Study of the 78A1 Isolate of Moloney Murine Sarcoma Virus. I. Molecular Cloning and Characterization

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SUMMARY

The 78A1 isolate of Moloney murine sarcoma virus (78A1 Mo-MuSV) was cloned from a genomic library obtained from virus producer rat cells, in the lambda vector L47. Among the recombinants hybridizing with a probe specific for the \textit{v-mos} sequences, we recovered a recombinant which contained leukaemia virus (MuLV) sequences and was able to transform both mouse and rat cells in transfection experiments. The cloned provirus could be rescued by both Mo-MuLV ecotropic and amphotropic viruses in mouse cells, but only with the amphotropic helper virus in rat cells. Comparative restriction mapping indicates that the 78A1 provirus is 200 bp longer than the HT1 provirus. The difference lies in the \textit{gag-pol} junction region of Mo-MuSV. Other minor differences were found in the \textit{gag} region, whereas the restriction patterns of the 3' parts of the proviruses were identical.

INTRODUCTION

The original Moloney murine sarcoma virus (Mo-MuSV) complex was isolated from a rhabdomyosarcoma induced in a BALB/c mouse after infection with high doses of Moloney murine leukaemia virus (Mo-MuLV) (Moloney, 1966). Several Mo-MuSV substrains have been derived from the original complex. This group of replication-defective viruses transforms fibroblasts in culture and induces fibrosarcoma \textit{in vivo}. Each strain displays a different set of deletions in the \textit{gag} and \textit{pol} regions (Donoghue et al., 1979). Four of them, namely HT1, m1, 124 MuSV and myeloproliferative sarcoma virus (MPSV), have been molecularly cloned (Vande Woude et al., 1979; Blair et al., 1980; Verma et al., 1980; Kollek et al., 1984) and extensively characterized in terms of structural organization and transforming potentials.

From nucleotide sequencing studies, it appears that the different isolates derive from the same parental MuSV which arose by recombination between Mo-MuLV and the proto-oncogene \textit{c-mos} present in the normal mouse genome (Brow et al., 1984; Reddy et al., 1981; Van Beveren et al., 1981). This cellular sequence in MuSV, termed \textit{v-mos}, is controlled by viral sequences and its expression is required for the induction and maintenance of viral transformation (Wood et al., 1983). The homologous \textit{c-mos} gene does not transform NIH-3T3 fibroblasts in DNA transfection assays, but can be activated by the addition of a long terminal repeat sequence (LTR) of the provirus either at the 5' or the 3' end, and it thereby gains transforming properties (Blair et al., 1981; Oskarsson et al., 1980).

The characterization of the MuSV strain 78A1 (Bernard et al., 1967) has been recently reported (Devaux et al., 1982). When the genomic RNA was compared with those of other MuSV isolates, it appeared that the 78A1 genome is smaller than the MPSV 7·0 kb RNA, but larger than the HT1 MuSV 6·1 kb RNA (Hamelin et al., 1983).

The Mo-MuSV/MuLV virus complexes induce sarcomas in newborn mice when injected intramuscularly. In addition, MPSV, derived from the same original Mo-MuSV complex,
exhibits a broader target cell specificity, since it also induces erythroid and myeloid leukaemia in adult mice (Le Bousse-Kerdiles et al., 1980). MPSV displays limited changes in the genome as compared with Mo-MuSV (Stacey et al., 1984).

In preliminary studies, we found that the viral complex produced by 78A1-infected rat cells induced morphological alterations of the haematopoietic system when injected into adult mice. To facilitate studies of the biological activity of 78A1 Mo-MuSV, we have molecularly cloned the integrated provirus of 78A1 cells in bacteriophage \( \lambda \). This paper reports on molecular cloning, characterization by restriction endonuclease mapping and transforming properties in cultured cells of the cloned Mo-MuSV 78A1 proviral DNA. The accompanying paper describes the biological activity of this isolate (Le Bousse-Kerdiles et al., 1985).

**METHODS**

**Enzymes.** Restriction endonucleases were purchased from New England Biolabs, Bethesda Research Laboratories and Promega Biotec, and digestions were performed according to the suppliers' specifications.

**Cells and transfection experiments.** All the cell lines were routinely grown on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum.

Transfections were performed using the calcium phosphate co-precipitation technique (Graham & Van der Eb, 1973). The day before transfection, the recipient cells were seeded at a density of either 10^6 cells per 10 cm dish or 3 \( \times 10^5 \) cells per 6 cm dish. Transforming potentialities of cloned DNA were tested with the equivalent of 200 to 300 ng of transforming insert. No carrier DNA was used. In some cases co-transfection was performed with the plasmid pMov-3 (Harbers et al., 1981) containing the MuLV helper virus (20 to 30 ng). Twenty-four h after transfection, the cells were washed, trypsinized and re-seeded in four dishes. Foci of morphologically transformed cells appeared within 2 to 3 weeks. This period of time was reduced to 5 or 6 days when pMov-3 was used as helper virus. The medium was changed twice a week.

**Cloning of the integrated proviral Mo-MuSV DNA in bacteriophage \( \lambda \).** Molecular cloning was performed essentially as described by Maniatis et al. (1982). A library of BamHI fragments of DNA larger than 8 kbp, from 78A1-infected cells, was constructed in bacteriophage \( \lambda \) L47.1. Recombinant phages were packaged in vitro (Williams & Blattner, 1980) and screened with a v-mos-specific probe. Plaques positive for v-mos were also hybridized with a MuLV probe. Plaques positive for both probes were assumed to contain a complete phage–MuSV provirus recombinant and were further purified.

**Southern blot analysis.** The restriction map of the phage–Mo-MuSV recombinant provirus positive for transformation on NIH-3T3 cells was established by Southern blot analysis, both with a 1.3.5 kbp BamHI insert, and a SacI proviral insert. Blots were hybridized with a 840 bp v-mos-specific probe (Devaux et al., 1982) consisting of three adjacent PstI fragments subcloned from the MuSV ml clone (Blair et al., 1980) and with a MuLV probe consisting of the entire genome of MuLV excised from plasmid pMov-3 (Harbers et al., 1981).

**RESULTS**

**Restriction analysis of unintegrated Mo-MuSV**

A recently cloned population of the MuSV-producing 78A1 cell line (clone A43) was used for preliminary restriction mapping studies and subsequent molecular cloning. Unintegrated viral replicative forms were obtained from NIH-3T3 cells freshly infected with 78A1 MuSV upon cocultivation with A43 producer cells. Restriction enzyme mapping did not reveal any convenient restriction site for cloning the circular molecules. Although EcoRI, BamHI, SacI and XhoI did not cleave the 78A1 MuSV genome, all other enzymes tested so far cleaved more than once (results not shown).

**Molecular cloning of the integrated Mo-MuSV DNA**

The high molecular weight DNA prepared from 78A1 cloned cells was digested with BamHI endonuclease. After separation on an agarose gel, blotting on nitrocellulose and hybridization with a nick-translated v-mos probe, two bands of 13 kbp and 10 kbp were detected with similar intensities (Fig. 1). Similarly, EcoRI digestion generated two bands of 14 kbp and 11 kbp (results not shown). Since there is a single copy of c-mos in rat cells we assumed that there was a single copy of integrated Mo-MuSV (Van der Hoorn et al., 1982). BamHI-restricted fragments larger than 8 to 9 kbp were prepared by sucrose density gradient fractionation, ligated with purified BamHI arms of bacteriophage \( \lambda \) L47.1 and packaged in vitro (Williams & Blattner, 1980).
Molecular cloning of the 78A1 Mo-MuSV isolate

Fig. 1. Southern blot of cellular 78A1 Mo-MuSV DNA digested with BamHI. The blot was hybridized with an 840 bp v-mos-specific probe consisting of three adjacent PstI fragments subcloned from the MuSV m1 clone (Devaux et al., 1982). These fragments are located entirely within the v-mos sequences. Two bands of 13 kbp (v-mos) and 10 kbp (c-mos) were revealed. λ DNA hydrolysed with HindIII gave the size markers (kbp).

Among 500,000 plaques screened, five hybridized with the v-mos-specific probe; two of these which also hybridized with the MuLV probe were assumed to contain a complete λ phage–MuSV provirus construct and were further purified.

Transforming efficiency of the cloned 78A1 Mo-MuSV DNA in murine cells
To test whether the Mo-MuSV 78A1 DNA cloned in λ retains the oncogenic potential of the virus, transfection experiments using NIH-3T3 cells, C127 mouse cells (Lowy et al., 1978) and Fisher rat FR3T3 cells (Seif & Cuzin, 1977) were performed (Graham & Van der Eb, 1973). From the two clones hybridizing with both the v-mos and Mo-MuLV probes, only one (clone A22) induced morphological transformation of both mouse and rat cells, suggesting that the other one could be a rearranged recombinant. The A22 MuSV recombinant containing the 13 kbp BamHI insert was therefore chosen for further analysis and cloned in a pBR322-derived vector (Lusky & Botchan, 1981). Transfection experiments were carried out either with the complete recombinant phage, or with the MuSV DNA insert excised from λ by BamHI hydrolysis, or with the total plasmid recombinant. There was no significant difference in the number of transformed foci obtained when identical amounts of MuSV DNA were transfected in NIH-3T3 or C127 cells. In parallel sets of experiments, we performed co-transfection of murine cells with 78A1 MuSV DNA and pMov-3, a recombinant plasmid which carries an infectious Mo-MuLV helper genome (Harbers et al., 1981). In both cases, the transforming activity of cloned 78A1 MuSV was similar to that of HT1 MuSV on the two different murine cell lines: 500 to 600 f.f.u./μg of MuSV insert. Cloned 78A1 MuSV DNA could also transform rat
FR3T3 cells, although the transforming efficiency was much lower: 40 to 50 f.f.u./µg of MuSV insert. Transfection of FR3T3 cells with 78A1 provirus was performed in parallel in the presence of an amphotropic helper virus (1504 A) (Hartley & Rowe, 1976), without any significant increase of transforming efficiency.

In the three cell types used for transfection experiments with suitable helper virus, the viruses released in the supernatant were able to transform NIH-3T3 cells with high efficiency, indicating that the proviral 78A1 MuSV had been rescued.

Restriction endonuclease mapping of Mo-MuSV 78A1 DNA

Restriction endonuclease mapping of the *BamHI* 13 kbp insert of 78A1 MuSV was carried out. As revealed by comparison with Mo-MuLV provirus, it appeared that integration of the *v-mos* sequences occurred in the *env* gene as for the other isolates, i.e. HT1, m1 and 124 MuSV (Donoghue & Hunter, 1983). In addition, the presence of a major deletion in the *pol* region of MuLV, encompassing the *SacI, KpnI, BamHI, SalI, EcoRI* and *BglII* sites was detected. The results were confirmed by studying heteroduplex molecules formed between Mo-MuSV 78A1 DNA and Mo-MuLV DNA; one deletion loop of 2.3 kb was observed at a distance of 2.3 kb from the 5' end, and a substitution loop of approximately 1.2 kb long at 1.3 kb from the 3' end (data not shown).

From the results of *SacI* hydrolysis, sizes of 4.2 and 1.5 kbp were assigned to the cellular rat sequences flanking the provirus at the 5' and 3' ends, respectively.

78A1 MuSV and HT1 MuSV restriction maps were compared after excision of the λ recombinant flanking sequences with *SacI*. Several endonuclease digestions were performed, DNA fragments were separated on an agarose gel and Southern blot analyses were carried out on two identical blots as exemplified in Fig. 2. Hybridization with specific *v-mos* and Mo-MuLV probes revealed that 78A1 Mo-MuSV is significantly larger than HT1 Mo-MuSV.

**DISCUSSION**

Detailed comparative restriction maps of the two isolates, HT1 Mo-MuSV and 78A1 Mo-MuSV proviral clones, showed that these two isolates are quite similar, 78A1 being 200 bp larger (Fig. 3). Comparison with the MPSV endonucleases pattern reported by Kollek et al. (1984) indicated that there was also a marked similarity. The restriction endonucleases which were used gave identical patterns for the HT1 and 78A1 Mo-MuSV isolates with three exceptions. (i) The *XhoI* site present in the *gag* region of HT1, as well as in MuLV and all the other MuSV isolates studied so far, is lacking in the 78A1-MuSV. (ii) Concomitantly, an additional *PvuI* site is observed in the same region of the genome. This might reflect discrete rearrangement(s) or mutation(s) in the *gag* sequence originating from MuLV that may be similar to small (5 bp and 20 bp) strain-specific deletions reported for the 124 Mo-MuSV variant (Van Beveren et al., 1981); interestingly, such a restriction site was noted in the same region of the *gag* gene in xenotropic viruses (Joseph, 1981). (iii) The 200 bp difference in length between 78A1 and HT1 MuSV DNA was found to be precisely confined within a single *PvuI–HindIII* restriction fragment, located in the *gag–pol* MuSV junction area; this fragment is 450 bp long in HT1 and 650 bp long in 78A1. Hybridization patterns indicate that this sequence, like all the others with the exception of the *v-mos* gene, originated from the MuLV parental virus. Size comparisons of the *BglII–HindIII* fragment that includes the *PvuI–HindIII* described above indicate that it is identical in MPSV and HT1, but is larger in 78A1. The *XhoI* site located in the *gag* region of the genome and already mentioned is also present in MPSV.

From the sequences reported for other MuSV isolates (Brow et al., 1984; Reddy et al., 1981; Van Beveren et al., 1981; Blair et al., 1980), it appears that HT1 MuSV has retained more of the parental Mo-MuLV and *c-mos* sequences than the other isolates, and therefore is probably the most closely related to the parental Mo-MuSV. 124 Mo-MuSV and m1 Mo-MuSV might have diverged from this common *MuLV–mos* recombinant (Donoghue & Hunter, 1983). In view of its similarities to HT1 and its larger size, 78A1 MuSV might be closer than HT1 to the ancestor MuSV.
Fig. 2. Comparative restriction patterns of 781 Mo-MuSV (clone A22) and HT1 Mo-MuSV. Pairs of lanes show comparison of 78A1 MuSV (clone A22) (lanes 1 and 3) and HT1 MuSV (lanes 2 and 4) digested with several enzymes. The same gel was blotted on two different nitrocellulose membranes by a sandwich method and identical duplicates were obtained. One was hybridized with the v-mos probe (lanes 1 and 2) and the other with the MuLV probe (lanes 3 and 4). Size markers are \( \lambda \) DNA digested with HindIII (kbp).
As reported recently (Stacey et al., 1984), only MPSV is larger than 78A1 MuSV. It is worth noting that MPSV deriving from the same original tumour consists of only Mo-MuLV and c-mos-related sequences, but possesses a distinctive biological activity. In addition to the ability to induce sarcomas as is common to all the other previously described Mo-MuSV isolates, MPSV causes a myeloproliferative syndrome in susceptible adult mice, involving drastic alterations in the erythroid and myeloid compartments of the haematopoietic system (Pragnell et al., 1981; Kollek et al., 1984). It would be interesting to know what sequences are involved in target cell specificity. In this respect, molecular cloning of the 78A1 isolate and comparison with MPSV and HT1 MuSV in reconstruction experiments might prove helpful. Comparative studies of the biological effects of the cloned provirus have been performed in vitro and in vivo and are reported in the accompanying paper (Le Bousse-Kerdlies et al., 1985).

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Molecular cloning of the 78A1 Mo-MuSV isolate


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