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Somatic Cell Genetic and Molecular Analysis of DNA-mediated Gene Transfer

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ABSTRACT

SOMATIC CELL GENETIC AND MOLECULAR ANALYSIS OF DNA-MEDIATED GENE TRANSFER

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Gene transfer in mammalian tissue culture cells has attained a new level of sophistication with the introduction of donor genes in the form of purified DNA into recipient cells. The structure and intracellular location of the donor genetic material can be analyzed by a combination of somatic cell genetic, biochemical, and molecular genetic techniques. The results presented here describe some of the events involved in DNA-mediated gene transfer (DMGT).

Specific donor genes can be transferred by DMGT using whole cell DNA. The donor genes present in transformant cell lines derived from whole cell DMGT often cannot be analyzed at the nucleic acid level due to lack of a specific probe. In one set of experiments I analyzed a series of transformant cell lines derived by treating TK^- mouse L cells with whole cell DNA from TK^+ Chinese hamster cells. The donor TK^+/HAT^r phenotype was expressed in either an unstable or stable fashion, and a correlation could be made between conversion from instability to stability and a decrease in TK activity of at least 50% on a per ug of cellular protein basis.

A second set of experiments involving the mixing of plasmid DNA with the DNA used to transfer the TK gene (referred to as cotransfer experiments) confirmed the results from the laboratories of Dr. R. Axel and Dr. S. Silverstein which demonstrated that recipient cells which

took up and expressed specific donor DNA sequences, e.g. a TK gene, and were thereby able to survive under particular selection conditions, e.g. HAT selection, also took up and maintained donor DNA sequences, e.g. plasmid DNA, which were unlinked to the selected gene. I observed coordinate segregation of originally unlinked donor sequences supporting a model of intracellular construction of high molecular weight DNA molecules involving the ligation of multiple donor DNA sequences. Restriction endonuclease digestion of cellular DNA isolated from independent cotransfer lines, followed by filter hybridization using ^{32}P-labeled β-globin plasmid DNA as probe, provided evidence for the existence of circular forms of the β-globin plasmid in the murine tissue culture cells.

The intent of the final set of experiments was to analyze the phenotypic and genotypic nature of the TK gene in transformant cell lines derived by treating TK^- mouse L cells with plasmid DNA containing the Herpes simplex virus type 1 TK gene cloned into pBR322 (pTKx-1). These DMGT experiments were performed without adding carrier DNA to the plasmid DNA, and the recovery rate for TK^+/HAT^r colonies was low, e.g. 1 colony per 0.5 ug of TK gene DNA. Six of seven carrier-free transformant cell populations were stable for TK gene expression, and two of seven showed evidence for the existence of circular pTKx-1 molecules after 25 cell doublings. Continued propagation of several of these transformants, in conjunction with back selection and subcloning experiments, revealed a number of mechanisms by which the recipient cell regulated donor gene expression. The most interesting of these was an example of gene amplification which correlated with gene

inactivation, followed by loss of amplification which correlated with gene re-expression.

All of these experiments have had as a unifying focus the understanding of the molecular and biochemical processes underlying DMGT. However, many of the observations have been sufficiently unusual in nature to suggest that with regard to a general model for recipient cell regulation of donor genetic material, unpredictibility is the rule rather than the exception.

Acknowledgements

My first acknowledgement most appropriately goes to Frank H. Ruddle. Both as an advisor and as a friend, he has played a central role in my introduction to scientific thought and to scientific research. I think that the most important notion he has conveyed to me is that of always seeing one's research as part of a bigger picture, and not as an end unto itself.

I would like to thank those who have helped to evaluate my work and suggest new avenues of experimentation. Among this group are my thesis committee members, Jerry Eisenstadt, Bernie Forget, and William Summers, as well as coworkers George Scangos and James Barbosa.

For assisting me in preparing figures for this thesis as well as for several papers I would like to thank Suzy Pafka.

On a personal note, to those who made life as one of a "clutch of graduate students" more sane and more enjoyable, I extend my gratitude to the numerous present and former members of Dr. Ruddle's laboratory with a special thank you to Vinnie Salerno, Ann Cunningham, Diana Landino, Marie Siniscalchi, and Mae Reger.

It goes without saying, although I will do so anyway, that I have the most appr eciation for the support I have received from my family and from my wife, Michele Roberts.

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Publications

Much of this thesis work, especially Chapters II and III and the Appendix, has been published or has been submitted for publication.

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. USA 76:5820-5824.

Huttner, K.M., J.A. Barbosa, G.A. Scangos, D.D. Pratcheva and F.H. Ruddle. 1981.

DNA-mediated gene transfer without carrier DNA.

Submitted to J. Cell Biol.

Huttner K.M. and F.H. Ruddle. 1981. Analysis of a Model for DNA-mediated Gene Transfer. Submitted to J. Natl. Can. Inst.

Introduction

The transfer of genetic material using purified DNA (DNA-mediated gene transfer, DMGT) was reported first in 1962 by Szybalska and Szybalski (1). These investigators attempted to transfer the gene for inosinic acid pyrophosphorylase (IMPase) between two human cell lines. Although this report included the effects of several parameters on the transfer process, e.g. spermine addition and donor DNA concentration, the absence of a technique for the demonstration of the donor provenance of IMPase activity in the "transformed" recipient cell lines was a hindrance to the acceptance of this result.

Eleven years later, the discovery by Graham and Van der Eb (2) that calcium phosphate-DNA coprecipitation and the use of microgram quantities of carrier DNA enhanced the frequency of viral transformation led to new attempts at DMGT in mammalian tissue culture cells. In 1977, 3 independent groups (3-5) published convincing evidence that DMGT could be used to transfer a virally-encoded thymidine kinase (TK) gene into TK deficient mouse L cells. One of these 3 groups (6) extended the use of DMGT to include the transfer of genomic genes, present in whole cell DNA, into mouse L cells. The genomic TK gene was transferred into mouse cells using as donor material mouse, Chinese hamster, chicken, calf, or human whole cell DNA. A 1980 catalog of genes transferred by DMGT using whole cell DNA includes: TK, adenosine phosphoribosyl transferase (APRT) (7), hypoxanthine-guanine phosphoribosyl transferase (HPRT) (8,9), dihydrofolate reductase (DHFR) (10), galactokinase (GALK) (11), and esterase D (Est D) (12).

Several groups have analyzed the effects of various parameters on the DMGT process itself (13,14). It appears that the donor DNA size, the donor DNA concentration, the length of time of recipient cell exposure to donor DNA, the pH of the phosphate-containing solution, the choice of recipient cell line, and the choice of gene to be transferred all have a strong influence on the transfer efficiency. Under optimal conditions and using a purified Herpes Simplex Virus TK gene (HSV-TK), one can treat 10^6 attached TK^- mouse L cells with donor DNA and recover on the order of 100 TK^+ transformant colonies (4,13) (as identified by cell growth in HAT selective medium which requires TK expression (15)). The actual transfer frequency may be as high as one colony per 10-40 pg of HSV-TK gene. Under the same conditions, but using whole cell DNA as the donor material, the best reported results are one colony per microgram of whole cell DNA (6).

The intracellular fate of donor DNA has been analyzed from a number of points of view. The use of techniques including autoradiography, cell fractionation, electron microscopy, Hirt fractionation, in situ hybridization, quantitative analysis of enzyme levels, and Southern blotting and filter hybridization has allowed researchers to begin to delineate the pathways and cellular constituents involved in the processing of donor DNA. We now know that most donor DNA is taken up into recipient cell lysosomes (16). Donor DNA can be shown to penetrate the nuclear membrane while still in a complex with precipitated calcium phosphate. At some stage donor DNA is released from its complex with calcium phosphate. Individual donor DNA molecules are altered and attached covalently to high molecular

weight DNA (17,18). The donor sequences then may be maintained either stably or unstably as measured by the retention (stable) or loss (unstable) of the donor phenotype (and usually donor genotype) in the absence of conditions requiring expression of the donor gene(s) (18).

In several cases donor DNA has been shown to be associated with a recipient cell chromosome (18-20), although in only two cases was there a correlation made between the chromosomal association and stability (18,19). In both cases the transformed recipient cell line was stable.

The experiments which have been most productive in analyzing the cellular processing of donor DNA involve the cotransfer of unlinked, nonselected but identifiable sequences along with a selectable gene (21). These cotransfer experiments have established that in a DMGT experiment the recipient cells which retain the selectable marker can retain additional donor DNA including as many as 20-100 copies of a donor DNA sequence which represented only 5% of the total donor DNA (21-23).

In order to examine the physical relationship between the selectable gene and additional donor DNA present in the transformed recipient cell lines, a number of experiments were designed whereby transformed cells were exposed to conditions selective for the loss of or amplification of the selectable gene, and the fate of the nonselected sequences was followed by DNA analysis. Selection against the TK gene led to concommitant loss of cotransferred sequences (24), and in some cases selection for amplification of a DHFR gene led to concomitant amplification of cotransferred sequences (10,24). In these experiments there were instances where some of the donor material was

retained despite loss of the TK gene, and instances where some of the donor material was not amplified despite amplification of the DHFR gene.

A final piece of evidence proving that donor DNA sequences which were unlinked prior to DMGT could become linked intracellularly came from experiments involving the reisolation of donor DNA sequences from transformed recipient cell lines. In three instances donor sequences which were known not to be linked prior to transfer were shown to be linked covalently inside of the recipient cell (24).

Many groups have taken advantage of this phenomenon of cotransfer in order to introduce various genes into mouse cells. DNA sequences including the rabbit and human β-globin genes (22,23,25), the chicken ovalbumin gene (26,27), pBR322 (21), the hepatitis B genome (28), and SV40 sequences (29) have been cotransferred with a selectable marker. In several cases the cotransferred material was attached covalently to the DNA containing the selectable marker prior to DMGT (25-29). Gene expression in the transformed cells was evaluated at the levels of transcription and translation. Qualitative abnormalities were detected in most cases, and the levels of expression at the RNA and protein levels varied between different cotransfer lines. No correlation could be made between the number of copies of the cotransferred sequences present in the transformed line and the level of transcription and/or translation of that sequence. It does appear to be the case that DMGT experiments involving cotransfer of sequences which were ligated to selectable genes prior to transfer more often resulted in transcription and translation of the cotransferred sequences (22,25).

In the series of experiments which will be described here, I have investigated the nature of the DMGT process, addressing the questions of recipient cell regulation of donor gene structure and expression.

Materials and Methods

Cell Culture

The murine TK^- cell line, Ltk^-, was a gift of S. Silverstein and was maintained in monolayer culture at 37°C, 10% CO_2, in Dulbecco's modified Eagle's medium (DVME, Gibco laboratories, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (HI FBS, Flow Laboratories, Inc., Rockville, MD). After performing the transfer experiments described in Chapters I and II, the Ltk^- cell line and all derivative cell lines were grown in alpha-minimal essential medium (α MEM, Gibco). This change was enacted to take advantage of the higher cloning efficiency and enhanced growth properties resulting from the use of a richer medium. The Chinese hamster TK^- cell line, RJK, was maintained in αMEM under the same conditions as above. Periodically cell lines were tested by Ann Cunningham for mycoplasma contamination as described by Barile et al (30).

DNA Sources

The plasmid pTKx-1 (Fig. 1) was a gift of W. Summers and consists of a 3.5 kb Herpes Simplex Virus type 1 Bam HI fragment inserted into the unique Bam HI site of pBR322 (31). This 3.5 kb fragment encodes a viral TK gene (HSV-TK) (4). The plasmid pTKx-1 was grown in LE 392 under P2-EK1 conditions in accordance with NIH guidelines. The plasmid Hβ1 (Fig. 2) was a gift of T. Maniatis to B.G. Forget and consists of a 4.4 kb fragment of human DNA, which contains the human β-globin gene (32), inserted into the unique Pst 1 site of plasmid pBR322. The

Figure 1. Restriction map of the plasmid pTKx-1. The 7.9 kb plasmid consists of a 3.5 kb Bam HI Herpes simplex virus fragment (heavy line) cloned into the unique Bam HI site in pBR322 (light line). The direction of transcription and approximate location of the Herpes-specific thymidine kinase gene are indicated. The numbers adjacent to each restriction site refer to the nucleotide distance from the unique Eco RI site in pBR322.

B:Bam HI; E:Eco RI; H:Hind III.

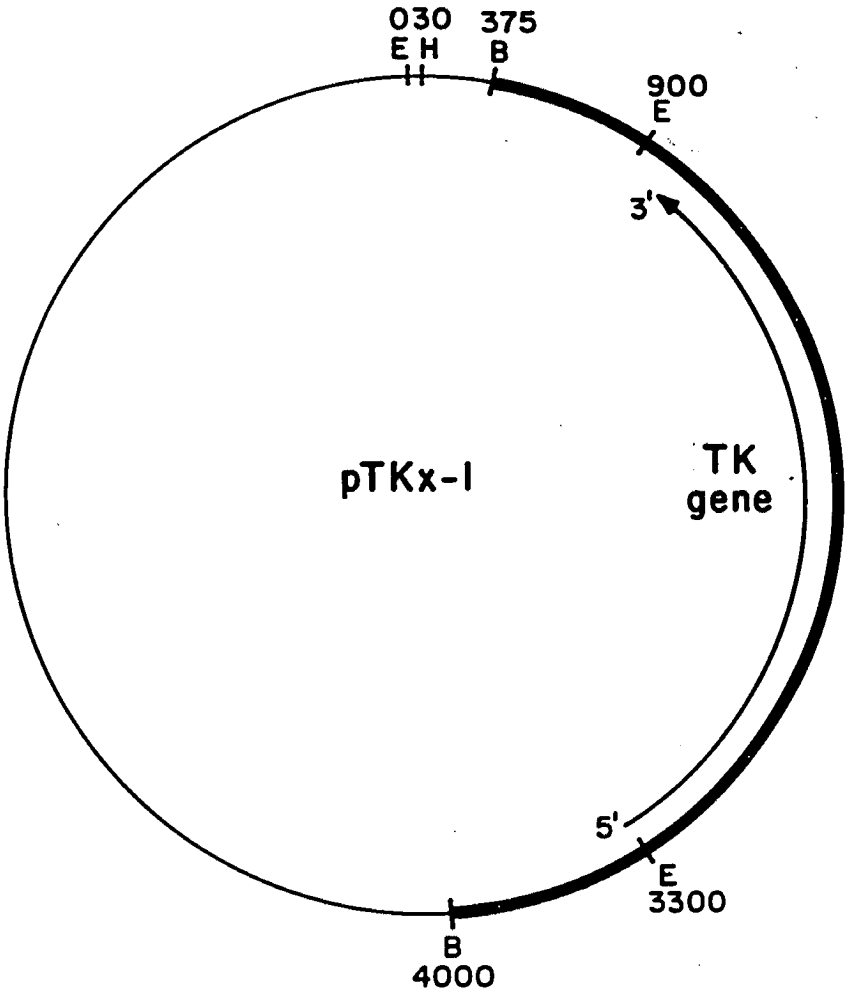
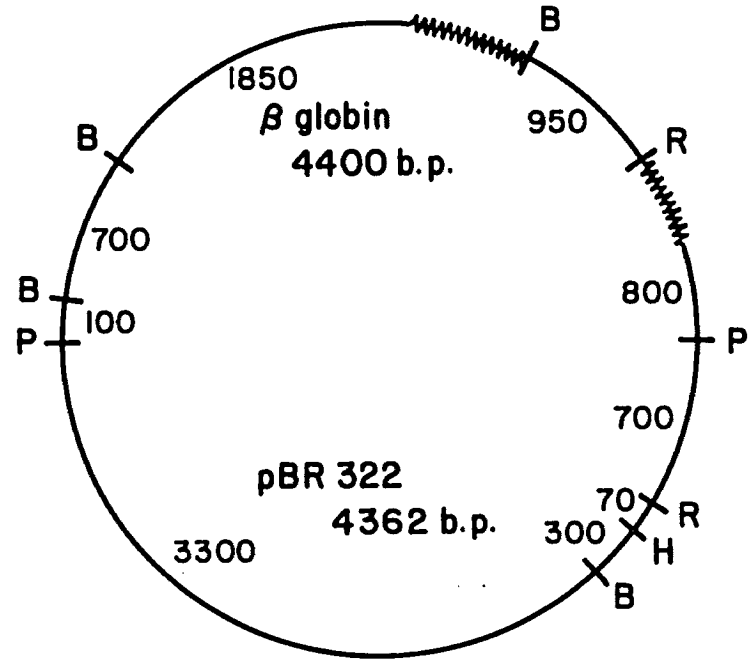


Figure 2. Restriction map of the plasmid Hβ1. This plasmid is a derivative of phage HβG 1 (32). Distances between restriction sites are indicated in number of base pairs.

B:Bam HI; E:Eco RI; H:Hind III; P:Pst I. Kpn I and Xba I do not cleave Hβ1. ⩙, globin coding sequences.



wwwww indicates globin coding sequences

plasmid HβA1 was grown in χ^{1776} under P2-EK2 conditions in accordance with NIH guidelines. Both plasmid DNAs were isolated by a standard procedure involving a CsCl gradient (33).

For use in DMGT, the HSV-TK gene fragment (3.5 kb Bam HI fragment) was isolated by G.A. Scangos from intact HSV-1 DNA (34). High molecular weight DNA was isolated from HeLa cells (human) and CH-Rel.22cA4 cells (Chinese hamster) by a standard procedure involving cell lysis with detergent, protease digestion for 4-10 hours at 37°C followed by phenol and chloroform: isoamyl alcohol extractions, ethanol precipitation, and resuspension in a low salt solution (21). Salmon sperm DNA for use as carrier DNA was purchased from Sigma.

For analysis of donor DNA content in transformed recipient cell lines two DNA isolation techniques were used. One involved Hirt precipitation of high molecular weight DNA using a detergent-high salt-4°C combination to fractionate cellular DNA into high molecular weight DNA (Hirt precipitate (ppt)) and low molecular weight DNA (Hirt supernatant (supt)) (35). The second involved whole cell lysis followed by steps similar to those for isolation of cellular DNA for use in DMGT (see above).

DNA-mediated Gene Transfer

DMGT was accomplished by the calcium phosphate coprecipitation technique as described by Wigler et al (6) with modifications. Briefly, the donor DNA solution was diluted with 250 mM $CaCl_2$ (Mallinkrodt, anhydrous), 10 mM Tris, pH 7.12 to twice the final DNA concentration. The DNA-Ca solution was added dropwise with agitation

to an equal volume of 280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4 pH 7.2. The DNA-CaP coprecipitate formed immediately and was kept undisturbed at room temperature for 30 minutes before 1 ml of DNA solution was added to the medium in each recipient flask. In the first transfer experiments (Chapters I and II) the recipient Ltk^- cells were plated in 10 mls of DVME, 10% HI FBS at 5 x 10^5 cells per 75 cm^2 plastic flask (Falcon) 24 hours prior to DNA addition. In the experiments outlined in Chapter I the donor DNA was whole cell Chinese hamster DNA and each flask received 20 ug of CaP-DNA coprecipitate. The CaP-DNA coprecipitate remained on the cells for 4 hours before being removed and replaced with 10 mls of fresh DVME. Thirty to thirty-six hours later this medium was removed and replaced with DVME supplemented with HAT (Hypoxanthine/ Aminopterin/Thymidine) (15). The components of HAT comprise a selective system requiring the presence of intracellular TK and HPRT activity for cell survival. In this case the Ltk^- cells could survive in HAT only if they had acquired and had expressed a donor TK gene, since no TK positive revertant of this cell line has been reported. The transfer flasks were fed every 2-3 days by replacing 50% of the medium with fresh medium. Macroscopic colonies were picked and expanded for further analysis.

In the experiments outlined in Chapter II the donor DNA was either 10 ng of the purified HSV-TK gene plus 20 ug of salmon sperm DNA, 10 or 30 ug of Chinese hamster whole cell DNA, or 30 ug of HeLa whole cell human DNA per flask. Mixed with these sources of TK gene was 1 ug of an Hβ1 DNA preparation composed of Form 1 (supercoiled) monomeric and multimeric molecules.

In the experiments outlined in Chapter III the donor DNA consisted of 1-10 ug of circular pTKx-1 DNA. The circular material was isolated as Form 1 DNA and was composed of a mixture of monomers and multimers. In this set of experiments the recipient murine Ltk^- cells were plated at 10^6 cells per 75 cm^2 flask 24 hours prior to DNA addition.

Stability Testing

The stability of the HAT-resistant phenotype was monitored by removing TK^+ transformed cell populations from HAT selective medium and growing them in nonselective medium (αMEM). At various intervals after transfer to nonselective conditions, e.g. every 7-10 days for 1 month, 400 cells from each line were plated in αHAT and in αHT (Hypoxanthine/Thymidine) nonselective medium. αHT was used as the nonselective growth condition to eliminate any growth advantage that HT may provide in the HAT selective system. Ten days after plating, the flasks were stained with Wrights stain (36) 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-resistant phenotype.

Back-selection

HAT-sensitive derivatives of HAT-resistant transformants were isolated as described by Smiley et al (19). TK positive cell lines were switched from growing in αHAT to αHT for 2-5 days, and then switched to αMEM + 30 ug/ml BUdR (αBU). After 2 days in αBU the medium was removed, 5 mls of phosphate buffered saline (PBS) was added to the

flasks, and cells were exposed to a Sylvanian F15T8 UV lamp at 10 cm for 60 minutes. After 60 minutes, the PBS was removed, fresh αBU was added back to the flasks, and the cells were grown for 48 hours followed by another round of UV-exposure. The cells then were grown in αBU for 2 weeks and surviving colonies were isolated and analyzed. All BUdR-resistant cell lines isolated in this manner were tested, and >99% of the cells in every back selectant line were HAT-sensitive.

Isozyme Analysis

Assays for Chinese hamster isozymes were performed on extracts of TK^+ transformed Ltk^- cells. These assays were carried out by E. Nichols as described in Nichols and Ruddle (37). The following isozymes were screened: glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49); phosphoglucomutase-1 (PGM1, E.C. 2.7.5.1); dipeptidase-1 (DIP1, E.C. 3.4.11); tripeptidase-1 (TRIP1, E.C. 3.4.11); malic enzyme (ME, E.C. 1.1.1.40); purine nucleoside phosphorylase (NP, E.C. 2.4.2.1); lactate dehydrogenase A (LDHA, E.C. 1.1.1.27); adenine phosphoribosyl transferase (APRT, E.C. 2.4.2.7); uridine phorphorylase (UP, E.C. 2.4.2.3); galactokinase (GALK, E.C. 2.7.1.6); triosephosphate isomerase (TPI, E.C. 5.3.1.1); glutathione reductase (GSR, E.C. 1.6.4.2); mannose phosphate isomerase (MPI, E.C. 5.3.1.8); glucose phosphate isomerase (GPI, E.C. 5.3.1.9); esterase-10 (ES10, E.C. 3.1.1.1); adenosine kinase (ADK, E.C. 2.7.1.20); glyoxylase-1 (GLO, E.C. 4.4.1.5).

Quantitative TK Assay

For quantitative analysis of TK activity, cell lines were grown on 150 cm^2 petri dishes. HAT-resistant cell lines were grown in αHAT while HAT-sensitive lines were grown in αHT. Cells were harvested in logarithmic growth phase, centrifuged, resuspended in saline on ice, centrifuged again, and stored at -70°C under 0.3 to 0.5 ml of 20 mM tris, 1 mM β-mercaptoethanol, 0.05 mM thymidine, pH 7.8 (38).

Frozen cell pellets were thawed rapidly and sonicated twice at 45% maximum power for 15 seconds using a Sonifier 350 (Branson). The homogenates were made up to 0.15 M KCl, 3 mM β-mercaptoethanol, 0.5% Nonidet P-40. These samples then were centrifuged at 10,000 RPM, 30 minutes, 4°C in a Sorvall RC2-B. The supernatants were removed and used immediately for the TK assay.

The TK assay was performed in a final volume of 100 ul containing 100 mM Tris (pH 8.0), 13 mM $MgCl_2$, 11.5 mM 3-phosphoglycerate, 10 mM ATP, 0.11 mM thymidine, 50 uCi [^{14}C] thymidine, and 30 ul of cell extract supernatant. Duplicate samples were analyzed after 0 and 30 minutes of incubation at 37°C. The reaction was stopped by placing the reaction tube on ice and removing immediately 50 ul for application to a DE81 (Whatman) cellulose disc. The discs were dried under an infrared lamp for 10 minutes. Subsequently these discs were washed 3 times for 5 minutes each in 1 mM ammonium formate (10 ml/disc), once for 5 minutes in distilled water, and once for 5 minutes in 95 % ethanol (39). Finally they were baked at 60°C for one hour and counted using a toluene based liquid scintillation mixture.

Heat inactivation studies to determine the species-specificity of cellular TK activity were performed by incubating supernatant samples

at 60°C for 0 or 1 minutes and then cooling on ice prior to adding the remaining components of the TK assay reaction.

Determination of total protein content in the supernatant samples was done by a Folin reaction (40). One ml of a solution of 0.5% $CuSO_4$, 1.0% Na citrate, was added to 50 ml of 2% Na_2CO_3, 0.1 N NaOH. Two ml of this mixture were added to 50 ul of cell extract + 350 ul of H_2O and allowed to incubate for 5 minutes at room temperature. After this incubation, 200 ul of 1N Folin reagent was added, the final solution was mixed thoroughly, and the mixture was allowed to incubate at room temperature for 30 minutes. Protein determination was made by measuring absorbance at 600 nm on a spectrophotometer and comparing the value for the sample to a standard curve determined from dilutions of bovine serum albumin (BSA) processed in the same fashion.

Electrophoresis and Filter Hybridization

Whole cell DNA and Hirt DNA samples isolated from cell lines were digested with restriction endonucleases Eco RI, Bam HI, Hind III, Hpa II, Kpn I, Msp I, Pvu II, and Xba I using assay conditions suggested by the suppliers. Over the course of several years involved in this work each of the enzymes was purchased from several different suppliers including New England BioLabs, Boehringer Mannheim, and Bethesda Research Laboratories, Inc. Samples were electrophoresed in horizontal agarose gels (Sigma) using one of two sets of conditions. In the experiments outlined in Chapter II, the electrophoresis buffer consisted of 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.7 (21). For the experiments in Chapter III the agarose gels were run in 160 mM Tris

(pH 8.0), 80 mM NaCl, 5 mM EDTA (41) which produced sharper bands. In all experiments the agarose concentration was 1% unless Kpn I or Xba I digests were being analyzed in which case 0.7 to 0.8% gels were used to achieve better separation of large DNA fragments (42).

After electrophoresis the gels were stained with a 25 ug/ml solution of ethidium bromide in water, and were photographed under UV illumination. Blotting of the gels was accomplished either by the Southern technique (43) or by a modification of this technique (44). The Southern technique involved denaturation of the DNA in the gel by soaking the gel in a 1.5 M NaCl, 0.5 NaOH solution for 30 minutes. Neutralization in preparation for blotting entailed a 90 minute soaking in a 3 M NaCl, 0.5 M Tris (pH 7) solution. Finally the gel was placed on top of a sheet of Whatman 1 paper lying in a reservoir of 20 x SSC (1 x SSC 0.15 M NaCl, 0.015 M NaCitrate). On top of the gel was placed a nitrocellulose filter (S and S) which had been presoaked for 20 minutes in 2 x SSC, and on top of the nitrocellulose were layered 2 pieces of Whatman 1 paper and a 2-3 inch pile of dry brown paper towels. The 20 x SSC solution in the reservoir was soaked up by the paper towels acting as a wick. As the 20 x SSC solution moved through the gel the DNA migrated out and was trapped by the nitrocellulose filter. Blotting of the DNA was continued for 12-18 hours at room temperature. Afterwards the nitrocellulose sheet was removed, soaked in 2 x SSC for 20 minutes, air dryed, and baked at 80°C for 2 hours under reduced pressure.

In the modified technique, the DNA-containing gels were treated twice for 15 minutes each time with a 0.25 N HCl solution, twice for 15

minutes each time with the standard denaturing solution, and twice for 30 minutes each time with the standard neutralizing solution. This procedure produces single stranded breaks in the DNA and therefore large DNA molecules will be broken into 1-2 kb fragments which transfer more efficiently during blotting (44). The blotting procedure and baking conditions were not altered.

^{32}P-labelled DNA molecules were produced by the nick-translation reaction as outlined by Maniatis (45). This reaction involves the production of single stranded nicks in a double stranded DNA molecule followed by replacement of one strand with new nucleotides, one or more of which contain ^{32}P at the alpha position. The replacement step is carried out by E. coli polymerase 1. The nick-translated molecules were denatured by boiling for 5 minutes to yield a single stranded probe. The specific activity varied, but reasonable hybridization results were obtained using probes ranging from 5×10^7 to 3×10^8 dpm/ug of DNA.

Filter hybridization was performed by the procedure of Wahl et al (44). The filters were preteated for 2-3 hours at 65°C in Sears "Seal-N-Save" bags using for each 10 cm^2 of filter surface area, 1 ml of a solution of 6 x SSC, 50 mM sodium phosphate pH 6.5, 10 x Denhardt's reagent (1 x Denhardt's reagent contains 0.02% (wt/vol) of each of the following: bovine serum albumin, ficoll, and polyvinyl pyrolidone), 1% glycine and 500 ug/ml sonicated, denatured salmon sperm DNA. For hybridization this solution was removed and replaced with an equal volume of 6 x SSC, 20 mM sodium phosphate pH 6.5, 2 x Denhardt's reagent, 100 ug/ml sonicated, denatured salmon sperm DNA, 10% sodium

dextran sulfate (Sigma), and 7 ng/ml ^{32}P-labelled, denatured probe DNA. The hybridization continued for 8-9 hours at 65°C.

Posthybridization treatment of the filters involved 3 room temperature washes for 5 minutes each in 2 x SSC, and 2 65°C washes for 15 minutes each in 0.1 x SSC. The filters were air dryed and exposed to X-ray film at -70°C using a Cronex intensifying screen (DuPont). Exposure times ranged from 2 hours to 3 weeks.

Microcell-mediated Gene Transfer

Micronucleation of cell populations was achieved by plating the cells at a 1:4 dilution on 8 bullet-shaped plastic pieces cut from 150 cm^2 tissue culture petris. Colcemid (Gibco) was added to the growth medium at a concentration of 0.07 ug/ml for 36 hours. After the colcemid treatment, the cells were enucleated by centrifugation of the bullets at 29,000 x g for 30 minutes at 34°C in medium containing cytpchalasin B (10 ug/ml) (Aldrich). The microcell pellets were resuspended and filtered through a sterile 5 micron polycarbonate filter (Bio-Rad Labs.). The filtrate was collected and fused with monolayer recipient cells as outlined by Fournier and Ruddle (46). Twenty-four hours following fusion the recipient cells were distributed into a number of 25 cm^2 flasks and HAT selection was initiated.

Chapter I

Stability and Quantitative Analysis of Donor Thymidine Kinase Activity

Results

High molecular weight whole cell DNA isolated from Chinese hamster tissue culture cells was used to transform Ltk^- mouse cells to the TK^+/HAT^r phenotype. In the absence of a method for detecting the presence and structural integrity of the hamster TK gene sequences, I used a heat inactivation assay to demonstrate that the TK^+ phenotype in 2 transformed (HAT^r) cell lines was similar to that of the donor cells (Fig. 3) (see discussion). In addition, 25 transformed cell lines were analyzed for the expression of 17 other Chinese hamster isozyme markers using gel electrophoresis (see Materials and Methods). None of these 25 transformants expressed additional Chinese hamster phenotypes.

As a second form of analysis, I studied the stability of the transferred phenotype. Individual colonies of HAT^r Ltk^- transformants were isolated and expanded. When these colonies had grown into cell lines of 5×10^7 to 10^8 cells (25-27 doublings) an aliquot of 10^4 cells was transferred to a flask containing medium which was nonselective for TK expression. After various lengths of time growing in nonselective medium the cells were tested for the retention of the HAT^r phenotype by replating into ~~&~~HAT. Twelve transformant cell lines were tested and they fell into 3 groups (Table 1, Fig. 4). There was one transformant cell line that was entirely stable (1613), i.e. 100% of cells retained the TK^+ phenotype over 30 days in the absence of selective pressure. Eight transformant cell lines were unstable (163, 164, 165, 166, 168, 169, 1612 and 1618), i.e. the percentage of HAT^r cells continued

Figure 3. Heat inactivation study of thymidine kinase activity. Cell extracts were heated at 60°C before adding the thymidine kinase reaction mixture. The controls are CDR (hamster) and A9 (mouse), and the two cell lines are 162 and 164.

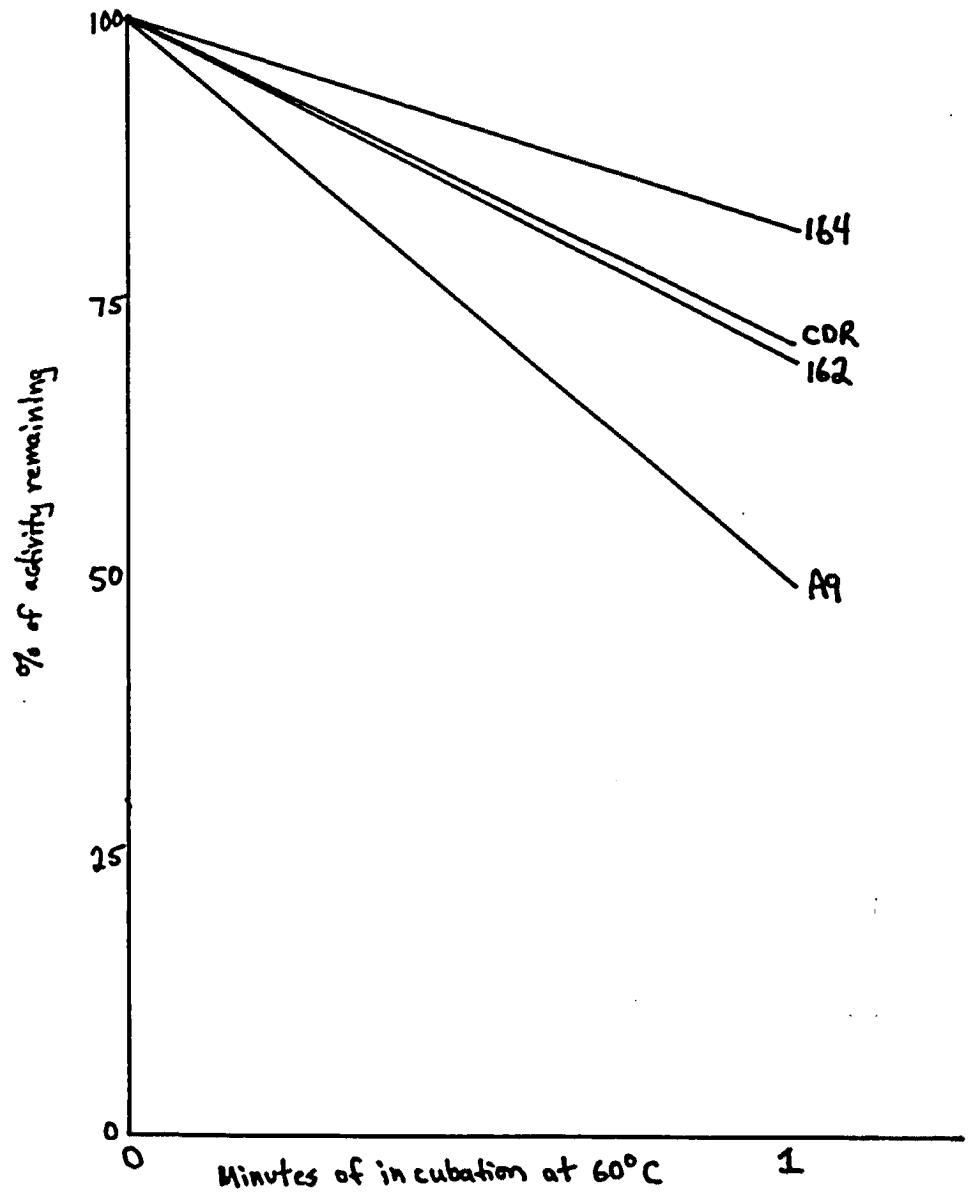
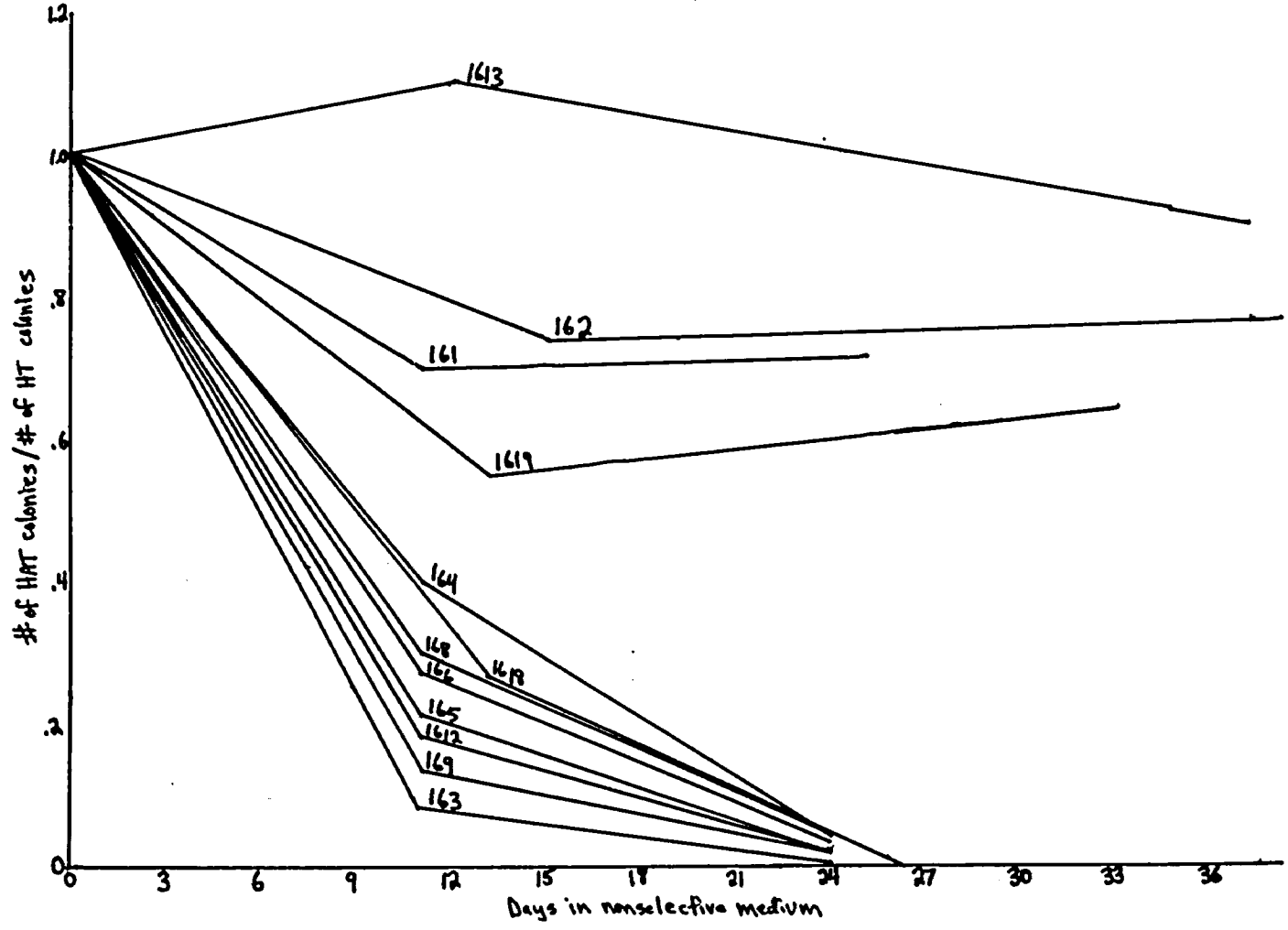


Table 1. Stability test data on TK transformants.

Cell Line	Day-(HAT/HT)		
161	0 (1.00)	11 (.70)	25 (.72)
162	0 (1.00)	15 (.74)	38 (.75)
163	0 (1.00)	11 (.08)	24 (0)
164	0 (1.00)	11 (.40)	24 (.04)
165	0 (1.00)	11 (.21)	24 (.01)
166	0 (1.00)	11 (.27)	24 (.03)
168	0 (1.00)	11 (.30)	24 (.04)
169	0 (1.00)	11 (.13)	24 (.01)
1612	0 (1.00)	11 (.18)	24 (.01)
1613	0 (1.00)	12 (1.10)	37 (.90)
1618	0 (1.00)	13 (.27)	26 (0)
1619	0 (1.00)	13 (.55)	33 (.65)

The stability tests were carried out as described in Materials and Methods. All of the cell lines were derived by transferring the Chinese hamster TK gene into TK^- mouse L cells. The results are expressed as the number of colonies in HAT divided by the number of colonies in HT. All time points for a given cell line are normalized with the value at the zero time point considered to be 100%.

Figure 4. Stability tests on TK transformants. This figure presents the data from Table 1 in graphic form.



dropping over the 30 day period. In three of the transformant cell lines the percentage of HAT^r cell dropped for 1-2 weeks and then leveled off (161, 162 and 1619) as if there were some unstable cells which lost the TK^+ phenotype rapidly and some stable cells which retained the TK^+ phenotype.

Analysis of transformed cell lines resulting from chromosome-mediated gene transfer has suggested that unstable transformants may have elevated levels of TK activity as a result of an increase in TK gene copy number (47,48). I had no nucleic acid probe for the hamster TK gene, and therefore could assay only the TK protein activity. The quantitative assay I used was a combination and modification of several techniques (38,39). Since the various transformant cell lines were assayed for TK activity on different days, aliquots of TK^+ Chinese hamster donor cells and TK^- mouse recipient cells were stored at $-70^{\circ}C$ and used in each set of assays as internal positive and negative controls. In order to compare results from assays completed on separate days, the TK activity of a given cell line was expressed as the percentage of the value for the positive control tested on the same day. A correction was made for variation in cell number in samples from different transformant cell lines by dividing the number of counts of ^{14}C-thymidine incorporated into thymidine monophosphate by the number of micrograms of protein in the cellular extract.

The TK activity values ranged from 41% to 417% of the positive control (Table 2). In general, unstable transformant cell lines had higher TK activity than mixed or stable cell lines. The TK activity of many transformants was equal to or greater than that of the hamster cell line used as a positive control.

Table 2. TK activity of transformant cell lines.

Cell line	TK Activity	ug protein	Phenotype
161	167%	62	Mixed
162	41%	73	Mixed
163	126%	57	Unstable
164	238%	45	Unstable
166	373%	72	Unstable
1612	417%	55	Unstable
1613	165%	85	Stable
1619	114%	37	Mixed

The activity values are expressed as a percentage of the positive control value assayed on the same day. The protein values are for 30 ul of cell extract.

I studied the transition from instability to stability by subcloning 2 unstable transformant cell lines, lines 166 and 169, and isolating and analyzing stable derivative lines (Table 3). The 3 stable derivatives of line 166 showed a decrease in TK activity of at least 50%. When line 169 was subcloned after 30 days in nonselective medium, the 3 subclones were unstable. One of the lines, 169a, was resubcloned after 30 more days in nonselective medium, and 4 subclones were isolated (Fig. 5). Three subclones were unstable and one was mixed (169a3). After 30 days in nonselective medium the 169a3 cell line was subcloned and 4 cell lines, 2 derived from each of two flasks, were analyzed (Table 3). All of these subclones were stable and all 4 showed a marked decrease in TK activity.

Discussion

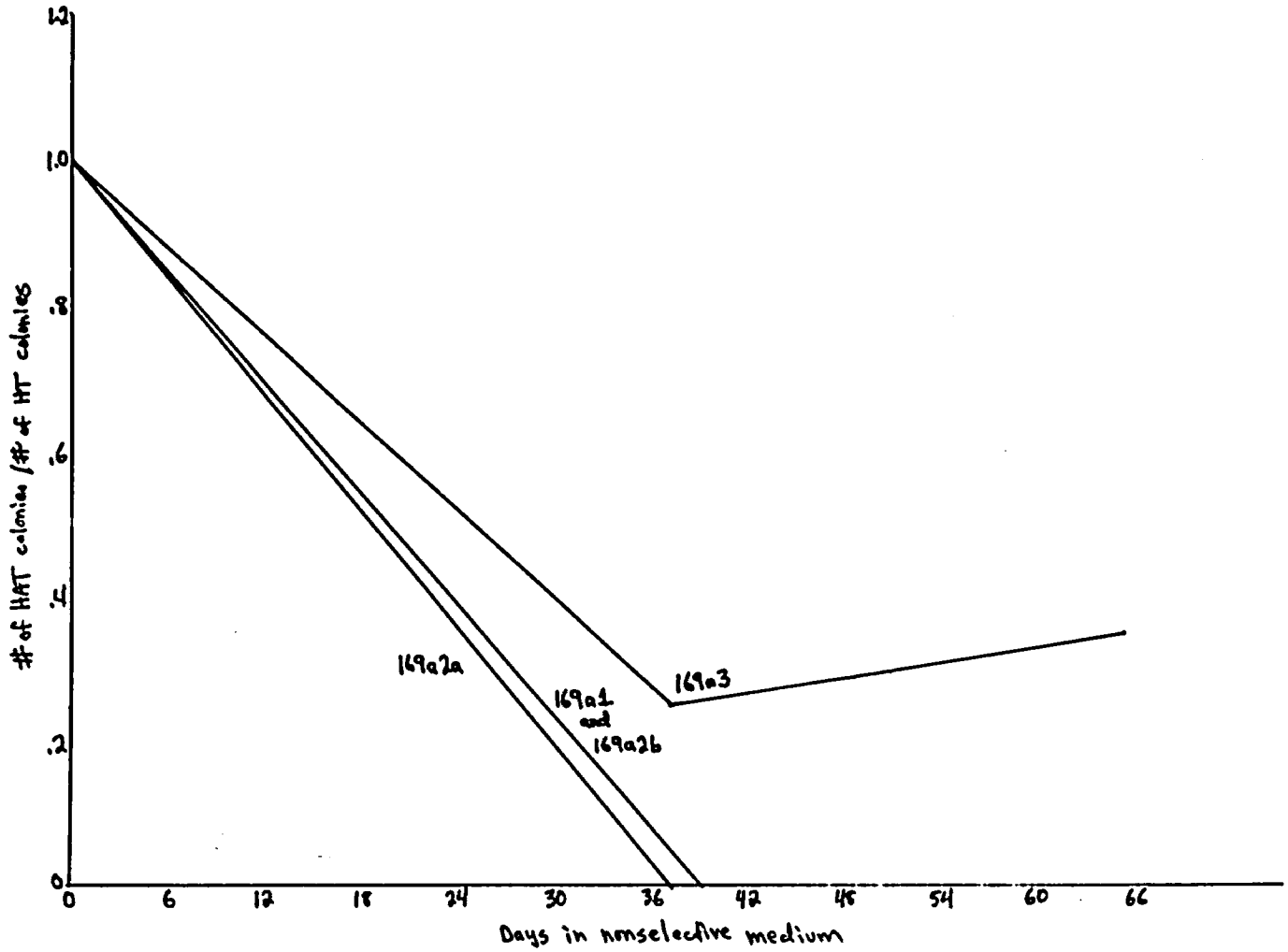
There are several published reports on DMGT which have included data on the stability and/or quantitative activity for donor phenotypes transferred to recipient cells (7,18). Although I had no nucleic acid probe to use in investigating the fate of the Chinese hamster TK gene, the combination of stability testing and quantitative TK analysis yielded some interesting results. The elevated TK activity generally present in unstable cell lines, and the decrease in activity observed in stable derivatives of these unstable lines, are consistent with results from CMGT (47,48). In one case of CMGT, there was a correlation made between the decreased TK activity after stabilization and a decrease in gene copy number (48).

Table 3. TK activity of 166 and 169 subclones.

Cell Line	TK Activity	Phenotype
166	373%	Unstable
166a1	156%	Stable
166a2	185%	Stable
166a3	162%	Stable
169a3	184%	Mixed
169a3Ia	29%	Stable
169a3Ib	18%	Stable
169a3IIa	53%	Stable
169a3IIb	43%	Stable

The TK activity is expressed as a percentage of the positive control (Chinese hamster donor line). The phenotype refers to the stability of the TK^{+}/HAT^{r} phenotype.

Figure 5. Stability tests on 169a subclones.



The stability test format used in this study has been used in analyzing both CMGT lines (47) and DMGT lines (18). CMGT lines in which no detectable fragment of donor material could be detected were unstable and fast losers (6.5-10% loss rate per day). Some unstable CMGT lines were slow losers, and karyotypic analysis demonstrated that the donor material was present on a chromosomal fragment which; (1) was detectable by light microscopy, (2) was not translocated onto a recipient cell chromosome, and (3) possibly contained donor centromeric function. Although investigators have reported the existence of slow loser unstable DMGT lines (7), their result was based on stability tests which involved only 1 time point. It is important to note, however, that only from multiple time points can one discriminate between mixed cell lines and lines which are unstable but have a slow loss rate. A population of transformed cells in which 2-3% of the cells lose the donor phenotype each generation may contain 40% TK^+/HAT^r cells after one month of culture in nonselective medium. Alternatively, a mixed transformed cell population in which 40% of the cells retain the donor phenotype stably and 60% lose it rapidly, i.e. 10% of these cells lose the donor phenotype each day, also would contain 40% TK^+/HAT^r cells after one month of culture in nonselective medium. The shape of the stability test curve can be used to sort out these two types of transformed populations (47). None of the DMGT lines that I tested were slow losers.

One assumption made in interpreting stability test data is that stabilization is a rare enough event that its occurrence during the course of the stability test does not produce enough stable cells to

alter the data. There were some lines that I tested in which the percentage of HAT^r cells remaining in the population did not level off after several weeks in non-selective medium, and the frequency of colonies able to grow in HAT became less than 1 in 4,000. In theory, one might be able to discriminate between the loss rate pattern for a mixed population, and the pattern for an unstable population in which stabilization was occurring in several percent of the population each day, by examining the kinetics of loss of donor phenotype from the population. However, the variation involved in cellular dilution and plating make this kind of exacting analysis quite difficult.

The results of the quantitative TK assay were expressed as a percentage of the control value in order to compare results between experiments. Although the control values were relatively reproducible between experiments, in examining a set of cell lines, e.g. the 169 subclones, all of the assays were performed in a single experiment. The amount of protein present in the 30 ul aliquot of cellular extract used for the TK assay varied considerably (37 ug to 189 ug). I did not analyze the total protein content per cell in independent transformant cell lines and therefore cannot be absolutely sure that the TK activity/ug protein can be extrapolated to TK activity/cell. The independent transformants were derived from a single recipient cell line and had similar morphologies. The fact that many of the transformant cell lines had a higher level of TK activity than the donor cell line raises some interesting questions. Before any of these questions are addressed, however, one must be able to demonstrate that this observation is not a simple reflection of gene copy number. If TK

activity is found not to correlate directly with TK gene copy number, one might begin to investigate the effects on TK activity of 5′ and 3′ nontranslated gene sequences as well as host sequences at the integrated site.

The results from the quantitative TK assay are compatible with several models for the instability to stability conversion. One model hypothesizes the presence of multiple, nonchromosomally associated TK genes in the unstable cells. Chromosomal integration of one active TK gene copy could lead to its propagation in a stable fashion, while the remaining free TK gene copies could be lost without affecting the HAT^r of that cell. There has been no definitive demonstration of autonomous TK genes in unstable or stable transformant cell lines. Chromosomal association of a transferred TK gene has been demonstrated in stable but not in unstable transformant cell lines (18). Other models for the instability to stability conversion which would involve changes in TK activity include chromatin structural rearrangement and gene sequence rearrangement.

Several experiments could be effective in discriminating among the various models. A correlation between gene copy number, TK activity, and stability/instability could be made if one analyzed a series of TK^+ transformant cell lines which received the HSV-TK gene. A metaphase chromosome fractionation technique which is capable of separating normal mouse chromosomes from subchromosomal fragments could be used to search for nonchromosomally associated TK genes in unstable cells (49). If one had a nucleic acid probe for the TK gene one might be able to study the unstable to stable transition using <u>in situ</u> hybridization.

Chapter II

Introduction of Plasmid DNA into Mouse Cells by Unlinked Cotransfer

Results

The introduction of Hβ1 DNA sequences into Ltk^- cells was accomplished by cotransformation using three sources of TK gene: HSV-1, human, and Chinese hamster DNA. The frequencies of recovery of HAT-resistant colonies in the cotransformation experiments using cellular DNA (Table 4) were in the range of those seen by other investigators (6). The input ratio of Hβ1 molecules to cellular TK genes was on the order of 20,000:1. Sixteen of the 52 transformants were expanded and analyzed. DNA was isolated as soon as each line grew to 10^8 cells.

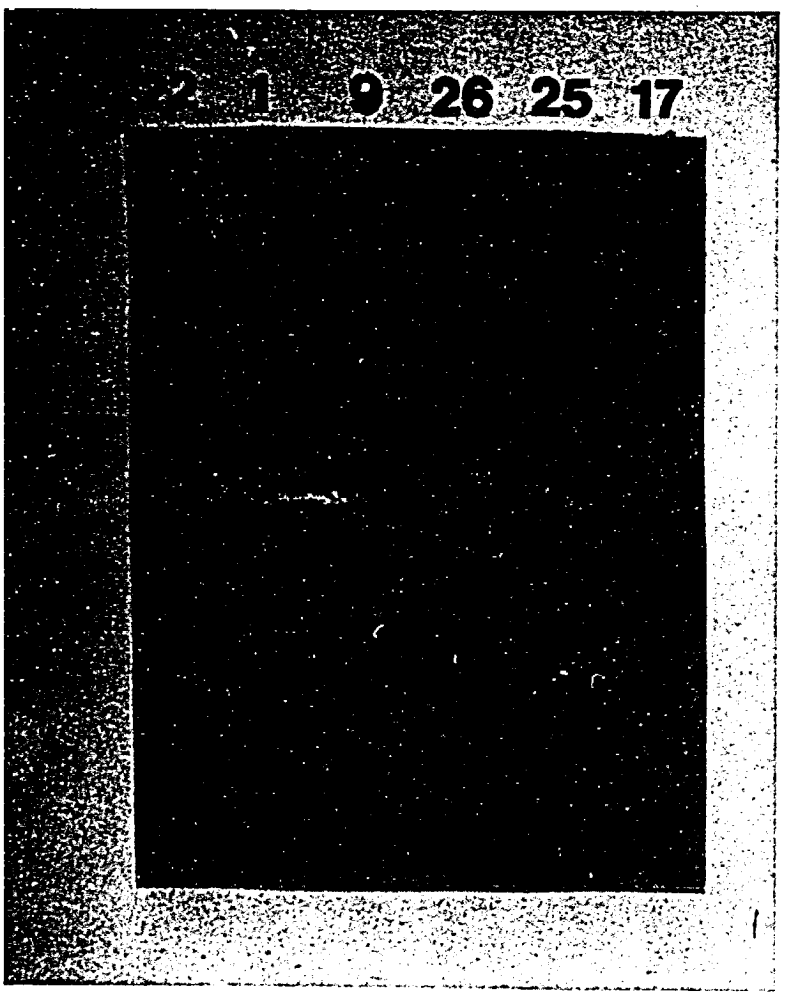
Examination of the restriction map of Hβ1 (Fig. 2) shows that Eco RI, Hind III, and Kpn I cleave the molecule twice, once, and not at all, respectively. Initial screening of the 16 TK^+ transformants by Eco RI DNA digestion and filter hybridization demonstrated that 13 (80%) of the lines contained Hβ1 sequences and that nearly half of these lines had five or more copies per cell. There was no significant correlation between the source of TK and either the frequency of cotransformation or the number of copies of Hβ1 sequences present. As judged by band intensities following filter hybridization, one line, 530, had approximately 30 copies of Hβ1 sequences per cell. The most striking finding was that Eco RI digestion gave the same two bands in many positive lines (Fig. 6). These two bands were present at the same positions as the bands produced by Eco RI digestion of the Hβ1 molecule (7.2 kb and 1.5 kb). Many of the digested transformant cell DNAs

Table 4. DNA-mediated gene transfer with Ltk$^-$ as recipient.

DNA	Colonies	Positive Flasks	Colonies/30 ug DNA
Hamster + Hβ1	6	2/4	1.5
Hamster + Hβ1	25	10/10	7.5
HeLa + Hβ1	10	7/10	1.0
Salmon sperm + HSV-TK + Hβ1	11	7/10	NA

Data indicate TK transformation frequency only. NA, not applicable to gene transfer with carrier DNA.

Figure 6. Eco RI digestion of DNAs from cotransformants. The lines are 522 (lane 22), 51 (lane 1), 59 (lane 9), 526 (lane 26), 525 (lane 25), and 517 (lane 17). The TK sources were HSV (525), Chinese hamster (51, 59, 517, 522), and human (526) DNAs. The two main bands present in almost every line (7.2 kb and 1.5 kb) are identical to the bands seen with Eco RI digestion of Hβ1. The DNA was analyzed by filter hybridization using ^{32}P-labeled Hβ1 DNA as probe.



showed additional larger bands representing Hβ1 sequences attached to high molecular weight DNA.

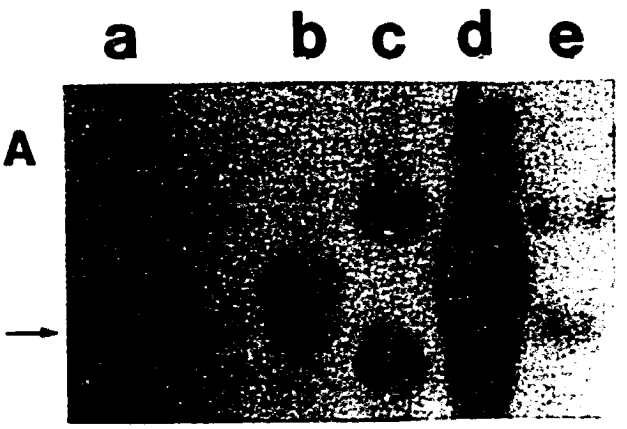
Hind III analysis of DNA from the transformants that demonstrated the two main Eco RI bands gave a consistent pattern (Fig. 7). Most lines had a band present at a position identical to that of linear Hβ1 (8.7 kb). Additional bands seen in these lines probably represent integrated Hβ1 sequences, analogous to the Eco RI results.

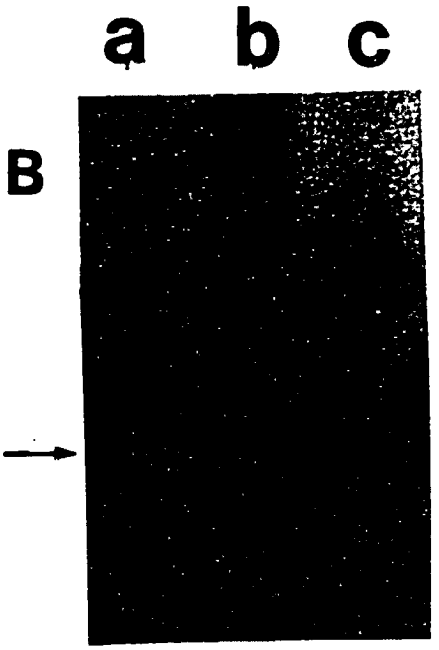
Kpn I and Xba I do not cleave Hβ1, and digestion of the plasmid DNA with either of these two enzymes gave bands corresponding to covalently closed monomers, open circular monomers, and some multimers. Kpn I and Xba I digested DNA samples were electrophoresed in 0.7-0.8% agarose gels and blotted using acid and alkali treatments to insure adequate fragment separation and successful blotting of supercoiled molecules. Digestion of transformant DNA with Kpn I followed by filter hybridization using ^{32}P-labelled Hβ1 as probe gave a variable pattern (Fig. 8). At least three lines (523, 525, and 526) out of five analyzed in detail contained a band whose electrophoretic mobility was indistinguishable from that of open circular monomeric plasmid in a background of Ltk$^-$ DNA. These lines contained other bands as well. In addition, line 523 DNA digested with Xba I gave a band migrating to the position of open monomeric plasmid circles (Fig. 8).

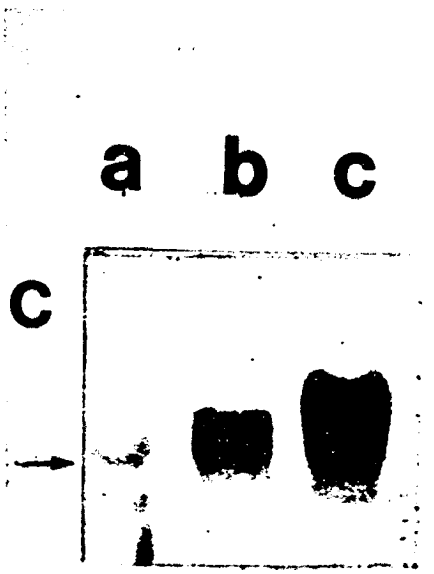
One transformant cell line, 526, was chosen for further analysis. After several months in culture in selective medium independent HAT^r subclones of line 526 retained the initial hybridization pattern of Hβ 1-containing fragments when their DNA was digested with Hind III (data not shown). 526 DNA fractions, derived using the Hirt fractionation

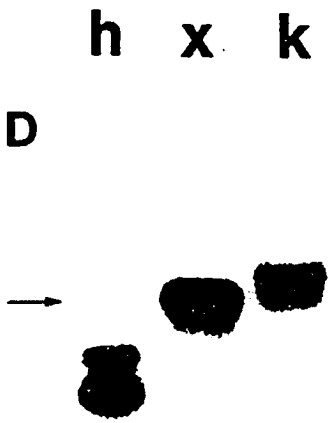
Figure 7. Hind III digestion of DNAs from cotransformants. Lanes: a, line 523; b, HeLa DNA control; c, 530; d, 517; e, Ltk$^-$ plus Hβ1; f, Hβ 1; g, 526. Hind III cleavage of Hβ1 produces a linear molecule (single band) seen in lanes e and f. The four cotransformants contained this main band (8.7 kb), and 523 (a), 526 (g), and 530 (c) contained additional bands. TK sources were HSV (530), Chinese hamster (517, 523), and human (526) DNAs. The DNA was analyzed by filter hybridization using ^{32}P-labeled Hβ1 DNA as probe.

Figure 8. Kpn I and Xba I digestion of DNAs from cotransformants. All of the digestions for A, B, and C were done with Kpn I. (A) Lanes: a, line 526; b, 523; c, Hβ1; d, 530; e, Ltk⁻ plus Hβ1. (B) Lanes: a, Ltk⁻ plus Hβ1; b, 526; c, Hβ1. (C) Lanes: a, Hβ1; b, 525; c, 526. (D) Lanes: h, Hind III 523; x, Xba I 523; k, Kpn I 523. The arrow in each photograph indicates the band position corresponding to open circular monomeric Hβ1 molecules when electrophoresed in a background of digested Ltk⁻ DNA (approximately 22 kb). Notice the altered mobility of open circular Hβ1 molecules when electrophoresed with Ltk⁻ DNA (B, lane c vs. lane a). The TK sources are listed in Figs. 6 and 7. The DNA was analyzed by filter hybridization using ^{32}P-labeled Hβ1 DNA as probe.







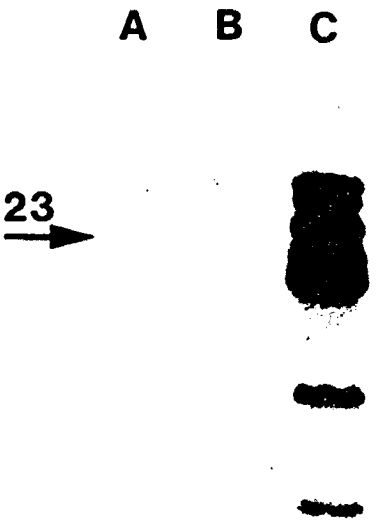


technique, were analyzed and Hβ1 sequences were present only in the Hirt ppt (Fig. 9). Back selection on a population of 526 cells using BUdR with UV exposure (see Materials and Methods) produced BUdR/UV-resistant colonies at a frequency of 1 in 10 to 1 in 100 cells. Five independent HAT^S backselectants of line 526 were analyzed, and all of these lines had lost all of the Hβ1 sequences (Fig. 10). The faint bands seen in the back selectant tracks represents hybridization to a mouse DNA sequence, possibly repetitive in nature, since it is present in negative control tracks (Fig. 11).

The stability of the TK^+/HAT^r phenotype in line 526 was analyzed and the population was found to contain both stable and unstable transformants. I isolated a stable subclone, 526H1a, which contained all of the original Hβ1 sequences. Back selection on 526H1a cells produced BUdR/UV-resistant colonies at a frequency of 1 in 10^5. Again, each of the 6 independent HAT^S lines had lost all of the Hβ1 sequences (data not shown).

The concommitant loss of HAT^r (presumably through loss of the human TK gene) and loss of all Hβ1 sequences is in agreement with other published reports that unlinked sequences can become linked with and segregate with the selected gene (21,24). In an attempt to analyze the physical proximity of the human TK gene and the cotransferred Hβ1 sequences in 526H1a DNA, I used high molecular weight whole cell DNA isolated from 526H1a cells as donor material in a second round of DMGT into Ltk^- cells. A total of ten independent secondary transformants derived from 2 separate experiments were analyzed (Fig. 12). None of these secondary transformants contained Hβ1 sequences.

Figure 9. Xba I digests of line 526 Hirt fractionated DNA. Digests of 526 Hirt supt DNA (A), Ltk^- DNA (negative control, B) and 526 Hirt ppt DNA (C) were electrophoresed on a horizontal 0.8% agarose gel, blotted onto nitrocellulose paper, and hybridized using ^{32}P-labeled Hβ1 DNA as probe.



Xba I

Figure 10. Hind III digestion of DNAs from line 526 and from 526 back selectants. Lane C contained 526 DNA; lanes A,B,D,E, and F contained DNAs from 5 independent back selectants of line 526. The 8.7 kb arrow represents the point to which linear Hβ1 molecules would migrate. The probe was ^{32}P-labeled Hβ1 DNA.

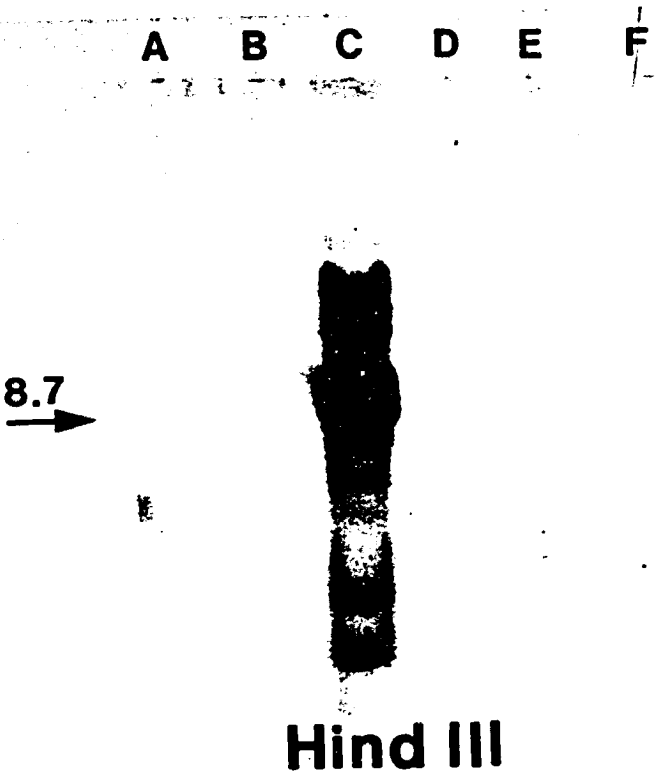
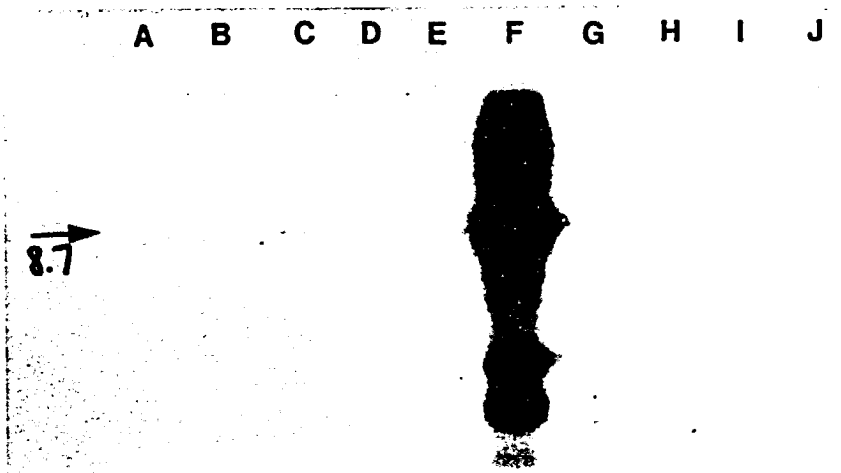


Figure 11. Bam HI digestion of DNAs from line 526H1a and from derivative lines. Lane D contained DNA from line 526H1a, a HAT^r subclone of line 526. Lanes A and E contained DNAs from 2 back selectants of 526. Lanes B and F contained DNAs from 2 secondary transformants derived using 526H1a DNA (see Fig. 12). Lanes C and G contained Ltk^- DNA. The 4 arrows represent the positions of bands derived from Bam HI digestion of Hβ1 DNA. The DNAs were hybridized using ^{32}P-labeled Hβ1 DNA as probe.

Figure 12. Hind III digestion of DNAs from 526Hla secondary transformants. Lane F contained DNA from line 526Hla. Lane E contained Ltk$^-$ DNA. Lanes A-D and G-J contained DNAs from 8 independent secondary transformant lines derived using whole cell 526Hla DNA as donor material. All DNAs were probed with ^{32}P-labeled Hβ1 DNA. The arrow at 8.7 kb indicates the position of linear Hβ1.



Hind III

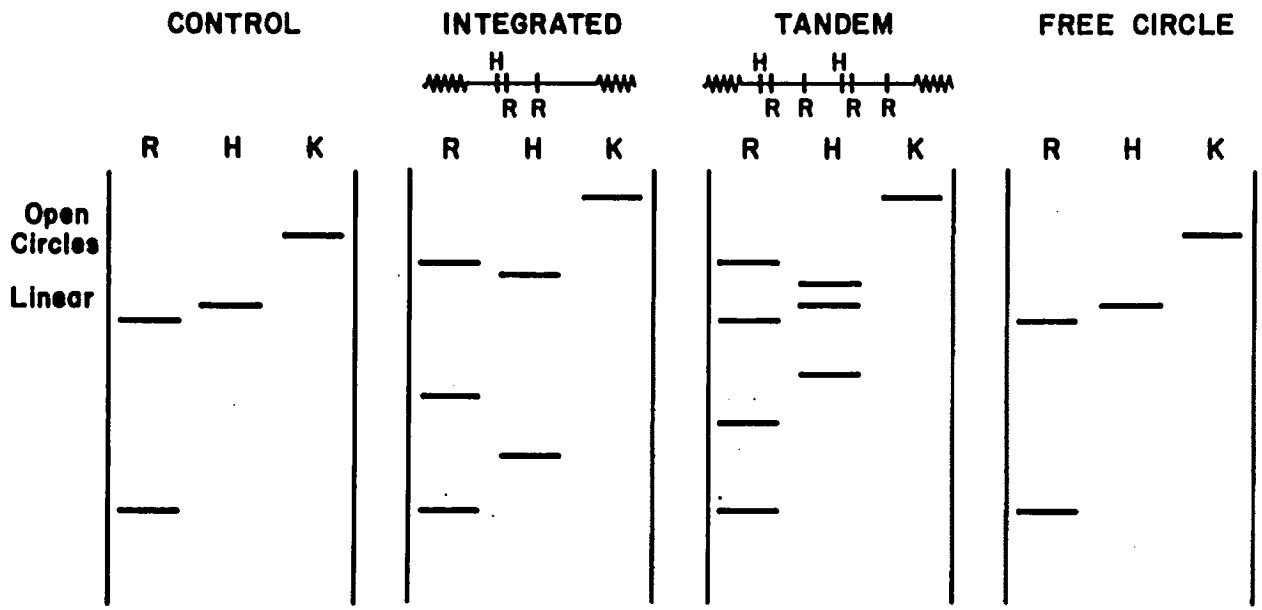
Discussion

I have analyzed Hβ1 sequences transferred by DMGT into mouse tissue culture cells. The presence of multiple copies of Hβ1 in the transformant lines corroborates the findings of Wigler et al. (21). Hβ 1 DNA represented 3-10% of the total donor DNA used in the transformation and was found in 80% of the TK^+ lines analyzed, at up to 30 copies per cell. These data indicate that cells which take up and express the TK gene incorporate additional DNA, and that cotransformation is an efficient method for the introduction of nonselected genes into mammalian cells.

Evidence for the propagation of unintegrated DNA molecules has been reported (50). In several transformant lines containing copies of Hβ1 DNA we have seen a pattern of Hβ1-containing bands, produced by several restriction enzymes, which is indistinguishable from the pattern produced by digestion of authentic circular plasmid DNA.

Hind III digestion of integrated monomeric plasmid would yield one or two fragments of varying size, depending upon the retention of the Hind III site, with each fragment consisting of a portion of the Hβ1 molecule attached to flanking host sequences. Hind III digestion of cellular DNA from many transformants produced a band migrating at the position of linearized plasmid (Fig. 7). This can be explained only by site-specific integration at the Hβ1 Hind III site, the presence of tandem Hβ1 repeats, or the presence of free circular molecules (Fig. 13).

Figure 13. A diagrammatic representation of band patterns expected after endonuclease digestion of Hβ1 sequences present in transformed cells. The labels "open circles and "linear" refer to the position of bands representing open circular or linear Hβ1. Control lanes represent circular plasmid which yields: two fragments after Eco RI (R) digestion, a full-length linear band after Hind III (H) digestion, and open circular molecules after Kpn I (K) digestion (covalently closed molecules are visualized poorly due to nicking by Kpn I and difficulties in blotting). Integrated lanes represent the expected pattern from a single copy of Hβ1 integrated into cellular DNA. Eco RI gives one of the two control bands (here the smaller) and two additional bands representing Hβ1 sequences attached to cellular DNA (tail fragments). Hind III gives two bands, and Kpn I gives one band. Integrated tandem repeats (dimer shown here) digested with Eco RI would yield several copies of the two control bands plus two tail fragments. Hind III digestion would give an 8.7 kb fragment (identical to linear Hβ 1) and two tail fragments. Kpn I would give one high molecular weight band. Free circles would give the same pattern as the control, Hβ1 DNA.



The presence of free circles is favored by the finding that digestion of the DNA of several lines with Kpn I, which does not cleave the Hβ1 plasmid, gave a band migrating at the position of open circular plasmid (Fig. 8). Open circular plasmids migrate with an apparent molecular weight of 22 kb. One cannot rule out the possibility that Kpn I-digested monomeric or dimeric plasmid molecules attached to cellular DNA coincidentally migrate at this molecular weight, but the presence of bands at 22 kb in several independent lines makes this interpretation unlikely. However, cell lines that gave a Hind III band characteristic of linear Hβ1, and gave no Kpn I band at 22 kb, probably contain some form of tandem Hβ1 repeat. Analysis of DMGT (17) has produced no evidence for site-specific integration.

In many of the transformant cell lines the presence of multiple copies of the plasmid sequences could confuse the interpretation of band patterns. One cell line with a relatively simple restriction pattern is 523. Eco RI digestion (not shown) gave two intense bands corresponding to those derived from plasmid DNA, plus two additional faint bands. The intensity of the main bands was consistent with the presence of less than five copies of Hβ1 sequences per cell. Hind III digestion produced three bands; one migrating to the position of linear Hβ1, one larger by several kilobases, and a third very faint band slightly smaller than linear Hβ1 (Fig. 7). Kpn I digestion gave two bands, one migrating to the same location as open circular Hβ1 and one larger (Fig. 8). Finally, Xba I digestion gave one intense band at the position of a free circle (Fig. 8). My interpretation of these data is that line 523 contains both free circular and integrated copies of Hβ1.

The free circles produced bands indistinguishable from authentic plasmid bands after Hind III, Kpn I, and Xba I digestion, whereas the integrated sequences were represented by the other two Hind III bands and by the larger Kpn I band. The Xba I band resulting from digestion of the integrated sequence may have been too large to detect. Alternatively, it may have a molecular weight of approximately 22 kb and therefore would comigrate with open circular molecules.

In summary, this evidence suggests that plasmid Hβ1 is capable of existence as an independent circular molecule in mammalian cells. Based solely on restriction analysis with four enzymes, however, one cannot rule out the possibility that all of the bands were derived from integrated (or free) tandem repeats of Hβ1. The observation that the Hβ1 sequences could not be demonstrated in the Hirt supt fraction of line 526 DNA may support this latter interpretation. However, another laboratory has conducted cotransformation experiments using the same human β globin-containing plasmid and has had similar results (50). Only from the Hirt ppt DNA fraction were they able to rescue plasmid molecules by transformation of bacterial cells (Anderson, W.F. Presented at the conference on Construction and Use of Mammalian Vectors. Cold Spring Harbor, December, 1980). The existence of circles in the transformed cells does not necessarily indicate that the circles were replicating. It remains to be shown that these autonomous circular molecules were not being generated on a regular basis from integrated sequences, possibly concatamers.

The results of back selection experiments on lines 526 and 526Hla agree with other published results which indicate that unlinked donor

DNA sequences can become linked to form larger molecular units (18,24), known variously as pekalasomes (51) and transgenomes (18), and that loss of a portion of a transgenome molecule, e.g. the selectable gene, can be accompanied by loss of some or all of the remaining transgenome sequences. Stability testing of the 526 cell population indicated that there were HAT^S cells being generated at a rate of 8% per day. During the course of the back selection experiment, which involved up to a week of cell growth in non-HAT containing medium prior to UV exposure, a large number of cells in the 526 population would become HAT^S. If the donor TK gene had become part of a transgenome which consisted of all or most of the donor material present in each cell in the 526 population, and if this change to HAT^S involved physical loss of the donor TK gene, then concommitant loss of all $H\beta 1$ sequences could be explained on the basis of loss of the entire transgenome. This result would be compatible with the model of an unstable transgenome being nonchromosomally associated and easily lost as a unit. However, line 526H1a, which was a stable, HAT^r 526 subclone, also lost all $H\beta 1$ sequences upon back selection. There are at least two reasonable hypotheses to explain this result. The first is that back selection involving BUdR/UV exposure led either to chromosomal loss or chromosomal fragmentation as a means of producing a HAT^S cell. Established tissue culture cell lines are known to be aneuploid and to contain chromosomal rearrangements. Detailed karyotypic analysis of the 526 HAT^r cells and the HAT^S derivatives would be necessary to test this hypothesis, but a number of control experiments involving BUdR without UV, UV without BUdR, and subcloning with neither BUdR not UV

treatment would be required in order to prove a relationship between any karyotypic change and loss of the TK gene.

The second hypothesis is based on the observation that the donor gene in stable DMGT transformants is less stable than genomic genes (9). The evidence for this comes from fluctuation analysis on a human HPRT gene transferred into mouse recipient cells. Unpublished results (Barbosa, J.A., K.M. Huttner and F.H. Ruddle. Unpublished observations) extend this observation to the TK system. If this relative instability is caused by the TK gene sequence excising from a host chromosome, the size of the excision unit relative to the size of the transgenome would determine the frequency of coordinate loss of cotransferred sequences in conjunction with the loss of the selected gene. This second hypothesis may overlap with the first in that excision without proper chromosomal rejoining could lead to loss of the distal chromosomal segment.

The fact that none of the Hβ1 sequences cotransferred with the human TK gene in the secondary transformation experiments suggests that the Hβ1 sequences were not immediately adjacent to the TK gene. However, analysis of transformation experiments in which the TK gene was known to be linked directly to pBR322 sequences in the donor DNA demonstrated that in less than 20% of the resulting transformants did the TK gene-pBR322 linkage remain intact (52). Therefore, the possibility that some of the Hβ1 sequences in 526Hla were located within 10-20 kb of the TK gene is not ruled out. If cotransfer experiments can be used to produce transgenomes in which probeable DNA sequences, e.g. Hβ1 DNA, are within 20-30 kb of selectable genes, e.g.

TK, then cloning of transformant cell line DNA using random fragments of cellular DNA, 20-40 kb in length, could produce a recombinant molecule which contains both the probeable sequence and the gene of interest. This molecule could be identified by a combination of nucleic acid hybridization (probeable portion) and a gene transfer bioassay (selectable gene) (53).

Chapter III

DNA-mediated Gene Transfer Without Carrier DNA

Results

DMGT using pTKx-1 DNA without carrier DNA produced HAT^r colonies at a frequency of 1-10 colonies/10 ug pTKx-1 DNA/10^6 recipient cells. The frequency of HAT^r colonies produced by using nanogram quantities of pTKx-1 DNA with 20 ug of Ltk^- carrier DNA was 1 colony/1 ng pTKx-1 DNA/10^6 recipient cells in control experiments. I isolated 7 independent transformants from DMGT experiments using circular pTKx-1 DNA. Each transformant colony was expanded to 10^8 cells (approximately 27 generations) before analysis of its phenotypic and genotypic properties. Six of seven transformants were stable at first testing. Initially, one circular transformant, 101, was found to contain a mixture of cells of stable and unstable phenotypes (Fig. 14). After one month of growth in non-selective medium, a HAT^r population derived from this line was retested and was entirely stable within the limits of resolution of the assay.

DNA was isolated from all transformant cell lines using a standard procedure for whole cell DNA isolation, and was isolated from 2 cell lines using the Hirt fractionation procedure. Analysis of Hirt fractionated DNA from one circular transformant line, 101 (see above), is shown in Fig. 15. The band pattern present in the Hirt supt DNA (lanes S) was distinct from that seen in the Hirt ppt fraction (lanes P). I compared Hirt supt and Hirt ppt DNA digests to digests of circular pTKx-1 DNA. Hind III-cut, linear pTKx-1 DNA migrated to a position of 7.9 kb, and bands at this position were present in Hind III

Figure 14. Stability of the HAT^r phenotype in carrier-free transformant lines. Two lines are shown. 13a represents a stable population and 101 represents a mixed population composed of stable and unstable cells.

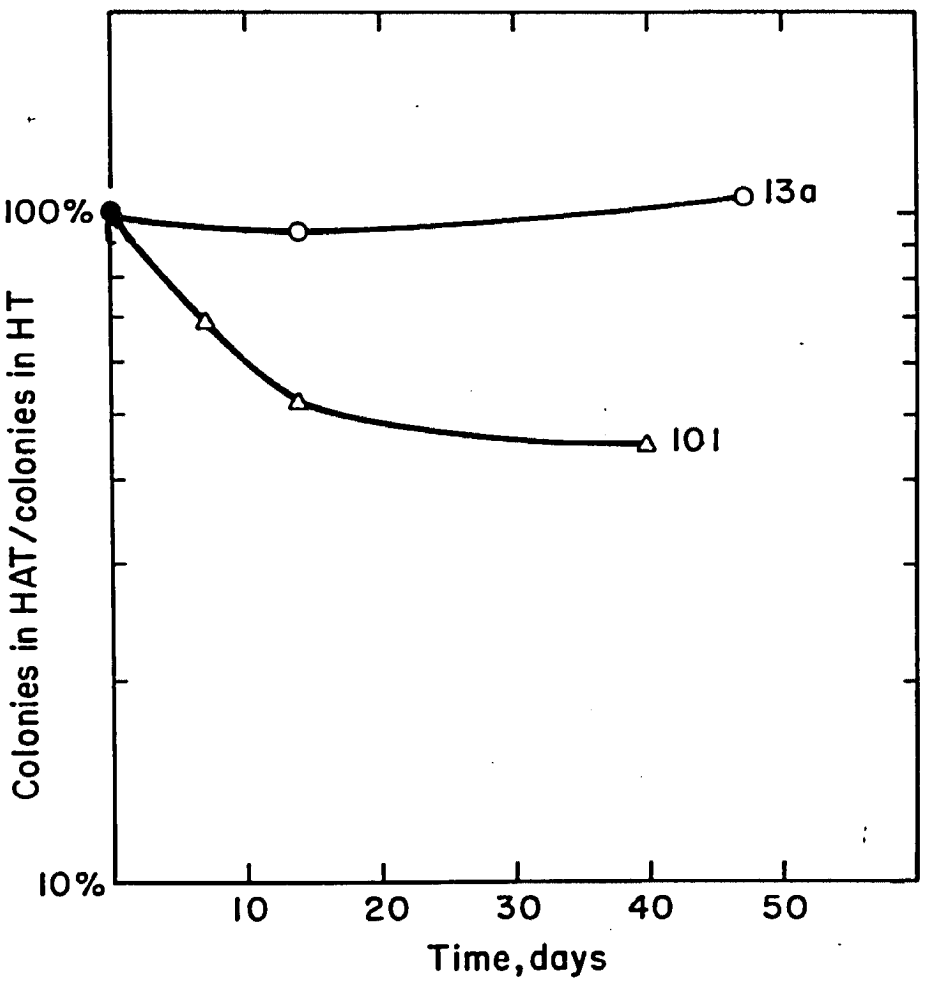
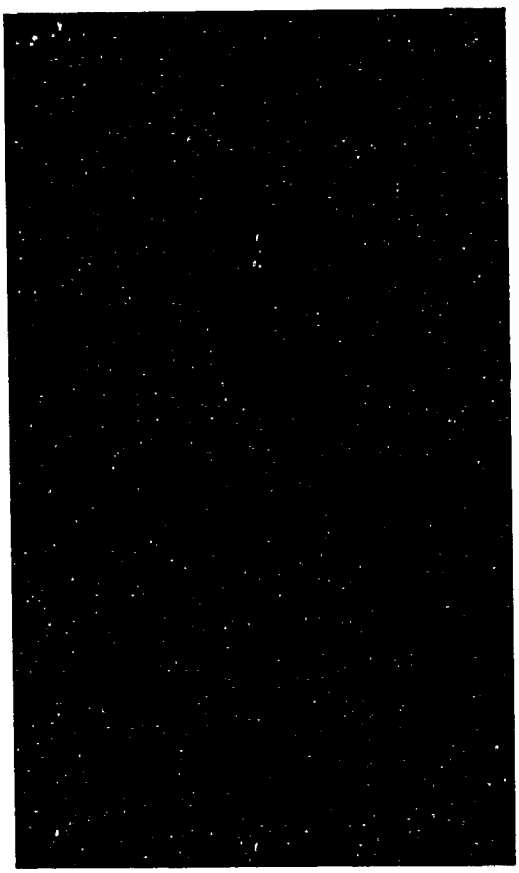


Figure 15. Hirt fractionation of DNA from a circular transformant line. DNA was isolated from a circular transformant line, 101, and fractionated into Hirt ppt (P) and Hirt supt (S). DNA from these two fractions was digested with restriction endonucleases Xba I and Hind III, electrophoreses on a 0.8% agarose gel, blotted onto nitrocellulose paper, and hybridized to ^{32}P-labeled pTKx-1 DNA. 7.9 kb represents the size of linear pTKx-1 DNA which is generated by Hind III digestion. Lane C contained pTKx-1 plasmid DNA mixed with L cell DNA, digested with Xba I, and electrophoresed with the Hirt samples. The high molecular weight band (>23 kb) in Lane C is presumed to be open circular plasmid DNA generated by the nicking activity contaminating commercial Xba I preparations. A weak and variable band at approximately 3.5 kb is seen in all lanes as well as in Ltk$^-$ control lanes. This band appears to represent a hybridization to pBR322 sequences as it is absent when one uses the Bam HI Herpes TK fragment as a probe.



digests of both the ppt and supt DNAs. Digestion with Xba I, which recognizes no sites in pTKx-1 DNA, converted Form 1 DNA to open circular DNA (Fig. 15, lane c, band at >23 kb) by a non-specific nicking activity contaminating commercial Xba I. The fact that the open circular pTKx-1 (7.9 kb) band appeared to be higher in molecular weight that the open circular H 1 (8.7 kb) band (see Chapter II) is consistent with the presence of a large proportion of pTKx-1 molecules being multimers, and this was seen to be the case in subsequent analysis of the undigested pTKx-1 preparation. Xba I digestion of the supt but not the ppt fraction gave a band at the position of open circular pTKx-1 DNA (Xba I, lanes S and P). A similar pattern was seen in DNA from another circular transformant line, 3g, which was phenotypically stable. However, in DNA from this cell line, the only Hirt supt sequences detected were those corresponding in mobility to bands derived from control plasmid. After one month of growth in nonselective medium, 101 and 3g cells were reanalyzed. Hirt DNA fractionation now resulted in the retention of pTKx-1 sequences only in the Hirt ppt.

Digestion of 101 and 3g cellular DNAs with Hind III, followed by hybridization with labelled pTKx-1 DNA, produced many bands (Fig. 16, lane 101, Fig. 17, lane c). In both lines, cellular and Hirt ppt DNAs included a 7.9 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. In contrast to the many Hind III bands detected in DNA from lines 101 and 3g, only one or a few Hind III or Eco RI bands could be detected in DNA from other

Figure 16. Hind III digestion of DNAs from a circular transformant line and from several derivative lines. Lane 101 represents the Hind III pattern of donor DNA sequences in the original transformant; lanes UV1, UV2, and UV3 are DNAs from 3 101 back selectant lines; and lanes UV1H1, UV1H2, and UV3H1 are DNAs from 3 independent re-expressor lines derived from UV1 and UV3. All DNAs contained an intense Hind III band at the position of linear pTKx-1 DNA (7.9 kb) as well as several other bands. The donor sequences were identified by hybridization using ^{32}P-labeled pTKx-1 DNA as probe.

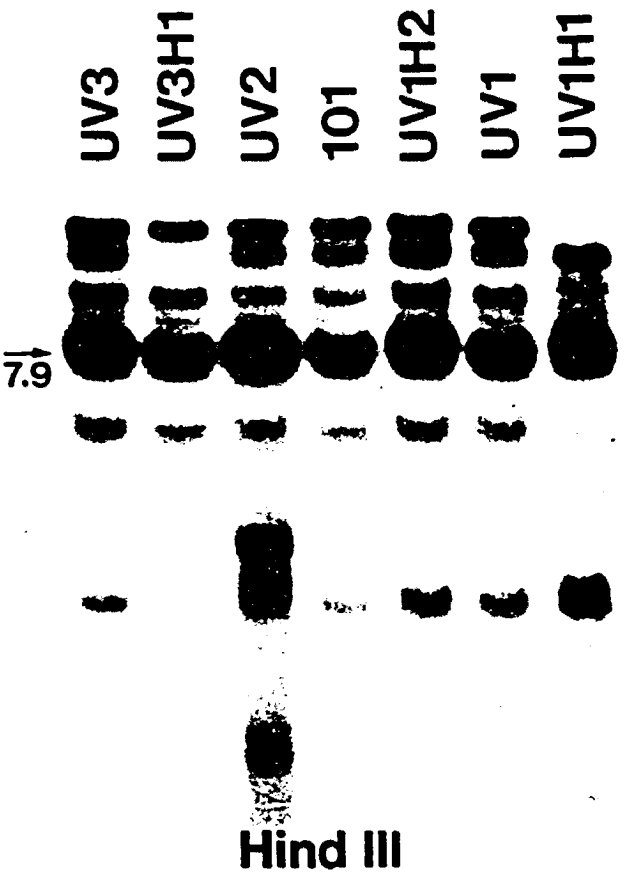
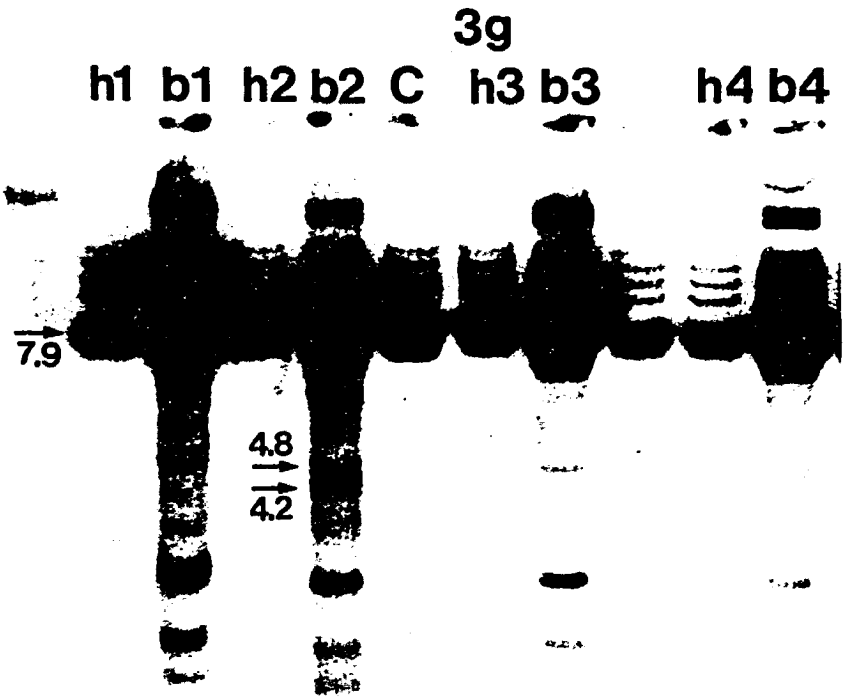


Figure 17. Hind III digestion of DNAs from line 3g and from derivative cell lines. Lane C contained DNA from the circular transformant, line 3g; lanes b1, b2, b3, and b4 contained DNAs from back selectants of line 3g; and lanes h1, h2, h3, and h4 contained DNAs from re-expressing, HAT^r lines derived from b1, b2, b3, and b4 respectively. Back selectant lines b3 and b4 were picked as colonies from one flask, with the remaining 6 lines (b1, b2, h1-h4) all picked from separate flasks. Amplification of the original sequences as well as the appearance of several new Hind III bands can be seen in all 4 back selectant DNAs. The largest two Hind III bands in each back selectant represent a doublet seen faintly in 3g DNA at longer exposure times. Donor sequences were detected by filter hybridization using ^{32}P-labeled pTKx-1 DNA as probe.



Hind III

circular transformant lines (data not shown) indicating that a relatively small amount of donor material had been retained. Based on the number and molecular weight of bands present upon Hind III or Eco RI digestion, I estimate that there were from 2 kb up to 50-65 kb of donor material present per cell in the circular pTKx-1 carrier-free transformants.

Digestion of 3g and 101 whole cell DNAs with Xba I, which recognizes no sites in pTKx-1 DNA, produced 1 high molecular weight band (>23 kb) (Fig. 18, lanes c, 101). One circular transformant contained several Xba I bands (data not shown). Since all Xba I sites were from the mouse recipient cell DNA, either multiple transgenomes integrated into recipient cell DNA, one transgenome integrated and then generated additional pieces of donor material with new Xba I sites, or one transgenome formed consisting of donor as well as recipient DNA with the latter providing the Xba I sites (see discussion).

A number of phenomena were seen in the course of subcloning and selection studies on these circular transformant cell lines. These phenomena underscore the complex host cell mechanisms available for regulating donor genetic material.

101 (summarized in Fig. 19)- The Hind III band pattern of 101 DNA was unaltered in 4 independent subclones of the original line (data not shown). Back selection of HAT^r 101 cells using BUdR+ UV selection produced 2 independent HAT^s populations in which no band pattern alterations were detected with Hind III (Fig. 16, Lanes UV1 and UV3) or Xba I digestion (Fig. 18, lane UV3). A third back selectant contained at least 3 additional Hind III bands (Fig. 16, lane UV2) and 5-6

Figure 18. Xba I digestion of DNAs from circular transformant lines. Lanes 101 and C, DNAs from the original circular transformants 101 and 3g; lanes UV3, b1, and b2, DNAs from back selectants of 101 (UV3) and 3g (b1 and b2); lanes H1 and h1, DNAs from re-expressor lines derived from UV3 (H1) and b1 (h1). The Xba I digests were electrophoresed in 0.8% agarose gels prior to blotting. Filter hybridization was performed using ^{32}P-labeled pTKx-1 DNA as probe.

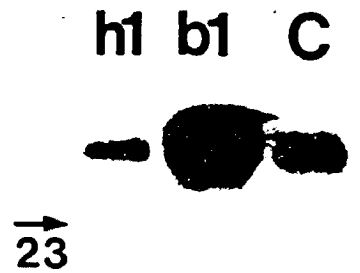
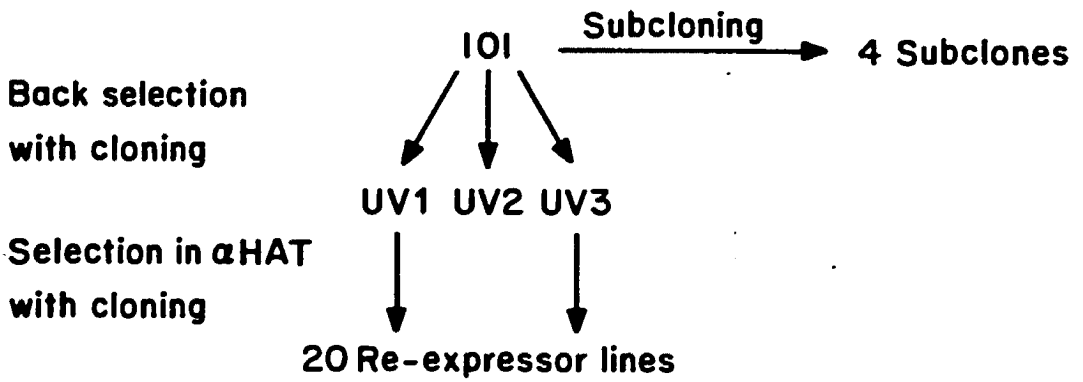


Figure 19. Summary of experiments involving transformant line 101.



additional Xba I bands (data not shown). None of the back selectants had lost any of the original sequences. The back selectant populations were returned to HAT selective medium, and at a frequency of 1 in 3 x 10^3 to 1 in 10^5 cells were able to develop HAT^r colonies. The DNA from 2/3 re-expressors showed loss of Hind III bands (Fig. 16, lanes UV3H1 and UV1H1). One of these re-expressor cell populations, UV1H1a, was subjected to another round of backselection and re-expression. DNA from both clonal back selectants and clonal re-expressor lines derived from these back selectants was analyzed following Hind III digestion (Fig. 20). No band pattern changes were detected.

In a series of cell lines derived from 101, where gene expression was modulated without detectable Hind III or Xba I band pattern changes, I investigated the role of DNA methylation in this process using the Hpa II (methylation sensitive)/Msp I (methylation resistant) isoschizomer pair (54). One limitation to the analysis is that Hpa II and Msp I recognize multiple sites in pTKx-1 DNA, and many of the restriction fragments one would expect to be produced were too small to be detected using standard blotting conditions. Although different band patterns were seen after Hpa II (one 5.7 kb band) and Msp I (one 3.3 kb band) digestion, no recognizable differences could be detected among the digests of 101b, 101bUV1 (back selectant), and 101bUV1H1 (re-expressor) DNAs (Fig. 21). This data does not suggest a role for DNA methylation in TK activity modulation in this series of cell lines.

Following up on the evidence from Xba I digestion of 101 DNA which produced only 1 band, suggesting the presence of only one integrated pTKx-1-containing transgenome, I attempted to use microcell-mediated

Figure 20. Hind III digestion of DNAs from line 101 from and a series of derivative lines. Lane O contained DNA from 101B, a HAT^r subclone of 101, whose Hind III band pattern is identical to that of 101. Lane N contained DNA from 101BUV1, a HAT^s back selectant of 101B. Lane M contained DNA from 101BUV1H1a, a HAT^r re-expressor line derived from 101BUV1. Lanes L and P contained DNAs from 2 independent back selectants, 101BUV1H1aUV1 and 101BUV1H1aUV3, derived from 101BUV1H1a. Lanes H-K contained DNAs from 4 independent re-expressor lines derived from 101BUV1H1aUV1 (lane L). Lanes Q-S contained DNAs from 3 independent re-expressor lines derived from 101BUV1H1aUV3 (lane P). The DNAs were probed with ^{32}P-labeled pTKx-1 DNA. The arrow at 7.9 kb represents the position of linear pTKx-1 DNA.

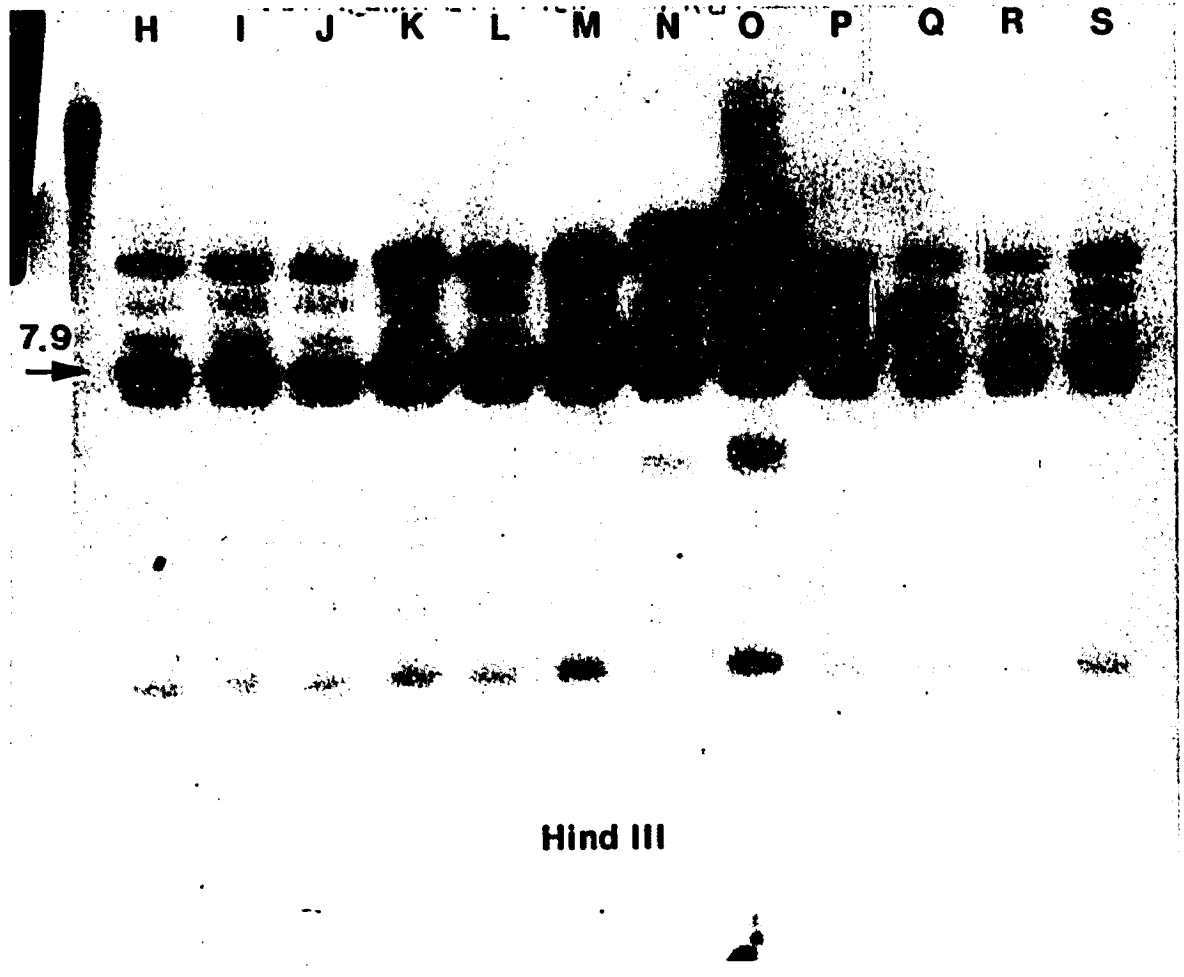


Figure 21. Analysis of methylation in DNAs from derivatives of line 101. Lanes A-D contained 15 ug each of DNA from line 101B. Lanes E-H contained 15 ug each of DNA from back selectant 101BUV1. Lanes I-L contained 15 ug each of DNA from re-expressor 101BUV1H1a. All DNAs were digested for 11 hours at 37°C. DNAs in lanes A, E, and I were digested with 0.8 units of Hpa II per ug DNA; DNAs in lanes C, G, and K were digested with 1.3 units of Hpa II per ug DNA; DNAs in lanes D, H, and L were digested with 4 units of Hpa II per ug DNA; and DNAs in lanes B, F, and J were digested with 2 units of Msp I per ug DNA. The DNAs were hybridized using ^{32}P-labeled pTKx-1 DNA as probe.

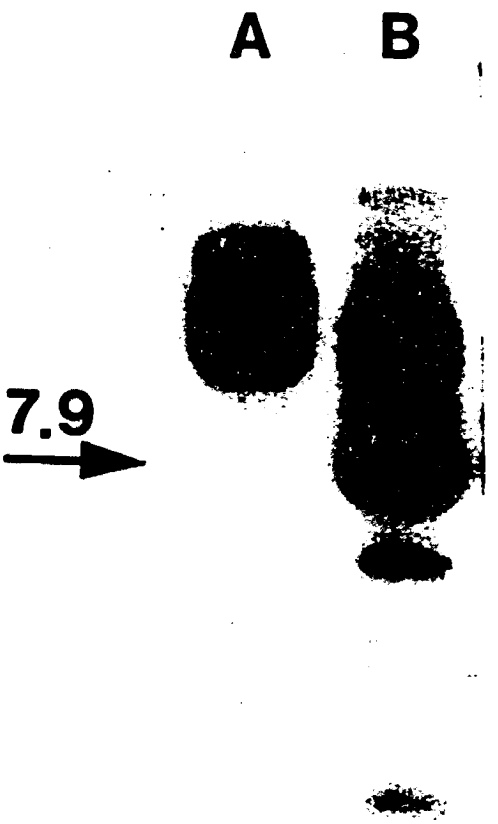
gene transfer to transfer the active HSV-TK gene(s) from transformant line 101 to RJK, a TK^- Chinese hamster recipient cell line. From several microcell experiments I was able to isolate only one microcell hybrid, 101Ru4. Isozyme analysis performed on 101Ru4 cell extracts indicated that 5 out of 12 mouse isozymes were being expressed in addition to all of the hamster isozymes (Table 5). There were two pairs of isozymes which are known to be syntenic in the normal mouse genome, yet only one of each pair was expressed in 101Ru4 cells (MPI and ME (9), GPI and LDHA (7)). I have not compared the karyotypes of LTAP and 101 cells and therefore have not determined whether 101 cells have a large number of rearranged chromosomes or whether the isozyme expression pattern reflects chromosomal breakage and rearrangement during or subsequent to the microcell transfer procedure. Analysis of pTKx-1 sequences present in 101Ru4 indicated that some of the original 101 Hind III bands were present, some were lost, and that some new bands were detected (Fig. 22).

3g (summarized in Fig. 23)- Digestion of 3g DNA with Hind III gave a large number of pTKx-1-containing bands ranging in size from 3 to 23 kb (Fig. 17, lane c). Back selection on populations of 3g cells was performed by growing the cells in BUdR for 17 days prior to UV treatment. This modification of the standard protocol was chosen in an attempt to isolate cells with different mechanisms of conversion to the $BUdR^r/HAT^s$ state. DNA digests from three of the four independent back selectants are shown in Fig. 17, lanes b1, b2, and b3. The most striking observation seen in all 4 independent back selectants was the 2-5 x amplification of nearly all of the Hind III bands present in the

Table 5. Isozyme data on 101Ru4 (See Materials and Methods).

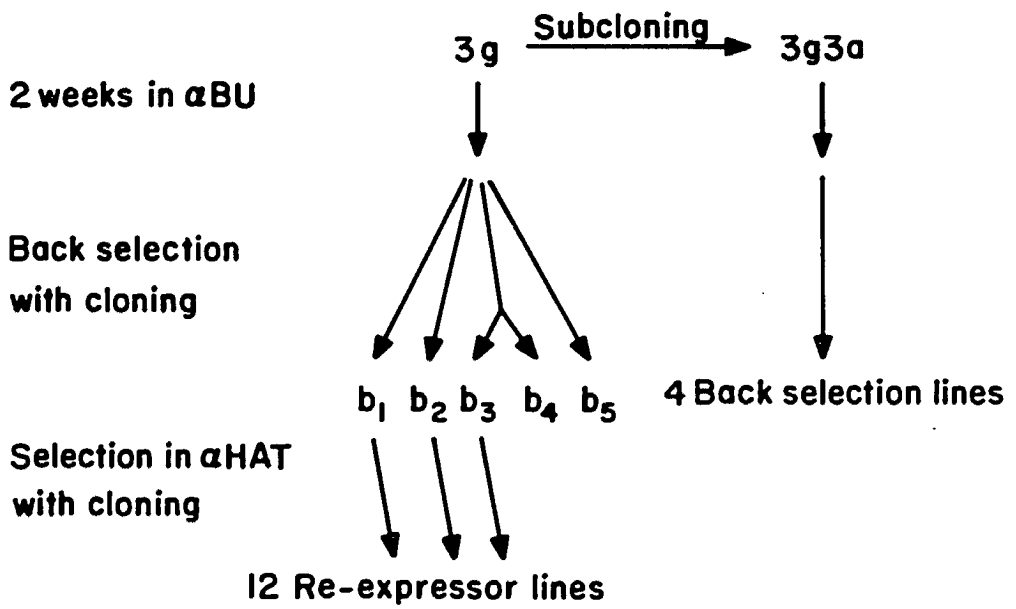
Enzyme	Mouse Isozyme	Hamster Isozyme
AdoK	+	+
APRT	-	+
DIP1	-	+
GalK	-	+
GLO	-	+
GPI	+	+
GSR	-	+
ME	-	+
MPI	+	+
PGM1	+	+
TPI	-	+
TRIP1	+	+

Figure 22. Donor DNA sequences present in microcell hybrid 101Ru4. Lane A contained Hind III digested 101Ru4 DNA. Lane B contained Hind III digested 101 DNA. The arrow at 7.9 kb represents linear pTKx-1. The DNAs were hybridized using ^{32}P-labeled pTKx-1 DNA as probe.



Hind III

Figure 23. Summary of experiments involving transformant line 3g.



original line. One of the back selectants, 3gb2 (lane b2), had several additional bands at 4.8 and 4.2 kb. Xba I digestion of DNA isolated from the back selectants produced one high molecular weight band which was somewhat diffuse and certainly of greater intensity than the 3g control band (Fig. 18, lanes c and b1). Line 3gb2 DNA contained at least 3 novel Xba I bands (Fig. 18, lane b2). Eco RI digestion of 3g and back selectant DNAs demonstrated that the degree of amplification was not homogeneous for all of the many Eco RI bands (Fig. 24).

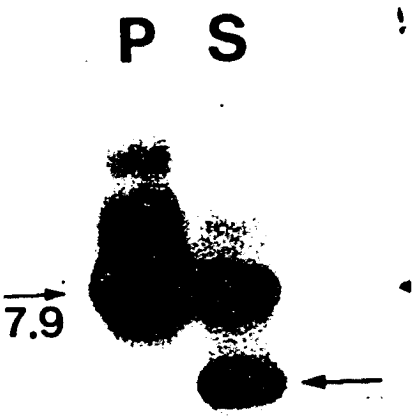
In order to examine the relationship between the gene amplification and the loss of TK activity in the 3g back selectants, I plated each of the back selectant populations back into HAT. Re-expressors, i.e. HAT^r colonies, were isolated from the 3 back selectants (b1, b2, and b3) at a frequency of 1 in 10^4 to 1 in 10^5 cells. DNA from a total of 12 independent re-expressor lines was analyzed with Hind III and Xba I digestion. All evidence of amplification was lost in the 12 re-expressor lines (e.g. see Fig. 17, lanes h1-h4, Fig. 18, lane h1). However, Eco RI digestion demonstrated that the re-expressor pattern of band intensities was not identical to that of 3g (Fig. 24, lanes A and C). Two of these re-expressor lines were examined in greater detail and were found to be: (1) phenotypically stable, i.e. HAT^r, when propagated in the absence of selection for 30 days; and (2) genotypically stable, i.e. no Hind III band changes, during the same period. All pTKx-1 sequences were found in the Hirt ppt and not in the Hirt supt fraction of DNA isolated from these 2 re-expressor lines (data not shown).

Figure 24. Eco RI digestion of DNA from 3g, a 3g back selectant, and 2 re-expressor lines. Lane A, 3g; lane B, 3gb1; lanes C and D, 2 independent re-expressors derived from 3gb1. Eco RI digestion of plasmid pTKx-1 Form 1 DNA yields 3 bands migrating at approximately 4.6, 2.4, and 1 kb. ^{32}P-labeled pTKx-1 DNA was used as a probe for donor sequences.

For further characterization of the amplification, I chose 2 back selectant lines, 3gb1 and 3gb4 (Fig. 17, lanes b1 and b4). The cell lines were grown in both neutral medium and BUdR-containing medium for a period of 30 days. DNA was isolated at 0, 14, and 30 days and fractionated by the Hirt procedure. Under both growth conditions the Hind III band patterns of Hirt ppt DNA isolated at all 3 time points showed no band pattern changes while retaining the amplification. At the end of the 30 days the cells were plated back into αHAT and re-expressors were isolated at the same frequency as determined originally, i.e. 1 in 10^4 to 1 in 10^5 cells. The Hirt supt fraction was unrevealing except for one time point, 3gb1 0 days, in which there was one major difference between the supt and ppt fractions (Fig. 25). Frequently I have found the Hirt supt fraction contaminated by a small amount of chromosomal DNA. In such instances, the supt hybridization pattern was similar to that of the Hirt ppt although of diminished intensity. In contrast, line 3gb1 Hirt supt DNA contained one very intense Hind III band at approximately 6 kb and 1 Pvu II band at approximately 6.3 kb which were not seen in the Hirt ppt DNA. These sequences were not detectable at 14 or 30 days in either the Hirt ppt or Hirt supt fractions of 3gb1 DNA.

Based on the observation that even after 30 days in culture the 3g back selectant populations re-expressed at a low frequency, 1 in 10^4 to 1 in 10^5, I subcloned line 3gb1 and tested for the ability of clonally derived 3gb1 subpopulation to re-express. Three independent subclones were analyzed, 3gb1A, 3gb1B and 3gb1C. The Hind III restriction pattern of pTKx-1-containing bands in these 3 DNAs was the same as for

Figure 25. Hirt fractionation of line 3gb1 DNA. Hirt ppt (P) and Hirt supt (S) DNA fractions from 3g back selectant line 3gb1 were digested with Hind III and analyzed by filter hybridization. The unlabeled arrow points to a 6 kb supt-specific Hind III band. Donor sequences were detected using ^{32}P-labeled pTKx-1 DNA as probe.



Hind III

3gb1 (Fig. 26). When 10^6 cells from each of these three BUdR^r subclones were plated back into HAT, 3gb1A produced no colonies, 3gb1C produced approximately 1000 colonies, and 30-50% of all of the plated 3gb1B cells survived. Two independent re-expressor colonies derived from 3gb1C as well as a mass population of cells derived from 3gb1B were expanded and analyzed. The Hind III pattern for DNA from each of these 3 cell populations showed a loss of amplification, and a pTKx-1-containing band pattern identical to that of all previous re-expressors studied (Fig. 26).

DNAs from 3g, 3gb1, and a re-expressor line derived from 3gb1 were digested with Hpa II and Msp I in order to examine the role of DNA methylation in gene regulation (Fig. 27). Msp I, which recognizes at least 30 sites in pTKx-1 DNA, produced no bands large enough to detected on Southern blot filters. Hpa II, which recognizes the same sites unless methylation is present, produced a large number of bands in all 3 DNAs indicating that multiple Hpa II/Msp I sites were methylated. Most of the bands were comparable in mobility in all 3 samples, with the bands in the back selectant DNA showing the 2-5 x amplification characteristic of the Hind III and Eco RI bands. As was the case in the 101 series, in the 3g series I saw no changes in band patterns or band intensities which might support a role for DNA methylation in modulating TK gene expression.

In a second attempt at back selection, using a population of 3g cells which had been frozen in liquid nitrogen, stored for 12 months, and then thawed for reculturing, BUdR^r lines were isolated using either 2 or 17 days of growth in BU prior to UV treatment. A parallel set of

Figure 26. Hind III digestion of DNAs from 3gb1 subclones and re-expressors. The 3g back selectant, 3gb1 (lane D), was subcloned and DNAs from 3 independent lines, 3gb1A, 3gb1B, and 3gb1C are represented in lanes B, E, and H. Lanes A and C contained DNAs from 2 independent re-expressor lines derived from 3gb1A (lane B). Lane G contained DNA from a mass population of re-expressor cells derived from 3gb1C (lane H). Lane F contained DNA from a 3gb1 re-expressor line derived in an earlier experiment. The arrow at 7.9 kb represents the position of linear pTKx-1 DNA. The DNA were hybridized using ^{32}P-labeled pTKx-1 DNA as probe.

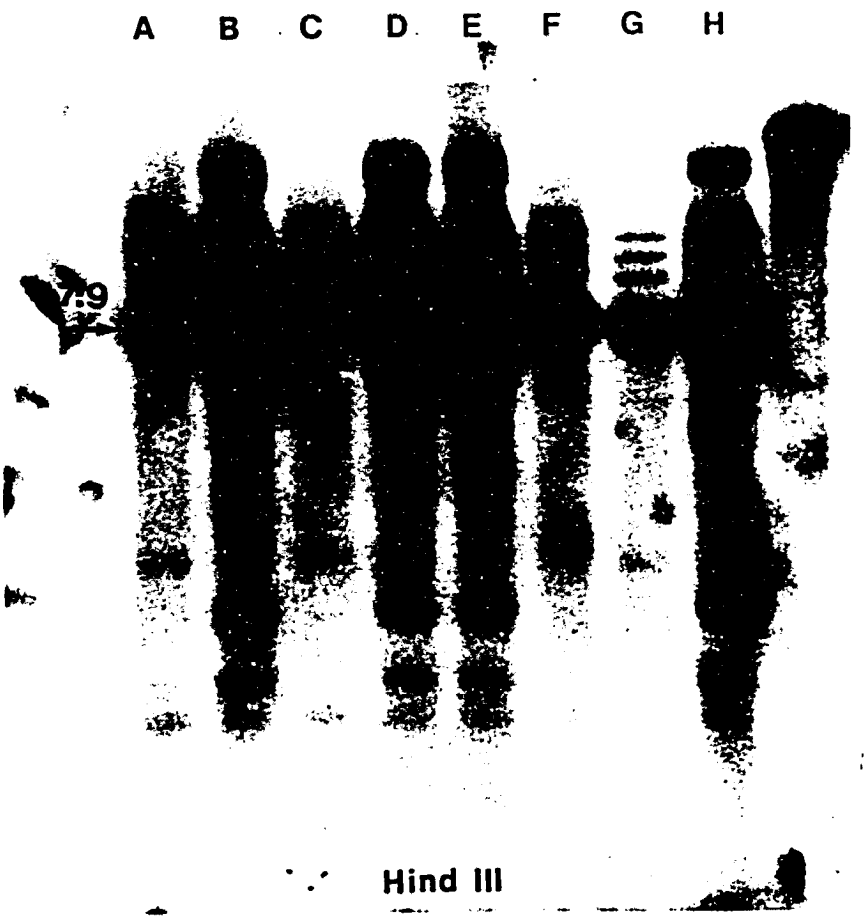
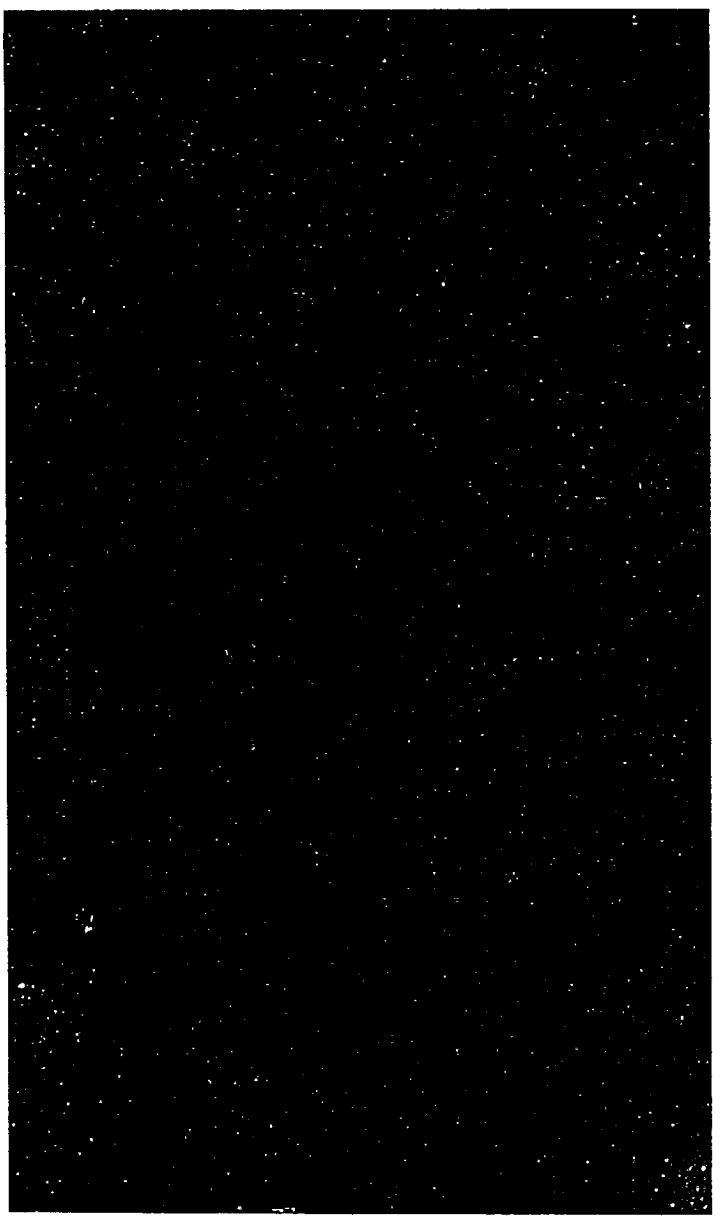


Figure 27. Analysis of methylation in DNAs from line 3g and from 3g derivative cell lines. DNAs from lines 3g, 3gb1, and 3gb1h8 were digested with Hpa II (lanes A, C, and E) and with Msp I (lanes B, D, F). The Hpa II digests were done at 1.6 units of enzyme per ug DNA for 11.5 hours. The Msp I digests were done at 2 units of enzyme per ug DNA for the same time. The DNAs were hybridized using ^{32}P-labeled pTKx-1 DNA as probe. Two exposures are presented to show the bands in all 3 Hpa II tracks.



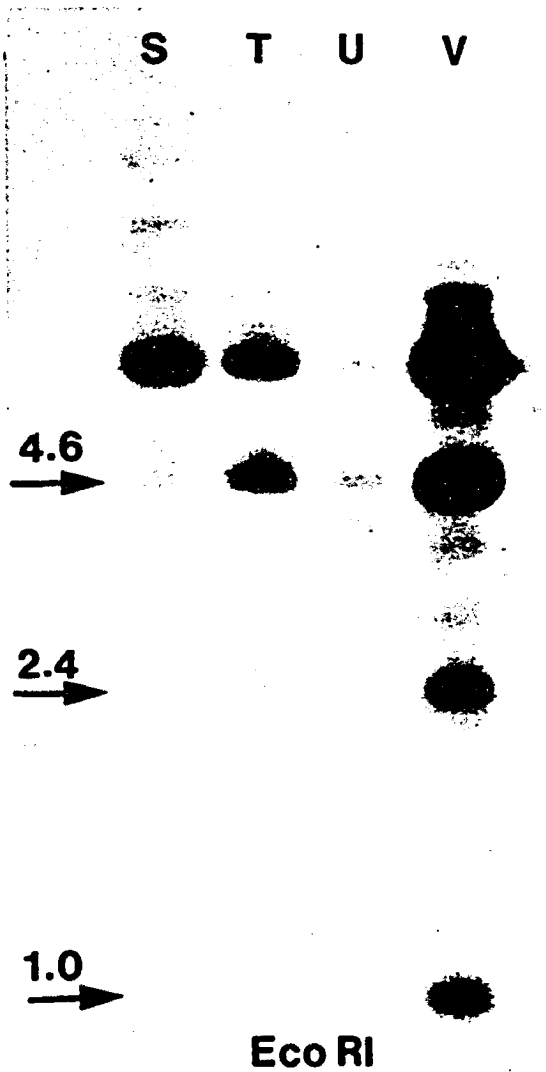
experiments was conducted with cells from a subclone of 3g, 3g3a. In both sets of experiments all HAT^S back selectants retained either all, some, or none of the original bands (Fig. 28), and there was no evidence of amplification.

Discussion

I have transferred the HSV-TK gene into murine cells using DMGT without added carrier DNA, and have analyzed seven independent transformant cell lines. A number of changes in the recipient cell content and organization of donor DNA sequences can be correlated with length of culture time post-transfer, loss of TK expression ($HAT^S/BUdR^r$), and re-expression of TK (HAT^r).

The availability of a nucleic acid probe for all of the donor DNA sequences (pTKx-1, no carrier DNA) allowed for the more accurate determination of the total amount and organization of exogenous DNA present in stable recipient cells. Based on several restriction endonuclease digestions, the donor DNA content of 7 carrier-free transformants was calculated to range from 2 up to 50-65 kb per cell. The restriction endonuclease Xba I does not recognize any sites in pTKx-1 DNA, and each piece of donor DNA integrated into high molecular weight host cell DNA should acquire flanking host Xba I sites and should appear as a single band after Xba I digestion. The presence of multiple Hind III bands in 3g and 101 DNAs, combined with the presence of a single Xba I band, suggests that multiple donor fragments have

Figure 28. Analysis of back selectants derived from thawed 3g cells. A frozen ampule of 3g cells was thawed and the 3g cell population was back selected by BUdR/UV treatment. Three independent back selectant cell lines were isolated. DNAs from 3g (lane V) and the 3 back selectants (lanes S-U) were digested with Eco RI and hybridized using ^{32}P-labeled pTKx-1 DNA as probe. The arrows represent the positions of bands derived from Eco RI digestion of pTKx-1 DNA.



been assembled into units (transgenomes) which retained a number of pTKx-1 Hind III sites in the absence of internal Xba I sites.

One major difference between transformants derived from carrier-free and carrier-containing DMGT is that whereas carrier-free transformants were almost always stable, carrier-containing transformants often are unstable at first analysis and rapidly lose HAT^r (10%/cell/day) (18). This observation has been seen in the transfer of Chinese hamster (see Chapter I) and HSV-TK (18) into mouse L cells. Stable lines were derived from unstable transformants at a frequency which indicated that stabilization itself may be an infrequent event (18). Continued growth in HAT would enrich for the stable cells in a mixed population of stable and unstable cells.

Earlier work has demonstrated that even in the unstable state, donor sequences were present in the Hirt ppt fraction, i.e. high molecular weight DNA (18). To date, no evidence has been presented which proves an autonomous existence (not chromosomally associated) for transgenomes in unstable populations. However, if unstable transgenome molecules are autonomous, perhaps certain sequences derived from the carrier DNA allow for the maintanence and replication of the donor material. Donor material lacking sequences which can function as origins of replication, chromatin assembly sites, etc. may be reorganized into transgenomes but may not be capable of being maintained in the absence of chromosomal integration. The limited variety of sequences present in pTKx-1 DNA may have made it difficult to maintain, in the unstable state, transgenomes composed of this material. If chromosomal integration were a rare event in itself, this

could account for the low frequency of recovery of transformant cell lines, as well as for the rapid stabilization in the carrier-free transformation system.

Hirt fractionation of 101 and 3g cellular DNA lead to the recovery of Hirt supt-specific sequences early in the culture history of these two lines. The Hind III pattern of Hirt supt sequences in 101 was the more complex, but in both lines I saw evidence based on restriction analysis suggesting the presence of circular material. This material was absent from later cultures of these two lines, as well as from all linear transformants. Although a limited number of lines have been analyzed, this observation is consistent with the presence of donor circles (the Hirt supt lane represents 25-50% of all Hirt supt DNA isolated from 5×10^7 cells). In conjunction with other reports (23,49,x), now there exists somewhat stronger evidence that donor circular material may survive transiently during the transfer process. The questions of possible replication or generation from integrated material are difficult to address but are crucial to answer before one can understand this phenomenon.

The results presented from lines 3g and 101 and all of their subclones, back selectants and re-expressors make one point clear: there is heterogeneity present and continuously being generated in these transformed cell populatons. This observation is supported by recent work involving SV40 T antigen expression (29). Selective systems act to isolate a subset of the original population with an altered phenotype. Although I have not tested directly for the possibility of selection-induced genotypic or phenotypic alteration, I

know of no report suggesting mutagenic activity for the components of HAT. The BUdR + UV selection involved 2 rounds of UV exposure to cells grown in the presence of 30 ug/ml of BUdR. The first UV exposure could have generated genotypic and subsequently phenotypic changes which allowed a cell to survive the second exposure. Also, extended growth in BUdR (2 weeks or more) has been shown to lead to gradual phenotypic evolution followed by genotypic evolution (55).

DNA isolated from 2 101 back selectant lines (UV1 and UV3) showed no band pattern changes. Phenotypic changes without DNA sequence rearrangement have been reported for HSV-TK expression (20) and complementation of defective viral infection with integrated viral fragments (56). However, in several re-expressor lines derived from these 2 lnes, I detected band pattern changes (Fig. 16). The simplest explanation is that random band pattern changes occurred unrelated to changes in phenotype. Another possibility is that TK gene inactivation occurred by some mechanism of localized DNA or chromatin modification without direct sequence rearrangement. Re-activation in this region required a second event involving DNA sequence rearrangement which produced detectable band alterations. In a second set of 17 re-expressors, I detected only one sequence rearrangement involving pTKx-1 DNA. Although this last result is not incompatible with the latter explanation, i.e. reactivation could have occurred with sequence rearrangement outside of the pTKx-1 sequences, it offers no supporting evidence. Initial analysis of the DNA methylation state of the unrearranged DNAs using Hpa II/Msp I did not produce any obvious explanation for the modulation of phenotype.

One of the most intriguing findings was the sequence amplification present in 3g back selectants (Fig. 17). Amplification of nearly all of the original 3g Hind III bands suggests that the 3g transgenome was amplified as a unit. A correlation between amplification and gene inactivation has been reported elsewhere. Rat hepatocyte rDNA genes were reported to be amplified but inactive secondary to DNA methylation (57). The donor sequences in line 3g, a 3g back selectant, and a re-expessor line contained several methylated Hpa II sites, but I have no evidence that DNA methylation played an important role in modulating gene expression. Amplification of donor material has been demonstrated in the transfer of a mutant dihydrofolate reductase gene into mouse cells, but only in response to an elevation in methotrexate levels (10). A second report of amplification in gene transfer lines involved the cotransfer of a SV40-containing plasmid with the HSV-TK gene using carrier-containing DMGT (29). No mechanism was demonstrated for the amplification of both SV40 and HSV-TK sequences, but one suggestion was that this event occurred in response to a selection for elevated TK gene expression. In our lines the amplification was shown to persist for at least 30 days in the presence or absence of BUdR. One clue to the mechanism of generation of the amplification was the transient presence of sequences recovered in the Hirt supt of 3gb1 cultures. Generation of non-integrated sequences followed by reintegration could account for the amplification.

The subcloning of back selectant 3gb1 and the demonstration that independent 3gb1 subclones, although identical in Hind III restriction patterns for donor DNA sequences, could generate re-expressor clones at

widely different frequencies suggests that there is a mechanism for switching on TK expression in 3gbl cells, and that this mechanism may operate at different frequencies in back selectant subpopulations which appear to be identical by DNA analysis. The observation that a total of 15/15 3g re-expressor populations lost the amplification, but retained the same basic Hind III donor DNA restriction pattern, argues that the amplified sequences influence the expression of a TK gene(s) present in all re-expressors. Whether or not the amplified pTKx-1 sequences are located on the same mouse chromosome as the sequences retained in the re-expressor populations may be answerable through use of the in situ hybridization technique. In situ amplification has been reported with integrated polyoma sequences (58).

The circular pTKx-1 carrier-free transformation experiments have been complemented by a series of linear pTKx-1 carrier-free transformation experiments done by James Barbosa in the laboratory of Dr. Frank Ruddle. Since the 2 types of transformants, circular and linear, were produced in independent experiments, it is difficult to deduce any generalities contrasting circular and linear transformants. Six of the seven linear transformants were stable and one was mixed. Donor plasmid DNA presented in either form was remodelled and propagated in recipient cell populations in a fairly wide range of amounts (2-100 kb). Linear transformants contained multiple Xba I bands (4/7 lines) significantly more ofter than circular transformants did (1/7 lines). However, the same range of amount of pTKx-1 DNA appeared in both types of transformants. One interpretation is that the linear pTKx-1 molecules were assembled into smaller transgenomes

but that the number which integrated was enhanced. This interpretation, if supported by further observations, may reflect a significant differnce 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. Another difference between the two sets of transformants was that only in circular transformants did we detect Hirt supt sequences, a subset of which suggested the presence of circular material.

Appendix

The overall theme of this work was the analysis of the DMGT process by a combination of cell culture and nucleic acid techniques. In one sense, this work has generated more questions than answers. As I and other investigators have analyzed DMGT experiments many unexpected observations have appeared. Among the unforeseen phenomena which require new explanations are concommitant segregation of originally unlinked markers, gene amplification, phenotypic instability, and evidence for free circular molecules. Rather than attempting to summarize anew all of the evidence and constructing a model for DMGT, I am including in the Appendix a review paper I have written in which a model for DMGT is presented and discussed.

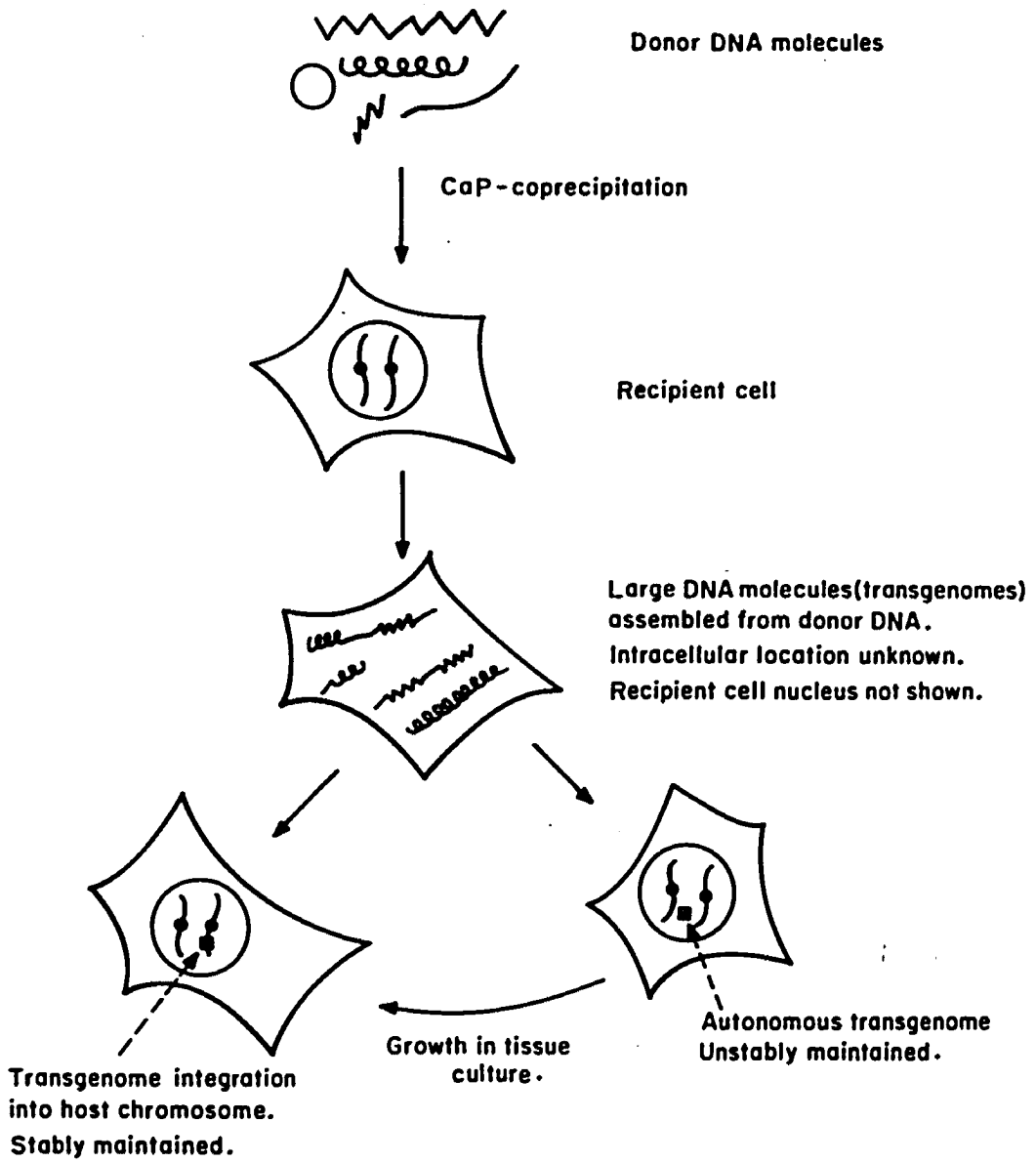
DNA-mediated gene transfer (DMGT) is a procedure for introducing donor genetic material into recipient cells. This technique involves the coprecipitation of purified donor DNA with calcium phosphate, followed by presentation of the precipitate to recipient cells in culture (3). Several genes have been transferred by this technique (4,6,7,9,10), and their expression demonstrated by the ability to complement genetic deficiencies in recipient cells.

The mechanism by which a recipient cell takes up donor DNA sequences and maintains them is under active investigation. Figure 29 presents a model for DMGT. In this paper we will consider the evidence supporting this model, and will attempt to use this model to explain a number of experimental results. I. Evidence for the alteration and assembly of donor DNA sequences into large molecules (transgenomes).

Evidence for the alteration of donor material has come from detailed analysis of integrated donor DNA sequences (17,18). As a rule, donor molecules integrated into the DNA of a recipient cell have lost a subset of their original sequences, with the amount retained varying between independent integrated molecules. This loss may have occurred through exonuclease digestion, endonuclease cleavage, random breaks, or recombinational events. The intracellular location where these changes occurred is not known. The observation that donor DNA introduced as circular molecules may undergo the same types of alterations as donor DNA introduced as linear molecules (Huttner KM, Barbosa JA, Scangos GA, et al: Submitted for publication) indicates that exonuclease activity is not in itself sufficient to account for the loss of donor material.

Both indirect and direct evidence support the notion that donor

Figure 29. A model for DNA-mediated gene transfer.



DNA molecules are assembled into larger molecules (transgenomes). Many investigators have experimented with DMGT using a mixture of different types of DNAs as donor material, with some of the donor DNA sequences detectable by nucleic acid hybridization. These probeable sequences entered the recipient cell unlinked to each other as well as to the other donor DNA used as carrier. Recipient cells were selected on the basis of the expression of a donor gene overcoming their own genetic deficiency and allowing them to survive in a selective medium. The system employed most often was the HAT selective system (15) in which cellular expression of TK is a requisite for survival. Scangos et al. (18) used a cloned Herpes simplex virus TK gene to transform TK^- mouse L cells (Ltk^-) to the TK^+/HAT^r state. Analysis of 3 transformants demonstrated that multiple copies of the TK gene sequences (up to 9) were present in each cell. Subcloning of the 3 original transformant cell lines produced cell lines which retained subsets of the multiple TK gene copies. Independent but complementary subsets were shown to exist in at least 2 transformants. For example, one line, LHI 3, contained 5 copies of the TK gene sequence. Seven independent subclones of LHI 3 contained either one group of 3 TK gene copies or the other group of 2 copies. No subclone contained one or more copies from both groups. Back selection on the TK^+ LHI 3 cells using BUdR led to loss of the TK^+ phenotype and loss of all TK gene copies.

One interpretation of these results is that in the initial stages of DMGT, donor TK gene sequences became linked to each other genetically. The segregation of separate groups of TK gene sequences can be interpreted as the retention of one transgenome by recipient cells, and the loss of others. Only those cells which retained at

least one transgenome, with an active TK gene inserted, would survive under the selective conditions. The multiple segregation units could have arisen independently or from one initial transgenome which broke into smaller transgenomes, each containing its own set of donor sequences. The independent segregation of donor sequences also has been reported in the case of transfer of unlinked adenovirus sequences into TK^- human cells (56).

The term cotransfer experiment refers to the addition of nonselected but usually probeable sequences to the carrier DNA used to facilitate transfer of a selected marker (21). Recipient cell lines are selected for the expression of the selected marker, and then screened for the presence and expression of the nonselected sequences. Cotransfer experiments using unlinked, nonselected DNA can be an efficent method of introducing from 0-100 copies of the nonselected sequences into recipient cells (20,21,23). Several laboratories have begun to study the structural and functional relationship between the selected gene and the multiple copies of nonselected genes present in recipient cell lines. Genetic linkage of the originally unlinked sequences is supported by 2 types of experiments. First, back selection against cells expressing the selected marker, e.g. selection for TK^- cells from a TK^+ transformed line, lead to the loss of all or most of the cotransferred sequences in cases where the selected gene was lost (24; Huttner KM, Scangos GA, Ruddle FH: Unpublished observations). Second, cotransfer into mouse cells of pBR322 sequences along with a Chinese hamster mutant dihydrofolate reductase (DHFR) gene, followed by amplification of the DHFR gene sequences, led to amplification of some of the pBR322 sequences (10,24).

Physical linkage of originally unlinked sequences is supported by at least 3 pieces of evidence. First, transformation of Ltk$^-$ cells with DNA from a Herpes TK gene-containing plasmid, in the absence of any carrier DNA, produced cell lines with multiple copies of the plasmid DNA (Huttner KM, Barbosa JA, Scangos GA, et al: Submitted for publication). DNA from 2 such cell lines was isolated and digested with Xba I, a restriction endonuclease which recognizes no sites in the TK plasmid DNA. Xba I would cleave at sites in the mouse DNA surrounding any integrated TK plasmid sequence, and would produce a separate restriction fragment for each donor sequence integrated at a separate and distant site. In these 2 cell lines the multiple gene copies were present in only one Xba I band, suggesting that the sequences were physically adjoined. Second, the technique of in situ hybridization has been used to demonstrate that multiple unlinked donor sequences can become integrated at one chromosomal location (20; Huttner KM, Ruddle FH: Unpublished observations).

The third result supporting physical linkage involves the most direct and compelling evidence. Ltk$^-$ mouse cells were transformed with a mixture of carrier DNA, DNA from derivatives of the plasmid pBR322, and Herpes TK gene DNA (24). Analysis of pBR322 sequences present in the DNA of transformed cell lines demonstrated that these donor sequences were linked covalently to carrier DNA.

All of the above results support the model of transgenomes formed by the recipient cell using as starting material unlinked fragments of donor DNA. Independent transformant lines may have multiple non-identical transgenomes as well as multiple copies of a single transgenome per cell (18). No transgenome has been analyzed from end

to end, and therefore it is impossible to state definitively that recipient cell sequences are present only at the end of the integrated transgenome and not internally. However, the arguments for donor DNA linkage into transgenomes are strong enough for us to assume in our model that transgenomes are composed internally only of donor DNA sequences.

II Transgenome size

One approach to calculating transgenome size, which appears to be the simplest and the most straight forward, is to mix nonselected, probeable DNA, e.g. plasmid DNA, with the carrier DNA used in DMGT. Theoretically, one can calculate the size of the transgenome by analyzing the probeable DNA sequences present in the recipient cell, assuming that all donor DNA sequences have an equal probability of representation in the transgenome. If the probeable sequences represented 5% of the total donor DNA presented to the recipient cells, and if a given recipient cell constructed a transgenome containing 10 kb of the probeable DNA, then one might predict that the total transgenome size (x) could be calculated from the formula:

Formula 1 Percentage of the total donor DNA which consists of probeable sequences equals the percentage of the transgenome DNA which consists of probeable sequences.

In this example, $5\% = (10\text{ kb})/x$, $x = 200$ kb. Since investigators have reported a wide range in the number of plasmid copies per recipient cell in cell lines resulting from a single DMGT experiment (0-30 (23), 1-20 (22), 3-100 (20)), the assumption that all donor sequences are

present on a single transgenome leads to the conclusion that the size of the transgenome must be variable over a range of at least one log. According to this formulation, the frequency of cotransfer of any nonselected sequence should depend only on its proportional representation among all donor sequences.

Although nonselected plasmid DNA molecules have been cotransferred when introduced in either linear (21) or circular (23) form, no report has compared the frequency of cotransfer for the linear and circular forms of a single molecule. Almost all cotransfer experiments have involved probeable DNA sequences which have no homology to the remainder of the carrier DNA. Therefore, if recombination involving homologous regions plays a role in transgenome formation, one might expect that the probeable sequences would be underrepresented in the transgenome on a weight per weight basis. Another important point is that the probeable molecules usually are 5-20 kb in size while the remainder of the carrier DNA may contain pieces of 100 kb or greater. If construction of the transgenome is piece by piece, and if either smaller or larger pieces have an increased probability of utilization in the transgenome construction, there may be a bias towards an over- or underrepresentation of the probeable sequences.

Although results from our lab agree with the data reported by others (22,25) concerning the range in cotransferred sequence copy number in independent transformants, a closer examination of the data indicates that there is a bias towards fewer copies per cell. In the cotransfer of the rabbit β globin gene into mouse cells, which did involve a ligation to the selected gene sequence prior to transfer, the globin gene sequences were present in transformant cell DNA in a range

of 0-20 copies/cell, but 16/21 transformants contained only 3 or fewer copies/cell (25). In our results with cotransfer of the non-linked human βglobin gene, the range was 0-30 copies/cell, but 9/16 transformants contained only three or fewer copies/cell. These results can be interpreted as supporting the idea that there is some modal size for the transgenomes but occasionally the transgenome may be larger. A second interpretation is that transgenomes with a larger number of certain types of cotransferred sequences may be relatively unstable as compared to those containing a smaller number. A third possibility is that all integrated transgenomes are small initially, but that additional integration events at the same integration site can act to enlarge the integrated transgenome. Based on this possibility, those few cell lines in which integration occurred early might contain larger transgenomes. This remains to be tested.

In the earliest work on non-linked cotransfer (21) there was some discussion of the observation that in the transformed cell lines the ratio of the number of copies of the cotransferred material, e.g. ϕx174 DNA, to the number of copies of the selected gene, e.g. TK, never approached the ratio of these two sequences in the input donor DNA. This point is consistent with the notion that the proportional representation of the ϕx174 sequences in the total donor DNA determined the proportional representation of the ϕx174 sequences in the transgenome. There has been no systematic study reported which involved cotransfer of a sequence at several concentrations, e.g. 5%, 0.5%, and 0.05% of the carrier DNA, and subsequent analysis of the cotransfer frequency as well as copy number/cell in a large number of independent transformants.

It is our view that with the present state of knowledge, calculation of transgenome size by Formula 1 produces a rough guesstimate of unknown accuracy and standard error. A reliable calculation of transgenome size awaits the results of additional basic experiments designed to elucidate the mechanisms involved in transgenome assembly.

Our laboratory has analyzed transgenome size in a special case of DMGT, i.e. transfer of the HSV-TK gene into mouse cells in the absence of whole cell carrier DNA (Huttner KM, Barbosa JA, Scangos GA, et al: Submitted for publication). Carrier-free transformants were produced using circular or linear plasmid molecules containing the TK gene (pTKx-1). 1-10 ug of pTKx-1 DNA was coprecipitated with calcium phosphate and presented to 10^6 recipient cells under standard transfer conditions. The pTKx-1 DNA content per cell ranged from 2 to 100 kb in independent TK^+ transformants. Although we examined a limited number of transfer lines, 7 with circular pTKx-1 DNA and 7 with linear pTKx-1 DNA, the 2 types of transformants (linear and circular) had similar ranges in pTKx-1 sequence content. The presence of small amounts of donor DNA in transformants resulting from carrier-free DMGT has been observed elsewhere (59).

One major difference between the linear and circular transformants was that in 6/7 circular transformants all donor material appeared to be present in a single transgenome, while in 4/7 linear transformants there was evidence from restriction endonuclease digestion and filter hybridization that multiple non-identical integrated transgenomes were present. Although we could not prove that the several transgenomes in these 4 linear transformants were totally independent in derivation, the evidence is suggestive that linear and circular molecules were

processed by the recipient cells in different fashions.

One final point concerning the estimation of transgenome size involves the use of cotransfer studies in which one assays the phenotype rather than the genotype of the cotransferred sequence. Two examples of this type of cotransfer experiment involved cotransfer, with the genomic Chinese hamster TK gene, of the Chinese hamster Galactokinase (GalK) (11) and Esterase D (Est D) (12) genes. Based on the known chromosomal linkage to the TK gene (38), one cannot rule out the possibility that the GalK gene was cotransferred with the TK gene on a large intact segment of hamster chromosomal DNA. However, gene transfer experiments using 2 sequences that were known to be immediately adjacent and covalently linked in the donor material resulted in a cotransfer rate on the order of 10% (52). A reported frequency of 1/87 suggests to us either that the GalK and TK genes are within 20-30 kb of each other on the hamster chromosome or that cotransfer occurred as a result of the genes being present on two independent segments of donor DNA which became part of a single transgenome. The cotransfer of the hamster Est D gene, which is asyntenic with the hamster TK gene (Scangos GA, Ruddle FH: Unpublished observations), supports the latter hypothesis. The cotransfer in 1 out of 15 transformants of a gene (Est D) which represented only $1/10^5$ to $1/10^6$ of the donor material suggests either that; (1) some transgenomes can be very large, on the order of thousands of kilobases, (2) the Est D gene is associated with sequences which promote its transfer, e.g. transposable elements, or (3) this cotransfer was a very fortuitous finding. Peterson and McBride (11) have screened 87 lines for cotransfer of 1 marker, Warrick et al (12) have screened 15 lines for

cotransfer of 25 markers, and we have screened 25 lines for cotransfer of 15 markers (23). Out of a total of 837 assays reported, only 2 cotransfer events were detected. On the surface, this cotransfer frequency of 1/400 for genes estimated to be 1-10 kb in size, and present at $1/10^5$ to $1/10^6$ part of the donor DNA, would predict, using Formula 1, that the transgenome has an average size of $(1/10^5$ to $1/0^6) =$ (1-10 kb/ x), x = (10^5 to 10^7 kb) times a 1/400 cotransfer frequency gives a figure of 2.5 to 250 x 10^3 kb.

The primary assumption made in this calculation, and in all calculations involving cotransfer of a phenotype is that the mere presence of a gene sequence guarantees that the gene will be expressed and produce a functional protein. As has been shown with the globin (22,25), ovalbumin (26,27), and SV40 early region genes (29), recipient cells containing intact donor genes either may not transcribe them, transcribe them at a low level and incorrectly, or transcribe them and produce a protein somewhat different from the normal gene product. This is not to say that donor genes introduced by DMGT cannot be transcribed and translated properly. Clearly this is true for the selected markers that have been transferred by DMGT, e.g. TK, adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase, and dihydrofolate reductase. The reservation in calculating transgenome size from experiments involving evaluation of a cotransferred phenotype is that such calculations are minimum estimates. In addition, when calculating overall cotransfer rates for genomic genes, it may be ill-advised to use negative cotransfer data for markers which have never been transferred. There may be characteristics of certain genes or their gene products, e.g. gene size, which will make it virtually

impossible to transfer the phenotype of these genes using the current techniques for DMGT.

III Intracellular localization of the transgenome

Cell lines which have been selected for the transfer of donor genes by DMGT can be classified according to the maintanence of the selected donor phenotype when the cells are removed from selective conditions. There are reports of transformed cell lines which lose the selected donor phenotype in the absence of selection (unstable), and reports of transformed cell lines which do not lose the selected donor phenotype (stables) (3,7,9,25). Recent work from our laboratory evaluated several unstable lines and indicated that stable lines could be derived from unstable lines after prolonged propagation either in selective or nonselective medium (18). It is our feeling that all stable lines resulting from DMGT have passed through a phase where the donor material was unstable. The period of instability may range from hours to months. When one isolates transformed colonies from a flask of DNA-treated cells, one should consider that stabilization early in the life of a colony would give that colony a growth advantage over unstable colonies. Therefore, always selecting the largest colony from a transfer flask might lead one to miss the unstable lines.

Analysis of the donor material in stable transfer lines, using microcell mediated gene transfer (19) or <u>in situ</u> hybridization (20), has shown in several cases that the transgenome was associated with a recipient cell chromosome. The chromosome was different for different transformed cell lines indicating a lack of site specificity, at the chromosomal level, for general transgenome integration. It would be

informative to derive several independent stable lines from a single unstable transfer line and test for integration of a single transgenome into several different chromosomal sites.

Fluctuation analysis on the expression of an HPRT gene transferred into mouse cells demonstrated that even in transformed cell populations considered to be stable, the rate of loss of the transferred phenotype was several logs higher than the spontaneous rate of loss for phenotypes encoded in endogenous cellular genes (9). This observation is supported by work from other laboratories (20; Huttner KM, Scangos GA, Ruddle FH: Unpublished observations). One explanation for this result may be a small degree of instability occurring at a site of donor DNA integration into a recipient cell chromosome, especially if that integration event altered the complex folding of DNA required for normal chromosomal structure.

With regard to the unstable lines, no definitive evidence exists concerning the intracellular location of the unstable transgenome. Whether the unstable transgenome resides in the nucleus or cytoplasm, and whether the unstable transgenome is associated with a chromosome or is autonomous, are questions requiring clarification. We have attempted to use microcell mediated gene transfer to transfer the TK gene from unstable transformed mouse L cells to TK^- Chinese hamster cells but without success. The technique of <u>in situ</u> hybridization could be used to address the question of localization, or one could attempt to fractionate the DNA of metaphase cells using a double minute isolation technique (26), and use filter hybridization to test the chromosomal and nonchromosomal fractions for the presence of the transgenome sequences.

If the unstable transgenome is non-chromosomally associated, and if stabilization involves integration, the recombinational event between transgenome and chromosome may result in a diminishing of the transgenome size. For this reason, transgenome size estimates based on data from cotransfer experiments should include data on the stability of the transformed cell lines.

IV Role of the transgenome

It is our experience that chromosomal integration and continued expression of donor DNA is a rare event and one which can be associated with instability in the affected chromosome (18,19; Huttner KM, Scangos GA, Ruddle FH: Unpublished observations). Integration into a heterochromatic region of chromosomal DNA could lead to extinction of donor gene expression, while integration into the DNA of an essential recipient cell gene could be a lethal event for the cell. Formation of a transgenome may offer the recipient cell an opportunity to survive under conditions selective for the presence and expression of a donor gene, without requiring chromosomal integration of the donor material. The transformed recipient cell line can survive in the unstable state for several months (Huttner KM, Scangos GA, Ruddle FH: Unpublished observations).

If the unstable transgenomes are autonomous, then their sequence organization and content must allow for replication, resistance to recipient cell nucleases, and expression of the selected gene. Our experience with DMGT without carrier DNA supports this model. The pTKx-1 plasmid DNA contains no known eukaroytic origin of replication. Transformation with plasmid DNA alone gave rise to lines which were

stable at first analysis in almost all cases. The frequency of transformation was quite low, i.e. several logs below similar experiments using carrier DNA. These results are consistent with the organization of pTKx-1 sequences into transgenome molecules incapable of efficient autonomous replication. Colonies would survive under selective conditions only if chromosomal integration and stabilization occurred rapidly, and hence the low transformation rate yet high frequency of stable lines. If transgenome assembly occurs over a period of several days, and is continuing at the time that selection is imposed, the rapid stabilization of donor material required in carrier free transformants might result in the retention of transgenomes smaller than those seen in carrier-containing transfer experiments.

The ability of different cell lines to construct transgenomes may be reflected in a correlation between their rate of transformation and their rate of cotransfer. The strongest evidence in favor of this comes from work of DMGT into mouse teratocarcinoma cells (TCC) (60). TK^- TCC were transformed to the TK^+ phenotype at a rate 4-5 logs lower than TK^- mouse L cells. In a separate set of experiments, cotransfer of a human β globin gene, which was present as 14% of the total donor DNA, occurred in only 2 out of 10 transformed TCC lines. This compares to 80-90% cotransfer rates in mouse L cells when the probeable DNA represented only 5% of total donor DNA (21,23). Since there are many other mechanisms which could underlie this correlation between low transformation rate and low cotransfer frequency, it remains to be determined whether differences in transfer rates between cell lines and between species can be accounted for on the basis of differential transgenome construction.

V Conclusion

Definitive evidence for our model of DMGT will hinge on conclusive demonstration of two phenomena; transgenome assembly and the initial autonomous nature of unstable transgenomes. Work by our laboratory and several others has provided indirect evidence for transgenome assembly, while work by (24) has provided direct evidence. Additional work on the analysis of transgenome structure by isolation of portions of the transgenome should confirm this part of our model. The possible autonomous nature of the transgenome in unstable transformants is being analyzed in our laboratory by the procedures mentioned above. Definitive evidence regarding the mechanisms underlying DMGT will enable us to design better experiments testing gene regulation, site specificity of donor DNA integration, and possibilities for genetic therapy.

Literature Cited

(1) Szybalska E.H. and W. Szybalski. 1962. Genetics of human cell lines, IV. DNA-mediated heritable transformation of a biochemical trait. Proc. Natl. Acad. Sci. USA 48:2026-2034.

(2) 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.

(3) Bacchetti, S. and F.L. Graham. 1977. Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified Herpes simplex viral DNA. Proc. Natl. Acad. Sci. USA 74:1590-1594.

(4) Wigler, M., S. Silverstein, L.S. Lee, A. Pellicer, Y.C. Cheng and R. Axel. 1977. Transfer of purified Herpes virus thymidine kinase gene to cultured mouse cells. Cell 11:223-232.

(5) Maitland, N.J. and J.K. McDougall. 1977. Biochemical transformation of mouse cells by fragments of Herpes simplex virus DNA. Cell 11:233-241.

(6) Wigler, M., A. Pellicer, S. Silverstein and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell 14:725-731.

(7) Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub and L. Chasin. 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA 76:1373-1376.

(8) Willecke, K., M. Klomfab, R. Mierau and J. Dohmer. 1979. Intraspecies transfer via total cellular DNA of the gene for

hypoxanthine phosphoribosyltransferase into cultured mouse cells. Molec. Gen. Genet. 170:179-185.

(9) Graf Jr., L.H., G. Urlaub and L.A. Chasin. 1979. Transformation of the gene for hypoxanthine phosphoribosyltransferase. Somat. Cell Genet. 5:1031-1044.

(10) Wigler, M., M. Perucho, D. Kurtz, S. Dana, A. Pellicer, R. Axel and S. Silverstein. 1980. Transformation of mammalian cells with an amplifiable dominant-acting gene. Proc. Natl. Acad. Sci. USA 77:3567-3570.

(11) Peterson J.L. and O.W. McBride. 1980. Cotransfer of linked eukaryotic genes and efficient transfer of hypoxanthine phosphoribosyltransferase by DNA-mediated gene transfer. Proc. Natl. Acad. Sci. USA 77:1583-1587.

(12) Warrick, H., N. Hsiung, T.B. Shows and R. Kucherlapati. 1980. DNA-mediated cotransfer of unlinked mammalian cell markers into mouse L cells. J. Cell Biol. 86:341-346.

(13) Graham, F.L., S. Bacchetti, R. McKinnon, C. Stanners, B. Cordell and H.M. Goodman. 1980. Transformation of mammalian cells with DNA using the calcium technique. In Introduction of macromolecules into viable mammalian cells. Ed. by R. Baserga, C. Croce, and G. Rovera. Liss, New York. Pages 3-26.

(14) Lewis, W.H., P.R. Srinivasan, N. Stokoe and L. Siminovitch. 1980. Parameters governing the transfer of the genes for thymidine kinase and dihydrofolate reductase into mouse cells using metaphase chromosomes or DNA. Somat. Cell Genet. 6:333-348.

(15) Littlefield, J.W. 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145:709-710.

(16) Juricek, D.K., D.R. Keene, A. Loyter, A. Lalazar and F.H. Ruddle. 1980. Early events of DNA-mediated gene transfer: electron microscopy. J. Cell Biol. 87:SG2213.

(17) Pellicer, A., M. Wigler, R. Axel and S. Silverstein. 1978. The transfer and stable integration of the HSV thymidine kinase gene into mouse cells. Cell 14:133-141.

(18) Scangos, G.A., K.M. Huttner, D.K. Juricek and F.H. Ruddle. 1981. Molecular mechanisms of gene transfer in mammalian cells: the transgenome in unstable transformants. J. Cell. & Molec. Biol. In press.

(19) Smiley, J.R., D.A. Steege, D.K. Juricek, W.P. Summers and F.H. Ruddle. 1978. A Herpes simplex virus 1 integration site in the mouse genome defined by somatic cell genetic analysis. Cell 15:455-468.

(20) Pellicer, A., D. Robins, B. Wold, R. Sweet, J. Jackson, I. Lowy, J.M. Roberts, G.K. Sim, S. Silverstein and R. Axel. 1980. Altering genotype and phenotype by DNA-mediated gene transfer. Science 209:1414-1422.

(21) Wigler, M., R. Sweet, G.K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein and R. Axel. 1979. Transformation of mammalian cells with genes from procaryotes and eukaryotes. Cell 16:777-785.

(22) Wold, B., M. Wigler, E. Lacy, T. Maniatis, S. Silverstein and R. Axel. 1979. Introduction and exxpression of a rabbit β-globin gene in mouse fibroblasts. Proc. Natl. Acad. Sci. USA 76:5684-5688.

(23) 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. USA 76:5820-5824.

(24) Perucho, M., D. Hanahan and M. Wigler. 1980. Genetic and physical linkage of exogenous sequences in transformed cells. Cell 22:309-317.

(25) Mantei, N., W. Boll and C. Weissman. 1070. Rabbit β-globin mRNA production in mouse L cells transformed with cloned rabbit β-globin chromosomal DNA. Nature 281:40-46.

(26) Breathnach, R., N. Mantei and P. Chambon. 1980. Correst splicing of a chicken ovalbumin gene transcript in mouse L cells. Proc. Natl. Acad. Sci. USA 77:740-744.

(27) Lai, E.C., S.L.C. Woo, M.E. Bordelon-Riser, T.H. Fraser and B.W. O'Malley. 1980. Ovalbumin is synthesized in mouse cells transformed with the natural chicken ovalbumin gene. Proc. Natl. Acad. Sci. USA 77:244-248.

(28) Dubois, M.F., C. Pourcel, S. Rousset, C. Chany and P. Tiollais. 1980. Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. Proc. Natl. Acad. Sci. USA 77:4549-4553.

(29) Hanahan, D., D. Lane, L. Lipsich, M. Wigler and M. Botchan. 1980. Characteristics of an SV40-plasmid recombinant and its movement into and out of the genome of a murine cell. Cell 21:127-139.

(30) Barile, M.F., G.P. Bodey, J. Snyder, D.B. Riggs and M.W. Grabowski. 1966 J. Natl. Cancer Inst. 36:155-168.

(31) Enquist, L.W., G.F. Vande Woude, M. Wagner, 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 7:335-342.

(32) Lawn, R.M., E.F. Fritsch, R.C. Parker, G. Blake and T. Maniatis. 1978. The isolation and characterization of linked δ- and β-globin genes from a cloned library of human DNA. Cell 15:1157-1174.

(33) 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 Res. 5:563-581.

(34) Wagner, M.M. and W.C. Summers. 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. J. Virol. 27:374-387.

(35) Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.

(36) Wrights stain is a commonly used hematologic stain. It is composed of a mixture of methylene blue and eosin in methanol.

(37) Nichols, E.A. and F.H. Ruddle. 1973. A review of enzyme polymorphism, linkage, and electrophoretic conditions for mouse and somatic cell hybrids in starch gels. J. Histochem. Cytochem. 21:1066-1081.

(38) McBride, O.W., J.W. Burch and F.H.Ruddle. 1978. Cotransfer of thymidine kinase and galactokinase genes by chromosome-mediated gene transfer. Proc. Natl. Acad. Sci. USA 75:914-918.

(39) Migeon, B.R., S.W. Smith and C.L. Leddy. 1969. The nature of thymidine kinase in the human-mouse hybrid cell. Biochem. Genetics 3:583-590.

(40) Lowry, O.H., N.J. Rosegrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

(41) Shinnick, T.M., E. Lund, O. Smithies and F.R. Blattner. 1975. Hybridization of labeled RNA to DNA in agarose gels. Nucleic Acid Res. 2:1911-1929.

(42) McDonell, M.W., M.N. Simon and F.W. Studier. 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. 110:119-146.

(43) Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

(44) Wahl, G.M., M. Stern and G.R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA 76:3683-3687.

(45) Maniatis, T., A. Jeffery and D.G. Kleid. 1975. Nucleotide sequence of the rightward operator of phage . Proc. Natl. Acad. Sci. USA 72:1184-1188.

(46) Fournier, R.E.K. and F.H. Ruddle. 1977. Microcell mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc. Natl. Acad. Sci. USA 74:319-323.

(47) Klobutcher, L.A., C.L. Miller and F.H. Ruddle. 1980. Chromosome-mediated gene transfer results in two classes of unstable transformants. Proc. Natl. Acad. Sci. USA 77:3610-3614.

(48) Scangos, G.A., K.M. Huttner, S. Silverstein and F.H. Ruddle. 1979. Molecular analysis of chromosome-mediated gene transfer. Proc. Natl. Acad. Sci. USA 76:3987-3990.

(49) Stubblefield, E., S. Linde, F.K. Franolich and L.Y. Lee. 1978. Analytical technique for isolated metaphase chromosome fractions. Methods Cell Biol. 17:101-113.

(50) Anderson, W.F., L. Killos, L. Sanders-Haigh, P.J. Kretschmer and E.G. Diacumakos. 1980. Replication and expression of thymidine kinase and human globin genes microinjected into mouse fibroblasts. Proc. Natl. Acad. Sci. USA 77:5399-5403.

(51) Wigler, M., M. Perucho, D. Levy and D. Hanahan. 1980. "DNA mediated gene transfer: theory and application", Ninth Annual ICN-UCLA Symposia, Animal Virus Genetics, B. Fields and R. Jaenish, eds. Academic Press.

(52) Perucho, M., D. Hanahan, L. Lipsich and M. Wigler. 1980. Isolation of the chicken thymidine kinase gene by plasmid rescue. Nature 285:207-210.

(53) Lowy, I., A. Pellicer, J.F. Jackson, G.K. Sim, S. Silverstein and R. Axel. 1980. Isolation of transforming DNA: cloning the hamster aprt gene. Cell 22:817-823.

(54) Waalwijk, C. and R.A. Flavell. 1978. Msp I, an isoschizomer of Hpa II which cleaves both unmethylated and methylated Hpa II sites. Nucleic Acid Res. 5:3231-3236.

(55) Harris, M. and K. Collier. 1980. Phenotypic evolution of cells resistant to bromodeoxyuridine. Proc. Natl. Acad. Sci. USA 77:4206-4210.

(56) Grodzicker, T. and D.F. Klessig. 1980. Expression of unselected adenovirus genes in human cells co-transformed with the HSV-1 tk gene and adenovirus 2 DNA. Cell 21:453-463.

(57) Tantrahvahi, U., R.A. Katz, R.V. Guntaka, B.F. Erlanger and O.J. Miller. 1980. Amplified ribosomal RNA genes are inactivated by DNA methylation. European J. of Cell Biol. 22:10 (G28).

(58) Colantuoni, V., L. Dailey and C. Basilico. 1980. Amplification of integrated viral DNA sequences in polyoma virus-transformed cells. Proc. Natl. Acad. Sci. USA 77:3850-3854.

(59) Limenbach, A., K. Huebner and C.M. Croce. 1980. DNA-transformed murine teratocarcinoma cells: Regulation of expression of simian virus 40 tumor antigen in stem versus differentiated cells. Proc. Natl. Acad. Sci. USA 77:4875-4879.

(60) Pellicer, A., E.F. Wagner, A.E. Kareh, M.J. Dewey, A.J. Reuser, S. Silverstein, R. Axel and B. Mintz. 1980. Introduction of a viral thymidine kinase gene and the human β-globin gene into developmentally multipotential mouse teratocarcinoma cells. Proc. Natl. Acad. Sci. USA 77:2098-2102.