Mutational analysis of Moloney murine leukaemia virus surface protein gp70

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Ten mutations were generated in the env gene of Moloney murine leukaemia virus DNA. The mutations were made by site-directed mutagenesis to alter basic amino acids (lysine or arginine) in the surface glycoprotein gp70. Mutants were investigated following transfection into NIH/3T3 cells. All 10 mutants released virion particles into the medium, suggesting that none of the mutations affected overall viral gene expression or virion budding. Two mutants were positive in XC plaque assay, reverse transcriptase assay and re-infection experiments, showing that these mutations occurred in parts of the molecule not essential for infection. Three mutants were negative in both the XC plaque assay and re-infection experiments, suggesting that they make non-infectious virus particles. The results indicate a defect in the early phase of infection, perhaps in receptor binding or in the fusion of virion and host membranes. The other mutations resulted in reduced infectivity of released virion particles.

Introduction

Moloney murine leukaemia virus (Mo-MLV) is a type C retrovirus that, when inoculated into newborn mice, gives rise to leukaemias of mostly T cell origin (Gross, 1970). The Mo-MLV genome contains 8332 nucleotides which encode gag, pol and env proteins. Translation of the envelope gene produces an 80K glycosylated precursor that is cleaved to form a transmembrane protein, p15E, and an external envelope glycoprotein, gp70. Pinter & Honnen (1983) have shown that it is probable that the C terminus of gp70 is attached to the N terminus of p15E by disulphide bridges and possibly by hydrophobic interactions. The p15E and gp70 probably form multimers (Einfeld & Hunter, 1988). Recently, the nature of the cell surface component used as a receptor for Mo-MLV was determined (Albritton et al., 1989) and this is probably a membrane protein used for basic amino acid transportation, having no homology to CD4 (Kim et al., 1991; Wang et al., 1991). The CD4 glycoprotein, the receptor for human immunodeficiency virus (HIV), is a member of the immunoglobulin family (Maddon et al., 1986). During infection the envelope proteins of type C retroviruses bind to host receptors, mediate fusion between host and viral membranes, and deliver the virion core into the cytoplasm of the cell.

The detailed functions of the murine retrovirus surface glycoprotein gp70 and the transmembrane protein p15E are incompletely known but it is believed that the Mo-MLV gp70 is involved in mediating membrane fusion. Antibodies acting against gp70, but not those against p15E, inhibit membrane fusion (Zarling & Kesket, 1979). This is similar to that found for HIV where gp120 is believed to be involved in steps leading to fusion (Ratner, 1992). In HIV, the hydrophobic domain in the N-terminal part of the transmembrane protein gp41 is active in fusion (Gallaher, 1987). The p15E has a similar hydrophobic domain (amino acids 5 to 24 in p15E of Mo-MLV). Although p15E apparently mediates fusion, perhaps gp70 is necessary for p15E to act. The highly glycosylated gp70 carries most of the antigenic determinants and is responsible for interference (De Larco & Todaro, 1976). Other investigators have introduced deletion and insertion mutations into the env gene of murine leukaemia viruses to examine the role of the envelope proteins in the viral life cycle (Granowitz et al., 1991; Heard & Danos, 1991; Battini et al., 1992).

To investigate further the importance of gp70 in the infection process we used site-directed mutagenesis to alter arginine and lysine amino acids in gp70 of Mo-MLV. The basic amino acids were chosen in order to make drastic changes through altering molecular charge. Furthermore, these basic amino acids are likely to occur on the surface of the protein (Birdi, 1989). We have previously shown that cleavage of gp70 might be important for membrane fusion and infection (Andersen, 1987; Andersen & Skov, 1989). Similar results have been indicated for HIV gp120 (Clement et al., 1991). The
putative cleavage may be caused by a trypsin-like protease as infection can be inhibited by leupeptin and antipain (Andersen, 1983). Expression of the mutated Mo-MLV genome is analysed by transfection into NIH/3T3 cells.

**Methods**

**Recombinant DNA constructs.** Plasmid pMov-3 contains a Mo-MLV provirus with flanking host sequences in the vector pBR322 (Schneike et al., 1983). Plasmid pMov-3 was obtained as a gift from K. Harbers, Heinrich-Pette Institute, Hamburg, Germany. The plasmid was mutated by standard molecular cloning techniques according to Sambrook et al. (1989). An AflIII fragment of pMov-3 was deleted, extending from ori in pBR322 into the flanking murine DNA to a position approximately 0.5 kb from the right long terminal repeat (LTR). As a result of the AflIII deletion a number of restriction sites surrounding the env gene became unique (Fig. 1). Furthermore, the deletion of the left part of ori removed the negative controlling rop region (Scott, 1984), giving a better yield of plasmid in *Escherichia coli*.

The 2.8 kb HindIII–ClaI restriction fragment in pKA1558 containing the env gene was cloned into the phagemid vector Bluescript KS+ (Stratagene). Specific nucleotide changes were introduced by oligonucleotide-directed mutagenesis, using the Bio-Rad Muta-Gene Kit based on the method of Kunkel (1985). The presence of the mutation was confirmed by detection of a novel restriction endonuclease site and by DNA sequencing.

**DNA transfection.** NIH/3T3 and XC cells were grown in Dulbecco’s MEM, supplemented with 10% fetal calf serum, non-essential amino acids, penicillin and streptomycin. NIH/3T3 cells were plated at 2.3 × 10^4 cells per cm^2-culture dish 1 day before transfection. The cells were transfected with 0.07 μg plasmid DNA, using the calcium phosphate precipitation technique of Graham & van der Eb (1973) as modified by Corsaro & Pearson (1981). Culture supernatants were harvested 48 to 65 h after transfection, tested for reverse transcriptase (RT) activity and used for reinfection. The transfected cells were used for XC plaque assay and Moloney murine sarcoma virus (Mo-MSV) interference assay. The establishment of stable virus-producing NIH/3T3 cells was accomplished by calcium phosphate-mediated co-transfection of viral DNA and pSV2neo DNA (Southern & Berg, 1982). Recipient cells were isolated after selection with medium containing the aminoglycoside G418 (400 μg/ml).

**Reinfection.** The infectivity of progeny virus generated from transfection experiments was assayed in NIH/3T3 cells. Supernatants were collected 48 to 65 h post-transfection and centrifuged at 400 g for 5 min to remove cells and debris. After adjustment for the level of RT activity, identical amounts of mutant or wild-type virus were used to infect NIH/3T3 cells. Eighteen hours post-infection, the medium was removed, the cells were washed and fresh medium was added. Every 3 or 4 days, the supernatants of the infected NIH/3T3 cultures were harvested and the cells split 1:4 into new dishes. The RT activities in the supernatants were monitored.

**XC plaque assay.** Cells producing virus after DNA transfection were scored by the XC plaque assay according to Rowe et al. (1970). At 48 to 65 h post-transfection, the NIH/3T3 cells were killed by u.v.-irradiation and overlaid with XC cells. Two days later, the cells were fixed and stained with Giemsa and XC-positive cells were scored.

**XC co-cultivation.** Two days after transfection, the NIH/3T3 cells were reduced to a tenth of their cell density. Then 6 to 8 h later 3 × 10^4 XC cells were added directly to the cells. Two days after XC cell addition, the cells were fixed and stained as for the XC plaque assay.

**Reverse transcriptase assay.** Supernatants were harvested 48 to 65 h after transfection and then centrifuged for 5 min at 400 g to remove cells and debris. The supernatants were then centrifuged for 60 min at 30000 g. RT activity in the pellets was measured essentially according to Roy-Burman et al. (1976).

**Mo-MSV infection.** Two days after transfection the cells were divided 1:5. The next day the cells were challenged with a transforming Mo-MSV (Forchhammer & Turnoch, 1978). Mo-MSV foci were scored at 5 to 6 days.

**Analysis of viral proteins.** Transfected NIH/3T3 cells were labelled overnight with 10 μCi [35S]methionine (Amersham) in medium containing the non-radioactive methionine at 1:40 of its normal concentration. The cell supernatants were centrifuged as for the RT assay above and the virus pellets were either lysed and subjected to electrophoresis directly as previously described (Andersen, 1987) or the pellets were dissolved in detergents and precipitated with antisera against p30 or gp70. Immune complexes were collected with formaldehyde-fixed *Staphylococcus aureus* (Nexo & Ulrich, 1983). The

![Fig. 1. Construction of plasmid pKA1558. The plasmids are shown opened in at EcoRI site for simplicity. Plasmid pKA1558 is constructed from pMov-3 by cutting with AflIII and re-ligation. The unique HindIII (H), SphI (S), NdeI (N) and Clal (C) sites surrounding the env gene are shown. Other restriction sites shown are: EcoRI (E), AflIII (A), SalI (L), XhoI (X) and KspI (K).](image-url)
Proteins were analysed by SDS-PAGE (Laemmli, 1970). The protein bands were revealed by standard fluorographic techniques.

Results

Experimental design

A series of mutations was introduced into pKA1558 by site-directed mutagenesis. The structure of the plasmid pKA1558 is shown in Fig. 1. We have replaced arginine or lysine residues in gp70. Mo-MLV has two or more arginine or lysine amino acids in close proximity at several sites, including the gp70–p15E cleavage site. In five of the mutants, two basic amino acids were altered, either adjacent to each other or with one unaltered intervening amino acid. The oligonucleotides used in the mutagenesis were based on the published sequence for pKA1558.

Mo-MLV gp70 mutational analysis (Shinnick et al., 1981). The sequence of the env region in pMOv-3 was determined, and no differences were found (result not shown).

The viability of pKA1558 (wild-type) was similar to that of the parental plasmid pMOV-3 which is known to produce biologically active virus particles (Schneike et al., 1983). This was tested by transfection into NIH/3T3 cells. Infectious virus was produced and could readily be detected by the XC plaque assay and particulate RT activity in culture supernatants (results not shown).

The structures of the env mutants are shown in Fig. 2. The gp70 can be divided into domains (Dickson et al., 1984). Eight mutations (p102G/104Q, p124G/126A, p177I, p194A/195Q, p208Q, p223G/225G, p232A and p249Q/251A) were placed in the region determining receptor binding and host specificity. One mutation (p249Q/251A) was placed in the proline-rich region and two mutations (p280Q and p299Q) were in the carboxy terminus domain.

The viability of the mutants was assessed in comparison to pKA1558. An env deletion (pKA1680, deleted from the HindIII site in the pol gene to the Clal site in p15E), a gag deletion (pKA1826, deleted from a Xhol site in p30 to SalI in the pol gene) and a plasmid (pKA1704, in which the env fragment used for mutagenesis was removed and re-inserted) were included as controls. The viability of pKA1704 was comparable with that of pKA1558, and pKA1680 and pKA1826 were negative in all assays as expected (data not shown).

Biological activity of the mutants

Introduction of Mo-MLV DNA into NIH/3T3 cells by transfection made an artificially integrated provirus. By measuring the release of virions from transfected cells we could investigate whether the latter part of the virus life cycle, i.e. transcription, translation and virion assembly, occurs normally.

Transfection of the 10 site-specific mutations showed that all mutants released virion particles into the medium as measured by particle-associated RT activity (Table 1). The level of expression of RT activity ranged from below 20% (p102G/104Q, p124G/126A and p223G/225G) to the same amount as that of pKA1558 (p177I, p208Q, p232A, p280Q and p299Q). Two plasmids (p194A/195Q and p249Q/251A) showed 20 to 70% of the amount of RT activity of pKA1558.

In order to investigate whether the low RT activity was due to a low virus production or a defective enzyme, cell proteins were metabolically labelled with [35S]methionine overnight, 72 h after transfection. The virus particles in the supernatants were harvested by centrifugation and analysed by gel electrophoresis. All mutants released virion particles as shown by the recovery of the viral core...
protein p30 in cell supernatants. The mutants low in RT activity after transfection (102G/104Q, 124G/126A and 223G/225G) had low amounts of p30 also, showing that virus production was reduced (Fig. 3).

The difference found in the amount of virus particles released from the transfected cells can be explained by differences in either transfection efficiencies or the spread of virus particles. To analyse this the mutants with low RT activity and p30 production were labelled with [35S]methionine at different times post-transfection (Fig. 4). The virion particles in the supernatants were harvested and precipitated with antisera against both p30 and gp70. The amount of pelletable p30 was nearly the same in the mutant virions 24 to 48 h post-transfection, showing that the transfection efficiencies were alike. Two to three days post-transfection the amount of p30 in the wild-type was increasing and 3 to 4 days post-transfection the amount of p30 was higher for both wild-type and mutant virions of 249Q/251A. This shows that viral spread was poor in cultures transfected with plasmids p102G/104Q and p124G/126A. Labelling and immunoprecipitation of transfected cells showed that p30 was present in cell lysates

Table 1. Biological activity of mutants in transfection

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>RT activity†</th>
<th>XC plaque assay‡</th>
<th>XC cocultivation assay</th>
<th>Mo-MSV infection (no. of foci)</th>
</tr>
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<tr>
<td>No plasmid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>90</td>
</tr>
<tr>
<td>pKA1558 (wild-type)</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>0</td>
</tr>
<tr>
<td>p102G/104Q</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>p124G/126A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>120</td>
</tr>
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<td>+ +</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
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<td>+</td>
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<td>-</td>
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</tr>
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<td>p299Q</td>
<td>+ + +</td>
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<td>+</td>
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</table>

* The results are given in comparison to pKA1558 on a relative scale. Assays were performed several times.
† For RT activity, the four grades are defined as: -, less than background ± 3 S.D.; +, from background ± 3 S.D. to 20%; + +, 20 to 70%; + + +, above 70% of the values obtained with pKA1558.
‡ For XC plaque assay: -, negative; +, weak; + +, moderate; + + +, extensive.
§ Small plaques.
in amounts comparable to those of virus particles released (results not shown), indicating that no mutants were retained in the cells.

In order to test the XC fusion abilities of the mutants, the XC plaque assay was performed on the transfected NIH/3T3 cells. For a given retrovirus the XC plaque assay level and RT activity were proportional. However, cultures of cells transfected with the different mutant DNAs did not always show a proportional relationship between these parameters. The altered DNAs (p102G/104Q, p124G/126A and p223G/225G) were unable to induce XC plaques after transfection (Table 1). This was expected because of their low RT activity. Mutants p177I, p194A/195Q, p232A, p249Q/251A and the wild-type showed moderate to extensive XC plaque formation which correlated with their moderate or high RT activity. Mutants p208Q, p280Q and p299Q, however, showed a low efficiency of XC plaque formation despite their high RT activity, indicating slow syncytium development.

The development of XC plaques was analysed further by dilution and spreading of transfected cells together with XC cells. The results in XC plaque assays and XC co-cultivation were essentially the same for mutants of plasmids p102G/104Q, p124G/126A, p177I, p194A/195Q, p223G/225G and p232A and the results correlated with the RT activity. The size of the XC plaques induced by plasmids p208Q, p249Q/251A, p280Q and p299Q in XC co-cultivation was small, with syncytia of about three nuclei only. In contrast, XC plaques induced by the other mutants were indistinguishable from Mo-MLV-induced plaques, consisting of large syncytia with 10 to 15 nuclei (Fig. 5).

Leukaemia virus-producing cultures normally make excess envelope glycoproteins which block the receptor. Upon superinfection by a murine sarcoma virus with same envelope specificity, foci will only appear in uninfected cultures. Focus formation was not seen for cells transfected with p177I, p208Q, p232A, p249Q/251A, p299Q or the wild-type, showing that the viral spread was complete (Table 1). Only a few foci were seen with p194A/195Q and p280Q, probably because of a delay in viral spread or a weak binding of mutant virions. Extensive focus formation was seen in cells transfected with p102G/104Q, p124G/126A and p223G/225G indicating insufficient viral spread or lack of competition with Mo-MSV gp70.

To study whether virus infectivity was affected by alterations in the env gene of Mo-MLV, we re-infected NIH/3T3 cells with equal amounts of mutant or wild-type virus. The RT activities in the supernatants of the infected cultures were monitored (Fig. 6). After 7 days, viral spread was complete in cultures infected with mutant virions of p177I, p208Q, p232A, p249Q/251A, p299Q or wild-type virus. A delay in viral spread, as monitored by RT activity, was observed in cultures infected by virions of p194A/195Q or p280Q, this delay being more pronounced with virions of p280Q. For mutant virions of p102G/104Q, p124G/126A and p223G/225G, no RT activity was detected in infected cultures even after 45 days.
activity, but p102G/104Q(neo) and p124G/126A(neo) were still unable to induce XC plaques whereas p223G/225G(neo) produced XC plaques (Table 2).

Mo-MSV cannot infect co-transfected cells producing virions of p124G/126A, p177I, p223G/225G or the wild-type virus, indicating excess gp70 production which blocks the receptor. Cells producing virions of p102G/104Q cannot interfere with the receptor binding of Mo-MSV. As expected, p102G/104Q(neo) and p124G/126A(neo) virions could not re-infect cells. However, p223G/225G(neo) virions could.

**Discussion**

The data reported here showed that substituting one or two basic amino acids in gp70, thereby removing surface charges and possible cleavage sites for trypsin-like proteases, influences the XC fusion ability and/or the replication ability of the mutant viruses. All 10 mutant plasmids produced virions in transfected and co-transfected cultures, though in different amounts. This is not surprising since removal of the envelope gene does not block virion production (Dickson et al., 1984).

Mutant virions from p102G/104Q and p124G/126A could not infect cells and were unable to induce XC cell fusion. However, they are not alike. Co-transfected cells producing virions of p102G/104Q failed to interfere with Mo-MSV infection. This could be interpreted as a lack of binding to the receptor (Weiss, 1985), either because of no production or poor activity of gp70. Virions of p124G/126A interfered with Mo-MSV infection and therefore produced gp70.

Cells transfected with plasmid p223G/225G were XC-negative, unable to interfere with Mo-MSV infection and p223G/225G virions could not infect cells. After co-transfection and G418 selection of cells producing virions of p223G/225G, the cells became positive in the XC plaque assay, able to interfere with Mo-MSV and able to replicate. This unexpected result is unlikely to be explained by a reversion of this mutation, because the same results were obtained after three separate co-transfections and G418 selections of cells with this plasmid. Plasmids p177I and p232A behaved in all respect like the wild-type, indicating that lysine 177 and arginine 232 do not influence virus particle formation or infectivity.

Virions of p194A/195Q and especially p280Q showed a delay in infectivity despite high virus production, indicating a slightly impaired infection process. This delay corresponds with the few Mo-MSV foci developed in the Mo-MSV interference assay. Plasmid p280Q also showed a weak XC cell fusion. Plasmids p208Q, p249Q/251A and p299Q behaved essentially as the wild-type except in the XC plaque assays. In those cases, we
have XC fusion-defective mutants that are not impaired in their replication, showing that the XC fusion property is not necessarily linked to replication. It can therefore be questioned whether the XC cell fusion property is relevant for the virus–cell fusion, that is believed to occur during infection. Perhaps fusion can occur both at the host cell surface and in the vesicles after endocytosis, and only one of the paths is affected by the mutations. It will be interesting to pursue this question.

By comparing different retroviruses, the envelope glycoprotein can be divided into domains. The C-terminal half, which is well conserved (Dickson et al., 1984), presumably attaches to the N terminus of the transmembrane protein p15E (Pinter & Honnen, 1983). A proline-rich region (amino acids 231 to 274 in Mo-MLV) links the N-terminal half. The conformation of this half of gp70 is probably important as it contains intramolecular disulphide bridges (Pinter & Honnen, 1984). The N-terminal half is rather conserved except for the regions VRA (amino acids 51 to 130 in Mo-MLV) and VRB (amino acids 169 to 179 in Mo-MLV). The receptor-binding area of gp70, which determines the host range, is thought to be located within the first 160 amino acids, most probably in the VRA region (Battini et al., 1992). The virions of p102G/104Q and p124G/126A which are totally unable to replicate are mutated within the VRA region. The defect of p102G/104Q virions can be explained by poor receptor binding since cells producing these virions do not interfere with Mo-MSV. p124G/126A virions appear to bind, but possibly in a non-productive manner.

The receptor for ecotropic murine retroviruses has been shown to be a transporter for basic amino acids. It can be speculated that the basic amino acids in the VRA region of gp70 bind to the same sites of the receptor as those used in the amino acid transport.

The mutation made in the VRB region (p177I) had no influence on infectivity, indicating a minor role for this region as suggested by Battini et al. (1992). Except for those in p223G/225G, the mutations downstream of VRB, whether before, in, or after the proline-rich region, had weaker effects. The altered properties, slow infection and/or weak XC cell fusion, did not relate to specific areas, indicating different functional domains or a conformation where the domains are located in close proximity. Upon comparison, it can be seen that only a few of the 10 mutants behave identically. This is of interest, as it possibly reveals several steps in the infection process that might be worth investigating.

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References


Furchhammer, J. & Turnock, G. (1978). Glycoproteins from murine C-type virus are more acidic in virus derived from transformed cells than from nontransformed cells. Virology 88, 177–182.


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