Transfer of Murine Leukaemia and Murine Sarcoma Virus Genetic Information by Transfection with Isolated Metaphase Chromosomes

By JUDITH G. LEVIN, BARBARA F. HUGHES, JANELLE S. GRAETER, ALAN REIN, ELAINE RANDS and ANIL B. MUKHERJEE

1Laboratory of Molecular Genetics and 2Pregnancy Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205, U.S.A., 3Biological Carcinogenesis Program, Frederick Cancer Research Facility, Frederick, Maryland 21701, U.S.A. and 4Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, U.S.A.

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SUMMARY

Chromosome-mediated transfer of murine leukaemia (MuLV) and murine sarcoma (MuSV) virus genetic information to uninfected recipient cells was investigated. Metaphase chromosomes from AKR MuLV-infected SC-1 mouse cells were incubated with NIH/3T3 cells. After several passages (1 to 3 weeks), infectious virions exhibiting reverse transcriptase activity and the characteristic host range of ecotropic, N-tropic AKR virus appeared in the supernatant fluids of the treated cells. Restriction endonuclease analysis of genomic DNA from transfected cells indicated that AKR proviral DNA was associated with the high molecular weight DNA of the host. These results demonstrate that the AKR MuLV genome can be stably transferred to uninfected recipient cells via isolated metaphase chromosomes. Although AKR virions are not able to infect heterologous cells, chromosome-mediated transfection resulted in the establishment of productive AKR MuLV infection in mink cells. Thus, the use of chromosomes to transfer virus genes can circumvent the natural host restriction barrier. In other experiments, it was shown that normal NIH/3T3 cells were transformed after exposure to metaphase chromosomes isolated from an MuSV-infected, non-producer line. Foci were detected 14 to 21 days after chromosome treatment and were shown to contain true viral transformants since transforming virus was produced after superinfection with MuLV.

INTRODUCTION

The use of isolated metaphase chromosomes to transfer genetic information into cultured mammalian cells has been demonstrated by a number of different laboratories (Burch & McBride, 1975; Cassingena et al., 1978; Lewis et al., 1980; McBride & Ozer, 1973; Miller & Ruddle, 1978; Mukherjee et al., 1978; Shani et al., 1974; Shih et al., 1979; Willecke & Ruddle, 1975). Although the genome of the DNA tumour virus, SV40, has been transferred by this technique (Cassingena et al., 1978; Shani et al., 1974), successful transfer of retrovirus genomes with this procedure has not been reported to date. The importance of the retrovirus system for studying the regulated expression of cellular genes is emphasized by recent evidence suggesting that these viruses resemble transposable elements (Dhar et al., 1980; Shimotohno et al., 1980; Shoemaker et al., 1980; Sutcliffe et al., 1980; Van Beveren et al., 1980), which upon insertion into host chromosomes can disrupt the normal function of cellular genes and in some instances lead to induction of a tumour (Neel et al., 1981; Payne...
(Lueders et al., 1982). Since chromosomal material from infected cells presumably contains not only virus DNA sequences but also additional regulatory elements of either cellular or viral origin which affect virus expression, it seemed of interest to investigate the potential application of chromosome-mediated gene transfer to the retrovirus system.

In the present study, we show that treatment of uninfected NIH/3T3 cells with metaphase chromosomes from AKR murine leukaemia virus (MuLV)-infected mouse cells results in the production of infectious particles with the characteristic host range of AKR MuLV. Transfection with metaphase chromosomes also permits the establishment of productive infection in heterologous mink cells, thereby overcoming the natural host restriction barrier (Besmer & Baltimore, 1977; Ishimoto et al., 1978) to AKR virus replication. In addition, we find that normal NIH/3T3 cells become transformed following exposure to metaphase chromosomes from a Harvey murine sarcoma virus (Ha-MuSV)-infected, non-producer cell line. The induced foci contain authentic viral transformants since they produce transforming virus upon superinfection with MuLV.

**Methods**

**Materials.** [3H]TTP was purchased from New England Nuclear. Seakem agarose and the restriction endonucleases *PstI* and *BamHI* were purchased from Bethesda Research Laboratories (Gaithersburg, Md., U.S.A.). Serum and colcemid were obtained from Grand Island Biological Co. (Grand Island, N.Y., U.S.A.). Mitomycin C was obtained from Sigma.

**Cells and viruses.** SC-1 cells (Hartley & Rowe, 1975), NIH/3T3 cells, and the AKR-L1 and Moloney isolates of MuLV were graciously provided by Ms M. Lander (National Cancer Institute). Ha-MuSV-infected NIH/3T3 cells were a gift of Dr D. Lowy (National Cancer Institute). AKR MuLV-infected SC-1 cells and mink cells (line CCL64) were maintained in McCoy 5A medium and Dulbecco's modified Eagle's medium respectively, each containing 10% foetal calf serum and antibiotics (streptomycin, penicillin, aureomycin and mycostatin). NIH/3T3 cells and Ha-MuSV-infected NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics (see above). Moloney MuLV was propagated in SC-1 cells; virus pools were prepared from 18- to 24-h harvests which were clarified by centrifugation at 800 g for 15 min and then stored in liquid nitrogen.

**Isolation of metaphase chromosomes.** The method for the isolation and purification of metaphase chromosomes has been described by Burkholder & Mukherjee (1970). Essentially, the procedure was as follows: eight 1585-cm² roller bottles (Corning) with cells in the exponential phase of growth were exposed overnight to colcemid at 0.1 μg/ml. The rounded, metaphase cells were dislodged from the plastic surface by gently shaking the roller bottles with 25 ml of warm medium. Cells were collected by centrifugation at 600 g for 10 min at 4 °C, washed three times with Hanks' balanced salt solution (HBSS) and then exposed to 15 ml hypotonic solution (1:4, HBSS:distilled water) for 30 min at 37 °C. The cells were collected by centrifugation at 580 g for 15 min and resuspended in 10 ml cold chromosome medium I [2% (w/v) citric acid, 0.1 M-sucrose, 0.1 mM-CaCl₂, 0.1 mM-MgCl₂, 0.1% (v/v) Triton X-100]. The cell suspension was drawn up into a 12 ml plastic syringe and ejected forcibly through a 20-gauge needle. This procedure was repeated until microscopic analysis revealed that almost all cells had been disrupted. The lysate was then resuspended in chromosome medium II [chromosome medium I with Triton X-100 at 0.5% (v/v) instead of 0.1%]. This mixture was then filtered through a sintered-glass filter (pore size 20 to 30 μm) to separate nuclei from the chromosomes. Final concentration of the chromosomes was achieved by centrifugation at 70 g. DNA was estimated spectrophotometrically (Shih et al., 1979).
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Treatment of recipient cells with metaphase chromosomes from AKR MuLV-infected cells. Recipient cells in a T-75 flask were allowed to reach 70 to 75% confluency and were then treated for 3 h with metaphase chromosomes (300 to 500 µg DNA) contained in 2 to 3 ml of medium without serum. The cells were gently rinsed once, refed and after re-incubation for 24 to 48 h, were trypsinized and divided between three flasks. Upon reaching confluency, the cells from each of the three flasks were combined and subdivided for further passage. In experiments with mink cells as the recipient, the original flask with chromosome-treated cells was divided into two T-150 flasks (passage 1) and incubated until reaching confluency (1 week). Because ecotropic virus spread cannot occur in mink cells, the confluent cells were next treated with mitomycin C (10 µg/ml) for 2 h, and were then trypsinized and co-cultivated with NIH/3T3 cells (passage 2). Mitomycin C prevents the mink cells from undergoing further cellular division; however, since there is only a gradual cytocidal effect, virus production (if initiated) may continue for several days (Aaronson & Dunn, 1974). The ratio of mink cells to NIH/3T3 cells could be varied and ratios of 3:1, 1:1 and 1:3 were equally effective (data not shown). Prior to treatment with chromosomes and at each succeeding step, fluids were collected for assay of reverse transcriptase activity (Levin & Rosenak, 1976) and, in some cases, also for assay of infectivity (Rowe et al., 1970).

Treatment of recipient cells with metaphase chromosomes from Ha-MuSV-transformed cells. NIH/3T3 cells were treated with metaphase chromosomes (50 to 100 µg DNA) from an Ha-MuSV-infected non-producer cell line, as described above. Addition of carrier (metaphase chromosomes from normal NIH/3T3 cells) had no effect on induction of foci. After incubation for 24 h, the recipient cells were trypsinized and divided between five 100 cm² plates. Foci were scored after 14 to 21 days. In order to determine whether the foci contained transformants which could be rescued by MuLV, cultures containing foci were trypsinized and passaged three or four times, until most of the cells appeared to be transformed. Fluids from the transformed cells had no activity in the XC (Rowe et al., 1970) and reverse transcriptase assays respectively (data not shown). The cells were then infected with Moloney MuLV at an m.o.i. of 10 and, after two to four passages, the fluids were collected and used to infect NIH/3T3 cells. Foci were scored after 7 to 14 days.

Cellular DNAs and virus DNA probe. High molecular weight cellular DNA was isolated as described by Lowy et al. (1978). The virus DNA probe used for hybridization was a subgenomic fragment of molecularly cloned integrated AKR MuLV DNA (Lowy et al., 1980). This fragment, 1.4 kilobases (kb) in length, represents a pol-env segment which includes sequences from a SmaI site 5.1 kb from the left end of virus DNA to another SmaI site 6.5 kb from the left end. It was isolated by electroelution from an agarose gel and subsequently cloned in pBR322 (Chattopadhyay et al., 1982). A ³²P-labelled DNA probe (sp. act. 5 × 10⁷ ct/min/µg) was synthesized by nick translating (Maniatis et al., 1975) the cloned fragment. Hybridization was carried out in 0.6 M-NaCl-0.06 M-sodium citrate (4 × SSC) at 65 °C for 30 h, using about 10 × 10⁶ ct/min of virus probe.

RESULTS

Chromosome-mediated transfer of MuLV genes to NIH/3T3 cells

The potential use of metaphase chromosomes to transfer MuLV genes to uninfected cells was investigated by treating NIH/3T3 cells with chromosomes from AKR MuLV-infected SC-1 cells. Appearance of virus particles in the supernatant fluids was monitored by measuring reverse transcriptase activity; infectivity was determined by the XC plaque assay (Rowe et al., 1970). As may be seen from Fig. 1, virus production was detected at the sixth passage following chromosome treatment. Both infectivity and reverse transcriptase activity increased dramatically as the cells continued to be passaged and, by passage 10 or
Fig. 1. Transfection of NIH/3T3 cells with metaphase chromosomes from AKR MuLV-infected SC-I cells. NIH/3T3 cells were treated with metaphase chromosomes as described in Methods. The control flask was treated with supernatant fluid which was collected from the cells used for the chromosome preparation, filtered, and then subjected to the entire chromosome isolation procedure. Prior to treatment and at each passage, fluids were collected, concentrated, and assayed for reverse transcriptase activity as measured by TMP incorporation (Levin & Rosenak, 1976). The reverse transcriptase activity data (○) are given for \(10^{-1}\) portions of concentrated fluid (total vol. 100 \(\mu\)l). Infectivity (▲) was assayed on SC-1 cells by the XC plaque test (Rowe et al., 1970) and the data are given in p.f.u./ml of original supernatant fluid. Only reverse transcriptase data are shown for the control (■). However, control fluids were also tested for infectivity and were found to be XC-negative for 13 or more passages (see text).

11, the titre was approx. \(1 \times 10^7\) p.f.u./ml. In other experiments (e.g. see Fig. 3), virus production was apparent one or two passages following treatment, and reached a plateau approx. four or five passages later. Variation in onset of virus production is probably due to differences in the degree to which DNA remains intact during chromosome isolation (McBride & Ozer, 1973).

Controls were designed to exclude the possibility that virus particles or intact cells contaminating the chromosome preparation were responsible for virus production by the NIH/3T3 cells. In the experiment shown in Fig. 1, the culture medium from the infected donor cells was collected at the time the chromosomes were prepared. This fluid, which contained infectious virus (data not shown), was filtered, subjected to the chromosome isolation procedure, and then added to recipient NIH/3T3 cells. After 13 passages (and in other experiments, up to 15 passages) no reverse transcriptase activity (Fig. 1) or infectivity (data not shown) could be detected in the culture fluids. Concentrating the medium by centrifugation at 100,000 \(g\) and then subjecting it to the chromosome isolation procedure also gave negative results. Finally, isolation of chromosomes in the presence of 1% (v/v) Triton X-100, a concentration which is sufficient to disrupt virions and cells, did not affect the activity of the chromosome preparation.

The cells used for isolation of chromosomes were producing the AKR strain of MuLV, which is classified as an ecotropic N-tropic virus. In assessing the validity of the transfection experiments, it was important to determine whether virions produced after chromosome treatment had the properties of this class of MuLV. The data of Fig. 1 show that the virus
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Table 1. *Host range of MuLV produced after treatment with chromosomes from AKR MuLV-infected cells*

<table>
<thead>
<tr>
<th>Virus source</th>
<th>3T3FL</th>
<th>DBA</th>
<th>Balb/3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid, chromosome-treated cells*</td>
<td>$5 \times 10^6$</td>
<td>$2 \times 10^6$</td>
<td>$2 \times 10^5$</td>
</tr>
<tr>
<td>N-tropic MuLV†</td>
<td>$7 \times 10^5$</td>
<td>$9 \times 10^5$</td>
<td>$1 \times 10^6$+</td>
</tr>
<tr>
<td>B-tropic MuLV</td>
<td>$1 \times 10^6$</td>
<td>$1 \times 10^6$+</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>NB trophic MuLV</td>
<td>$2 \times 10^4$</td>
<td>$7 \times 10^4$</td>
<td>$5 \times 10^4$</td>
</tr>
</tbody>
</table>

* Passage number 10.
† WN1802N strain of MuLV.
‡ Restricted viruses titred at a dilution of $10^{-7}$.

is ecotropic, since only ecotropic strains of MuLV form syncytia in the XC plaque assay. To determine the tropism or host range of the virus with respect to the cellular Fv-1 locus (Hartley *et al.*, 1970; Pincus *et al.*, 1971), however, it was also necessary to measure the ability of the virus to replicate on three different types of mouse cell lines: 3T3FL (Gisselbrecht *et al.*, 1974), permissive to both N- and B-tropic MuLV; DBA (Benjers *et al.*, 1979), which contains the Fv-1^N gene, rendering it permissive to N-tropic MuLV and restrictive to B-tropic MuLV; Balb/3T3, which contains the Fv-1^B gene, rendering it permissive to B-tropic MuLV and restrictive to N-tropic MuLV. As illustrated in Table 1, MuLV from chromosome-treated cells exhibited a titre on Balb/3T3 cells which was more than 100-fold lower than that observed on 3T3FL or DBA cells. This pattern was the same as that obtained with a standard laboratory strain of N-tropic MuLV (WN1802N), but different from that for B- or NB-tropic MuLV. These results, as well as the data of Fig. 1, strongly suggest that the AKR MuLV genome was transferred to uninfected cells in the original transfection.

Further evidence for this conclusion was obtained from restriction endonuclease analysis of genomic DNA from transfected cells (Fig. 2). A molecularly cloned *pol-env* AKR MuLV DNA probe (Chattopadhyay *et al.*, 1982) was used for the hybridization. Fig. 2 gives the results of digestion with *PstI* and *BamHI*; the expected restriction sites and the segment corresponding to the probe are illustrated below the blot. As can be seen, *PstI* digestion of DNA from chromosome-treated and virus-infected cells (lanes 1 and 3 respectively) resulted in the expected band at 8.2 kb (Rands *et al.*, 1981); this band was not present in DNA from uninfected cells (lane 2). Similarly, a 2.9 kb band generated by *BamHI* digestion, which is diagnostic for the presence of the AKR MuLV genome (Steffen *et al.*, 1979), was not present in the sample from uninfected cells, but was very prominent in the restriction digest of DNA from the virus-producing cells. These data confirm that the virus replicating in the chromosome-treated cultures (Fig. 1) is identical to AKR MuLV.

**Chromosome-mediated transfer of MuLV genes to heterologous mink cells**

The results presented thus far indicate that metaphase chromosomes from MuLV-infected cells can be used in place of intact virions to establish chronic MuLV infection. It was next of interest to determine whether chromosome treatment would be effective under conditions where the virus itself is not infectious, e.g. where there is a host restriction barrier to infection. Thus, mouse ecotropic viruses (such as AKR MuLV) replicate to high titre in mouse cells and to a lesser extent in rat cells, but cannot infect hosts of heterologous origin. The basis for this restriction is thought to reside in the lack of appropriate receptors on the surface of the foreign host (Besmer & Baltimore, 1977; Ishimoto *et al.*, 1978).

To test the possibility that chromosome treatment can circumvent this type of block, metaphase chromosomes were isolated from ecotropic MuLV-infected cells and were then added...
Fig. 2. Hybridization of PstI- and BamHI-digested cellular DNAs to a subgenomic AKR MuLV DNA probe. Restriction endonuclease-digested cellular DNAs were subjected to electrophoresis in a 0.7% agarose gel (35 V, 20 h), transferred to a nitrocellulose filter (Southern, 1975) and hybridized to a $^{32}$P-labelled 1.4 kb Smal fragment (Chattopadhyay et al., 1982) derived from cloned AKR MuLV DNA (Lowy et al., 1980). The DNA samples were: lane 1, NIH/3T3 treated with chromosomes from AKR MuLV-infected SC-1 cells (passage 15); lane 2, NIH/3T3; lane 3, NIH/3T3 infected with AKR MuLV. The restriction endonuclease map of 8.8 kb linear AKR MuLV DNA showing the restriction sites for PstI and BamHI (Rands et al., 1981) is illustrated below the blot. Distances between sites are given in kb. The segment corresponding to the 1.4 kb probe is also shown. Hybridizable bands which appear in DNA digests from uninfected NIH/3T3 cells as well as from MuLV-producing cells result from homology of the probe with endogenous mouse virus DNA sequences (Steffen et al., 1979).

to uninfected mink cells. A portion of the same chromosome preparation was also used to transfect NIH/3T3 cells. Because secondary infection of MuLV does not occur in mink cells, it was necessary to amplify the potential number of virus particles produced by treating transfected mink cells with mitomycin C and then co-cultivating with NIH/3T3 cells. As shown in Fig. 3, reverse transcriptase activity was detected in the fluids of co-cultivated mink cells as well as in the chromosome-treated NIH/3T3 cells. In each case, the fluids were also positive in the XC plaque assay (Rowe et al., 1970), and titres of approx. $1 \times 10^7$
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Fig. 3. Transfection of mink cells with metaphase chromosomes from AKR MuLV-infected SC-1 cells as measured by reverse transcriptase activity (●). Mink cells were treated with metaphase chromosomes from AKR-infected SC-1 cells and, following mitomycin C treatment, were co-cultivated with NIH/3T3 cells (ratio of mink:NIH/3T3, 3:1). 'Passage 1' refers to the first passage after chromosome treatment (i.e. 1 week after treatment) and prior to co-cultivation with NIH/3T3 cells ('passage 2'). For comparison, the same chromosome preparation was used to transfect NIH/3T3 cells (▲). The control (■) was mitomycin C-treated mink cells which were co-cultivated with NIH/3T3 cells as above. Incorporation was monitored as described in the legend to Fig. 1.

p.f.u./ml were reached in the later passages (data not shown). These results indicate that ecotropic MuLV was being produced.

To be certain that mitomycin C treatment was not activating an endogenous virus in mink cells which would register in the reverse transcriptase assay, mink cells which were not treated with chromosomes were exposed to mitomycin C and co-cultivated with NIH/3T3 cells. As can be seen, there was no evidence of virus production in the absence of chromosome treatment (Fig. 3). In addition, the fluids from the control mink cells remained XC-negative (data not shown).

Chromosome-mediated transfer of Ha-MuSV genes to NIH/3T3 cells

Since the experiments involving chromosome-mediated transfer of MuLV genes depend upon virus spread for detecting transfection of uninfected cells, it is not clear whether stable association of the transgenome with recipient chromosomes (Klobutcher & Ruddle, 1979; McBride & Ozer, 1973; Willecke & Ruddle, 1975) occurs as a result of the initial transfer or only after one or more cycles of secondary infection. It was therefore important to employ a system in which the virus genome could be transferred to recipient cells, but not expressed as infectious virus particles. For this purpose, we decided to test the ability of metaphase chromosomes from an Ha-MuSV-transformed, non-producer line to induce foci in normal NIH/3T3 cells. An earlier attempt to perform this type of experiment which involved incubation of primary hamster embryo cells with chromosomes from an MuSV-transformed hamster cell line (HT-1) was unsuccessful, although some foci were obtained when recipient cells were co-cultivated with cells producing Rauscher MuLV (Ebina et al., 1974). In the present study, foci were consistently produced following chromosome treatment alone and
were detected within 14 to 21 days. Untreated cultures (Fig. 4a) or cultures treated with chromosomes from normal NIH/3T3 cells (data not shown) did not exhibit foci. Fig. 4(b) illustrates a typical primary focus induced in chromosome-treated cultures. On average, a frequency of 5 to 10 foci per $10^7$ recipient cells was observed. Variation of chromosomal DNA concentration over a wide range (1 μg to 1 mg of DNA) had no significant effect on the number of foci obtained. A similar observation was made by Burch & McBride (1975) for small variations in the input ratio of chromosomes to recipient cells. However, Lewis et al. (1980) have noted some effect of chromosome dosage.

To determine whether the foci produced after chromosome treatment represent true Ha-MuSV transformants and not the spontaneous foci which sometimes arise in normal cells (Shih et al., 1979), the treated cultures were tested for their ability to form transmissible Ha-MuSV(MuLV) pseudotype particles. The cultures were superinfected with Moloney MuLV and the supernatant fluids were assayed for transforming activity on normal NIH/3T3 cells. Fig. 4(d) shows an example of foci produced under these conditions. The foci were morphologically indistinguishable from those formed by authentic Ha-MuSV(MuLV) pseudotype virions (data not shown) and were typically more diffuse than the primary foci formed during the original transformation (compare with Fig. 4b), presumably because the presence of pseudotype particles allows virus spread within the culture. No foci were detected in cells treated with fluids from either mock-infected Ha-MuSV chromosome-treated cultures (Fig. 4c) or from MuLV-infected cells originally treated with chromosomes from non-transformed cells (data not shown).

These results demonstrate that chromosomes can be used to transfer the sarcoma virus genome to normal cells and to transform these cells stably under non-selective conditions. Thus, the transferred virus genome must be replicated by host cell enzymes and must be efficiently distributed to both daughter cells at mitosis. In addition, the data support the previous conclusion based on transfection studies with naked Ha-MuSV DNA (Lowy et al., 1978) indicating that helper leukaemia virus is not required for induction of transformation.

**DISCUSSION**

The demonstration that retrovirus genes can be transferred to recipient cells via isolated metaphase chromosomes should provide an important experimental approach for future work on the interaction between cellular and viral genomes and it complements studies involving transfection with proviral DNA (Hill & Hillova, 1972) or cloned virus DNA fragments (Hager et al., 1979; Lowy et al., 1980). Although naked DNA has been used quite successfully to elucidate the structure of the retrovirus genome and other aspects of virus replication, it should be emphasized that in DNA-mediated transfection only relatively small DNA fragments are transferred, whereas chromosome-mediated gene transfer involves transfection of a larger piece of biologically active material, including viral and nonviral DNA sequences as well as chromosomal proteins. In view of current interest in protein–DNA interactions (Igo-Kemenes et al., 1982) and evidence that the site of integration of virus DNA sequences may influence the outcome of virus infection (Neel et al., 1981; Payne et al., 1981), it would seem that chromosomal transfer will continue to be a valuable experimental tool, particularly in conjunction with new techniques for chromosome fractionation (Carrano et al., 1980).
et al., 1979; Padgett et al., 1977) and detection of unique DNA sequences by in situ hybridization (Tereba et al., 1979).

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