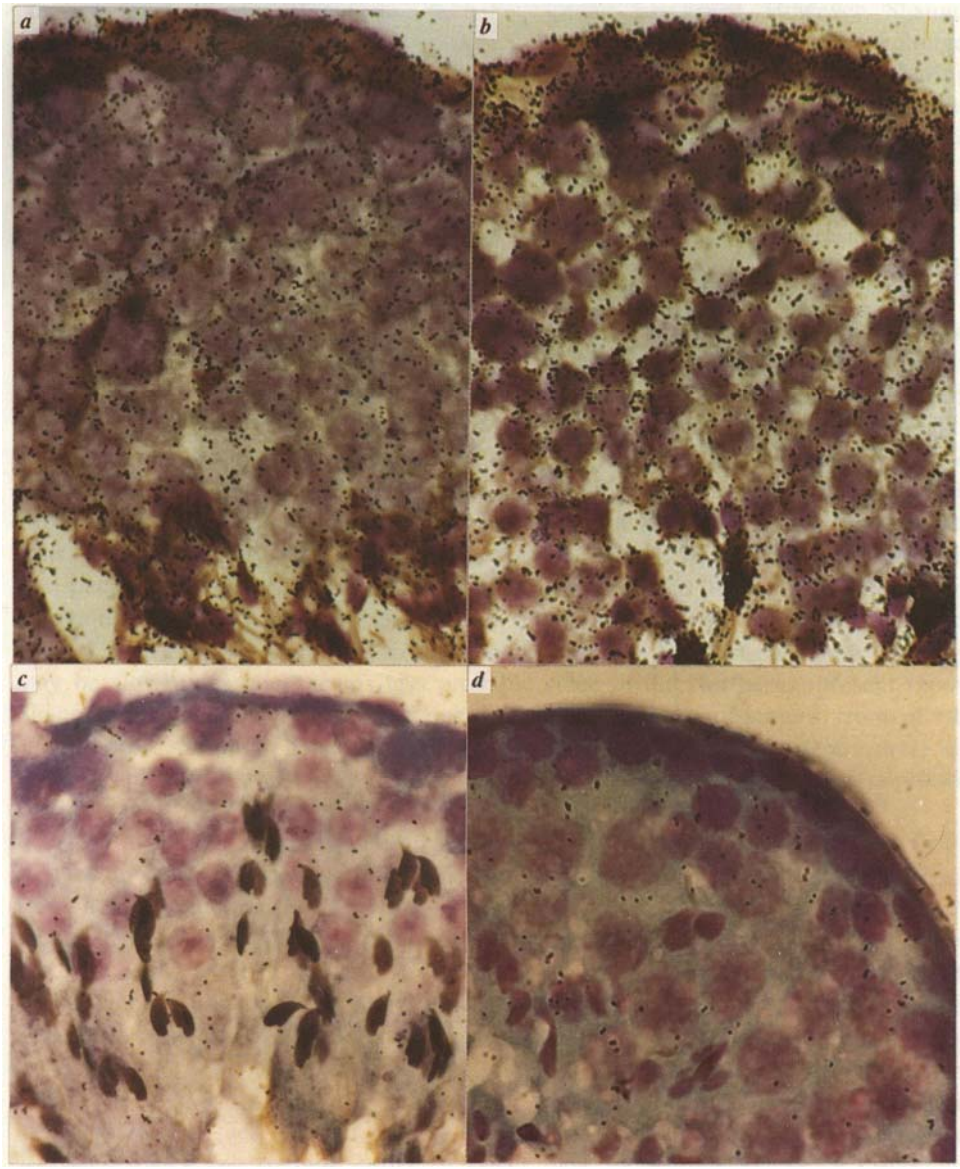

Figure 2B. RNA *in situ* hybridizations with (**a**) HPRT, **(b)** Ldh-X, (**c**) pGEM-3z and (**d**) mdx. Note that while (**a**) and **(b)** are heavily labelled in all the cell types (**c**) and **(d)** are unlabelled.

4. Discussion

Previous studies on the functional status of HPRT enzyme in the whole testis of rats of various ages and adult human, showed its presence in different samples giving the impression that the enzyme was active throughout spermatogenesis (Adams and Harkness 1976; Allsop and Watts 1985, 1986). However, in a recent

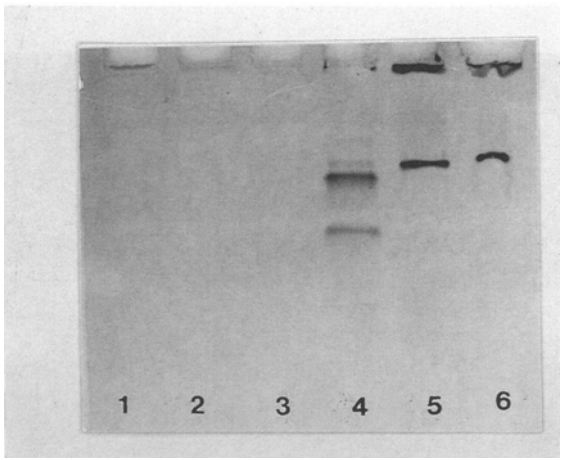

Figure 3. Potyacrylamide gel showing activity of the enzyme G6PD in pachytene (lane 1) genial (2) spermatic (3) fractions, whole testis (4), male liver (5) and female liver (6). Note the absence of G6PD activity in the lanes 1, 2 and 3.

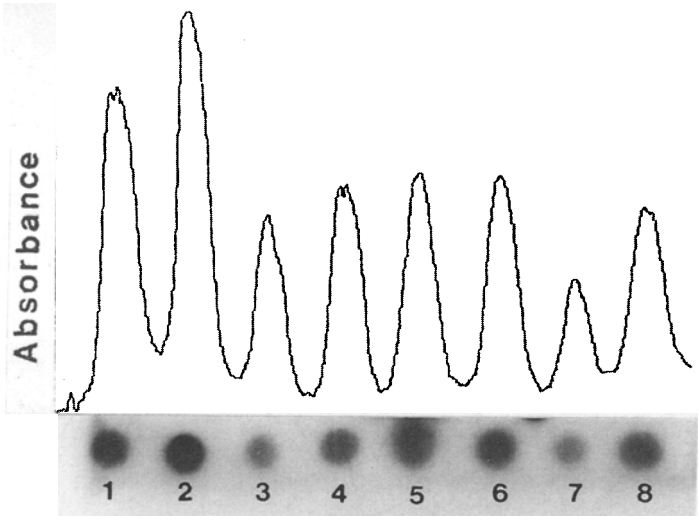

Figure 4. Dot blot hybridization of liver (Li) and testis (Ts) RNA with pGD-T-5B (G6PD) showing presence of G6PD specific transcripts. Relative abundance of the transcripts is shown above by absorbance plot detected by densitometric scanning of the autorndiogram at 540 am with 0·2 mm slit in 6d Li (1), 6d Ts (2), 16d Li (3), 16d Ts (4), 21d Li (5) 21 d Ts (6), adult Li (7), adult Ts (8).

study on fractionated cell types in mouse the enzyme activity was found only up to pachytene (Shannon and Handel 1993). All these studies were conducted on cell lysates. Since the present study was done at cellular level the evidence is unequivocal that the enzyme is active in Sertoli cells, spermatogonials and early meiotic prophase, but gets inactivated some time during pachytene and remains so thereafter. Thus,

our results agree substantially with those of Shannon and Handel (1993), particularly the low level of enzyme activity in the pachytene cell lysate. However, Shannon and Handel (1993) recorded lower activity in spermatid, which despite prolonged exposures we did not see either in spermatid or sperm. There could be various reasons for this disparity, (i) low native level of the substrate, phosphoribosyl pyrophosphate (PRPP), in spermatids (which was not provided in our study), (ii) our monitoring the activity at the level of polymerized nucleic acid rather than at the level of IMP, the first product of the reaction, or (iii) the difference in the strain of mouse used. It is, however, clear from our cytological results that at zygotene/pachytene, when the X is already heterochromatinized into X–Y body, the HPRT enzyme is functional.

The three independently carried out studies on the HPRT transcripts, though employing different methodologies—PCR (Singer-Sam *et al* 1990), Northern (Shannon and Handel 1993; present report) and RISH (present report), collectively confirm that the HPRT transcripts are present in different cell types, even in post-meiotic cells, though at this stage the enzyme is either inactive (present report – lack of [^{3}H]hypoxanthine uptake in spermatid) or lowly active (Shannon and Randel 1993). However, there is good indication that the transcript level declines in post-meiotic cells. The extremely low level of polysome bound HPRT transcripts in the post-pachytene cells has led to the suggestion that the RNA is stabilized and maintained in nonfunctional state in the adluminal zone of testis (Shannon and Handel 1993). Singer-Sam *et al's* (1990) suggestion that the post-pachytene transcript may be slowly degraded seems less probable since in the Northerns (present report, Shannon and Handle 1993), the same one band is seen in samples from all the age groups. Although none of the three studies, just mentioned, demonstrate the synthesis of HPRT transcript in testis, its temporal coincidence with enzyme product suggests that the gene may be active at least up to pachytene.

While a number of studies have previously been made on activity of the G6PD enzyme in different mammals, there is no report yet on its transcript level in testis. Here again, some reports showed complete lack of the enzyme activity in testis (Hotta and Chandley 1982; also see Luzzato and Battistuzzi 1985), but others reported a low level in whole testis (Shen and Lee 1976; Jones and Andrew 1978). These authors attribute this activity to the somatic component of the testis, i.e., Sertoli and interstitial cells. Since we too detected the enzyme activity in whole testis, but not in individual germ cell types we also conclude that G6PD enzyme should be functional only in the somatic component of testis. This is further indicated by the fact (present report) that the level of enzyme activity in testis of the 6 day mice (no meiotic cells) is higher than in adults. However, a rather low activity of G6PD in pachytene, has been detected by Erickson (1976) and Brock (1977). In a later paper, Erickson *et al* (1993) suggested that the enzyme synthesized in the somatic cells may have "leaked" into the downstream cells, a process common in testis. On the technical level, since the cell types were separated on STAPUT columns where each enriched fraction is contaminated by varying proportion of other cell types (15–25%; Sertoli cells mostly sedimenting with pachytenes), it is possible that the low activity observed in pachytenes by these authors is partly due to such contamination.

The dot blots of RNA (20 µg) extracted from whole testis of different ages show presence of the transcript. Since the cells were not fractionated, which specific cell

types made the transcript could not be established, but distinctly higher signal in the day 6 testis, which has only Sertoli and the gonial cells, strongly argues that the activity might derive from the somatic compartment of testis. The signal in other testis samples could be accounted to the presence of Sertoli cells in the testis of all ages.

The study on the mdx is constrained by the fact that we had access to only a partial probe from the 5' side of the cDNA, whereas truncated transcripts of mdx in nonmuscle cell types utilizing downstream promoters are known. We infer that the transcript utilizing the 5' promoter is not synthesized in testis. Whether or not the brain and kidney-specific 6·2 kb transcript, utilizing a downstream promoter is made, cannot be stated at this stage.

In brief, the most likely conclusion that could be drawn from the diverse approaches is that while G6PD is inactivated in premeiotic cells before the formation of XY body, HPRT is inactivated during pachytene but after the formation of the body. The muscle specific transcript of mdx does not reveal in testis. However, the quiescent of mdx may not have to do with XCI, instead with its differentiated function.

The formation of the heteropycnotic, condensed XY (or X) body in meiotic prophase has generally been identified with the XCI in testis (Kay *et al* 1993; Handel *et al* 1994). This has led to the impression that the entire X-chromosome is inactivated in testis at the zygotene/pachytene stage of meiosis. However, a review of the stage of inactivity of different genes in testis (table 2) argues for

Table 2. Summarized observations made so far (including the present study, PS) on X-Iinked gene expression during spermatogenesis of mouse.

Gene	Protein product (reference)	Transcript (reference)
HPRT	At least up to pachytene (Shannon and Handel 1993; present study)	All the cell types, frequency declines following pachytene (Shannon and Handel 1993; Singer-Sam *et al* 1990; present study)
mdx	Not studied	Absent in testis (present study)
Zfx	Not studied	Spermatogonia (autosomal Zfa may start spermatocyte stage onward) (Erickson *et al* 1993)
G6PD	At least in the premeiotic compartment of testis (Brock 1977; Erickson 1976; Jones and Andrews 1978; Shen and Lee 1976; present study)	In premeiotic testis; in lower concentration in whole testis of adult (present study)
Xist	Not identified (No ORF)	Sertoli (?) spermatogonia, primary spermatocytes declines pachytene onwards (Kay *et al* 1993; McCarray and Dilworth 1992)
Pgk-1	Present in somatic cells of testis (interstitial and Sertolis) (Kramer 1981; Kramer and Erickson 1981)	Up to preleptotene, marked decrease from leptotene/zygotene (Goto *et al* 1990; McCarrey *et al* 1992; Singer-Sam *et al* 1990)
Pdha 1 (X-linked form)	Present in spermatogonia, Leydig and Sertolis (Takakubo and Dahl 1992)	Present in spermatogonia, Leydig and Sertolis (Takakubo and Dahl 1992)
Sts	Present throughout spermatogenesis (Raman and Das 1991)	Not studied (due to lack of compatible probe)

stage-specific inactivation of individual genes which does not coincide with the time of formation of the XY body. Since most genes inactivate either before or after the heterochromatinization of the X, equating the XY-body formation with the genic inactivation of the X appears to be simplistic view. Nevertheless, XY body must be taken as gross, chromosomal modification that puts the chromatin into "potentially inactive" conformation.

4.1 *The inactivation of X-chromosome in male and females*

As stated earlier, the XCI in both male and female, has been suspected to be evolutionarily related (Cooper 1971, see Lyon 1992). However, the most curious feature of the XCI in testis is the temporal heterogeneity in the inactivation of genes. Similar heterogeneity has not been seen in female, rather it is tacitly assumed that the entire X is inactivated more or less concurrently. This raises two interrelated questions: (i) Is there a difference in the temporal regulation of intrachromosomal inactivation between the male and female X, and (ii) why are certain genes inactivated earlier (or later) than the others?

We suggest that multiple factors *viz.*, the organisation of chromatin domains, the effective concentration of the inactivation principle (e.g. Xist transcripts), and physical proximity of the genes to the inactivation centre, jointly contribute to determining the stage of inactivation of individual genes. For instance, there is good correlation of the expression of Xist and XCI in female embryos (Borsani *et al* 1991; Brockdorf *et al* 1991; Brown *et al* 1991; Kay *et al* 1993). It is also known that Xist is required only for the initiation and not maintenance of XCI in the somatic cells (Brown and Willard 1994). The Xist transcripts are unusual in that they lack an extended ORF that could produce a protein. But their binding directly with the sex chromatin has been demonstrated in human female which could so modify chromatin that it could lead to XCI (Brown *et al* 1992). Alternative suggestion assumes that the transcript could activate the Xce locus through which the XCI is regulated (Brockdorf *et al* 1992).

The Xist is also expressed in the premeiotic compartment of testis (Brockdorf *et al* 1992; Brown *et al* 1992; Salida *et al* 1992) but its concentration is about 1000 times less than in female (Kay *et al* 1993). If Xist transcript is assumed to be the inactivating principle in testis, it is possible that because of lower concentration the transcript will take longer to spread through the length of the X-chromosome, and to achieve the threshold level on each locus. This coupled with the prolonged cell cycles in male germ cells would cause a temporal difference in inactivation of different genes.

We further contend that low level of the Xist transcript would also establish the temporal scheme of inactivation of individual genes. The stipulated high to low gradient of the Xist transcript from its site of origin could affect proximal genes earlier than those placed progressively distal. Figure 5 gives a cartoon of the mapped position of the X-linked genes and their stage of inactivity in testis and a hypothetical gradient of the Xist. The fact that in the male germ cells G6PD is inactivated earlier than Zfx (present report; Brockdorf *et al* 1991; Erickson *et al* 1993), which apparently inactivates earlier than the genes occurring further down, *viz.*, HPRT (Singer-Sam *et al* 1990; Shannon and Handel 1993; present report) and

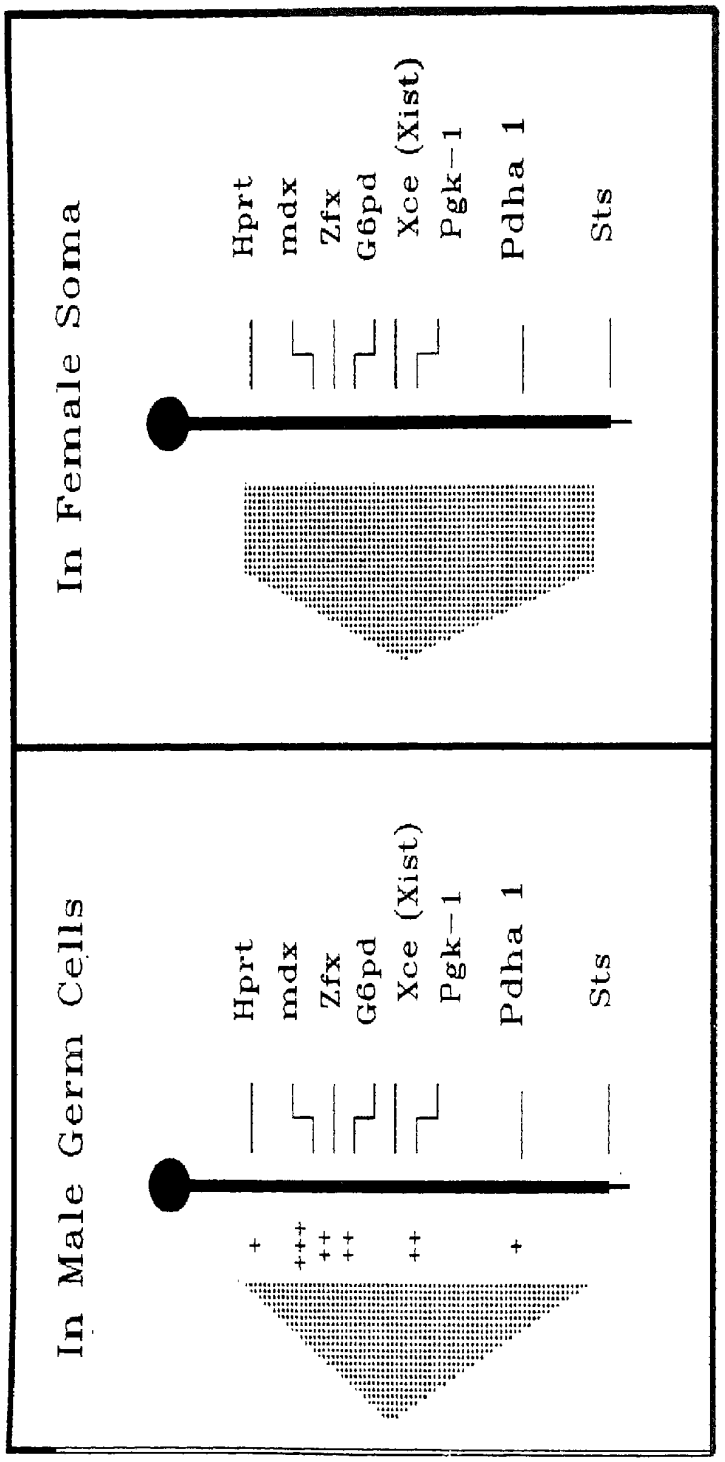

Figure 5. Hypothetical scheme for differential regulation of X-inactivation in male and female by the Xist product (location of genes on the X after Nadeu *et al* 1992; position of Pdh a1 after Blair *et al* 1993). The dots provide a quantitative estimate of the Xist transcript and its hypothetical gradient of distribution in male and female. The pluses (+) against a gene indicate its time of inactivation; those inactivated earlier show more pluses (for details see the text).

Pdh El (Takakubo and Dahl 1992) may support the proximity concept. Interestingly, Grant *et al* (1992) have invoked similar explanation (spread of Xist from proximal to the distal) to reconcile the stepwise rnethylation of the inactive X genes during early development in female mouse.

However, the activity of genes like Pgkl in spermatogonials (Kramer 1981; Kramer and Erickson 1981; Goto *et al* 1990; Singer-Sam *et al* 1990; McCarrey *et al* 1992) despite its extreme proximity to Xist make it clear that at least two other factors, chromatin conformation and tissue-specific potential of the gene or domains of genes will be important in XCI in male germ cells. Inactivation of the cell type specific genes, *viz.*, mdx may be a consequence of differentiation rather than the XCI. Whether or not the Xist concentration and proximity to this locus as well as modulations of chromatin organization have a causal influence on the XCI is required to be experimentally tested.

Heterochromatinization of the X-chromosome in male germ cells occurs not only in mammals but throughout the male heterogarnety taxa. However, it is obvious from the foregoing that in mammalian testis not all the genes are inactivated prior to heterochromatinization, More curious is the fact that following the inactivation of Pgk-2, Pdh El and Zfa their autosomal retroposon-like counterparts initiate activity exclusively in testis. Inactivation of these genes in testis despite their requirement during spermatogenesis indicates that the XCI in testis must impart strong selective advantage. Jablonka and Lamb (1988) and later, McKee and Handel (1993) have proposed that significance of the testicular heterochromatinization of the X-chromosome lies in its forestalling illegitimate X–Y or X-autosome recombination, and for protecting the unpaired X from the meiotic endonucleases. Hotta *et al* (1979) have demonstrated that in the mouse pachytene, while the unpaired regions are highly sensitive to endonucleases, heterochromatic regions are resistant. We tend to support the contention of these authors, and further suggest that in testis, heterochromatinization of the X as a whole was a more critical step than the inactivation of individual genes.

In summary, it appears that the XCI in the mammalian male testis and female soma may have a number of common features, and that the Xist transcript may be important in initiating the inactivation in both the conditions. We have proposed that the physical proximity to the Xist locus may be one of the factors that determine the stage of inactivity in testis. We support the contention that XCI by heterochromatinization of the chromosome evolved as a means to eliminate illegitimate recombination and nucleolytic digestion during meiosis.

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