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The search for the mouse X-chromosome inactivation centre

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Summary

The phenomenon of X-chromosome inactivation in female mammals, whereby one of the two X chromosome present in each cell of the female embryo is inactivated early in development, was first described by Mary Lyon in 1961. Nearly 30 years later, the mechanism of X-chromosome inactivation remains unknown. Strong evidence has accumulated over the years, however, for the involvement of a major switch or inactivation centre on the mouse X chromosome. Identification of the inactivation centre at the molecular level would be an important step in understanding the mechanism of X-inactivation. In this paper we review the evidence for the existence and location of the X-inactivation centre on the mouse X-chromosome, present data on the molecular genetic mapping of this region, and describe ongoing strategies we are using to attempt to identify the inactivation centre at the molecular level.

1. Introduction

X-chromosome inactivation is the genetic inactivation of one of the two X chromosomes present in each cell of the female mammalian embryo, whereby (with a few exceptions) all the genes on the inactive X chromosome become transcriptionally silent (Lyon, 1961). As a result of X-chromosome inactivation, one X chromosome remains active in all cells and gene-dosage for X-linked loci is similar in males and females. Although the mechanism of X-chromosome inactivation remains unknown, it is considered to have three components: firstly, initiation of X-chromosome inactivation in cells of the early female embryo; secondly, spread of inactivation along the chromosome; and thirdly, maintenance of the inactive state of the X chromosome throughout subsequent cell divisions.

Since the description of the phenomenon of X-chromosome inactivation by Mary Lyon in 1961, increasing evidence has pointed to the presence of a single *cis*-acting inactivation centre on the X-chromosome which is involved in the first stage, initiation of X-chromosome inactivation, and possibly also the second and third stages. In this paper we review the evidence for the existence and location of the X-inactivation centre in the mouse and describe ongoing strategies which we hope will culminate in the identification of the X-inactivation centre at the molecular level.

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2. X-autosome translocations

The main evidence for the presence of a major single *cis*-acting inactivation centre has come from mouse X-autosome translocations. In these translocations inactivation can spread from the X-chromosome into the physically contiguous autosomal material and cause inactivation of coat-colour genes in the attached autosomal segment which can be detected by variegation in expression of the coat colour genes in heterozygous individuals (Russell, 1963; Russell, 1983). As early as 1963, Russell and colleagues (Russell, 1963) established that variegation only occurs for genes in one of the two autosomal segments involved in a reciprocal X-autosome translocation, i.e. the segment which is attached to the part of the X-chromosome carrying the inactivation centre. However, inactivation of the X-autosome insertion, Is(X;7)1Ct (Cattanach, 1966), in which an inverted piece of chromosome 7 carrying the wild-type alleles of the *c*, *p* and *ru-2* loci is inserted into the X chromosome, has been used to support the concept of at least two inactivation centres, on either side of the insertion. Eicher (1970) concluded that inactivation spreads into the *ru-2* side of the insertion as well as the

c side when she found a line of females heterozygous for the translocation on a $p\ c^{ch}/p\ c^{ch}$ background that showed *p*-variegation without c^{ch} variegation. Since then, however, Cattanach (1974) has shown that subsequent reactivation of initially inactivated autosomal loci is possible in Is1Ct. There is therefore no *a priori* reason why one inactivation centre should not be capable of inactivating both parts of the X separated by an autosomal insertion if the inactivation is capable of initially spreading through the autosomal material. Subsequent reactivation of some of the inserted autosomal material would explain the results of Eicher (1978) and also the cytogenetic evidence of Disteche *et al.* (1979) and Rastan (1983) for an early-replicating or euchromatic autosomal segment in an otherwise inactivated Is1Ct chromosome. All other cytogenetic studies have shown that for all the X-autosome translocations examined, only one of the two segments into which the X-chromosome is broken can undergo inactivation as detected by late-replication (Russell & Cacheiro, 1978) or by dark Kanda staining (heterochromatinization) (Rastan, 1983).

Furthermore, genetic evidence for the inactivation of only one of the two segments of X chromosome involved in reciprocal translocation was provided by Lyon *et al.* (1986) in the different expression in the translocations T(X;4)37H and T(X;11)38H of the X-linked *sparse-fur* mutation, *spf*, which causes deficiency of the gene for ornithine carbamoyl transferase (OCT) (De Mars *et al.* 1976). The OCT locus has been shown, by *in situ* hybridization, to lie distal to the translocation-breakpoint in T38H and proximal to the translocation breakpoint in T37H (Lyon *et al.* 1986). When mice were bred which were heterozygous for *spf* and for either T38H or T37H (with the normal alleles of OCT on the translocated chromosome in each case) (Fig. 1) the livers of the T38H + / + *spf* mice showed, on histochemical staining, the expected mosaic pattern of positive and negative expression of OCT as seen in chromosomally normal *spf*/+ heterozygotes (Fig. 1*a*). However, the livers of the T37H + / + *spf* mice all showed uniform positive staining for OCT in all cells (Fig. 1*b*). This was because in T38H the OCT locus is in physical continuity with the inactivation centre and therefore undergoes normal random inactivation, whereas in T37H the OCT locus is separated from the inactivation centre and so remains active in all cells.

By the mid nineteen eighties the accumulated evidence from the known X-autosome translocations placed the inactivation centre somewhere between the breakpoint in Searle's translocation, T(X;16)16H, the proximal limit of the X-inactivation centre region (Rastan, 1983) and the Oak Ridge translocation T(X;7)6Rl, the distal limit (Russell & Cacheiro, 1978).

3. Deletions

The distal limit of the X-inactivation centre region of

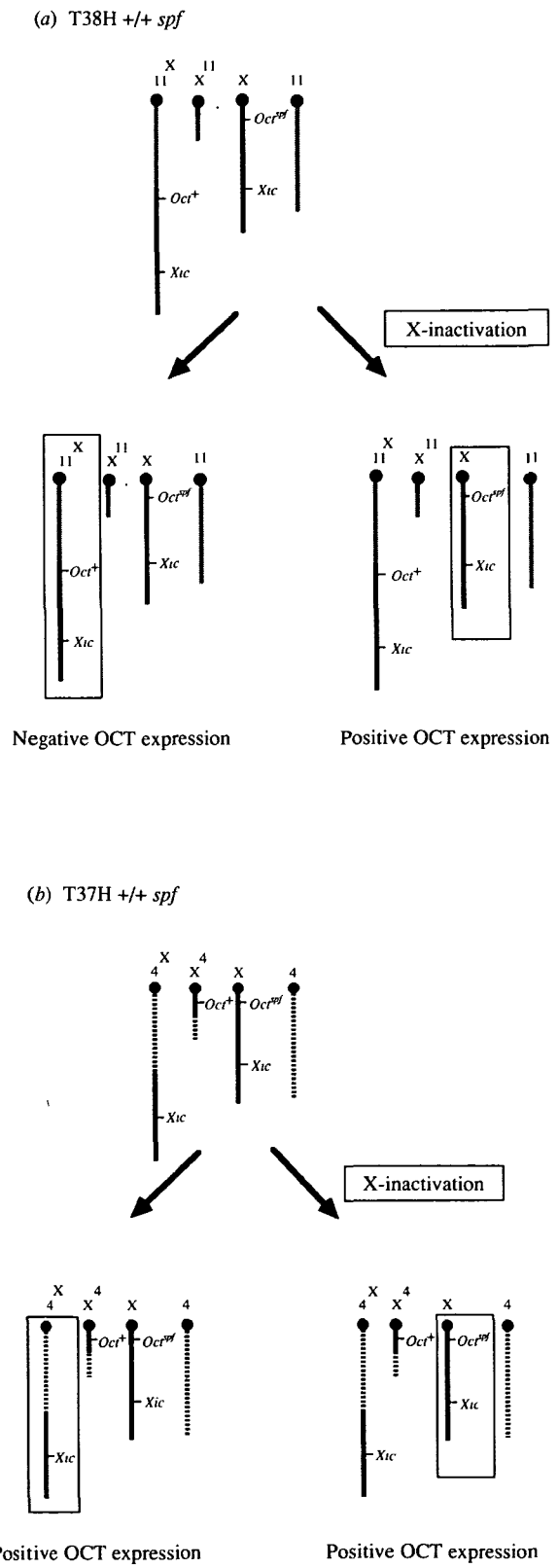

Fig. 1. OCT expression in T38H + / + spf and T37H + / + spf females. The position of the *Oct* locus with respect to the translocation breakpoints and the X chromosomal inactivation centre (*Xic*) is shown for T38H (*a*) and T37H (*b*). In each case the top panel shows the chromosomal constitution and genotype of T38H + / + *spf* females (*a*) and T37H + / + *spf* females (*b*), and the lower panel shows the effect of random X-inactivation on OCT expression.

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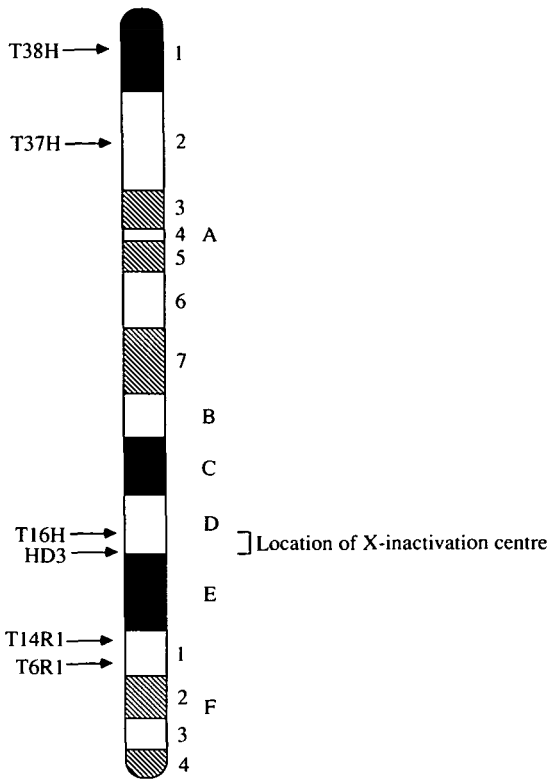

Fig. 2. Cytogenetic location of the X-inactivation centre. The G-banded diagram of the mouse X-chromosome is shown with the positions of some of the X-autosome translocations which have been used to assign the X chromosome inactivation centre. The inactivation centre is located somewhere between the breakpoints of the translocation T16H and the deletion HD3. The relative positions of T14Rl and T6Rl, while not definitively assigned cytogenetically, have been placed according to genetic data from Avner *et al.* (1987).

the mouse X-chromosome was further delineated cytogenetically in 1985 by Rastan & Robertson using a series of female embryonic stem (ES) cell lines (Evans & Kaufman, 1981) carrying deletions of one of the two X-chromosomes (Robertson *et al.* 1983*a*, *b*). X-chromosome inactivation was detected cytogenetically by dark Kanda staining. Cell line HD3 contains one normal X-chromosome and a deleted X-chromosome with the breakpoint in the proximal part of band E (see Fig. 2). Cell lines carrying deletions with breakpoints proximal to that in line HD3 failed to demonstrate any X-inactivation on differentiation; however the HD3 cell line, and cell lines with deletions distal to the HD3 breakpoint, underwent random X-chromosome inactivation normally on differentiation (Rastan & Robertson, 1985).

These results localize the X-inactivation centre to the small cytogenetic region between the T16H breakpoint, in the distal half of band D, and the HD3 breakpoint, at the band D/band E junction (Fig. 2).

4. The *Xce* locus

In 1965 Cattanach described a locus, the X-chromosome controlling element (*Xce*) locus, different alleles of which appeared to affect the degree of inactivation of the autosomal insertion in Cattanach's translocation, Is(X;7)1Ct (Cattanach & Isaacson, 1965, 1967) and certain X-linked coat colour/texture genes (Cattanach *et al.* 1969). Initially it was thought that alleles at the *Xce* locus altered the spread of inactivation into the autosomal insertion in Is1Ct, but later, when it was shown that true X-linked genes were also affected, it was suggested that alleles at the *Xce* locus exerted their effects by influencing the spread of inactivation into the X-chromosome itself (Cattanach *et al.* 1969). Eventually, after testing the X chromosomes from a number of different origins in translocation heterozygotes for their effect upon the translocation-associated variegation, evidence for an interaction between X chromosomes was found and it was postulated that alleles at the *Xce* locus act by influencing the probability of the whole X-chromosome on which they are carried being either expressed or inactivated.

Three alleles of the *Xce* locus are known, Xce^a, Xce^b and Xce^c. Using appropriate chromosomal marker genes it has been shown that in Xce^a/Xce^b heterozygotes, cells with an active Xce^b chromosome tend to predominate over those with an active Xce^a chromosome, and that in Xce^b/Xce^c heterozygotes, cells with an active Xce^c chromosome tend to predominate over cells with an active Xce^b chromosome. In addition, the Xce^a/Xce^c compound shows a more extreme departure from random X-inactivation than the Xce^b/Xce^c compound. (Cattanach & Johnston, 1981; Johnston & Cattanach, 1981).

Finally, Rastan (1982) showed that alleles at the *Xce* locus exert their effect at the chromosomal level by biasing the randomness of primary X-inactivation early in development. In addition, Rastan & Cattanach (1983), using dark Kanda staining to detect the inactive X chromosome, showed that the non-random inactivation of the paternal X chromosome caused by imprinting in certain extraembryonic tissues can be overriden to a certain extent by appropriate manipulation of alleles at the *Xce* locus. It was therefore suggested that it may be the *Xce* locus itself which undergoes imprinting. However, for reasons that are not yet clear, a similar effect could not be detected when levels of PGK-1 protein isozymes were used as the yardstick of X-chromosome inactivation (Bücher *et al.* 1985).

Classical genetic mapping of *Xce* with respect to *Ta*, *Mo*, *Pgk-1* and *Bpa* (Cattanach & Papworth, 1981; Cattanach, 1983; Cattanach *et al.* 1989*a*) has demonstrated tight linkage to the *Ta* locus, which is located just distal to the T16H breakpoint. In view of the effect of *Xce* alleles on the randomness of X-chromosome inactivation, and because of its genetic map position, very close to the physical region which has functionally been shown to contain the inactivation centre, the *Xce* locus has been implicated as a

candidate gene for the X-chromosome inactivation centre in the mouse. Russell (1971), Grahn *et al.* (1970) and Falconer & Isaacson (1972) have also described similar controlling sites which appear to map to the same region on the X chromosome, and a further mutation, *Ohv*, has been described (Ohno *et al.* 1974; Drews *et al.* 1974) that maps very close to the *Xce* locus or is an allele of it.

5. Long range molecular genetic mapping of the region containing the mouse X-inactivation centre

Recent genetic mapping of the mouse X chromosome using interspecific *Mus domesticus/Mus spretus* interspecific backcrosses (Brockdorff *et al.* 1987*a*, 1987*b*; Cavanna *et al.* 1988; Mullins *et al.* 1988) has provided a detailed and extensive molecular genetic map of the mouse X chromosome. The advantage of interspecific backcrosses is the multilocus nature of the mapping approach (Brown, 1989). The wide evolutionary separation of the parental species allows the easy detection of Restriction Fragment Length Variants (RFLVs) for DNA probes. Each backcross progeny from the interspecific cross is scored for the segregation of *spretus* and *domesticus* RFLVs for every DNA probe. DNA markers are ordered along the chromosome by minimising the number of observed crossovers. These crossovers define a number of small genetic intervals determined by adjacent crossover events. New probes are rapidly positioned to a particular genetic interval on the chromosome utilizing a pedigree analysis in a limited number of progeny carrying appropriate crossover events. In this manner, a large number of DNA markers have already been assigned to the mouse X chromosome in the vicinity of the *Ta* locus (Brockdorff *et al.* 1987*a*; Cavanna *et al.* 1988) and in the general region of the mouse X-inactivation centre.

We have recently produced CpG rich (HTF) island libraries (*Eag*I and *Not*I linking libraries) from the mouse X chromosome containing over 250 X-chromosome specific CpG-rich island clones (Brockdorff *et al.* 1990) and have mapped 75 of the clones on the mouse X chromosome using somatic cell hybrids (Avner *et al.* 1987) carrying the proximal part of various X-autosome translocations. Seventeen of these CpG-rich island clones map to the region of the X chromosome lying between the T16H and T14Rl breakpoints and encompassing the X-inactivation centre. Along with other existing probes in the vicinity of the mouse X-inactivation centre, they provide the basis for the provision of a detailed genetic map of the X-inactivation centre region.

Utilizing two interspecific backcrosses segregating for the X-linked genetic markers *Ta*, *Hq* and *mdx* (Brockdorff *et al.* 1987*a*; Cavanna *et al.* 1988), we have mapped a number of gene probes, microclones and *Eag*I linking clones distal to the T16H breakpoint and in the vicinity of the X-inactivation centre (Keer

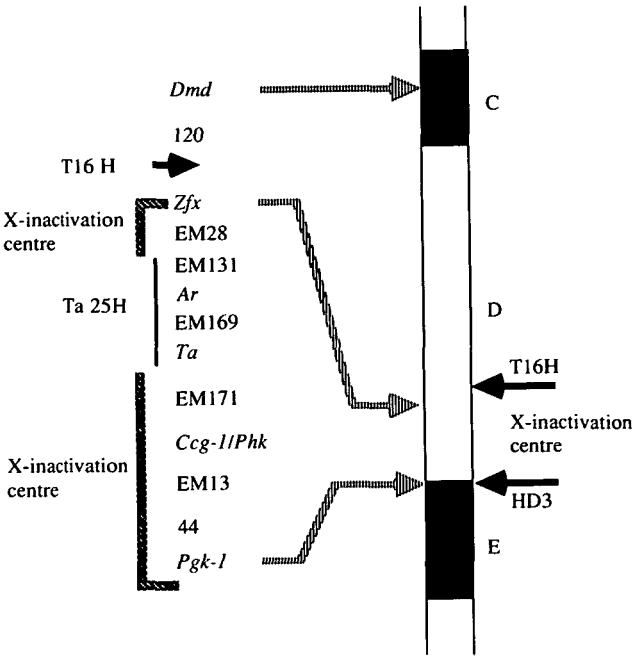

Fig. 3. Summary of genetic and physical analysis of probes mapped in the region of the mouse X-inactivation centre. Probes mapped in the *Dmd* to *Pgk-1* interval are shown. The physical positions of the T16H and HD3 breakpoints, the proximal and distal limits of the mouse X-inactivation centre region, are indicated. The approximate extent of the Ta^{25H} deletion is also shown, although its exact limits are unknown. The two regions of the X chromosome that may contain the X-inactivation centre itself are similarly shown with their approximate limits.

et al. 1990). The map of the X-inactivation centre region (see Fig. 3) extends from the T16H breakpoint to the *Pgk-1* locus and crosses a small deletion, Ta^{25H}, which removes the *Ta* and *Ar* (*Tfm*) loci and yet retains the X-inactivation centre (Cattanach *et al.* 1989*b*). The genetic map of the X-inactivation centre region provides a number of new genetically-ordered markers for this region and, in addition, defines two possible regions for the location of the mouse X-inactivation centre: (1) between T16H and the proximal limit of Ta^{25H} deletion, proximal to *Ar* and in the vicinity of the *Zfx* locus; or (2) between the distal limit of the Ta^{25H} deletion and the HD3 breakpoint, in the region encompassing the markers *Ccg-1* and *Pgk-1* (see Fig. 3).

In order to delineate further the most probable location of the mouse X-inactivation centre, it is pertinent to consider the comparable location of the human X-inactivation centre (Fig. 4). Studies of human X chromosome deletions and X-autosome translocations have defined a region of the long arm of the human X chromosome that appears to harbour an X-inactivation centre (Allerdice *et al.* 1978; Mattei *et al.* 1981; Tabor *et al.* 1983). Briefly, the cytogenetic evidence appears to indicate that the human X-inactivation centre lies in band Xq13, between the proximal breakpoint of Xq13–Xq21.3 deletion that

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leaves the X-inactivation centre intact and an X;14 translocation breakpoint. The derivative (14) translocation product is subject to X-inactivation and further molecular analysis indicates that while the derivative (X) translocation product retains the AR (Lubahn *et al.* 1988) and CCG1 (Brown *et al.* 1989) loci, both the inactivation centre and the PGK1 locus (Brown & Willard, 1989) have been translocated to chromosome derivative (14). Taken together, the molecular and cytogenetic data indicate that the human X-inactivation centre maps on the long arm distal to CCG1 and in the vicinity of the PGK1 locus.

Comparison of the genetic maps of mouse and human X chromosomes (Fig. 4) indicates that the X-inactivation centre in both species lies in the conserved segment containing *Ar* and *Pgk-1* loci. The proximal limit of this conserved linkage group on the mouse X chromosome lies somewhere between the *Ar* and *Zfx* loci, given their juxtaposition on the mouse X chromosome and their widely separate positions on the human X chromosome. For this reason, one location for the mouse X-inactivation centre is between *Zfx* and *Ar* loci, as *Ar* lies within the Ta^{25H} deletion and *Zfx* lies beyond the border of the conserved segment that contains the X-inactivation centre. Alternatively, the mouse X-inactivation centre may lie between the distal limit of the Ta^{25H} deletion and the *Pgk-1* locus (see Fig. 3). Comparison of the mouse X chromosome genetic map in this region with the human map location for the X-inactivation centre (Fig. 4) favours the second alternative, i.e. the comparative data would indicate that the mouse X-inactivation centre lies distal to *Ccg-1* and in the region of *Pgk-1*. Two new molecular markers have been mapped into this region: the microclone DXSmh44 and the *Eag*I linking clone EM13 (see Fig. 3). Further markers will be mapped into the *Ccg-1/Pgk-1* genetic interval employing pedigree analysis of backcross progeny carrying appropriate closely-spaced crossover events.

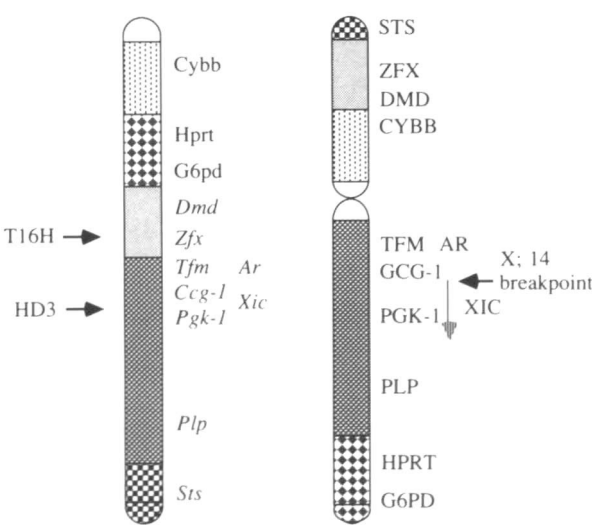

Fig. 4. Comparative maps of the mouse and human X chromosome. The position of the mouse X-inactivation centre (*Xic*) and the human X-inactivation centre (XIC) with respect to the various linkage groups conserved between the two X chromosomes is indicated. Figures indicated in bold highlight those loci most important in determining the relative positions of the mouse and human X-inactivation centres.

The provision of a detailed genetic map encompassing a large number of DNA probes over the X-inactivation centre region of the mouse X chromosome is the first step towards the construction of a detailed long-range physical map. From the microclone DXSmh120 to *Pgk-1*, a genetic distance of approximately 8 cM, twelve DNA probes have been mapped (Fig. 3). With the genetic mapping of further probes into this interval, physical linkage of tightly-linked clones utilizing pulsed-field gel electrophoresis (PFGE) will provide long-range skeleton restriction maps of the likely genetic regions containing the X-inactivation centre. PFGE analysis has already been successfully employed to establish a 1·5 Mb long-range restriction map encompassing the closely-linked G6pd, Factor VIII and P3 loci on the mouse X chromosome (Brockdorff *et al.* 1989). Subsequently, it will be necessary to overlay the long-range restriction map with YAC (yeast artificial chromosome) clones. YAC vectors allow the cloning of substantial segments of mammalian genomes (> 300 kb) and provide the necessary access to all of the underlying sequences of any mapped region (Burke *et al.* 1987). A variety of linked DNA markers on the pulsed-field restriction map can be used for screening mouse YAC libraries in order to provide the first YAC clones. These clones act as start points for the establishment of a complete set of overlapping YAC clones covering the mapped region by chromosome walking.

6. Future strategies

(i) *X-inactivation assay*

Identification of candidate sequences responsible for the initiation of X-inactivation raises a number of problems not usually associated with the identification of classical gene sequences. Whether or not the X-inactivation centre encodes an identifiable protein product is unknown. It is not inconceivable that the X-inactivation centre is composed of a protein recognition sequence or encompasses a DNA sequence or chromatin structure responsible for attachment or interaction with some cytoskeletal structure e.g. a membrane attachment site. For this reason, it is preferable to test candidate sequences by using a biological assay that demonstrates the ability of such a sequence to initiate X-chromosome inactivation. We have devised such a functional assay using $Hprt^-$ embryonic stem (ES) cells and vectors which have been specially designed to test the inactivating capacity of any candidate sequences.

Undifferentiated pluripotent ES cells have not yet undergone X-inactivation. However, following differentiation, the cells undergo X-inactivation and in

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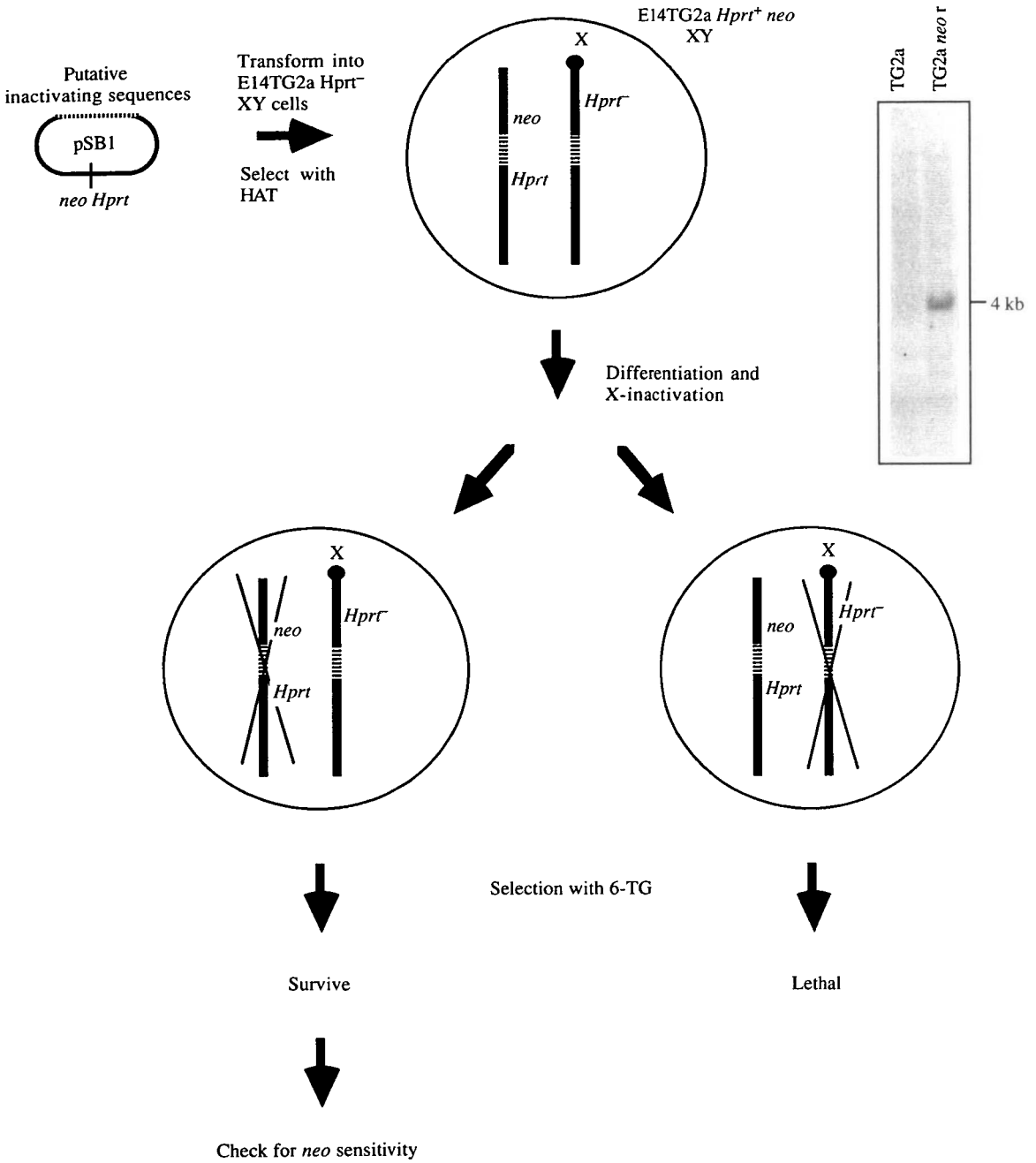

Fig. 5. Strategy of a functional assay for X-inactivation sequences. Sequences introduced in the pSB1 or pSB2 vector are transfected into E14TG2a *Hprt*⁻ cells. Following forward selection with HAT, differentiation and back selection with 6 thioguanine (6-TG), the presence of G418 sensitive surviving cells indicates a potential X-inactivation sequence (see text). The photographic insert demonstrates transfection of E14TG2a cells with pSB1 and selection with G418. G418 resistant colony (E14TG2a neo^r) and untransfected cells (E14TG2a) were digested with *Bam*HI and hybridized to pMC1 *neo*. A single band at 4 kb demonstrates a single pSB1 integrant in the E14TG2a neo^r cells that is not present in the E14TG2a control.

female ES cells one or other X chromosomes becomes inactivated (Rastan & Robertson, 1985). Introduction of an X-inactivation centre into a male (XY) ES cell provides the cell with two X-inactivation centres and, following differentiation, it would be expected that one or other of the centres would be inactivated: either the introduced foreign centre or, alternatively, the endogenous inactivation centre on the single X chromosome. This provides a route for the identification of X-inactivation sequences.

We have constructed three vectors, pSB1, pSB1cos and pSB2, to explore the principles of our assay system (Fig. 5). Each vector contains a *neo* gene providing resistance to G418 and an *Hprt* minigene. Both vectors also carry unique sites for the introduction of foreign DNA. A candidate inactivation sequence can be introduced into these vectors and transfected into Hprt⁻ male ES cells, E14TG2a (Thompson *et al.* 1989). E14TG2a cells carry a deletion of the X-linked *Hprt* gene. Stable integration of the

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construct can be demonstrated by forward selection with HAT on the introduced *Hprt* gene. Subsequently, transfected HAT resistant cells can be allowed to differentiate and X-inactivation occurs. Cells are then back-selected with 6-thioguanine (6-TG). There are two possible outcomes depending on whether the introduced sequence is able to initiate X-inactivation or not. If not, then all the differentiated cells are susceptible to 6-TG and die. However, if the introduced construct does contain sequences that can initiate X-inactivation then, as a result of random X-inactivation, either the single X chromosome is inactivated from the endogenous X-inactivation centre or the introduced inactivation centre undergoes inactivation. If the former occurs, this will presumably be lethal to the cell. However, if inactivation occurs from the introduced sequences in the construct then this will inactivate the neighbouring *Hprt* gene and the cells will be resistant to 6-TG. Thus, following back selection with 6-TG, cells only survive if they now carry an X-inactivation centre introduced on the transfected construct. In addition, surviving cells should also now be G418 sensitive since inactivation would also affect the *neo* gene introduced with the transfected construct. It is important to note that we do not expect the autosomal monosomy that may result from inactivation of chromosome regions adjacent to the integration site to be lethal to cells growing *in vitro*. Nevertheless, it may be necessary to test a number of different integrants of each potential inactivation sequence.

Ultimately, with no knowledge of the nature or the extent of the sequence region necessary for X-inactivation, it may be wise to test extremely large sequence constructs of several hundred kilobases or more through the X-inactivation assay using YAC variants of the pSB1/pSB2 vector system.

(ii) Trans-*acting factors*

To date the work undertaken to elucidate the mechanism of X-chromosome inactivation has involved progressively delineating the physical and genetic limits of the *cis*-acting X-inactivation centre with a view to cloning the inactivation centre by the reverse genetic approach. However, in addition to the inactivation centre, models for the initiation of X-chromosome inactivation involve *trans*-acting factors which interact with the *cis*-acting inactivation centre to either block or initiate X chromosome inactivation (Rastan, 1983). Evidence for the existence of such *trans*-acting factors comes from the link between initiation of X-chromosome inactivation and differentiation in both early embryos, teratocarcinoma stem cells and embryonic stem (ES) cells (see above). There is now considerable evidence that in early embryos and in undifferentiated teratocarcinoma stem cells and ES cells, both X chromosomes are in the active state and that the process of X-inactivation is triggered by the onset of differentiation (Monk & Harper, 1979; Paterno *et al.* 1985; Takagi & Martin, 1984; Rastan & Robertson, 1985). Thus embryonic stem cells will provide a good model system for searching for such *trans*-acting factors, the discovery of which will be essential for the complete description and understanding of the mechanism of X-chromosome inactivation.

The authors owe a great debt of gratitude to Mary Lyon, FRS, who first described the phenomenon of X-chromosome inactivation nearly thirty years ago. S.R. was a research student of Mary Lyon from 1977 to 1980.

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