

**Fig. 5** Transactivation of the chloramphenicol acetyltransferase (CAT) gene linked to the HIV-1 LTR when transfected into MOLT-3 cells which were uninfected (a), infected with HIV-1 (b) or infected with HHV-6 (c). Plasmids were pC15CAT (lanes 1; ref. 21) containing the HIV-1 LTR linked to the bacterial CAT reporter gene, and the control pSVOCAT (lanes 2; ref. 22) that lacked promoter-enhancer sequences.

**Methods.**  $10^7$  cells were transfected with 10  $\mu$ g plasmid DNA by the DEAE-dextran technique<sup>23</sup>, collected after 40 h and processed to obtain 100  $\mu$ l cellular extract; aliquots of 20  $\mu$ l were subsequently used for CAT assays in a 3-h incubation<sup>24</sup>. Different percentages of cell viability were found in the various cultures at the time of collection: a1, 79%; a2, 89%; b1, 51%; b2, 49%; c1, 26% and c2, 29%. The acetylation of chloramphenicol (%) as estimated from liquid scintillation counting was a1, 0.19; a2, 0.24; b1, 2.5; b2, 30; c1, 0.27 and c2, 19.7. Cm, chloramphenicol; AcCm, acetylated chloramphenicol.

The accelerated virus expression and cytopathicity in dually infected cultures suggested that a direct interaction occurs between HIV-1 and HHV-6. We therefore studied the transactivation of the HIV-1 LTR by HHV-6 using the human CD4<sup>+</sup> T-cell line MOLT-3. As illustrated in Fig. 5, there was a consistent transactivation in HHV-6-infected cells which was not found in uninfected cells. We obtained similar results using the CD4<sup>+</sup> T-cell line CEM (data not shown).

The part played by cofactorial mechanisms in the pathogenesis of AIDS and in particular the relevance of other potentially immunosuppressive agents are still to be clarified. We have previously demonstrated that HHV-6 exerts a direct lytic effect on human CD4<sup>+</sup> T lymphocytes<sup>18</sup>. Here we have shown that HHV-6 can co-infect individual T cells with HIV-1 and can transactivate the expression of HIV-1. The simultaneous presence of both viruses in the same cell emphasizes the potential importance of this transactivation. Replication of HHV-6 might in turn be enhanced by co-infection with HIV-1, thus generating a vicious cycle. Our observations, although they do not prove the aetiological involvement of HHV-6, are consistent with a possible cofactorial role of this virus in one of the critical pathological features of AIDS, namely the CD4<sup>+</sup> T-cell depletion. Prospective long-term seroepidemiological and *in vivo* investigations, using animal models or two-probe hybridization to detect dual infection in individual patient cells, will help evaluate the contribution of potential cofactors to the course of HIV-1 infection.

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## Genetically haploid spermatids are phenotypically diploid

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**Because chromosomal homologues segregate from one another during meiosis, spermatids are genetically different. Post-meiotic gene expression could lead to gametic differences, some of which might lead to preferential transmission of certain alleles over others. In both insects and mammals, however, all the cells derived from a single spermatogonial cell develop within a common syncytium formed as a result of incomplete cytokinesis at each of the mitotic and meiotic cell divisions<sup>1-3</sup>. It has been proposed that the intercellular bridges connecting the cells, which are about 1  $\mu$ m in diameter<sup>4</sup>, permit the sharing of cytoplasmic constituents, thus ensuring the synchronous development of a clone of cells and gametic equivalence between haploid spermatids<sup>2,5,6</sup>. By analysing the product of a transgene which is expressed exclusively in post-meiotic germ cells in hemizygous transgenic mice, we have shown that genetically distinct spermatids share the product of the transgene and hence can be phenotypically equivalent.**

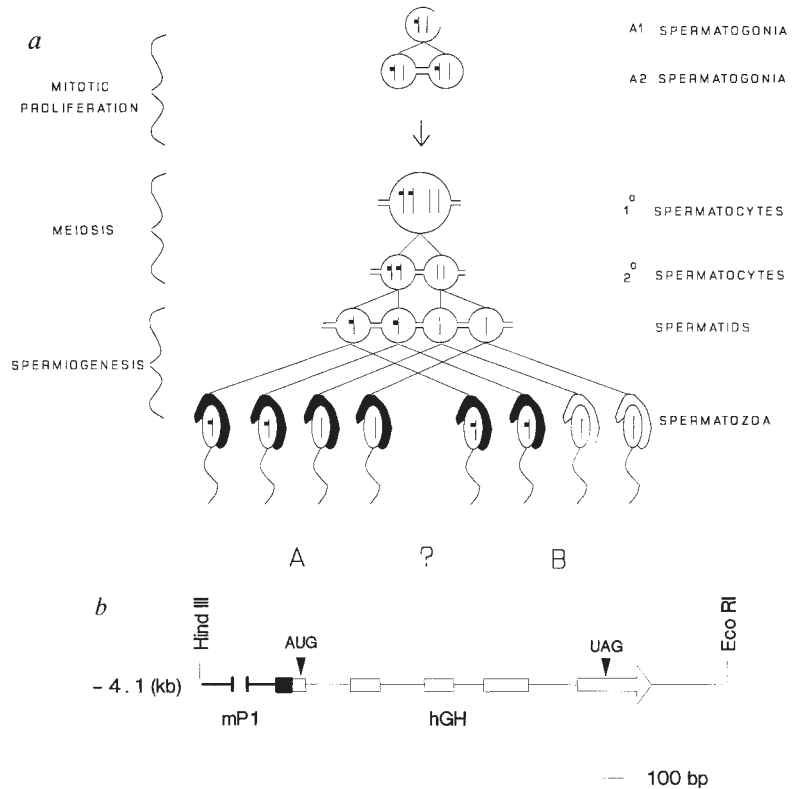
The experiments described here were designed to determine whether either RNA or protein can pass through the intercellular bridges which connect spermatids. To address these questions we have generated transgenic mice that carry a chimaeric gene consisting of the mouse protamine 1 (*mP1*) transcriptional regulatory sequences, fused to the human growth hormone (*hGH*) structural gene. The advantage of using transgenic mice for such an analysis is that mice can be generated which are hemizygous for the transgene, that is, the introduced DNA is present on only one of the two chromosomal homologues, so that after meiosis only half of the spermatids carry the transgene (Fig. 1a). The *mP1* gene is transcribed exclusively during spermiogenesis, the haploid phase of spermatogenesis<sup>7</sup>. Previous studies have shown that 4.8 kilobase (kb) of 5' sequence is sufficient for the proper tissue-specific and temporal regulation of *mP1* in transgenic mice<sup>8</sup>. The construct used in this study contains 4.1 kb of *mP1* 5' sequence fused to the *hGH* gene (Fig. 1b).

Two independent lines of transgenic mice harbouring the *mP1-hGH* gene were established (Fig. 1b). To determine the transmission frequency of the transgene, tail DNA from

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**Fig. 1 a**, Illustration of the syncytial nature of spermatogenesis and the three general stages of development<sup>20,21</sup>. The spermatogonial cells are drawn with a pair of homologous chromosomes inside them with one chromosome containing the introduced transgene (shown by a solid box). After the commitment of A1 spermatogonial cells to differentiate there are five or six rounds of mitotic proliferation. The first cell division generating A2 spermatogonial cells is shown. Primary spermatocytes contain a 4N complement of DNA, that is, each chromosome is associated with a sister chromatid. Each primary spermatocyte gives rise to four spermatids, two that contain a wild-type chromosome and two that carry a chromosome with the transgene. During spermiogenesis, round spermatids are transformed into mature spermatozoa. The question is whether all of the spermatozoa contain the product of the transgene (in this case the hGH protein localized to the acrosome), as shown in A, or whether only those spermatozoa that contain the transgene will have the product, as shown in B. **b**, Plasmid *mP1-hGH* contains approximately 4.1 kb of *mP1* 5' sequence, including the promoter and 91 base pairs (bp) of 5' untranslated sequence fused to a genomic clone for the *hGH* gene. Boxes represent exons and lines represent either non-transcribed DNA or introns. Bold lines and the solid box represent *mP1* DNA whereas thin lines and open boxes represent *hGH* DNA. The break in the line that corresponds to *mP1* sequence denotes that portion of the transgene that is not drawn to scale. The positions of the translational start and stop codons in the first and last exon, respectively, are shown above the gene structure. Transgenic mice were constructed by microinjecting a 6.3 kb *HindIII-EcoRI* fragment containing the *mP1-hGH* gene, free of plasmid vector sequences, into the pronuclei of fertilized C57BL/6 × SJL F2 mouse eggs<sup>22</sup>. Two lines of transgenic mice, 314-4 and 315-6, were generated and used for this study. Both lines contain multiple copies of the transgene integrated on one chromosome.



offspring was analysed for the presence of the transgene by hybridization with an *hGH*-specific radiolabelled DNA probe. Analysis of the progeny from three generations of outcrosses of transgenic with nontransgenic animals showed that the mice were hemizygous for the transgene (data not shown).

*In situ* hybridization was used to determine the developmental stage in which the transgene is first transcribed. Figure 2a and b shows serial sections of a testis prepared from a hemizygous transgenic adult male, and hybridized with <sup>35</sup>S-labelled antisense RNA probes specific for the *mP1-hGH* transgene and the endogenous *mP1* gene, respectively. In both cases expression was detected exclusively in the haploid spermatids and not in spermatogonial cells or in spermatocytes.

Northern blot analysis of RNA isolated from the testis of prepubertal transgenic animals of different ages supported the conclusion that the transgene was expressed exclusively in post-meiotic cells. No transcripts were detected from the transgene or the endogenous gene in testis from 19-day-old animals, an age at which cells in the first cycle of spermatogenesis have progressed to the pachytene spermatocyte stage<sup>9,10</sup>, whereas transcripts from both genes were detected, in about equal abundance, in 25-day-old animals, an age at which some cells have progressed into the haploid stage of development (data not shown).

To determine the phenotype of spermatids with respect to the product of the *mP1-hGH* transgene we used immunocytochemical analysis. Spermatozoa from transgenic mice harbouring the *mP1-hGH* construct contain the hGH protein localized in the acrosomal compartment (R.E.B. *et al.*, in preparation). Visualization of the hGH protein in developing acrosomes in early-stage seminiferous tubules (Fig. 3a), and in later-stage tubules (Fig. 3b), showed that the majority of the spermatids within a tubule of a hemizygous mouse were immunopositive for the hGH protein. Immunocytochemistry performed on the caput epididymis (the proximal portion of the epididymis where newly formed spermatozoa reside), showed that the hGH protein

was stable in the acrosome and that, once again, most of the spermatozoa were immunopositive (Fig. 3c).

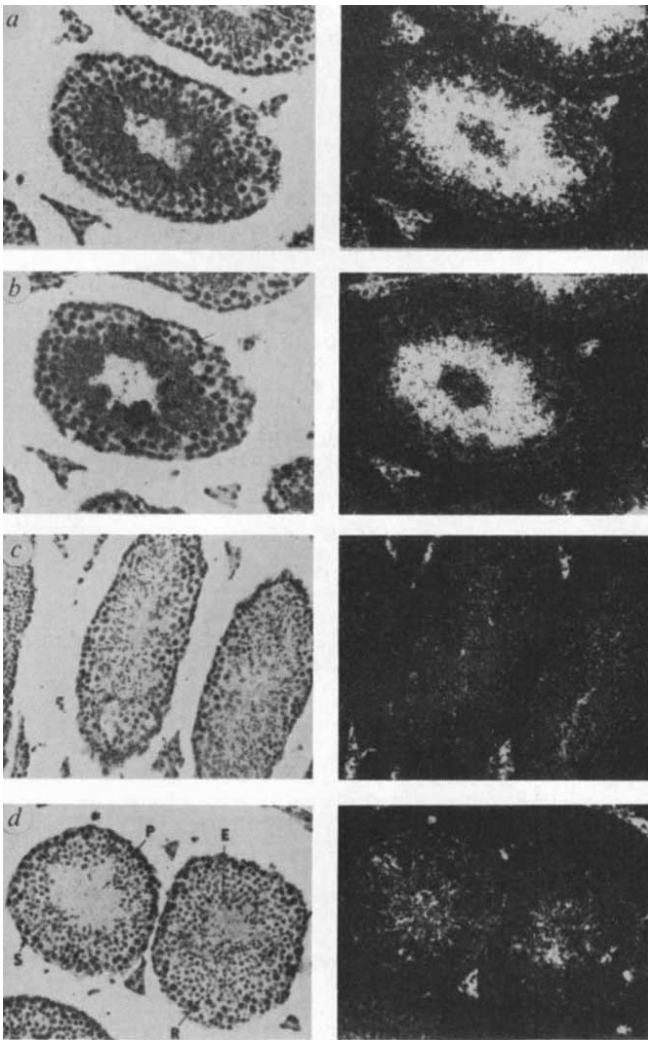
To quantitate the percentage of hGH-immunopositive spermatozoa, we isolated sperm from the caput epididymis (Fig. 3d) and determined directly the percentage that were immunopositive. Approximately 90% of all caput sperm analysed from hemizygous animals contained hGH-immunopositive acrosomes (Table 1), despite the fact that the gene was transmitted to only 50% of the offspring, showing that post-meiotic gene products can be shared.

Failure to detect hGH protein in 9% of the spermatozoa could be due to incomplete movement of RNA or protein between spermatids, nondetectable levels of expression of the transgene in some spermatids, or technical problems such as loss of the acrosome during the sperm isolation. To distinguish between incomplete movement of RNA or protein between the intercellular bridges and the other possibilities, we analysed caput sperm from mice homozygous for the transgene. Even in homozygous mice only 92% of the sperm were immunopositive (Table 1),

**Table 1** Quantitation of the percentage of hGH-immunopositive spermatozoa

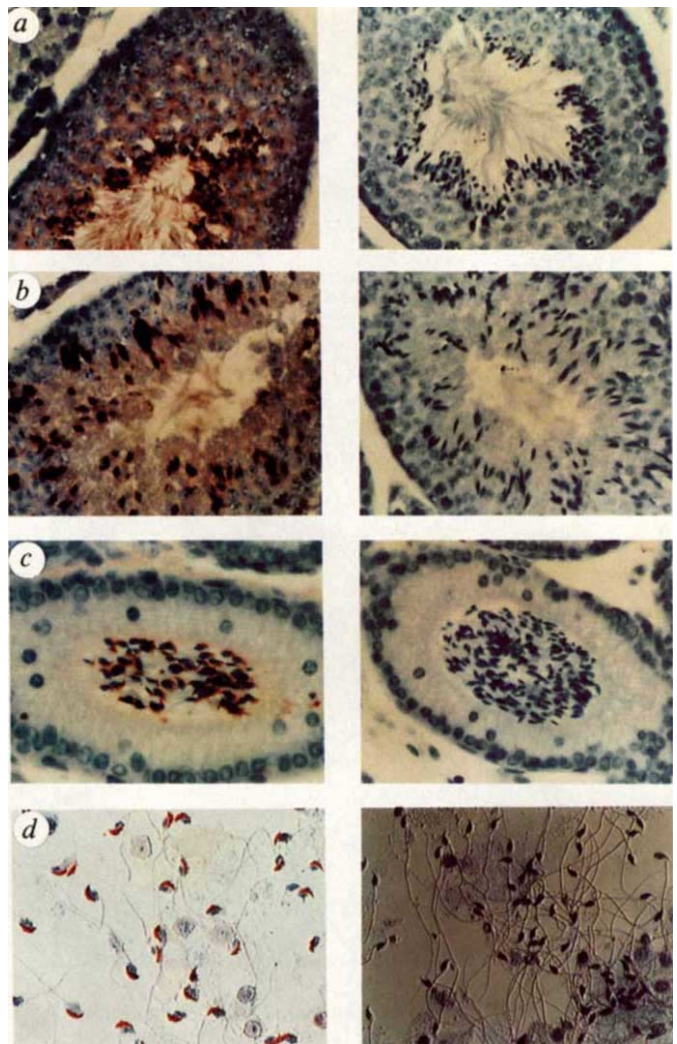
Genotype	Total sperm analysed	Percentage immunopositive
Hemizygous	2,185	91%
Homozygous	2,768	92%

Spermatozoa were isolated from the caput epididymis and immunocytochemistry was performed as described in the fig. 3 legend. These results were obtained by analysing spermatozoa isolated from three hemizygous mice (two from the 315-6 line and one from the 314-4 line) and two homozygous mice (both from the 314-4 line). Homozygous mice were obtained by crossing hemizygous transgenic animals and screening the offspring by quantitative DNA dot hybridization with an *hGH*-specific probe. Cells were counted using differential interference contrast microscopy at ×400 magnification.



**Fig. 2** *In situ* hybridization of testis sections from a transgenic mouse harbouring the *mPI-hGH* gene (line 314-4). Serial sections were hybridized with  $^{35}\text{S}$ -labelled probes: *a*, *hGH* antisense RNA; *b*, *mPI* antisense RNA; *c*, *hGH* sense RNA; and *d*, *mPI* sense RNA. Photographs on the left were taken with bright-field microscopy and those on the right with dark-field microscopy. Examples of the different cell types found within a seminiferous tubule are shown by arrows and are labelled as follows: S, spermatogonial cell; P, pachytene spermatocyte; R, round spermatid; and E, elongated spermatid.

**Methods.** Single-stranded  $^{35}\text{S}$ -containing sense and antisense riboprobes were made *in vitro* with linear templates and either SP6 or T7 RNA polymerase. The *mPI* template was made by cloning a 250-bp restriction fragment containing a portion of *mPI* exon 1, the intron, and a portion of exon 2 into pGEM-2. The *hGH* template was constructed by cloning a 170 bp restriction fragment containing *hGH* exon 5 into pGEM-2. Testes were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5  $\mu\text{m}$  sections. Hybridizations were as described<sup>23</sup>. Probes were used at a concentration of about 0.2 ng  $\mu\text{l}^{-1}$  with a specific activity of approximately  $5 \times 10^5$  c.p.m.  $\text{ng}^{-1}$ . Hybridizations were at 45° overnight. The most stringent washing conditions were at 45° in 0.1  $\times$  SSC. Slides were developed after one to three days. Sections were counterstained with haematoxylin.

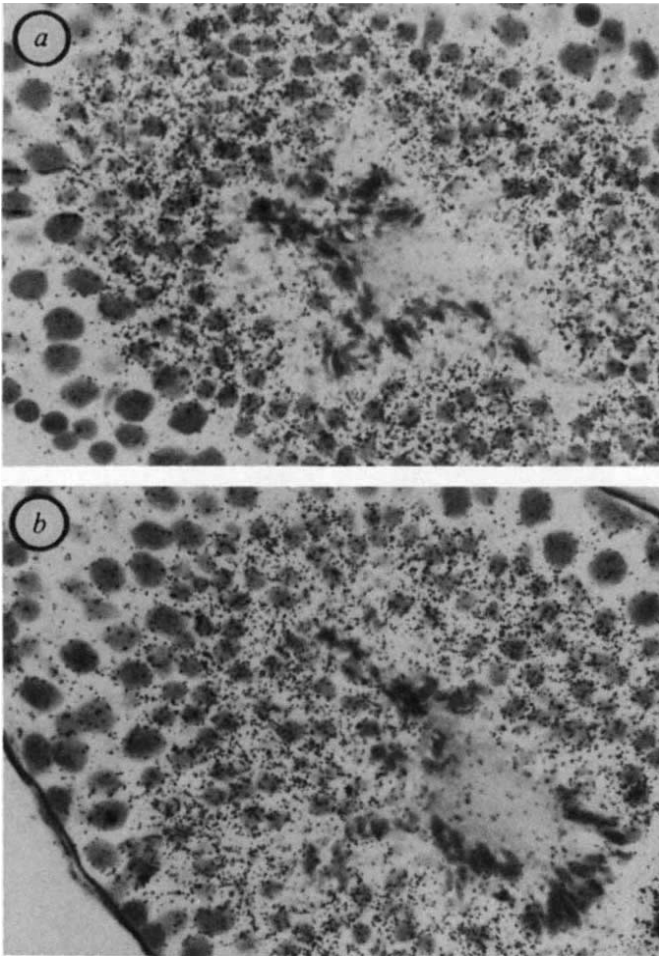


**Fig. 3** Immunocytochemistry of *hGH* protein in *mPI-hGH* transgenic mice (line 314-4). Photographs on the left show sections incubated in the presence of both the primary antibody (rabbit anti-*hGH*), and secondary antibody (biotinylated goat anti-rabbit IgG). Photographs on the right show sections incubated only in the presence of the secondary antibody. *a*, An early-stage seminiferous tubule with round spermatids at the CAP phase of acrosome development<sup>9</sup>. *b*, A late-stage tubule with many elongated spermatids and fully developed acrosomes. *c*, A section through the caput epididymis. *d*, Spermatozoa isolated from the caput epididymis. The *hGH* protein is seen as a red precipitate.

**Methods.** Testes were fixed in Carnoy's, embedded in paraffin and cut into 5  $\mu\text{m}$  sections. Sections were deparaffinized with xylene and re-hydrated using standard procedures. Indirect immunocytochemistry was performed using the reagents and suggested protocols of Vectastain ABC and Zymed SABC kits. The primary antibody, rabbit anti-*hGH*, was obtained from the National Hormone and Pituitary Program, Baltimore, Maryland, and used at either a dilution of 1/100 or 1/500. Aminoethyl carbazole (Zymed Lab. Inc.) was used as the substrate chromogen. Sections were counterstained with haematoxylin. Additional negative controls included the use of both the primary and secondary antibodies on testes isolated from nontransgenic animals. For the isolated epididymal sperm, transgenic animals were killed, the caput epididymis dissected out and cut into 8–10 pieces, and incubated for 30 min at 30 °C in EKRB media<sup>24</sup>. After 30 min the supernatant was removed, centrifuged for 5 min at 1,000 r.p.m., and the pellet resuspended in a small volume of EKRB media. A few  $\mu\text{l}$  of sample were spread onto a microscope slide, allowed to dry for about 1 min, and fixed in Carnoy's. After incubation in PBS for 30 min, immunocytochemical analysis was performed as described above.

suggesting that it is not incomplete sharing of post-meiotic gene products that leads to the immunonegative cells.

Development of the acrosome occurs adjacent to the nucleus during spermiogenesis<sup>9,11,12</sup>. Juxtaposed to the acrosome is the cellular machinery responsible for synthesizing the acrosomal enzymes, the Golgi apparatus and the rough endoplasmic reticulum (RER)<sup>12,13</sup>. Because *hGH* protein is localized to the



**Fig. 4** *In situ* hybridization of testis serial sections hybridized with either an *hGH* antisense probe (a) or an *mPI* antisense probe (b). Sections were photographed at  $\times 500$  magnification. See Fig. 2 legend for details of hybridization conditions.

acrosome, it is probably synthesized on the RER, at the site of acrosome formation, and is therefore not free in the cytoplasm<sup>14</sup>. We first detect *hGH* immunocytochemically in the developing acrosome (Fig. 3a). Although it is unlikely that vesicles bud off from the Golgi and move between the syncytial bridges, electron microscopy studies have shown that the endoplasmic reticulum does sometimes extend through the bridges<sup>15</sup> suggesting that growth hormone protein could be traversing the bridges through the ER. Comparison of serial sections hybridized with *hGH*-specific and *mPI*-specific RNA probes (Fig. 4a, b), however, showed that within a seminiferous tubule there seemed to be an equal distribution of silver grains over all of the round spermatids regardless of the probe used. These results suggest that there could be movement of *mPI-hGH* mRNA, presumably through the intercellular syncytial bridges. Experiments designed to test unequivocally for movement of mRNA are currently in progress.

These results provide evidence against functional differences between sperm arising from post-meiotic gene expression. Our results are supported genetically, with one exception, by the absence of any cases of non-mendelian inheritance in the mouse<sup>16</sup>. The one exception involves preferential transmission of the *t* locus in male mice heterozygous for the *t* chromosome<sup>17</sup>. Male mice heterozygous for a complete *t*-haplotype can transmit the *t*-haplotype chromosome with a frequency as high as 99%<sup>18</sup>. Although transmission ratio distortion in *t*-haplotypes might be due to preferential retention of mRNA and/or protein in spermatids in which they are synthesized<sup>16,19</sup>, it has yet to be demon-

strated that post-meiotic transcription is definitely involved in the transmission ratio distortion. Our data do, however, clearly show that, at least for the product of the *mPI-hGH* transgene, either mRNA and/or protein is free to move between the intercellular bridges which connect developing spermatids, and suggest that the products of most genes may be equally distributed among spermatids.

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## Tnt1, a mobile retroviral-like transposable element of tobacco isolated by plant cell genetics

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Transposable elements can be identified by their ability to induce mutant alleles at new loci. The retrotransposon family is thought to transpose through an RNA intermediate and has many similarities to vertebrate proretroviruses<sup>1,2</sup>. In plants, retrotransposons have been described in maize<sup>3,4</sup>, *Arabidopsis*<sup>5</sup> and wheat<sup>6</sup>, and non-viral retrotransposons in maize<sup>7</sup>. Most of these elements, however, have been found as non-mobile integrated units. Here, we report the isolation of the first tobacco (*Nicotiana tabacum*) transposable element, Tnt1, which seems to be the most complete mobile retrotransposon characterized in higher plants. Tnt1 has been isolated after its transposition into the nitrate reductase (NR) structural gene of tobacco, and transposition events have been detected through *in vitro* selection of spontaneous NR-deficient (NR<sup>-</sup>) mutant lines in cell cultures derived from tobacco mesophyll protoplasts. Tnt1 is 5,334 nucleotides long, contains two 610-base-pair-long terminal repeats and a single open reading frame of 3,984 nucleotides. Comparison of the Tnt1 open reading frame coding potential with those of the *Drosophila melanogaster copia*