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Expression-based assay of an X-linked gene to examine effects of the X-controlling element (*Xce*) locus

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Through an incompletely understood process, one of the two X chromosomes (Chrs) in mammalian XX female cells is inactivated, thereby achieving dosage equivalence with XY males (Lyon 1961). Studies of X;autosome translocations and of chromosomally abnormal embryos suggest that *trans*-acting autosomal factors interact with *cis*-acting X-linked elements to "choose" one X to remain active; all additional X Chrs subsequently undergo X inactivation (Russell 1963; Lyon 1971; Rastan 1983). Despite the expected complexity of these events, thus far only two genetic loci, the X-controlling element (*Xce*) locus (Cattanach and Isaacson 1967) and the *Xist* gene (Brockdorff et al. 1991; Brown et al. 1991), are known to influence this process (reviewed in Brockdorff and Duthie 1998; Carrel and Willard 1998; Goto and Monk 1998; Panning and Jaenisch 1998).

The *Xce* locus influences the choice process such that there is an equal probability that either parental X Chr will be inactivated in *Xce* homozygotes, whereas one parental X is preferentially inactivated in *Xce* heterozygotes (Cattanach and Isaacson 1967). The relative strengths of the known *Xce* alleles are $Xce^a < Xce^b < Xce^c$, with an X carrying the stronger allele demonstrating an increased probability of being the active X (Cattanach et al. 1969; Cattanach and Williams 1972; Johnston and Cattanach 1981). *Xce* allele strength was originally defined and has primarily been assayed by counting vibrissae in mice heterozygous for an X-linked mutation, Tabby, which affects vibrissae number (Cattanach et al. 1969). Alternative assays have examined protein polymorphisms (West and Chapman 1978), chromosomal markers (Nesbitt and Gartler 1970; Kanda 1973), gene expression (Singer-Sam et al. 1992; Buzin et al. 1994; Avner et al. 1998), or DNA methylation patterns (Courtier et al. 1995; Avner et al. 1998) as a measure or correlate of *Xce* allele strength. Using these assays, the *Xce* alleles of several inbred strains have been reported as Xce^a (CBA/H, C3H/HeH, and BALB/cH), Xce^b (C57BL/6H and DBA/2H), and Xce^c-like (CAST/Ei) (Cattanach et al. 1969; West and Chapman 1978; Johnston and Cattanach 1981; Cattanach and Rasberry 1991, 1994). The AKR/H *Xce* allele has been reported to be intermediate between Xce^a and Xce^c (Fowlis et al. 1991), although rigorous *Xce* typing has not been performed in this strain.

Xist is a key gene in the X inactivation pathway. Both knockout and transgenic studies have shown that *Xist* expression, in the correct developmental context, is both necessary and sufficient for X inactivation (Lee et al. 1996; Penny et al. 1996; Herzing et al. 1997; Marahrens et al. 1997; Clerc and Avner 1998). While *Xist* itself may be the inactivation signal, additional developmentally regulated factors appear to be required for X inactivation (Panning et al. 1997; Sheardown et al. 1997; Clemson et al. 1998; Hansen et al. 1998; Tinker and Brown 1998). Mutations in or including the *Xist* gene cause preferential inactivation of one parental X Chr in both the mouse (Clerc and Avner 1998; Marahrens et al. 1998) and human (Plenge et al. 1997), implying that it is involved in the choice process (Carrel and Willard 1998). *Xce* appears to map close to, but downstream of, the *Xist* gene itself (Simmler et al. 1993). It is unclear how (or whether) the two loci interact to control the choice process, although recent work has focused on a series of differentially methylated repeated sequences downstream of the *Xist* gene as a possible candidate for *Xce* (Courtier et al. 1995; Avner et al. 1998). Further, an antisense transcript, termed *Tsix,* which initiates in this region and continues through the *Xist* locus on the opposite strand, has been proposed to be an antisense regulator of *Xist* (Lee and Lu 1999). Targeted deletions downstream of *Xist* suggest that this region is also involved in the choice process (Clerc and Avner 1998; Lee and Lu 1999).

As a necessary prelude to attempts to define further the role of these loci and to search for additional factors in the X inactivation pathway, a robust assay is needed to measure and quantify X inactivation patterns early in embryogenesis. One useful approach is to measure the proportion of cells expressing alleles from one or the other X Chr (i.e., the X Chr inactivation pattern) in adult somatic cells. Current assays, however, are limited by their inability to detect subtle differences in X inactivation patterns or by insufficient throughput necessary for large-scale screens (Cattanach and Isaacson 1967; Cattanach et al. 1969; Nesbitt and Gartler 1970; Cattanach and Williams 1972; Kanda 1973; West and Chapman 1978; Singer-Sam et al. 1992). Although several expression-based assays have been developed (e.g., Singer-Sam et al. 1992; Buzin et al. 1994; Avner et al. 1998), limited characterization of relative *Xce* allele strength has been performed at the level of transcription or RNA abundance.

To overcome these limitations and to further characterize the *Xce* locus, we have developed a quantitative assay that measures allele-specific RNA levels of a ubiquitously expressed gene subject to X Chr inactivation, *Pctk* (Carrel et al. 1996). Our data establish the *Pctk* X inactivation assay as a quantitative expression-based assay to measure X inactivation patterns in the mouse and provide the foundation for future experiments to search for additional X inactivation genes.

Pctk X inactivation assay. At position +1752 in the *Pctk* cDNA sequence, a single nucleotide polymorphism distinguishes the *Mus castaneus* inbred mouse strain, CAST/Ei, from six other inbred strains of laboratory mice (Carrel et al. 1996). The polymorphism is a "G" in CAST/Ei, creating a *Pvu*II restriction site and is an "A" in the other inbred strains. Primers to distinguish cDNA from DNA were designed to span the *Pvu*II restriction site and an intron: PctkF (5′-TCCATATTTGCACTAAAGGAGG-3′) and PctkR (5′-CAACAAGCAGGGAGGATTGC-3′). Total RNA was extracted from mouse tissues with TRIzol (GibcoBRL) according to manufacturer's specifications. Tissues were sampled from 2- to 3-week-old mice and placed immediately on dry ice. Approximately 2 μg of RNA was reverse transcribed in a 20-μl reaction containing

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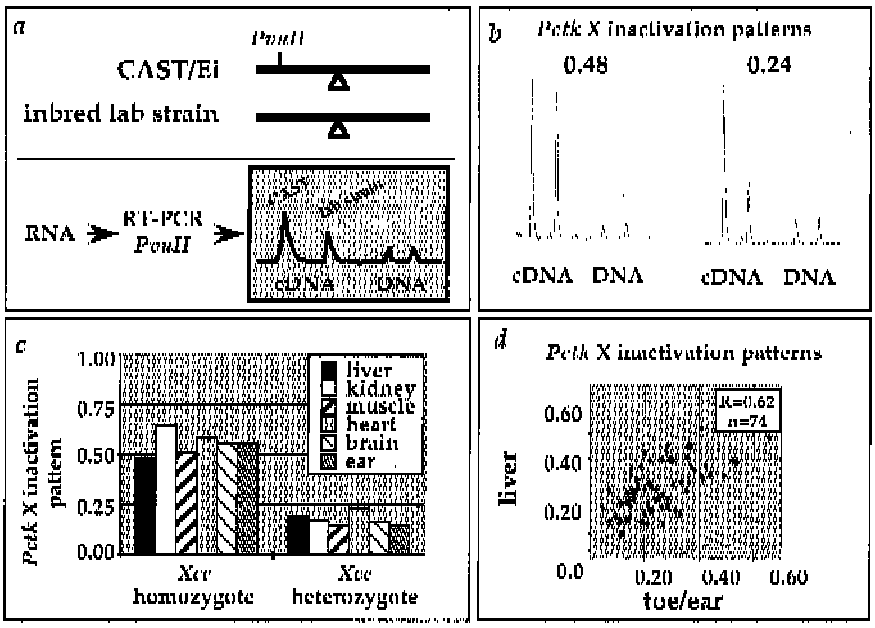

Fig. 1. The *Pctk* X inactivation assay. **a.** Upper panel: A single base pair change creates a *Pvu*II restriction site within the CAST/Ei *Pctk* gene (Carrel et al. 1996). This site is adjacent to an intron (triangle). Lower panel: RNA derived from mice heterozygous at *Pctk* is subject to RT-PCR followed by *Pvu*II digestion. The products are resolved on a polyacrylamide gel, and the relative intensity of the larger inbred lab strain allele (lab strain) is compared with the smaller CAST/Ei allele (CAST), thus generating an X inactivation pattern for a given mouse. **b.** Examples of the X inactivation patterns and traces from toe/ear (tissue from toe and ear were mixed together) for two mice homozygous ((BALB × CAST)F2) and heterozygous ((BALB × CAST)F1) at *Xce*. **c.** Examples of the X inactivation patterns from multiple tissues for two mice homozygous ((129 × CAST)G4) and heterozygous ((129 × CAST)G4) at *Xce*. **d.** Correlation of X inactivation patterns between liver and toe/ear for G4 female mice heterozygous at *Xce* (n = 74).

dNTP (100 μM each), 1× First Strand Buffer (GibcoBRL; 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 200 units M-MLV RT (GibcoBRL), and 20 units RNase Inhibitor (GibcoBRL). Reverse transcription was performed for 1.0 h at 37°C, followed by a 10-min inactivation step at 95°C. Amplification, with 1.5 μl of cDNA for the PCR reaction, was performed with unlabeled PctkF and PctkR primers in a 25-μl reaction volume [20 mM Tris (pH 8.4), 50 mM KCl, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.5 μM each primer, and 2.5 units *Taq* DNA Polymerase (GibcoBRL)] for 28 cycles (94°C for 15 s, 55°C for 10 s, and 72°C for 20 s). Following the 28th cycle, 5 μl of a fresh PCR cocktail containing the fluorescently labeled PctkR primer (1 μM final concentration) was added to the reaction, and a single primer extension step performed (94°C for 2 min, 55°C for 5 s, and 72°C for 7 min). As heteroduplex formation during PCR amplification interferes with restriction enzyme digestion, this final primer extension step is necessary to ensure complete digestion of the PCR products. Five microliters of a *Pvu*II digestion cocktail, containing 5 units *Pvu*II and 1× NEB #2 buffer, was added to the completed PCR reaction and incubated at 37°C for 1 h. A 1.5 μl aliquot of this reaction was run on a 6% polyacrylamide gel, and products were analyzed with ABI 672 Genescan software, based on relative peak heights of the two alleles. Samples were assayed in duplicate and were highly correlated (r = 0.98).

Because the *Pctk* gene is ubiquitously expressed and is subject to X inactivation (Carrel et al. 1996), the proportion of cells that express one *Pctk* allele relative to the proportion of cells that express the other *Pctk* allele should directly reflect the X inactivation pattern of a given mouse sample (Fig. 1a, b). Herein, all data are reported as the relative expression of the inbred lab strain *Pctk* allele; thus, 0.50 represents equal expression of both alleles, 0.05 represents 5% expression of the inbred lab strain *Pctk* allele, and 1.0 represents monoallelic expression of the inbred lab strain *Pctk* allele. To establish the parameters of the assay, DNA, RNA, or cDNA from CAST/Ei and 129/Sv was mixed across a range of relative template concentrations (0:100 to 100:0); linear and quantitative amplification was consistently observed (data not shown). The detection limit was determined to be 0.02, that is, the assay could detect as little as 2% expression of one or the other *Pctk* allele.

Previous assays showed that the X inactivation patterns among different tissues in the mouse are relatively consistent (Nesbitt 1971; Johnston and Cattanach 1981; Krietsch et al. 1986). To assess potential variability by use of the *Pctk* X inactivation assay, we measured the X inactivation patterns in six different tissues from mice either heterozygous or homozygous at *Xce* (as described below). Representative examples are shown in Fig. 1c. For the 18 animals assayed, there was approximately ten times more variation between animals than among tissues within a given animal (Fig. 1c, ANOVA $p = 10^{-7}$). Thus, X inactivation patterns from a given tissue appear to reflect X inactivation patterns in the entire mouse. The amount of sample variation between two tissue types within a given animal was also measured by the correlation coefficient (r) for animals heterozygous at *Xce* (Fig. 1d, r = 0.62, n = 74). These results are similar to those reported by others using other assays (Nesbitt 1971; Johnston and Cattanach 1981; Krietsch et al. 1986). Equivalent data were obtained when comparing different RNA preparations from the same tissue (r = 0.72, n = 13). From these results, we chose to determine the X inactivation patterns from a single tissue sample (toe/ear mixture) for the remainder of our study.

Intercrosses were performed to further validate the *Pctk* expression assay and to search in segregating progeny for novel genetic factors that influence the X inactivation choice process. Females from two inbred lab strains of the Xce^a genotype (C3H/HeJ and BALB/cByJ) and two of the Xce^b genotype (C57BL/6J and DBA/2J) were crossed to CAST/Ei males. F_1 animals were intercrossed to generate F_2 females, which were then genotyped with markers adjacent to *Pctk* (*Pctk* at 5.4 cM and *DXMit53* at 4.3 cM) and flanking the *Xist-Xce* critical region (*DXMit18* at 41.8 cM, *Xist-Xce* at 42.0 cM, and *DXMit97* at 49.0 cM) (Whitehead Institute for Biomedical Research and MIT Center for Genome Research, ⟨http://carbon.wi.mit.edu:8000/cgi-bin/mouse/index⟩). The distribution of *Pctk* X inactivation patterns from the four crosses is shown in Fig. 2a. Among F_1 *Xce* heterozygotes, the distribution of X inactivation patterns follows a trend consistent with the previously reported *Xce* allele strengths for these strains. Importantly, though, the *Pctk* assay provides a robust measure that expresses quantitatively the extent of X inactivation skewing seen in *Xce* heterozygotes.

F_2 females were compared to see whether the *Pctk* X inactivation assay could differentiate between *Xce* homozygotes and *Xce* heterozygotes. As shown for the (B6 × CAST)F_2's and (DBA × CAST)F_2's in Fig. 2b, there is a clear distinction between the distribution of X inactivation patterns of *Xce* homozygotes and *Xce*

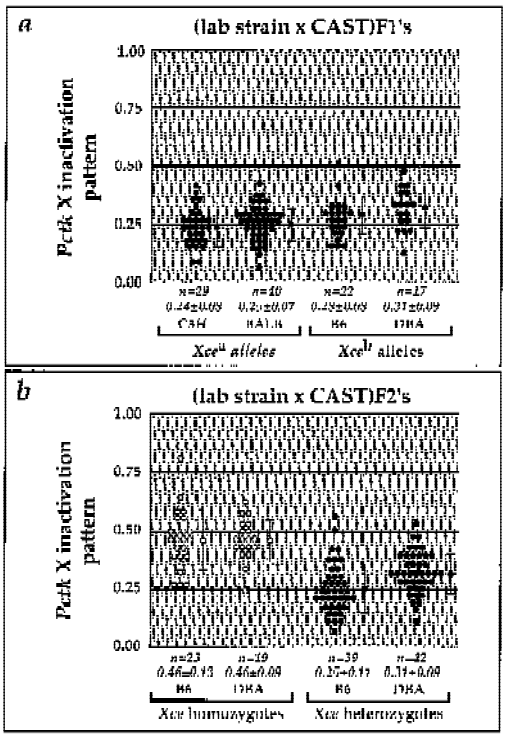

Fig. 2. Comparison of *Xce* alleles with the *Pctk* X inactivation assay. **a.** Distribution of *Pctk* X inactivation patterns from toe/ear samples for F1 (lab strain × CAST/Ei) females (mean ± S.D.). X inactivation patterns are expressed as the relative expression of the lab strain *Pctk* allele to the CAST/Ei *Pctk* allele. Each dot represents the X inactivation pattern for a given mouse. **b.** Distribution of *Pctk* X inactivation patterns from toe/ear samples for F2 *Xce* homozygotes and *Xce* heterozygotes. The *Xce* homozygotes demonstrate *Pctk* X inactivation patterns close to 50:50 (0.46 for both strains shown), while the *Xce* heterozygotes demonstrate marked skewing from a 50:50 mean (0.25 and 0.31). Abbreviations: C3H/HeJ (C3H); BALB/cByJ (BALB); C57BL/6J (B6); DBA/2J (DBA).

heterozygotes (*t* statistic −6.9 and −5.8, respectively; $p < 10^{-6}$). Similar results were obtained with two additional intercrosses (mean ± S.D.: (C3H × CAST)F_2, *Xce* homozygotes, 0.41 ± 0.20; n = 3 and *Xce* heterozygotes, 0.18 ± 0.07; n = 19; (BALB × CAST)F_2, *Xce* homozygotes, 0.46 ± 0.11; n = 9 and *Xce* heterozygotes, 0.21 ± 0.08; n = 26).

If genetic factors in addition to *Xce* influence the choice process, one would predict more variation to be present in the genetically heterogeneous F_2 female population than in the genetically identical F_1 population. To assess whether genetic variants other than *Xce* influence *Pctk* X inactivation patterns, we compared the means and variance of F_1 and F_2 animals heterozygous at *Xce* (Fig. 2a vs. 2b). For all four strains tested, the amount of variation observed in the F_2's compared with the F_1's was not statistically different [range of F-test statistic and *p* values: 0.53 and $p = 0.06$ (B6) to 0.97 and $p = 0.50$ (DBA)]. This suggests either that genetic loci affecting choice (other than *Xce*) are not variable between these mouse strains or that the effects are minimal and not detectable with our screen. The slight difference between the means of the F_1 and F_2 populations could be due to statistical fluctuation or to imprinting (Takagi and Sasaki 1975; Forrester and Ansell 1985; Fowlis et al. 1991; Bittner et al. 1997).

Because there does not appear to be additional genetic variation affecting *Pctk* X inactivation patterns within these strains, we generated a reference stock of mice with CAST/Ei alleles along the entire length of the X Chr. (CAST/Ei males are poor breeders, and introduction of non-*castaneus* alleles increases fecundity.) The (129 × CAST)G3 reference males were generated by backcrossing a (129/Sv × CAST/Ei)F_1 female to a CAST/Ei male. The N(2) females were backcrossed to a 129/Sv male, and G3 males were selected for CAST/Ei alleles along the entire length of the X Chr with markers from the MIT database (*DXMit53–DXMit75–DXMit18–Xist/Xce–DXMit97–DXMit152–DXMit184,* ⟨http://carbon.wi.mit.edu:8000/cgi-bin/mouse/index⟩). In total, six inbred lab strains were crossed to the G3 males, and the results are shown in Table 1.

Table 1. *Pctk* X inactivation patterns by use of reference males.

Xce alleles	Strain	Mean ± S.E.
Xce^a	C3H/HeJ	0.22 ± 0.01 ($n = 28$)
	BALB/cByJ	0.23 ± 0.02 ($n = 32$)
	CBA/J	0.24 ± 0.01 ($n = 39$)
Xce^b	C57BL/6J	0.29 ± 0.02 ($n = 28$)
	DBA/2J	0.33 ± 0.02 ($n = 27$)
$Xce^?$	AKR/J	0.37 ± 0.09 ($n = 17$)

The average X inactivation pattern of the Xce^a strains (mean ± S.E., 0.23 ± 0.01; n = 99) is statistically different from the average of the Xce^b strains (mean ± S.E., 0.31 ± 0.01; n = 55; $p = 2 \times 10^{-7}$). The *Xce* allele status of the AKR/H strain of mice has been reported to be intermediate between Xce^a and Xce^c (Fowlis et al. 1991); our results with the AKR/J strain suggest that it does not carry an Xce^a allele ($p = 10^{-7}$) and probably does not carry an Xce^b allele ($p = 0.04$). Thus AKR/J could carry either an Xce^c allele or an Xce^b-like allele. Direct comparison of the *Xce* alleles (for example, Xce^b vs Xce^c), as has been done previously with other assays, will most likely be necessary to further classify *Xce* allele strength with this assay. In paired comparisons, the mean X inactivation patterns of these *Xce* heterozygous G4 female mice were not statistically different from the F_1 intercross mice (all *p*-values > 0.24).

Several lines of evidence indicate that the *Pctk* expression differences reported here are due to X inactivation and do not reflect, for example, inter-strain allelic differences at *Pctk*. First, *Pctk* expression patterns are consistent among different tissue types within a given animal (Fig. 1). Second, F_2 females homozygous at *Xce* demonstrate mean *Pctk* X inactivation patterns that are close to 50:50 (Fig. 2b), a finding that is unexpected if, for example, *Pctk* levels differed characteristically between the strains tested here. Third, a striking difference is observed between *Xce* homozygotes and *Xce* heterozygotes (Fig. 2b). And fourth, we are able to differentiate between populations of mice (though admittedly not individual mice) carrying either an Xce^a or Xce^b allele (Table 1), again an unexpected finding if the expression differences are unrelated to X inactivation.

In conclusion, we have developed a quantitative assay to directly measure X inactivation patterns in the mouse and have characterized the *Xce* alleles of six inbred laboratory mouse strains relative to the inbred wild-derived mouse strain CAST/Ei at the level of *Pctk* transcription. Using this assay, we were able to easily detect differences between *Xce* homozygous and *Xce* heterozygous animals and between Xce^a and Xce^b alleles and the *Xce* allele carried by CAST/Ei. With an intercross, it does not appear that additional allelic variation within these strains affects the choice process. Thus, this *Pctk* X inactivation assay should be useful for future studies to model the choice process in the mouse with additional genetic screens.

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