STABLE TRANSFECTION OF PROVIRUS OF HUMAN IMMUNODEFICIENCY VIRUS INTO A MURINE PACKAGING CELL LINE

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Summary. – In order to generate HIV (murine leukemia virus (MuLV)) pseudotypes, HIV genome was transfected into the ecotropic murine packaging cell line (GP+E86) and four of the nine transfected clones were extensively characterized. One clone (801), harbouring a full copy of integrated HIV sequences, exhibited a detectable level of intracellular HIV p24 antigen expression. Northern blot analysis revealed that clone 801 expressed all three classes of HIV mRNAs. Multispliced 2kb mRNAs were detected in another clone (8.14). Two other clones (1.31 and 1.32) also exhibited a complete HIV provirus, but did not show any viral expression, as evaluated by Northern blot analysis or HIV p24 ELISA. Reverse transcription-polymerase chain reaction (RT-PCR) experiments revealed the presence of full length genomic RNA in four transfected clones, which were extensively characterized. A co-cultivation of clone 801 with human $CD4^+$ cells resulted in syncytia formation. By electron microscopy, mature HIV particles were observed after co-cultivation of uninfected C8166 cells with 801 cells. These results demonstrated that the murine clone was stably transfected with the complete HIV genome and was capable of shuttling infectious HIV to human cells. Clone 801 was co-cultivated with murine NIH-3T3 fibroblasts. In several experiments, HIV infection of NIH-3T3 cells was revealed by PCR technique. Thus, 801 cells appear to produce low levels of HIV (MuLV) pseudotypes capable of transferring the HIV genome into mouse cells.

Key words: human immunodeficiency virus; provirus; transfection; packaging cells; pseudotypes; murine leukemia virus

A considerable progress has been achieved in our knowledge of the epidemiology of acquired immunodeficiency syndrome (AIDS) and its etiologic agent (HIV), but little is known about the pathogenesis of the infection (Pantaleo and Fauci, 1995). Therefore, it is important to establish *in vivo* model systems to study some aspects of the pathogenesis of AIDS and to define the antiviral therapy. In this respect, a small and well characterized laboratory animal, such as the mouse, could be especially useful (Ausiello *et al.*, 1994).

It is widely accepted that the primary determinant of susceptibility to HIV infection is the expression of the human CD4 protein on the cell surface (Leonard *et al.*, 1988). Mouse cells do not express functional HIV receptors similar to the human CD4 protein. A possible approach for an extension of the host range of HIV to murine cells could be represented by the generation of HIV/MuLV pseudotypes. Phenotypic mixing is a well-known biological phenomenon that involves enveloped viruses (Weiss *et al.*, 1982). Several authors reported a pseudotype formation by co-infecting human cells with HIV and amphotropic or xenotropic retroviruses (Chesebro *et al.*, 1990; Canivet *et al.*, 1990;

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Abbreviations: AIDS = acquired immunodeficiency syndrome; ELISA = enzyme-linked immunosorbent assay; HIV = human immunodeficiency virus; MuLV = murine leukemia virus; PCR = polymerase chain reaction; RT = reverse transcriptase

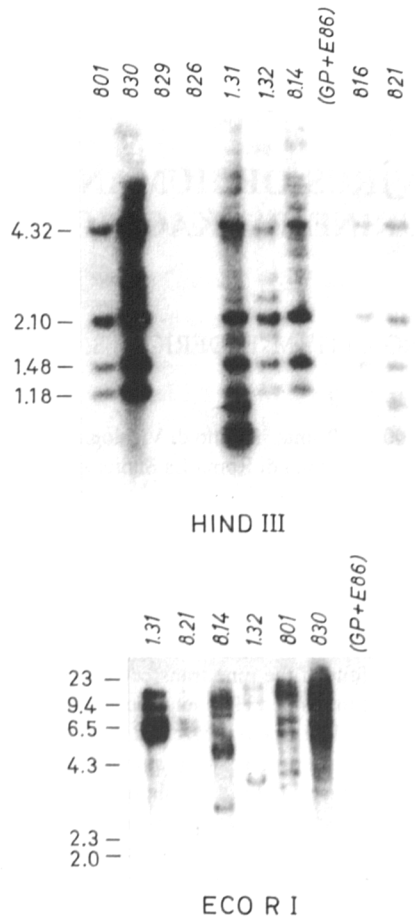

Fig. 1
Southern blot analysis of high molecular mass DNAs extracted from HIV-transfected murine clones

Twenty µg samples of high molecular mass DNAs extracted from the G418-resistent clones were digested with either *Hind*III or *Eco*RI and applied to horizontal 0.7% agarose gels, electrophoresed at 24 V for 48 hrs, blotted to Nylon-N membranes, and hybridized for 24 hrs with 1 x 10^6 cpm/ml of a full length ^{32}P-random-labelled HIV genome. *Hind*III-digested DNAs: the numbers on the left indicate M_r of the fragments. *Eco*RI-digested DNAs: the numbers on the left indicate M_r of the lambda DNA-*Hind*III markers. It should be pointed out that the Southern blot analysis, performed by our protocol, cannot be considered a quantitative assay.

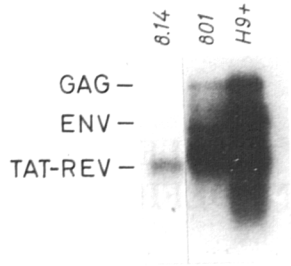

Fig. 2
Northen blot analysis of total cellular RNA from H9/HTLVIIIB-HIV- infected H9'cells and clones 801 and 8.14

Total cellular RNA was prepared by RNAzol B as described by the manufacturer (Cinna/ Biotecx), electrophoresed in 1.0% agarose-formaldehyde gel, blotted to a Nylon-N membrane, and probed with the same ^{32}P-labelled probe as in Fig. 1 under high stringency conditions. GAG, ENV, TAT-REV: bands of mRNAs of the respective genes.

Lusso *et al.*, 1989; Spector *et al.*, 1990). This experimental approach had some disadvantages as the pseudotype preparation led to the production of replication-competent murine retroviruses.

In the present study, we attempted to generate HIV (MuLV) pseudotypes by transfecting the complete HIV genome in the ecotropic murine packaging cell line (GP+E86) (Markowitz *et al.*, 1988). In this approach, we could use HIV as the only replication-competent virus. In fact, packaging cell lines already contain all the genes necessary to generate complete murine retrovirus virions, but are devoid of the corresponding retroviral genome (Kriegler *et al.*, 1990). The packaging cell line (GP+E86) was generated by co-transfecting two plasmids encoding the *gag/pol* and *env* genes of the Moloney MuLV, respectively, into NIH-3T3 cells (gift of Dr. A. Bank, Markovitz *et al.*, 1998).

Clone (GP+E86) was transfected with the pNL4-3 plasmid, containing the complete infectious genome of HIV (Adachi *et al.*, 1986). Transfection assays were carried out by the calcium phosphate precipitation technique (Graham and Van der Eb, 1973; Pulciani *et al.*, 1982). Transient expression experiments were performed to assess HIV expression in the (GP+E86) cells. The packaging cells were seeded at a density of 6.5 x 10^4 cells per 10 cm Petri dish and, one day later, were transfected with 50 µg of pNL4-3 plasmid with 40 µg of salmon sperm DNA as a carrier, or with 40 µg of the carrier alone as a control. At days 5 and 8 after DNA transfection, the cell pellets and supernatants of the test and control samples were collected and analyzed for HIV production. By ELISA, HIV p24 antigen was detected in the cellular lysates of (GP+E86) cells transfected with pNL4-3 plasmid, collected 8 days after DNA transfection. These results demonstrated that the (GP+E86) packaging cell line was able to express and synthesize HIV proteins once transfected with HIV genome, and thus proved suitable for generating HIV (MuLV) pseudotypes by the transfection procedure (Rozera *et al.*, 1991).

The experiments to isolate (GP+E86) clones stably transfected with HIV genome were performed following similar procedures, using the SV2neo plasmid as a selection marker, enabling the recipient eukaryotic cells to grow in the presence of the drug G418 (500 µg/ml). The pNL4-3 and SV2neo plasmids were co-transfected to cells at a ratio of 10:1, and several G418-resistant colonies were isolated. The

established clones were analyzed to assess the presence of HIV genome (Sambrook *et al.*, 1989).

The HIV genetic sequences were detected by Southern blot analysis, using the provirus cloned from the F12 cell line (which has 98% homology with the HIV provirus used in the transfection experiments) as a probe (Carlini *et al.*, 1992). *Hind*III cuts NL4-3-HIV sequence at 5 sites, thus generating 4 fragments of 4.32, 2.10, 1.48 and 1.18 K which in total represent the complete viral genome, except for two fragments of 0.3 and 0.6 K in the LTR regions. *Eco*RI, on the other hand, cuts at a single site only.

The DNAs extracted from several clones showed 4 bands of M_r corresponding to the expected viral genome *Hind*III-fragments (Fig. 1). The same DNAs presented several bands when digested with the *Eco*RI (Fig. 1). These results showed that the clones had integrated the HIV-transfected provirus in their chromosomes.

The selected clones produced high levels of extracellular murine Mn^{2+}-dependent RT activity, but no detectable Mg^{2+}-dependent RT activity. There was no evidence of virus production, except for one of the clones, as evaluated by p24 ELISA (Aldovini and Walker, 1990). Low levels of intracellular HIV p24 antigen were detected only in clone 801 (data not shown).

All three classes of viral mRNA were detected in clone 801 by Northern blot analysis (Ratner *et al.*, 1985). Fully spliced mRNA was detected in clone 8.14 (Fig. 2). On the other hand, using the RT-PCR procedure it was possible to detect the full length viral mRNA in clone 8.14 and two other clones (1.31 and 1.32), both containing a full copy of the transfected provirus (Fig. 3). The full length genomic mRNA was detected using the primers SK38 and SK39, and the probe SK19, located in the inner *gag* gene (Ou *et al.*, 1988). The RNAs amplified by RT-PCR were always treated with DNAase and then tested with the appropriate oligonucleotides, primers and probes for murine β-globin genes to exclude the presence of contaminating DNA. It should be pointed out that the RT-PCR procedure performed according to our protocol cannot be considered a quantitative assay.

In order to evaluate the capability of clone 801 to produce HIV particles which could infect human cells, the HIV-transfected clone was co-cultivated with the human cell line C8166 ($CD4^+$). These co-cultivation experiments were performed by seeding 5 x 10^4 mouse cells per 3.5 cm Petri dish, allowing them to attach and thereafter adding the human cells at a concentration of 1 x 10^4 cells/ml. The HIV production was first monitored by syncytia formation at various times after co-cultivation: the human cells, co-cultivated with 801 cells, showed relevant syncytia formation on day 10. Moreover, the co-cultivated human cells showed Mg^{2+}-dependent RT activity in the medium and the presence of intracellular and extracellular p24 viral antigen. After

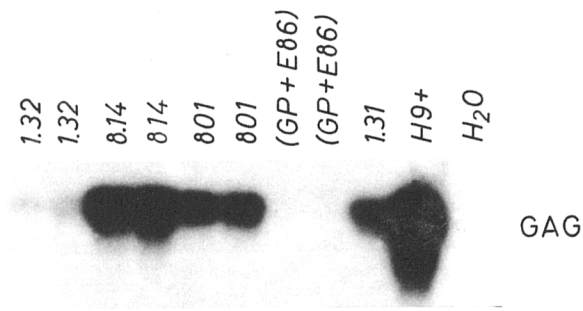

Fig. 3
RT-PCR analysis of four HIV-transfected clones
Total RNA was extracted from the selected clones, treated with DNase and amplified by RT-PCR according to the manufacturer's protocol (Perkin-Elmer Cetus) using primer pairs specific for *gag* sequences.

co-cultivation with 801 cells, C8166 cells proved to express and produce mature HIV particles, as revealed by electron microscopy (Fig 4A,B). In fact, numerous HIV virions were found in close proximity to the cell surface of the co-cultivated C8166 cells, while no HIV particles could normally be detected near the plasma membrane of 801 cells. In particular, the electron microscopy of 801 cells showed only murine retroviral particles (Fig. 4C,D,E), which closely resembled a C type oncovirus. Both mature (Fig. 4E) and immature (Fig. 4C, arrow) retroviral particles were observed in the extracellular space of 801 cells, along with typical retrovirus budding profiles at the cell surface (Fig. 4C, arrowhead; 4D). The fact that no apparent production of HIV particles could be detected by electron microscopy of 801 cells (despite of their capability to infect human cells in the co-culture experiments) suggests that HIV-transfected 801 cells express very low levels of HIV particles capable of infecting permissive cells. This would most likely happen by cell-to-cell transfer.

To determine whether the infectious HIV particles expressed by 801 cells had acquired the capability to infect mouse cells, we co-cultivated the HIV-transfected cells with the murine fibroblasts NIH-3T3. These co-cultivation experiments were carried out by use of microporous membranes (Falcon 3090). These membranes are specifically designed to allow co-cultivation procedures with exchange of fluids or fluid components, but without cell-to-cell contact. NIH-3T3 cells were seeded at a concentration of 5 x 10^2 cells/well. One day later, 801 cells were seeded (5 x 10^3 cells/well) on the porous membranes and added to each well, containing the murine fibroblasts. Once 801 cells had reached the confluency, the porous membranes were replaced with new ones containing exponentially growing cells. On average, the porous membranes were changed every 3-4 days, until NIH-3T3 cells reached confluency. Then they were collected and analyzed for the presence of acquired HIV sequences. DNA was ex-

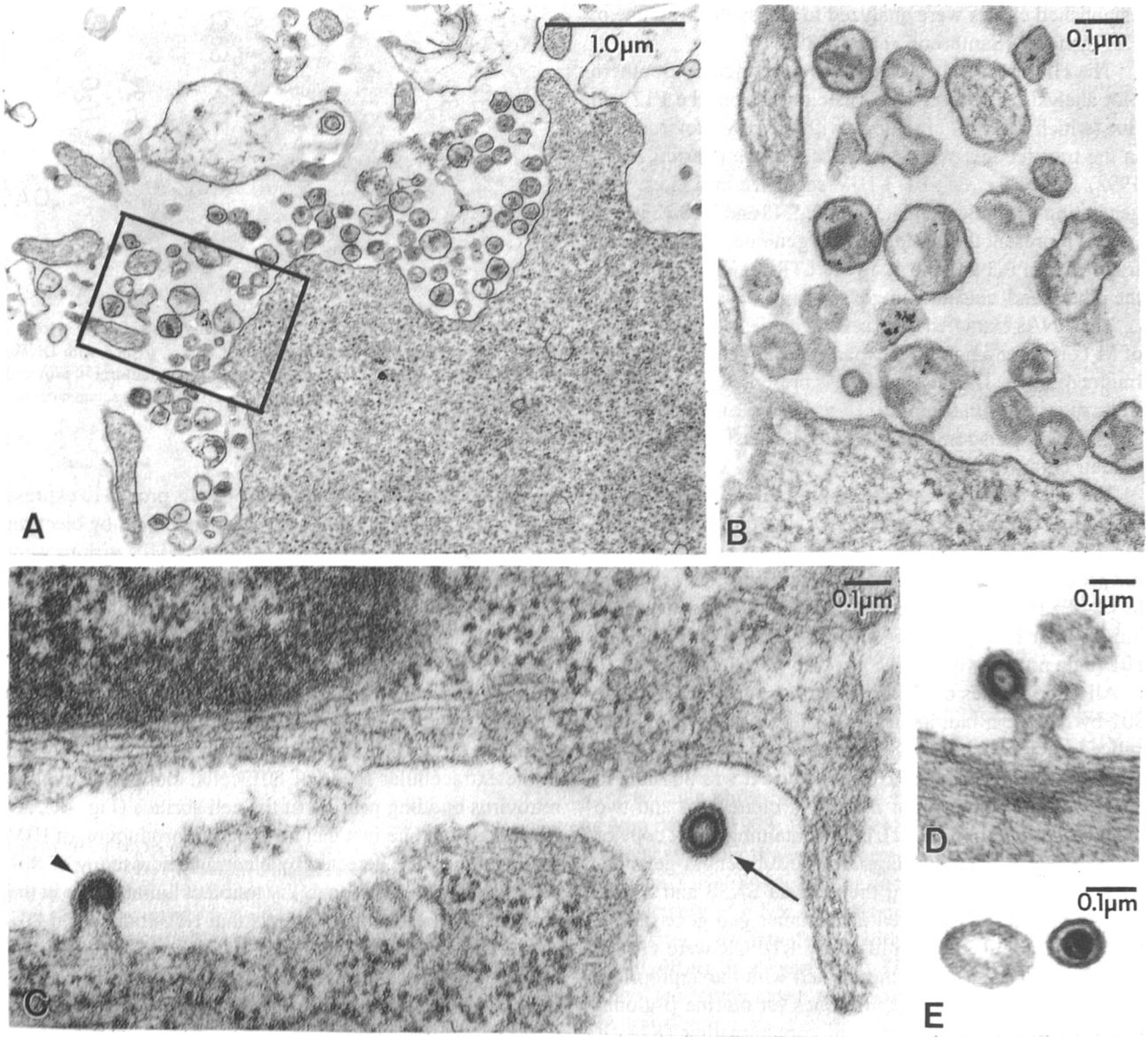

Fig. 4

Thin sections of C8166 cells co-cultivated with 801 cells

Numerous HIV particles are present around the C8166 cell surface as evident at low (A) or high magnification (detail in B). In 801 cells alone, production of MuLV particles is shown by budding profiles at the cell surface (C, arrowhead and D), and by immature (C, arrow) and mature particles (E) in the extracellular space. Cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline for 1 hr at room temperature, washed extensively with distilled water and postfixed with 1% osmium tetroxide in a cacodylate buffer pH 7.2 for 1 hr at 4°C. To increase the overall contrast, specimens were treated with 0.1% tannic acid, stained with 2% uranyl acetate for 1 hr, dehydrated in graded ethanol and embedded in Epon 812. Thin sections were cut with an Ultracut S ultramicrotome (Reichert Jung), mounted on copper grids, poststained with lead citrate and observed in a Philips CM 10 electron microscope. Magnification 20,000 x (A) and 70,000 x (B).

tracted from the co-cultivated NIH-3T3 cells and analyzed by PCR technique, always using the primers SK38 and SK39, and the probe SK19 specific for the *gag* gene. A HIV infection of the murine cell lines was shown by PCR in eight co-cultivation experiments (Fig. 5); in two experiments, no evidence of HIV infection of mouse cells was observed (Fig. 5). Thus 801 cells produced pseudotypes capable to shuttle the HIV genome into mouse cells.

In conclusion, by stable transfection we isolated several murine cell clones having integrated the complete HIV genome. RT-PCR tests demonstrated that the acquired viral sequences were expressed in four of the selected clones. There-

fore, our Northern blot data confirmed that a major block to HIV expression occurred in murine cells: the full length viral mRNA was expressed in these clones, but not efficiently (Pomerantz *et al.,* 1992). Our results regarding the HIV-specific gene expression agree with data reported by other groups which demonstrated a block in Rev function in murine cells (Trono and Baltimore, 1990). It has been proposed that Rev affects the stability and transport of the unspliced and partially spliced mRNA to the cytoplasm, and the utilization of viral mRNA at the level of translation. However, the Rev function is not totally repressed in the murine clone 801, since the latter produced some p24 antigen translated from the full length viral mRNA. The so far published data suggest that the HIV expression in murine cells is not stable since the virus producing cells die (Adachi *et al.,* 1986). Our data, instead, indicate that it was possible to isolate one clone which expressed the HIV-integrated genome at a low level. The selected clone might be considered resistant to HIV replication, or it might produce a level of HIV viral particles compatible with the cell survival.

Moreover, the experiments on the co-cultivation of 801 cells with murine fibroblasts NIH-3T3 demonstrated that it is possible to generate HIV (MuLV) pseudotypes by transfecting the complete HIV provirus into the murine ecotropic packaging cell line (GP+E86). It is worth highlighting the fact that these pseudotypes infect only murine cells and are devoid of any murine replication-competent retroviruses. In addition, the murine retrovirus, providing the virion structural proteins, may infect mainly T and B cells *in vivo* (Weiss *et al.,* 1982). Therefore, the HIV (MuLV) pseudotypes produced by 801 cells represent a reproducible and safe experimental tool for a development of murine animal models in which most of the animals could be infected.

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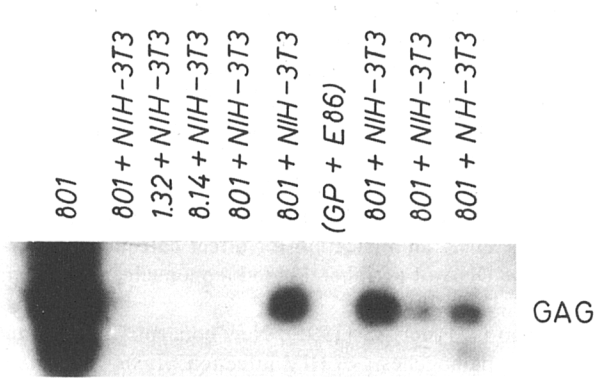

Fig. 5
PCR analysis of DNAs extracted from NIH-3T3 cells co-cultivated with clones 801, 1.32 and 8.14
Clones 1.32 and 8.14 were used as the negative controls. The DNAs were amplified by PCR according to the manufacturer's protocol (Perkin-Elmer Cetus) using the primer pair specific for the *gag* sequence. Four of the eight positive samples, derived from the experiments on co-cultivation of NIH-3T3 cells with clone 801, are demonstrated.

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