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Molecular correlates of the murine *Xce* locus

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Summary

The murine *Xce* locus, first identified by Bruce Cattanach, influences the primary choice of the X chromosome to be inactivated. Methylation of a GC-rich region (*DXPas34*) that includes multiple 34 bp repeats and lies some 15 kb 3′ to *Xist* has been shown to vary with *Xce* haplotype. The degree of methylation on the active X chromosome at this locus represents one of the few molecular correlates of *Xce* action currently available. Data relating to the specificity and other characteristics of this association are presented.

1. Introduction

In mammals, females differ from males by the presence of a second copy of the X chromosome. Inactivation of one of the two X chromosomes ensures the dosage compensation of X-linked gene expression between XX females and XY males.

X-inactivation in the female occurs during early embryogenesis and is tightly linked to the differentiation of the embryo occurring at this time. In the extra-embryonic lineages that first appear at around 3·5 days post coitum (dpc) X-inactivation is imprinted, resulting in the exclusive inactivation of the paternally inherited X chromosome. In embryonic lineages that arise later (5·5–6·5 dpc) the paternally and the maternally derived X chromosomes have an equal likelihood of being inactivated.

The X-inactivation process has been divided somewhat arbitarily into three phases: (1) initiation, (2) propagation or spreading, and (3) maintenance. Initiation is under the control of a *cis*-acting element known as the X-inactivation centre or *Xic* (Avner, 1996; Heard *et al*., 1997). The *Xic* is thought to be involved not only in the initiation of inactivation but also in the counting process, which senses the number of copies of the X chromosome in the cell and ensures that only a single X remains active per diploid cell, all other X chromosomes being inactivated.

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Evidence for the *Xic* has been provided by studies of X chromosome rearrangements in both man and mouse (Lyon 1983; Russell, 1983). The efforts of several investigators and in particular of Hunt Willard and his colleagues has led to the localization of the human *XIC* locus to an 800–1200 kb region lying within the *PHA–PGK1* region in band Xq13 (Brown *et al*., 1991*b*; Leppig *et al*., 1993; Lafrenière & Willard, 1993). The corresponding syntenic region in the mouse, which appears to have been subject to several chromosomal inversions, is somewhat smaller (Debrand *et al*., 1998).

The *Xist* (X-inactive specific transcript), gene which was first isolated in 1991 and lies within the *Xic* in both man and mouse, has been shown to play an important role in the initiation process (Brown *et al*., 1991*a*, 1992; Borsani *et al*., 1991; Brockdorff *et al*., 1991, 1992). Of particular significance are the perturbations in the inactivation process observed in female ES cells and embryos carrying null mutations within the *Xist* gene (Penny *et al*., 1996; Marahrens *et al*., 1997) and the capacity of YACs and cosmid transgenes carrying the *Xist* gene to induce X-inactivation (Lee *et al*., 1996; Herzing *et al*., 1997). The close association or 'decoration' of the inactive X chromosome by the non-protein coding transcript of the *Xist*/*XIST* gene (Clemson *et al*., 1996) has led to suggestions that the *Xist* RNA may have a chromatin remodelling role during the establishment of the inactive state.

Another locus intervening in the X-inactivation process, *Xce* (X-controlling element), has been charac-

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terized genetically by Bruce Cattanach (Cattanach, 1970, 1974). The *Xce* locus influences in an as yet undefined manner the random nature of the X-inactivation process in the mouse (Cattanach & Williams, 1972). Although non-random X-inactivation can result from several distinct causes, in the case of *Xce*, primary non-random inactivation is involved, implying distortion of the random nature of the X-inactivation process itself. The distortion induced results most often in a 20–30% skew in the ratio of the products of the two X chromosomes.

Three alleles at the *Xce* locus have been defined using the *Tabby*/vibrissae system of phenotyping (Cattanach & Rasberry, 1994): Xce^a, present in the C3H/HeH, 1O1/H, 129/Sv, CBA/N and A/H inbred strains; Xce^b, present in the JU/Ct and C57BL/6 strains; and Xce^e in mice carrying the X-linked *Pgk-1a* gene derived from a feral *Mus musculus musculus* strain (Simmler *et al.*, 1993, and references therein). In Xce^a/Xce^b heterozygotes, the X chromosome carrying the Xce^a allele is more likely to be inactivated than that carrying the Xce^b allele; similarly in Xce^b/Xce^c heterozygotes, the X carrying the Xce^b allele is more likely to be inactivated in most cells. Most extreme skewing is seen in Xce^a/Xce^c heterozygotes. Both *Mus castaneus* and *Mus spretus* carry an Xce^c-type allele although the possibility that one or other may be carrying a more extreme allele cannot be ruled out using the *Tabby*/vibrissae test system (Cattanach & Rasberry, 1994).

Non-random coat colour chimaerism observed by Falconer & Isaacson (1979), using animals heterozygous for the brindled mutation at the X-linked *Mottled* locus, was also shown to be due to X-inactivation differences. In this case there was some suggestion that expression of the brindled phenotype was greater after maternal transmission of the mutant allele than when transmission of this allele was paternal, suggesting that the skewing process might be subject to imprinting. Fowlis *et al.* (1991) have similarly obtained evidence for imprinted or parental specific modification of *Xce* action using PGK-1 enzyme activity as a marker for X chromosome activity. The inactivation of the *albino* locus inserted into the X chromosome in the Is1Ct translocation has also been shown to be influenced by *Xce*, or an *Xce*-like effect (Cattanach, 1970), and possible imprinting effects on the degree of skewing again noted (Cattanach & Perez, 1970). The translocated X chromosome was in this case more strongly inactivated when paternally derived.

Whilst the ability of the *Xce* locus to influence the random X-inactivation characterizing somatic tissues derived from the embryo proper is clear, its ability to influence imprinted inactivation in extra-embryonic tissues remains controversial (Rastan & Cattanach, 1983; West & Cattanach, 1985). It may indeed be logical to imagine that *Xce* would not influence X-inactivation when the choice of X chromosome to be inactivated has already been predetermined by imprinting.

Clear evidence that the *Xce* locus exerts its effect by primary non-random X-inactivation or chromosome choice, rather than through a secondary cell selection effect, was provided by Rastan (1982), using differential cytological staining of embryos at the time when X-inactivation is first seen in the embryo proper.

If *Xce* acts to modify the choice of chromosome that will be inactivated, all X-linked markers subject to inactivation would be expected to reflect its action and direct analysis at the transcriptional level should demonstrate the effect of *Xce*. In this article we describe our efforts to establish molecular correlates for the action of *Xce* and present additional data concerning a previously established association between the methylation status of the *DXPas34* locus and the *Xce* locus.

2. Materials and methods

(i) *Mice*

The origins of the 129.Pgk1a congenic strain are described in Courtier *et al.* (1995). The *Xce* recombinant animals T3958, T4165, T3995 and T4079 were obtained by Bruce Cattanach and his colleagues from crosses of F1 Xce^c/Xce^a heterozygote females to Xce^c males. Recombinants for the *Xce* region were obtained by selecting animals having recombined the *Tabby* and Mottled flanking markers used to mark the parental stocks. These flanking markers define an approximately 4–6 cM region of the mouse X chromosome (Cattanach *et al.*, 1991). Phenotypic analysis for the *Xce* locus was as described in Cattanach & Williams (1972).

(ii) *Cell lines and culture conditions*

The male ES cell line D3 was obtained from R. Kemler and is described in Doetschmann *et al.* (1985). The HP3.10 and HP3.11 female ES cell lines were derived from ($Hprt^{bm1}Pgk1a \times 129$/Sv)F1 embryos and will be described in detail elsewhere. All ES cells were grown on irradiated mouse fibroblast feeder layers in medium containing 15% foetal calf serum (FCS), 10^3 units/ml of lymphocyte inhibitory factor (LIF), 2×10^{-4} M/ml 2-mercaptoethanol, 2 mM glutamine. Differentiated ES cultures were obtained by forming aggregates using standard techniques and replated for 6 days prior to harvesting (Robertson, 1987).

(iii) *Sequencing*

The 1·6 kb of cloned genomic sequence containing the 34 bp repeat was manually double-strand sequenced

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using the Sanger dideoxy sequencing method and the Sequenase enzyme.

(iv) *Primers*

Primers were designed using the Oligo software programme. The primers used were P2:(5′CTAAAATGCCTGCCACCG3′) designed from within the repeat sequence and P1:(5′CCTCGGAAATAAACGGAACA3′) situated 514 bp from the 5′ end of the repeat sequence.

(v) *Minisatellite repeat variation amplification by PCR*

One hundred and fifty nanograms of genomic DNA were amplified in a 25 μl reaction volume using 1 mM of primers P1 and P2, 2·5 units of Taq Polymerase (Amersham) and the PCR buffer system previously described by Jeffreys *et al.* (1991). After denaturation for 5 min at 95 °C, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 5 min were followed by a final 8 min elongation step at 72 °C. Fifteen microlitres of the PCR product was electrophoresed through a 1% agarose gel and alkali transferred onto a Hybond N membrane (Amersham). Blots were hybridized using a ^{32}P-labelled oligonucleotide designed from the repeat sequence for 3 h at 50 °C in 0·45 M sodium phosphate buffer pH 7·2, 1 mM EDTA and 7% SDS, washed twice at 50 °C in 5 × SSC, 0·1% SDS and exposed at −80 °C with intensifying screens. Changes in annealing temperature from 50 °C to 60 °C, or in the internal oligonucleotide used, altered both the number and the distribution of the detectable amplification products, but rarely changed the overall length of the repeat-containing array. The hybridization intensity of the amplified products varied with the oligonucleotide probe used, probably reflecting the high degree of sequence degeneracy of the repeat units in the array.

(vi) *Methylation analysis*

Methylation analysis of the Sal* site within the *DXPas34* locus was carried out by *Eco*RI/*Sal*I and *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I digestion followed by Southern blot analysis as described in Courtier *et al.* (1995) on spleen DNAs from males. Methylation analysis of the 3′ region of the *Xpct* and *Bpx* genes was carried out using *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I digested DNA within a similar overall protocol. Blots were probed for *Xpct* using the 123E2 cDNA described in Debrand *et al.* (1998) and for *Bpx* using the PO324 probe described in Rougeulle & Avner (1996).

(vii) *Transcript profiling: estimation of relative RNA levels of in heterozygotes*

Kidney RNA was prepared according to the method first described by Auffray & Rougeon (1980) and first strand cDNA was synthesized from 2 μg of total RNA, using M-MLV reverse transcriptase (Gibco BRL), in a 20 μl reaction volume. To estimate the amount of each allelic *Atp7a* transcript fluorescent RT-PCR was performed on region VI of the *Atp7a* cDNA that contained a previously defined 34 bp length polymorphism situated in the 3′UTR (Cecchi & Avner, 1996). One microlitre of cDNA was used for the RT-PCR reaction, using fluorescent primers moF3930 and moF4812 in a volume of 50 μl as previously described (Cecchi *et al.*, 1997). PCR conditions were 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, followed by a single 10 min elongation step at 72 °C.

The relative amounts of allelic transcripts were quantified for each strand of the short and the long PCR products of the *ATP7a* region VI after separation on an automated ABI373A sequencer using the GENESCAN software.

3. Results and Discussion

(i) *Molecular characterization of transcript levels in* Xce *heterozygotes*

In most cases the skewing of X-inactivation produced by the *Xce* locus has been assessed using mutations in X-linked genes that influence phenotypes associated with coat colour or vibrissae number, and can easily be carried out on the whole mouse. Less information is available regarding the effects of *Xce* at the level of transcription. Evaluation of *Xce* action at this level requires the identification of polymorphisms within the X-linked gene to be analysed, so that the transcription levels of the two X chromosomes within the female cell heterozygous for *Xce* can be individually assessed.

Increased levels of *Smcx* transcripts derived from an X chromosome carrying an Xce^c allele have been correlated, as expected, with lower levels of *Xist* expression from the same chromosome (Penny *et al.*, 1996; Sheardown *et al.*, 1996). A correlation between the degree of skewing seen for *Pgk1* gene expression in Xce^c/Xce^a heterozygotes and the skewing of *Xist* have also been reported (Buzin *et al.*, 1994). We have found similar results when analysing transcript levels by RT-PCR at the nearby *Atp7a*(*Mo*) locus in F1 101/SEG animals. Allele-specific amplification of transcripts was obtained by anchoring the PCR product within a 34 bp sequence occurring only once within the C3H/He- and 101-derived *Atp7a* alleles but as two copies in the *M. spretus*-derived SEG strain, *Mus*

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5' GGCAGGGATTTTAGCGATCTCCCCAGGTCCCTGGCGGC
   GGCAGGCATTTTAGTGATAGCCCAGGTCCCCGGT
   GGCAGGCATTTTAGCGATAGCCCAGGTCCCCGGT
   GGCAGGCATTTTAGCGATCTTCCAGATCCCCAGT
   GGCAGACATTTTAGTGATAGCCCAGGTCCCCGGT
   GGCAGGCATTTTAGTGATAGCCCAGGTCCCCGGT
   GGCAGGCATTTTAGCGAT.....3'
```

Fig. 1. The sequence of part of the 34 bp minisatellite repeat unit containing the HpaII sites whose methylation is altered depending on *Xce* haplotype. The repeats containing the first four *Hpa*II sites (indicated by arrows) are shown. The underlined sequence represents the repeat primer P2.

musculus musculus strains carrying the Xce^c allele and other feral strains (Cecchi & Avner, 1996). Sixty per cent of total *Atp7a* transcripts were found to be derived from the chromosome carrying the Xce^c type allele (i.e. SEG) and 40% from the Xce^a chromosome (101/H) (data not shown).

(ii) *Methylation patterns and* Xce

The degree of methylation of a region lying 15 kb distal to the 3′ end of *Xist* has been shown to vary in different *Xce* strains (Courtier *et al.*, 1995). The hypermethylation of this region, like that of the 5′ region of *Xist*, is specifically associated with the active rather than the inactive X chromosome. This differentially methylated region is composed of a *Sal*I site (referred to as Sal*) and a series of *Hpa*II sites lying within an imperfect 34 bp direct tandem repeat spanning 1·0–1·2 kb of genomic DNA almost immediately adjacent and distal to the Sal* site (Courtier *et al.*, 1995; Simmler *et al.*, 1996). The structure of part of this repeat is shown in Fig. 1.

We were interested in the possibility that the Pas34 repeat unit itself might be directly implicated in the *Xce* haplotype through alterations in the primary sequence. Allelic differences at the *Xce* locus affecting the choice of X chromosome to be inactivated have, for instance, been suggested to be due to the affinity of an activation molecule for multiple binding sites such as might be furnished by a repeat element (Gartler & Riggs, 1983). Of the nine inbred mouse strains that have been typed for their *Xce* allele by Bruce Cattanach and his colleagues (West & Chapman, 1978; Cattanach & Williams, 1972; Kay *et al.*, 1993), eight have been examined for their repeat structure and number using a PCR amplification assay based on that outlined by Jeffreys and his colleagues for

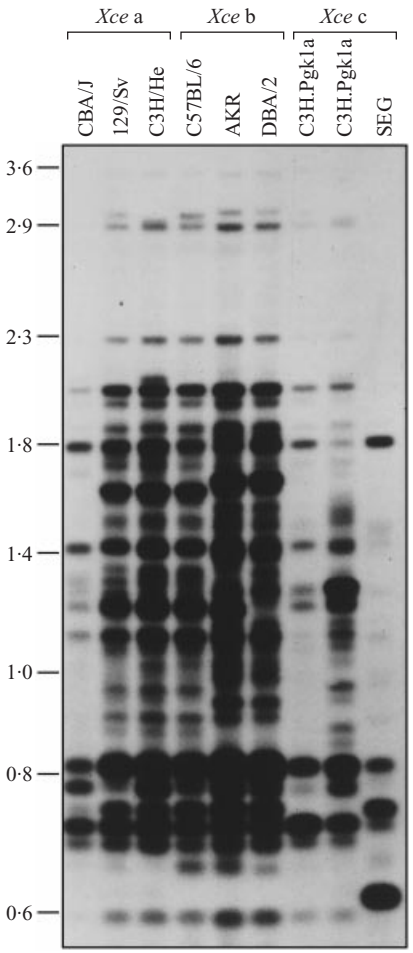

Fig. 2. PCR-based analysis of the minisatellite repeat array in various inbred strains carrying different alleles at the *Xce* locus detected using the P2 oligonucleotide as probe. PCR was carried out on male genomic DNA in each case. Product sizes are shown in kilobases.

minisatellite analysis (Jeffreys *et al.*, 1991, Tamaki *et al.*, 1993) (Fig. 2). Considerable variation between strains was seen in the total length of the repeat array, the degree of degeneracy between repeats and the internal organization of the array (Fig. 2). Whilst the Xce^c strains are highly divergent from the majority of Xce^a and Xce^b strains, there is no obvious consensus organization associated with any of the individual *Xce* haplotypes. Strains carrying the Xce^a haplotype, such as CBA/J, 129/Sv and C3H/He, can be seen to diverge in their internal organization close to the proximal end of the array (Fig. 2).

The original experiments involving the characterization of the methylation status of the *DXPas34* locus and *Xce* allelism were largely dependent on the use of congenic strains for the central part of the X chromosome which had been derived on the 129 background. The 129.Pgk1a congenic strain derived by crossing males of the C3H.Pgk1a mouse strain Xce^c (Johnston & Cattanach, 1981) to 129/Sv (Pgk1b

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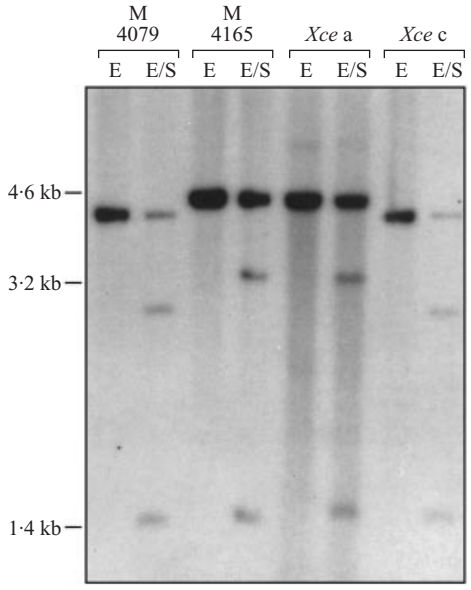

Fig. 3. Methylation patterns associated with the Sal* site as determined by *Eco*RI (E) and *Eco*RI/*Sal*I (E/S) digestion analysis in male mice recombinant for the *Tabby–Mottled* region and carrying different *Xce* alleles.

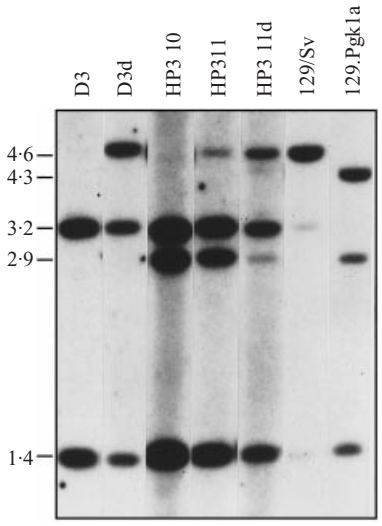

Fig. 4. Methylation patterns associated with the Sal* site in undifferentiated ES cell lines D3 (male), HP310 (female), HP311 (female) and their differentiated derivatives (D3d and HP311d). The 129/Sv and 129.Pgk1a lines are male spleen DNA controls. *Eco*RI/*Sal*I digestion was carried out in each case and the *DXPas34* probed used. The sizes indicated are in kilobases.

Xce^a) as the recurrent parent were selected at each generation for retention of the *Pgk1a* locus which is closely linked to the *Xce* locus. The differential segment carried by the 129/129.Pgk1a congenic pair was expected to extend both centromeric and telomeric to *Pgk1*, the differential locus. Whilst the distal breakpoint in the 129.Pgk1a strain was found to lie relatively close to the *Pgk1* locus (between the latter and *DXMit117*) the differential segment extends proximally to at least the *DXMit6* locus, lying centromeric to the *Tabby* locus. The differential segment in these congenics must therefore extend over at least 10 cM.

In order to address more precisely whether the variation in methylation pattern of the *DXPas34* region was correlated with allelism at the *Xce* locus, a series of DNAs from *Xce* recombinant animals that had been obtained from crosses involving parental strains of the Xce^a and Xce^c haplotypes were analysed. These animals were obtained in the framework of a much larger investigation of the *Xce* locus being carried out in collaboration with Bruce Cattanach and his colleagues. In each case the methylation pattern of the Sal* site (Fig. 3) was that predicted by the *Xce* haplotype carried by the recombinant. Detailed analysis of the recombination breakpoints carried by these and other recombinants should enable us to evaluate whether the *Xce* locus directly controls methylation at the *DXPas34* locus (M. Prissette, C. Rasberry and B. Cattanach, unpublished data).

The association between *Xce* haplotype and methylation status observed for the *DXPas34* locus led us to evaluate whether a similar association could be detected for other methylated sequences lying within the *Xic* candidate region. No variation in the methylation profiles of sites 3′ to the *Xpct* and *Bpx* genes, which are normally methylated on both the active and inactive X chromosomes, was found between *Xce* alleles (data not shown). Furthermore methylation of several other sites within the *DXPas34* locus but centromeric to the Sal*-34mer repeat region have previously been shown not to be subject to *Xce* modification (Courtier *et al*., 1995). Taken together these results suggests that *Xce*-associated methylation modification at the *DXPas34* locus is unlikely to be an indirect reflection of some chromosome-wide *Xce* effect.

To examine the developmental control of the hypermethylation of *DXPas34* on the active X chromosome we have exploited the ES cell system in an initial set of experiments. Female ES cells, in which both X chromosomes are active, are known to undergo X-inactivation upon *in vitro* differentiation (Rastan & Robertson, 1985) like that seen during early mammalian embryogenesis. The Sal* site in undifferentiated male and female ES cells was observed to be unmethylated (Fig. 4) but to become methylated on the active X chromosome after differentiation. The presence of an active X-specific methylation pattern on this region in both differentiated male and female cells and the absence of methylation for this region in undifferentiated ES cells suggests that such *Xce*-sensitive methylation precedes X-inactivation itself and may be associated with the choice of X chromosome to be inactivated (Courtier *et al*., 1995). Experiments involving early embryonic stages are

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currently underway in collaboration with Jörn Walter (MPIMG, Berlin) to establish the exact timing of the appearance and pattern of methylation at the *DXPas34* locus during mouse development.

It is worth noting that the hypermethylation of certain sequences on the active X chromosome and their hypomethylation on the inactive chromosome, as is seen for the *DXPas34* locus, is more highly conserved between mammals than is the inactive X-specific methylation associated with the 5′ ends of housekeeping genes (for references see Heard *et al*., 1997).

Skewing of X-inactivation is not restricted to the mouse and has been observed in the normal human population. Up to 20% of the female population without known X-linked disease appears to be subject to such skewing effects (Fey *et al*., 1994; Belmont, 1996), with approximately 10% of the population showing skewing as extreme as 90/10 (Naumova *et al*., 1996). Such studies rely mainly on the analysis of methylation patterns of polymorphic markers to distinguish the active from the inactive X chromosome. In some cases familial clustering suggestive of genetic determinism has been observed. Naumova *et al*. (1996) reported a very interesting three generation family in which both the paternal grandmother and all seven grand-daughters showed markedly skewed X-inactivation. Like some of the cases reported by Azofeifa *et al*. (1995) the direction of skewing could vary in successive generations. Skewing was associated with the inactive X in the grandmother but with the active X chromosome in all seven of the grand-daughters. The apparent absence of recombination events involving the centromere–Xq13 region in this family suggests that it is not involved in the skewing effect. Since, on the basis of the synteny between man and mouse, it is precisely this region that would be expected to contain a human homologue of the *Xce* locus, it seems unlikely that the skewing seen in this particular family involves a human homologue of *Xce*.

Overall such studies would suggest that primary skewing may be caused by a variety of different genetic elements, several of which may localize to the *Xic* region. The recent observation of apparent primary non-random inactivation associated with a deletion in the mouse *Xist* gene between exons 1 and 5 could represent one such case (Marahrens *et al*., 1998). The absolute skewing introduced by the deletion of a 65 kb region extending 3′ and distal to *Xist* exon 6 could represent yet another example (Clerc & Avner, 1998). Human families showing exceptionally high skewing ratios that have been correlated with changes in the *XIST* promoter region might represent a third such case (Plenge *et al*., 1997). In this perspective the mild skewing associated with the *Xce* locus would represent a series of naturally occurring polymorphisms at yet another element participating in the initiation of X inactivation. It may be interesting to speculate whether the more extreme forms of skewing that can be induced experimentally might be selected against in natural populations by the burden of increased penetrance of eventual X-linked mutations.

As the characterization of the *Xic* region in the mouse becomes more precise, and functional exploration of the initiation of X-inactivation reveals more clearly the mechanisms of action not only of *Xist* but of other elements implicated in the process, one may reasonably expect the secrets of the *Xce* locus to be unravelled. When this happens it will be yet another textbook example of the fundamental power of genetics for the understanding of complex biological processes. It will also provide yet another reminder of the incredibly productive energy that Bruce Cattanach has brought to a wide range of subjects over the course of his career. It has been both a pleasure and a privilege for our laboratory to be associated with Bruce and his colleagues over the past few years, in the fine mapping and molecular characterization of the *Xce* locus.

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