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# The *Mus cervicolor* MuLV Isolate M813 Is Highly Fusogenic and Induces a T-Cell Lymphoma Associated with Large Multinucleated Cells

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M813 is a type-C murine leukemia virus (MuLV) isolated from the Asian rodent *Mus cervicolor.* We have recently demonstrated that M813 defines a distinct MuLV receptor interference group. Here we show that M813 rapidly induces fusion of MuLV-expressing fibroblasts from "without," with syncytia being observed within 1 h after exposure to virus. Infection of fibroblasts with MuLV from all tested receptor-interference groups imparts susceptibility to M813-induced fusion, provided the cells also express the M813 receptor. Syncytium induction is also observed *in vivo;* mice infected with M813 develop a peripheral T-cell lymphoma, which is associated with large multinucleated cells of macrophage origin. A recombinant Moloney MuLV/M813 chimeric virus demonstrated that syncytium induction is a function of the Env SU protein. We postulate that the highly fusogenic property of M813 is attributable to either its unique receptor usage or sequences in the proline-rich domain of the Env protein. 

*Key Words:* type-C MuLV; syncytium formation; fusogenicity; Env protein; *Mus cervicolor;* endogenous retrovirus; T-cell lymphoma.

## INTRODUCTION

Retroviruses are associated with a wide variety of diseases, including leukemias, immunodeficiencies, and neurological disorders. The study of these diseases has uncovered a wide variety of pathogenic mechanisms, either shared by several different retroviruses or uniquely associated with a particular virus (for a review see Rosenberg and Jolicoeur, 1997). For instance, although oncogene activation by insertional mutagenesis is a key event in the generation of leukemia by murine leukemia viruses (MuLVs), leukemia induction by the human T-cell leukemia viruses (HTLV) is most likely initiated by *cis*-activation of cellular regulatory genes by the accessory protein Tax (Mesnard and Devaux, 1999). Similarly, the induction of an immune deficiency by the human immunodeficiency virus (HIV) has been attributed to the interaction of its accessory protein Nef with cellular proteins (Harris, 1996). In contrast, a truncated version of the structural Gag protein has been shown to be the critical component of the MAIDS virus, responsible for the induction of a lymphoproliferative and autoimmune disease in mice (Huang *et al.,* 1989; Pozsgay *et al.,* 1993). A glycosylated form of Gag has also been shown to alter disease latency and specificity of MuLV (Corbin *et al.,* 1994; Fujisawa *et al.,* 1998; Portis *et al.,* 1994).

The importance of *env* gene products in retroviral pathogenicity is found at several different levels. First of all, retroviral entry, and thus target cell specificity, is mediated by the Env protein complex, composed of a surface subunit (SU), implicated in receptor recognition, and a transmembrane subunit (TM), which harbors a fusion peptide (Hunter, 1997). Thus the disease specificity of HIV is determined by its restricted tropism to cells of the immune system. In the case of MuLV pathogenicity, it has been shown that generation of recombinant *env* gene viruses through recombination of ecotropic MuLVs with endogenous polytropic sequences is an important step in leukemia induction, presumably by increasing *both* the number of infections per cell (by overcoming the block to superinfection) and the number of available target cells (by stimulating lymphohematopoietic hyperplasia of the spleen) (Brightman *et al.,* 1990; Li and Fan, 1991)—the consequence of which is an increase in the probability of oncogene activation. The direct interaction of the Env protein with either its own cellular receptor or other transmembrane proteins has also been shown or postulated in disease pathogenicity, by either disrupting or activating the normal function of the protein (e.g., stimulation of the erythropoietic receptor by the truncated Env protein of the Friend spleen focus-forming virus, ultimately resulting in the induction of erythroleukemia) (Hoatlin *et al.,* 1990; Li *et al.,* 1990) or a proposed

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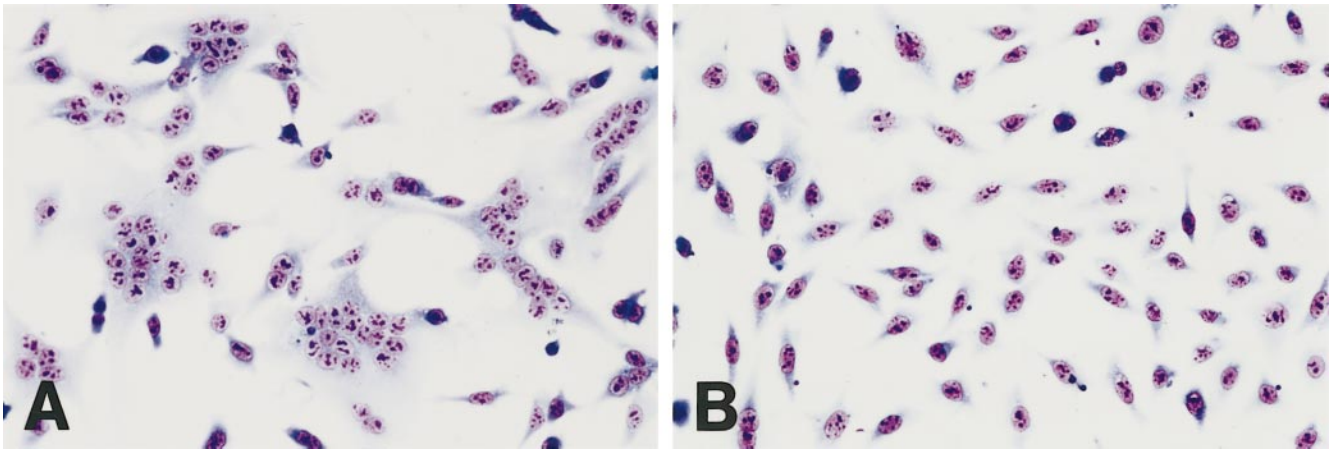


FIG. 1. M813 infection induces fusogenicity of MuLV-infected cells *in vitro*. (A) PA317 cells incubated with M813 for 4 h. (B) Uninfected PA317 cells. Giemsa staining. Magnification, 150×.

disruption of the transporter function of either ecotropic or amphotropic MuLV receptors in glial cells of the central nervous system, leading to the observed spongiform encephalomyelopathy (Kay *et al.,* 1993; Münk *et al.,* 1998). Finally, Env-induced syncytium formation has been postulated to be a mechanism by which HIV induces cell death of uninfected cells, which is supported by the observation that the appearance of syncytium-inducing HIV variants accelerates the course of the disease (Jurriaans *et al.,* 1994; Tersmette *et al.,* 1988). Multinucleated giant cells are also a hallmark of encephalitis induced by both HIV and simian immunodeficiency virus (Geleziunas *et al.,* 1992; Simon *et al.,* 1994). The ability of retroviruses to induce cell fusion is thought to be regulated by the same mechanisms that govern viral cell entry: receptor recognition by the SU induces conformational changes that unmask fusion determinants in the TM.

M813-MuLV was originally isolated from lung cells derived from the Asian rodent *Mus cervicolor.* We have recently shown that M813 defines a unique MuLV interference group (Prassolov *et al.,* 2001). The lack of infection interference with other MuLVs supports the use of a unique cellular receptor for cell entry. Molecular cloning and sequencing of the *env* gene further support this contention: the variable region A (VRA) in the SU domain, important in determining receptor binding, is distinct from that of other MuLVs. The host range of M813 is quite restricted, with efficient infection being observed only in rodent cells derived from *Mus musculus* (Benveniste *et al.,* 1977; Prassolov *et al.,* 2001).

In the course of characterizing M813, we observed a high fusogenic potential of the virus. The studies presented here were designed to more closely define the conditions necessary for induction of syncytium formation by M813 and to determine whether this fusogenic potential of M813 also contributed to the pathogenicity of the virus. We established that M813 induces a T-cell lymphoma *in vivo,* similar to but distinct from that observed by other MuLVs. Significantly, large multinucleated cells were often associated with the tumor, demonstrating that M813 fusion induction is not limited to *in vitro* cultures. Based on a M813 *env* recombinant virus, we postulate that the high fusogenicity of M813 is attributable to either its unique receptor usage or unique sequences in the proline-rich domain of the SU.

## RESULTS

### M813-MuLV induces syncytium formation in PA317

In an experiment initially designed to generate M813(Ampho)-MuLV pseudotypes to infect human cells, PA317 amphotropic packaging cell lines were inoculated with supernatant from murine SC1 cells expressing M813. Within 1 h after exposure to retrovirus, clear signs of syncytium formation between PA317 cells were observed in the cultures. After 4 h, almost all cells in the culture were fused (Figs. 1A and 1B), resulting in the death of the culture within 24 h. Although the addition of polybrene to the culture accelerated the fusion process, it was neither necessary nor singly able to induce fusion of PA317 cells (data not shown).

To obtain a closer view of the cell fusion process, electron microscopic methods were employed. Cell culture monolayers were incubated in the cold with cell culture supernatants rich in M813 virus particles. After extensive washing, the cultures were either fixed immediately or incubated further for 30 to 120 min at 37°C. Replicas prepared immediately after exposure of PA317 cells to virus-bearing supernatant demonstrated numerous cells with irregular contours and complex surface structures. The average length of the elongated cells that exhibited slightly folded cell membranes bearing numerous microvilli was 25–30 mm (Fig. 2a). Adhering virus particles could be detected primarily at the glass surface, but were also seen attached to the microvilli (Fig. 2a). After incubation of the virus-exposed cell cultures for

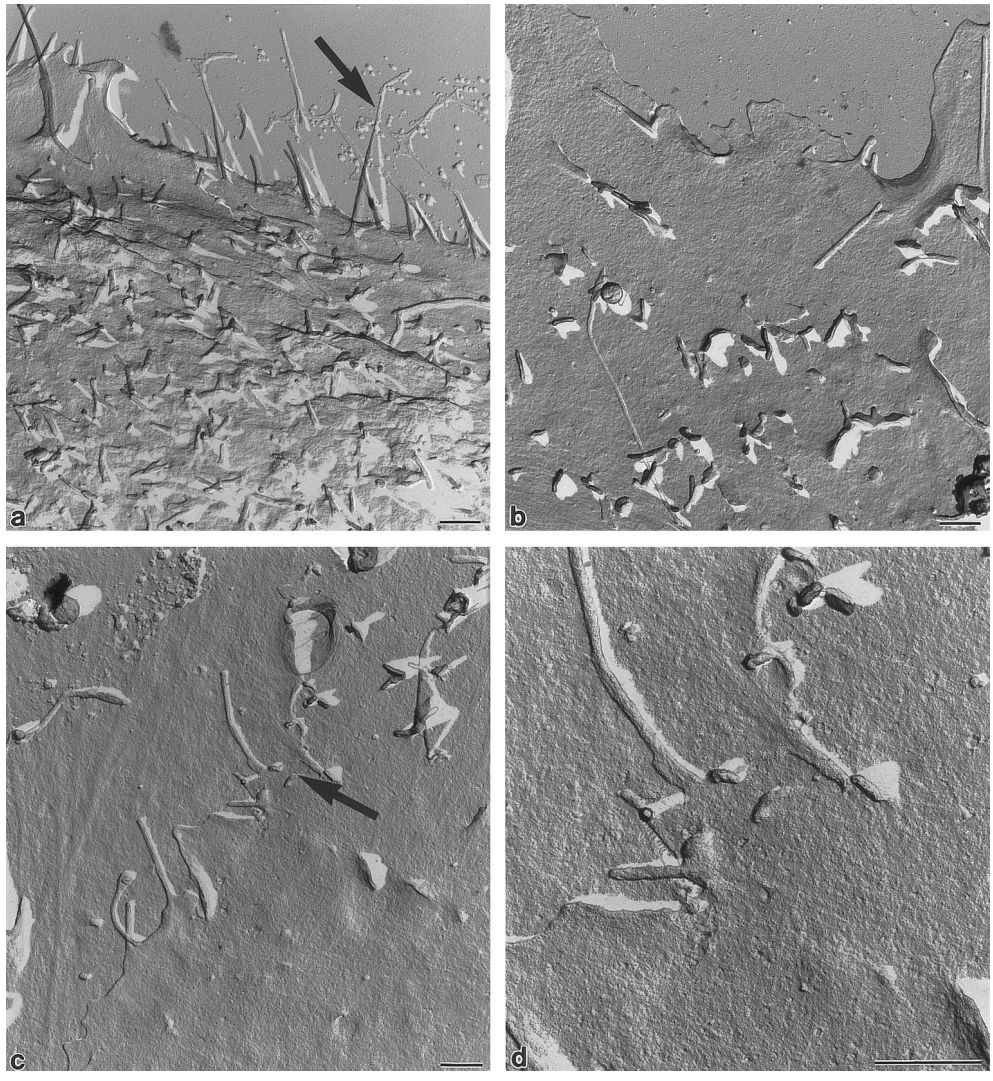


FIG. 2. Ultrastructural aspects of M813 virus-induced cell fusion as shown by replica preparation technique. (a) Cell surface replica of a PA317 fibroblast after incubation for 30 min at 4°C with medium containing M813 virus. Numerous microvilli extend from the slightly folded cell surface. The arrow points to virus particles attached to microvilli. (b) Further incubation for 120 min of PA317 cell cultures at 37°C results in a flattened cell body and considerable reduction of microvilli. Note the prominent membrane ruffles. (c) This electron micrograph shows partially fused plasma membranes of two adjacent cells (extending from the arrow to the upper margin of the picture). (d) Higher magnification of the cell membrane fusion process indicated by the arrow in C. Magnification bar represents 1.0 mm.

more than 60 min at 37°C, a striking alteration of the cell morphology occurred: the cells became flattened and their processes gradually disappeared. Two hours after the temperature shift, only a few prominent short membrane ruffles and very few microvilli were present (Fig. 2b). Moreover, delimitation between individual cells at contact points became obscured due to partial membrane fusions, with the consequence that cell borders were more and more difficult to distinguish (Figs. 2c and 2d).

These kinetics indicate that fusion occurs from "without," i.e., when a single virion simultaneously fuses with two cells, in contrast to the fusion observed when XC sarcoma cells are infected with Moloney MuLV (Mo-MuLV) (Klement *et al.,* 1969), which occurs from "within," i.e., when an infected cell expressing Env on its cell surface fuses with an adjacent cell (White *et al.,* 1983).

### M813-MuLV and not MuLVs of other interference groups induces fusion of PA317 cells

Several experiments were carried out to determine whether the observed fusogenicity with M813-infected PA317 cells was a particular characteristic of the PA317 cells, the M813 virus, or both. In all experiments, equal numbers of the target cell were plated and exposed to virus supernatants with defined titers. After 4 h, cultures were assessed for syncytium formation and the fusion index (FI) was determined using the formula $(N - S)/T$,

TABLE 1

M813, but Not Other MuLVs, Is Highly Fusogenic in PA317 Cell Cultures

| Virus | Titer[a] (GTU) | Fusion index (%) on target cell[b] NIH 3T3 | PA317 |
|---|---|---|---|
| M813-MuLV | $1.4 \times 10^4$ | 6 ± 4.2 | 78 ± 12 |
| 10A1-MuLV | $7.8 \times 10^4$ | 3 ± 1.4 | 4 ± 0.3 |
| Mo-MuLV | $3.6 \times 10^4$ | 4 ± 0.6 | 25 ± 2.8 |

[a] The virus titer of the supernatant used to inoculate the cell cultures was determined by marker rescue using the retroviral vector MPEV-neoR. The number of G418-resistance transfer units (GTU) was determined by end-point dilution of virus, infection of SC1 cells, and selection under G418.

[b] The fusion index (FI) was determined using the formula $(N - S)/T$, where $N$ is the number of nuclei in a syncytium, $S$ is the number of syncytia, and $T$ is the total number of nuclei (Andersen, 1994). Over 500 nuclei were counted in each experiment to obtain the FI value. The average values with standard deviations (±SD) from two independent experiments are shown.

where $N$ is the number of nuclei in a syncytium, $S$ is the number of syncytia, and $T$ is the total number of nuclei (Andersen, 1994). Over 500 nuclei were counted in each experiment to obtain the FI value.

We first established that, whereas the PA317 cells readily formed syncytia after exposure to M813, no syncytium induction was observed in NIH3T3 cells, from which the PA317 cells are derived (an FI of 78% for PA317 versus an FI of 6% for NIH3T3 cells; average of two independent experiments). This suggested that the expression of amphotropic Env proteins or other viral proteins may be important for fusion induction.

In a second set of experiments, we tested whether MuLVs of other receptor groups, namely, the ecotropic Mo-MuLV, which uses mCAT1, or alternatively, 10A1-MuLV, which uses Pit1 and Pit2 for cell entry, would also induce syncytium formation of PA317 cells. No cell fusion was observed when PA317 cells were exposed to 10A1-MuLV. Some fusion was observed with the ecotropic Mo-MuLV, but significantly less than that observed with M813 (Table 1). Whereas syncytia with up to 18 nuclei were observed with M813, at most 2 to 3 nuclei per syncytium were observed with MoMuLV. Thus, both the PA317 cell line and the M813 virus itself contribute to the high degree of fusogenicity observed.

## M813 induces fusion of cells infected with different MuLVs, but infection of M813-expressing cells with other MuLVs does not result in syncytium formation

It is likely that expression of the amphotropic Env protein in PA317 cells contributes to the altered fusogenic properties of the cell line. It was therefore of interest to determine whether this was a particular property of amphotropic Env sequences or whether the expression of other MuLV Envs would also alter the fusogenicity of the cell. SC1 mouse fibroblasts expressing different MuLVs representing four different receptor interference groups (e.g., ecotropic, polytropic, amphotropic, and 10A1) were tested for their fusogenicity after incubation with M813. Although SC1 fibroblasts did not form syncytia upon exposure to M813-MuLV, all cell lines expressing any one of the other MuLVs were highly fusogenic when exposed to M813 (Table 2). These results show that expression of the amphotropic Env is not unique in its ability to "prime" cell fusion, but rather either all MuLV Env proteins have this function or other viral proteins (e.g., Gag proteins) common to the MuLVs used in this assay are responsible for the priming of cell fusion in NIH 3T3 fibroblasts.

We next sought to determine whether SC1 fibroblasts infected with M813 would become susceptible to fusion with other MuLVs. SC1-M813 cells were thus incubated with either Mo10A1V or MoMuLV and monitored for cell fusion. No fusion above background levels was observed in these cultures, even when observed 48 h after infection (data not shown). In conclusion, expression of M813 in SC1 cells does not make them permissive for fusion from either within or without with either ecotropic or 10A1 MuLVs.

## M813 susceptibility is a prerequisite for fusion induction

Human cells are not permissive for M813 infection, presumably because they lack the M813 receptor for cell entry. It would be expected that M813-induced fusion is mediated through interaction with its cell receptor. To test this, human cells were incubated with M813. To increase the likelihood that fusion would be observed, retroviral-packaging cell lines of human origin expressing MoMuLV Pol and Gag proteins, as well as the Env proteins of MoMuLV (TE-FLY-Mo), amphotropic 4070-MuLV (293-Phoenix-Ampho), or the gibbon ape leukemia virus (TE-FLY-GALV), which shares a common receptor

TABLE 2

MuLV Expression Makes SC1 Cells Permissive to M813-Induced Fusion

| Target cell in culture | Fusion index (%)[a] Uninoculated | M813 inoculated |
|---|---|---|
| SC1 | 2.4 ± 0.7 | 5.4 ± 0.3 |
| SC1 + MoMuLV | 0.8 ± 0.14 | 94.0 ± 5.7 |
| SC1 + MoMCFV | 1.6 ± 0.6 | 73.2 ± 15.6 |
| SC1 + MoAmphoV | 2.0 ± 0.6 | 70.1 ± 8.0 |
| SC1 + Mo10A1V | 2.8 ± 0.01 | 77.4 ± 11.9 |

[a] The fusion index was calculated as stated in Table 1. The average ± SD of two independent experiments is shown.

TABLE 3

Human Cells Expressing MuLVs Do Not Form Syncytia after M813 Infection

| Target cell in culture | Fusion index (%) | |
|---|---|---|
| | Uninoculated | M813-inoculated |
| TE671 | 1.6 ± 0.2 | 4.2 ± 0.15 |
| TE-FLY-GALV | 7.8 ± 0.3 | 6.5 ± 0.4 |
| TE-FLY-MO | 8.4 ± 0.57 | 6.8 ± 0.32 |
| 293 | $<10^b$ | $<10^b$ |
| 293-Phoenix-Ampho | $<10^b$ | $<10^b$ |

[a] The fusion index was calculated as stated in Table 1. The average ± SD of two independent experiments is shown.

[b] The fusion index was not calculated in this set of experiments, but was similar to that of other cell lines. No difference was observed between noninfected and M813-infected cultures.

with 10A1-MuLV, were tested. As shown in Table 3, M813 did not induce fusion of any of these cells.

### M813 SU sequences are sufficient to impart high fusogenicity to the ecotropic MoMuLV

To ascertain whether the unique receptor usage of M813 was important in the fusogenic property of M813, a chimeric virus was tested in which the MoMuLV sequences encoding the majority of the mature SU protein [including the complete binding recognition domain (VRA, VRB, and VRC) and the proline-rich domain] but not the TM protein were replaced with those of M813 (Fig. 3). Although titers were somewhat lower than in previous assays ($2 \times 10^3$ G418-resistance transfer units per milliliter), syncytium formation was readily observed when PA317 cells were incubated with the recombinant virus. A fusion index of 61.3 was calculated (average of two independent experiments). Although somewhat lower than that observed for wild-type M813 (79.3), this most likely reflects the lower virus titer.

### M813-MuLV-infected mice develop T-cell lymphomas containing multinucleated syncytia

The high fusogenicity of M813-MuLV observed *in vitro* prompted us to determine whether this property would also be reflected in its pathogenicity *in vivo.* Within 36 h after birth, either DBA/2J or DDD mice were infected with M813-MuLV produced from SC1 cells ($n = 8$ and $n = 12$, respectively). Between 9 and 12 months after infection for DDD mice and 13 and 18 months for DBA/2J, approximately 75% of the mice were moribund, in contrast to uninfected controls that remained asymptomatic within this time frame. Examination of sacrificed animals revealed hepatosplenomegaly (spleen weights between 0.7 and 1.5 g, as compared to 0.08 g in uninfected controls) accompanied by enlarged, tumorous lymph nodes. Large mesenteric lymphomas (>600 mg) were observed in all animals that were more closely examined (6/6). Significantly, no enlargement of the thymus was observed in any of these animals.

Histological examination of M813-infected DDD and DBA mice revealed a peripheral T-cell lymphoma of lymph nodes (Fig. 4A) and spleen, metastasizing preferentially to the liver (Fig. 4B). Involvement of other organs was rare. The lymphomas presented with effacement of lymph node and splenic architecture, a nodular to diffuse growth pattern, and invasion of perinodular tissue. The neoplastic cells ranged from small lymphocytes to large bizarre cells, but appeared mostly as medium to large lymphoid cells with nuclear atypias and abundant cytoplasm (Fig. 4A). Apparently, the peripheral T-cell lymphomas transformed to an anaplastic large cell lymphoma in the final stages of the disease (Fig. 4B). The lymphocytic tumor cell population consistently exhibited a positive CD3 immunoreactivity (Fig. 4C), confirming the T-cell origin. A distinct feature of T-cell lymphomas of M813-infected DDD mice—as revealed by immunocytochemical tagging—was the admixture of numerous mature and immature granulocytes, preplasma and plasma cells, dendritic cells, and histiocytes to the neoplastic lymphoid cells (data not shown).

Strikingly, very large, multinucleated syncytia with nondistinctive cell boundaries (Fig. 4D) and more typical multinucleated giant cells (Fig. 4E) were observed in both the medullary and the extracapsular invasion zones of these lymphomas, as well as in the expanded periarterial lymphocyte sheath of the splenic white pulp. Occasionally, more than 50 nuclei could be counted per

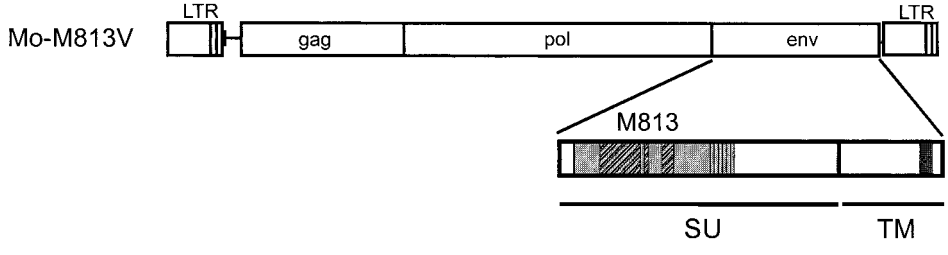


FIG. 3. Schematic diagram of the MoMuLV/M813 (MoM813V) chimeric retrovirus used to determine the sequences responsible for the fusion induction of M813. Shaded areas in SU are those derived from M813, whereas all other sequences are derived from MoMuLV. The variable regions (VRA, VRC, and VRB, respectively) are boxed with diagonal lines, and the proline-rich domain is boxed with vertical lines. Transmembrane domain of TM is indicated by a black box.

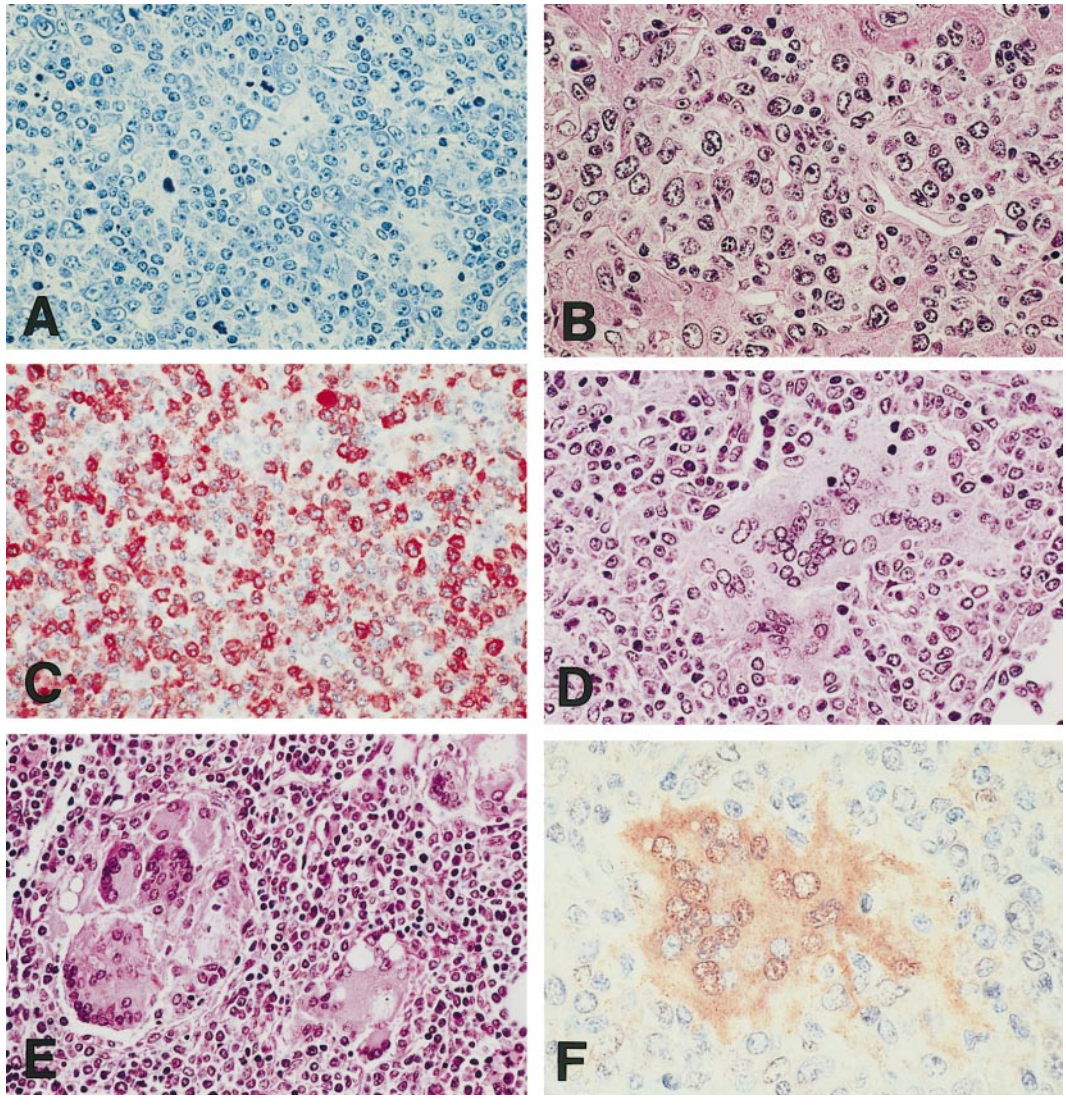


FIG. 4. Histopathology of M813 virus-induced lymphomas. (A) Peripheral T-cell lymphoma of a lymph node. (B) Anaplastic large cell lymphoma infiltrating the liver. (C) CD3 immunostaining of peripheral T-cell lymphoma. (D) Large multinucleated syncytium of peripheral T-cell lymphoma. (E) Multinucleated giant cells of a peripheral T-cell lymphoma. (F) S100 immunostaining of multinucleated giant cells. Stains were Giemsa (A) and hematoxylin and eosin (B, D, and E). Magnification: 380× (A, B, and D), 240× (C), 290× (E), and 580× (F).

syncytium. Syncytia formation seemed to be a transient phenomenon, since syncytia undergoing necrosis (apoptosis) could be observed, indicating their limited viability. Because of their positive S100 and lysozyme immunostaining, the multinucleated structures are probably of macrophage and/or dendritic cell origin (Fig. 4F). However, the extensive syncytia are morphologically clearly distinct from conventional multinucleated giant cells, which are a well-known reactive form of mononuclear phagocytes in response to diverse stimuli. Using antibody against $SU^{gp70}$, MuLV env expression could be detected in CD3-positive lymphoma cells and macrophages, as well as in the syncytia, but in no other cell types (data not shown). This confirms that both the lymphoid transformation and the syncytia formation were induced by M813-MuLV and/or MCF recombinants generated by the M813 infection.

## DISCUSSION

M813 is a type-C MuLV that defines a novel receptor interference group. Similar to ecotropic MuLVs, the M813 host range is restricted to rodent cells. However, our studies have clearly shown that M813 uses a cellular receptor distinct to both the CAT1 protein, used by the ecotropic MuLVs, and receptors used by other characterized MuLVs (Prassolov *et al.,* 2001). In the studies presented here, we have shown that M813 readily induces cell fusion *in vitro* and *in vivo.* Electron microscopy revealed the adherence of virus particles to the fusing membranes and demonstrated the rapid changes in the

cellular membrane following exposure to the virus. Syncytium formation was observed only between cells that expressed the M813 receptor and that were made permissive for cell fusion by expression of an MuLV. Analysis of a chimeric Env protein showed that the M813 SU protein dictated its high fusogenicity. This unique property of M813 was also reflected in its pathogenicity. Histological examination of lymphomas induced by M813 in infected DDD mice revealed large multinucleated cells. Unlike lentiviral and oncoviral infection of humans, retroviral MuLV infections of mice are normally not associated with the development of multinucleated syncytia. Thus, whereas the transformation of the lymphoid cells is probably the result of oncogene activation by insertional mutagenesis of M813-MuLV or MCF recombinants, the multinucleated syncytia are most likely induced by direct binding of M813 to its receptor on these cells, providing evidence that the fusogenic capacity of the M813 retrovirus is not restricted to cell culture conditions but is also effective *in vivo.*

The ability of a virus to induce cell fusion is dictated by several factors, inherent both to the virus itself and to the cellular environment. Retroviruses reported to induce cell-to-cell fusion include feline viruses [feline leukemia virus and RD-114 (Hampar *et al.,* 1973)], the avian reticuloendotheliosis virus (Delwart and Panganiban, 1989), and both the human viruses HIV and HTLV (Freed *et al.,* 1990; Poon and Chen, 1998; Tersmette *et al.,* 1988). For the murine retroviruses, fusion induction has been reported for the mouse mammary tumor virus and the ecotropic MuLVs under limited conditions (Andersen, 1994; Jones and Risser, 1993; Klement *et al.,* 1969; Pinter *et al.,* 1986; Siess *et al.,* 1996; Wilson *et al.,* 1992).

What are the Env determinants of M813 that render it highly fusogenic? Hydrophobic fusion peptides that share many features of the well-characterized fusion peptide of influenza virus (White, 1992) have been defined in the extracellular domain of the TMs of HIV and MuLV (Freed *et al.,* 1990; Jones and Risser, 1993). In addition, sequences in the cytoplasmic domain of the TM and at the amino-terminus of the SU have been shown to be essential for virus–cell fusion (Bae *et al.,* 1997; Januszeski *et al.,* 1997). It is believed that binding of the SU to its receptor results in conformational changes in the TM that activate the fusion peptide. Conceivably, a unique structure of the M813 TM may increase its fusogenic capacity. Our data found this unlikely, as a MoMuLV/M813 Env recombinant, in which the entire TM was derived from MoMuLV, was also fusogenic. These results are also consistent with earlier results comparing the increased fusogenicity of the ecotropic MoMuLV with amphotropic MuLV; swapping TMs did not alter the fusogenic potential of either virus (Ragheb *et al.,* 1995). In contrast, sequences in the proline-rich region of SU in MoMuLV were shown to impart high fusogenicity to an amphotropic Env (Andersen, 1994; Lavillette *et al.,* 1998). This region has been proposed to participate in the fusion process by regulating the transition between the conformational changes that occur in the Env glycoprotein before and after receptor binding (Lavillette *et al.,* 1998). Domain swapping between other MuLV Env proteins is required to determine whether the M813 proline-rich domain can impose increased fusogenicity to other Env proteins.

The number of receptors expressed on the cell surface is also a critical parameter in virus-induced cell fusion. The level of syncytium formation was found to correlate with either mCAT1 or Pit1 receptor levels in CHO cells infected with either ecotropic or amphotropic viruses, respectively (Siess *et al.,* 1996). These results suggest that receptor clustering is the limiting step in syncytium formation, which can be modulated by both receptor numbers and unknown cellular factors. The cellular receptor of M813 is unknown, but could be a molecule that is expressed at high levels in fibroblasts or in macrophages and thus account for the observed fusion *in vitro* and *in vivo.* The primary receptor binding domain of other MuLVs resides in the variable regions VRA and VRB in the amino-terminus of the SU (Battini *et al.,* 1992, 1995; Fass *et al.,* 1997; Heard and Danos, 1991). Substitution of only this domain from M813 with that of another MuLV will help determine whether the receptor itself is important in the cell fusion process.

In addition to parameters dictated by the virus, several cellular factors are also important in determining the fusogenic potential. Earlier studies have clearly shown that oncogenic transformation can make a cell more permissive for virus-induced fusion (Kaufman and Ehrbar, 1980; Klement *et al.,* 1969; Wilson *et al.,* 1992). The reason for this is unclear, but may be related to increased levels of receptor expression or, alternatively, altered posttranslational processing of the receptor, such as glycosylation. Glycosylation levels on the receptor have been shown to have a dramatic effect on the ability of a virus to infect a cell (Miller and Miller, 1992; Wilson and Eiden, 1991). Furthermore, the level of receptor glycosylation has been found to correlate with syncytium formation in several studies (Andersen, 1994; Jones and Risser, 1993). Alternatively, sensitivity to cell fusion in transformed cells may be due to changes in the plasma membrane associated with malignant transformation, including changes in the cytoskeleton.

In the study presented here, the expression of MuLV proteins imparted sensitivity to M813-induced syncytium formation. As Env expression appeared to be a likely explanation for this altered phenotype, we tested whether Env proteins from different MuLV receptor groups had the same effect. No difference in the acquired fusogenicity was observed when Envs of amphotropic, ecotropic, 10A1, or polytropic viruses were used. How might Env expression impart an increased fusogenicity to fibroblasts? Expression of the Env protein on the

cell surface would allow it to interact with receptor expressed on neighboring cells. Normally, this interaction is probably not sufficient to induce cell fusion, as activation of the fusion peptide in MuLV requires not only binding of the SU with the cellular receptor, but also cleavage of the p2E peptide on the TM precursor (pre15E) by the viral protease to yield the mature TM protein (p15E) (Ragheb and Anderson, 1994; Rein and Schultz, 1984; Zhao *et al.,* 1998). As this occurs within the viral particle, only the immature form is expressed on the cell surface. However, this receptor–Env interaction either may facilitate cell–cell interaction, thereby increasing the likelihood that the M813 virus particle comes in contact with more than one cell, or may already have induced changes in the cell membrane that generally make it permissive to cell fusion. Importantly, we cannot currently exclude the possibility that other viral proteins, such as Gag, that assemble at the cell membrane also impart changes to the cell membrane and thus its sensitivity to cell fusion.

The increased fusogenicity of M813 prompted us to determine whether this property would be reflected in its pathogenicity. Similar to the other MuLVs, we found that M813 induced T-cell lymphomas in infected mice. This disease is distinct from that induced by the ecotropic MoMuLV, in that the retarded course of the tumor disease is associated with the development of a peripheral instead of a central T-cell lymphoma. A remarkable feature of the M813-induced T-cell lymphoma is the pronounced infiltration of nonneoplastic cells, such as granulocytes, dendritic cells, and macrophages, the last of which apparently give rise to extensive yet transient syncytia formation. The admixture of various other hematopoietic cell elements to neoplastic lymphocytes is also a hallmark of the human lymphoma category of "peripheral T cell lymphomas, unspecified," as recently defined in the new WHO classification of human lymphomas (Jaffe *et al.,* 1999). Noteworthy was the occurrence of large multinucleated cells in the periphery of the tumorigenic growth, demonstrating that the fusogenicity of M813 also influences its pathogenicity. Similar findings have been found for the TR1.3 MuLV, which induces syncytium formation of endothelial cells both *in vitro* and *in vivo;* inoculation of neonatal mice uniformly induces cerebral infarctions and hemorrhages due to fusion of cerebral vessel endothelial cells (Park *et al.,* 1994a,b).

The long latency of M813 disease induction (>1 year) may reflect the importance of secondary mutations and/or the generation of recombination events with endogenous MuLVs. Indeed, preliminary analysis of genomic DNA derived from tumors has revealed M813-recombinant proviruses (C.S., unpublished results). We postulate that recombination with endogenous polytropic sequences generates replication-competent MuLVs that synergize with M813 in inducing lymphomas. Cells expressing a polytropic MuLV may be primed for fusion induction by M813 or M813 recombinants carrying the M813 SU domain, as we have observed *in vitro.*

In conclusion, we have shown that M813 can readily induce syncytium formation in permissive cells. This unique property of M813 resides in the SU domain of the Env glycoprotein. Molecular cloning of the M813 receptor should shed light on its role in fusion induction. In addition, the generation of Env chimeras will lead to the identification of sequences in the SU domain that facilitate this process. Numerous attempts have been made to alter the tropism of type C MuLVs (Cosset and Russel, 1996). Although extension of the amino-terminus of MuLV Envs with different ligand types, such as single-chain antibodies and cytokines, has been generally successful, infectivity has been intrinsically poor due to low fusogenicity. Understanding the mechanism by which M813 SU sequences increase fusogenicity may help eliminate this problem.

## MATERIALS AND METHODS

### Viruses, cell lines, and cell culture

SC1 fibroblasts were used as virus producers in all experiments. SC1 cells expressing M813 were obtained by infection of SC1 cells with the supernatant of M813 (NIH), kindly provided by U. Rapp (Rapp and Marshall, 1980). SC1 or SC1-MPEVneoR cells expressing MoMuLV, MoAmpho, Mo-MCFV, and Mo10A1V have been previously described (Prassolov *et al.,* 2001). The NIH3T3-based packing cell line PA317 was obtained from A. D. Miller and produces an amphotropic Env (Miller and Buttimore, 1986). The human TE671 cell line is the basis of the two packaging cell lines, TE-FLY-GA16 and TE-FLY-MO, producing the Env proteins from GALV and Mo-MuLV, respectively, and were kindly provided by L. F. Cosset (Cosset *et al.,* 1995). The Phoenix-Amphotropic packaging cell line, provided by G. Nolan, is based on the human embryonic kidney 293 cell line and expresses the amphotropic Env protein. All cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The MoM813V recombinant used in this study has been previously described (Prassolov *et al.,* 2001).

### Virus titers and syncytia assays

Virus titers were determined either by end-point dilution (1:5) on SC1 cells and detection by immunohistochemistry or by a marker rescue assay. A rabbit antibody against $CA_{p30}$ (1:150) was used to detect positively infected cells (kindly provided by P. Nobis, Munich, Germany), and was visualized by FITC-labeled goat anti-rabbit secondary antibody (1:80; Biosource International, Camarillo, CA) using a modified method of Wilson *et al.* (1994). Marker assays were performed in the case where the virus was produced from SC1-MPEVneoR cells,

which carry a retroviral vector conferring resistance to G418 (Laker *et al.,* 1998). Two days after infection of SC1 cells, G418 (400 $\mu$g/ml) was added. After 10 to 14 days, neoR colonies were counted.

To determine the fusion index, cells were seeded at a concentration of 2–3 $\times$ $10^5$ cells per well in six-well plates. Twenty-four hours later, medium was replaced with virus-containing supernatant. Syncytia formation was visible after 1 h and cells were generally fixed after 4 h and stained with Giemsa's solution (Sigma). Assays were normally performed with polybrene (8 $\mu$g/ml), unless noted otherwise. At least 500 nuclei were counted for each experiment.

### Mouse infections

Within 36 h after birth, DBA/2J or DDD ($Fv2^s$) mice were infected intraperitoneally with 75 $\mu$l supernatant from SC1-M813 fibroblasts. Virus titers were determined in parallel on SC1 cells and were 3.9 $\times$ $10^5$ per milliliter. Mice were monitored weekly for clinical symptoms. Moribund mice were sacrificed and subjected to histopathological analysis.

### Histologic and immunohistologic methods

Tissue specimens were fixed in 4% formaldehyde containing 1% acetic acid. Deparaffinated sections were stained with hematoxylin–eosin, periodic acid-Schiff reaction, and Giemsa stain according to standard protocols. To demonstrate immunohistochemically the expression of immunoglobulins, T-cell receptor, myeloperoxidase, lysozyme, S100 protein, or the MuLV $SU^{env}$ by the avidin–biotin complex method (Hsu *et al.,* 1981), a panel of polyclonal antibodies was used: rabbit anti-mouse $\kappa$ and $\lambda$ light chains (Nordic, Tilburg, The Netherlands), goat anti-mouse $\mu$ heavy chain (Pel-Freez, Rogers, AZ), rabbit anti-human CD3 (Dako Diagnostica, Hamburg, Germany), rabbit anti-human myeloperoxidase (Dako), rabbit anti-bovine S100 protein (Dako), rabbit anti-lysozyme (Biogenex, San Ramon, CA), and goat anti-$SU^{gp70}$ (gift from W. Schäfer, Tübingen, Germany). For antigen retrieval, deparaffinated sections were treated with a commercial "target unmasking fluid" (Dianova, Hamburg, Germany) in a microwave oven. Incubation with primary antibodies was done overnight at 4°C. Bound antibodies were detected with a biotinylated secondary antibody and subsequent incubation with phosphatase- or peroxidase-conjugated streptavidin (Biogenex). Visualization of phosphatase activity was done with naphthol AS-BI phosphate in combination with hexazotized new fuchsine (Merck, Darmstadt, Germany). Peroxidase activity was revealed with $H_2O_2$ and 3,3-diaminobenzidine-tetrahydrochloride (Merck). Endogenous avidin-binding activity was reduced by pretreatment of the sections with avidin and biotin solutions (Zymed Laboratories, San Francisco, CA).

### Replica preparation for electron microscopy (EM)

PA317 cells were seeded on glass coverslips measuring 8 $\times$ 50 mm at a density of 5 $\times$ $10^5$ cells/ml. Sixteen hours later, the cultures were washed twice with PBS and incubated with SC1 tissue culture supernatant containing M813 virus particles for 30 min at 4°C. After extensive washing, the cultures were either fixed immediately with a cold solution or incubated further for 30, 60, 90, and 120 min at 37°C. For EM preparations, specimens were washed with ice-cold 0.1 mol/L PIPES buffer (pH 6.9) and fixed *in situ* for 30 min with 2.5% glutaraldehyde in 0.1 mol/L PIPES supplemented with 20 mM $CaCl_2$. Fixed cells were rinsed with the same buffer and postfixed in PIPES-buffered 1% osmium tetroxide for 30 min at 4°C. After extensive washing in PIPES, the cultures were treated for 10 min with 1% tannic acid in water, washed, and dehydrated in ethanol. Dehydration was followed by two short washes with tetramethylsilane (Fluka, Taufkirchen, Germany) (Ting-Beall *et al.,* 1995). After being dried for 30 min at 37°C, coverslip fragments with appropriate cell density were cut out. Shadow-casting was done with platinum/carbon at a 45° angle and carbon at a 90° angle in a Bioetch 2005 (Leybold-Heraeus, Cologne, Germany) cooled at −100°C. The shadowed cells were detached from glass in hydrofluoric acid and the organic material was digested with a sodium hypochlorite solution. The replicas were mounted on grids without supporting films and examined in a Philips CM 120 electron microscope at 80 kV (Hohenberg, 1989).

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