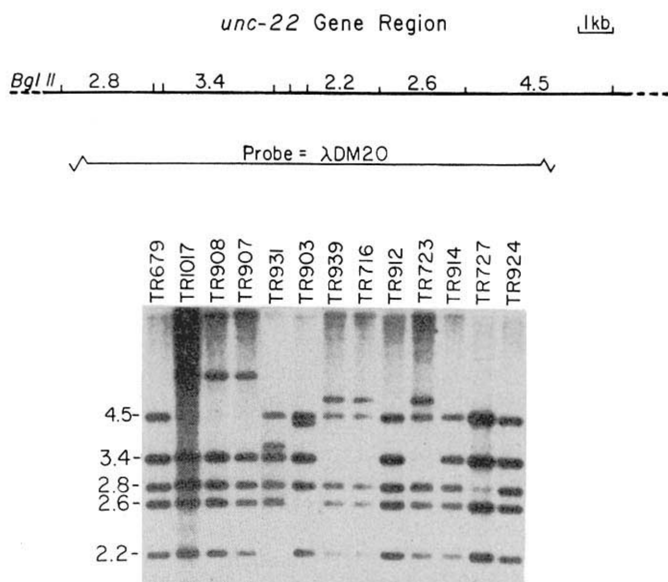


**Fig. 1** Southern blot analysis of mutator-induced *unc-22* mutations and their revertants. Each *unc-22* mutation results from an insertion of ~1.6 kb, and each revertant no longer contains the inserted material. Total genomic DNAs were digested with *Bgl*II and the restriction fragments were separated on a 0.8% agarose gel. The radiolabelled probe DNA was recombinant λ phage DM20, which contains ~14 kb of genomic material from the *unc-22* gene region[14]. Strain TR679 (genotype *mut-2(r459)* shows the wild-type pattern of *unc-22* restriction fragments. Strains TR1017, TR908, TR907, TR931, TR903, TR939, TR716, TR723 and TR727 each contain a spontaneous *unc-22* mutation induced by *mut-2(r459), mut-2(r459), mut-2(r459), mut(r458), mut(r457), mut-3(r456), mut(r454), mut-3(r456)* and *mut*-3(*r*456), respectively. For each restriction fragment affected by Tc*1* insertion, the 'empty site' products of somatic excision are detected as a minor fraction of DNA having wild-type mobility[15]. Strains TR912, TR914 and TR924 are spontaneous *unc-22*$^+$ revertants of strains TR716, TR723 and TR727, respectively.

transposition, mutator mutants show increased frequencies of X-chromosome nondisjunction. In *C. elegans*, sex is determined by a chromosomal XX/XO mechanism[17]. The proportion of male offspring arising from a hermaphrodite parent is a measure of the X-chromosome nondisjunction frequency[18]. Table 2, column 3, shows that each mutator mutant has a mild Him (*h*igh *i*ncidence of *m*ales) phenotype. We do not know if the Him phenotype is a direct result of high levels of Tc*1* transposition and excision, or whether it is due to pleiotropy of the *mut* mutations. We tested whether existing *him* mutants[18] are active for Tc*1* transposition. If *him* mutants have been activated for Tc*1* transposition, then the number and/or position of Tc*1* elements in their genomes should be altered. We performed total genomic Southern blots of *him-1(e879), him-2(e1065), him-3(e1147), him-4(e1267), him-5(e1467), him-6(e1423), him-7(e1480)* and *him-8(e1489)* using a Tc*1* hybridization probe. The number and position of Tc*1* elements in these strains are identical to that of the wild-type Bristol strain from which they were derived.

The number of different loci that can be mutated to yield a mutator phenotype is unclear. The Him phenotype of mutator mutants is semi-dominant, and this makes interpretation of complementation tests difficult. Genetic mapping experiments demonstrate that *mut-3(r456)* is linked to *unc-54* on chromosome I but that *mut-2(r459)* is not (data not shown). At least two loci, therefore, are represented among our mutants. We recovered mutator mutations at a frequency that is equal to or greater than the frequency of EMS-induced mutations affecting an average sized *C. elegans* gene[19]. This high frequency suggests that mutator mutations are loss-of-function alleles. Yet the semi-dominance of the Him phenotype suggests that mutator mutations are gain-of-function alleles. We suggest two explanations for these observations. Firstly, the genes affected by mutator mutations could display a haplo-insufficiency phenotype (such as mutations affecting a repression function). Secondly, a large number of genes could each be mutable to yield a mutator phenotype (such as mutations affecting the Tc*1* element itself).

Mutator mutants should prove to be an important tool for *C. elegans* molecular genetics. Because Tc*1* insertion causes most mutator-induced mutations, mutant genes can be cloned using 'transposon-tagging' techniques. Mutator mutants have high frequencies of spontaneous mutations affecting many different genes. Spontaneous mutations affecting over 50 visible, developmental, and behavioural genes have been identified from *mut-2(r459)* alone (*C. elegans* workers, personal communications).

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# The HIV '*A*' (*sor*) gene product is essential for virus infectivity

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**The genome of the human immunodeficiency virus (HIV) contains several open reading frames (ORFs) not present in other viruses. The '*A*' gene[1], also known as $Q^2$ $P'^3$, *ORF-1*[4] or *sor*[5], partially overlaps the *pol* gene; its protein product has a relative molecular mass of 23,000 ($M_r$ 23K) and is present in productively infected cells[7–10]. The function of this protein is unclear; mutant viruses deleted in '*A*' replicate in and kill CD4$^+$ lymphocyte lines[8], but the high degree of conservation of the deduced amino-acid sequence in nine different HIV isolates (80%) and the presence of analogous genes in HIV-2[11] and other lentiviruses suggest that the gene**

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**function is an important one. Here we describe a mutant virus deficient in the '*A*' gene which produces virion particles normally; however, the particles are ~1,000 times less infective than wild type. Transcomplementation experiments partially restore infectivity. The mutant virus spreads efficiently when virus-producing cells are co-cultivated with CD4$^+$ lymphocytes, however, indicating that HIV can spread from cell to cell in a mechanism that does not require the '*A*' gene product and probably does not require the production of infective virus particles.**

We have created a plasmid, pΔA, containing a mutant '*A*' gene by eliminating 620 base pairs (bp) between the *Nde*I site at position 5,158 and the *Eco*RI site at position 5,779 (Fig. 1). This plasmid and the infectious plasmid pNL432 were introduced into SW480 cells, which efficiently express transfected DNA[12,13], using the calcium phosphate coprecipitation technique[14,15]. Virus production could be detected within 24 h (data not shown), and no significant difference in the transient expression of either wild-type or the ΔA mutant plasmid was observed by immunoblotting or by electron microscopy and the reverse transcriptase (RT) activity in the culture fluids were indistinguishable (see Fig. 2, Sup [Day1], for example). These results indicate that the HIV '*A*' gene product is not important in the regulation of viral gene activity or in the assembly and release of progeny virions.

Although SW480 colon carcinoma cells can be directly infected with HIV[16], virus propagation is inefficient compared to CD4$^+$ lymphocyte cell lines. Thus transfection of SW480 cells measures virus production, not virus infectivity. The latter can be measured, by either cocultivating CD4$^+$ lymphocytes with virus-producing SW480 cells or infecting CD4$^+$ lymphocytes with cell-free supernatant from transfected SW480 cells. Initially, we used coculture with the CD4$^+$ lymphocytic leukaemia line A3.01[17]. The ΔA virions could infect A3.01 cells in this system (Fig. 2, top), but unlike pNL432 infection, which peaked between days 7 and 9 and persisted for an additional 10 days, the A3.01 cells continued to produce ΔA virus particles for at least 82 days (data not shown). Neither wild-type nor ΔA progeny could infect cells of the CD4$^-$ A2.01 line[18] (data not shown), indicating that expression of the CD4 molecule is required to spread virus by cocultivation.

Cell-free samples of ΔA and wild-type virus particles were prepared from the supernatants of SW480 cells transfected with pΔA and pNL432 by low-speed centrifugation (1,000 *g* for 10 min) and filtration through a 0.22 μm filter. A3.01 cells were infected with equivalent (RT units) amounts of each virus preparation and HIV replication was monitored (Fig. 2, bottom). Plasmid pNL432 gave a spreading infection detected on day 5 with a peak of RT activity occurring on day 11, but ΔA particles failed to infect the A3.01 cells during the same observation period, indicating that the mutant virions contain an intrinsic defect affecting an early event in the infectious cycle.

We used the pNLA3 plasmid, a derivative of the 1-11 '*A*' cDNA clone (Fig. 1), to attempt to complement the defect in pΔA. Equal amounts of pΔA and pNLA3 were cotransfected into SW480 cells; the infectious proviral DNA clone pNL432 and pΔA were used as positive and negative controls, respectively. Supernatants were harvested after 24 h, filtered and tested for infectivity on A3.01 cells. Approximately equivalent amounts, measured by RT activity, were used. As shown in Fig. 3, progeny particles were detected by day 8 in cells infected with the filtrate from the pNL432 transfection, but not when ΔA was used. Complementation of the ΔA defect by the pNLA3 clone was observed; virus was detected in infected A3.01 cells by day 19, after which gradually increasing amounts of progeny were synthesized. The slower kinetics observed can be explained if complementation generates infectious particles containing the mutant genome, which can initiate an exogenous infection but, like the ΔA mutant, can only spread via a cell-to-cell mechanism. Because this is not as efficient as infection induced by wild-type particles (see Fig. 2), the infection spreads with delayed kinetics.

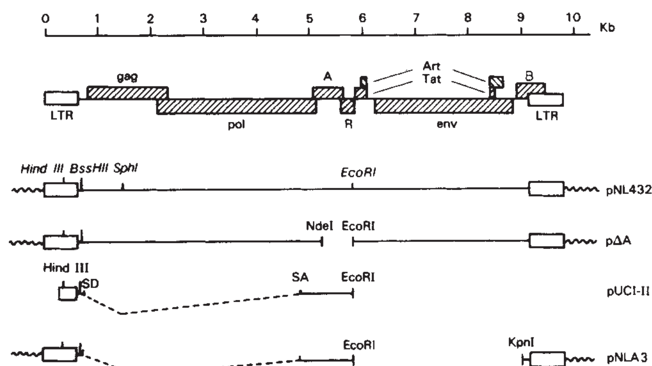


**Fig. 1** Structure of HIV clones used to characterize the '*A*' gene. Top, genomic structure of the HIV proviral DNA. The infectious cloned proviral DNA, pNL432 (ref. 12), was initially cleaved with *Nde*I. *Eco*RI linkers were ligated to the digestion products after the 5' overhanging *Nde*I ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I. The mixture was digested with *Sph*I and *Eco*RI generating the 3.7-kb fragment which maps from the *Sph*I site at position 1,443 to the *Nde*I site (containing the *Eco*RI linker) at position 5,158. This fragment was isolated from a preparative agarose gel and inserted into *Sph*I/*Eco*RI-digested pNL432, generating the pΔA plasmid, which contains only the amino-terminal 29 amino-acid residues of the '*A*' ORF. This deletion also eliminated 126 bp immediately 3' to the '*A*' gene, extending into the region designated R[20]. An oligo-dT primed λgt10 cDNA library was constructed from lysates of virus-producing H9 cells, which express the 5.5- and 5.0-kb '*A*' gene RNAs[6]. One clone (1-11) was obtained containing a 1.1-kb insert which was subcloned into pUC18 [pUC1-11] and M13mp19. The exact structure of the 1-11 cDNA clone was determined by nucleotide sequencing using the Sanger chain-termination method[22]. The cDNA began 16 nucleotides 3' to the cap site of all HIV mRNAs, extended an additional 273 bp to a splice donor at nucleotide 289, and was joined to nucleotide 4,459 in the *pol* ORF. Clone 1-11 therefore contains a 402 bp 5' untranslated leader sequence that precedes an ATG at nucleotide 4,587; this ATG is followed by an ORF encoding 192 amino acids. The cDNA terminates at the *Eco*RI site, 126 bp 3' to the end of the *A* ORF. The portion of the cDNA clone located between the *Bss*HII and the *Eco*RI sites was inserted into the infectious molecular plasmid pNL432, thereby placing a complete HIV LTR on both sides of the cDNA and retaining the splice characteristic of the '*A*' gene mRNA. The resulting plasmid (pNLA1, not shown) was digested with *Eco*RI and *Kpn*I to eliminate sequences between positions 5,779 and 9,026 encoding *tat, art, env* and parts of *R* and *B* and religated to generate pNLA3.

We wished to ascertain whether complementation or recombination was responsible for the appearance of progeny particles in these experiments. We prepared DNA by the Hirt procedure[19] from A3.01 cells which had been infected with 'complemented' virus, (pΔA+pNLA3), two weeks after the initial appearance of RT activity. The DNA was digested with *Hin*dIII and subjected to Southern blot hybridization using the 6.5-kilobase (kb)pBenn6 probe[17] (mapping between positions 1,712 and 8,188). Two fragments of 3.7 and 2.1 kb were found, consistent with the presence of ΔA proviral DNA; wild-type pNL432 proviral DNA gives fragments of 4.4 and 2.1 kb. Further, progeny virus particles, generated in A3.01 cells after inoculation with the 'complemented' filtrate behaved exactly like the ΔA mutant, failing to initiate a spreading infection in the A3.01 cells. Both of these results are compatible with transcomplementation but not recombination.

Higher multiplicities of ΔA virus give detectable infection of CD4$^+$ cells but with slow progeny particle synthesis compared to parental virus. The relative infectivity of ΔA particles, as determined by limiting dilution, is ~1,000-fold less than wild type (data not shown). Cell death and syncytia formation in ΔA-infected cells was observed, but at significantly lower levels than for wild-type HIV.

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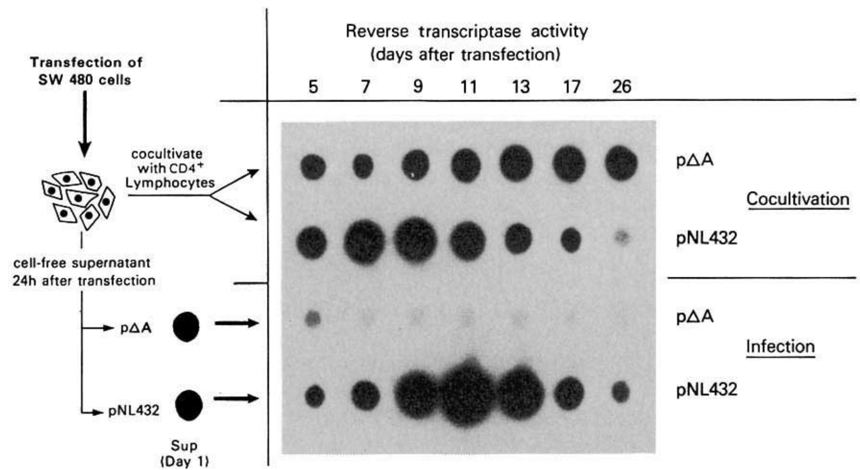


**Fig. 2** Effect of the '*A*' gene deletion on the infectivity of virus particles. Plasmid DNA (15 μg) from the infectious molecular HIV clone (pNL432) or the '*A*' gene deletion proviral DNA (pΔA) were transfected into SW480 cells growing in 25-cm$^2$ tissue culture flasks, using the calcium phosphate precipitation procedure[14,15]. In the cocultivation experiments, $2\times10^6$ A3.01 cells were added to the SW480 monolayer 24 h after transfection and the cultures were maintained in 5 ml of RPMI1640 medium containing 10% fetal calf serum for 26 days. Infectivity of progeny virions, present in the supernatants of the transfected cells, was evaluated following filtration and determination of RT activity[13]. Comparable amounts (pNL432: $1.1\times10^5$ c.p.m.; pΔA: $9.2\times10^4$ c.p.m.) of cell-free virus were used to infect $2\times10^6$ A3.01 cells propagated in 5 ml of RPMI medium. Aliquots were tested for RT activity at the indicated times. The cell density in all cultures was maintained at $\sim1\times10^6$ ml$^{-1}$.

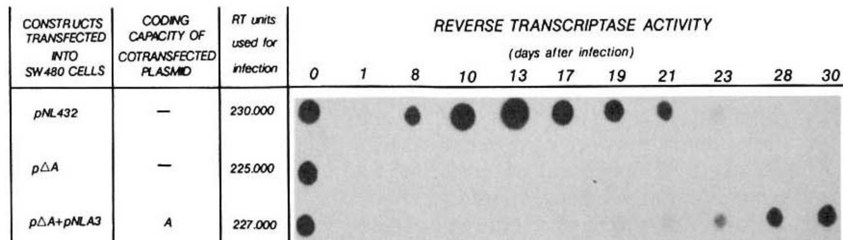


**Fig. 3** Complementation of the '*A*' gene mutation with the cDNA clone. SW480 cells were transfected with the indicated plasmids (15 μg). In the case of pNL432 and pΔA, the amount of DNA was adjusted to 30 μg by the addition of pUC18. Virus stocks were harvested from SW480 cells 24 h after transfection. Comparable amounts of virus particles, as determined by RT activity, in the cell-free filtrates were added to $2\times10^6$ A3.01 cells as described in Fig. 2. Cells were cultivated at a density of $\sim1\times10^6$ cells ml$^{-1}$ and aliquots of the culture fluid were tested for RT activity at the indicated times.

As virus lacking the '*A*' gene can spread in cocultures, some property of the mutant virions must preclude efficient initiation of productive infection. The fact that replication-competent virus particles can be generated by transcomplementation raises the possibility that the 'A' protein may be required for adsorption, penetration, endocytosis, or uncoating and suggests that it is virion-associated. If the 'A' protein is not an integral component of HIV particles, it could serve a scaffolding function required during packaging. Preliminary results showing that ΔA particles can support an endogenous RT reaction *in vitro* suggest that the mutant virions contain RNA (data not shown).

The existence of the short *R* ORF in both the HIV and visna virus genomes was recently reported[20]. As the 620-bp deletion present in pΔA also eliminates 63 codons from the amino terminus of the putative *R*-gene product, it was important to show that the ΔA phenotype reflected changes only in the HIV '*A*' gene. We therefore made a mutant containing an alteration in the *R* ORF (pNLΔRI) by digesting the infectious HIV clone at the single *Eco*RI site (see Fig. 1), filling in the termini with the Klenow fragment of *E. coli* DNA polymerase I, and ligating the flush ends with T4 DNA ligase. This generated a frame shift that terminates the 96 codon *R* gene at codon 63. The ΔR particles resulting from the transfection of SW480 cells with pNLΔRI, did not show the defect characteristic of ΔA virions. Furthermore, complementation of pΔA with pNLA3 (Fig. 3), which contains only a portion of the *R* ORF, indicates that the ΔA phenotype is not due to a concomitant deletion in the *R* gene. Cotransfection of pΔA with pAR(*tat*)[21], a plasmid which expresses the HIV tat protein, does not complement the ΔA mutation (data not shown).

The mechanism by which the ΔA infection spreads from cell to cell is presently unclear. As the recipient cells must express the CD4 epitope it is very likely that fusion is involved. HIV structures passed to recipient cells may include replication-competent RNA-protein complexes or unintegrated proviral DNA. Integration of the latter into chromosomal DNA would sustain a spreading productive infection and avoid many of the early events associated with an exogenous infection.

Cell-to-cell spread of HIV has been suggested as a possible explanation for the chronic nature of the viral infection *in vivo* which persists despite the antibody response. We have shown here that for the ΔA mutant this is the principal mechanism of virus spread. Although not formally demonstrated, it must be assumed that wild-type HIV can also spread cell-to-cell. This result has implications for the epidemiology of HIV infection as well as strategies of antiviral and vaccine intervention.

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