

DNA-mediated Gene Transfer without Carrier DNA

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ABSTRACT DNA-mediated gene transfer is a procedure which uses purified DNA to introduce new genetic elements into cells in culture. The standard DNA-mediated gene transfer procedure involves the use of whole cell DNA as carrier DNA for the transfer. We have modified the standard DNA-mediated gene transfer procedure to transfer the Herpes simplex virus type 1 thymidine kinase gene (TK) into TK⁻ murine recipient cells in the absence of whole cell carrier DNA. The majority (8/10) of carrier-free transformant lines expressed the TK⁺ phenotype stably, in sharp contrast to our results with carrier-containing DNA-mediated gene transfer. There was a wide range in donor DNA content among independent transformants. Further analysis on one transformant line using DNA restriction digests and *in situ* hybridization provided evidence that, in the absence of whole cell carrier DNA, multiple donor DNA sequences became integrated at a single chromosomal site.

DNA-mediated gene transfer is a procedure for introducing donor DNA into recipient cells. This procedure involves DNA-calcium phosphate coprecipitation and requires high concentrations of DNA (up to 20 $\mu\text{g}/\text{ml}$) to produce a precipitate effective in transforming tissue-culture cells (1). In DNA-mediated gene transfer experiments involving small amounts of plasmid DNA, the use of whole cell DNA (carrier DNA) to achieve a high concentration of DNA raises several questions concerning the function(s) of this carrier DNA in the transfer process itself. The carrier DNA may function simply to increase the quantity or quality of DNA precipitation. Alternatively, the carrier DNA sequences may provide intracellular function(s) which facilitate recipient cell expression and propagation of donor DNA sequences (2, 3).

As one approach to investigating the role(s) of carrier DNA, we have performed DNA-mediated gene transfer experiments without using whole cell carrier DNA. The Herpes simplex virus type 1 thymidine kinase gene (HSV-TK), cloned in pBR322 (pTKx-1) (4), was used as donor material in the transformation of TK⁻ murine L cells. Transformation was carried out by the method of Wigler et al. (5) using microgram quantities either of linear or of circular pTKx-1 DNA. Independent transformant colonies were isolated, and these colonies were analyzed for stability of donor gene expression and organization of donor DNA sequences.

MATERIALS AND METHODS

Cell Culture

The TK⁻ murine cell line, Ltk⁻, (a gift of S. Silverstein, Columbia University)

was maintained in monolayer culture at 37°C, 10% CO₂, in minimal essential medium, alpha modification (α MEM, Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% heat-inactivated fetal calf serum (HI-FCS; Flow Laboratories, Inc., Rockville, Md.). Thymidine kinase-positive, hypoxanthine/aminopterin/thymidine-resistant (HAT^r) (6) cell lines were maintained in α MEM, 5% HI-FCS, 1 \times HAT (α HAT).

DNA Sources

Plasmid pTKx-1 was a gift of W. Summers and was constructed by cloning the TK-containing *Bam* HI fragment of HSV type 1 DNA into the unique *Bam* HI site in plasmid pBR322. Plasmid DNA was isolated as described by Wilson et al. (7). High molecular weight cellular DNA was isolated as described by Wigler et al. (8).

DNA-mediated Gene Transfer

Recipient cells were plated in 10 ml of α MEM, 10% HI-FCS at 10⁶ cells/75 cm² flask 24 h before DNA addition. The donor material, pTKx-1 DNA, was used in circular or *Hind* III-cut linear form. The circular material was isolated as Form I DNA and was composed of a mixture of monomers and multimers. The linear material was generated by *Hind* III digestion of circular DNA, and the conversion to linear form was confirmed by gel electrophoresis. The donor DNA was diluted with 250 mM CaCl₂ (Mallinkrodt, anhydrous) to a concentration of 2–20 $\mu\text{g}/\text{ml}$. The DNA-Ca solution was added dropwise with agitation to an equal volume of 280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.1, giving a final DNA concentration of 1–10 $\mu\text{g}/\text{ml}$. The DNA-CaP coprecipitate was kept undisturbed at room temperature for 30 min before being added to the medium in each recipient flask (1 ml added per flask). The flasks containing DNA precipitate were incubated at 37°C. The medium with precipitate was removed from the flask after 4 h of incubation and fresh medium was added. At 30–60 h post-DNA treatment, the medium was changed again (still in 10% HI-FCS) and HAT selection was initiated. The cells were fed every 2–3 d and colonies were detected at 2 wk postselection. Independent HAT^r colonies were

isolated, grown to 10^8 cells, and analyzed for phenotypic stability as well as for the presence of donor sequences.

Stability Testing

The stability of the HAT^r phenotype was monitored by removing cell lines from HAT-selective medium and growing them in nonselective medium. At various intervals after transfer to nonselective conditions, 400 cells from each line were plated in HAT-selective medium and in hypoxanthine/thymidine (HT) nonselective medium. 10 days after plating, the flasks were stained with Wright's stain and colonies were counted. The ratio of colonies in selective to nonselective medium was used as an indication of the percentage of the population which retained the HAT^r phenotype.

DNA Analysis

Cellular DNA was digested with restriction endonucleases *Eco* RI, *Hind* III, and *Xba* I using assay conditions suggested by the suppliers. The DNA samples were electrophoresed in horizontal 0.7 or 1.0% agarose gels (Sigma Chemical Co., St. Louis, Mo.) using a high-salt electrophoresis buffer consisting of 160 mM Tris (pH 8.0)/80 mM NaOAc/ 80 mM NaCl/ 5 mM EDTA (9). This buffer was found to produce sharper bands in our filter hybridization. 15 μ g of digested DNA was electrophoresed in each lane. DNA blotting and filter hybridization were performed as described previously (10). pTKx-1 DNA was nick-translated to a specific activity of $1-3 \times 10^8$ dpm/ μ g using the nick-translation kit supplied by New England Nuclear, Boston, Mass.

In Situ Hybridization

Metaphase chromosome spreads fixed on glass slides were baked for 15 h at 65°C. After the baking, the slides were treated with acetic anhydride (11) in an attempt to minimize background. The slides were then incubated in 70% formamide, 2 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M NaCitrate, pH 7) at 70°C for 2-3 min to denature the DNA, followed by 3-min incubations in room temperature 75, 95, 95, and 100% ethanol solutions. Nick translation of pTKx-1 DNA, using ¹²⁵I-dCTP (New England Nuclear), produced a probe labeled to a specific activity of $1-2 \times 10^8$ dpm/ μ g DNA. The hybridization solution contained 50% formamide, 2 \times SSC, 10% dextran sulphate, 1 mM sodium phosphate, Denhardt's reagent (0.02% each of bovine serum albumin, ficoll, and polyvinyl pyrrolidone), 100 μ g/ml denatured sonicated salmon sperm DNA, and 5×10^4 dpm of denatured probe DNA per microliter. 8.5 μ l of hybridization solution was placed on a slide and covered by a 22-mm cover slip which was sealed along the edges with a 50:50 mixture of paraffin:vaseline. Hybridization was carried out for 18-20 h at 39-42°C. Posthybridization washes were: (a) 2 h at 50°C in 2 \times SSC, (b) 40 min at 65°C in 0.1 \times SSC, (c) 3 min at room temperature in 70, 95, and 100% ethanol consecutively (d) 3 min at room temperature in xylene, and (e) 3 min at room temperature in 100% ethanol. At this stage the slides were air-dried and coated with NTB-2 autoradiographic emulsion. Slides were exposed for 11 d before being developed and stained. For chromosome identification, metaphase chromosome spreads were banded by the trypsin technique (12), stained, photographed, and destained before the 65°C baking. The overall rate of disruption of the chromosomal spreads during the in situ hybridization procedure was markedly elevated in the prebanded slides. In addition, chromosome spreads which had been trypsin-banded before hybridization were visualized poorly posthybridization, even after a 30-min staining in a 10% Giemsa solution.

RESULTS

Transformation

The transformation frequency was reduced greatly in the absence of whole cell carrier DNA. DNA-mediated gene transfer using pTKx-1 plasmid DNA without whole cell carrier DNA produced HAT^r colonies at a frequency of 1-10 colonies/ μ g pTKx-1 DNA/ 10^6 recipient cells. In parallel cultures of the same recipient line, the frequency of DNA-mediated gene transfer using pTKx-1 DNA with whole cell carrier DNA was 1 colony/1 ng pTKx-1 DNA/ 10^8 recipient cells.

We isolated five independent transformants from DNA-mediated gene transfer using only circular pTKx-1 DNA (circular transformants) and five independent transformants from DNA-mediated gene transfer using only *Hind* III linearized pTKx-1 DNA (linear transformants). These ten independent

transformants were picked from ten separate flasks. In nine of the flasks, only one colony was detected after 2 wk in HAT medium. In the tenth flask (circular DNA) five colonies were picked and analyzed (101-105). The stability characteristics and the organization of donor DNA sequences were identical in all five lines. This result is consistent with the derivation of these five colonies from one initial colony, and therefore we are presenting the data only for clone 101. Each transformant colony was expanded to 10^8 cells (~26 generations) before analysis of its phenotypic and genotypic properties.

Stability

The ten transformant cell lines were tested for stability of the HAT^r phenotype (see Materials and Methods). We define as stable those cell lines in which at least 95% of the TK⁺ HAT^r-transformed cells retained the ability to grow in HAT after one month of culture in nonselective medium. Unstable cell lines are those in which >5% of the TK⁺/HAT^r-transformed cells lost the HAT^r phenotype in nonselective conditions. The 5% figure was chosen based on the average range of values for stability tests on TK⁺ control lines, such as human HeLa cells, probably reflecting errors in diluting and pipetting small numbers of cells. A cell population which consisted of a mixture of stable and unstable cells would show a loss of HAT^r for a percentage of the population (unstable), followed by a leveling off in the number of cells remaining HAT^r (stable). In our transfer experiments using whole cell DNA as carrier DNA, 11/15 lines were unstable, 3/15 were mixed, and 1/15 were stable at first testing (G. A. Scangos, K. M. Huttner, and F. H. Ruddle. Manuscript in preparation). The frequency of stable transformants was increased substantially in the absence of whole cell carrier DNA. 4/5 of the circular transformants and 4/5 of the linear transformants were stable at first testing. Initially, one circular transformant, 101, and one linear transformant, 1c, were found to contain a mixture of cells of stable and unstable phenotypes (Fig. 1). After one month of growth in nonselective medium, HAT^r populations from both lines were retested and were stable within the limits of resolution of our assay.

Molecular Analysis of Transformants

DNA was isolated from the transformant cell lines using a standard procedure for whole cell DNA isolation. pTKx-1 DNA contains one *Hind* III site, 3 *Eco* RI sites, and no *Xba* I sites. The majority of the carrier-free transformants contained one to two copies of the pTKx-1 sequences as judged by *Hind* III and *Xba* I DNA digests. *Hind* III digestion of cellular DNAs from two circular transformants, 101 and 3g, followed by hybridization with ³²P-labeled pTKx-1 DNA, produced at least five bands (e.g., Fig. 2, lane 3g, H). In both lines, cellular DNAs contained a 7.9 kilobase (kb) *Hind* III band (size of linear pTKx-1), probably present in the cell as part of donor DNA concatamers or multimeric plasmids integrated into high molecular weight DNA. 3g and 101 whole cell DNAs were digested with *Xba* I, which recognizes no sites in the pTKx-1 DNA sequence. The *Xba* I digested DNAs were run out in 0.7% agarose gels until the dye front, migrating at the position of 0.5 kb, had moved 12-14 cm. In many independent experiments we were able to demonstrate only one *Xba* I band in both 3g and 101 DNAs (Fig. 2, lane 3g, X).

DNA from the linear transformants contained from 3 to 13 *Eco* RI bands which hybridized with pTKx-1 DNA (e.g., Fig.

2, lanes 1a-1c, E). Since all donor molecules in linear transfers were monomers of pTKx-1, the presence in line 1b of 13 *Eco* RI bands and only 2 *Xba* I bands (Fig. 2) provides strong evidence for intracellular lineage of independent donor sequences. *Xba* I digestion of DNAs from 4/5 linear transformants, including 1b and 1c (Fig. 2, lanes 1b and 1c, X), and from 1/5 circular transformants contained several *Xba* I bands.

If each of the *Hind* III bands in line 3g DNA represented a separate plasmid molecule integrated at an independent chromosomal site, then *Xba* I digestion, which would cleave at mouse *Xba* I sites surrounding each intergrated plasmid sequence, would produce multiple pTKx-1 containing bands.

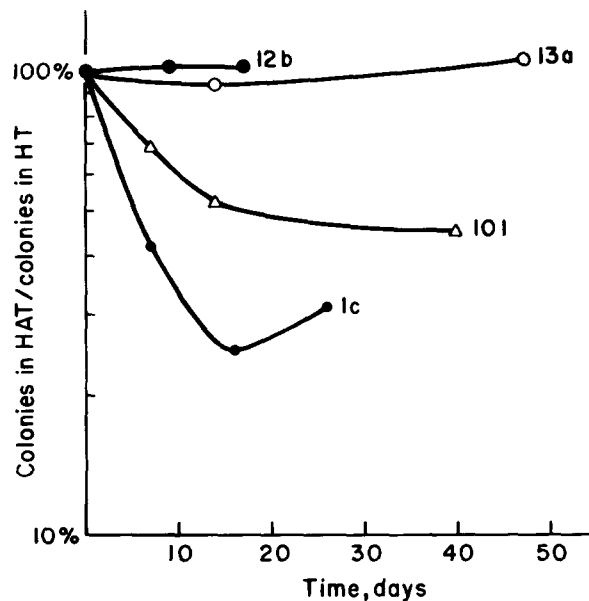


FIGURE 1 Stability of the HAT⁺ phenotype in carrier-free transformant lines. Mouse Ltk⁻ cells, transformed to the TK⁻/HAT⁺ phenotype by DNA-mediated gene transfer without carrier DNA, were released from selective pressure at day 0 and monitored at subsequent time-points for the retention of the ability to grow in HAT. Results from four lines are shown. 12b, a circular transformant, and 13a, a linear transformant, represent stable populations. 101, a circular transformant, and 1c, a linear transformant, represent mixed populations of stable and unstable cells.

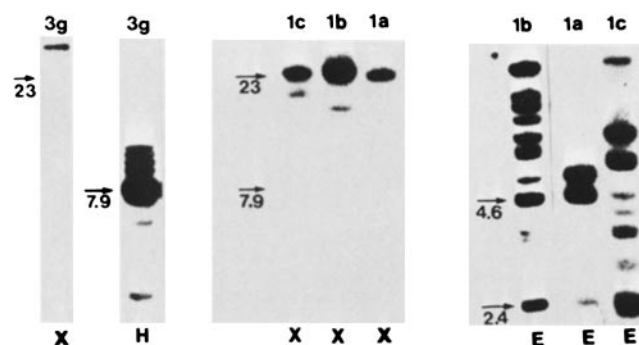


FIGURE 2 Restriction analysis of DNA from lines 3g, a circular transformant, and lines 1a-1c, linear transformants. H, *Hind* III; X, *Xba* I; and E, *Eco* RI. The arrow at 7.9 kb represents the position of linear pTKx-1 DNA. 15 μ g of digested DNA was run in each track. The *Hind* III and *Eco* RI-digested DNA were run on a 1.0% agarose gel, while the *Xba* I-digested DNA were run on a 0.7% agarose gel. After transfer onto nitrocellulose paper, the DNA were hybridized using ³²P-labeled pTKx-1 DNA as a probe.

The detection of only one *Xba* I band in transfer line 3g supports the idea of physical linkage of donor DNA molecules, and integration of this linked unit into the host DNA. To demonstrate more conclusively that the multiple *Hind* III bands represented multiple plasmid sequences integrated at one chromosomal location, we performed *in situ* hybridization on metaphase spreads from line 3g. In 100 nonbanded chromosome spreads, 90 contained grains over only one chromosome (Fig. 3), and this chromosome could be shown by a chromosome banding technique to be an apparently normal mouse chromosome 15 (K. M. Huttner, D. D. Pratcheva, and F. H. Ruddle. Manuscript in preparation). 4/100 spreads showed no hybridization, and 6/100 spreads contained grains over two chromosomes. One of these two chromosomes was the mouse chromosome 15 and the other was different in each of the six spreads. The intrachromosomal location of the donor sequences could be identified in several of the hybridized spreads, and the donor sequences mapped to a position, starting from the centromere, approximately one-third of the way down the length of the chromosome.

DISCUSSION

We have transferred the HSV-TK gene into murine cells using DNA-mediated gene transfer without added whole cell carrier DNA. The availability of a nucleic acid probe for all of the donor DNA (pTKx-1, no carrier DNA) allows us to determine more accurately the total amount and organization of exogenous DNA present in stable transformed cells. Each transformant cell contained at least one copy of the HSV-TK gene to survive HAT selection. Therefore, in the transformant lines with only one *Hind* III or *Eco* RI band, the total donor DNA content may be as low as 2 kb per cell (13). In transformant line 3g, which contains multiple *Hind* III bands, the theoretical maximum amount of donor DNA present can be calculated by assuming that each band consists entirely of donor sequences, and then summing the molecular weights of the various bands as determined by comparison with molecular weight standards run in a control in lane. In this calculation, one assumes that each band represents a DNA fragment present at one copy per cell. The relative intensity of the 7.9 kb band in 3g DNA suggests that this particular fragment might be present at two to three copies per cell. Therefore, in line 3g, with all those assumptions, the maximum amount of donor DNA present is at least 60 kb. An analogous calculation made for the linear transformant 1b is at least 75 kb.

One major difference between transformants derived from carrier-free and carrier-containing DNA-mediated gene transfer is that, although we found the majority of carrier-free transformants to be stable, carrier-containing transformants are often unstable at first analysis and rapidly lose HAT⁺ (6-10%/cell/d) (14) (G. A. Scangos, K. M. Huttner, and F. H. Ruddle. Manuscript in preparation). Within our assay system, transformants which are unstable but losing the donor phenotype at a rate of $<10^{-3}$ /cell generation (e.g., those described by Graf et al. [15]) would be classified as stable transformants. Since we examined all HAT⁺ clones in each flask, this stability result was not an artifact produced by analyzing only the fastest growing (and presumably stable) colonies. We are pursuing further experiments in an attempt to delineate the role of whole cell carrier DNA in the establishment of unstable vs. stable propagation of donor sequences.

Linear transformant lines contained multiple *Xba* I bands (4/5 lines) significantly more often than did circular transform-

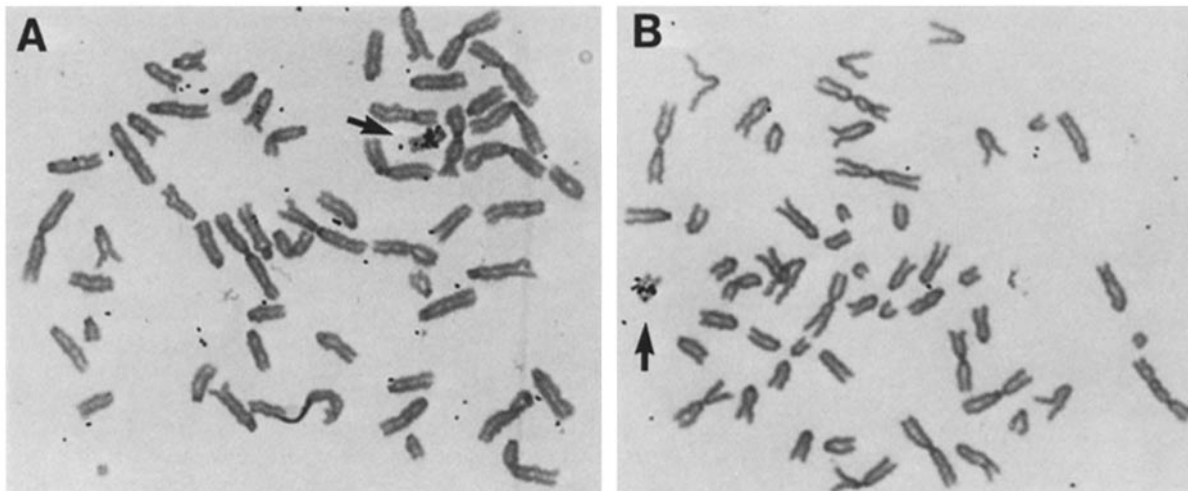


FIGURE 3 A and B, in situ hybridization on nonbanded metaphase spreads from line 3g. The probe was ^{125}I -labeled pTKx-1 DNA.

ant lines (1/5 lines). However, the same range of amounts of pTKx-1 DNA appeared in both types of transformants. One interpretation of this result is that the use of linear molecules as donor DNA enhanced the frequency of integration of multiple donor DNA molecules at independent sites. This interpretation, if supported by further observations, may reflect a significant difference in the cellular mechanism of recognition and recombination of exogenous DNA when the DNA is present in a form either containing or lacking free ends.

The presence of multiple *Hind* III bands in 3g DNA combined with the presence of a single *Xba* I band is consistent with the interpretation that multiple donor fragments have been assembled into a single unit (transgenome) which retained a number of pTKx-1 *Hind* III sites in the absence of internal *Xba* I sites contributed by mouse DNA. Theoretically, it is possible that all the 3g bands were derived from one large integrated pTKx-1 multimer. It also is possible that multiple 3g *Xba* I bands were present, but that the electrophoretic techniques employed were incapable of resolving them. The analysis of linear transformant 1b adds supporting evidence to the notion that unlinked donor molecules may become lined within the recipient cell.

We used in situ hybridization which demonstrated that most, if not all, of the donor sequences in carrier-free line 3g were associated with one site on one recipient cell chromosome, an apparently normal mouse chromosome 15. Our *in situ* hybridization result from a carrier-free transformant complements that of Robins et al. (16) in which multiple donor sequences were shown to be associated with single chromosomal locations in carrier-containing transformants.

In summation, DNA-mediated gene transfer using pTKx-1 plasmid sequences in the absence of whole carrier DNA produced HAT^r colonies at a relatively low frequency, but most colonies expressed the donor phenotype stably. In both linear and circular transformants there was a range in the number of copies of the pTK-1 sequences. In one case, 3g, we showed that the donor material was integrated within an intact mouse chromosome. These results should assist investigators in designing genetic engineering experiments in which one wishes to transfer purified cloned sequences into intact organisms.

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REFERENCES

- Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*. 52:456-467.
- Scangos, G. A., and F. H. Ruddle. 1981. Mechanisms and applications of DNA-mediated gene transfer in mammalian cells—a review. *Gene* (Amst.). 14:1-10.
- Huttner, K. M., and F. H. Ruddle. 1981. Analysis of a model for DNA-mediated gene transfer. *J. Natl. Cancer Inst. Monogr.* In press.
- Enquist, L. W., G. F. Vande Woude, M. Wanger, J. R. Smiley, and W. C. Summers. 1979. Construction and characterization of a recombinant plasmid encoding the gene for the thymidine kinase of *Herpes simplex* type 1 virus. *Gene* (Amst.). 7:335-342.
- Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. *Cell*. 14:725-731.
- Littlefield, J. W. 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* (Wash. D. C.) 145:709-710.
- Wilson, J. T., J. B. Wilson, J. K. deRiel, L. Villa-Komaroff, A. Efstratiadis, B. G. Forget, and S. M. Weissman. 1978. Insertion of synthetic copies of human globin genes into bacterial plasmids. *Nucleic Acid Research*. 5:563-581.
- Wigler, M., R. Sweet, G. K. Sim, B. Wjold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from prokaryotes and eukaryotes. *Cell*. 16:777-785.
- Shinnick, T. M., E. Lund, O. Smithies, and F. R. Blattner. 1975. Hybridization of labeled RNA to DNA in agarose gels. *Nucleic Acids Research*. 2:1911-1929.
- Huttner, K. M., G. A. Scangos, and F. H. Ruddle. 1979. DNA-mediated gene transfer of a circular plasmid into murine cells. *Proc. Natl. Acad. Sci. U. S. A.* 76:5820-5824.
- Hayashi, S., I. C. Gillam, A. D. Delaney, and G. M. Tener. 1978. Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiographs from hybridization with ^{125}I -labeled RNA. *J. Histochemistry and Cytochemistry*. 26:677-679.
- Wang, H. C., and S. Fedoroff. 1972. Banding of human chromosomes treated with trypsin. *Nat. New Biol.* 235:52-54.
- Colbere-Garapin, F., S. Chousterman, F. Horodniceanu, P. Kourilsky, and A. Garapin. 1979. Cloning of the active thymidine kinase gene of herpes simplex virus type 1 in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. U. S. A.* 76:3755-3759.
- Willecke, K., M. Klombaf, R. Mierau, and J. Dohmer. 1979. Intraspecies transfer via total cellular DNA of the gene for hypoxanthine phosphoribosyltransferase into cultured mouse cells. *Mol. Gen. Genet.* 170:179-185.
- Graf Jr., L. H., G. Urlaub, and L. A. Chasin. 1979. Transformation of the gene for hypoxanthine phosphoribosyltransferase. *Somatic Cell Genet.* 5:1031-1044.
- Robins, D. M., S. Ripley, A. S. Henderson, and R. Axel. 1981. Transforming DNA integrates into the host chromosome. *Cell*. 23:29-39.