Localization of the X inactivation centre on the human X chromosome in Xq13

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X-CHROMOSOME inactivation results in the strictly *cis*-limited inactivation of many but not all genes on one of the two X chromosomes during early development in somatic cells of mammalian females[1]. One feature of virtually all models of X inactivation is the existence of an X-inactivation centre (*XIC*) required in *cis* for inactivation to occur[2–5]. This concept predicts that all structurally abnormal X chromosomes capable of being inactivated have in common a defineable region of the X chromosome[6–8]. Here we report an analysis of several such rearranged human X chromosomes and define a minimal region of overlap. The results are consistent with models invoking a single *XIC* and provide a molecular foothold for cloning and analysing the *XIC* region. One of the markers that defines this region is the *XIST* gene[9], which is expressed specifically from inactive, but not active, X chromosomes. The localization of the *XIST* gene to the *XIC* region on the human X chromosome implicates *XIST* in some aspect of *X* inactivation.

Previous cytogenetic analyses of rearranged X chromosomes that are subject to inactivation have clearly ruled out a requirement for either the short arm or the distal long arm of the X chromosome in X inactivation, thus delimiting the possible location of the *XIC* to a region in the proximal long arm, which is present in all cases of structurally abnormal, inactive X's examined to date[6–8]. To extend these cytogenetic observations to the molecular level, we have isolated a number of X-chromosome rearrangements with breakpoints in the proximal long arm in rodent–human somatic cell hybrids. The abnormal X chromosomes used in this study, the somatic cell hybrids containing them, and their X-inactivation status[10–15] are shown in Fig. 1.

DNA from hybrid cell lines containing these structurally abnormal X chromosomes was analysed using probes for DNA markers previously known to map to the Xq11–Xq21 region[16]. As controls, all probes were hybridized to human, mouse, and hamster genomic DNAs, to DNA from a hybrid containing a normal human X chromosome as its only human component, and to DNA from a hybrid (A63-1A) containing essentially the entire long arm of the X (ref. 17; Fig. 2*a*). Results of these Southern blot hybridizations are shown in Fig. 2*b*. The region from the A63 breakpoint in Xq11 to below the 81 deletion breakpoint in Xq13 can be subdivided into five intervals, as shown in Fig. 2*a*. Considering all of the data, the order of loci in Xq13 is: DXS132–DXS227–*XIST*, DXS128–DXS171, DXS56, *PGK1*–DXS72 (Fig. 2*a*).

The localization of the *XIST* gene to this region was independently confirmed by *in situ* hybridization. The 8A11 *XIST* cDNA (see accompanying article[9]) was labelled with [^{3}H]dCTP and hybridized to human metaphase chromosomes. In 92 male metaphases examined, the only significant peak of grains observed (14% of the total and 50% of those on the X) was located in band Xq13, at the interface of bands Xq13 and q21.1 (data not

CELL LINE	KARYOTYPE	SOMATIC CELL HYBRID	PORTION OF X INACTIVATED	EVIDENCE FOR INACTIVATION	LOCATION OF *XIC*
68 (GM4628)	46,X,t(X;22)(q13;p11)	A68-2A	q13->qter	Late-replication[13]	distal to break
4 (GM0074)	47,Y,t(X;14)(q13;q32)+der(14)mat	W4-1A	q13->qter	Late-replication[16]	distal to break
81	46,X,del(X)(pter>q13:)	t81-az1b	pter->q13	Late-replication[17]	proximal to break
A.G.	45,X/46,X,idic(Xp)(pter->q13::q13->pter)	tAG-1Baz1b	pter->q13	Late-replication[18] Gene inactivation*	proximal to break

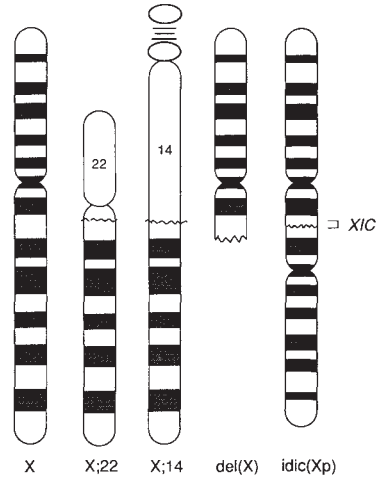
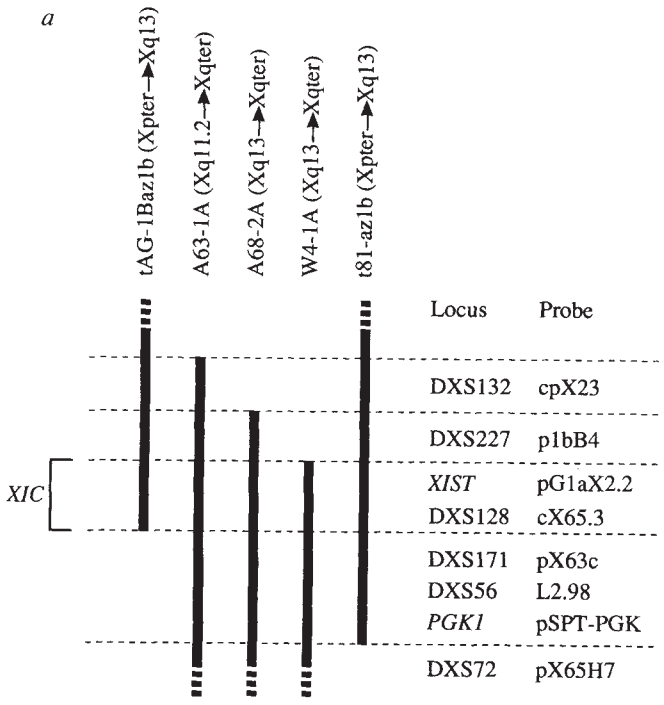


FIG. 1 Structurally abnormal human X chromosomes, each capable of being inactivated. For reference, a normal human X chromosome is also shown (X). For the X; 22 (case 68) and X; 14 (case 4) translocations involving band Xq13, the autosomal portion of the translocation chromosomes is indicated in white. The derivative X; 22 translocation chromosome, isolated in hybrid A68-2A, is from a female with a balanced translocation with the X breakpoint in proximal band Xq13. The subject has a daughter who inherited the der(22) chromosome (containing the X long arm material), but not the der(X) chromosome. In the daughter, the der(22) chromosome was observed to be late-replicating[10], the principal cytogenetic manifestation of X inactivation[11,12]. The derivative X; 14 chromosome was isolated from a male with both reciprocal products of a X; 14 translocation, as well as a second copy of the der(14), chromosome that is late-replicating and inactive[13]. The del(X), which is late-replicating, was observed in a female with a deletion of much of the long arm of one X (ref. 14). The idic(Xp) chromosome (Xpter→Xq13::Xq13→Xpter) was derived from a female patient (A.G.) with symptoms of Turner's syndrome[15]. This patient is mosaic for a 45, X cell line and a second cell line with the late-replicating isodicentric X chromosome[15]. Because the X inactivation centre must be located on all abnormal X chromosomes that are subject to inactivation, *XIC* maps distal to the breakpoints in the X; 22 and X; 14 translocations, but proximal to the breakpoints in the del(X) and the idic(Xp), as indicated to the right. Asterisk, *TIMP* and *POLA* are not expressed, see Fig. 3.



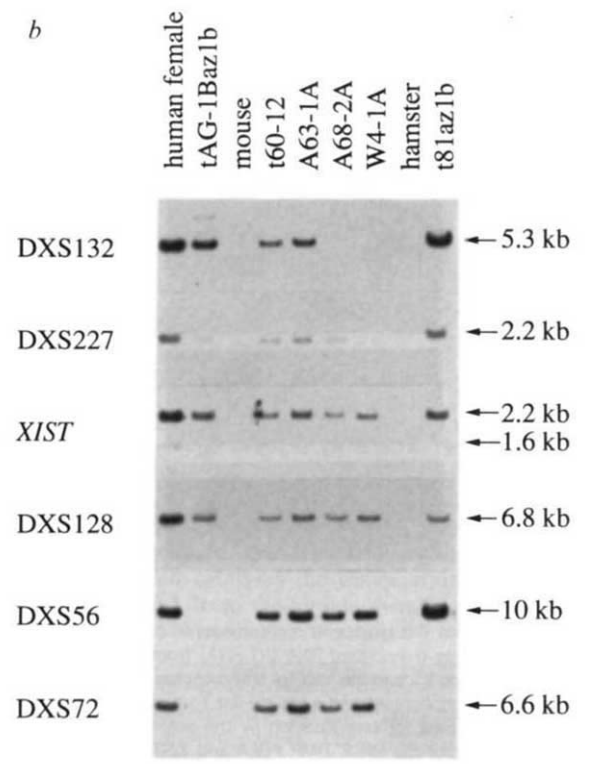


FIG. 2 Regional localization of DNA markers in the proximal long arm of the human X chromosome. *a*, Schematic illustration of the region of overlap in the structurally rearranged X chromosomes contained in hybrids indicated at the top. Locus and probe designations for the DNA markers tested are given at the right. The position of the *XIC* is indicated to the left. *b*, Representative results of Southern hybridization of probes from the Xq13 region to *Hin*dIII-digested DNA from control human, mouse and hamster lines and from somatic cell hybrids containing different portions of the X chromosome. Sizes of resulting bands are indicated at the right. Figure is a composite from six separate hybridization experiments.

METHODS. Mouse–human hybrids t60–12, A63-1A, A68-2A, and t81az1b and hamster–human hybrid W4-1A have been described[17–19,27]. Hybrid tAG-1Baz1b was formed by fusion of lymphoblasts from patient A.G.[15] and mouse cell line tsA1S9, as described[19,27]. The active X chromosome was removed by back-selection in medium containing 8-azaguanine. Presence of the inactive idic(Xp) and not the normal X was confirmed by extensive karyotyping (by standard methods) and by fluorescence *in situ* hybridization, using a specific alpha satellite DNA probe for the centromere of the X chromosome[32]. Southern hybridization with ^{32}P-labelled probes was performed by standard methods[18,19,22]. Full details on probes can be found in ref. 16. For the *XIST* gene, the pG1aX2.2 probe is a 2.2-kb *Xba*I genomic fragment at the 5′ end of the known transcribed sequences and was isolated from one of the genomic phages described in ref. 9.

shown). No significant signal was detected on the Y chromosome or on any of the autosomes, consistent with *XIST* being a single-copy locus.

By combining the map in Fig. 2*a* with the knowledge of which portion of the X in these cell lines is able to be inactivated, it is possible to identify the region containing the *XIC* (Fig. 1). Of the probes tested, only the *XIST* and DXS128 loci are common to all of the rearranged X chromosomes subject to inactivation. Thus, as predicted by the single *XIC* model of X inactivation, there is a single region within Xq13 which is present on all inactive X chromosomes studied. In conjunction with other mapping data using these hybrids (refs 16, 18, 19, and data not shown), the order of loci around the *XIC* on the human X chromosome is: centromere-*AR-CCG1-PHKA-XIC-PGK1*-telomere. As our data specifically demonstrate that the *XIC* region lies proximal to *PGK*1, a locus previously associated with *XIC* in humans or with the homologous *Xic* (or *Xce*) locus (loci) in mouse[20,21], this information should also help guide efforts to localize the murine X-inactivation centre.

The proximal limit for the *XIC* region is currently defined by the breakpoint in a X; 14 translocation, in which the der(14) chromosome, containing the Xq13→qter portion of the X chromosome, is inactivated[13] (Fig. 1). The distal limit for the *XIC* region is defined by the breakpoint in a late-replicating isodicentric X (abbreviated idic(Xp))[15]. This idic(Xp) chromosome does not contain most of the region of the long arm present in the inactivated der(14) chromosome (Fig. 1). Thus, providing that the idic(Xp) chromosome is inactive, *XIC* must lie within the small region of overlap in Xq13 between the der(14) and idic(Xp) chromosomes, a region defined by the loci DXS128 and *XIST* (Fig. 2).

To establish that this idic(Xp) chromosome is subject to inactivation, we isolated this chromosome (without the normal X present) in a mouse–human somatic cell hybrid for analysis of transcription of two X-linked genes known to be subject to inactivation[22,23]. As shown in Fig. 3, neither the *TIMP* gene nor the *POLA* gene is expressed in the idic(Xp) hybrid. These data demonstrate that the idic(Xp) chromosome is inactive and so contains the *XIC*. As shown in Fig. 2*b*, the *XIST* gene is present on this chromosome and, as expected for an inactive X chromosome, *XIST* is expressed in idic(Xp) hybrid cells (Fig. 3).

The concept of a single X-inactivation centre has long been a part of most X-inactivation models[2,3,24]. At least two key events in X inactivation have been proposed to involve the *XIC*. First, the *XIC* must be marked before inactivation as a means of distinguishing between which X remains active and which X becomes inactive[24,25]. Second, an inactivation signal which leads to the *cis*-limited transcriptional inactivation of genes located on either side of *XIC*, but which must be capable of 'skipping' over portions of the chromosome containing genes that escape inactivation[26–28], has been suggested to spread from this site[28–31]. The data reported here provide support for the existence of a

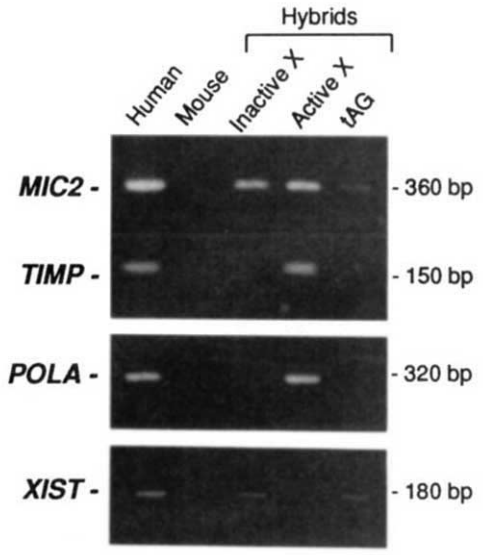


FIG. 3 Evidence that the isodicentric chromosome of A.G. is subject to X inactivation. Reverse-transcribed RNA (RT-RNA) from somatic cell hybrids containing an X_i, and X_a, and the idic(Xp) chromosome (tAG), as well as from a human female lymphoblast and a mouse line (the parental mouse line for the hybrids) was used for amplification by the polymerase chain reaction (PCR) with primers for the *MIC2*, *TIMP*, *POLA*, and *XIST* genes. The *TIMP* and *POLA* genes have previously been demonstrated to be subject to X inactivation, while the *MIC2* gene is known to escape X inactivation[22,23]. The *MIC2* primers amplified a 360-bp product for all RT-RNAs except for the mouse. The *TIMP* primers were used in a duplex reaction with the *MIC2* primers. They amplified a 150-bp product in the human RT-RNA and the X_a hybrid RT-RNA. The *POLA* primers also only amplified their 320-bp product in the female and X_a hybrid RT-RNA. The 180-bp *XIST* product was amplified from the female, the X_i hybrid, and the tAG hybrid. Therefore, the tAG hybrid behaved identically to the X_i hybrid.
METHODS. RT-PCR was performed as described[9,22], except that 10 times the input RT-RNA was used for the *POLA* primers. The *MIC2* and *TIMP* primers have been described[22]. The *POLA* primers are 5′-TGGCCATTTCATCACCCAGT-3′ and 5′-ACTGCCATACTGAAATACAT-3′ which 'amplify a predicted 320-bp product[33]. The *XIST* primers were 1→ and 2← as described in the accompanying article[9]. The X_a and X_i hybrids used were as described[9,22].

single *XIC* on the human X chromosome and, accordingly, should significantly refine efforts to clone and analyse this locus. That the *XIC* region is coincident (at the current level of mapping resolution) with the location of the *XIST* gene[9], whose expression pattern is specifically and uniquely affected by the inactivation status of the X chromosome on which it lies, strongly implicates *XIST* in some aspect of the X inactivation process. □

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The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the *Tme* locus

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T-ASSOCIATED maternal effect (*Tme*) is the only known maternal-effect mutation in the mouse[1,2]. The defect is nuclear-encoded[3] and embryos that inherit a deletion of the *Tme* locus from their mother die at day 15 of gestation[4]. There are many genomically imprinted regions known in the mouse genome[5,6], but so far no imprinted genes have been cloned. The *Tme* locus is absent in two chromosome-17 deletion mutants, T^{hp} and the t^{Lub2}, and its position has been localized using these deletions to a 1-cM region[7–10]. We report here that the genes for insulin-like growth factor type-2 receptor (Igf2r) and mitochondrial superoxide dismutase-2 (Sod-2) are absent from both deletions. Probes for these genes and for plasminogen (Plg) and T-complex peptide 1 (Tcp-1) were used in pulsed-field gel mapping to show that *Tme* must lie within a region of 800–1,100 kb. We also demonstrate that embryos express Igf2r only from the maternal chromosome, and that Tcp-1, Plg and Sod-2 are expressed from both chromosomes. Therefore Igf2r is imprinted and closely linked or identical to *Tme*.

The position of the T^{hp} and t^{Lub2} deletions relative to cloned DNA markers[1,8–10] is shown in Fig. 1. Of these marker loci, only *Tcp-1* is deleted in the t^{Lub2} chromosome, so the distance between the flanking marker loci *D17Rp17* and *D17Leh66D* gives the closest approximation of the limits of the region containing the *Tme* gene. Cumulative mapping data indicate that these markers are separated by at least one centimorgan[7].

Tcp-1 (ref. 11) and a gene from the *D17Leh66D* locus, *Tcp-10* (ref. 12), map in the human to chromosome 6q21–27 in close linkage to the plasminogen, insulin-like growth factor type-2 receptor and superoxide dismutase-2 loci[13]. These last three genes have been assigned to mouse chromosome 17 (refs 14–16) but not mapped with respect to the *Tme* locus. Figure 2 shows the mapping of these three genes in the t^{Lub2} deletion chromosome and the Tt^{Orl} chromosome that is presumed to contain a duplication of the *Tme* locus (see refs 10 and 17 for a description