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Expression of Genes from the Human Active and Inactive X Chromosomes

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Summary

X-chromosome inactivation results in the cis-limited inactivation of many, but not all, of the genes on one of the pair of X chromosomes in mammalian females. In addition to the genes from the pseudoautosomal region, which have long been anticipated to escape inactivation, genes from several other regions of the human X chromosome have now been shown to escape inactivation and to be expressed from both the active and inactive X chromosomes. The growing number of genes escaping inactivation emphasizes the need for a reliable system for assessing the inactivation status of X-linked genes. Since many features of the active or inactive X chromosome, including transcriptional activity, are maintained in rodent/human somatic-cell hybrids, such hybrids have been used to study the inactivation process and to determine the inactivation status of human X-linked genes. In order to assess the fidelity of inactivation status in such hybrids, we have examined the expression of 33 X-linked genes in eight mouse/human somatic-cell hybrids that contain either the human active (three hybrids) or inactive X (five hybrids) chromosome. Inactivation of nine of these genes had previously been demonstrated biochemically in human cells, and the expression of these genes only in hybrids retaining an active X, but not in those retaining an inactive X, confirms that expression in hybrids reflects expression in human cells. Although the majority of genes tested showed consistent patterns of expression among the active X hybrids or inactive X hybrids, surprisingly, 5 of the 33 genes showed heterogeneous expression among the hybrids, demonstrating a significantly higher rate of variability than previously reported for other genes in either human somatic cells or mouse/human somatic-cell hybrids. These data suggest that at least some X-linked genes may be under additional levels of epigenetic regulation not previously recognized and that somatic-cell hybrids may provide a useful approach for studying these chromosomal phenomena.

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Introduction

Early in the development of mammalian females, one of the pair of X chromosomes is inactivated, presumably to achieve dosage equivalence with males, who have only a single X chromosome. The initial hypothesis of X-chromosome inactivation (Lyon 1961; Beutler et al. 1962) was quickly followed by direct evidence for inactivation of the human glucose-6-phosphate dehydrogenase gene (*G6PD*), based on clonal expression in heterozygous females (Davidson et al. 1963). Subsequently, many human genes have been shown to be subject to X inactivation (reviewed in Willard 1995). However, although it had been widely accepted that all X-linked genes except those in the pseudoautosomal region would be subject to X inactivation, recent evidence has shown that there are many genes that "escape" X inactivation, being expressed from both the active X chromosome (Xa) and the inactive X chromosome (Xi). The majority of these genes have Y-chromosome homologues and appear to cluster together in regions of the short arm of the X chromosome. However, genes without Y-linked homologues and genes on the long arm of the X chromosome have also been shown to escape X inactivation (reviewed in Disteche 1995). Knowledge of whether a gene is expressed solely from the Xa is important for the clinical assessment of females heterozygous for X-linked diseases, and genes that escape X inactivation are candidates for being involved in the phenotype of Turner syndrome (Zinn et al. 1993). Furthermore, determination of which genes escape the inactivation process will help in the elucidation of the chromosomal mechanisms involved in X-chromosome inactivation.

Evidence for inactivation of a gene has been derived either from the observed mosaic expression of the gene product in heterozygous females or from equivalent expression of a gene product (RNA or protein) in individuals with different numbers of inactive X chromosomes. Although such direct evidence has been reported for only a few dozen X-linked genes, indirect evidence for inactivation has been reported for a large number of loci, on the basis of criteria such as (1) mosaic or variable expression of an X-linked disease phenotype in heterozygous females; (2) clonal selection, resulting in nonrandom inactivation of the X chromosome with the mutant allele; and (3) expression of the disease in females with

an X; autosome translocation disrupting the gene and accompanied by nonrandom inactivation of the intact X chromosome (reviewed in Brown and Willard 1993; Willard 1995). Such analyses require a detectable product, rare chromosomal rearrangement, expressed polymorphism, or careful dosage analyses and therefore are not generally applicable to the growing number of genes now being identified on the human X chromosome by molecular and genomic techniques. Furthermore, these indirect analyses can be misleading if there is decreased expression from the Xi (as observed for *STS* [Migeon et al. 1982*a*]), putative modifiers of expression (e.g., the *BGN* gene [Geerkens et al. 1995]), or pseudomosaicism (such as has been reported for *G6PD* [Papayannopoulou and Stamatoyannopoulos 1964]). As an alternative and/or complementary approach, the use of rodent/human somatic-cell hybrids that segregate the human Xa and Xi in a rodent background is a straightforward technique for systematically assessing the inactivation status of genes that are expressed in the hybrid cell line.

Expression studies have shown that hybrids retaining the human Xa or Xi reflect the activity of a limited number of X-linked genes in human cells (Migeon 1972; Migeon et al. 1974; Kahan and DeMars 1975; Graves and Gartler 1986). However, since a large number of newly isolated genes are now being assayed routinely by this approach—and since, often on the basis primarily or solely of the observed expression from the Xi in somatic cell hybrids, the number of genes described as escaping inactivation increases—it becomes important to address whether mouse/human somatic-cell hybrids accurately reflect the expression of a large number of X-linked genes in human cells. Therefore, we undertook a survey of expression of 33 X-linked genes in a series of somatic-cell hybrids, to evaluate the stability of X-linked gene expression in both Xa- and Xi-containing hybrids. For those genes for which there was prior evidence of inactivation status, the somatic cell–hybrid panel showed complete concordance with prior data. Overall, however, ~15% of the genes tested showed heterogeneous expression within the panel of hybrids, suggesting that gene activity in somatic cells or somatic-cell hybrids is more variable than generally believed, perhaps reflecting additional levels of epigenetic control of X-linked gene expression.

Material and Methods

Somatic-Cell Hybrids

The isolation and culture of the mouse/human somatic-cell hybrids retaining either the human Xa or the human Xi has been described elsewhere (Brown and Willard 1989; Willard et al. 1993). A panel of eight independent hybrids was used for this study, three containing an Xa and five containing an Xi. Two of the Xa-containing hybrids (t60-12 and AHA11aB1) and one of the Xi-containing hybrids (LT23-1E2Buv5Cl26-7A2) do not retain any other identified human chromosomes. The other four Xi-containing hybrids retain 1–12 human autosomes, with no autosome being common to all four (Willard et al. 1993). Hybrids A23-1aCl5 and LT23-1E2Buv5Cl26-7A2 were derived from fusion of mouse cells with the same human parental line, and, on the basis of analysis of polymorphic loci, the hybrid lines appear to retain the same X chromosome, in the active state and inactive state, respectively (Carrel et al. 1996). Although four of the Xi-containing hybrids were maintained under selective pressure for the Xi (Brown and Willard 1989), LT23-1E2Buv5Cl26-7A2 is the only hybrid for which there is no selection for retention of the active or inactive X chromosome. Cytogenetic analysis indicated that this hybrid retains an X chromosome in ~15%–20% of cells. Where studied, DNA methylation analysis, enzyme assays, and replication-timing studies were consistent with the active or inactive nature of the X chromosome in each hybrid (Brown and Willard 1989; Carrel and Willard 1996).

Reverse-Transcriptase–PCR Analysis (RT-PCR)

Cells were harvested, at confluence, with trypsin-EDTA, and RNA was prepared with RNAZOL (BiotecX), according to recommended procedures. The RNA was quantitated spectrophotometrically and was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), with random-hexamer priming, as described elsewhere (Brown et al. 1990). The primers used to amplify products for each gene are listed in table 1. Each primer pair was designed specifically to amplify human cDNA and to not amplify mouse cDNA. As controls, all primer pairs were checked for mouse cDNA amplification of the same size, and all cDNAs were demonstrated to be free of DNA contamination at the highest concentration of cDNA used by amplification of RNA without reverse transcription. Amplification generally consisted of 30–35 cycles at 94°C for 15 s, 54–55°C for 15 s, and 72°C for 40 s, in a Perkin Elmer 9600 thermocycler, with the following exceptions: *AR* and *FMR1* were amplified for 30 cycles of 94°C for 15 s, 58°C for 15 s, and 72°C for 40 s.

Results

Expression of the genes listed in table 1 was examined in the panel of three Xa- and five Xi-containing human/mouse somatic-cell hybrids, by RT-PCR amplification of cDNA. The complete results are summarized in table 2 and include several genes that have been analyzed elsewhere in only a subset of the full panel shown here (see references in table 1). In most instances, a series of fivefold cDNA dilutions from each of the hybrids was

amplified, and, for each gene from which expression was detected, amplification was observed in at least two dilutions. Individual hybrids were generally consistent in the number of dilutions for which amplification was detectable for a particular gene. However, as expected, expression levels varied significantly among the genes tested, presumably reflecting differences in their steady-state RNA levels.

The amplification products observed for several of these genes are shown in figure 1. In other studies, the *XE169* gene has been suggested to escape X inactivation, on the basis of expression from two Xi-containing hybrids (Agulnik et al. 1994; Wu et al. 1994). Consistent with those data, this gene is amplified from all hybrids containing either the Xa or the Xi in the current study (fig. 1). Similarly, primers for an expressed sequence tag (EST), WI-12682, amplified cDNA from all hybrids, whereas the primers for the *RP3, CCG1, DDP, OCRL, IDS,* and *G6PD* genes amplify only cDNA from those hybrids that retain an Xa. Among other controls, it should be noted that two other genes known to escape inactivation (*MIC2* and *RPS4X;* Goodfellow et al. 1984; Schneider-Gadicke et al. 1989) were expressed in all eight hybrids, whereas *XIST,* which is transcribed only from the Xi (e.g., see Brown et al. 1992), was expressed in the five Xi-containing hybrids but not in the three Xa-containing hybrids (table 2).

For 28 of the genes tested, expression in the hybrids was concordant, being present in all three Xa-containing hybrids (or absent in the case of *XIST*) and present (6 genes) or absent (22 genes) in all five Xi-containing hybrids. Thus, on the basis of this analysis, 22 genes were deemed to be subject to X inactivation, whereas 6 genes can be said to escape inactivation (table 2).

For the remaining five genes analyzed, amplification was not consistently present or absent among all of the Xi- or Xa-containing hybrids (fig. 2 and table 2). Four of these genes (*FMR1, TIMP1,* DXS423E, and *ALD*) were expressed in one or more, but not all, of the five Xi hybrids tested, whereas one gene (*AR*) was expressed in only two of the three Xa-containing hybrids.

Discussion

In order to gain insight into the organization of the human X chromosome, with respect to X inactivation, as well as to evaluate objectively the use of a somatic cell–hybrid system for studying X inactivation, we have examined the expression of 33 X-linked genes in a series of eight mouse/human somatic-cell hybrids, three containing different active X chromosomes and five containing different inactive X chromosomes. Although some of these genes have been examined elsewhere, by us or by others, in a limited number of hybrids, these analyses have been extended to a common set of hybrids, to more completely test the hypothesis that X chromosomes in somatic-cell hybrids faithfully retain the properties of an active or inactive X in female somatic cells.

X Inactivation in Somatic-Cell Hybrids

For nine of the genes examined here, there was prior evidence of X inactivation in human tissues. In other studies, the *PDHA1, AR, PGK1, HPRT, IDS, ALD,* and *G6PD* genes have been shown to be subject to X inactivation, by the demonstration of mosaic expression in heterozygous females (Davidson et al. 1963; Rosenbloom et al. 1967; Migeon et al. 1968, 1977; Gartler et al. 1972; Meyer et al. 1975; Capobianchi and Romeo 1976; Brown et al. 1989; Kirchgessner et al. 1995), whereas the analysis of expression of *FMR1, IDS,* and *OCRL* in individuals with X-chromosome rearrangements has shown these genes to be subject to X inactivation (Attree et al. 1992; Kirchgessner et al. 1995). With the few exceptions noted in table 2, all of these genes were also found to be expressed only from the Xa in the somatic cell–hybrid panel examined here. This confirms that, to a very substantial degree, expression in somatic-cell hybrids reflects expression in human somatic cells.

Of the additional genes examined that were subject to inactivation, nine had been demonstrated, by earlier studies, to be subject to inactivation, by the analysis of transcription or protein expression in a limited number of somatic-cell hybrids; these include the *PRPS1* and *PRPS2, POLA,* and *ANT2* genes examined by others (Wang et al. 1985, 1992; Scheibel et al. 1993), as well as the *ARAF1, TIMP1, ELK1, ZXDA/B,* and *XPCT* genes, which we elsewhere had analyzed in a subset of the hybrids analyzed here (Brown et al. 1990; Greig et al. 1993; Lafreniere et al. 1994; Carrel et al. 1996). On the basis of cDNA-dilution experiments performed for most of the genes in this study, for genes subject to X inactivation, expression from the Xi was $<5\%$ of that seen from the Xa, and, for many such genes, expression from the Xi was demonstrated to be $<0.2\%$ that of the Xa, confirming the transcriptional basis for X inactivation (Graves and Gartler 1986; Brown et al. 1990).

Notably, among the transcribed sequences that we have analyzed are three X-linked ESTs. Although definitive proof that these correspond to actual genes is currently lacking for most such ESTs, the large number of ESTs currently being described and mapped to chromosomes, as part of the human genome project (e.g., see Schuler et al. 1996), makes them an attractive source of potential genes for expanding these analyses to the level of the entire X chromosome. That some ESTs appear to escape inactivation whereas others are subject to inactivation (table 2; Miller et al. 1995) argues that assaying the status of their transcription from inactive X chromosomes is meaningful, notwithstanding their current state as unproved genes.

Table 1

Human X-Linked Genes Analyzed for X-Inactivation Status

Gene[a]	Gene Product/Disease	Primers	Reference	Product Size (bp)
MIC2	Cell-surface antigen	1: ACCCAGTGCTGGGGATGACT 2: TCTCCATGTCCACCTCCCCT	Darling et al. (1986)	360
PRPS2	Phosphoribosyl pyrophospate synthetase 2	A: CTCCGCCACCTCCTCCGC B: TGATGAGGAGTTCCATCAGG	Roessler et al. (1989)	270
PDHA1	Pyruvate dehydrogenase subunit; lactic acidosis	3: CGAATGGAATGGGAACGTCTGT 4: CGACTTCTCGTGTACGGTAACT	Dahl et al. (1987)	237
POLA	DNA polymerase alpha	1: AGATTAAACAGAGCAGGCTT 2: ACTGCCATACTGAAATACAT	Wong et al. (1988)	320
RP3	Retinitis pigmentosum 3	1: ACAACACCAAGCAAAGACATG 2: AGGGAGAATTTGAGATACACA	Meindle et al. (1996)	457
UBE1	Ubiquitin-activating enzyme subunit E1	1: GAGCGGGGACTTTGTCTCCT 7: CTTTGACCTGACTGACGAT	Carrel et al. (1996)	150
PCTK1	PCTAIRE-1; cdc2-related protein kinase	h4: CACGCCAACATCGTTACGCT h7: TGGGATTGACTTGGCTCGG	Carrel et al. (1996)	286
ARAF1	ARAF-1 proto-oncogene	B: TCAGCAAAATCTCCAGCAAC 3: TGGAGATGGAGGAGCTCCCA	Mark et al. (1986)	482
TIMP1	Tissue inhibitor of metalloproteinases 1	1: AGATCCAGCGCCCAGAGAGA 2: CCCTGATGACGAGGTCGGAA	Brown et al. (1990)	147[b]
ELK1	ELK1 proto-oncogene	A: GGACCTAGAGCTTCCACTCA B: AGAGCATGGATGGAGTGACC	Rao et al. (1989)	388
XE169/SMCX	X-linked homologue of H-Y antigen	1: ACCTGAGGAGCCTCCTAACT 2: CAGTCAACTGTGGCAACAGCG	Wu et al. (1994)	195
DXS423E	Anonymous member of condensation protein family	3A: AGGCATAGTGATGCTCCTGT 4A: CGATGTTTTTGAGATCTGTGC	Brown et al. (1995)	179
ZXDA/B	Zinc-finger proteins A and B	C: CTCTTACAAGCTCAAGAGGC D: ACATGAACCTCCGGTCATCG	Greig et al. (1993)	510
AR	Androgen receptor; androgen insensitivity; spinal bulbar muscular atrophy	1A: AGGAAAGCGACTTCACCGCA 1B: GAGCTCCATAGTGACACCCA	Tilley et al. (1989)	280
p54nrb	Nuclear RNA-binding protein	f: AGAGGCCGTGTAGCGTCG r: CTCCGCTAGGGTTCGGGT	Dong et al. (1993)	485
CCG1	P250 subunit of TATA factor TFIID	h1: ACCAAGTGGCGTTTTCTTTC h2: GAATAAGGTTTACATCATCC	Sekiguchi et al. (1991)	220
RPS4X	Ribosomal protein S4	1: AGCATCTGAAGCGGGTGGCA 2: AGCGGATGGTGCGGGCATCA	Wiles et al. (1987)	425
PHKA1	Phosphorylase kinase, alpha subunit	1: GGCCTGGCTGAGTGTTCATT 2: TTGCAGAAGTGTCATGGACT	Lafreniere et al. (1993)	600
XIST	Inactive X-specific transcripts	s1: CTCCAGATAGCTGGCAACC s2: AGCTCCTCGGACAGCTGTAA	Brown et al. (1992)	240
XPCT	PEST-containing transporter	A3.2: TGGTGCAACGGCTCCATCCT A5.2: GCCCAAACGGTCAGTGAATA	Lafreniere et al. (1994)	180
PGK1	Phosphoglycerate kinase; hemolytic anemia	1: TCGGCTCCCTCGTTGACCGA 2: AGCTGGGTTGGCACAGGCTT	Michelson et al. (1985)	395
PRPS1	Phosphoribosyl pyrophospate synthetase 1; X-linked gout; uric acid urolithiasis	A: CTCTGCAGCAGCCGTGAT B: CATGATCAAAAGCTCCATTAAA	Roessler et al. (1989)	270
WI-12682	EST	f: TACTAAGGATAATTCTGGTGGTCTG r: GATAAAGGTACATGTTCTGCATTCT	Schuler et al. (1996)	125
WI-6537	EST	f: AAGTTAAGTGAAATTTGCAGTTTT r: TATATGTTGGGGTTATGTTCAAATG	Schuler et al. (1996)	202
SGC33825	EST	f: CTTAAAGGTCATCAAATGCAAGC r: CCCTTACATTTCCAAATATGCC	Schuler et al. (1996)	139
DDP	Deafness-dystonia protein; Mohr-Tranebjaerg syndrome	r: AGCAAATCATCATATAGGAAAGG f: TTGATAGTGGGACCACATACG	Jin et al. (1996)	222
ANT2	ATP/ADP translocase 2	1: GGGTTGACTTCCTATCCATT 2: GCTTCCCATTTTCAACCAGT	Houldsworth and Attardi (1988)	353

(*continued*)

Table 1 (continued)

Gene[a]	Gene Product/Disease	Primers	Reference	Product Size (bp)
OCRL	Protein related to inositol polyphosphate-5-phosphatase; Lowe oculocerebrorenal syndrome	F: TCCTCAAACGACACGCAG R: AAGCCCTGAAAAAACAGAAGG	Attree et al. (1992)	549
HPRT	Lesch-Nyhan syndrome	C1: TCCTCCTGAGCAGTCAGC C2: GGCGATGTCAATAGGACTC	Jolly et al. (1983)	800
FMR1	Fragile X mental retardation	1: GGCGCTAGCAGGGCTGAA 2: CCGTAAGTCTTCTGGCACA	Kirchgessner et al. (1995)	437
IDS	Iduronate synthase; Hunter syndrome	1: GAGTTTTGCCAACCATGGAT 2: CGTATCCAAAGGTATGACAT	Wilson et al. (1990)	215
ALD	Adrenoleukodystrophy	F: CACACACACTTGCTACAGTTCG R: AAGGGTTTTCTAGGAGGAGGG	Mosser et al. (1993)	359
G6PD	Glucose-6-phosphase dehydrogenase; G6PD deficiency	C: GATGATGACCAAGAAGCCGG E: TTCTCCAGCTCAATCTGGTG	Perisco et al. (1986)	220

[a] Ordered pter-qter, on the basis of physical map positions from Nelson et al. (1995). Specific map positions are given in table 2.
[b] *TIMP1* primers described elsewhere (Brown et al. 1990) amplify a 325-bp product (Scheibel et al. 1993), whereas the primers listed here amplify the 147-bp product identified elsewhere (Brown et al. 1990).

Although, on the basis of the general agreement between results reported here and those from analysis of human tissue samples described elsewhere, the use of somatic-cell hybrids to determine X-inactivation patterns appears to be valid, one limitation of the hybrid approach is that only genes that are expressed in the hybrids can be analyzed. This limits the analysis to genes (or ESTs) expressed in fibroblasts, including all "housekeeping" or ubiquitously expressed genes but excluding many tissue-specific genes. However, the sensitivity of the RT-PCR approach allows the detection of a low level or "illegitimate" expression for tissue-specific genes in all tissues, as demonstrated for the expression of dystrophin in numerous tissues (Chelly et al. 1988). Since it has recently been demonstrated that such illegitimate expression of the dystrophin gene is also subject to X inactivation (Gardner et al. 1995), analysis of low-level expression by RT-PCR may provide a means to extend these analyses to tissue-specific genes or ESTs.

Heterogeneous Gene Expression in Some Hybrid Cell Lines

Five of the 33 genes examined showed heterogeneous expression among the Xa- or Xi-containing hybrids, complicating the assessment of inactivation status. For three of the genes (*AR,* DXS423E, and *FMR1*) a single hybrid, either one of three Xa-containing hybrids or one of five Xi-containing hybrids, is expressed differently from the other hybrids tested. Although it may appear parsimonious to make, on the basis of the remaining hybrids, conclusions regarding inactivation, unequivocal assessment of inactivation status may be impossible and, in fact, may be without meaning.

The number of discordant hybrids observed here seemed surprisingly high. Indeed, previous studies of the stability of X inactivation have shown that gene reactivation (e.g., the gain of expression from an Xi) is a very rare event in human cells (Migeon et al. 1982*b*). Although the frequency is higher in somatic-cell hybrids, localized reactivation events are still rare, being detected at frequencies generally $<1 \times 10^{-6}$ (Kahan and DeMars 1975, 1980; Hellkuhl and Grzeschik 1978). This frequency can be dramatically increased by treatment with demethylating agents such as 5-azadeoxycytidine (Mohandas et al. 1984; reviewed in Gartler and Goldman 1994), showing the importance of DNA methylation in repressing expression of genes from the Xi. Repression of genes by DNA methylation has been shown to be partially dependent on the CpG density of the promoter (Boyes and Bird 1992), and most studies of X-chromosome reactivation frequencies have been restricted to analysis of the *HPRT, PGK1, G6PD*, and *GLA* genes, all of which have extensive CpG islands and show concordant results in our hybrid panel. Correlation with the presence or absence of a CpG island is not complete, however, since the *ALD* gene, which is expressed in two Xi-containing hybrids, also has a large CpG island (Sarde et al. 1994).

Although numerous studies have examined the gain of expression from the Xi, little is known about the loss of expression. Extinction of gene expression in somatic-cell hybrids can often be correlated with the chromosome composition of the hybrid, since expression is influenced by the dosage of transcriptional regulatory factors (Peterson and Weiss 1972). Loss of gene expression could be due to mutation resulting in loss of the gene,

Table 2

Expression of 33 X-Linked Genes in Panel of Active X and Inactive X Hybrids

Map Position[a] (Mb)	Genes Expressed in[b]			Heterogeneous Results
	Active X Hybrids Only	Inactive X Hybrids Only	Active X and Inactive X Hybrids	
2			*MIC2*	
13	*PRPS2*			
20	*PDHA1*			
26	*POLA*			
40	*RP3*			
45			*UBE1*	
45			*PCTK1*	
47	*ARAF1*			
47				*TIMP1* (2/5 inactive X hybrids)
47	*ELK1*			
53			*XE169*	
54				DXS423E (4/5 inactive X hybrids)
58	*ZXDA/B*			
cen				
64				*AR* (2/3 active X hybrids)
70	*p54nrb*			
70	*CCG1*			
72			*RPS4X*	
72	*PHKA1*			
74		*XIST*		
74	*XPCT*			
78	*PGK1*			
. . .	*PRPS1*			
. . .			*WI-12682*	
. . .	*WI-6537*			
. . .	*SGC33825*			
100	*DDP*			
117	*ANT2*			
127	*OCRL*			
132	*HPRT*			
150				*FMR1* (1/5 inactive X hybrid)
153	*IDS*			
157				*ALD* (2/5 inactive X hybrids)
158	*G6PD*			

[a] Approximate physical location, based on a megabase scale from pter-qter (Nelson et al. 1995); loci without entries have been mapped between flanking loci but have not yet been placed on the megabase physical map.

[b] Based on RT-PCR results in a panel of three active X and five inactive X hybrids.

loss of promoter activity, or loss of the primer-annealing sites. For both the *AR* and DXS423E genes, multiple pairs of primers were shown to amplify products from genomic DNA of the hybrids that fail to express the gene, thus precluding either a large deletion of the gene or mutation of the primer-annealing sites. Given the relatively high frequency of heterogeneous gene expression in different hybrids, one hypothesis would be that the gain or loss of expression reflects epigenetic, rather than mutational, events. Whether such events are related to X inactivation itself is unknown and will require additional study, which should be facilitated by the somatic cell–hybrid system described here.

The observed discordancies were found in four of the eight hybrids examined, demonstrating that this phenomenon is not restricted to a particular hybrid, although it is perhaps noteworthy that three hybrids showed discordant expression for two different genes. Furthermore, the genes showing discordant expression are not clustered together, ruling out a regional effect. In fact, the DXS423E gene, which is not expressed in one Xi-containing hybrid, has been shown to be located $<\sim$200 kb from the *XE169* gene that is expressed in this hybrid. Similarly, the *ARAF1* gene maps $<$100 kb from the *TIMP1* gene (Derry and Barnard 1992; Coleman et al. 1994) and is not expressed

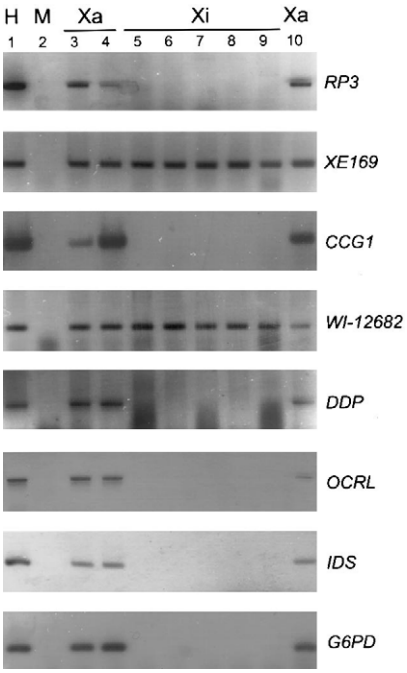

Figure 1 Expression of genes from active or inactive X-containing hybrids. cDNA from eight human/mouse somatic-cell hybrids retaining the human active X chromosome (Xa) or the human inactive X chromosome (Xi) was amplified with primers as listed in table 1. Shown is a negative image of ethidium bromide–stained products separated by agarose gel electrophoresis. From left to right, the 10 lanes refer to the following: human female, mouse tsA1S9az31b cell line, t60-12, AHA11aB1, t86-B1maz1b-3a, t11-4Aaz5, t48-1a-1Daz4a, t75-2maz34-4a, LT23-1E2Buv5Cl26-7A2, and A23-1aCl5. The primers amplify products for the genes listed to the right of each panel.

in either of the Xi-containing hybrids that express *TIMP1*.

The heterogeneous expression observed among hybrids may reflect events that occurred within the somatic-cell hybrids themselves or, alternatively, may reflect heterogeneity in the original human cells used to generate the hybrids. Although it is facile to conclude that gene reactivation has occurred in the cells in culture, since such reactivation is generally considered to be more frequent in hybrids than in diploid cells (Gartler and Goldman 1994), the frequency at which we observed discordant expression is much higher than that previously detected for reactivation of genes from the Xi, and it is thus important to consider whether the discordant expression detected here may reflect a different phenomenon. Although gene expression is generally considered to be stable and consistent from cell to cell within a cell population, there are in fact very few data that have addressed this question directly for human diploid cells. Indeed, among X-linked genes, such heterogeneity would only have been appreciated in studies designed to detect either *G6PD* heterodimers in samples from *G6PD* A/B heterozygotes (Migeon and Kennedy 1975; Migeon et al. 1982*b*) or rare HAT-resistant cells among clonal populations of cells from Lesch-Nyhan carriers with the normal *HPRT* allele on the inactive X (Migeon 1971; Migeon et al. 1988).

Thus, the heterogeneity of X-linked gene expression noted here among Xi-containing hybrids may reflect a more general phenomenon. The instability of expression of a subset of X-linked genes in human somatic cells would be of substantial biological importance, and analysis of allele-specific expression of these genes in the parental human cell lines will be required in order to address this possibility.

Genes That Escape X-Chromosome Inactivation

It is generally believed that most genes are subject to X inactivation. However, the recent description of a number of genes that escape inactivation (reviewed in Disteche 1995; also see fig. 3) raises the issues of (*a*) how common such escape from inactivation is and (*b*) what allows these genes to be expressed from the otherwise inactive X chromosome. For less than one half of the genes shown (in fig. 3) as escaping X inactivation is there evidence for expression from the Xi other than expression in somatic-cell hybrids. This includes both analysis of expression in heterozygous females and analysis of RNA in human cells with multiple X chromosomes (Race and Sanger 1975; Shapiro et al. 1979; Migeon et al. 1982*a;* Schneider-Gadicke et al. 1989; Fisher et al. 1990; Slim et al. 1993; Smith et al. 1993; Carrel et al. 1996). The relatively large number of genes described as escaping inactivation is partially reflective of

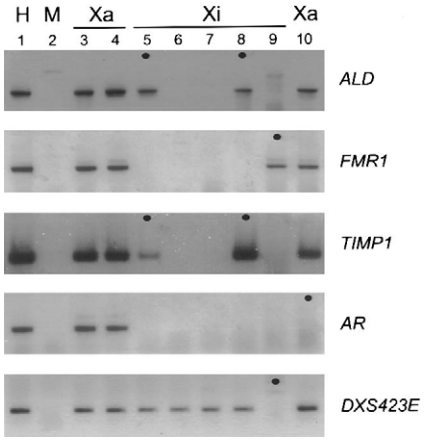

Figure 2 Heterogeneous expression for some genes in some active or inactive X-containing hybrids. cDNA from eight human/mouse somatic-cell hybrids retaining the human active X chromosome (Xa) or the human inactive X chromosome (Xi) was amplified with primers as listed in table 1. The lanes are the same as in figure 1. The primers amplify products for the genes listed to the right of each panel. Hybrids showing discordant results are indicated by a black dot.

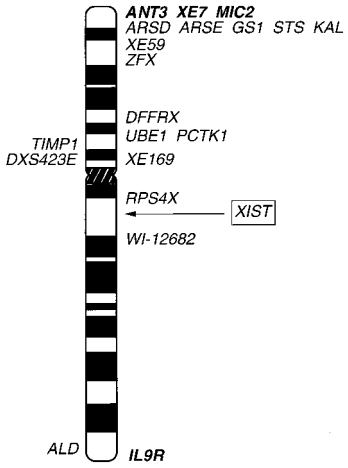

Figure 3 Summary of expression of genes from the inactive X chromosome. The genes listed on the right of the schematic chromosome have been demonstrated, in this and/or previous studies, to escape X inactivation, on the basis of consistent expression from multiple inactive X hybrids, whereas those on the left show expression in at least two, but not all, inactive X hybrids. Genes from the Xp/Yp or Xq/Yq pseudoautosomal region are indicated in boldface. Recent studies, by others, of some X-linked genes are described: the *DFFRX* gene, by Jones et al. (1996); the *ARSD* and *ARSE* genes, by Franco et al. (1995); and the *IL9R* gene, by Vermeesch et al. (1997). Other studies have been summarized by Disteche (1995). Cytogenetic locations are from Nelson et al. (1995) and L. Carrel (data not shown).

the extensive mapping and analysis of inactivation status within the pseudoautosomal and adjacent region and within other X-Y homologous regions, as well as of specific strategies to detect genes on the basis of their expression from the inactive X chromosome (Ellison et al. 1992). Nonetheless, although many of the genes analyzed in our studies were chosen because of prior knowledge about their inactivation status, $\geq$17 of the genes or ESTs were selected without any obvious bias in this respect. Notably, 4 of these 17 escape inactivation. Extrapolated to the entire chromosome (a step that may or may not be valid), this finding suggests that a significant proportion of all X-linked genes may escape inactivation. It is clear from this and other studies that many genes do escape inactivation, and therefore it seems no longer justified to assume a priori that any given gene is subject to inactivation.

The majority of the genes currently known to escape X inactivation are clustered in and around the pseudoautosomal region and in Xp11. The clustering observed may be indicative of X inactivation being a regional phenomenon, whereby entire blocks of genes are coordinately regulated (Willard et al. 1993; Miller et al. 1995; Carrel et al. 1996). The appeal of this hypothesis aside, there are several genes that escape inactivation but that have not been demonstrated to be part of a region of genes escaping inactivation. Notably, one of the ESTs studied here that escapes X inactivation maps distal to the X-inactivation center, one of very few genes that both map to the so-called ancestral X chromosome (Graves and Watson 1991) and escape inactivation (fig. 3). Although this may indicate that relatively few ancestral X-linked genes escape inactivation, it may in part also reflect a tendency to examine a larger number of Xp genes in this regard. Resolution of this question, as well as the issue of gene-specific versus regional control of X inactivation, will await determination of inactivation status of a greater number of genes that are more densely clustered along the length of the X chromosome, efforts that will clearly be aided by the Human Genome Project.

Genes that escape X inactivation may show patterns of expression that are quite different from the classical patterns of X-linked gene expression. X-linked inheritance is characterized by an excess of affected males and by no male-to-male transmission, with female carriers showing a range of expression from unaffected to completely affected, depending on the pattern of X inactivation. However, for XY-homologous genes, if a gene escaping inactivation shows equivalent expression in males and females (such as is the case for *RPS4X*), then females may be affected as frequently as males (depending on the relative mutation rates of the X- and Y-linked copies of the gene), since both males and females will be affected only by homozygous recessive mutations. For genes that are expressed from the Xi and that do not have a Y homologue, there will normally be more expression in females than males (in the absence of some other dosage-compensation mechanism). Unless this overexpression is required for normal development, heterozygous females will only very rarely manifest an X-linked disorder, which will be a true recessive trait. Last, characterization of genes that escape inactivation may permit their assessment as candidates for a role in determining phenotypic effects associated with X-chromosome aneuploidy (Zinn et al. 1993; Willard 1995).

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