ErbB2 that bind the PTB domain in vitro are autophosphorylation sites in vivo and are involved in ErbB2-mediated transformation (*11–13*, *23*).

Our data indicate that PTB domains recognize tyrosine-phosphorylated targets through a different mechanism than do SH2 domains. PTB domains bind to phosphotyrosine within a motif containing an essential asparagine NH_2-terminal to the phosphotyrosine, whereas SH2 domains recognize residues COOH-terminal to the phosphotyrosine (*3*, *5*, *6*, *19–22*). The SH2 and PTB domains of Shc do not recognize the same sequence motifs or phosphorylated proteins (Fig. 2) (*7*). Further, the apparent affinity of the Shc PTB domain for peptide pY1221-pY1222 is as much as 100 times greater than that of a typical SH2 domain–peptide interaction in similar assays (*6*). Finally, multiple residues on both sides of the phosphotyrosine in the PTB domain binding site contribute to overall affinity. Thus, unlike SH2 domains, the PTB domain may make physical contacts with many residues in the phosphorylated peptide, or a specific conformation adopted by the phosphorylated peptide may contribute to binding. The former possibility is unlikely, given the variability of these residues outside the N-X_1-X_2-pY consensus in peptides that bind the PTB domain (Table 1). N-P-X-Y motifs adopt tight reverse-turn conformations which are dependent on the asparagine and tyrosine residues for structural stability and which are important for the function of these motifs as internalization signals (*24*, *25*). The asparagine and tyrosine side chains are closely juxtaposed in these structures. Thus, PTB domain binding to tyrosine-phosphorylated targets appears to require the motif N-X_1-X_2-pY within a specific secondary structure and to provide a mechanism for regulated protein-protein interactions during signaling by growth factors and oncogenes.

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27. Peptides were biotinylated during synthesis and purified by HPLC. GST-PTB domain or GST-Shc SH2 domain (*7*) proteins (100 ng) were incubated in 1× hybridization buffer with 500 nM biotinylated peptide for 1 hour at 4°C, immunoprecipitated as in Fig. 1, and washed once. The sedimented material was incubated with 0.25 units of streptavidin–alkaline phosphatase for 5 min at 4°C. The sedimented material was washed twice more, incubated for 3 min at room temperature with *p*-nitrophenol phosphate (1 mg/ml) in 100 mM glycine (pH 10.1), 1 mM $ZnCl_2$, and 1 mM $MgCl_2$, and absorbance at 405 nm was measured.

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Inducible Gene Expression in Trypanosomes Mediated by a Prokaryotic Repressor

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An inducible expression system was developed for the protozoan parasite *Trypanosoma brucei*. Transgenic trypanosomes expressing the tetracycline repressor of *Escherichia coli* exhibited inducer (tetracycline)-dependent expression of chromosomally integrated reporter genes under the control of a procyclic acidic repetitive protein (PARP) promoter bearing a *tet* operator. Reporter expression could be controlled over a range of four orders of magnitude in response to tetracycline concentration, a degree of regulation that exceeds those exhibited by other eukaryotic repression-based systems. The tet repressor–controlled PARP promoter should be a valuable tool for the study of trypanosome biochemistry, pathogenicity, and cell and molecular biology.

The medical importance of *Trypanosoma brucei* and its numerous departures from higher eukaryotic strategies for genomic organization and regulation [reviewed in (*1*)] have established it as a singular model system among the lower eukaryotes. The unorthodox biology of this parasite has both increased the intrinsic interest of the group and suggested potential targets for chemotherapy. The ability to gain insight from the evolutionary perspective that this system offers on higher eukaryotic biology, or to evaluate candidate drug targets, depends critically on an ability to address functional questions about in vivo roles of specific gene products. Whereas DNA-mediated stable transformation of trypanosomatids was accomplished several years ago (*2*), the study of genes essential for parasite growth has remained problematic in the absence of a means to control gene expression exogenously.

We report the development of an inducible expression system for *T. brucei* that

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allows precise control of the expression of introduced genes through a range of four orders of magnitude. This should greatly facilitate exploration of key functional questions by permitting mutational analysis of essential genes and overexpression of toxic gene products. In the absence of any known inducible trypanosome promoter, the system is based on the import into *T. brucei* of the regulatory elements of the tetracycline resistance operon (*3*) of *Escherichia coli*. The tetracycline-responsive Tet repressor (TetR) has served as the basis for the establishment of heterologous repression-based inducible systems in fungal, plant, and mammalian cells, yielding 10- to 500-fold regulation of transcription by RNA polymerase II or III (Pol II and Pol III) (*4–9*). In *T. brucei* the TetR mediates very tight transcriptional control of gene expression, yielding regulation factors that exceed 10,000 fold, much higher than those reported for other repression-based eukaryotic systems (*4–10*).

To generate trypanosomes expressing the Tet repressor, we targeted a copy of the Tn10-encoded *tetR* gene (*11*) modified for expression in eukaryotes (*9*) to the tubulin locus of *T. brucei* using construct pHD360 (Fig. 1), a derivative of the targeting expression vector pHD330 (*12*). Hygromycin-resistant pHD360 transformants were tested functionally for repressor expression.

In the higher eukaryotic systems the interference of TetR-operator complexes with Pol II transcription depends on the appropriate positioning of the operator relative to the basal promoter and transcription initiation site (*4–7, 13*). We therefore investigated whether the TetR would block transcription initiation when bound very near the transcription start site of a trypanosome promoter. We used the well-characterized promoter of the procyclic acidic repetitive protein (PARP) genes (*14–17*), one of the few trypanosomatid promoters for which the transcription start site has been mapped with any degree of precision (*17–20*). Although the PARP promoter drives expression of protein-coding genes, it recruits a polymerase that exhibits α-amanitin resistance characteristic of RNA polymerase I (Pol I) (*21, 22*). Additional biochemical (*23*) and genetic (*24*) evidence has supported the view that in *T. brucei*, in which trans-splicing (*25*) effectively uncouples Pol II transcription and mRNA capping (*12, 26*), transcription of the PARP genes and the variant surface glycoprotein genes (*27*) is mediated by Pol I.

We placed potential repressor binding sites within the PARP promoter by inserting single or tandem Tet operator sequences at a position +2 or –2 relative to the transcription start site (Fig. 2A) (*20*). Reporter plasmids in which these operator-bearing PARP promoters drove luciferase expression were then tested by transient transfection into transgenic trypanosomes stably expressing the TetR. All operator-bearing PARP promoters produced luciferase activities comparable with that of the operatorless parent reporter, pHD33, in wild-type trypanosomes but produced only back-

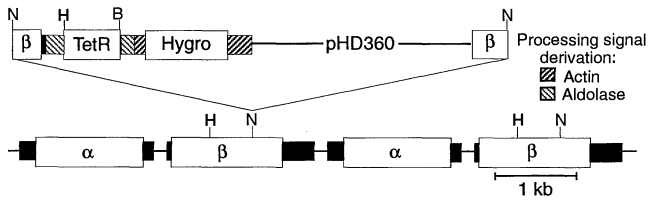

Fig. 1. Construct and targeting strategy used for the stable expression of TetR in *T. brucei*. The plasmid pHD360 is shown aligned above the repetitive αβ-tubulin locus (*35*) of *T. brucei*. The Eco RI–Bam HI fragment of pUHD14-1 (*9*) was subcloned (after filling of the Eco RI overhang) into the Bam HI–Hind III sites of the *T. brucei* tubulin targeting vector pHD330 (*12*). pHD360 was linearized at the Not I site within the tubulin targeting segment and transfected into procyclic forms of AnTat 1.1 with 10 μg of DNA per 10^7 cells per cuvette. Twelve hours after transfection, hygromycin was added to a final concentration of 50 μg/ml, and the trypanosomes were serially diluted in microtiter plates with conditioned medium (*36*). Clones which grew out of selection were then tested functionally for repressor expression by transient introduction of pHDTop4 (Fig. 2A). Four randomly selected clones all exhibited tight operator-dependent repression (>150-fold) of luciferase expression. One of these, clone 3, was used for construction of additional cell lines. Southern (DNA) blot analysis confirmed that in clone 3, two to four copies of pHD360 had integrated at the tubulin locus. N, Not I; H, Hind III; B, Bam HI; Hygro, hygromycin resistance gene.

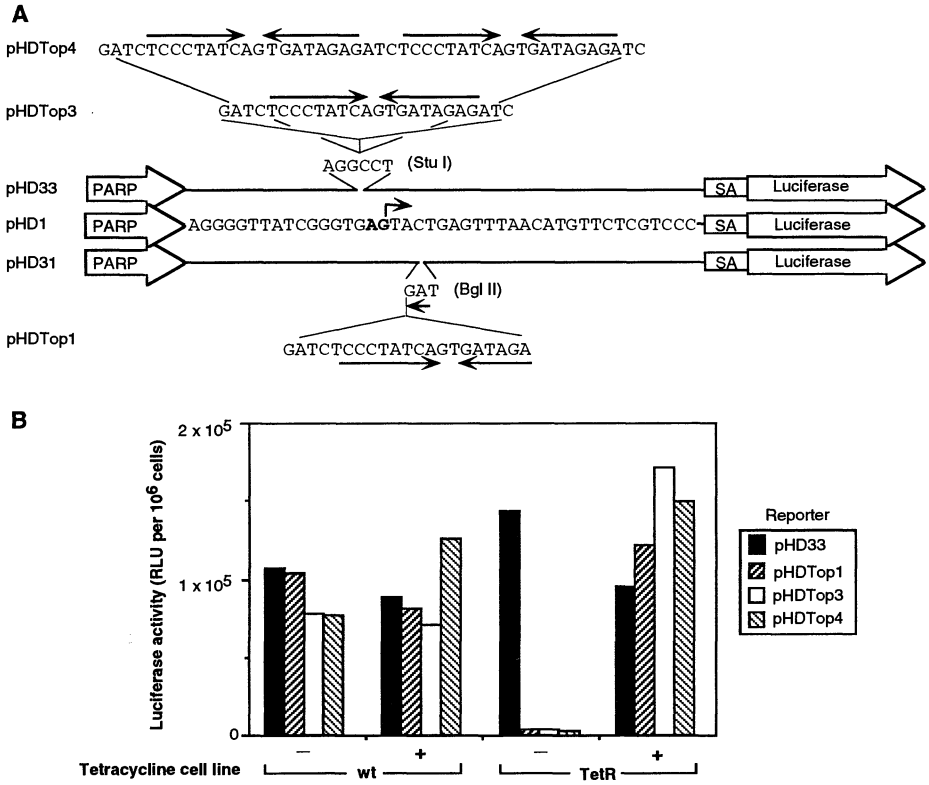

Fig. 2. The TetR-operator complexes proximal to the transcription start site block transcription driven by the PARP promoter. (**A**) Anatomy of the reporter constructs showing positions of operator insertions within the PARP promoter region. The cloning sites for the operator sequences were generated in the wild-type PARP promoter of pHD1 (*16*) either by insertion of 6 base pairs (bp) at a position – 2 relative to the start site (*20*) (shown in bold) to form a Stu I site (pHD33), or by insertion of 3 bp at a position +2 relative to the start site to form a Bgl II site (pHD31). An operator cassette corresponding to the O2 operator of *E. coli* (*3*) was made by annealing a pair of synthetic oligonucleotides (*37*) and inserting them into the restriction sites as shown. The inverted repeats comprising the repressor binding sites are over- or underlined with arrows. SA, splice acceptor site. (**B**) Results of transient expression assays. TetR-expressing procyclic forms (clone 3 from cell line 360) or wild-type (wt) AnTat 1.1 parent strain trypanosomes were transfected (*38*) with pHD33, Top1, Top3, or Top4 with 10^7 cells and 5 μg of plasmid per cuvette. Cells were cultured overnight with or without Tc (10 μg/ml), and then lysates were prepared and luciferase activity was assayed (*38*). Luciferase activities are given as average relative light units (RLU) per 10^6 cells from duplicate transfections.

ground levels of luciferase when transfected into a TetR-expressing cell line (Fig. 2B). Expression was fully inducible by tetracycline (Tc). Down-regulation of luciferase in the cell line containing pHD360 (cell line 360) was clearly due to repression of transcription because it required both the repressor and the operator. Differences in operator position or number did not lead to large differences in repression or inducibility. Thus, the TetR reversibly blocked transcription driven by the PARP promoter in the context of transient expression. We next explored the feasibility of regulating chromosomally integrated genes.

Maintenance of regulatability in a chromosomal context requires integration into a transcriptionally silent region of the genome. The region upstream of the PARP promoter has been reported to be quiescent (*19*, *22*), although a very low level of Pol II transcription has been seen (*15*), and no transcription has been detected in the intergenic spacer of the ribosomal RNA (rRNA) locus (*28*). Constructs bearing a luciferase gene under the control of the Tc-responsive promoter of pHDTop4 (Fig. 2A) linked to a phleomycin resistance cassette (*29*) were adapted for targeting into these loci. One construct, pHD404 (Fig. 3A), was designed for stable integration in the sense orientation into the upstream region of a PARP locus. The second, pHD430 (Fig. 3C), was designed for stable integration in the reverse orientation into the ribosomal DNA (rDNA) intergenic spacer. Each construct was linearized at a unique Not I site and transfected into the TetR-expressing cell line. Because transcription of the linked phleomycin resistance gene was expected to be dependent on transcriptional induction, Tc was included in the selective medium. No phleomycin-resistant parasites were obtained in the absence of Tc. After selection, phleomycin was washed away and the Tc-dependence of luciferase expression monitored.

The two constructs yielded cell lines exhibiting quantitatively different regulation. In both cell lines, upon withdrawal of Tc, the amount of luciferase declined gradually over a few days, possibly because of the stability of the protein within the trypanosome microbodies into which it is imported (*30*). For the construct integrated in a PARP locus, luciferase activity declined to a stable level approximately 1/30 that of the fully induced state (Fig. 3B). This noninduced basal level of expression approximates that obtained from a luciferase gene integrated in the tubulin locus and transcribed by Pol II (*31*). In the cell lines containing pHD404, therefore, expression

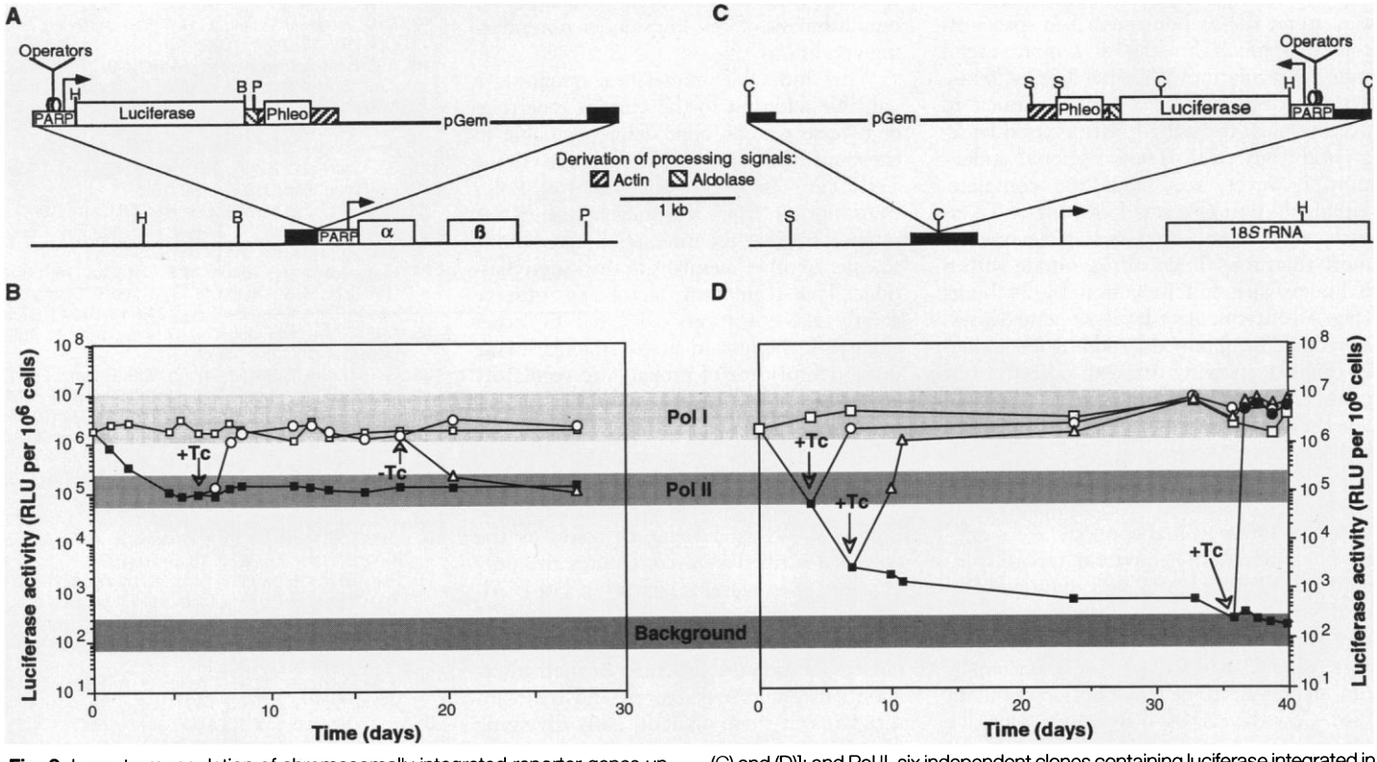

Fig. 3. Long-term regulation of chromosomally integrated reporter genes under the control of the Tc-dependent promoter. (**A**) The plasmid pHD404 is shown above the wild-type PARP A locus. The targeting segment of pHD404 (black box) corresponds to the region upstream of the PARP promoter (*39*), and the operator-bearing PARP promoter (open box) is derived from pHDTop4 (Fig. 2A) (*40*). TetR-expressing procyclic forms were transfected with linearized pHD404 and cloned immediately upon addition of phleomycin (*41*). The cell line showing the greatest Tc-dependent regulation of luciferase expression, clone 5D, contained two to four copies of pHD404 integrated in the PARP A locus as a tandem repeat. H, Hind III; B, Bam HI; P, Pst I; Phleo, phleomycin resistance gene; pGem, plasmid vector sequence pGem4 (Promega); α and β are the endogenous PARP genes. (**B**) Tc-dependent expression in the PARP locus. Luciferase activities were determined with 1/10 of the lysate from 2×10^6 cells. At time zero and after removal of the first sample, phleomycin was withdrawn. At the times indicated cultures were split and Tc added back or withdrawn. The shaded bars represent the range of luciferase activity measured from different cell populations: Pol I, fully induced cells containing pHD430 and pHD360 [see (C) and (D)]; and Pol II, six independent clones containing luciferase integrated in the tubulin locus [made with pHD421 (*31*)]. RLU, average relative light units. (**C**) Targeting a regulatable luciferase gene to the ribosomal locus spacer. pHD430 is shown aligned above an rRNA locus of *T. brucei*. It is identical to pHD404 except that the targeting fragment is a segment of the nontranscribed spacer of the rRNA locus, upstream of the transcription initiation site (black box) (*42*). Repressor-expressing trypanosomes were transfected with pHD430 (*41*). Four clones selected at random showed greater than 50-fold repression of luciferase expression after 4 days in the absence of Tc. Clone 3 showed the highest repression rate and contained two to four tandem copies of the vector integrated in an rRNA spacer. C, Cla I; S, Stu I. (**D**) Tc-dependent expression of luciferase in clone 3 of cell line 430/360. Details as for (B). Symbols: □, original culture maintained in the presence of Tc; ■, population derived from this original culture by withdrawal of tetracycline at time 0. (– Tc); ○, population derived from this –Tc culture by addition of Tc at approximately 4 days; △, population derived from the –Tc culture by addition of Tc at approximately 7 days; ●, population derived from the –Tc culture by addition of Tc at approximately 36 days.

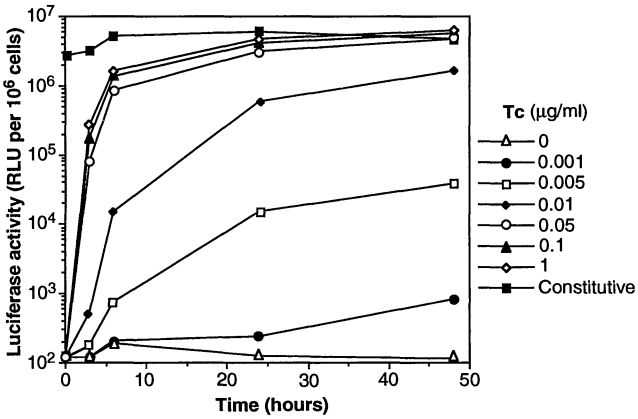

Fig. 4. Luciferase induction kinetics in cell line 430/360 at different Tc concentrations. A culture of clone 3 from cell line 430/360 which had been continously passaged in the absence of Tc and phleomycin for over 1 month was divided into seven subcultures. These were grown overnight. At time zero, Tc was added at the indicated concentrations. Luciferase activities were determined at the times indicated with 1/10 of the lysate from 2×10^6 cells. The constitutive values refer to a 430/360 clone 3 culture maintained in Tc (5 μg/ml). RLU, average relative light units.

could be modulated only through the range between Pol II– and Pol I– mediated levels.

Cell lines that contained the pHD430 construct integrated in the reverse orientation in an rDNA nontranscribed spacer afforded a much broader and more useful range of regulation. Within 4 days luciferase activity decreased 98% and continued to decline until it reached instrumental background (Fig. 3D). Transcriptional induction, however, was rapid and complete. Upon addition of a small amount of Tc, as little as 50 ng/ml, activity was elevated by more than four orders of magnitude within 6 hours, with full induction by 24 hours (Fig. 4). Intermediate levels of gene expression could be obtained by adding less inducer (Fig. 4), showing that expression of test genes can be modulated to achieve a desired level of expression. These results show that transcription initiation by the PARP polymerase (probably Pol I) is sensitive to the binding of the prokaryotic repressor, even within a chromosomal context.

The differential behavior of reporters integrated into the PARP and ribosomal loci could be attributable to differences in the transcriptional status of the two target sites. Whereas the rRNA locus spacer appears by run-on analysis to be transcriptionally silent (*28*), low levels of α-amanitin–sensitive transcription have been detected in the region flanking the PARP promoter (*15*), presumably arising from readthrough from the Pol II–transcribed microtubule-associated protein locus situated immediately upstream (*32*). TetR-operator complexes were incapable of interfering with elongating Pol II in the plant system (*6*, *7*, *13*).

Preliminary results with transgenic bloodstream form trypanosomes expressing TetR indicate that the system is functional in this medically relevant stage of the parasite. The current system is limited where expression of toxic gene products is desired, because the regulated promoter in the pHD430 construct is used to drive expression of the selectable marker in addition to that of the reporter or test gene. A second downstream promoter to drive constitutive expression of the resistance gene will be needed to enable the establishment of cell lines under noninducing conditions.

This inducible expression system is a valuable addition to the current repertoire of reverse genetic approaches available in these medically important protozoa. If the TetR can also be used to control Pol I transcription from trypanosomatid rRNA promoters, this technology should readily transfer to other members of the kinetoplastida. The regulation factor we observe greatly exceeds those exhibited by other eukaryotic repression-based systems, being more reminiscent of prokaryotic regulatory systems. In eukaryotes, only an activation-based system has yielded regulation factors in this range (*33*). The extent to which this is a consequence of the class of polymerase under regulation (Pol I compared with Pol II or Pol III) is difficult to assess as the system described here constitutes the only example of exogenous control of Pol I. Alternatively, it may represent a reflection of differences between the transcriptional apparatus, chromatin structure, or their interaction in higher eukaryotes, and their antecedents in these ancient, early diverging (*34*) lower eukaryotes.

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39. A targeting segment of pHD404 (black box in Fig. 3) corresponding to the region upstream of the PARP promoter was generated by polymerase chain reaction (PCR) with cloned PARP A locus DNA as the template and the primers TACGGTACCGCGGCCGCAACAAAGGCTGACTGCACCT and TACGAGCTCTTTCAGACTTCTTCGATGCC (introduced Kpn I, Not I, and Sac I restriction sites are underlined).
40. To construct a PARP promoter with internal Kpn I and Not I sites, we cloned the PARP A upstream targeting segment into pHD63 cut with Kpn I and Sac I (*29*). This was transferred as a Sac I–Xho I fragment to pHD330 (*12*), replacing the tubulin homology and yielding pHD383. The hygromycin resistance gene of pHD383 was then replaced by the phleomycin resistance gene of pHD63, producing pHD403. Finally the Spe I–Bam HI fragment of pHD403 spanning the downstream region of the wild-type PARP promoter and CAT gene was replaced with the Spe I–Bam HI fragment of pHDTop4, supplying the operator-bearing PARP promoter, splice acceptor, and luciferase gene and yielding pHD404.
41. Clone 3 trypanosomes expressing the TetR (see Fig. 1) were transfected with 10 μg of Not I–linearized pHD404 or pHD430 (per 10^7 cells per cuvette). After overnight culture in hygromycin (50 μg/ml) and Tc (10 μg/ml), phleomycin was added to a final concentration of 5 μg/ml, and trypanosomes were diluted serially in microtitre plates.
42. This fragment was generated by PCR by using a cloned fragment from the rRNA locus intergenic region as template (*28*) and primers GATCTCGAGATGGGTACCGGTGTGTTGCCAAAGACATT and GCAGAGCTCAGGCTTTCGGACATGAATTTG (introduced Xho I, Kpn I, and Sac I sites are underlined); a Not I linker was inserted into the unique Cla I site.
43. We thank H. Bujard, F.-B. Wang, A. Bonin, and M. Gossen (Zentrum für Molekulare Biologie der Universität Heidelberg) for the plasmid pUHD14-1 and for helpful discussions; S. Beverley (Harvard) for discussions and commmunicating unpublished results; G. Rudenko (Netherlands Cancer Institute) for calling our attention to the rDNA nontranscribed spacer as a potential integration site and for providing plasmids containing cloned fragments from the rRNA locus; G. Hobbs for grammatical insight; and J. Zutt for the serene environment. This work was partially supported by the Deutsche Forschungsgemeinschaft (SFB 229 Teil D5).

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Absence of Polymorphism at the *ZFY* Locus on the Human Y Chromosome

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DNA polymorphism in the Y chromosome, examined at a 729–base pair intron located immediately upstream of the *ZFY* zinc-finger exon, revealed no sequence variation in a worldwide sample of 38 human males. This finding cannot be explained by global constraint on the intron sequence, because interspecific comparisons with other nonhuman primates revealed phylogenetically informative sequence changes. The invariance likely results from either a recent selective sweep, a recent origin for modern *Homo sapiens*, recurrent male population bottlenecks, or historically small effective male population sizes. A coalescence model predicts an expected time to a most recent common ancestral male lineage of 270,000 years (95 percent confidence limits: 0 to 800,000 years).

The human Y chromosome, nonrecombining along most of its length and paternally inherited, should be extremely useful for the reconstruction of genetic and evolutionary history. However, relatively little is known about the patterns of polymorphism between human Y chromosomes. Polymorphism has been reported for certain regions of the chromosome; with few exceptions (*1*, *2*) these reports involve either the use of anonymous probes of uncharacterized sequence (*3*) or represent variable numbers or locations of Alu or other repetitive elements (*4*).

Here we report a study of sequence variation at a well-characterized human Y-linked locus: a 729–base pair (bp) intron located between the third exon and the zinc-finger–encoding fourth exon of the ZFY locus (*5*). This gene, located in interval Yp-1A2, is actively transcribed in males and appears to be involved in sperm or testes maturation (*6*). We carried out a detailed survey of this region in a worldwide sample of humans in order to provide a preliminary picture of sequence polymorphism on the Y chromosome. We surveyed 38 individuals, chosen to represent a cross section of geographic origins, and sequenced the entire intron in all of them (*7*). In addition, we sequenced part or all of the 3′-most zinc-finger exon in 12 of those individuals, as well as the homologous intron in three other nonhuman primates—chimpanzee, gorilla, and orangutan (*8*).

Surprisingly, we detected no intraspecific polymorphism whatsoever, in either the intron or the exon, in our human sample. Such an absence of variation across the 729-bp intron in a sample of this size (a total of ~28,000 bp sampled) is unexpected, because intron sequences appear to be subjected to few sequence-specific constraints. Selection at this intron cannot account for the absence of variation, as interspecific comparisons of the sequences of this intron in other primates show that variable sites are distributed throughout the intron and include at least 21 unambiguous transitions, 14 unambiguous transversions, and 4 insertions or deletions (*8*). Furthermore, these data suggest that the absence of recombination in this region of the Y chromosome does not detectably slow rates of interspecific divergence; indeed, Y-linked sequences have been shown to exhibit accelerated rates of evolution (*9*). Table 1 summarizes the interspecific differences at this region, which were then used to construct a parsimony tree of these sequences (Fig. 1). These Y chromosome sequences yielded a completely unambiguous shortest-length tree (Fig. 1) uniting the human and chimpanzee sequences on the basis of two synapomorphic changes (*10*).

The interspecific data predict a level of Y chromosome polymorphism in *Homo sapiens*, assuming clock-like behavior of these sequences. The divergences listed in Table 1 correspond to an approximate mean mutation fixation rate of 0.135% per million years (My) of elapsed time along a single branch for this intron (*11*). Given the total human intron sequence examined in this study (28,000 bp), we would expect 19.5 segregating sites if human lineages were to trace back 500,000 years on average. If the origin of modern *Homo sapiens* is more recent (on the order of 150,000 years), the expectation for segregating sites declines to 5.5.

There are three general classes of explanation for the lack of variation of a chromosomal region: purifying selection, chance absence of segregating sites, or recent common ancestry. The interspecific comparisons mentioned previously rule out a selectively mediated global conservation of the intron sequence. Could the sampling variation in the distribution of polymorphic sites among individuals produce the observed monomor-

Table 1. Absolute (below diagonal) and mean (above diagonal) interspecific distances for the final *ZFY* intron, averaged over all possible states. Distances calculated according to the Kimura two-parameter model (*18*) are listed in parentheses. Mean sequence length compared = 729 bp.

	Human	Chimp	Gorilla	Orang
Human		0.007 (0.007)	0.014 (0.013)	0.041 (0.042)
Chimp	5		0.015 (0.015)	0.043 (0.043)
Gorilla	10	11		0.044 (0.046)
Orang	30	31	32	

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