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Stage-Specific Transcription of Distinct Repertoires of a Multigene Family During *Plasmodium* Life Cycle

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Members of a multigene family in the rodent malaria parasite *Plasmodium yoelii yoelii* code for 235-kilodalton proteins (Py235) that are located in the merozoite apical complex, are implicated in virulence, and may determine red blood cell specificity. We show that distinct subsets of *py235* genes are expressed in sporozoites and hepatic and erythrocytic stages. Antibodies to Py235 inhibited sporozoite invasion of hepatocytes. The switch in expression profile occurred immediately after transition from one stage to another. The results suggest that this differential expression is driven by strong biological requirements and provide evidence that hepatic and erythrocytic merozoites differ.

Invasive stages (oökinete, sporozoite, and merozoite) of the malaria parasite penetrate specific host cell types at different stages of the life cycle. The 235-kD rhoptry proteins (Py235) of the rodent parasite *Plasmodium yoelii yoelii* are implicated in the type of erythrocyte (normocyte or reticulocyte) invaded by merozoites and in parasite virulence (*1*–*3*). There are ~35 copies of *py235* genes in the parasite genome. Analysis of the transcription pattern of *py235* in blood stages has revealed a mechanism of clonal phenotypic variation (*4*): Merozoites from a single infected erythrocyte differ with respect to Py235 in their rhoptries, suggesting a unique survival strategy (*4*, *5*). Homologs of Py235 are found in other malaria species (*6*–*13*), and antibodies to both Py235 and a *P. falciparum* homolog inhibit merozoite invasion (*1*, *2*, *13*). We investigated the transcription pattern of *py235* during the different stages of the parasite's life cycle and the effect of specific antibodies on cell invasion.

We used a panel of antibodies specific to Py235 to establish that Py235 proteins are found in sporozoites and infected hepatocytes. A 235-kD protein was detected in extracts of sporozoites (Fig. 1A). By immunofluorescence, staining was only obtained with the pAb-S6 and pAb-F sera, indicating that Py235 proteins in pre-erythrocytic stages differ from those in erythrocytic parasites. All sporozoites were labeled, with diffuse staining outlining each cell and regions of more intense label at both ends (Fig. 1B). Infected hepatocytes were labeled with a patchy pattern that may correspond to developing merozoites (Fig. 1C). Evidence that sporozoite Py235 proteins have a functional role was obtained from antibody inhibition of sporozoite invasion of cultured primary hepatocytes (*14*). A Py235-specific antibody reactive with the proteins in sporozoites inhibited invasion, but an antibody to Py235 expressed only during blood stages did not inhibit invasion (Fig. 1D).

We analyzed *py235* transcripts using nested reverse transcription–polymerase chain reaction (RT-PCR). The size-polymorphic 3′-end of *py235* (*4*, *15*) (Fig. 2A) was amplified with RNA purified from 10 to 100 oocysts (found on a single midgut) or from 10,000 to 100,000 salivary gland sporozoites (265BY line) (Fig. 2B). A single-sized fragment was consistently amplified from early (5-day) and mature (10-day) oocysts (Fig. 2B, + lanes) and from different batches of salivary gland sporozoites (Fig. 2B, + lanes); in contrast, multiple-sized products were obtained with RNA purified from an equivalent number of erythrocytic parasites (Fig. 2C). Sequencing of about 200 different cloned fragments derived from at least three independent RT-PCRs showed that these single products all had the same sequence (Fig. 2D, type IIb). A single band was also consistently amplified from RNA extracted from liver-stage parasites grown in vitro or in vivo (Fig. 2C), and sequence analysis of about 100 cloned products also showed that they had the identical sequence. No transcript was detected in very early hepatic trophozoites (in liver biopsies 3 hours after sporozoite inoculation), indicating that the sporozoite *py235* mRNA is degraded very soon after hepatocyte invasion.

Although multiple *py235* genes are transcribed in the erythrocytic stages (*4*, *15*), it is not known how soon this pattern is established after initiation of blood infection by hepatic merozoites. Therefore, we prepared RNA from blood samples of sporozoite-in-

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fected mice, collected at closely spaced intervals before and after maturation of the liver schizonts. As expected, no RT-PCR products were obtained from the samples obtained 24 and 44 hours after sporozoite inoculation, because no merozoites would be released into the blood before 45 hours. Multiple transcripts were detected at 66 hours (Fig. 2C), corresponding to the first erythrocytic schizogony.

The analysis was extended to two additional cloned lines, YM and 1.1, derived independently from the 17X isolate. For clone 1.1, the pattern of *py235* transcription was identical to that in the 265BY line (*16*). For the YM clone, transcription of two different single genes in the pre-erythrocytic parasites was suggested by the size of the RT-PCR products (Fig. 3A). Sequencing established that a single type of 3′-end variant is present in each of these stages; types IIb and I were expressed in the sporozoite and the hepatic parasite, respectively (Fig. 2B). Multiple transcripts were detected during the first erythrocytic schizogony of YM parasites (*16*). Although no differences could be detected in the size of the RT-PCR products from the 3′ ends of transcribed *py235* in the 1.1 and 265BY parasites, different *py235* genes can share the same 3′-end repeat sequence (*17*, *18*), and the repertoire of *py235* genes differs among parasite lines (*15*). Therefore, to refine the RT-PCR analysis, we used a variable region, vr (Fig. 2A), found at the 5′ end of the *py235* genes (*17*). The products were of the expected size [264 or 267 base pairs (bp)] (Fig. 3B) from the different stages of YM parasites, and restriction fragment length polymorphism (RFLP) Southern blot analysis (Fig. 3C) of the individual products demonstrated a specific subset of the *py235* family expressed at each stage. Sequencing of approximately 100 cloned vr regions derived from at least three independent RT-PCR reactions per stage showed that *py235* genes transcribed in the sporozoite and hepatic forms differed from those transcribed in the erythrocytic parasite (Fig. 3D). For the sporozoite, two vr types (vr1 and vr3) were detected equally, whereas for hepatic schizonts the vr3 type predominated and the vr2 type was observed once. Whether or not Py235 expression at these stages undergoes phenotypic variation (*4*) could not be determined, because it could not be established that sporozoites or hepatic merozoite progeny were derived from a single oocyst or hepatic schizont, respectively. Two vr types (vr4 and vr5) were detected in blood-stage parasites. All the vr sequences detected by RT-PCR, except vr2 and vr3, were also found in fragments amplified from genomic DNA, and two other (vr6 and vr7) were detected by direct amplification of genomic DNA but not in any of the RT-PCR products. It is therefore unlikely that the different vr sequences detected in the RNA arose as a result of RNA editing.

Our results showed that a distinct subset of *py235* genes was expressed at each of the following stages: sporozoite, hepatic schizont, and erythrocytic schizont. The expression of the *py235* family was reset for each of the three invasive forms of the parasite. Invariably, the same *py235* repertoire was detected in samples from independent experimental infections with a cloned parasite line. Additional preliminary analysis of the vr region from RNA obtained from the uncloned 265BY parasite was consistent with the conclusion that nonoverlapping sets of *py235* genes are expressed during the three developmental stages studied. All together, these observations demonstrate that differential transcription of *py235* is a general feature in *P. y. yoelii*.

Little is known about the mechanisms regulating the transcription of multigene families in *Plasmodium*. The two or three different ribosomal RNA genes of malaria parasites (depending on species) are differentially expressed in the insect and vertebrate stages (*19*); however, expression of the insect stage–specific type begins in the vertebrate host, and this rRNA can still be detected in the newly invaded hepatocyte of the next cycle. Of the extensive multigene families coding for antigenically variant proteins, only the *var* gene family of *P. falciparum* has been investigated in detail (*20*–*22*). PfEMP1, the *var* gene product, is only detected in erythrocytic-stage asexual and sexual parasites, and the pattern of *var* transcription differs from that of *py235* (*4*). The expression of the *py235* family differs in two major respects from that of the trypanosome variant surface glycoprotein genes (*23*, *24*), because the *py235* repertoire expressed at a given stage is immediately switched off at transit to the next stage; and, in blood-stage parasites, expression from the *py235* repertoire does not appear to be sequential, with several transcripts observed in individual multinucleate parasites (*4*). The tightly regulated stage-specific expression of different subsets of *py235* may thus be a newly discovered type of transcriptional regulation in protozoa.

The demonstration of a distinct set of merozoite rhoptry protein genes expressed only in hepatic schizonts is molecular evidence for a difference between hepatic and erythrocytic merozoites. The activation or repression of *py235* expression appears to be mediated by the cellular environment of the parasite. However, the biological require-

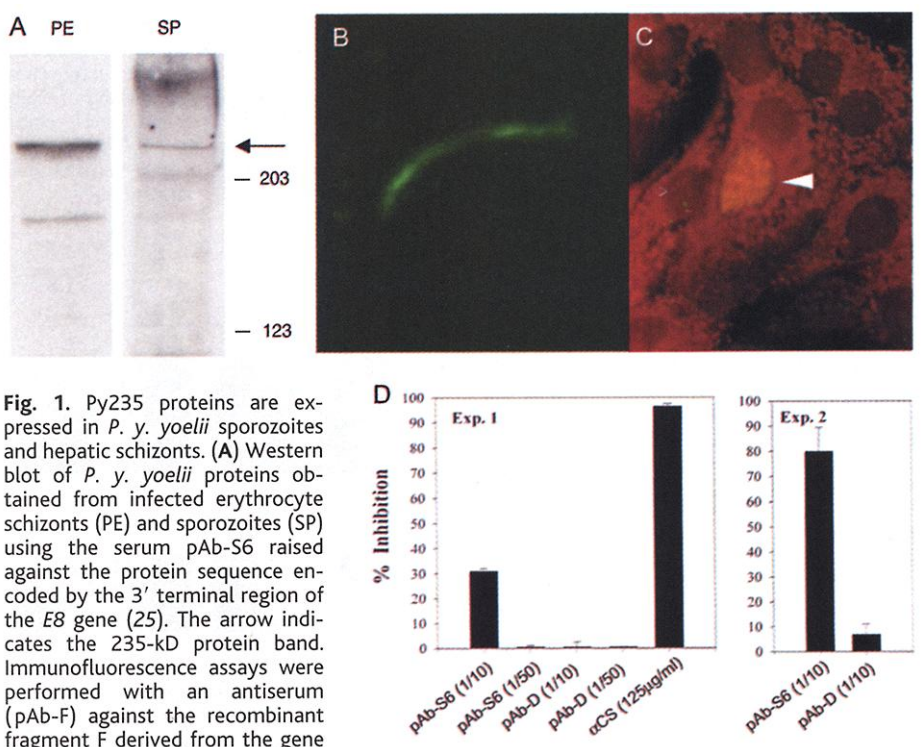
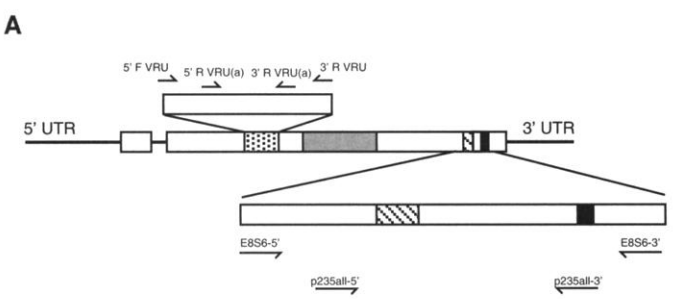
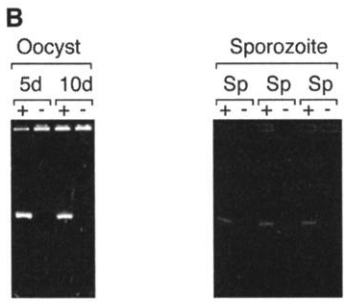
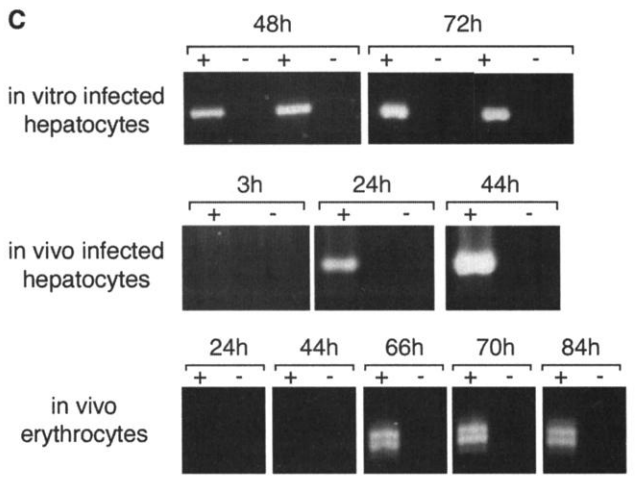


Fig. 1. Py235 proteins are expressed in *P. y. yoelii* sporozoites and hepatic schizonts. (**A**) Western blot of *P. y. yoelii* proteins obtained from infected erythrocyte schizonts (PE) and sporozoites (SP) using the serum pAb-S6 raised against the protein sequence encoded by the 3′ terminal region of the *E8* gene (*25*). The arrow indicates the 235-kD protein band. Immunofluorescence assays were performed with an antiserum (pAb-F) against the recombinant fragment F derived from the gene *E8* (*26*). Immunoreactivity with (**B**) air-dried and methanol-fixed sporozoites (×1000 magnification) and (**C**) methanol-fixed 48-hour liver-stage schizonts (×750 magnification) is shown (*27*). Identical results were obtained with the serum pAb-S6. The arrowhead indicates a liver schizont. (**D**) Sporozoite invasion inhibition assays (*14*) on two occasions (Exp. 1 and Exp. 2) with two different preparations of polyclonal antisera pAb-S6 (with different levels of Py235 antibody titers), pAb-D (*26*, *28*), or a monoclonal antibody (αCS) specific to circumsporozoite protein. The full range of Py235-specific antibodies tested is presented in (*29*).





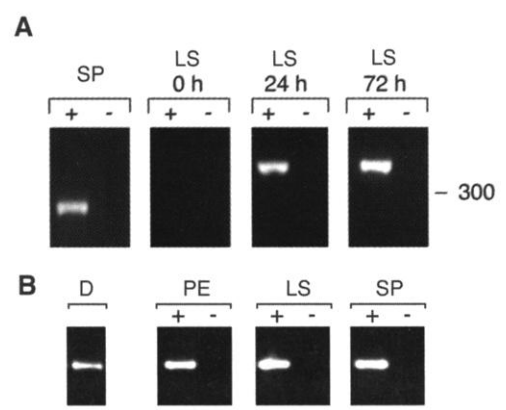


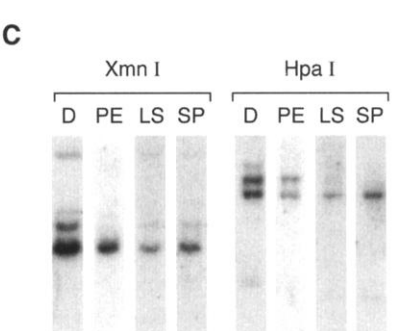
D

3' Repeat types

								YM
DIN	DIN	DVI	DIN	DIN	DTN	DIN	I	PE/LS
DIN	DIN		DIN	DIN	DTN	DIN	IIa	PE
DIN	DIN			DIN	DTN	DIN	IIb	SP
DIN	DIN	DVN		DIN	DTN	DIN	IIIa	PE
	DIN	DVN		DIN	DTN	DIN	IIIb	-
DIN				DIN	ETN	DIN	IV	-
DIN					ETN	DIN	V	+
		AVN			DTN	NIN	VI	+

Fig. 2. Expression of a single 3′-end repeat type in the mosquito and hepatic stages of *P. y. yoelii* and multiple types in blood stages. (**A**) Schematic structure of *py235*. The gray area indicates a region that has significant homology with the reticulocyte-binding protein of *P. vivax* (*30*). The black box indicates the transmembrane domain, and the cross-hatched area shows the repeat region, which was amplified with the nested primers E8S6-5′/E8S6-3′ and p235all-5′/p235all-3′. vr (*17*) (stippled area), was amplified with the nested primers 5′F-VRU/3′R-VRU and 5′F-VRU(a)/3′R-VRU(a). UTR indicates 5′ and 3′ untranslated regions. (**B**) Nested RT-PCR 3′-end repeat region products obtained from 5-day (5d) and 10-day (10d) oocysts and from salivary gland sporozoites (SP) (*29*). RT reaction (*4*) was performed in the presence (+) or absence (−) of reverse transcriptase (*29*). Products were cloned and sequenced as previously described (*15*). (**C**) In vitro infected hepatocytes panel: RT-PCR products from in vitro infected hepatocytes 48 (48h) and 72 (72h) hours after sporozoite infection (*29*). Complete maturation of the parasite in this in vitro system takes 72 hours (*31*), whereas this phase is completed in 45 hours in vivo (*32*). In vivo infected hepatocytes panel: RT-PCR products from in vivo infected hepatocytes 3 (3h), 24 (24h), and 44 (44h) hours after sporozoite infection. Five- to 8-week-old female BALB/c mice (Harlan Laboratories, Orléans, France) were injected intravenously with 20,000 sporozoites. At different times after inoculation, liver biopsies were removed from three infected mice and immediately immersed in lysis buffer for further RNA extraction. In vivo erythrocytes panel: RT-PCR products from circulating erythrocytes taken at 24 to 84 (24h to 84h) hours after sporozoite infection. Blood was obtained from groups of three similarly infected mice at different times after sporozoite inoculation and processed for RNA extraction. Blood-stage parasitemia was ascertained by examination of Giemsa-stained blood smears. We also indicate the RT reaction in the presence (+) or absence (−) of reverse transcriptase. (**D**) A list of the translations of the 3′-end repeats identified at the time of manuscript preparation, indicating those detected in the cloned parasite line YM. PE, SP, and LS refer to transcripts detected in erythrocytic parasites, sporozoites, and hepatic parasites, respectively. (+) indicates the presence of *py235* in the genome but not its detection as a transcript, and (−) indicates *py235* not being detected at either the DNA or RNA levels in this parasite line.





D

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YTDKIKKINDDIMAVSQQIDQHINGLDDIQKKSESYVSEMKEQIDKLEKVSDTEISNDNVEGIEKKQQIIVTK  Majority
          10        20        30        40        50        60        70
YANEVNKIKDDIKTVSQQIDHHINKLEDIKKKSKSYVGEMKEQIDKLEKVPDTAISNDTVEGIEKKHKIIVTQ  VR1  SP/D
YNNEVNKIKDDIKNVSQQIDHHINELEEIKKKSENYVDEMKAQINKLEKVADTAISNDNVEGIEKKQQNIVTK  VR2  LS
YTDKIKKINNDIMAVSQQIDQHINGLDDIQKKSESYVSEMKEHIDKLEKVSDTEISNDNVEGIEKKQQIVVKK  VR3  LS/SP
YTDKIKKINDDIMAVSQQIDQHINGLDDIQKKSESYVSEMKEQINKLEKVSNTEISNDNVEGIKKKQQIIVTK  VR4  PE/D
YTDKIKKINDDIMAVSQQIDQHINGLDDIEKKSESYVSEMKEHINKLEKVSKTEISNDNVEGIKKKQQHIVTK  VR5  PE/D
YTDKIKKINDDIMAVSQQIDQHINGLDDIQKKSESYVSELKEQINNLEKVSNTELSYGYVEGIKKKQQFLVTN  VR6  D
YINEI-KIKNDINTLDQKIDKSIETLTKIKKTSESHIGEIKGQIDKLEKVADINTYNEDPKEIEKKIENVVKK  VR7  D
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Fig. 3. Unique expression of multiple *py235* genes at different stages of parasite development. (**A**) RT-PCR analysis of the 3′-end repeat region from the different stages of the cloned parasite line *P. y. yoelii* (YM). Salivary gland sporozoites (SP) as well as uninfected 0- (0h), 24- (24h) and 72- (72h) hour-old in vitro infected hepatocytes (LS) are shown. The location of the 300-bp marker is indicated. (**B**) PCR and RT-PCR of the vr region (*29*) using genomic DNA (*33*) (D) or RNA obtained from hepatic stages (LS), sporozoites (SP), or erythrocytic parasites (PE). (**C**) RFLP Southern blot analysis of genomic parasite DNA probed with radiolabeled PCR or RT-PCR product obtained from D, PE, LS, or SP was carried out as previously described (*34*). A maximum of four bands was detected for Xmn I– (9.0, 4.1, 3.4, and 2.7 kb), or Hpa I– (10.2, 7.4, 6.0, and 2.25 kb) digested DNA. (**D**) vr amino acid sequences. Sequence analysis of products was performed as previously described (*15*). Because PCR is known to introduce mutations, a vr allele was only considered as a variant when it was obtained from two independent PCR amplifications or when it differed at several sites from other variants. Amino acids differing from the consensus sequence (Majority) are boxed, and differences among closely related sequences that are expressed in different stages are shaded.

ments for the complex pattern of transcriptional regulation of the *py235* genes remain to be elucidated. Py235 proteins have previously been shown to be involved in red blood cell invasion. Because a subset of these proteins is expressed in the sporozoite and is the target of antibodies that inhibit hepatocyte invasion, these proteins may be important in the recognition and/or invasion of the mosquito salivary glands and the liver. Merozoites released from both the liver and the infected erythrocyte invade red blood cells, so the need to express a distinct set of *py235* genes in the infected hepatocyte is puzzling. This differential expression of *py235* in the hepatic schizont reinforces the idea that the obligatory passage of the parasite through the liver not only amplifies the number of parasites injected by the mosquito but also preadapts the parasite to invade red blood cells. The presence of distinct rhoptry proteins in the sporozoite and the liver-stage malaria parasite may form the basis of an efficient vaccination strategy to target these pre-erythrocytic–stage parasites, which are present in small numbers and are at their most vulnerable. Conserved regions of the rhoptry proteins that are the target of protective immune responses may also form the basis of a vaccine against both pre-erythrocytic– and erythrocytic-stage parasites.

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CTCF, a Candidate *Trans*-Acting Factor for X-Inactivation Choice

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In mammals, X-inactivation silences one of two female X chromosomes. Silencing depends on the noncoding gene, *Xist* (inactive X-specific transcript), and is blocked by the antisense gene, *Tsix*. Deleting the choice/imprinting center in *Tsix* affects X-chromosome selection. Here, we identify the insulator and transcription factor, CTCF, as a candidate *trans*-acting factor for X-chromosome selection. The choice/imprinting center contains tandem CTCF binding sites that function in an enhancer-blocking assay. In vitro binding is reduced by CpG methylation and abolished by including non-CpG methylation. We postulate that *Tsix* and CTCF together establish a regulatable epigenetic switch for X-inactivation.

Dosage compensation ensures equal expression of X-linked genes in XX females and XY males. In mammals, this process results in inactivation of one female X chromosome (XCI) (*1*) in a random or imprinted manner. In the random form (eutherian), a zygotic counting mechanism initiates dosage compensation and enables a choice mechanism to randomly designate one active (Xa) and one inactive (Xi) X [reviewed in (*2*)]. In the imprinted form, zygotic counting and choice are superseded by parental imprints that direct exclusive paternal X-silencing (*3, 4*). Imprinted XCI is found in ancestral marsupials (*3*) but vestiges remain in the extraembryonic tissues of eutherians such as mice (*4*).

An epigenetic mark for random and imprinted XCI has long been postulated (*2*). The marks are placed at the X-inactivation center (*Xic*) (*5*), which includes the *cis*-acting noncoding gene, *Xist* (*6, 7*), and its antisense counterpart, *Tsix* (*8*). *Xist* RNA accumulation along the Xi initiates the silencing step (*9, 10*), whereas *Tsix* represses silencing by blocking *Xist* RNA accumulation (*11, 12*). A *cis*-acting center for choice and imprinting lies at the 5′ end of *Tsix*, as its deletion abolishes random choice in epiblast-derived cells to favor inactivation of the mutated X (*11, 13*) and disrupts maternal *Xist* imprinting in extraembryonic tissues (*14, 15*). Thus, while imprinted XCI is parentally directed and random XCI is zygotically controlled, both work through *Tsix* to regulate *Xist*.

To date, only X-linked *cis*-elements have been identified as XCI regulators. Yet, virtually all models invoke *trans*-acting factors which interact with the X-linked sites. In one model for imprinted XCI, a maternal-specific *trans*-factor confers resistance to XCI (*16*). In models for random XCI, an autosomally expressed "blocking factor" protects a single X from silencing (*2*). We have proposed that *Tsix* is the *cis*-target of both *trans*-factors (*11, 14*).

To isolate candidate *trans*-factors, we now used computational analysis (Fig. 1) to identify mouse-to-human conserved elements within the 2- to 4-kilobase (kb) sequence implicated in choice and imprinting (*11, 13–15*), a region including *DXPas34* (*17*). We found that the region is composed almost entirely of 60– to 70–base pair (bp) repeats with striking resemblance to known binding sites for CTCF, a transcription factor with a 60-bp footprint and 11 zinc fingers that work in various combinations to generate a wide range of DNA-binding activities (*18*). CTCF functions as a boundary element at the globin locus (*19*), regulates enhancer access to the *H19-Igf2* imprinted genes (*20–23*), and associates with CTG/CAG repeats

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