which might have escaped detection by the run-on transcription assay. The sensitivity of the S_1 mapping assays was estimated by counting the radioactivity in the corresponding gel slices (not shown). In nuclear RNA from Mov13 and P19 cells we would have been able to detect a 100-fold lower concentration of $\alpha 1$(I) collagen transcripts as compared with wild-type cells. Assuming that the nuclear RNA used contained predominantly primary transcripts, we can conclude that initiation of transcription at the $\alpha 1$(I) collagen promoter in Mov13 cells is reduced by at least 100-fold relative to wild-type cells and is comparable to P19 cells which normally do not transcribe $\alpha 1$(I)collagen mRNA. The hybridization of restriction fragment 1F with *in vitro* elongated RNA from Mov13 cells (Fig. 1*b*) can therefore not be due to transcripts initiated at the $\alpha 1$(I) collagen promoter. It may be the result of activation of cryptic promoter sequences within the $\alpha 1$(I) collagen gene due to the provirus insertion, or cross-hybridization of this section of the gene with type III collagen mRNA or with other unknown repetitive sequences. The presence of a member of the mouse *B1* family of middle repetitive sequences in the $\alpha 1$(I) collagen gene has in fact recently been shown[7].

Probe A did not detect any transcripts initiating in the proviral 3′ LTR and extending into cellular sequences (Fig. 2). This was not unexpected because indirect evidence indicates that this proviral copy in Mov13 cells is transcriptionally inactive[4], and because intact retroviral proviruses usually do not use their 3′ LTR for initiation of transcription[8].

Our results show that the provirus-induced change of chromatin structure of the $\alpha 1$(I) collagen gene in Mov13 cells inhibits initiation of transcription, probably by preventing RNA polymerase and/or other transcription factors from binding to the DNA template. The most striking difference in chromatin structure of the wild-type and mutant gene is the absence of the transcription-associated DNase-hypersensitive site in the latter[4]. DNase-hypersensitive sites within eukaryotic genes are thought to be regions where transcription factors bind to regulatory DNA sequences[9–12]. They may have an important role in developmental gene activation[13]. Because the virus insertion in Mov13 cells has not changed the DNA sequence containing the presumptive binding site but exerts its effect over a distance of ~500 bp, the binding of such factors must be controlled by structural features of the chromatin rather than DNA sequence alone. Our results therefore suggest that the promoter-associated hypersensitive site is a prerequisite for rather than a consequence of gene activity. The virus seems to cause the mutation by long-range and indirect effects which may prevent correct activation of the $\alpha 1$(I) collagen gene by interfering with the developmentally regulated induction of the hypersensitive site.

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The *trans*-activator gene of HTLV-III is essential for virus replication

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Studies of the genomic structure of human T-lymphotropic virus type III (HTLV-III) and related viruses, implicated as the causal agent of acquired immune deficiency syndrome (AIDS), have identified a sixth open reading frame in addition to the five previously known within the genome (*gag*, *pol*, *sor*, *env* and 3′*orf*)[1–4]. This gene, called *tat*-III, lies between the *sor* and *env* genes and is able to mediate activation, in a *trans* configuration, of the genes linked to HTLV-III long terminal repeat (LTR) sequences[5–8]. We now present evidence that the product of *tat*-III is an absolute requirement for virus expression. We show that derivatives of a biologically competent molecular clone of HTLV-III[9], in which the *tat*-III gene is deleted or the normal splicing abrogated, failed to produce or expressed unusually low levels of virus, respectively, when transfected into T-cell cultures. The capacity of these *tat*-III-defective genomes was transiently restored by co-transfection of a plasmid clone containing a functional *tat*-III gene or by introducing the TAT-III protein itself. As HTLV-III and related viruses are the presumed causal agents of AIDS and associated conditions[10–12], the observation that *tat*-III is critical for HTLV-III replication has important clinical implications, and suggests that specific inhibition of the activity of *tat*-III could be a novel and effective therapeutic approach to the treatment of AIDS.

Transcriptional activation of viral genes by viral regulatory elements acting in *cis* and *trans* configurations was first described for DNA tumour viruses[13–18], and *trans*-activator genes have since been found in retroviruses of man[19–21], cattle[22,23] and sheep[24]. Knowledge that *trans*-acting factors can specifically augment the expression of viral genes has led to intense speculation that *trans*-activator genes may have crucial roles in the biology of these viruses.

The *tat*-III gene, as characterized by functional mapping studies, consists of three exons[6,7]. The first exon is non-coding and extends 287 base pairs from the 5′ LTR. The second exon, located between the *sor* (for short open reading frame) and *env* (envelope) genes, encodes 72 amino acids and is crucial for *trans*-activation. The third exon encodes only 14 amino acids and is located within the *env* gene in a different reading frame. To investigate whether *trans*-activation is an essential feature of HTLV-III replication, a panel of HTLV-III plasmid clones containing modifications in the *tat*-III gene were generated (Fig. 1). These constructs were derived from a plasmid clone pHXB2D which contains full-length HTLV-III provirus and produces infectious HTLV-III virions and cytopathic effects when transfected into normal human T lymphocytes[9]. Clone pHXB2gpt, which contains an *Xba*I/*Hpa*I viral DNA fragment from λHXB2D inserted into plasmid vector pSP65gpt, was used to generate genomic constructs lacking either the major coding region (pHXB2ΔSal-Sst) or the splice acceptor sequences

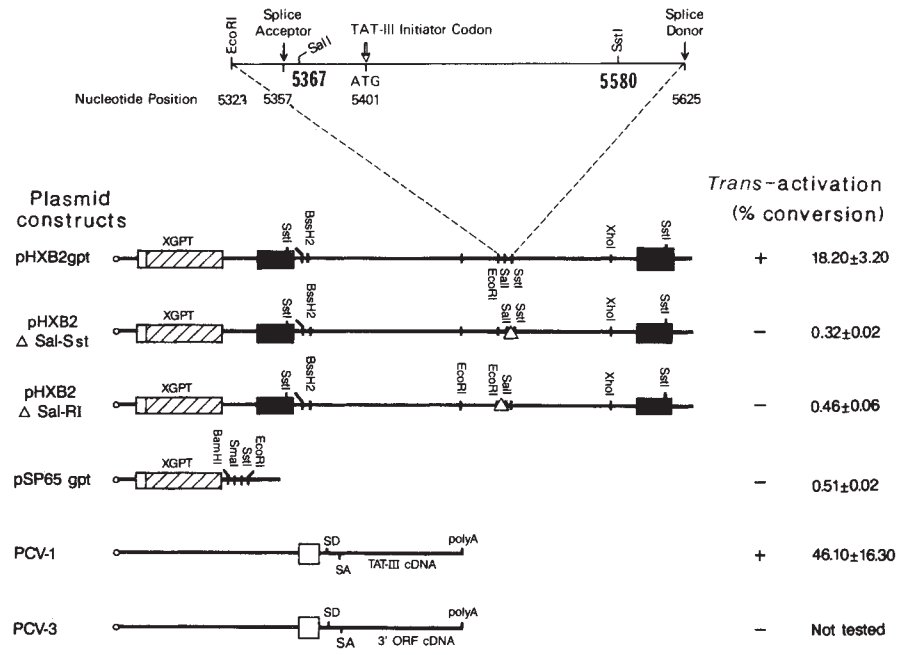

Fig. 1 Construction of HTLV-III plasmids and analysis of CAT activity. The precise nature of the deletions shown was confirmed by nucleotide sequence analysis. The nucleotide positions shown correspond to those published for BH10 (ref. 1). Closed boxes depict LTR sequences, cross-hatched boxes depict the *Escherichia coli* xanthine-guanine phosphoribosyl transferase gene (*xgpt*)[29] and open triangles show regions deleted from the HTLV-III genome. Open squares shown in plasmids pCV-1 and pCV-3 (ref. 6) represent adenovirus middle late promoter (MLP) sequences[30].

Methods. An 11.5-kb *Xba*I/*Hpa*I fragment containing full-length HTLV-III proviral DNA was excised from the phage clone λHXB2-D (ref. 31) and inserted into the *Bam*HI and *Eco*RI sites of the plasmid pSP65gpt. Clone pHXB2gpt contains the viral insert in the same transcriptional orientation as the *E. coli xgpt* gene. pHXB2gpt was used for the construction of pHXB2ΔSal-Sst and pHXB2ΔSal-RI plasmids. Construct pHXB2ΔSal-Sst is deleted of nucleotides 5,367–5,580 and hence lacks the entire first coding exon of *tat*-III (exon 2), including the initiating methionine. It was generated from pHXB2gpt by a *Sst*I partial restriction digest followed by digestion to completion with *Sal*I, end repair with T4 DNA polymerase and re-closure with T4 DNA ligase. Plasmid pHXB2ΔSal-RI is deleted of nucleotides 5,323–5,367 and hence lacks the splice acceptor sequence thought to be preferentially used to generate mature *tat*-III mRNA[6], while the entire *tat*-III coding sequence is unaffected. The clone was constructed by an *Eco*RI partial restriction of pHXB2gpt, followed by complete digestion with *Sal*I, repair with the Klenow fragment of *E. coli* DNA polymerase and re-closure with T4 ligase. Plasmids pCV-1 and pCV-3 contain the complete cDNA of the spliced *tat*-III and *3'orf* mRNA, respectively[6]. The ability of plasmid clones to *trans*-activate genes linked to HTLV-III LTR sequences was assessed as follows: 10 μg of DNA from the recombinant plasmid pCD12CAT (containing the bacterial gene for CAT under the regulation of the HTLV-III LTR[26]) was mixed with 10 μg of DNA from each test plasmid individually and transfected into 1.1×10^7 H9 lymphoid cells. The transfections were performed by incubating the cells in RPMI 1640 media containing 250 μg ml^{-1} DEAE-dextran, 50 mM Tris-HCl *p*H 7.3 for 1 h at 37 °C. Cells were washed with complete media (RPMI 1640, 10% fetal calf serum (FCS) and 50 μg ml^{-1} gentamicin) and maintained for 48 h at 37 °C in complete media. The cells were collected, washed in phosphate-buffered saline and resuspended in 1 ml of 0.04 M Tris-HCl *p*H 7.4, 0.15 M NaCl and 0.001 M EDTA, transferred to Eppendorf tubes and pelleted. Cells were resuspended in 0.25 M Tris-HCl *p*H 7.8 and lysed by freeze-thawing three times. After pelleting cell debris (5 min in Beckman microfuge), supernatants were transferred to new Eppendorf tubes and heated to 60 °C for 10 min. CAT activity was determined on 20-μl aliquots incubated with ^{14}C-chloramphenicol and acetyl CoA as previously described[23]. Chloramphenicol and acetylated metabolites were separated by ascending TLC and visualized by autoradiography. Radiolabelled chloramphenicol and derivatives were cut from the plates and quantitated by liquid scintillation counting. CAT activity was determined as counts per min (c.p.m.) of acetylated metabolites of chloramphenicol expressed as a percentage of the total c.p.m. The results shown are the means and standard deviation of three independent co-transfections for each test plasmid. Transfection of RSVCAT[32], SVOCAT[32] and pCD12CAT alone gave values of 48.0±6.9, 0.31±0.036 and 1.41±0.13, respectively. RSVCAT thus served as a positive control for each experiment, while SVOCAT, which lacks a functional promoter, is a negative control. Levels of CAT activity significantly above the values for pCD12CAT alone were indicative of *trans*-activating activity.

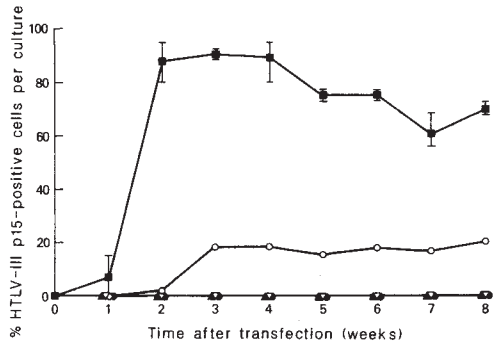

Fig. 2 Comparison of virus expression in H9 cultures following transfection with HTLV-III plasmid derivatives. The percentage of cells expressing HTLV-III p15 antigen was assessed by immunofluorescence using the monoclonal antibody BT2 and standard protocols[33]. The results shown are the mean value and range obtained following three independent transfections with plasmids pHXB2gpt (■), pHXB2ΔSal-Sst (▲), pSP65gpt (▽) and pHXB2ΔSal-RI (cultures 1 (●), 2 and 3 (○)).

Methods. H9 cells (2.5×10^6) were transfected with 10^{10} bacterial protoplasts using a protoplast fusion technique[9]. The cells were maintained in culture at a density of 5×10^5 to 2×10^6 per ml for 8 weeks in RPMI 1640 media containing 20% fetal calf serum/antibiotics, and HTLV-III virus production assayed by electron microscopy 1, 2, 4, 6 and 8 weeks after transfection.

(pHXB2ΔSal-RI) of *tat*-III. In pHXB2ΔSal-Sst, nucleotides 5,367–5,580 (from the *Sal*I/*Sst*I sites) have been deleted, removing most of the *tat*-III coding sequences of the second exon together with sequences upstream of the *tat*-III initiation codon (position 5,401) (Fig. 1). Plasmid pHXB2ΔSal-RI has lost nucleotides 5,323–5,367 (from the *Sal*I/*Eco*RI sites) and hence lacks the splice acceptor (position 5,357) thought to be preferentially used to generate mature *tat*-III messenger RNA[6], while the entire coding sequence remains unaffected.

The extent to which these deleted genomes are capable of *trans*-activating genes linked to the HTLV-III LTRs was assessed in a series of DEAE-mediated co-transfection experiments[25] using pCD12CAT as an indicator[26]. Chloramphenicol acetyltransferase (CAT) activity was determined 48 h after transfection as the amount of cytoplasmic ^{14}C-labelled chloramphenicol converted to acetylated metabolites, expressed as a percentage of the total. Transfection of H9 cells with pHXB2gpt + pCD12CAT significantly augmented CAT activity (18.2%) as compared with transfection using pCD12CAT alone (0.03%) or pCD12CAT + pSP65gpt (0.5%) (Fig. 1), consistent with pHXB2gpt containing a biologically functional *tat*-III gene[26]. Co-transfection of pCD12CAT with pHXB2ΔSal-Sst or pHXB2ΔSal-RI gave values of 0.3% and 0.4%, respectively. Thus, in standard assays, *trans*-activation was not detected using genomes that lacked either the second exon coding sequences or the splice acceptor site of the *tat*-III gene. These results are consistent with reports of the location of *trans*-activator gene of HTLV-III and its normal splicing[6,7].

To assess the capacity of *tat*-III-defective genomes for productive virus replication, constructs pHXB2ΔSal-Sst and

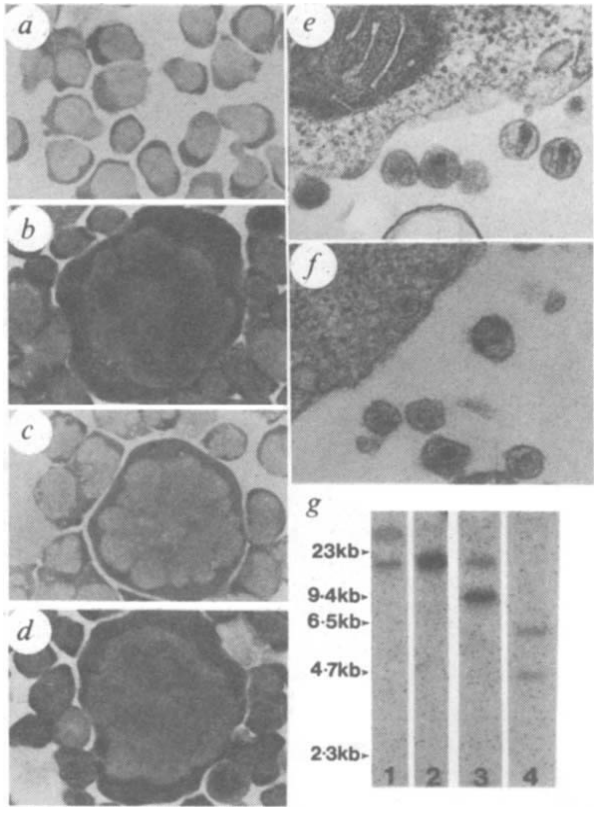

Fig. 3 Demonstration of multinucleated cells, virus particles and HTLV-III DNA sequences in cultures transfected with HTLV-III plasmid derivatives. Three to five days after transfection, H9 cells were removed from culture, cytocentrifuge preparations made and stained with Wright-Giemsa stain to reveal nuclear morphology. *a–d*, Samples from cultures transfected with pSP65gpt (*a*), pHXB2gpt (*b*), pHXB2ΔSal-Sst (*c*) and pHXB2ΔSal-RI (*d*). Multinucleated cells at frequencies of >1 to 5% of total were observed in samples *b*, *c* and *d*. *e*, *f*, Electron micrographs of virions produced 2 weeks after transfection with pHXB2gpt and pHXBΔSal-RI, respectively. In the case of pHXB2gpt, the virions had typical HTLV-III morphology, were abundant and budding particles readily observed. In the case of pHXB2ΔSal-RI, both characteristic HTLV-III virions and particles with abnormal morphology (irregular shape and cores) were evident and very few particles were found. Budding virus was not evident in these cultures 1–4 weeks after transfection but was occasionally demonstrated thereafter. *g*, Southern blots of DNA prepared from H9 cells 2 weeks after transfection with pHXB2ΔSal-Sst (lanes 1–3) or pHXB2gpt (lane 4) DNA of high M_r was isolated using standard protocols[34]. DNA (5 μg) was digested separately with the enzymes *Xba*I (lane 1), *Bam*HI (lane 2) or *Sst*I (lanes 3, 4) electrophoresed in 0.8% agarose gels, blotted and hybridized to probe prepared from nick translation of the *Sst*I/*Sst*I fragment of the phage clone λ BHIO[31]. Nitrocellulose acetate filters were washed in 0.5 × SSC, 0.1% SDS at 65 °C for 2–4 h before exposure. Digestion of H9/pHXB2ΔSal-Sst DNA with *Xba*I (an enzyme that does not cut the viral genome but has a single site in the plasmid) yields a single band at 17 kb, corresponding to linearized plasmid in these cells, and a high-M_r smear (>23 kb), suggesting integration of the transfected DNA. Digestion with *Bam*HI (an enzyme that cuts at a single site in the HTLV-III sequences of the plasmid) reveals a single 17-kb band, consistent with the presence of plasmid DNA in transfected cells. *Sst*I digestion of DNA from pHXB2ΔSal-Sst-transfected cultures shows an intense band of 9.5 kb and a less intense band of 17 kb. The former corresponds to the *Sst*I/*Sst*I viral insert which is predicted following the construction of pHXB2ΔSal-Sst from pHXB2gpt. The latter probably represents residual partially digested plasmid. *Sst*I digestion of H9/pHXB2gpt DNA (lane 4) shows two bands at 5.8 and 3.7 kb, representing the fragments predicted from digestion of unintegrated proviral DNA with this enzyme.

pHXB2ΔSal-RI, pHXB2gpt and pSP65gpt, were introduced into H9 cells by protoplast fusion. This technique has been used by our own and other laboratories to transfect and stably express genes in lymphoid cells[9,27]. Figure 2 summarizes time-course experiments in which the frequency of cells expressing HTLV-III p15 (*gag*-related protein) was assessed following protoplast fusion. One week after transfection with pHXB2gpt, 1–15% of H9 cells expressed HTLV-III p15 and extracellular and budding virions were evident. The number of HTLV-III-expressing cells increased to 80–90% within the first 2 weeks and was maintained at a high level throughout the experiment. In contrast, no virus- or p15-expressing cells were detected after transfection with pHXB2ΔSal-Sst. These results indicate that *tat*-III is crucial for HTLV-III production. Southern blots (see Fig. 3*g*) prepared from these cells and probed for HTLV-III sequences showed the presence of linearized unintegrated plasmid DNA (a 17-kilobase (kb) band, lanes 1, 2) and integrated HTLV-III sequences (a high relative molecular mass (M_r) smear >23 kb, lane 1). Hence, the failure of H9/pHXB2ΔSal-Sst cultures to produce virus was not due to failure to introduce this DNA into cells. Notably, digestions with enzymes that do not cut in the HTLV-III sequence (*Xba*I, Fig. 3*g*, lane 1) or cut at a single site in the plasmid (*Bam*HI, lane 2) failed to reveal a 9.5-kb band, corresponding to unintegrated HTLV-III DNA in pHXB2ΔSal-Sst-transfected cells. The absence of unintegrated viral DNA may be an indication of lack of virus replication.

Transfection of clone pHXB2ΔSal-RI resulted in virus expression in two of three cases. In these, low-level expression (<2% of cells) of p15 was demonstrated 2 weeks after transfection. Electron micrographs revealed small numbers of exclusively extracellular particles, many of which had unusual morphology compared with typical virions (see Fig. 3*f* and *e*, respectively). These aberrant particles may represent true defective particles or simply degenerating virions. The proportion of cells expressing p15 increased to ~30% by week 3 and reached a plateau at 10–20% in successive weeks.

These results have two implications. First, the molecular clone pHXB2ΔSal-RI is capable of generating HTLV-III virus, but at markedly reduced levels compared with the parental pHXB2gpt plasmid (we estimate that cultures transfected with the former contained 10 times fewer particles). Hence, we suggest that this clone, deprived of the normal splice acceptor used to generate *tat*-III, is able to use alternative splice acceptor sites in the 5′ region of the genome to generate mature *tat*-III mRNA. Alternatively, we can postulate that *tat* is being read off a polycistronic message which includes *sor-tat-3′orf*. Complementary DNA clones corresponding to these sequences have been identified and shown to *trans*-activate in the CAT assay (S. K. Arya *et al.*, in preparation). An apparent inconsistency with these hypotheses is the failure to demonstrate *trans*-activation with plasmid pHXB2ΔSal-RI. To clarify this point, we performed extended CAT assays on the virus-positive and -negative pHXB2ΔSal-RI-transfected cultures. Cultures 1 (virus negative) and 2 (virus positive) (Fig. 2), assayed 8 weeks after transfection, gave conversions of ^{14}C-chloramphenicol of 0.534 ± 0.068% and 52.47 ± 11.07%, respectively, in 13-h assays. The latter value is significantly greater than the conversion rates seen in uninfected cultures (0.5%), consistent with low levels of *trans*-activation in virus-positive, pHXB2ΔSal-RI-transfected H9 cultures. Second, the frequency of HTLV-III p15-positive cells in pHXB2ΔSal-RI-transfected cultures did not increase to approach 100%. Using an *in situ* technique capable of detecting infrequent cells expressing as few as 20–60 HTLV-III RNA transcripts per cell[28], we determined that 0% and 25% of cultures 1 and 2 (week 8) and 20% of culture 3 (week 4) expressed viral RNA. These results are comparable to immunofluorescence values and support the contention that virus generated from pHXB2ΔSal-RI is either defective or produced in too low amounts to spread efficiently through the culture. As the splice acceptor deleted in pHXB2ΔSal-RI may be used to generate *tat*-III and 3′*orf* mRNA, the reduced infectivity might be attributable to compromising either or both of these genes.

Figure 3*c*, *d* demonstrates the capacity of *tat*-III-defective constructs transiently (3–5 days after protoplast fusion) to induce H9 cell multinucleation at frequencies exceeding 1% (1–5%) and suggests that shortly after transfection with

a

PLASMID USED FOR TRANSFECTION	COMPLEMENTATION PARTNER	RELATIVE FREQUENCY OF HTLV-III EXPRESSING CELLS IN H9 TRANSFECTED CULTURES (time/days after transfection)								
		1	2	3	4	5	6	7	14	21
pHXB2 gpt	None	–	–	+	++	++	+++	++++	++++	++++
pSP65 gpt	None	–	–	–	–	–	–	–	–	–
pHXB2 ΔSal-Sst	None	–	–	–	–	–	–	–	–	–
pHXB2 ΔSal-Sst	pHXB2 gpt	–	–	–	+	+	++	+++	+++	++++
pHXB2 ΔSal-Sst	pCV-1	–	–	–	–	–	+	+	+++	–
pHXB2 ΔSal-Sst	pCV-3	–	–	–	–	–	–	–	–	–
pHXB2 ΔSal-Sst	tat-III protein	–	–	–	–	–	–	–	–	–

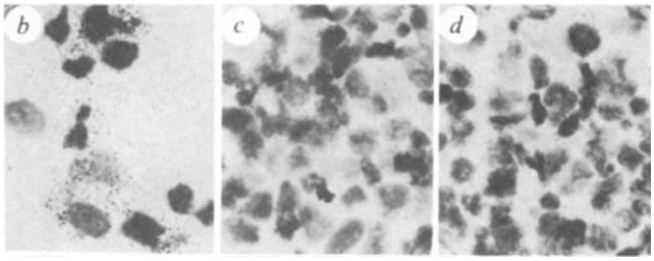

Fig. 4 Complementation of *tat* activity in H9 cells transfected with HTLV-III plasmids deleted in the *trans*-activator gene. ***a***, Time course of relative frequency of HTLV-III-expressing cells in H9-transfected cultures. Expression of viral *gag* protein p15 was assessed on a daily basis and the proportions of expressing cells are indicated on an increasing scale where – = none detected, + = few expressing cells, ++ = 0.1–1.0% positive cells, +++ = 1.0–10% positive cells and ++++ = 10–>99% positive cells. ***b–d***, Autoradiographs of transfected cells labelled by *in situ* hybridization for HTLV-III RNA. The number of grains (intensity of labelling per cell) indicates the relative abundance of HTLV-III RNA. *b*, *c*, H9 cell cultures 16 days after transfection with pHXB2ΔSal-RI. In the case of *b*, 48 h before *in situ* assay these cultures were transfected with protoplasts containing TAT-III protein. Comparison with parallel cultures not transfected with TAT-III protein (*c*) shows that expression is clearly elevated in the former, 30% and 1% of cells in cultures, corresponding respectively to *b* and *c*, being judged as positive (>20 grains per cell). H9 cells were also transfected with pHXB2ΔSal-Sst and 14 days later transfected with protoplasts containing TAT-III protein. In this case (*d*), we were unable to detect HTLV-III RNA 48 h later.

Methods. *a*, Using the protoplast fusion technique as described previously[9], 10^{10} bacterial protoplasts containing plasmids pHXB2ΔSal-Sst, pSP65gpt or pHXB2gpt were separately transfected into 3×10^6 H9 cells. In addition, 5×10^9 protoplasts containing pHXB2ΔSal-Sst were separately combined with equivalent amounts of pCV-1, pCV-3 and pHXB2gpt or bacterial protoplasts containing TAT-III protein, and the mixed preparations fused with 3×10^6 H9 cells. Bacterial protoplasts containing TAT-III protein were prepared as follows. *E. coli* strain AR120 carrying the plasmid pOTS-*tat*-III (ref. 35) were grown exponentially and induced to synthesize TAT-III protein by the addition of 60 μg ml^{-1} nalidixic acid[36], for 5 h at 37 °C. 10^{10} bacteria were collected, washed, treated with lysozyme to produce protoplasts and fused with H9 cells. The efficiency of TAT-III production following nalidixic acid treatment was checked by subjecting bacterial lysates to SDS-polyacrylamide gel electrophoresis and visualization of the separated proteins by Coomassie brilliant blue staining (data not shown). We estimate that the TAT-III protein accounts for 2–5% of the total cellular protein in the induced bacteria. ***b–d***, Cells were cytocentrifuged on to microscope slides, fixed and hybridized *in situ* according to Harper *et al.*[28]. The ^{35}S-labelled RNA probe was synthesized by transcription of clone pBH10-R3[28] and was specific for the 3′ portion (1–2 kb) of the HTLV-III genome.

pHXB2ΔSal-Sst or pHXB2ΔSal-R1, small numbers of cells transcribe HTLV-III envelope genes. This observation can in part be explained by reports that HTLV-III LTRs can act as efficient promoters in lymphoid cells[5,26], in the absence of *tat*-III.

Complementation experiments were performed to determine whether the failure of TAT-defective clones to generate HTLV-III efficiently could be compensated by the provision of a functional *tat*-III gene or the TAT-III protein itself (see Fig. 4). H9 cells transfected with pHXB2gpt alone or in combination with pHXB2ΔSal-Sst expressed HTLV-III p15 and virus particles 3–4 days after transfection, indicating that the simultaneous fusion of protoplasts containing different plasmids does not alter the efficiency of DNA transfection *per se*. HTLV-III-expressing cells were not detected following transfection of pHXB2ΔSal-Sst alone or in combination with pCV-3. However, following co-transfection of pHXB2ΔSal-Sst and pCV-1, HTLV-III p15-positive cells were apparent 6 (<0.01%) to 14 (2.8%) days after fusion and HTLV-III RNA-expressing cells confirmed in parallel *in situ* studies. These results show that the capacity for virus expression can be restored to a genome deprived of *tat*-III by the provision of an unlinked, functional *tat*-III gene. No virus-expressing cells were seen in the same cultures 21 days after transfection. This transient expression infers that complementation was achieved in *trans* and that decreased expression was coincident with reduced numbers of cells which stably retained both pHXB2ΔSal-Sst and pCV-1. Analysis of DNA prepared from these cells on day 14 showed that the proviral DNA corresponds exactly to that predicted from clone pHXB2ΔSal-Sst (data not shown), and excludes the generation of a competent virus by recombination.

The introduction of bacterially derived TAT-III protein into H9/pHXB2ΔSal-Sst cultures did not result in virus expression, whereas its addition to H9/pHXB2ΔSal-RI cultures caused a marked increase in virus expression within 48 h; approximately 1% of cells expressed HTLV-III RNA before the introduction of *tat*-III (Fig. 4*c*), compared with 30%, including heavily labelled (>1,000 grains per cell) multinucleated cells, 2 days after its introduction (Fig. 4*b*). Thus, a single introduction of TAT-III protein appears insufficient to restore virus production to a genome devoid of *tat*-III coding sequences, but sufficient to boost virus production in a genome containing a functionally compromised *tat*-III gene. This discrepancy may reflect a short biological half-life of the TAT-III protein. The observation that *tat*-III is essential for HTLV-III replication parallels a report that *tat*-II (otherwise called the *x* or *x-lor* gene of HTLV-II) mediates *trans*-activation of viral genes and is essential for replication of HTLV-II[21]. These data support the notion that *trans*-activation control systems are fundamental to the biology of this newly identified group of retroviruses. In addition, as *tat* activity seems to be essential for virus production, the development of inhibitors that specifically block the activity of these *trans*-acting factors provides a new approach to the treatment of AIDS patients and individuals infected with HTLV-III.

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A model of synthetase/transfer RNA interaction as deduced by protein engineering

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The recognition of transfer-RNA by their cognate aminoacyl-tRNA synthetases is the crucial step in the translation of the genetic code. In order to construct a structural model of the complex between the tyrosyl-tRNA synthetase (TyrTS) from *Bacillus stearothermophilus* and $tRNA^{Tyr}$, 40 basic residues at the surface of the TyrTS dimer have been mutated by site-directed mutagenesis and heterodimers created *in vitro* by recombining subunits derived from different mutants. As reported here a cluster of basic residues (Arg 207-Lys 208) in the N-terminal domain of one TyrTS subunit interacts with the acceptor stem of $tRNA^{Tyr}$ and two separated clusters of basic residues (Arg 368-Arg 371; Arg 407-Arg 408-Lys 410-Lys 411) in the C-terminal domain of the other subunit interact with the anticodon arm. The TyrTS would thus clamp the tRNA in a fixed orientation. The precise alignment of the flexible ... ACCA 3′ end of the tRNA for attack on the tyrosyl adenylate is made by contacts closer to the catalytic groups of the enzyme, such as with Lys 151.

TyrTS catalyses the aminoacylation of $tRNA^{Tyr}$ in a two-stage reaction. The tyrosine is first activated with ATP to form tyrosyl adenylate and pyrophosphate, then the adenylate is attacked by the 3′-terminal ribose of the tRNA to form Tyr-$tRNA^{Tyr}$ and AMP. TyrTS is a dimer which shows 'half-of-the-sites' reactivity, forming one tyrosyl adenylate and binding tightly one tyrosine and one $tRNA^{Tyr}$ per dimer in solution. The two subunits are related by symmetry through a 2-fold axis. Each subunit has an N-terminal domain (residues 1-319), which makes all the interactions with the tyrosyl adenylate and forms the subunit interface, and a C-terminal domain (residues 320-419), which is disordered in the crystal (reviewed in ref. 1). By creating a truncated TyrTS at the level of the gene, Waye *et al.*[2] have shown that the N-terminal domain of TyrTS catalyses the formation of tyrosyl adenylate with unchanged k_{cat} and K_M but does not charge and does not bind $tRNA^{Tyr}$; this shows that the C-terminal domain of TyrTS is essential for tRNA binding.

To identify residues of TyrTS that interact with $tRNA^{Tyr}$, we chose the following strategy: (1) We assumed that basic residues of the synthetase could form salt bridges with the phosphates of the tRNA backbone or hydrogen bonds with the nucleotide bases. (2) Because the *B. stearothermophilus* and *Escherichia coli* TyrTS have homologous sequences and similar properties[3,4], we considered mainly the conserved basic residues. (3) We changed the arginine and histidine residues to glutamine, and lysine to asparagine; such changes remove the charge but not the hydrophilic character of the residue. We therefore mutated 40 basic residues of the *B. stearothermophilus* TyrTS by oligonucleotide-directed mutagenesis of the encoding gene (*tyrS*) (see Fig. 1 legend).

To test the overall activity of the mutant synthetases, we devised an *in vivo* genetic complementation assay. In this assay, the *B. stearothermophilus tyrS* gene is carried by and expressed from a recombinant M13 phage[5]. The host is HB2111, an *E. coli* strain which harbours a thermosensitive mutation in its own *tyrS* gene, which is an essential gene. The HB2111 cells can grow at 42 °C, the non-permissive temperature, only if they are infected by a phage which directs the production of an active *B. stearothermophilus* TyrTS. Most of the 40 mutant phages could complement HB2111 and were therefore eliminated. However, 13 mutants were either unable to complement—KN82, RQ86, KN151, KN208, KN230, KN233, RQ368, RQ407, KN410 and KN411—or did so weakly—RQ207, RQ371 and RQ408 (Fig. 1).

The 13 TyrTS mutants identified from the complementation assay and the wild-type TyrTS were purified from phage-infected cells. All the mutant enzymes were able to form enzyme-bound tyrosyl adenylate, albeit slowly for mutants RQ86 and KN233 (half life, $t_{1/2} = 8$ and 27 min respectively, compared with 2 s for the wild-type enzyme)[6]. The pyrophosphate exchange assay showed that the mutant and wild-type enzymes had similar activities (4.6 s^{-1} at 2 mM ATP, 50 μM Tyr and 2 mM pyrophosphate) except for mutants KN82, RQ86, KN230 and KN233 (<0.23 s^{-1}) (see Table 1). Thus, although these four mutants are able to form tyrosyl adenylate and are presumably correctly folded, there is nevertheless a lesion in the activation step. Additional experiments are needed to determine whether the

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Table 1 Kinetic parameters for $tRNA^{Tyr}$ charging

Mutant	k_{cat} ($s^{-1} \times 10^3$)	K_M (μM)	k_{cat}/K_M ($s^{-1}\,M^{-1} \times 10^{-3}$)	ΔG_{app} (kcal mol^{-1})	Complementation
Wild type	450	1.4	315	0	+
KN151	3	1.2	2.3	2.9	−
RQ207	ND	>100	19.4	1.7	+/−
KN208	ND	>28	11.3	2.0	−
RQ368	ND	>100	2.4	2.9	−
RQ371	ND	>100	17.1	1.7	+/−
RQ407	ND	>100	11.9	1.9	−
RQ408	ND	>100	16.5	1.8	+/−
KN410	ND	>100	9.3	2.1	−
KN411	ND	>100	12.8	1.9	−

$\Delta G_{app} = -RT\ln(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}$ (mut, mutant; wt, wild type)[11]. For genetic complementation assay, see Fig. 1 legend. ND, not determined. Purification of the wild-type and mutant enzymes from phage-infected cells[7], active-site titration, pyrophosphate exchange and tRNA charging assays[11] were done as described elsewhere. We obtained different values for the wild-type TyrTS with pure *E. coli* $tRNA^{Tyr}$ (Boehringer: 1,000 pmol of tyrosine incorporation per A_{260} unit) and crude *E. coli* tRNAs ($K_M = 1.43$ versus 2.57 μM and $k_{cat} = 0.45$ versus 1.17 s^{-1}). The rate of charging by the wild-type enzyme gave a straight line in the Eadie-Hofstee plot (see ref. 12) only for pure $tRNA^{Tyr}$ concentrations less than 6 μM and was inhibited for higher values. We therefore used pure *E. coli* $tRNA^{Tyr}$ for the mutant enzymes, in the range 0.375-6 μM.