Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells

(somatic cell genetics/gene mapping)

R. E. K. FOURNIER AND F. H. RUDDLE

Department of Biology, Kline Biology Tower, Yale University, New Haven, Connecticut 06520

Contributed by Frank H. Ruddle, October 21, 1976

ABSTRACT In this report, we describe the production and characterization of proliferating hybrid cell populations generated by fusion of murine microcells with intact mouse, Chinese hamster, and human recipient cells. The microcell hybrids so produced contained one to five intact murine chromosomes derived from the microcell donor. These transferred chromosomes were maintained as functioning genetic elements in the hybrid cells. Our results firmly establish subnuclear particle-mediated chromosome transfer as a valid somatic cell genetic tool.

Somatic cell genetics represents a useful approach to mammalian genetics and epigenetic studies. These experimental systems depend on the analysis of proliferating hybrid cell populations which contain only a subset of the genetic material of one of the parents. Until recently, such a result was universally achieved via cell/cell hybridization and subsequent chromosome elimination. A more direct approach to the generation of cell lines possessing a limited amount of genetic material of one parent would be the introduction of only a small number of donor cell chromosomes into the recipient at the time of fusion. Such a technique would permit the experimental control of the direction of chromosome segregation, and would also make possible more meaningful studies of the genetic regulation of epigenotype expression. It is now possible to implement these kinds of experiments by fusing subnuclear particles with whole cells.

The potential usefulness of subnuclear particles in hybridization experiments was first shown by Ege and Ringertz (1) by using murine donor material. Johnson and coworkers (2) have independently developed a system for transferring limited numbers of human chromosomes from one cell to another. Both systems are capable of generating small cell-like structures ("microcells" and "mini-segregants," respectively) containing a limited amount of genetic material packaged in a micronucleus. The micronucleus is surrounded by a rim of cytoplasm and an intact plasma membrane, the entire particle thus serving as an efficient vector for the transfer of small numbers of chromosomes.

In this report, we describe the successful transfer of small numbers of murine chromosomes into intact mouse, Chinese hamster, and human recipient cells. These proliferating hybrid cell populations so produced represent successful fusions of rodent-derived microcells with intact recipients. Our experiments firmly establish that chromosomes introduced by microcells can be maintained indefinitely as functioning genetic elements in hybrid cells.

MATERIALS AND METHODS

Three mouse L-cell derivatives were used in this study: A9 lacks hypoxanthine phosphoribosyltransferase (HPRT, IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) and adenine phosphoribosyltransferase (APRT, AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) (3). B82 is deficient in thymidine kinase (TK, ATP:thymidine 5'-phosphotransferase, EC 2.7.1.75) (3), and CT11Cl is an HPRT- derivative (4). Human and Chinese hamster recipient cell lines were HeLa S3 (5) and E36 (6), respectively. Primary mouse embryo fibroblasts were produced by using standard procedures (7). All cell lines were maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 µg/ml). Cell reagents were obtained from Gibco. Parental cell lines and hybrid clones were found to be free of mycoplasma by the culture method of Hayflick as modified by Barile (8).

Micronucleation of the cells was achieved by exposing cultures growing in logarithmic phase to colcemid (Gibco) for 1.5-4 days. Populations of A9 or CT11Cl in which 80-90% of the cells had become micronucleate were produced using colcemid (0.1 µg/ml) for 48 hr. Micronucleation (60-70%) of mouse embryo fibroblasts was observed after treating the cells with 0.05 µg/ml colcemid for 36 hr.

Micronucleation of micronucleate cells closely followed the procedure used by Ege and Ringertz (1). Plastic discs to which the cells were attached were placed cell-side-down in centrifuge tubes containing complete medium and spun at 12,000 × g for 10 min at 34°C to remove loosely attached cells. The discs were then centrifuged at 39,000 × g for 20 min at 34°C in complete medium containing cytochalasin B (10 µg/ml) (Aldrich). This step resulted in micronucleation of >95% of the cells on the discs. The microcell pellets were pooled and resuspended in 2 ml of 0.5% bovine serum albumin (fraction V, Sigma) in phosphate-buffered saline at pH 7.2. At this point, the preparation typically contained 50-60% microcells of varying sizes, 40-50% small cytoplasmic fragments, and 0-10% whole cells.

The crude microcell preparation was purified by unit gravity sedimentation on a linear 1-3% bovine serum albumin gradient (9). Total volume of the gradient was 50 ml, and the preparation was allowed to settle for 3.5 hr at room temperature. The particle composition of a typical gradient fractionation is shown in Table 1. A purified microcell preparation was obtained by collecting the top 20 ml (excluding the sample zone) of the gradient and resuspending in 0.5 ml serum-free medium.

For fusion, the purified preparation was added to a confluent monolayer of recipient cells (3 to 9 × 10^6 recipiensts, depending on cell line employed). The microcell-to-cell ratio varied from 1:1 to 1:3. β-Propiolactone-inactivated Sendai virus was added to a final titer of 500 hemagglutination units/ml of serum free medium. The flask was incubated at 4°C for 30 min and at 37°C for 60-90 min, after which virus and nonadhering microcells were removed from the monolayer with three washes of complete medium. The cells were allowed to incubate 18-24 hr at
320 Genetics: Fournier and Ruddle

Table 1. Purification of A9 microcells by sedimentation at unit gravity on a linear 1–3% bovine serum albumin gradient

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% of particles</th>
<th>Cytoplasts</th>
<th>Intact cells</th>
<th>Microcells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>49</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>62</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>67</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>54</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>48</td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>39</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>22</td>
<td>6</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>21</td>
<td>6</td>
<td>73</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>23</td>
<td>8</td>
<td>69</td>
</tr>
<tr>
<td>10 (bottom)</td>
<td></td>
<td>7</td>
<td>29</td>
<td>64</td>
</tr>
<tr>
<td>1–4 pooled</td>
<td></td>
<td>55</td>
<td>0</td>
<td>45</td>
</tr>
</tbody>
</table>

A9 cells were exposed to colcemid (0.1 µg/ml) for 48 hr. The cells were enucleated as described under Materials and Methods, and the crude microcell preparation was applied to a 1–3% bovine serum albumin gradient. After 3.5 hr at room temperature, 10 fractions (5 ml each) were collected. The preparations were stained with 0.4% aceto-orcein and the particle composition of each fraction was determined by microscopic examination. The values shown are averages of duplicate counts of 200 particles each.

37°C and then distributed into a number of small flasks. The heterokaryon frequency as determined by autoradiography or by Hoechst 33258 staining (10) typically approached 10–2. Selection pressure was applied either immediately [HAT hypoxanthine-aminopterin-thymidine selective system (11)] or after 24–48 hr [ouabain selective system (12)].

Hybrid clones appeared 2–4 weeks after fusion, were picked by the ring technique, and expanded. Electrophoretic analyses were carried out by procedures described previously (13, 14). Chromosomes for karyotype analysis were prepared after arresting the cells in mitosis for 1–3 hr with colcemid (0.1 µg/ml). Hoechst 33258 centric heterochromatin staining was performed essentially as described (15).

RESULTS

The time of exposure of the microcell donors to the mitotic arrest agent used to induce micronucleation was a critical factor influencing the recovery of viable hybrid clones from microcell X cell fusions (Table 2). A9 (HPRT–) cells were micronucleated by exposure to colcemid (0.1 µg/ml) for 2, 3, or 4 days. Cell populations exposed to the drug for 4 days had a higher percent of micronucleate cells (96 versus 88% for cultures treated for 2 days) and a slightly higher average number of micronuclei per cell (11 versus 9). Microcell preparations were isolated from each of the three micronucleate A9 populations and purified by unit gravity sedimentation. Fusion conditions were carefully controlled so that equal numbers (5 × 10⁶) of similarly-sized 2, 3, or 4 day microcells were fused with 8 × 10⁶ intact B82 (TK+) recipients. Heterokaryon analyses demonstrated that the frequency of microcell X cell fusion in each experiment was virtually identical (about 10⁻²; see Table 2). In spite of this finding, there was a greater than 10-fold enhancement of hybrid yield in experiments employing 2 day microcells versus fusions involving microcells derived from A9 populations exposed to colcemid for 3 or 4 days.

Similar results have been observed in interspecific microcell X cell hybridizations. Our results showing similar heterokaryon frequencies in all these experiments ruled out a possibly more efficient fusion of “young” microcells with recipients as an explanation for these findings. Rather, we believe that the genetic material of microcells isolated from donors incubated in the presence of colcemid for extended periods was in a state incompatible with its subsequent maintenance and/or expression in the recipient cell. In all further experiments, conditions for micronucleation of the donor cells were carefully controlled.

Chromosome spreads were prepared from 30 of the A9 microcell X B82 hybrid clones, and representative data are shown in Fig. 1. The modal chromosome numbers of the hybrid clones were consistent with the hypothesis that these populations were the products of microcell X cell fusion events. However, to more firmly establish this point and to more fully characterize the products of microcell X cell fusions, we performed interspecific hybridizations.

Table 2. Hybrid yield of A9 (HPRT–) microcell x B82 (TK+) fusions

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Duration of colcemid treatment of A9 (days)</th>
<th>No. of primary hybrid colonies after 3 weeks</th>
<th>Fraction of isolation flasks with colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2</td>
<td>&gt; 60</td>
<td>20/20</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>5</td>
<td>4/20</td>
</tr>
<tr>
<td>III</td>
<td>4</td>
<td>3</td>
<td>3/20</td>
</tr>
</tbody>
</table>

A9 was treated with colcemid (0.1 µg/ml) for the time period indicated. Microcells were isolated and purified by unit gravity sedimentation as described under Materials and Methods. A9 microcells (5 × 10⁶) were fused with B82 recipients (8 × 10⁶) and HAT-resistant colonies were scored and isolated. The heterokaryon frequencies in experiments I, II, and III were determined by observing 5000 individual cells and were 0.96 × 10⁻², 1.14 × 10⁻², and 0.88 × 10⁻², respectively.

Fig. 1. Chromosome distributions of A9, B82, and four representative interspecific microcell hybrids generated by fusion of A9 (HPRT–) microcells with intact B82 (TK+).
Mouse CT11G (HPRT\(^+\), APRT\(^-\)) cells were micronucleated with colcemid (0.1 \(\mu\)g/ml) for 48 hr. Microcells were prepared, purified, and fused with E36, a nonreverting HPRT\(^-\) Chinese hamster cell line which grows vigorously in culture (6). Two hybridizations were performed according to procedures described under Materials and Methods, and seven independent hybrid clones were isolated after 2–3 weeks in HAT selective medium. In addition, two HAT-resistant colonies which strongly resembled the microcell donor were isolated. Subsequent analyses showed that these two populations were in fact clones of CT11G. It is noteworthy that CT11G is HPRT\(^+\), TK\(^+\), and thus HAT-resistant. The recovery of only two clones resulting from intact mouse cells contaminating the microcell preparation attests to the efficiency of the purification procedure employed. The practical advantages of this include: (i) one-sided selection can be used in microcell \(\times\) cell fusions because in our procedure intact donors are virtually eliminated, and (ii) the potential problem of cell \(\times\) cell fusion occurring between intact donors and recipients is avoided.

The presence of intact mouse chromosomes in the hybrid clones resulting from CT11G microcell \(\times\) E36 fusions (ECm and HCm hybrid series) was unambiguously demonstrated with the use of the fluorochrome Hoechst 33258. This dye stains preferentially the AT-rich centric heterochromatin of mouse chromosomes (16), and the bright chromocenters so produced are diagnostic of the presence of murine chromosomes in both metaphase spreads and interphase nuclei (10, C. Koza\(k\) and F. H. Ruddle, manuscript in preparation). Fig. 2A shows a typical metaphase spread prepared from a mouse microcell \(\times\) Chinese hamster hybrid clone and stained with Hoechst 33258. In addition to the Chinese hamster chromosomes characteristic of E36, four mouse chromosomes with brightly stained centromeric regions are evident in this particular preparation. In Fig. 2B a Hoechst-stained interphase nucleus of the same clone is illustrated; four high fluorescent intensity chromocenters are clearly visible.

By using this staining protocol, we were able to analyze the content of mouse chromosomes in each of the seven hybrid clones. Fig. 3 shows the distribution of introduced murine chromosomes in four of the hybrids when analyzed 2 weeks after isolation. These data were obtained by determining the number of chromosomes with highly-fluorescent centromeres in each of 50 metaphase spreads (hatched bars) and the number

![Figure 2](image1.png)

**Fig. 2.** (A) Fluorescence photomicrograph of a Hoechst 33258-stained metaphase spread of ECm-3, a mouse microcell \(\times\) Chinese hamster hybrid cell. Four murine chromosomes with brightly-stained centromeric regions are present in addition to the Chinese hamster chromosomes characteristic of the E36 recipient. \(\times\)990. (B) Photomicrograph of a Hoechst 33258-stained interphase nucleus of ECm-3. The four high-fluorescent-intensity chromocenters are indicative of the presence of four mouse chromosomes in this hybrid cell. \(\times\)990.

![Figure 3](image2.png)

**Fig. 3.** Distribution of introduced mouse chromosomes in four mouse microcell \(\times\) Chinese hamster hybrid clones. For explanation, see text.
of bright chromocenters in each of 50 interphase nuclei (solid bars). Several points are illustrated in the figure. First, the modal number of introduced mouse chromosomes varied from one to four. Furthermore, the highest number of murine chromosomes observed in any single hybrid cell was six. Finally, it was also clear that in hybrid lines containing a limited number of mouse chromosomes, a 1:1 correspondence existed between the number of metaphase chromosomes with brightly-staining centromeres and the number of chromocenters per interphase nucleus. While it is known that heteroploid mouse cell lines may contain a few chromosomes that do not exhibit brightly-staining centromeres (15), this staining technique nonetheless provides a useful tool for monitoring the input of murine chromosomes in interspecific hybridizations involving mouse microcells.

The presence of functioning mouse genetic material in the ECm and HCm hybrids was confirmed by electrophoretic analysis of murine isozymes. All seven clones were resistant to HAT medium by virtue of their expressing the CT11C5-derived form of HPRT. Electrophoretic analysis of 16 additional isozymes representing at least eight murine linkage groups was performed, and the results are summarized in Table 3. Most of the mouse isozymes assayed were absent from the hybrid clones. Four of the seven clones, however, clearly expressed the murine form of at least one isozyme in addition to the selected marker, HPRT. These clones possessed a modal number of three to four mouse chromosomes. In those hybrid lines containing fewer mouse chromosomes (e.g., ECm-2 and ECm-3 which contained only one and two mouse chromosomes, respectively), no nonselected murine isozymes were detected among the 16 markers assayed. This was not an unexpected result because the presence of 13 mouse linkage groups would not have been revealed by our isozyme analysis. These observations were consistent with the chromosome analysis of the hybrid lines and provided further evidence that relatively few chromosomes had been transferred from the mouse donor to the Chinese hamster recipient.

To demonstrate the practical utility of microcell X cell fusion procedures for gene mapping, we fused microcells derived from normal, diploid mouse embryo fibroblasts with human HeLa S3 cells. A human recipient was chosen because a large body of information exists concerning the discrimination of homologous murine and human phenotypes. This information has been acquired over the last several years in the course of human gene mapping studies. Because human cell lines containing limited numbers of mouse chromosomes can now be constructed using microcell technology, numerous genetic markers for which reliable assays have already been developed can be expeditiously mapped in the mouse genome.

Primary mouse embryo fibroblast cultures were prepared in collaboration with C. Kozak and micronucleated by exposure to 0.05 μg/ml of colcemid for 36 hr. These cells were more sensitive to cytotoxic effects of the mitotic arrest agent than the established lines. After mitotic arrest, 60–70% of the cells had become micronucleate. The cells were enucleated as described under Materials and Methods. The crude microcell preparation was largely free of intact cells (<0.1%), presumably due to the strong attachment of the fibroblasts to the substratum, but contained significant numbers of "minicells" (17)—intact nuclei surrounded by scant cytoplasm and a plasma membrane. These structures were separated from the microcells by unit gravity sedimentation. Mouse embryo fibroblast microcells were fused with intact HeLa S3 cells as described above, and clones resistant to 1 μM ouabain (12) selected.

Twelve independent hybrid clones were isolated and ana-
lyzed by using the strategy employed to characterize the ECm and HCm hybrid series. Hoechst 33258 centric heterochromatin staining demonstrated that the hybrid clones contained modal numbers of introduced mouse chromosomes which varied from one to five. Murine isozymes were present in some but not all of the clones. All hybrid lines were resistant to 1 μM ouabain, and detailed karyotyping revealed the presence at high frequency of a particular mouse chromosome in all the clones. This analysis, which has made possible the assignment of the murine gene conferring ouabain resistivity to man-mouse hybrids to a particular mouse chromosome, will be the subject of a subsequent report (C. Kozak, R. E. K. Fournier, and F. H. Ruddle, manuscript in preparation).

**DISCUSSION**

The studies described in this report firmly establish subnuclear particle-mediated chromosome transfer as a valid somatic cell genetic procedure. There are now three operationally distinct modes of genetic manipulation which can be applied to mammalian cells. These are somatic cell hybridization, subnuclear particle-mediated chromosome transfer, and chromosome-mediated gene transfer (4, 18), techniques which allow the whole genome, one or a few chromosomes, or small pieces of genetic material to be transferred from one cell to another. These distinctly different modes of genetic transfer can also be used profitably in combination.

In our experiments, small numbers of murine chromosomes have been introduced into mouse, Chinese hamster, and human recipient cells. By using the smallest size classes of microcells as fusion material, we have observed between one and five resident mouse chromosomes in the resulting hybrid populations. It is reasonable to expect that by using larger microcells, it would be possible to introduce more chromosomes into the recipient, and to determine the practical utility of such an approach for controlling the input of foreign genetic material.

The hybrid lines we have prepared closely resemble the recipient cell parent in both morphology and growth characteristics. Of the seven mouse microcell × Chinese hamster hybrid clones described in this report, five had a single Chinese hamster chromosome complement (15 hybrids) and two a double set of chromosomes (25 hybrids). The 15 hybrids were virtually identical to the Chinese hamster parent in terms of their growth properties in culture. The 25 hybrids offered a potential advantage in that they tended to retain more introduced mouse chromosomes than their 15 counterparts (for example, see Fig. 3). These considerations make it possible to generate hybrid cell lines particularly suited to a given experimental goal by judiciously choosing the recipient cell line.

Another major advantage of subnuclear particle-mediated chromosome transfer is that it enables one to predetermine the direction of chromosome segregation in the hybrid cells. That chromosome segregation did in fact occur in microcell hybrids was indicated by two observations. First, the number of mouse chromosomes varied between individual cells of a clonal population. Second, 25 hybrids tended to retain more foreign chromosomes than 15 hybrids. These properties are also characteristic of segregating populations of hybrid cells derived from cell × cell hybridizations (19, 20).

The question of whether particular chromosomes or combinations of chromosomes tend to be cotransferred with the specific chromosome carrying the selected gene remains open. The occurrence of such a phenomenon might have implications regarding the organization of metaphase chromosomes during mitosis. If such were the case, then it might be possible to gain some measure of control over which chromosomes were transferred simply by employing different selective conditions for the isolation of the microcell hybrids.

We believe that subnuclear particle-mediated chromosome transfer represents an important advance in somatic cell genetic technology. The intrinsic advantages of this approach make it a particularly attractive experimental tool for the study of a wide range of problems of cell and developmental biology. The greatest promise of the system, however, would seem to stem from the possibilities it offers for both genetic analysis and the construction of novel cell populations which contain one or several additional chromosomes of a specified type.

We gratefully acknowledge the technical assistance of Ms. C. Colmenares, Ms. E. Nichols, and Ms. J. Lawrence, and the preparation of the manuscript by Ms. M. Siniscalchi. R. E. K. F. is a Leukemia Society of America Fellow. These studies were also supported by Grant GM 9966 from the National Institutes of Health.