COMPLEMENTATION OF MUTANT PHENOTYPES AND GENOTYPES OF CULTURED MAMMALIAN CELLS

De foto op de omslag toont een aantal HPRT-deficiënte muizecellen, waarvan sommigen zijn geinjecteerd met een extract van HPRT-proficiënte cellen. De aktiviteit van het HPRT-enzym is zichtbaar gemaakt als zwarte korrels boven de cellen met behulp van autoradiografie.

COMPLEMENTATION OF MUTANT PHENOTYPES AND GENOTYPES OF CULTURED MAMMALIAN CELLS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
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OFFSETDRUKKERIJ KANTERS B.V.,
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De boom der kennis is niet de boom des levens.

Lord Byron.

CONTENTS

ABBREVIATIONS

α-Gal A	alpha galactosidase A
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CMGT	chromosome-mediated gene transfer
DEAE	diethylamino ethyl
DMGT	deoxyribonucleic acid-mediated gene transfer
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
GAA	acid alpha glucosidase
GALK	galactokinase
G6PD	glucose-6-phosphate dehydrogenase
HAT	hypoxanthine + aminopterin + thymidine
HPRT	hypoxanthine phosphoribosyl transferase
L-N	Lesch-Nyhan
MI	microinjection
ml	milliliter
mRNA	messenger ribonucleic acid
PEG	polyethylene glycol
PGK	phosphoglycerate kinase
RNA	ribonucleic acid
RNAse	ribonuclease
T4	bacteriophage T4
TK	thymidine kinase
tRNA	transfer ribonucleic acid
UDS	unscheduled deoxyribonucleic acid synthesis
UV	ultraviolet light
XP	xeroderma pigmentosum

PREFACE - VOORWOORD

Allen die op enigerlei wijze hebben bijgedragen aan de totstandkoming van dit proefschrift, bedank ik daarvoor van harte. Enkele mensen wil ik met name noemen. Prof.Dr. D. Bootsma dank ik voor de geboden mogelijkheden het hier beschreven onderzoek uit te voeren. Ik ben hem zeer erkentelijk voor de zorgvuldige manier waarop hij het werk en de rapportage daarvan heeft begeleid en het geduld waarmee hij de voltooiing van dit boekwerk heeft afgewacht. Ik waardeer bijzonder de gelegenheden die ik gekregen heb om in de laboratoria van Prof. Ruddle (New Haven, U.S.A.) en Prof. Graessmann (West Berlijn) voor ons nieuwe technieken te gaan bestuderen. Mede dankzij de vrijheid die ik gekregen heb in het kiezen van de experimentele benadering van probleemstellingen is dit proefschrift tot stand gekomen.
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Bram

1. GENERAL INTRODUCTION

This dissertation describes experiments aimed at the complementation of a genetic mutation in cultured mammalian cells in order to investigate several aspects of the structure and functioning of the human genome. Complementation is indicated by the correction of a biochemical function in which the mutant cells are deficient. Where appropriate in this text, a synonymous use of the terms 'complementation' and 'correction' is made. Complementation at the level of the cellular phenotype was studied as well as complementation at the level of the cellular genotype.

The phenotype of a cultured mammalian cell can be changed by the introduction of protein molecules which are not normally produced by that cell or messenger RNA molecules which direct the intracellular synthesis of such molecules. Since both protein and RNA molecules are not self perpetuating, a transient change in cell phenotype is usually observed. We have used phenotypic correction to investigate proteins for their ability to correct the deficiency in DNA repair displayed by human excision deficient xeroderma pigmentosum (XP) cells. For this purpose we developed an assay procedure in which prokaryotic DNA repair enzymes of known specificity were introduced into living XP cells by microinjection (MI) via glass microneedles and the complementation to a repair proficient phenotype was investigated (Appendix paper I). In addition, extracts of repair proficient human cells were assayed for activities that are able to complement the deficiency in XP cells. Appendix paper II describes the identification of a protein factor which specifically corrects the deficiency in one class of XP cells but not in others. These papers demonstrate the use of the living cell as part of a microinjection assay system to investigate the biological activity of proteins.

The genotype of a cultured mammalian cell can be supplemented by the introduction of genetic material (gene transfer). For DNA-mediated gene transfer (DMGT) using genomic DNA and chromosome-mediated gene transfer (CMGT) using metaphase chromosomes isolated from eukaryotic cells, the genetic material is usually administered as a co-precipitate with calcium phosphate. For DMGT via viral or plasmid DNA molecules, MI has also been found useful. Gene transfer can be detected as a transient or a more or less permanent change in cell phenotype if the genetic material is expressed correctly. The transfer and continued expression of genes generally oc-

curs at such a low frequency that it is necessary to use marker genes which confer viability on complemented cells in selective culture conditions. Although transient genotypic complementation has been studied occasionally, the more permanent mode of correction has been investigated extensively and used in a number of celgenetic studies.

We have used the genotypic complementation of cultured mammalian cells to compare the DMGT and CMGT processes. In addition, two aspects of the structure and expression of the human genome were investigated. Firstly, a contribution was made to the mapping of genes to human chromosomes by the regional localization of the human gene for acid alpha glucosidase (a lysosomal enzyme) on human chromosome 17, as deduced from the pattern of co-transfer with syntenic genes (Appendix paper III). Secondly, the nature of X-chromosome inactivation was investigated in DMGT experiments. It is demonstrated in Appendix paper IV that DNA isolated from inactive human X-chromosomes can be expressed efficiently after gene transfer.

Various aspects and applications of MI, DMGT and CMGT, including the experimental work, will be discussed in chapters II and III of this dissertation. For such a discussion, a distinction can conveniently be made between the donor cell providing the material transferred, the recipient cell which receives the donor material and -in the case of gene transfer- the resulting transformant cell containing the recipient cell genome plus a variable amount of donor genetic material (usually referred to as the transgenome). Part of this text has appeared in a review of chromosome and DNA-mediated gene transfer (de Jonge and Bootsma, 1984).

2. TECHNICAL ASPECTS OF MI, DMGT AND CMGT

2.1 Microinjection (MI)

Microinjection techniques have the aim of introducing material directly into the cytoplasm or nucleus of cultured mammalian cells without permanently damaging vital cellular functions. Effects of the microinjected material can then be studied in the living cell. Common to the techniques is that the injected material is first isolated and can therefore be manipulated (eg. purified, biochemically characterized or modified, or provided with a traceable label) at will by the investigator.

Basically, three methods can be distinguished for the introduction of material into living cells: direct physical injection into the cytoplasm or the nucleus of individual recipient cells in culture via a glass microcapillary needle, fusion of recipient cell populations with vesicles containing the material to be introduced and reversible permeabilization of cells in the presence of that material.

2.1.1 Injection via a glass microcapillary needle

Microneedle injection is probably the most versatile method available so far. The techniques were originally developed by Diakumakos et al. (1970) and Graessmann and Graessmann (1971) and have been published in detail (Diakumakos 1973, 1980; Graessmann et al., 1980[a]; Graessmann and Graessmann, 1983). Injection needles are drawn from borosilicate capillary tubes on commercial or home-made pullers to a tip diameter of 1 micrometer or less. In Diakumakos' method the microneedle is engineered further on a microforge and loaded from the rear. Stacey and Allfrey (1976) use a micropipette to deposit the sample very near the tip of the needle, Kreis et al. (1979) describe needles with an inner filament to facilitate the passive transfer from the back to the tip of the needle. Ansorge (1982) combines the two methods. Back-loaded microneedles are usually filled up with an inert liquid such as silicone oil and then connected to a hydrolic pressure system.

In the less laborious method of Graessmann, the needle produced by the puller is ready for use and connected to a pneumatic pressure system. Sample loading is performed by aspiration after dipping the needle tip into a small drop (1-5 microliters) of the sample solution. To prevent evaporation, the sample drop is usually kept cool and in a humid atmosphere.

All methods include a short high-speed centrifugation of the sample immediately prior to loading in order to remove fine particulate material which could clog the needle tip. For the same reason, the capillary tubes are very thoroughly washed before use. Other possible treatments of the needles include siliconization to minimize interactions between the glass and the sample and etching to obtain a very smooth needle tip (Graessmann et al., 1980[a]).

The loaded needle is mounted in a micromanipulator and the injection of individual cells is performed using a phase contrast microscope to monitor each injection. A cell is injected by gently introducing the needle into either the cytoplasmic or the nuclear region and ejecting a small volume of sample by increasing the needle pressure. Introduction of material generates a temporary change in the refractive properties of the cell content and can thus be verified visually (see Figure 1). Per hour, 400 to 1,000 cells can be injected.

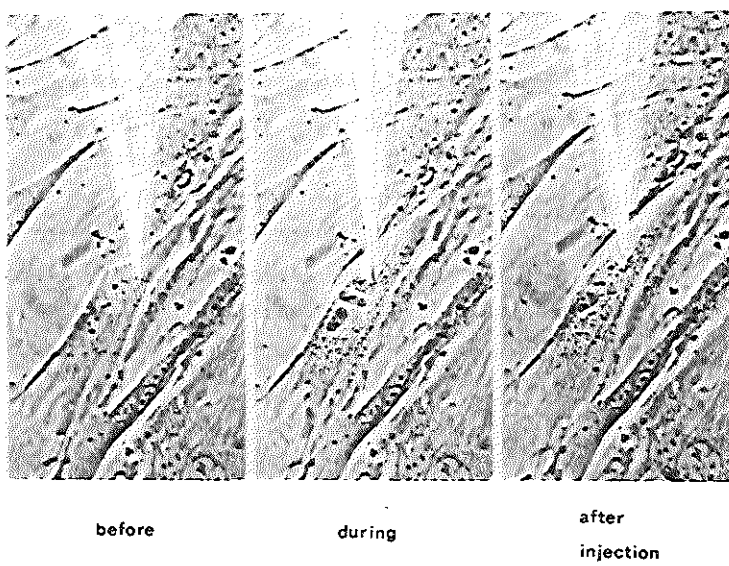

Fig. 1. Sequence of events in microneedle injection as seen using phase-contrast optics.
During injection, the contrast between nuclei and cytoplasm is increased.

Stacey and Allfrey (1976) measured an injection volume of about 10^{-10} ml per HeLa cell using ^{125}I-labeled bovine serum albumin and Graessmann et al. (1980[a]) estimated $1\text{-}5\times10^{-11}$ ml for fibroblasts from volumetric measurements of the needle tip. Generally, 5 to 10% of the cell volume can be injected without any significant effect on cell viability. The actual volume injected per cell cytoplasm can be enlarged to about 2×10^{-6} ml by injecting giant multinucleated cells generated by fusion of confluent cell monolayers (Graessmann et al., 1979).

Practically no restrictions have so far been encountered regarding the type of material or recipient cell used for microinjection. Intact cell organelles, virions, biological macromolecules (proteins, RNAs and DNAs), low molecular weight metabolites or substances unrelated to cellular metabolism can be introduced into various types of recipient cells. Non-adherent cells (for example cells of the lymphoid lineage) can be attached to a substratum via linker molecules such as phytohaemagglutinin, Concanavalin A, polylysine or immunoglobulins (Graessmann et al., 1980[b]). Although microneedle injection may seem to be a rather traumatic event, there is ample evidence indicating that cellular functions in microinjected cells are not seriously disturbed. Properly injected cells generally retain a healthy appearance, degrade microinjected proteins and RNA's at usual rates, support the expression of nucleic acids and can grow out to transformant cell clones at a high frequency (Graessmann and Graessmann, 1976; Stacey and Allfrey, 1976, 1977; Capecchi, 1980; Anderson et al., 1980).

A consequence of the limited number of cells that can reasonably be injected in an experiment is that the results have to be studied with single-cell or microassays. Many biological activities and cellular processes can be investigated using autoradiographic or immunofluorescent assays on the single cell and biochemical analyses of cellular proteins can be performed on as few as 100 cells (Bravo and Celis, 1980). Also, injection into giant fused multikaryons can be performed if necessary.

Recently, a modification of the microneedle injection technique was applied by Lo (1983) who introduced plasmid DNA molecules into the nuclei of cultured cells and embryos by electrophoresis via a micro-electrode. Although the general applicability of this iontophoretic microinjection procedure has yet to be established, several interesting features can be mentioned. Since there is no net fluid displacement and molecules are transported by virtue of their charge, the amount of material introduced is not

limited by the injection volume cells can tolerate nor should the use of a highly concentrated, viscous solution be a problem. It should therefore be possible to introduce very large amounts of material with this technique. A drawback is the reported low rate of injection (2 to 5 minutes per cell).

2.1.2 Fusion with loaded vesicles

Material can be introduced into the cytoplasm of a large number of recipient cells simultaneously via fusion with previously loaded membrane-bound carrier vesicles such as resealed erythrocyte ghosts (Furusawa et al., 1974; Loyter et al., 1975; Schlegel and Rechsteiner, 1975; Kaltoft and Celis, 1978; Antman and Livingston, 1980), artificial liposomes (Papahadjopoulos et al., 1974; Ostro et al., 1977, 1978; Uchida et al., 1979[a]; Fraley et al., 1980), reconstituted virus particles (Slilaty and Aposhian, 1983; Vainstein et al., 1983) and bacterial protoplasts (Schaffner, 1980).

Erythrocytes are usually loaded by hypotonic dialysis in a solution containing the molecules to be sequestered, liposomes and virus particles are loaded respectively by production and reconstitution in such solutions while bacterial protoplasts can be prepared directly from bacteria containing the material to be transferred.

For fusion of the carrier vesicles with recipient cells, inactivated Sendai virus or a high concentration of polyethylene glycol (PEG) is generally used. The efficiency of PEG-mediated fusion can be substantially enhanced by preincubation with suitable linker molecules such as phytohemagglutinin (Mercer and Schlegel, 1979) or specifically tailored immunoglobulins (Godfrey et al., 1981, 1983; Hashimoto et al., 1983) to agglutinate vesicles and cells before the fusogen is added.

Erythrocyte ghosts have been used as carriers for a variety of proteins as well as some species of tRNA (for references, see Celis et al., 1980). Recent modifications have made the delivery of small mRNA (Boogaard and Dixon, 1983[b]) and plasmid DNA molecules (Iino et al., 1983) also possible with this method. The use of nucleated avian erythrocytes (Jonak and Mora, 1980; McClung and Kletzien, 1984) allows an evaluation of the fusion efficiency and a rough estimation of the amount of material introduced into a recipient cell.

Liposomes have been used to deliver substances of low molecular weight as well as macromolecules into recipient cells in a biologically active form (for a summary of early work, see Papahadjopoulos, 1978). In these

earlier studies using liposomes as carriers, multilamellar or small unilamellar vesicles were usually prepared, which resulted in a low trapping efficiency and uptake of a substantial fraction of the sequestered material via an endocytotic pathway leading to a lysosomal degradation of the material (Poste et al., 1977). Advances include the use of large unilamellar vesicles with a higher trapping efficiency (Straubinger and Papahadjopoulos, 1983). Treatment with various chemical facilitators, for example dimethyl sulphoxide, glycerol or polyethylene glycol has been found to enhance the delivery and expression of liposome-sequestered viral or plasmid DNA (Straubinger and Papahadjopoulos, 1983) and may also stimulate the uptake of other macromolecules.

Uchida et al., 1979[a] showed that the contents of liposomes with Sendai virus spike protein molecules is delivered into recipient cells with receptors for Sendai virus much more efficiently than the contents of liposomes without the spikes. The incorporation of charged molecules or other ligands such as specific immunoglobulins (Weinstein et al., 1982; Hashimoto et al., 1983) into liposomes may also improve the efficiency as well as the specificity of liposome-recipient cell interaction. However, even large unilamellar vesicles cannot be used to deliver substantial volumes to recipient cells. As Deamer and Uster (1980) have calculated, the addition of 10% to a cell volume, easily accomplished by microneedle injection or by fusion with one erythrocyte ghost, would require fusion with 24,000 large liposomes. Straubinger and Papahadjopoulos (1983) have calculated that under saturating conditions the content of 1,000 vesicles is bound or internalized by a recipient cell.

Reconstituted particles prepared from Polyoma virus (Slilaty and Aposhian, 1983) or Sendai virus (Vainstein et al., 1983) have been used on a limited scale as carrier vesicles. Loading is performed by mixing purified viral envelope phospholipids and glycoproteins solubilized in a non-ionic detergent, with the aqueous sample solution and removal of the detergent by dialysis. The reassociating envelope components form vesicles containing some of the sample solution. The reconstituted virus particles attach to specific receptors on the surface of susceptible cells and fuse with the plasma membrane in a manner similar to intact virions, thereby delivering their content to the cellular cytoplasm with a relatively high efficiency (Vainstein et al., 1983). Protein molecules have been entrapped in complete Sendai virus particles by prolonged sonication of virions suspend-

ed in sample solution (Uchida et al., 1979[b]). The method seems unsuited to nucleic acids which are damaged by sonication.

Finally, PEG-mediated fusion of cultured eukaryotic cells with protoplasts of bacteria harbouring recombinant plasmid DNA molecules has been used for gene transfer (Schaffner, 1980; Robert de Saint Vincent et al., 1981; Robert de Saint Vincent and Wahl, 1983; Sandri-Goldin et al., 1983).

2.1.3 Cell permeabilization

The plasma membrane of recipient cells can be reversibly permeabilized without substantial loss of cell viability by incubation in hypertonic solutions (Castellot et al., 1978) or by treatment with Sendai virus (Tanaka et al., 1975), lysolecithin (Miller et al., 1978; Myers et al., 1983) or trypsin (Burr, 1980). Hypertonic, lysolecithin and trypsin treatment of recipient cell populations results in uptake from the medium bathing the cells of normally excluded compounds of low molecular weight (MW 1,000 D) such as the dye trypan blue and nucleoside triphosphates. At higher concentrations of lysolecithin, passage of larger (protein) molecules (MW 10,000-40,000 D) across the plasma membrane occurs but cell viability is lost (Miller et al., 1979). Treatment with Sendai virus allows introduction of protein molecules with a MW of about 10,000 D without seriously compromizing cell viability (Tanaka et al., 1977).

Yamamoto et al. (1981) describe a novel method which combines features of cell permeabilization and microneedle injection: cell pricking. A small amount of external medium (about 10^{-12} ml, 1-10% of the volume usually injected with a microneedle) is introduced into individual recipient cells by piercing them with an empty microinjection needle with a very fine tip (about 0.1 micrometer). Larger proteins such as Horse Radish peroxidase (MW 44,000 D) and immunoglobulins (MW 160,000 D) can be introduced. Also injection of plasmid DNA molecules can be performed successfully (Kudo et al., 1982). The use of a microscope especially adapted for microneedle injections (Injectoscope, Yamamoto and Furusawa, 1978) and the fact that a quick piercing of the recipient cell suffices have allowed cell pricking to be performed at a speed of 2,000 to 7,000 cells/hour.

Finally, exogenous plasmid DNA molecules have also been introduced by exposing cultured recipient cells suspended in the DNA solution to a series of short electric pulses which induce transient structural changes in the

plasma membrane without permanently damaging the cell (Neuman et al., 1982). Genetic transformation with the plasmid DNA is described. The simplicity of the method is attractive and it may be useful for other macromolecules and cells not suited to other methods of gene transfer.

2.2 DNA-mediated gene transfer (DMGT)

For DMGT, high molecular weight DNA can be obtained from any population of cells, eukaryotic as well as prokaryotic, using standard isolation procedures consisting of cell lysis, protease and RNAse treatments as well as extraction and precipitation of the DNA. The average size of DNA molecules isolated from eukaryotic cells is usually $50\text{-}80 \times 10^3$ base pairs. Szybalska and Szybalski (1962) found evidence for genetic transformation in an intraspecific cell system using DNA donor and recipient cells of human origin. Although reversion of the mutation in the recipient cells could not be completely excluded in this system, gene transfer was the most plausible explanation on the basis of a quantitative comparison with appropriate controls. DMGT in cultured mammalian cells became a reproducible technique (Wigler et al., 1978) when the importance of co-precipitating the DNA with calcium phosphate, as developed by Graham and Van der Eb (1973) for the assay of viral DNA infectivity, was realized. There has since been a rapid increase in both the number of genes transferred and the applications of the DMGT methodology to study several aspects of the genetic organization of mammalian cells. The increasing interest in DMGT may be attributed at least in part to the relative simplicity of the methodology, the fact that DNA can be isolated from virtually every population of cells (prokaryotic as well as eukaryotic) and the possibilities of manipulating the DNA (for instance with recombinant DNA procedures) before it is added to cultured recipient cells.

DMGT is usually performed by treating mass populations (10^6) of cells with a DNA-calcium phosphate co-precipitate and reported transfer frequencies for one gene using total donor cellular DNA range from 10^{-5} to less than 10^{-7}. Comparisons of transfer frequencies obtained in different laboratories are difficult because of variations in technique and cell lines used and because the independent origin and true transformant nature of every clone obtained has not always been established. Using cloned selectable marker genes inserted in viral or plasmid DNA molecules, transfer

frequencies up to 10^{-3} can be obtained. Three dominant vector systems should be mentioned in this respect: vectors containing the prokaryotic dihydrofolate reductase gene (DHFR) introduced by O'Hare et al. (1981), the xanthine-guanine phosphoribosyl transferase gene (XGPRT; Mulligan and Berg, 1981) and the phosphotransferase gene (PTR; Colbère-Garapin et al., 1981). These genes confer to recipient cells a resistance to methotrexate, mycophenolic acid and the antibiotic G-418 respectively. The viral or plasmid DNA is usually co-precipitated with calciumphosphate after mixing with carrier DNA. Several adjuvants have been used to enhance the transformation frequency. Treatment with dimethylsulfoxide (DMSO) resulted in an increase in the number of transformants using the transfer of the hamster thymidine kinase (TK) gene into mouse LTK^- cells (Lewis et al., 1980). It did not significantly increase the transfection frequency in a gene transfer system using Chinese hamster ovary (CHO) cells (Abraham et al., 1982). Recently a high efficiency of transfection with Polyoma virus DNA was observed after treatment of the recipient cells with the lysosomal enzyme inhibitor chloroquine (Luthman and Magnusson, 1983). Its applicability for the transfer of non-viral DNA sequences to mammalian cells has to be demonstrated.

Various other methods have been developed for the introduction into mammalian cells of eukaryotic genes carried on viral or plasmid vectors. Gene transfer was acccomplished after the addition, as a calciumphosphate co-precipitate, of intact bacteria carrying plasmids (Schaffner, 1980) or of intact recombinant bacteriophages (Lowy et al., 1980; Ishiura et al., 1982) to recipient cells.

By microneedle injection of recipient cell nuclei with solutions of viral (Graessman et al., 1977) or plasmid (Capecchi, 1980) DNA, where a relatively large fraction of the DNA molecules consists of the sequences of interest, many copies of a gene can be introduced into each nucleus. Expression of the donor DNA can then be observed in virtually every injected cells and up to 20% may grow out to transformant clones (Capecchi, 1980; Anderson et al., 1980). Other methods of microinjection have also been used for DMGT. Efficient gene transfer was obtained after fusion of recipient cells with protoplasts of bacteria containing recombinant plasmids (Schaffner, 1980; Robert de Saint Vincent et al., 1981; Robert de Saint Vincent and Wahl, 1983; Sandri-Goldin et al., 1983). DMGT based on infection of susceptible cells with *in vitro* assembled Polyoma-like particles (Slilaty

and Asposhian, 1983) or reconstituted Sendai virus particles containing plasmid or viral DNA (Loyter et al., 1983; Vainstein et al., 1983) has also been described.

Microinjection of DNA has mostly been applied in studied of the organization and expression of viral genomes. With the increasing number of plasmid and viral vectors carrying selectable markers and eukaryotic genes, there are several potentially useful applications in studies of the eukaryotic cellular genome as well. The applicability of DMGT may be extended to cells not amenable to other methods of gene transfer and to genes for which no selective system is available. Since only small amounts of DNA are needed for microneedle injection (per cell a few femtoliters of a 1 mg/ml DNA solution are injected), the technique may be applied to assay the biological activity of the DNA when only small amounts can be obtained, for example in DNA fractionation studies.

In this context it should also be mentioned that microneedle injection has been used to introduce DNA sequences into early embryos of Drosophila, frog or mouse origin. One of the aims of such procedures is to generate strains of transgenic laboratory animals carrying specifically constructed foreign DNA sequences in all their cells (Rusconi and Schaffner, 1981; Costantini and Lacy, 1981; Wagner et al., 1981; Rubin and Spradling, 1982; Gordon and Ruddle, 1983). This constitutes a promising new approach in the study of developmental biology. With the methods mentioned so far, transfer frequencies at least as high as those obtained with the calciumphosphate technique have been observed and a stable mode of gene expression can be achieved. Gene transfer using a DEAE dextran-DNA solution, a technique originally used in DNA-mediated viral infection of cells, has been reported to result in only a transient expression of donor genes in 0.1-1% of the cells during the first days after transfer (Milman and Herzberg, 1981). Stable transformants which could be isolated under selective conditions were not obtained.

2.3 Chromosome-mediated gene transfer (CMGT)

For CMGT, complete eukaryotic chromosomes can be obtained from populations of suitable donor cells arrested in the metaphase of the cell cycle. Rupture of the cellular membrane liberates the chromosomes which are separated from other cellular material using centrifugation techniques. Only a

relatively small number of established or transformed cell lines has been used as CMGT-donor cells because they meet the requirements of a high proliferative activity and the desired respons to mitotic inhibitors (arrest in the metaphase). When isolated metaphase chromosomes are incubated with cultured recipient cells, they can be taken up by the cells and broken down into chromosomal fragments. Occasionally, a fragment is transported into the nucleus where expression of donor genes can take place to generate a transformant cell. Since the first convincing report of CMGT (McBride and Ozer, 1973), a number of investigators have used this technique to transfer various selectable marker genes into suitable recipient cells.

In most early CMGT experiments, transfer frequencies of about 10^{-7} were obtained by incubating isolated chromosomes with a suspension of recipient cells in the presence of poly-L-ornithine to enhance the association of chromosomes and cells. Comparisons with controls omitting the polycation were not made. Wullems et al. (1975) found a 10-fold increase in the number of clones arizing in selective medium when the fusogen Sendai virus was added to the mixture of chromosomes and recipient interphase cells. With recipient cells in mitosis, a 30-fold increase was observed. In later experiments using the same transfer system we have not been able to confirm these results.

Most efficacious (CMGT frequencies of about 10^{-5} in several laboratories) has been the administration of chromosomes to recipient cell monolayers as a chromosome-calcium-phosphate coprecipitate with a subsequent treatment of the recipients with DMSO, as introduced in CMGT by Miller and Ruddle (1978) and further analyzed by Lewis et al. (1980).
This procedure has since become general practice so that isolated metaphase chromosomes and purified genomic DNA are now introduced into recipient cells with the same coprecipitation technique. For a number of genes, CMGT occurs at a strikingly higher frequency than DMGT in the same donor and recipient cell system. We have repeatedly transferred the gene coding for hypoxanthine phosphoribosyl transferase (HPRT) into mouse A9 ($HPRT^-$) cells using chromosomes isolated from human HeLa cells at frequencies of 10^{-6}. In later experiments using HeLa-DNA, transfer occurred only in a few experiments at a frequency of about 10^{-8} (Appendix paper III). Similar results were reported by Lewis et al. (1980) in a comparative study of CMGT and DMGT of Chinese hamster genes encoding thymidine kinase or methotrexate resistance into mouse LTK^- cells. It is possible that non-DNA chromosomal con-

stituents (RNA or protein), or the manner in which the DNA is packaged in metaphase chromosomes, offer some protection against degradation in the recipient cell. Aternatively, the higher transfer frequency may be due to a larger size of the DNA molecules in isolated chromosomes. It is also possible that a fraction of purified DNA may not be active in transfer of genetic information due to damage introduced by the DNA isolation procedure.

2.4 Choice of recipient cells in MI, DMGT and CMGT

Virtually all types of cells are amenable to microneedle injection although there will obviously be limits to the practicability of specific experimental designs. For example, we have experienced considerable difficulties with microneedle injections into the cytoplasm of small spherical cells attached to a substratum via polylysine. The cell swelling due to introduction of material caused detachment in many cases. Also, nuclear injections were not infrequent and when cells were approached horizontally with the needle instead of from above, many were dislodged and blocked the needle orifice (unpublished observations with Dr. D.J. Halley).

A prerequisite for a successful use of carrier vesicles is the ability of the recipient cells to undergo fusion. A preliminary agglutination of cells and vesicles can be performed to lower the concentration of polyethylene glycol required for a satisfactory fusion efficiency of PEG sensitive cells. For virus-mediated fusion and for the use of loaded viral envelopes as carriers, the recipient cells need to display the suitable virus receptor molecules. Reversible cell permeabilization using lysolecithin can be accomplished with a variety of cell types (Miller et al., 1979). Thus, microinjection techniques generally allow a considerable freedom in the choice of recipient cell, permitting the selection of the cell type best suited to the investigation undertaken and also extending the applicability of the techniques.

In contrast, the calcium phosphate technique for the transfer of genes via isolated cellular DNA or metaphase chromosomes imposes restrictions on the choice of recipient cells since high frequencies of gene transfer using this donor genetic material have only been obtained with a limited number of cell lines. In most studies gene transfer have been performed at reproducible transfer frequencies of 10^{-5} to 10^{-6} into cells derived from the murine L929 cell line (for example LTK, A9, B82). DMGT and CMGT into reci-

pients of other origin has generally occurred at substantially lower frequencies.

An important case in point is the gene transfer into cells of Chinese hamster ovary (CHO) origin. CHO cells is one of the most widely used experimental tools in somatic cell genetic analysis and a variety of characterized mutant cell lines is available. However, CHO cells have been poor recipients in CMGT and in DMGT using total genomic DNA, usually transforming at 50-100 fold lower frequencies than LTK^- cells. Elaborate studies attempting to improve this (Lewis et al., 1980; Linsley and Siminovitch, 1982; Abraham et al., 1982; Nairn et al., 1982) have been partially successful (Nairn et al., 1982). Important for the enhancement of transfer frequencies has been the discovery of clonal variation in the transformability of murine (Corsaro and Pearson, 1981) as well as CHO cells (Nairn et al., 1982). Even sister subclones of LTK^- can show a substantial (10-20 fold) difference in DMGT of the same gene (Corsaro and Pearson, 1981). In view of this clonal variation, it may be worthwhile to attempt the selection of high transfer subclones of more cells lines valuable in gene transfer. Such selection could be attempted by transfer of a dominant acting selectable gene such as ouabain, mycophenolic acid or methotrexate resistance and testing the transformant clones generated for their transformability in a second round of gene transfer. Alternatively, the isolation of suitable mutant sublines of a high transfer cell line such as LTK^- may suffice. We have isolated several LTK^-HPRT^- double mutant cell lines and have used one of these, LTH1, in DMGT of the human HPRT-gene (Appendix papers III and IV).

Another complication in gene transfer is the varied response recipient cells of different origin show towards a DMSO treatment after the addition of DNA or chromosomes. In mouse L-derived cells, the treatment enhances transformation efficiency, in human cells it is useful at shorter treatment times with a higher concentration (Gross et al., 1979; Gross Lugo and Baker, 1983) while in CHO cells no effect is apparent (Srinivasan and Lewis, 1980; Abraham et al., 1982). Furthermore, we have repeatedly observed 8-10 fold reductions in CMGT of the human gene for thymidine kinase (TK1) into Swiss mouse $3T3TK^-$ fibroblasts when a DMSO posttreatment is given (Appendix paper III). It appears necessary to determine the optimal transfer conditions, including the effects of adjuvants such as DMSO, for each recipient cell line used.

3. THE INTRODUCTION AND BIOLOGICAL ACTIVITY OF DONOR MACROMOLECULES IN RECIPIENT CELLS

3.1 Phenotypic complementation by the introduction of donor proteins

Microinjection techniques have been used very effectively to introduce proteins into cultured cells. All three methods described above have been applied but the use of microneedle injection and erythrocyte fusion predominate. There are numerous indications that proteins can function normally after microinjection. For example, catalytic activity is retained by enzymes such as thymidine kinase (Schlegel and Rechsteiner, 1975), Horse radish peroxidase (Yamamoto et al., 1981) and hypoxanthine phosphoribosyl transferase, (Kaltoft and Celis, 1978).

Proteins other than enzymes also appear to function normally after microinjection even when conjugated with fluorochromosomes or radioactive tracers. For instance, purified non-histone chromosomal proteins injected into the cytoplasm quickly accumulated in the nucleus (Yamaizumi et al., 1979; Rechsteiner and Kuehl, 1979), isolated cytoskeletal proteins are incorporated into intracellular filamentous networks (for a review, see Kreis and Birchmeier, 1982) and antibodies can bind and inactivate intracellular antigens (Antman and Livingston, 1980) after microinjection.

A normal biological functioning of microinjected proteins is also indicated by studies which have shown that the rate and manner of their degradation can be similar to those found for endogenous cytosolic proteins (Stacey and Allfrey, 1977; Neff et al., 1981). Microinjection therefore seems a very useful technique to introduce proteins into cultured cells and study their biological activity as well as the cellular process(es) they are involved in.

3.1.1 The study of a DNA repair mechanism by microinjection of protein molecules

The lack of suitable assay systems has been a serious limitation in the isolation and characterization of factors involved in the repair of DNA damage in mammalian cells. Despite extensive studies of the genetic defect in the human skin disease xeroderma pigmentosum (XP), no gene or gene product involved in this DNA repair deficiency syndrome has so far been isolated and characterized. This may be attributed at least in part to the extreme complexity of the substrate of such gene products, human

chromatin. With microinjection, assay systems using human chromatin in its natural form (i.e. in the living cell) can be developed to study factors involved in XP.

Excision repair deficient XP cells show a reduction in UV-induced unscheduled DNA synthesis (UDS) as a concomitant of the impaired capacity of these cells to remove UV-induced pyrimidine from their DNA (see Figure 2). UDS can be measured autoradiographically in single cells. So far, 9 genetic complementation groups, designated A through I have been identified on the basis of an increase in the rate of UDS in heterokaryons generated by fusion of different XP fibroblast strains. As reported in Appendix paper I, increased rates of UV-induced UDS were also observed in cells of these XP complementation groups after microneedle injection of partially purified preparations of the prokaryotic DNA repair enzymes Micrococcus luteus UV-endonuclease or T4 endonuclease V. These experiments verified and extended the results of Tanaka et al. (1975, 1977) and Hayakawa et al. (1981) who respectively introduced T4 endonuclease V into cells of XP complementation groups A through E and F by permeabilization with Sendai virus and also found restoration of the UV-induced UDS. Therefore, the action of a prokaryotic DNA repair enzyme can compensate at least in part for the deficiency in cells of all known XP complementation groups.

The two prokaryotic repair enzymes we have injected into XP cells both specifically incise DNA at the site of a pyrimidine dimer. Our results therefore give further support to the hypothesis that in XP fibroblasts with an excision repair deficiency, the defect resides before or at the incision step (see figure 2). In addition, the feasibility of this experimental approach to the study of DNA repair in mammalian cells was demonstrated.

In order to identify human factors which can complement the repair defect in XP cells, we next injected crude concentrated extracts of repair proficient human cells. Appendix paper II reports the discovery of an activity which is abundantly present in the extracts and specifically complements the deficiency in cells of XP complementation group A. The activity was stable on storage, still detectable 8 h. after injection and found to be sensitive to the action of proteinase K, which suggests that it is a protein. Subsequent experiments (W. Vermeulen and J.H.J. Hoeijmakers, pers. comm.) indicate that this factor can be purified by ammonium sulphate precipitation and DEAE-cellulose chromatography and that it binds to UV-irradiated double-stranded DNA.

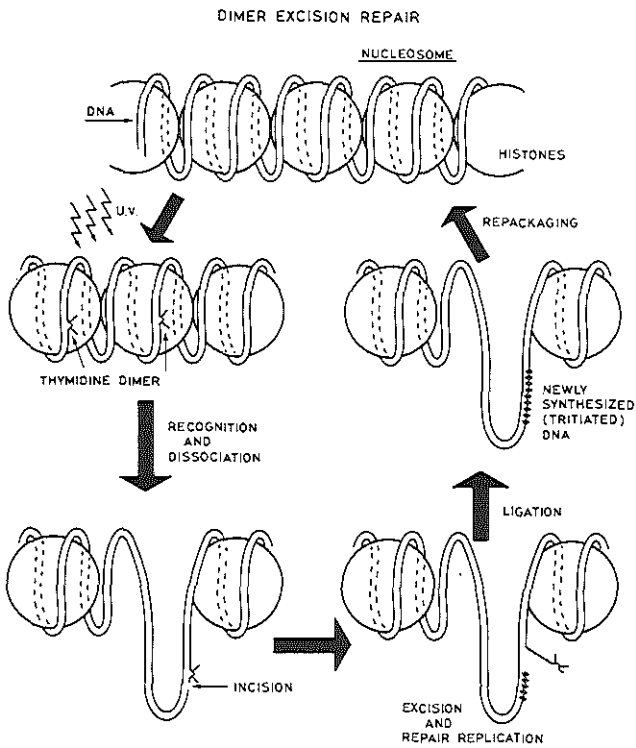

Fig. 2. Schematic representation of the steps thought to be involved in the removal of pyrimidine dimers induced in DNA by ultraviolet light. DNA, newly synthesized during UDS in the presence of (^{3}H)-thymidine can be recognized by autoradiography.

Using a modified, more sensitive assay for UDS, Vermeulen and Hoeijmakers (pers.comm.) have recently also found correction of the repair defect in cells of XP complementation groups B through I, although not in all cases to a high level of UDS, after microinjection of human repair proficient cell extracts or extracts of heterologous XP cell strains. Specificity of the phenotypic complementation was indicated by the absence of correction after injection of homologous extracts.

Our microinjection assay of DNA repair has also been used to investigate the effect of Saccharomyces cerevisiae or Anacystis nidulans photoreactivating enzyme on UV-induced UDS in normal human cells (J.C.M. Zwetsloot

et al., Mutation Res., in press) and XP cells. Crude extracts as well as the purified enzymes were active in the normal human cells and cells of some XP complementation groups, as measured by a substantial decrease in the normal respectively residual level of UDS.

In conclusion, these experiments show that microinjection can provide a useful assay system for the biological activity of protein activities which function in human DNA repair. At the same time, the method seems a promising approach to study DNA repair processes in the living cultured cell.

3.2 Phenotypic complementation by the introduction of donor messenger RNA molecules

Since the first demonstrations of the translation of messenger RNA (mRNA) microinjected into cultured cells (Graessmann and Graessmann, 1971, 1976; Stacey and Allfrey, 1976), numerous reports testify to the feasibility of this approach to study the mRNA or its product (Green et al., 1983; Franke et al., 1984; Burke and Warren, 1984) as well as to provide bioassays for mRNA activity during purification and biochemical characterization (Liu et al., 1979; Lin et al., 1982; Fainsod et al., 1984; Legerski et al., 1984). In most studies mRNAs have been injected using microneedles but the use of liposomes (Ostro et al., 1980) and erythrocyte ghosts (Boogaard and Dixon, 1983[a,b]) as transport vesicles has also been reported.

Microinjection assays appear especially useful in combination with mRNA purification methods to identify messages specifying the synthesis of proteins whose biological activity can best be studied in the living cell. For such a study detailed knowledge of an encoded protein is not necessary; a suitable bioassay of the protein's activity suffices.

This was very recently demonstrated by Legerski et al. (1984) who reported the transient restoration of unscheduled DNA synthesis (UDS) in excision repair deficient XP cells by microneedle injection of cytoplasmic poly(A)$^+$ RNA isolated from repair proficient human cells. XP cells of complementation groups A and G were corrected, cells of the two other complementation groups tested (D and F) were not measureably corrected. It was calculated that an approximate 30-fold excess of XP correcting mRNA's is present in HeLa cells, a figure similar to the 20 to 30-fold excess we calculated for the XP-A correcting protein in HeLa cells (Appendix paper II).

Assays for the RNA obtained from sucrose gradient fractions showed that the messengers correcting XP-A and G group cells have different sedimentation rates which suggests that different genes are involved. The lengths of these mRNA molecules were roughly estimated to be 690 (group A) and 880 (group G) nucleotides, which could encode polypeptides of about 16,000 and 23,000 Daltons respectively. Since we have observed that the XP-A correcting protein activity does not pass Amicon XM 100 or XM 300 ultrafilters and is excluded by Sephadex G-50 (Hoeijmakers et al., 1983), the biologically active form would seem to be a multimer.

3.3 Genotypic complementation by the introduction of donor DNA molecules or metaphase chromosomes (gene transfer).

So far, expression of donor genes in cultured cells microinjected with total genomic DNA isolated from eukaryotic cells has not been reported. Moreover, only one report (Mukherjee et al., 1978) has appeared claiming the use of liposomes to introduce metaphase chromosomes into cultured cells. Therefore, microinjection techniques have so far not been very useful for the transfer of genes via donor cellular DNA or metaphase chromosomes.

In contrast, DMGT and CMGT via precipitation of the donor genetic material with calcium phosphate has been extensively executed and analysed. Studies addressing the state of donor genetic material in the recipient cell (usually referred to as the transgenome), which have been reviewed previously by Willecke (1978); McBride and Peterson (1980); Pellicer et al., (1980); Klobutcher and Ruddle (1981) and Scangos and Ruddle (1981), have revealed an extensive heterogeneity with respect to size as well as organization and mode of propagation of transgenomes. These and more recent results will be summarized below.

3.3.1 Transgenome size and gene copy number

The largest reported transgenome is probably an apparently complete human X-chromosome, transferred in our laboratory by CMGT into Chinese hamster-human cell hybrids as recipient cells (Wullems et al., 1976) with selection for the expression of HPRT. In all 16 transformants analyzed, the human X-linked genes for HPRT, glucose-6-phosphate dehydrogenase (G6PD), α-galactosidase A (α-Gal A) and phosphoglycerate kinase (PGK) were expressed and karyotypic analysis revealed the presence of the human X-chromosome.

In subsequent elaborate attempts using the same cell system, we have not been able to generate clones and other laboratories have not reported on CMGT into rodent-human cell hybrids.

A minority of CMGT transformants carry a macrotransgenome detectable using alkaline Giemsa staining of metaphase spreads (Miller and Ruddle, 1978; Klobutcher and Ruddle, 1979; Klobutcher et al., 1980; Olsen et al., 1981; Appendix paper III). The transgenome carried by most CMGT transformants and all DMGT transformants studied so far is apparently too small to be detected with the alkaline Giemsa staining method. In a few transformants, obtained after DMGT of cloned selectable donor-genes, microtransgenomes have been located with the more sensitive in situ hybrization technique using radiolabeled plasmid DNA as a probe (Huttner et al., 1981; Robins et al., 1981; Robert de Saint Vincent and Wahl, 1983). An example of the visualization of a transgenome present in Swiss mouse 3T3TK$^+$ transformant cells generated by CMGT using human HeLa chromosomes is shown in Figure 3. In this case (transformant 60C5), the transgenome could not be distinguished by chromosome staining techniques. However, in situ hybridization with radio-labeled reiterated human DNA sequences (Cot-1 DNA) demonstrated the presence of a large piece of human DNA carried independent of a mouse chromosome. Further refinements of the in situ hybridization methodology which allow detection of single-copy DNA sequences (Malcolm et al., 1982) should make detection of even the smallest transgenomes possible.

In addition to these methods of visualization, transgenome sizes have also been estimated from frequencies of cotransfer of closely linked syntenic donor genes (see section 3.3.3) and from analytical DNA-DNA hybridization of radiolabeled purified unique sequence donor cell DNA with transformant cell DNA (Olsen et al., 1981). With appropriate probes, the latter method allows accurate estimates of the amount of donor genetic material retained in transformant cells as well as providing data on the copy number of the donor cell sequences. Such studies should provide further insight in the structure of transgenomes generated by DMGT and CMGT. Many transformants generated by DMGT of plasmid-borne selectable genes have been shown to carry concatanated multiple copies of plasmid sequences. Concatanates appear to be the most frequently generated type of DMGT-derived transgenomes. In some CMGT-generated transformants, the presence of multiple copies of donor genes, but not their mutual linkage, has been demonstrated (Scangos et al.,1979).

Furthermore, the accumulation of multiple copies has been proposed to

account for the overexpression of donor genes and as a mode of propagation of the selectable marker gene in unstable transformants. However, various observations indicate that in many CMGT-transformants, donor genes are present (or expressed) at single-copy level (Miller and Ruddle, 1979; Klobutcher et al., 1980; Olsen et al., 1981). In conclusion, a variety transgenomes with regard to the amount as well as the organization of the donor genetic material has been observed. No fundamental differences between DMGT- or CMGT-derived transgenomes are evident but the larger size of some CMGT-derived transgenomes and the apparent preponderance of single copy transgenomes in CMGT may indicate that donor chromosomal material has a larger average size and/or a reduced susceptibility to degradation and reconstruction in comparison to isolated donor DNA.

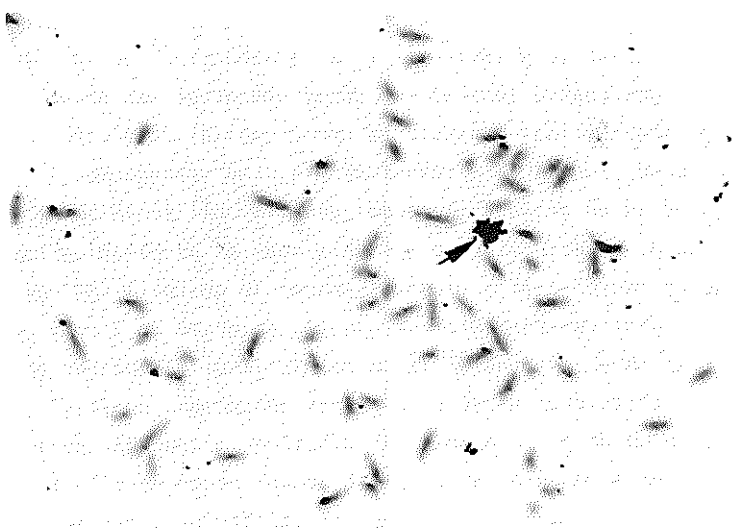

Fig. 3. Visualization of a human transgenome in the genome of transformant 60C5 by in situ hybridization.
This thymidine kinase (TK) positive transformant of TK⁻ Swiss mouse 3T3 cells was selected in HAT medium after chromosome-mediated gene transfer using isolated human HeLa chromosomes. ^{3}H-labeled human repetitive DNA (Cot-1) was hybridized to metaphase spreads of transformant cells. One chromosome contains sequences that hybridize with the Cot-1 DNA (arrow). This chromosome is absent after selection for loss of expression of the human TK1 gene in medium with bromodeoxyuridine. Furthermore, it was established by electrophoretic and immunological procedures that in addition to the selected human TK1 marker gene, this chromosome also carries the non-selected genes coding for human galactokinase (GALK) and acid alpha glucosidase (GAA), all three genes being located on chromosome 17 in human cells (see appendix paper III).

3.3.2 Transgenome stability and linkage to host cell chromosomes

Transient expression in recipient cell populations has been observed to take place during the first days after DMGT of various mammalian genes. The phenomenon is reminiscent of the abortive transformation observed in transfection of viral DNA. It is presumed to reflect the presence of the genes on donor DNA sequences which lack elements necessary for their continued propagation in a relatively large fraction of the recipient cell population. Such sequences would therefore be progressively lost during continued culture, with a concomitant loss of gene expression. Ultimately, only the few cells carrying the donor genes in a propagatable fashion would continue expression and be selectable as transformant cells. Both stable and unstable modes of propagation have been observed. When cultured in non-selective medium, stable transformants retain the expression of donor genes indefinitely in all progeny cells. In unstable transformants, expression is progressively lost at a rate of about 10^{-2} to 10^{-1} per cell generation. In some cases, loss of the donor phenotype was shown to be accompanied by the physical loss of the donor gene(s), but other modes cannot be excluded. In unstable transformants, the transgenome is believed to be propagated as an extra-chromosomal genetic unit reminiscent of the bacterial episome. Direct evidence for this mode of propagation has come from the observation of free macrotransgenomes in unstable CMGT-generated transformants. Such transformants are usually observed at 1 copy per cell and many of them have centromere-like constrictions. Klobutcher et al. (1980) have suggested that these free macrotransgenomes possess donor centromeres which provide for a normal distribution to daughter cells at mitosis and that the xenogenic nature of the donor centromere in the recipient cell could be responsible for the instability of the transgenome, analogous to the unstable retention of chromosomes of one of the parental cells which is often observed in interspecific cell hybrids.

Transformants carrying macrotransgenomes with centromere-like constrictions do not always express all syntenic donor genes known to be located proximal to the selected gene. An obvious explanation for this is the fragmentation of donor chromosomes and rejoining of fragments. Linkage of a selected gene to the centromeric region of another donor chromosome could occur.

Another indication of rearranged donor sequences in macrotransgenomes is the cotransfer of an asyntenic unselected donor gene, which segregates

with the selectable gene and the macrotransgenome (Klobutcher and Ruddle, 1979). Although other explanations, for example the transfer of a rearranged donor chromosome cannor be excluded, it is evident that the conservation of donor chromosomal sequence organization in macrotransgenomes cannot simply be assumed. Molecular analysis using nucleic acid probes specific for certain chromosomal regions should be useful in establishing the frequency with which such rearrangements occur.

The manner in which other free macrotransgenomes and microtransgenomes, which are believed to be acentric, are propagated in unstable transformants is much less clear. For such genetic elements there is no known mechanism of equal distribution over descendent cells. The favoured hypothesis propose that unequal segregation during successive rounds of replication and cell division early in the history of these transformant cell lines leads to the accumulation of multiple copies of the transgenome in some cells and no transgenomes in others. This would give the population as a whole the characteristic of instability. Evidence for the accumulation of donor genes in unstable transformants has been found in several laboratories (see for a review of this work: Klobutcher and Ruddle, 1981). Two lines of evidence indicate the appearance of cells that do not express the selectable donor gene in clones generated from unstable transformants. In selective medium the negative cells die and clones of highly unstable transformants would grow slower than clones of more stable transformants. Lewis et al. (1980) have proposed this explanation for the significantly larger clone size of CMGT transformants versus DMGT transformants they obtained with the same cell system. In nonselective medium, mozaic clones composed of positive and negative cells would be generated. Bacchetti and Graham (1977) have observed a number of such clones after subcloning unstable DMGT transformants in nonselective medium.

Both direct and indirect evidence has been obtained for the presence of multiple copies of the donor selectable gene in some unstable transformants grown under selective conditions. However, apart from a few transformants observed to carry duplicate macrotransgenomes, it is not clear whether the multiple copies are linked into one or a few concatanate transgenomes, or whether many separate copies are propagated although the observed instability is more easily understood by assuming concatanate transgenomes. Furthermore, concatanate transgenomes have been observed in many DMGT transformants while for most CMGT transformants studied, the data obtained are

consistent with the retention of only one copy of the donor sequence. Hence, the mechanisms for the generation and propagation of putative acentric transgenomes are still uncertain. Also, different mechanisms may be involved for the different types of transgenomes generated in DMGT and CMGT. A better understanding of these mechanisms is necessary to increase the experimental control over the gene transfer process and further extend the utility of transfer technology.

Unstable transformant cell populations have often been observed to convert to a stable mode of expression upon prolonged culture. The reverse process, conversion of stable into unstable expression, has not been described. The generally accepted interpretation of the stabilization process is that at a low frequency an unstable (free) transgenome becomes associated with a host cell chromosome, probably by covalent integration. Although such linkage is a rare event, the stable transformant generated would distribute the transgenome efficiently to all progeny cells and would, with time, become the predominant cell type of the population. Integration seems to occur at a random moment in the history of a transformant cell line as is indicated by the isolation of stable cell lines, in which linkage has apparently occurred at an early stage, and of unstable populations, in which it has been possible to study the stabilization process (Klobutcher and Ruddle, 1979). These studies and the abundant evidence for the integration of the transgenome into host cell chromosomes in stable transformants have been reviewed before (Mc Bride and Peterson, 1980; Klobutcher and Ruddle, 1981; Scangos and Ruddle, 1981). In summary, it has been shown that integration can take place in many different sites of the host genome. Rearrangement and loss of the transgenome sequences can occur during the integration process. Rearrangement of host cell chromosomes carrying transgenomes has also been reported. Although denoted as stable, integrated transgenomes do not seem to be carried as stably as endogenous host cell genes. As suggested by Fournier et al. (1979), host cell chromosomal lability may be a prerequisite for, or a consequence of, the association with transgenomes.

Mechanistic details of the integration process are unknown but recent evidence indicates that mammalian cells are capable of precise homologous recombination between donor and host cell chromosomal sequences (Goodenow et al., 1983). Further investigation will have to elucidate the general mechanisms operating in transgenome integration. Characterized nucleic acid

sequences constitute a powerful tool in such studies. Fundamental differences with regard to integration of DMGT and CMGT-derived transgenomes are not apparent at present. The preponderance of stable transformants isolated after DMGT of plasmid-borne selectable genes without genomic carrier DNA (Wigler et al., 1979; Huttner et al., 1981) may be explained by the necessity of transgenome integration into host cell chromosomes in order to aquire linkage to eukaryotic origins of DNA replication in such an experimental design. In most protocolls of DMGT using plasmid molecules, cellular genomic DNA is added as carrier and the selectable gene can then become linked to a carrier-DNA fragment containing an origin of replication and thus be propagated in an unstable fashion, at least for some time.

Since integration is often accompanied by the loss of transgenome sequences, the stabilization event can be used to construct deletion maps of large macrotransgenomes expressing syntenic donor genes which allow a regional mapping of those genes (Klobutcher and Ruddle, 1979).
In constructing such a deletion map, it is necessary to know on which side of the macrotransgenome the deletions have occurred. In addition to the chromosome banding technique used by Klobutcher and Ruddle (1979) to determine this, other methods such as in situ hybridization to metaphase chromosomes of nucleic acid probes specific for certain chromosomal regions, should extend the applicability of this method to chromosomal regions which lack a conspicuous banding pattern. A serious complication of the method is the occurrence of donor sequence rearrangements during gene transfer, which could lead to false localizations.

3.3.3 Cotransfer of non-selected genes

In DMGT as well as CMGT, there is ample evidence for the retention of more donor genetic material than only those sequences necessary for the propagation and expression of the selectable gene. DMGT using mixtures of plasmids results in concatanate transgenomes in which non-selected plasmid sequences have become linked to selected donor genes. This discovery has advantageously been used to permanently introduce nonselectable plasmid sequences into recipient cells by simply coprecipitating an excess of these plasmids with plasmids containing a selectable gene (Wigler et al., 1979; Wold et al., 1979).

Even in DMGT using genomic donor cell DNA, co-transfer and expression

of non-selected donor genes has been observed at a low frequency. Peterson and Mc Bride (1980) reported one out 87 LTK^- DMGT transformants selected for the expression of Chinese hamster TK, which also expressed the Chinese hamster donor gene for galactokinase (GALK) known to be linked to the TK-locus. Similarly, in a study of DMGT into LTK^- cells using human HeLa-DNA, we have found expression of human GALK in 2 independent transformants out of 17 in which human TK1 is expressed (Appendix paper III). As discussed by Peterson and McBride (1980), the average size of donor DNA molecules in DMGT (10^5 nucleotide base pairs) is at least an order of magnitude smaller than the estimated intergenic TK1-GALK distance in the donor genome and at least two explanations for the cotransfer are possible. First, the intergenic distance may be less than estimated and second, the cotransfer observed may well be the result of a fortuitous linkage of donor DNA molecules in the recipient cell. In a study of 15 TK^+ transformants of LTK^- cells, Warrick et al. (1980) did not find cotransfer of GALK or 23 other genes assayed, but one transformant expressed the asyntenic gene for esterase-D.

In CMGT, cotransfer of non-selected asyntenic genes has also been observed at similar low frequencies, but cotransfer of closely linked syntenic genes occurs at relatively high frequencies. Various laboratories have reported a cotransfer frequency of about 25% for the human TK1 and GALK loci and the average CMGT-derived transgenome was estimated to be 25-33% longer than the distance between these two loci (in different studies determined at 0.04% or 0.2% of the haploid genome). Thus the average size of CMGT-derived transgenomes may be estimated at 0.05 to 0.25% of the haploid human genome, or $2x10^6$ to $7x10^6$ nucleotide base pairs (see McBride and Peterson, 1980). Evidently further studies on the chromosome- and DNA mediated cotransfer of closely linked genes should establish the generality of these findings, but the data obtained so far indicate a considerable difference in the average sizes of the donor genetic material in CMGT and DMGT.

In CMGT transformants carrying macrotransgenomes, cotransfer of syntenic genes is often observed. Upon back-selection for loss of expression of the selectable gene, expression of the cotransferred gene(s) and the visible transgenome are usually also lost (for examples, see Appendix paper III). This concomitant loss has often been interpreted as an indication of transgenome integrity. However, in addition to the evidence of rearran-

gements in macrotransgenomes mentioned before, a few CMGT macrotransgenomes expressing normally asyntenic donor genes have also been observed, again indicating the recombination of donor chromosomal fragments. When complications such as these are taken into account, CMGT transformants expressing genes known to be syntenic can be used to regionally map those genes by determining the frequencies of cotransfer with the selected marker gene in primary transformants. These frequencies reflect the relative positions and distances of the genes in that linkage group (Klobutcher and Ruddle, 1981). Assuming that donor chromosome breakage and degradation occurs at random during CMGT, a gene located far from the marker gene should be cotransferred at a lower frequency than a gene close to the marker gene. In view of the evidence that previously separate donor sequences can become linked in the recipient cell, this method will probably be restricted to genes for which relatively high cotransfer frequencies are observed. Furthermore, the cotransfer frequency of a gene should be calculated on the basis of multiple independent cotransformants expressing that gene, which could necessitate the analysis of large numbers of transformants.

Appendix paper III reports on the analysis of a number of CMGT and DMGT transformants for the cotransfer of human acid alpha-glucosidase (GAA) with human thymidine kinase (TK1) and galactokinase (GALK). All 3 loci were mapped before to region 17q21-q25 (Human Gene Mapping 6, 1982). Our findings (Appendix paper III) indicate a close linkage of these loci and in combination with the results of Halley et al. (1984), who deduced a localization of the GAA locus distal to the TK1-GALK segment, the gene ordering of CENTROMERE-GALK-TK1-GAA on human chromosome 17 was tentatively established.

3.3.4 Transfer and expression of a gene on the inactivated human X-chromosome.

In somatic cells of eutherian mammals only one of the two X-chromosomes is genetically active; the other X-chromosome is maintained as condensed, phenotypically almost completely unexpressed chromosomal material (Lyon, 1961; Gartler and Andina, 1976). Inactivation occurs early in the embryogenesis of a female individual and arbitrarily affects either the maternal or paternal X-chromosome. Inactivated X-chromosomes are reactivated during normal oogenesis (Gartler et al., 1972; Gartler et al., 1975;

Kratzer and Chapman, 1981), indicating that the inactivated genes can be expressed but are kept in a repressed state in somatic cells. The molecular basis of X-chromosome inactivation has been investigated using DMGT.

We have compared the transformation efficiency of the gene for hypoxanthine phosphoribosyl transferase (HPRT) carried on the active and inactive human X-chromsome (Appendix paper IV). The HPRT gene is susceptible to inactivation and humans heterozygous for HPRT deficiency (the Lesch-Nyhan syndrome, L-N) show a mozaic pattern of HPRT expression in their cultured fibroblasts. After SV40 transformation of fibroblasts from a L-N heterozygote, we obtained clonally derived cell lines carrying the $HPRT^+$ allele either on the active of on the inactive chromosome. DNA isolated from $HPRT^+$ and $HPRT^-$ cells was used in DMGT of the active and inactivated HPRT gene respectively into HPRT deficient mouse LTH1 cells we have isolated. Transformants expressing human HPRT were obtained with both DNA's. The transformation frequency observed with the active and the inactivated HPRT gene was 10^{-6} and $5x10^{-7}$ respectively. These experiments demonstrate that the HPRT gene on the inactivated human X-chromosome can be expressed efficiently after DMGT. In similar experiments, Venolia and Gartler (1983) have found that the inactivated HPRT gene was transformed at least 25 times less efficient than the active HPRT gene (transformation frequencies of $2x10^{-8}$ and $5x10^{-7}$ respectively). Although there are differences in procedures and cell lines used, it is not obvious how these could account for the marked difference in transformation efficiency of the inactivated HPRT gene observed in these two studies.

In other experiments using DNA isolated from mouse-human hybrid cells with an active or an inactivated human X-chromosome, the inactivated X-DNA was ineffective while transformation with the active HPRT gene was obtained at frequencies ranging from $2.5x10^{-7}$ to $25x10^{-7}$. (Liskay and Evans, 1980; Lester et al., 1982; Venolia et al., 1982). In one experiment using inactivated X-DNA, Lester et al. (1982) did obtain 10 clones (frequency 10^{-7}) which aborted before they could be analyzed for expression of HPRT. In these colonies a transient expression of reactivated HPRT could have occurred. We have also obtained similarly abortive colonies using active as well as inactivated X-DNA (unpublished results). The true nature of such abortive colonies has not been established. However, they have been observed before in experiments aimed at transfer of HPRT (McBride and Ozer, 1973; Graf et al., 1979; Lester et al., 1980). Although a transient

expression of the HPRT gene could account for the generation of abortive colonies, other explanations unrelated to HPRT expression must be considered equally likely.

Chapman et al. (1982) reported that one out of 59 transformants isolated after DMGT of HPRT using DNA isolated from various tissues of adult mice expressed the inactivated allelic HPRT variant these mice carry. The lower frequency in DMGT of the inactivated HPRT has been interpreted to indicate a modification of the DNA in inactive X-chromosomes, such as methylation of cytosine residues. Evidence that methylation may be involved in maintenance of the inactivated state has come from studies using 5-azacytidine, a cytosine analogue which cannot be methylated at the 5 position and therefore leads to demethylation of the DNA in which it is incorporated.

From cultures of mouse-human cell hybrids with an inactive human X-chromosome only, clones expressing human HPRT can be isolated after treatment with 5-azacytidine at frequencies up to 10^3 times the spontaneous frequency (Mohandas et al., 1981; Lester et al., 1982; Marshall Graves, 1982; Jones et al., 1982). In some clones, expression of another X-linked gene (G6PD or PGK) was also observed. DNA isolated from hybrid cells with 5-azacytidine reactivated HPRT functions in DMGT of that gene (Venolia et al., 1982; Lester et al., 1982). This was interpreted to indicate that the 5-azacytidine treatment has caused a structural change, possibly hypomethylation, in the DNA at or near the formerly inactivated HPRT gene which results in expression of the gene.

Differences in the cell systems used in the studies mentioned may have contributed, at least in part, to the observed differences in the capacity of active, inactivated and reactivated HPRT to function in DMGT. Studies of X-chromosome methylation including unsuccessful attempts at 5-azacytidine reactivation in primary human fibroblasts have indicated that if there is a relation between methylation and X-chromosome inactivation, this relation could be complex (Wolf and Migeon, 1982).

4. CONCLUSIONS AND PROSPECTS

Injection techniques have been developed for the introduction of biologically active macromolecules (proteins, RNAs, DNAs) into living eukaryotic cells in culture without compromizing cell viability. Although procedures such as microneedle injection may seem rather traumatic for cells, there is ample evidence indicating that many cellular functions are not seriously disturbed by the treatment. Also, the injected material is usually biologically active. Thus the techniques offer novel methods to study the biological activity of a macromolecule as well as the cellular process-(es) it is involved in. Since isolated material is used, it is often possible to manipulate the molecules (e.g. providing them with a traceable label, purification, biochemical characterization or modification) to suit the specific investigation undertaken. Furthermore, microinjection techniques generally allow a considerable freedom in the choice of recipient cell, permitting the selection of the cell type best suited to the investigation. Microinjection techniques have thus evolved rapidly into very versatile experimental approaches useful for the study of various questions addressing the cell biology of eukaryotic cells and organisms.

However, with respect to gene transfer using genomic DNA or metaphase chromosomes isolated from eukaryotic cells as donor genetic material, microinjection has not been applied very successfully and incubation of cultured mammalian cells with that donor genetic material under appropriate conditions remains the method of choice in the transfer of functional eukaryotic genes. Transient expression of donor genes, measurable during the first days after transfer, can occur in a relatively large fraction of the recipient cell population. Cells with a more permanent mode of expression arise at such a low frequency that sensitive systems for their selection have to be employed. Thus, transfer has in general been restricted to selectable marker genes. Most details of the events in gene transfer are still unclear, but it is evident that recipient cells can process donor genetic material in various ways.

Besides degradation, ligation (Miller and Tamin, 1983) as well as homologous (Robert de Saint Vincent and Wahl, 1983; Small and Scangos, 1983) and non-homologous (Folger et al., 1983) recombination of donor DNA sequences mutually and with host cell chromosomal sequences (Goodenow et al., 1983) have been observed. In CMGT, these processes can involve long

donor sequences since cotransfer studies using genes of known proximity indicate that, on average, a sequence of about $5x10^6$ nucleotide base pairs can remain intact during transfer. However, intact sequences at least an order of magnitude longer (macrotransgenomes) have also been observed in some transformants and shorter sequences may be retained intact at a high frequency. The maximal size of donor sequences retained intact after DMGT is in the order of 10^5 nucleotide base pairs, a high estimate of the average size of isolated donor DNA molecules. The initial size and the structural composition of donor genetic material may influence the ultimate size of intact donor sequences as it is conceivable that non-DNA chromosomal constituents or helix folding offer some protection against breakage and degradation of donor chromosomal DNA. In both CMGT and DMGT, donor sequences can be ligated and recombined into compound transgenomes. Linkage appears to occur at random, creating novel gene sequences. These can be very useful (for example in generating stable cotransformants of a non-selectable gene) but they can also complicate linkage studies of syntenic genes by creating spurious synteny relationships.

A transformant may contain multiple identical or different transgenomes. A transgenome can be propagated independently (unstable transformant) or covalently linked to host cell chromosomes (stable transformant). In most unstable transformants, the mechanisms of propagation of the transgenome are obscure and a transgenome loss-rate of 10^{-1} to 10^{-2} per cell generation is observed. Stabilization appears to occur at a random moment in the history of the transformant by integration into a host cell chromosome. The integration process did not seem to have any site-specificity. However, recent evidence (Goodenow et al., 1983) indicates that very precise homologous recombination between exogenous and host cell chromosomal DNA sequences is possible but the generality of this phenomenon has yet to be established. It is plausible that a site specificity of transgenome integration does exist but is usually masked by the complexity of the eukaryotic genome, where multiple sequences of sufficient homology with transgenome sequences may exist. As has been proposed before (Klobutcher and Ruddle, 1981), the multiplicity of transgenome integration sites could be advantageously used in the development of panels of stable transformant cell lines with a selectable gene integrated into previously unselectable linkage groups. Such panels would be useful for further regional mapping of chromosomes, using CMGT and would also facilitate assignment of genes to

chromosomes in cell hybridization studies by providing a selectable marker on chromosomes which previously did not have such a marker.

Isolation of a number of mammalian genes has been accomplished via the cloning of a complementary DNA (cDNA) after immunoprecipitation of polyribosomes, or other methods, and subsequent use of this cDNA in screenings of genomic clone banks. The alternative approach using DMGT can be used for genes which are not represented in relative abundance in cellular mRNA and should allow the isolation of selectable or otherwise indentifiable genes which can be stably transferred (see for examples Perucho et al., 1980; Lowy et al., 1980; Bartram, 1984). In these experiments cotransfer with vectors containing dominant selectable marker genes (e.g. Westerveld et al., 1984) may facilitate the selection of high transfer clones. These transfer techniques not only provide a tool to isolate eukaryotic genes, but also allow these genes - manipulated at will by the investigator - to be introduced into cells of various genetic make-up in order to investigate the regulation of gene expression.

A wide range of applications of the gene transfer methodology is evident but the generality of many phenomena observed will have to be established and the rather narrow base of selectable genes and suitable recipient cell lines will have to be extended if this methodology is to be used to its full potential. Substantial variation has been observed in both transformability, even for closely related cell lines, as well as in the effect of adjuvants such as DMSO. Therefore, the conditions for optimal transfer will have to be determined separately for every new cell system introduced. The use of cloned dominant-acting resistance markers should be helpful in this respect.

Although many details of the gene transfer process remain to be elucidated, this technique has, in combination with recombinant-DNA and other molecular biological techniques, evolved in less than a decade from the first basic methodological studies to exciting applications in the analysis of the genetic organization of mammalian cells. Further understanding of the cellular processes involved and refinement of the gene transfer techniques will extend the utility and experimental control of the methodology.

SUMMARY

This dissertation describes celgenetic investigations in which experiments were performed aimed at the complementation of genetic defects in mammalian cells cultured in vitro. Complementation at the level of the cellular phenotype was performed by the introduction of cell extracts or proteins. Complementation at the level of the cellular genotype was accomplished in gene transfer experiments using cellular DNA or metaphase chromosomes isolated from human cells. In chapters 1 through 4 a comparative review of the methods used and relevant literature is presented and the research performed is discussed in this context.

A further report of this research is given in the appended scientific papers. Protein solutions were microinjected into living xeroderma pigmentosum (XP) cells deficient in DNA excision repair in order to investigate the utility of the technique for the correction of the mutant phenotype displayed by these cells (a reduced rate of unscheduled DNA synthesis, UDS). Appendix paper I describes the development of a microinjection assay procedure in which solutions of the prokaryotic DNA repair enzymes Micrococcus luteus UV-endonuclease and T4 endonuclease V were tested for their ability to restore UDS in XP cells. The results show that the action of these enzymes can compensate at least in part for the UDS deficiency in cells of all 9 XP-complementation groups tested (A through I). Also, further support was given to the hypothesis that the excision repair process in these cells is impaired at or before the incision step.

Appendix paper II describes the use of the developed microinjection assay to investigate extracts of DNA repair proficient human cells for factors involved in XP. A putative protein activity was discovered which specifically corrects the UDS deficient phenotype of cells belonging to XP complementation group A.

Appendix paper III concerns gene transfer experiments designed to monitor a complementation of the mutant genotype of mouse cells deficient in thymidine kinase (TK) or hypoxanthine phosphoribosyl transferase (HPRT) by the introduction of cellular DNA or metaphase chromosomes isolated from human cells. It was found that chromosome-mediated gene transfer resulted in a higher transfer frequency of selected marker genes and a higher incidence of cotransfer of nonselected syntenic genes than when DNA-mediated gene transfer was performed in the same cell system. The analysis of trans-

formants expressing human thymidine kinase (TK1) for the co-expression of the syntenic genes for human galactokinase (GALK) and acid alpha-glucosidase (GAA) allowed the tentative ordering of CENTROMERE-GALK-TK1-GAA on human chromosome 17.

In somatic cells of female mammals, one of the two X-chromosomes present is genetically almost completely inactive. Appendix paper IV describes an investigation of the genotypic complementation of HPRT deficient mouse cells by gene transfer using DNA isolated from inactivated human X-chromosomes. Gene transfer experiments were performed in parallel with DNA preparations isolated from each of two populations of human cells which carried an intact HPRT gene on the active respectively the inactivated X-chromosome. The active HPRT gene and the inactivated HPRT gene were found to be expressed with almost the same frequency in transformants, indicating that the molecular structure of the DNA in the inactivated HPRT gene allows efficient expression after gene transfer.

SAMENVATTING

Dit proefschrift beschrijft celgenetisch onderzoek aan de hand van experimenten gericht op de komplementatie van genetische defekten in in vitro gekweekte zoogdiercellen. Enerzijds betrof dit de komplementatie op het niveau van het fenotype van de cellen door de introduktie van celextrakten of eiwitten. Anderzijds werd komplementatie op het niveau van het genotype van de cellen bewerkstelligd in genoverdrachts experimenten met cellulair DNA of met metafase chromosomen geïsoleerd uit humane cellen. In de hoofdstukken 1 tot en met 4 worden de gebruikte technieken en relevante literatuur gegevens vergelijkenderwijs besproken en wordt het uitgevoerde onderzoek in dit kader bediscussieerd.

In de als appendix toegevoegde publikaties wordt nader verslag gedaan van dit onderzoek. De microinjectie van eiwitoplossingen in levende xeroderma pigmentosum (XP) cellen deficiënt in DNA excisie herstel werd uitgevoerd om na te gaan of hiermee een korrektie kan worden verkregen van het mutante fenotype dat deze cellen vertonen (een verlaagd niveau van unscheduled DNA synthesis, UDS). Appendix publikatie I beschrijft de ontwikkeling van een microinjectie test waarin oplossingen van de prokaryotische DNA herstel enzymen Micrococcus luteus UV-endonuclease en T4 endonuclease V getest werden op hun vermogen om de UDS in XP cellen te herstellen. De resultaten tonen aan dat de werking van deze enzymen ten minste gedeeltelijk kan kompenseren voor de UDS deficiëntie in cellen van alle 9 onderzochte XP komplementatie groepen (A tot en met I). Ook werd de hypothese verder ondersteund dat deze cellen de incisiestap van het excisieherstel proces niet goed kunnen uitvoeren.

Appendix publikatie II beschrijft het gebruik van de ontwikkelde microinjectie test om extrakten van DNA-herstel proficiënte humane cellen te onderzoeken op faktoren die bij XP betrokken zijn. Een aktiviteit (vermoedelijk een eiwit) werd geïndentificeerd welke specifiek het UDS-deficiënte fenotype in cellen van XP komplementatiegroep A kan korrigeren.

Appendix publikatie III betreft gen overdrachtsexperimenten waarin de komplimentatie werd nagegaan van het mutante genotype van muizecellen deficiënt in thymidine kinase (TK) of hypoxanthine fosforibosyl transferase (HPRT) door introduktie van DNA of metafase chromosomen geïsoleerd uit humane cellen. Gevonden is dat genoverdracht via geïsoleerde chromosomen leidt tot een hogere overdrachtsfrequentie van geselekteerde markergenen

en een vaker vóórkomen van de gelijktijdige overdracht van gekoppelde niet-geselekteerde genen dan wanneer in hetzelfde celsysteem geïsoleerd DNA wordt gebruikt. Door transformanten, die geselekteerd waren op grond van de expressie van humaan thymidine kinase (TK1), te onderzoeken op expressie van de gekoppelde genen voor humaan galaktokinase (GALK) en zure alfa-glucosidase (GAA) werden gegevens verkregen waaruit de genvolgorde CENTRO-MEER-GALK-TK1-GAA op het menselijk chromosoom 17 kon worden afgeleid.

In de lichaamscellen van vrouwelijke zoogdieren is één van de twee aanwezige X-chromosomen genetisch vrijwel geheel inaktief. Appendix publikatie IV beschrijft onderzoek naar de genotypische komplementatie van muizecellen deficiënt in HPRT door genoverdracht via DNA van menselijke geïnaktiveerde X-chromosomen. Overdrachts experimenten werden in parallel uitgevoerd met DNA dat geïsoleerd was uit ieder van twee populaties humane cellen waarin zich een intakt HPRT gen bevond op respektievelijk het aktieve X-chromosoom dan wel het geïnactiveerde X-chromosoom. Het aktieve HPRT locus en het geïnaktiveerde HPRT locus werden met nagenoeg dezelfde frequentie tot expressie gebracht in transformanten, hetgeen aangeeft dat de molekulaire struktuur van het DNA in het geïnaktiveerde HPRT locus een efficiënte expressie na genoverdracht toelaat.

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CURRICULUM VITAE

Schrijver dezes is geboren op 27 oktober 1946 in Nijmegen. De middelbare schoolopleiding ving hij aan in 1958 aan het Gymnasium Haganum te 's-Gravenhage en in december van dat jaar emigreerde hij naar Nieuw Zeeland. In december 1963 werd het Endorsed School Certificate behaald aan het Avondale College, Auckland, Nieuw Zeeland, waarna de terugkeer naar Nederland in april 1964 volgde.

Van september 1964 tot januari 1966 was hij als analist werkzaam bij de (toenmalige) Kon. Nederlandse Gist- en Spiritusfabriek te Delft. De militaire dienstplicht werd van oktober 1966 tot mei 1968 vervuld als ziekenverpleger/laborant bij de Kon. Marine. Van augustus 1968 tot september 1970 werkte hij als researchassistent bij het Medisch Biologisch Laboratorium, destijds van de Rijksverdedigingsorganisatie TNO te Rijswijk, Z.H. In avondstudie zijn opleidingen gevolgd voor leerling analist chemische richting, leerling analist medische richting en biochemische laboratoriumassistent, welke diploma's respectievelijk in 1966, 1968 en 1970 zijn behaald.

In 1970 werd de studie Scheikunde aangevangen aan de Rijks Universiteit te Leiden. Het kandidaatsexamen S2 (biochemie) werd in juni 1974 afgelegd en het doctoraalexamen in januari 1977 met als hoofdvak Biochemie en bijvakken Moleculaire Genetica en Stralenbescherming. Het diploma Stralingsdeskundige C en de onderwijsbevoegdheid Scheikunde werden hierbij ook verworven.

Van maart 1977 tot december 1981 is onder leiding van Prof.Dr. D. Bootsma het in dit proefschrift beschreven onderzoek verricht op de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam met -gedurende de eerste vier jaren- financiële ondersteuning van FUNGO, Stichting voor Medisch Wetenschappelijk Onderzoek. Ook is in deze periode bijgedragen aan het onderwijs in de Celbiologie en Histologie.

Sinds maart 1982 wordt aan de Faculteit der Geneeskunde van de Vrije Universiteit te Amsterdam gewerkt aan de produktie van monoklonale antistoffen gericht tegen diverse antigenen.

APPENDIX PAPER I

MICROINJECTION OF MICROCOCCUS LUTEUS UV-ENDONUCLEASE RESTORES UV-INDUCED UNSCHEDULED DNA SYNTHESIS IN CELLS OF 9 XERODERMA PIGMENTOSUM COMPLEMENTATION GROUPS.

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SUMMARY

The UV-induced unscheduled DNA synthesis (UDS) in cultured cells of excision deficient xeroderma pigmentosum (XP) complementation groups A through I was assayed after injection of Micrococcus luteus UV-endonuclease using glass microneedles. In all complementation groups a restoration of the UV-induced UDS, in some cells to the repair proficient human level, was observed. Another prokaryotic DNA repair enzyme, T4 endonuclease V, restored the UV-induced UDS in a similar way after microinjection into XP cells. Since both enzymes specifically catalyse only the incision of UV-irradiated DNA, we conclude that this activity is impaired in cells of all 9 excision deficient XP complementation groups tested.

Mutation Res., in press.

INTRODUCTION

The autosomal recessive human disorder xeroderma pigmentosum (XP) is characterized by an extreme sensitivity of the skin to sunlight, predisposition to skin cancer and frequently neurological abnormalities (see Kraemer, 1983 for a review). Cultured fibroblasts of most XP patients are deficient in the removal of ultraviolet light (UV) induced pyrimidine dimers from their DNA and show a decreased rate of unscheduled DNA synthesis (UDS, Cleaver, 1968; Bootsma et al., 1970). Using cell fusion techniques, these excision deficient XP cells have been classified into 9 genetically distinct complementation groups, designated A through I. (Kleijer et al., 1973; de Weerd-Kastelein et al., 1974; Kraemer et al., 1975; Arase et al., 1979; Keijzer et al., 1979; Moshell et al., 1983; Fischer et al., submitted). This abundance of mutually complementary mutations suggests a considerable genetic (and biochemical) complexity of the process in which UV-induced pyrimidine dimers are removed from mammalian DNA. So far, no genes or gene products involved in XP have been isolated.

Tanaka et al. (1975, 1977) have reported that the UDS in fibroblasts of XP complementation groups A through E can be restored to the level of normal human cells by the introduction of T4 endonuclease V using cell-permeabilization by Sendai virus. Using the same technique, Hayakawa et al. (1981) subsequently found restoration of UV-induced UDS in XP-F group cells. However, in this case correction was less than 50% of the wild type level. Since T4 endonuclease V is known to catalyse the first step (incision) of the prokaryotic DNA repair process (Yasuda and Sekiguchi, 1970; Minton et al., 1975), these results support the conclusion that the excision deficient XP cells used are deficient in the incision of UV-damaged DNA. Cells of XP complementation groups G to I have so far not been investigated in this way.

We have recently used microneedle injection of crude human cell extracts into cultured XP fibroblasts to identify proteins in these extracts that are able to restore the UV-induced UDS of the injected cells (de Jonge et al., 1983; Hoeijmakers et al., 1983). Using the same technique we have also studied the effect exerted by various non-human DNA repair enzymes on the UV-induced UDS in excision deficient XP cells. This report concerns the restoration of UV-induced UDS in XP cells representative of XP complementation groups A through I by microinjection of Micrococcus luteus UV-endonuclease. We also report here that T4 endonuclease V introduced into excision deficient XP cells by microinjection can restore the UV-induced UDS of XP complementation groups A and C through G, thus confirming and extending the results of Tanaka et al. (1975) and Hayakawa and coworkers (1981).

MATERIALS AND METHODS

Information on the XP cell lines used in this study is given in Table 1. Cells were cultured in Ham's F10 medium (Flow) with 7.5% foetal calf serum (FCS), 7.5% newborn calf serum, 100 IU penicillin and 100 µg streptomycin per ml. Some fibroblast strains were grown in the same medium containing 15% FCS. Details of the microinjection procedure and subsequent assay for UV-induced UDS have been described previously (de Jonge et al., 1983). In short, homopolykaryons of each XP cell line were generated by cell fusion with the aid of β-propiolacton inactivated Sendai virus (de Weerd-Kastelein et al., 1972) and cultured for at least 3 days to allow completion of DNA replication (S-phase) before they were used for microinjection. For each experiment enzyme was injected into the cytoplasm of at least 50 homopolykaryons (2 to 8 nuclei per cell) using glass microneedles

Table 1.

RELEVANT INFORMATION ON THE CELL LINES USED

Cell line designation	XP complementation group	Relevant characteristics	Reference
XP25RO	A	Excision deficient	Kraemer et al. 1975
XP11BE	B	Excision deficient	Kraemer et al. 1975
XP21RO	C	Excision deficient	Kleijer et al. 1973
XP1BR	D	Excision deficient	W. Keijzer, unpublished results
XP2RO	E	Excision deficient	De Weerd-Kastelein et al. 1974
XP126LO	F	Excision deficient	W. Keijzer, unpublished results
XP2BI	G	Excision deficient	Keijzer et al. 1979
XPCS2	H	Excision deficient	Moshell et al. 1983
XP3MA	I	Excision deficient	Fischer, E., W. Keijzer, O. Popanda, E. Bohnert, L. Edler, E.G. Jung and D. Bootsma, accepted for publication.
C5RO	-	Normal human	

according to the procedure of Graessmann et al. (1980). Relevant data on the injection and injected cells were recorded during microinjection with the aid of a tape recorder.

The assay for UV-induced UDS was performed by UV-irradiation (20 J/m^{-2}), culture in the presence of (^{3}H)-thymidine and visualization of the radioactivity incorporated into repair patches of the DNA by autoradiography. Either assay procedure A, performed as described (de Jonge et al., 1983) was used, or a more sensitive procedure (procedure B) which differed from

procedure A on the following points: the culture medium contained dialysed foetal calf serum (15%), (^{3}H)-thymidine of high specific activity (46-80 Ci/mM^{-1}) and fluoro deoxyuridine (1 μM). These modifications result in a 6-fold increase in sensitivity.

The level of UV-induced UDS was calculated from the average number of silver grains per nucleus (± SEM) determined for the polynucleated cells that survived the microinjection treatment (usually more than 70% of the injected cells). For comparison, the level of UDS was also determined for a population of noninjected homopolykaryons on the same slide. The observed level of UDS was related to the UV-induced UDS of repair proficient C5RO fibroblasts assayed in each experiment as a standard. The wild type UDS level varied between experiments but was always higher than 50 grains per nucleus. The use of homopolykaryons has the advantage that confusion of grains due to UDS with grains due to a short period of S-phase incorporation of (^{3}H)-thymidine is avoided because the nuclei of polykaryons no longer enter S-phase three or more days after fusion (Jaspers et al., 1981; unpublished observations).

M.luteus UV-endonuclease, corresponding with fraction II in the purification method of Carrier and Setlow (1970), was generously provided by Drs. G. van der Schans and L. Roza, TNO, Rijswijk. T4 endonuclease V, purified and stored as described (Seawell et al., 1981), was a generous gift from Dr. A.A. van Zeeland, State University, Leiden. Immediately before use, the T4 enzyme preparation was dialysed for 30 minutes against 1000 volumes of reversed phosphate-buffered saline (RPBS, 4.05 mM Na_2HPO_4; 1.1 mM KH_2PO_4; 140 mM KCl; pH 7.2) in order to remove ethylene glycol (which proved to be lethal to the injected cells).

RESULTS

Homopolykaryons of XP complementation groups A through I were assayed for UV-induced UDS after microinjection of M.luteus UV-endonuclease in the cytoplasm. Quantitative data from these experiments are presented in Table 2. For all complementation groups the microinjected cells displayed a higher mean grain count than noninjected neighbouring homopolykaryons although the degree of stimulation varied between different complementation groups.

Table 2.

LEVELS OF UV-INDUCED UDS IN HOMOPOLYKARYONS AFTER MICRO-INJECTION OF M.LUTEUS UV-ENDONUCLEASE

Cell line[1]		UDS[2] (grains per nucleus) as % of wild type ± SEM		
		Non-injected	Injected	
C5RO		103 ± 4	100	(A)
XP25RO	(A)	1 ± 1	79 ± 4	(B)
XP11BE	(B)	9 ± 1	54 ± 3	(B)
XP21RO	(C)	20 ± 1	37 ± 3	(A)
XP1BR	(D)	17 ± 1	55 ± 2	(B)
XP2RO	(E)	50 ± 2	65 ± 5	(A)
XP126LO	(F)	19 ± 1	50 ± 2	(B)
XP2BI	(G)	2 ± 1	23 ± 2	(A)
XPCS2	(H)	36 ± 2	86 ± 4	(B)
XP3MA	(I)	14 ± 1	55 ± 3	(B)

1) In parenthesis, the XP complementation group.

2) The UDS procedure used (see Materials and Methods for details) is given between brackets.

The grain count also varied between different experiments with the same cells (compare Tables 2 and 3) as well as between individual homopolykaryons injected in the same experiment (data not shown). This variation probably reflects differences in the amount of enzyme injected.

Figure 1 shows a trinucleated XPCS2 cell (XP complementation group H) injected with M. luteus UV-endonuclease, together with 3 noninjected mononucleated XPCS2 fibroblasts (one of which is in S-phase) after UV-irradiation and the autoradiographic assay for UDS. Many autoradiographic grains can be seen above the nuclei of the injected tri-karyon, whereas considerably fewer grains are observed over the nuclei of the noninjected cells. For the tri-karyon shown, the average grain count per nucleus was 95% of the grain count of repair proficient C5RO cells used as a standard in this experiment.

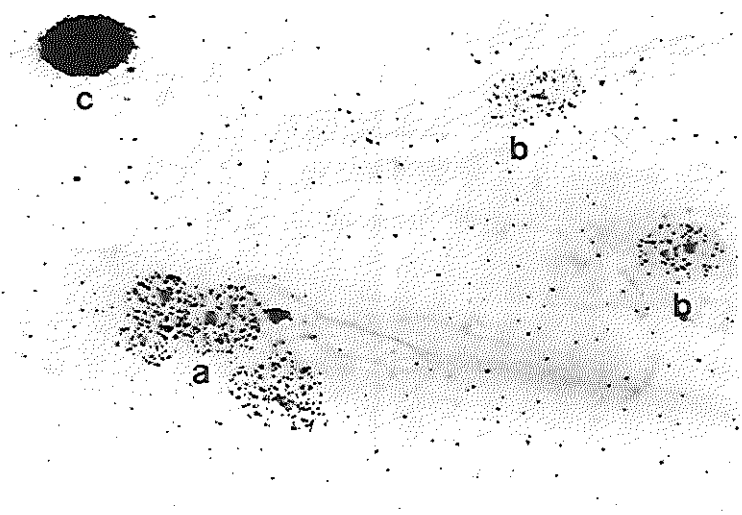

Fig. I Photomicrograph of a XPCS2 homopolykaryon (containing 3 nuclei) after microinjection of M. luteus UV-endonuclease, assay for UDS and autoradiography. See Materials and Methods for experimental details. A: injected homo tri-karyon, B: noninjected monokaryons. C: monokaryon in S-phase.

In a series of experiments carried out with cells from XP complementation groups A and G the specificity of the observed increase in grain count was investigated. As shown in Table 3, microinjection of M.luteus UV-endonuclease did not noticeably affect the UV-induced UDS in repair proficient C5RO fibroblasts, indicating that this is not a phenomenon which acts supplementary to the regular UDS. The appearance of grains above nuclei of injected XP cells was observed only after UV-irradiation of the cells. This demonstrates that the grains are due to UV-induced UDS and not

Table 3.

LEVELS OF UDS IN HOMOPOLYKARYONS AFTER DIFFERENT TREATMENTS

Cell line[1]	Microinjection	UV-irradiation	UDS[2] as % of wild type ± SEM
C5RO	-	+	100
C5RO	M.luteus UV-endonuclease	+	101 ± 4
XP25RO (A)	-	+	2 ± 1
XP25RO (A)	RPBS[3]	+	2 ± 1
XP25RO (A)	M.luteus UV-endonuclease	-	1 ± 1
XP25RO (A)	M.luteus UV-endonuclease	+	56 ± 6
XP2BI (G)	-	+	2 ± 1
XP2BI (G)	M.luteus UV-endonuclease	-	1 ± 1
XP2BI (G)	M.luteus UV-endonuclease	+	39 ± 4

1) The XP complementation group is given in parenthesis

2) UDS was measured as grains per nucleus using Assay procedure A.

3) RPBS = Reversed phosphate buffered saline, see Materials and Methods.

to incorporation of ^{3}H-TdR caused eg. by contaminating nuclease or DNA polymerase activities in the enzyme preparation. Furthermore, when buffer was injected instead of enzyme no increase in UDS was observed, showing the requirement of the endonuclease for stimulation of UDS. Identical results were obtained with mononucleated and polynucleated cells (data not shown) and the number of nuclei in an injected homopolykaryon did not have a noticeable effect on the level of UDS reached since the mean grain counts for cells with 2,3,4 or 5 and more nuclei did not differ significantly from the mean of the total population (Figure 2). These experiments demonstrate that the UV-induced UDS in excision deficient XP fibroblasts can be restored by microinjection of M. luteus UV-endonuclease.

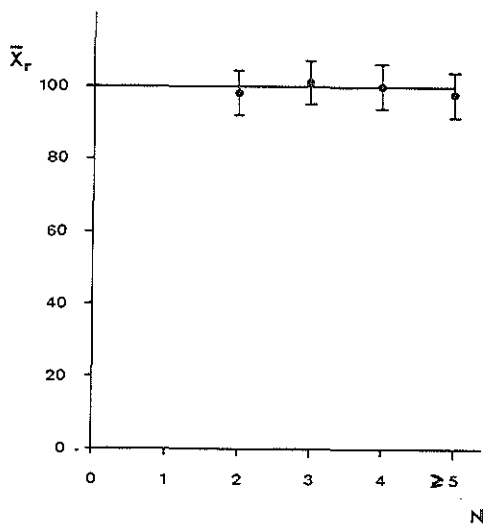

Fig. 2. The level of UV-induced UDS in relation to the number of nuclei in microinjected homopolykaryons. The grain count of an injected homopolykaryon was expressed as a percentage of the average grain count calculated for the total population, to obtain the relative grain count, X_r. Subsequently, a classification was made according to the number of nuclei per cell (N) and for each category (bi-, tri-, tetra- and polykaryons) the mean of the relative grain counts, $\bar{X}_r$, was determined ± SEM.
This method of calculation was followed because the results of different experiments (with different values for the average grain count of the total population) were combined in order to obtain sufficient cells of each category (bikaryons, 53 cells; trikaryons, 32 cells; tetrakaryons, 14 cells and polykaryons, 25 cells).

As shown in Table 4, T4 endonuclease V was also able to restore the UV-induced UDS after microinjection into homopolykaryons of the XP complementation groups tested. As was found with M.luteus UV-endonuclease, a number of individual cells was complemented to wild-type UDS level but the average number of grains was lower than the wild-type level.

Table 4.

LEVELS OF UV-INDUCED UDS IN XP HOMOPOLYKARYONS AFTER MICROINJECTION OF T4 ENDONUCLEASE V.

Cell line[1]	UDS[2] (grains per nucleus) as % of wild type ± SEM	
	Non-injected	Injected
XP25RO (A)[3]	2 ± 1	23 ± 1
XP25RO (A)[3,4]	2 ± 1	18 ± 1
XP21RO (C)	17 ± 1	31 ± 1
XP1BR (D)	28 ± 1	39 ± 2
XP2RO (E)	50 ± 2	65 ± 3
XP126LO (F)	17 ± 1	61 ± 3
XP2BI (G)	3 ± 1	56 ± 2

1) In parenthesis, the XP complementation group.

2) Unless stated otherwise assay procedure A was used (see Materials and Methods).

3) UDS assay according to procedure B (see Materials and Methods).

4) T4 endonuclease preparation 1:1 diluted with injection medium.

DISCUSSION

The experiments presented here demonstrate a correction of the excision repair deficiency in cells of all XP complementation groups after microinjection of the prokaryotic DNA repair enzyme Micrococcus luteus UV-endonuclease and of T4 endonuclease V in the cases tested. These results confirm and extend the findings of Tanaka et al. (1975, 1977) and Hayakawa et al. (1981). These authors reported a restoration of UV-induced UDS in XP cells of group A through F after introduction of T4 endonuclease V into XP cells permeabilized by concomitant treatment with Sendai virus. The correction of XP cells of group F was only to 50% of the wild-type (wt) UDS in spite of the fact that the amount of T4 enzyme used was 3-fold in excess of the concentration required for the correction of XP group A cells to the wt level (Hayakawa et al., 1981).

In our experiments the average level of UV-induced UDS varied between cell strains and to some extent also between experiments with the same cells. Usually UDS was restored to less than the level of normal human cells, although a number of individual injected cells did display wt grain numbers. When the results of Tanaka et al., (1975) are taken into account it seems probable that -at least in the case of XP-cells of group A through E- limiting amounts of enzyme have been injected into most of the cells. This is supported by the finding that dilution of the T4 endonuclease preparation yielded a lower level of UDS in XP-A fibroblasts than the non-diluted sample (Table 4). Although quantitative comparison of UDS levels obtained after microinjection is difficult due to the fact that the injected volume is not constant, this finding suggests that in the case of T4 endonuclease the amount of enzyme is at least one of the factors that limit the level of correction.

With cells of group F, UDS levels were obtained comparable to those found by Hayakawa et al. (1981). It has to be established whether the UDS found in XP cells of group G through I was limited by the amount of enzyme injected or whether the maximally obtainable level of correction was reached in those cells.

As mentioned by Hayakawa et al. (1981) in relation to XP group F cells, a possible explanation for a correction to less than the wt UDS level could be that not all lesions are readily accessible to the exogenous enzyme, e.g. because the lesions are masked by an inactive protein or repair complex. The work of Mortelmans et al. (1976) and Kano and Fujiwara (1983) suggests that the defect in cells of some XP complementation groups may reside in an altered chromatin structure. It is possible that in those cases the injected prokaryotic endonucleases are not able to reach some of the dimers during the assay period, despite their small size of only 16-18 kD (Nakabeppu and Sekiguchi, 1981; Grafstrom et al., 1982).

The fact that cells of all 9 excision deficient XP complementation groups are corrected at least partly by enzymes that incise DNA near pyrimidine dimers suggests that the defect in all these XP cell strains resides at stages before or at the incision step of the DNA excision repair pathway. If the number of complementation groups in XP actually reflects the number of polypeptides required in these early stages of repair, it is noteworthy that a function which involves at least 9 polypeptides in human cells can be performed by a prokaryotic enzyme on its own. However, in this respect it should be taken into account that the microbial endonucleases used here have only pyrimidine dimers as a substrate (Friedberg et al., 1981) whereas the excision repair process in human cells acts on a much broader spectrum of DNA lesions (Grossman, 1981).

Both M. luteus UV-endonuclease and T4 endonuclease V catalyse the incision of UV-irradiated DNA in a two-step reaction: the pyrimidine dimer is attacked successively by a dimer-DNA glycosilase which hydrolyses the 5' glycosyl bond of the dimer and by an apyrimidinic endonuclease which cleaves the phosphodiester backbone 3' of the apyrimidinic site (Haseltine et al., 1980; Nakabeppu and Sekiguchi, 1981; Grafstrom et al., 1982).
It has not been established whether one of these activities alone is sufficient for the correction of all XP excision repair defects, or whether the concerted action is required. The mechanism of action of the equivalent human UV-endonuclease is not known. It is possible that the normal human enzyme yields reaction products different from the microbial enzymes we have used, as has been found for the uvrA, B, C complex of Escherichia coli (Sancar and Rupp, 1983). Therefore, the UDS observed in excision deficient XP cells treated with M. luteus UV-endonuclease or T4 endonuclease V could reflect a DNA repair pathway involving steps not normally followed during excision repair in human cells.

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APPENDIX PAPER II

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Microinjection of human cell extracts corrects xeroderma pigmentosum defect

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Cultured fibroblasts of patients with the DNA repair syndrome xeroderma pigmentosum (XP) were injected with crude cell extracts from various human cells. Injected fibroblasts were then assayed for unscheduled DNA synthesis (UDS) to see whether the injected extract could complement their deficiency in the removal of u.v.-induced thymidine dimers from their DNA. Microinjection of extracts from repair-proficient cells (such as HeLa, placenta) and from cells belonging to XP complementation group C resulted in a temporary correction of the DNA repair defect in XP-A cells but not in cells from complementation groups C, D or F. Extracts prepared from XP-A cells were unable to correct the XP-A repair defect. The UDS of phenotypically corrected XP-A cells is u.v.-specific and can reach the level of normal cells. The XP-A correcting factor was found to be sensitive to the action of proteinase K, suggesting that it is a protein. It is present in normal cells in high amounts, it is stable on storage and can still be detected in the injected cells 8 h after injection. The microinjection assay described in this paper provides a useful tool for the purification of the XP-A (and possibly other) factor(s) involved in DNA repair.

Key words: xeroderma pigmentosum/microinjection/phenotypic correction/ DNA repair enzymes

Introduction

Xeroderma pigmentosum (XP), an autosomal recessive human disease, is characterized by an extreme sensitivity of the skin to sunlight, a very high incidence of skin cancer and frequently neurological abnormalities (for a review, see Kraemer, 1980). Cultured skin fibroblasts from most XP patients are deficient in the excision repair of u.v.-induced pyrimidine dimers from their DNA and this is thought to be the primary biochemical defect. As a consequence, excision-deficient XP cells show a decreased rate of unscheduled DNA synthesis (UDS), monitored as the incorporation of [^{3}H]TdR in cells in the G1 and G2 phase of the cell cycle after u.v. irradiation (Cleaver, 1968; Bootsma *et al.*, 1970).

Using cell hybridization, seven complementation groups have been identified so far within the XP syndrome (De Weerd-Kastelein *et al.*, 1972; Keijzer *et al.*, 1979). This extensive genetic heterogeneity indicates that the repair of u.v.-induced DNA lesions in mammalian cells follows a complex pathway. Efforts to unravel this pathway and to characterize the factors involved have been undertaken using essentially non-viable systems: such as isolated nuclei (Smith and Hanawalt, 1978), cell-free extracts obtained by osmotic disruption (Ciarrocchi and Linn, 1978), sonication (Mortelmans *et al.*, 1976) or permeabilized cell systems (Roberts and Lieberman, 1979; Dresler *et al.*, 1982). Up to now these studies have not resulted in the identification of such factors. We have chosen to use the living XP cell as a 'test tube' and to try to provide it with the lacking factor using microinjection. Microinjection into living cells *via* glass micro-needles has been applied successfully to study cellular processes (Kreis *et al.*, 1979; Burridge and Feramisco, 1980) and the activity of various biological macromolecules (Graessmann and Graessmann, 1976; Graessmann *et al.*, 1980b; Liu *et al.* 1979; Capecchi, 1980; Anderson *et al.*, 1980). In the cited studies, pure or purified material was injected. We have injected crude extracts prepared from human cells. Here we report the finding of an activity in these extracts which corrects the repair defect in cells belonging to XP complementation group A. This activity is abundantly present in normal cells, inactivated by the action of proteinase K and specific for XP complementation group A.

Results

Correction of the XP-A defect by microinjection

Crude extracts prepared from repair-proficient cells were injected into the cytoplasm of at least 3-days old XP25RO homopolykaryons (XP complementation group A) using standard microinjection procedures. Usually each extract was injected into at least 50 homopolykaryons of which >70% survived the injection. The ability of the surviving cells to perform excision repair was tested by determining their rate of UDS. Briefly, this is carried out as follows: after injection, the cells are irradiated with a saturating dose of u.v.; they are then cultured in the presence of [^{3}H]thymidine and the radioactivity incorporated in the repair patches is visualized by autoradiography.

A u.v.-exposed XP25RO homopolykaryon, injected with a HeLa cell extract is shown together with two non-injected monokaryons in Figure 1a, and repair-proficient control fibroblasts in Figure 1b (C5RO, non-injected). Quantitative data on UDS after injection of various extracts and treatments are summarized in Table I. Although there is considerable variation in the level of UDS with different extracts, the UDS of >90% of the surviving injected XP-A cells is significantly above that found without injection (i.e., at least 2x the highest level found in non-injected cells in the same preparation). With some extracts, the UDS of many injected cells was close to the wild-type (C5RO) UDS level (compare, for example, the cells of Figure 1a and b, see also some extracts in Table I and II). Even giant polykaryons with >20 nuclei were found that displayed wild-type UDS levels after a single microinjection. We attribute the spreading of UDS values with different HeLa extracts (see Table I), at least in part, to uncontrolled variation in the quality of extracts.

A number of control experiments confirmed that the grains observed above nuclei of injected cells were due to u.v.-

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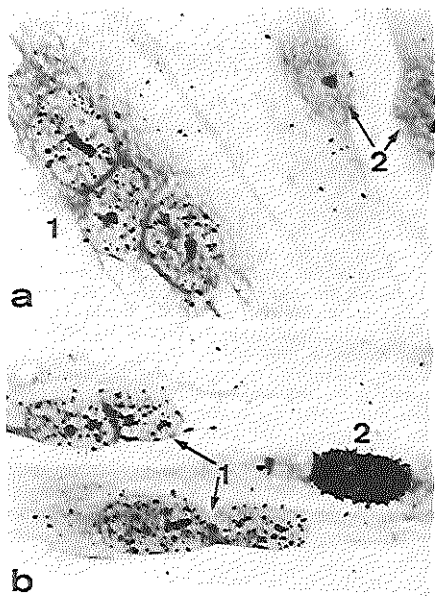

Fig. 1. (a) Micrograph of a XP25RO homopolykaryon (containing three nuclei) after microinjection of a HeLa extract, followed by assay for UDS. See Materials and methods for experimental details. (1) Injected XP25RO homotrikaryon. (2) Uninjected XP25RO monokaryons. Silver grains above nuclei indicates UDS. (b) Micrograph of a control (C5RO) homopolykaryon after assay for UDS. (1) Homobikaryons showing UDS. (2) Monokaryon showing S-phase labelling.

induced UDS and not to some artefact of the procedure. (i) The increase in the level of nuclear labeling was dependent on injection of the extract and on u.v. irradiation since no increase was observed when the extract was replaced by buffer (not shown) or when the u.v. irradiation was omitted (Table I). (ii) Neither the injection procedure itself nor the injected HeLa extract noticeably affected the u.v.-induced UDS of repair-proficient control polykaryons (Table I). (iii) A difference in UDS between injected monokaryons and injected homopolykaryons was not observed (data not shown). (iv) Finally, UDS was the same whether recipient cells were u.v. irradiated before or after injection (Table I) ruling out the possibility that the UDS was due to u.v. irradiation and repair of any injected DNA or chromatin present in the extract.

Characterization of the correcting activity

The factor in the HeLa extract responsible for the restoration of UDS is ubiquitous among human cells and acts specifically on the repair defect of complementation group A. No significant stimulation of UDS was found when the extract was injected into cells belonging to complementation groups C, D or F (Table I). On the other hand, HeLa factor did correct the defect in XP2CA, a XP-A cell line unrelated to XP25RO (Table I). Extracts prepared from an SV40-transformed XP-C cell line (XP8CA C SV2) and an SV40-transformed repair-proficient fibroblast (VH10 SV40) stimulated the UDS of XP25RO to the same extent as HeLa extracts (Table I). On the other hand, five extracts prepared from three different SV40-transformed XP-A cell lines [three from XP12RO SV40, one from XP25RO SV40 and one from XP2OS (SV)] were unable to induce UDS after injection into XP25RO fibroblasts. The result with one extract is presented in Table I. The absence of XP-A correction was not due to any inhibitors of UDS present in the XP-A extracts, since injection of these extracts in repair-proficient fibroblasts did not influence their u.v.-induced UDS (see Table I). Moreover, a 1:1 mixture of XP-A and HeLa extracts stimulated the UDS of XP25RO almost to the same extent as the HeLa extract alone (data not shown). From the foregoing data, we conclude that the correction observed is specific for XP complementation group A. We have also found XP-A correcting activity in extracts prepared from human placenta (Table I) demonstrating that this property is not limited to transformed or cultured cells.

The XP-A correcting factor is reasonably stable on storage. We have not found considerable loss of activity after storage of the extract for 7 weeks at 4°C or for longer periods at −70°C (Table I). In the injected cell its activity can still be detected 8 h after injection (Table I).

To determine whether the factor involved is a protein, the extract was incubated with proteinase K covalently linked to CNBr-activated Sepharose beads. After removal of the immobilized protease by centrifugation and injection of the supernatant into XP25RO polykaryons, the correcting activity was no longer detectable (see Table II). In contrast, activity was retained in a control incubation with beads to which bovine serum albumin (BSA) had been attached. Injection of a 1:1 mixture of proteinase K-incubated extract and untreated extract showed that the loss of UDS-correcting activity was not due to inhibiting factors generated during the proteinase K incubation. Furthermore, treated extract did not affect UDS in normal (C5RO) homopolykaryons. The proteolytic action of the proteinase K beads under these conditions was confirmed by the substantial reduction of two enzymatic activities present in HeLa extracts. The first was glucose-6-phosphate dehydrogenase (G6PD) quantitatively determined in an enzyme assay (see Table II). The second enzyme tested was hypoxanthine phosphoribosyl transferase (HPRT) assayed in a similar way to the XP-A factor, i.e., by microinjection into HPRT-deficient mouse cells. From the foregoing data, we conclude that the XP-correcting factor contains a protein moiety essential for its function.

Discussion

The experiments presented here demonstrate that biological activities in crude (cell) extracts can be assayed by microneedle injection into suitable recipient cells. Using this procedure, we have identified a protein in extracts of normal human cells which specifically corrects the XP-A repair defect. Although the identification of factors involved in DNA repair is possible with the microinjection assay, there are also limitations. One of these is that the activity to be assayed must be present in sufficient amounts in the injected extract. The XP-A correcting protein certainly fulfils this criterion. A single microinjection is sufficient to restore the UDS to the maximal level of repair-proficient cells. Even a

Table I. Levels of u.v.-induced UDS after microinjection of various human cells extracts into XP homopolykaryons

Injected cell line[a]	Extract injected	Experimental details[b]	UDS (grains per nucleus) % of wild-type (± SEM)[c]
C5RO	None	No injection	100
C5RO	HeLa extract-1		92 ± 7
C5RO	XP-A extract[d]		94 ± 7
XP25RO(A)	None	No injection	5 ± 1[e]
XP25RO	HeLa extract-2	No u.v.	3 ± 1
XP25RO	HeLa extract-2		83 ± 6
XP25RO	HeLa extract-2	u.v. before injection	79 ± 5
XP25RO	HeLa extract-3		26 ± 3
XP25RO	HeLa extract-3	UDS 8 h after injection	28 ± 3
XP25RO	HeLa extract-4		37 ± 5
XP25RO	HeLa extract-4	Extract >7 weeks at −70°C	48 ± 4
XP25RO	HeLa extract-4	Extract 7 weeks at 4°C	33 ± 3
XP25RO	XP-C extract[f]		60 ± 4
XP25RO	VH-10 extract[g]		43 ± 3
XP25RO	XP-A extract[d]		3 ± 1
XP25RO	Placenta extract		36 ± 3
XP2CA(A)	None	No injection	5 ± 1[e]
XP2CA	HeLa extract-3		47 ± 4
XP21RO(C)	None	No injection	23 ± 3[e]
XP21RO	HeLa extract-2		21 ± 2
XP3NE(D)	None	No injection	34 ± 3[e]
XP3NE	HeLa extract-3		38 ± 5
XP23OS(F)	None	No injection	15 ± 2[e]
XP23OS	HeLa extract-3		15 ± 3

[a]Between brackets the XP complementation group. For further details on the cell lines used see Table II.
[b]Unless indicated otherwise cells were u.v. irradiated immediately after injection.
[c]The UDS of injected cells is expressed as the % of the UDS of C5RO a repair-proficient control cell line, used as a standard in each experiment. This wild-type UDS level differed between individual experiments but was always >50 grains/nucleus.
[d]Extract prepared from XP12RO SV40.
[e]Residual repair activity.
[f]Extract prepared from XP8CA C SV2.
[g]Extract prepared from VH10 SV40.

3-fold diluted HeLa extract gave clearly detectable correction (unpublished observations). This in itself is an interesting observation, given the fact that the injected volume is very small relative to the volume of the injected polykaryon (we estimate <10%). Furthermore, the concentration of the factor in the extract is considerably lower than in the cells from which the extract was prepared (a factor of 2 to 3 is a minimal estimate). Even ignoring possible loss or inactivation of the XP-A factor during preparation of the extract, the activity of the protein in normal (non-u.v.-irradiated) cells must be at least 20- to 30-fold higher than necessary for maximal UDS activity. This apparent excess of the XP-A correcting component renders it unlikely that the factor is involved in a rate-limiting step in the normal repair process. Our observation that the UDS over the first 2 h after injection is already close to that of control cells indicates that the correcting protein can exert its function rapidly after introduction into the cell. This extends results obtained in cell hybridization (Matsukuma *et al.*, 1981; Giannelli *et al.*, 1982) and cybridization experiments involving XP and control cells (Keijzer *et al.*, 1982). Also, our finding that the activity of the XP-A correcting protein is still detectable 8 h after injection agrees well with data from cybridization experiments in which cytoplasts from normal cells were fused with XP-A fibroblasts (Keijzer *et al.*, 1982).

Phenotypic correction of the XP-A repair defect was also obtained by injection of the prokaryotic enzymes (*Micrococcus luteus* u.v.-endonuclease and T4 endonuclease V (A.J.R. de Jonge *et al.*, in preparation). In these cases correction was not specific: all XP complementation groups were corrected, in agreement with results reported by others who used a permeabilized cell system (Tanaka *et al.*, 1975, 1977; Hayakawa *et al.*, 1981). It appears that these prokaryotic enzymes cause a complete by-pass of all the repair defects in XP. This is not the case for the protein factor described in this paper since the correction was found to be XP-A specific. However, a by-pass of some steps in the repair process, including the step affected in XP-A cells, is not ruled out. In that case the correcting protein should be missing in XP-A as a direct or indirect consequence of the XP-A-deficient step. It is even possible that the injected extract provided two or more (protein) factors responsible for the XP-A correction. If so all these factors should be present in 20- to 30-fold excess in normal cells and all of them should be deficient in XP-A cells.

We favour, therefore, the more simple interpretation that the correction is due to just one component: the gene product deficient in XP complementation group A.

With the microinjection repair assay described here, we are now trying to further characterize and purify this protein.

Materials and methods

Cell lines and culture conditions

Relevant data on the cell lines used in this study are listed in Table III. All cells were cultured in Ham's F10 medium (Flow supplemented with 7.5% fetal and 7.5% newborn calf serum and penicillin and streptomycin (100 μg/ml). Cells to be microinjected were cultured on 0.6 x 0.8 cm pieces of a microscope slide with a 2 mm grid.

Table II. The effect of treatment with proteinase K on the XP-A correcting activity in HeLa extracts.

Treatment of extract	UDS		Enzymatic activity of	
	Grains per nucleus	% of wild-type	HPRT	G6PD
(i) No incubation	47 ± 4	77	+ +	100%
(ii) Proteinase K-beads[a]	3 ± 0.3	5	−	31%
(iii) BSA-beads	40 ± 2	66	+	73%
1:1 mixture (i) and (ii)	16 ± 2	26	+ +	n.d.

Sepharose beads with covalently attached proteinase K or BSA were incubated with aliquots of a HeLa extract. The beads were removed by centrifugation. The supernatant was microinjected into XP25RO polykaryons and UDS was assayed. The enzymatic activity of HPRT was assayed by microinjection into HPRT-deficient mouse LTH-1 cells as described in Materials and methods. The enzymatic activity of G6PD was assayed according to Jongkind (1967). The activity in the untreated aliquot was set at 100%.

[a]The proteinase K-treated extract, injected into control cells resulted in 50 ± 3 grains/nucleus, *versus* 51 ± 2 grains/nucleus observed in non-injected fibroblasts.

n.d. not determined.

Preparation of cell extracts and microinjection

Cultures of HeLa S3 cells (8 – 20 x 10^7 cells) in log phase were harvested by trypsinization or scraping (using a rubber policeman) and washed twice in Na-K reversed phosphate buffered saline (RPBS: 4.05 mM Na_2HPO_4; 1.1 mM KH_2PO_4; 140 mM KCl; pH 7.2). After the second wash the supernatant was removed and the 'dry' pellet (with a volume of 0.2 to >1 ml) was subjected to sonication (six pulses of 10 s with 10 s intervals, at 0°C, using the microtip of a MSE sonicator operating at maximum output). The sonicate was centrifuged for 40 min at 130 000 *g*, at 4°C in a type 50 fixed angle rotor, using a L5-65 Beckman ultracentrifuge. Aliquots of the supernatant were either used directly for microinjection or rapidly frozen in liquid nitrogen and stored at −70°C until use. Under these conditions, extracts can be stored for >7 months without notable loss of XP-A correcting activity. The activity of the extract is sensitive to repeated cycles of freezing and thawing. Crude extracts from other cells were prepared in the same way as described above. Placenta extract was made by sonication of finely cut fresh placental tissue and ultracentrifugation as specified for the HeLa extract.

The crude cell extracts obtained above were microinjected into the cytoplasm of homopolykaryons (see below) *via* glass micro-needles using the procedure described by Graessmann *et al.* (1980a). Relevant data on the injection and the injected cells were recorded during microinjection with the aid of a tape recorder.

Cell fusion and assay of repair activity

Only homopolykaryons were used for microinjection. These were obtained by fusion of cells of an (XP) cell strain in suspension using inactivated Sendai virus at a concentration of 200 HAU/ml as described by De Weerd-Kastelein *et al.* (1972). The fused cell population was cultured for at least 3 days after seeding to allow completion of DNA replication (S-phase) in the homopolykaryons (Jaspers *et al.*, 1981, and unpublished observations). This eliminates possible confusion of radioactive labeling due to a short period of normal DNA replication with that due to UDS. Moreover, the unique morphology of each polykaryon facilitates registration and subsequent re-identification of injected cells. Usually 50 – 100 homopolykaryons each containing 2 to >20 nuclei were injected with the same cell extract. The percentage of cells which died from the injection varied between 5 and 30. The ability of the surviving injected cells to perform UDS was assayed as follows. After injection, cells were irradiated with a saturating dose of u.v. (15 J/m^2; Jaspers and Bootsma, 1982), cultured for 2 h with 10 μCi/ml [^{3}H]thymidine (sp. act. 20 Ci/mmol) and washed, fixed and processed for autoradiography according to Zelle and Bootsma (1980). Exposure time was 1 week. After development, fixation and staining with Giemsa's solution the slides were mounted, the injected cells were relocated and the average number of grains per nucleus (±SEM) was determined to calculate the level of UDS. All data on UDS of injected cells refer to the fraction of cells which survived the microinjection treatment. In some experiments the u.v. irradiation was carried out prior to the microinjection.

Table III. Relevant information on the cell lines used

Cell line designation	XP complementation group	Characteristics	Reference
XP25RO	A	Primary fibroblast	Kraemer *et al.* (1975)
XP25RO SV40	A	SV40-transformed	Kramer *et al.* (1975)[a]
XP2OS (SV)	A	SV40-transformed	Takebe *et al.* (1974)
XP12RO SV40	A	SV40-transformed	De Weerd-Kastelein *et al.* (1972)[b]
XP2CA	A	Primary fibroblast	Hashem *et al.* (1980)
XP21RO	C	Primary fibroblast	Kleijer *et al.* (1973)
XP8CA C SV2	C	SV40-transformed	Hashem *et al.* (1980)[a]
XP3NE	D	Primary fibroblast	de Weerd-Kastelein *et al.* (1976)
XP23OS	F	Primary fibroblast	Arase *et al.* (1979)
C5RO	–	Repair-proficient primary fibroblast	
VH10 SV40	–	Repair-proficient, SV40-transformed fibroblast[a]	
HeLa S3	–		
LTH-1	–	Mouse cell line, HPRT$^-$	de Jonge *et al.* (1982)

[a]These cell lines were transformed with a SV40 *ori*$^-$ fragment (6 – 17, Gluzman *et al.*, 1980, and unpublished results).

[b]SV40 transformation of this cell line was carried out by G. Veldhuizen (Medical Biological Laboratory, Rijswijk).

Studies using proteinase K

Proteinase K (pretreated for 2 h at 37°C to destroy any contaminating DNase or RNase activity) or BSA was covalently linked to CNBr-activated Sepharose beads and after extensive washing with ice-cold RPBS to remove unattached protein, the beads were incubated at 37°C with aliquots of HeLa cell extracts. After 30 min, beads were removed by centrifugation, the supernatant was microinjected with XP25RO homopolykaryons and UDS was assayed as described. The effect of proteinase K on the enzymatic activity of HPRT in the cell extracts was studied by microinjection of the treated extracts into HPRT-deficient mouse LTH-1 cells (de Jonge *et al.*, 1982), followed by culturing in the presence of 10 μCi/ml [^{3}H]hypoxanthine (sp. act. 1 Ci/mmol) for 24 h. Further processing was as described above for injected XP cells. The enzymatic activity of G6PD in the treated extracts was quantitatively determined according to Jongkind (1967). The G6PD activity in the untreated aliquot was set at 100%.

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A P P E N D I X P A P E R III

Cotransfer of syntenic human genes into mouse cells using isolated metaphase chromosomes or cellular DNA

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Summary. Chromosome-mediated gene transfer (CMGT) of the human genes for hypoxanthine phosphoribosyl transferase (HPRT) and cytosol thymidine kinase (TK1) into HPRT deficient mouse A9 cells or TK deficient Swiss mouse 3T3TK$^-$ cells was found to occur at frequencies at least one order of magnitude higher than DNA-mediated gene transfer (DMGT). The frequency of CMGT into 3T3TK$^-$ cells was reduced by more than an order of magnitude by a posttreatment of the recipient cells with dimethyl sulphoxide (DMSO). After CMGT, expression of the non-selected genes coding for galactokinase (GALK) and acid alpha-glucosidase (GAA), both syntenic with TK1, was observed in a number of transformants. From the pattern of cotransfer, a tentative gene ordering of CENTROMERE-GALK-TK1-GAA on human chromosome 17 was deduced. Chromosome-mediated cotransfer of X-linked human phosphoglycerate kinase (PGK) with HPRT was observed in two out of 33 A9 transformants analysed. DNA-mediated cotransfer of a syntenic gene was only observed for GALK, cotransferred with TK1 in two out of 18 TK$^+$ transformants of mouse LTK$^-$ cells. Therefore, with murine cells as recipients of human donor genetic material, CMGT results in a higher frequency of transfer and a higher incidence of cotransfer of syntenic genes than DMGT using cellular DNA in the same cell system.

Introduction

Cotransfer of genes syntenic with the selected marker gene thymidine kinase (TK), or hypoxanthine phosphoribosyl transferase (HPRT) after chromosome-mediated gene transfer (CMGT) has been reported by several laboratories. For example, cotransfer of the gene coding for galactokinase (GALK, EC2.7.1.6) with the gene for cytosol thymidine kinase (TK1, EC2.7.1.21) has frequently been found in transformants generated with human chromosomes (Klobutcher et al. 1980; Willecke et al. 1976; Wullems et al. 1977), corroborating earlier gene mapping data that indicate a close linkage of these genes within region q21–q22 of human chromosome 17 (Elsevier et al. 1974; McDougall et al. 1973). Cotransfer of human TK1, GALK, and the gene for type I procollagen (PC1) has also been described and one cotransformant with expression of these genes was used to regionally map the three loci on human chromosome 17 (Klobutcher and Ruddle 1979). A gene order of CENTROMERE-GALK-(TK1, PC1) was deduced. Chromosome-mediated cotransfer of the human X-linked genes for phosphoglycerate kinase (PGK, EC2.7.2.3) and glucose-6-phosphate dehydrogenase (G6PD, EC1.1.1.49) with the gene for hypoxanthine phosphoribosyl transferase (HPRT, EC2.4.2.8) has also been reported (Miller and Ruddle 1978; Olsen et al. 1981; Wullems et al. 1976).

In DNA-mediated gene transfer (DMGT) using cellular DNA, only one case of cotransfer of syntenic genes (GALK and TK of Chinese hamster origin) has so far been reported (Peterson and McBride 1980). We have performed CMGT and DMGT, selecting for transfer of either human TK1 or HPRT into mouse fibroblasts deficient in TK or HPRT respectively. The cotransfer of genes syntenic with the selected marker was investigated in a number of the transformants obtained. This report presents a comparison of gene transfer frequencies using CMGT and DMGT in two transfer systems not previously analysed in this way. In addition, it presents the first evidence of chromosome-mediated cotransfer of the human gene for acid alpha-glucosidase (GAA, EC3.2.1.20) with TK1. This gene, involved in the lysosomal storage disease glycogenosis type II (Pompe disease; Hers 1963), has been mapped to region q22–q25 of human chromosome 17 (Sandison et al. 1982; Weil et al. 1979). Our data allow a tentative gene ordering of CENTROMERE-GALK-TK1-GAA on human chromosome 17. Finally, we have observed DNA-mediated cotransfer of human GALK with TK1 in two out of 18 transformants tested.

Materials and methods

a. Cell lines and culture conditions

Human HeLa S3 and Swiss mouse 3T3TK$^-$ cells were cultured in monolayer using Ham's F10 medium supplemented with 10% fetal calf serum, streptomycin, and penicillin. Mouse A9 (HPRT$^-$), LTK$^-$, and LTH-1 (HPRT$^-$, TK$^-$ double mutant; de Jonge et al. 1982) cells were cultured in monolayer using a 1:1 mixture of Ham's F10 and Dulbecco's modified or α-modified Eagles medium supplemented with 5% fetal calf serum, 10% newborn calf serum, and antibiotics. In order to eliminate revertants, TK-deficient cells were periodically cultured in the presence of 10 μg/ml 5-BUdR and HPRT-deficient cells in the presence of 10 μg/ml 6-thioguanine. At least three generations before transformation with chromosomes or DNA, the drug was removed. All cell lines used were free of

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mycoplasma infection, as judged in periodic screening using the Hoechst fluorescent staining method (Chen 1977).

b. Isolation of chromosomes or DNA, transformation, and selection of transformants

Metaphase chromosomes were isolated from HeLa S3 cells and purified as described by Maio and Schildkraut (1967), with some modifications. After mitotic arrest (16 h with 0.012 µg/ml colchicine), 60–70% of the cells were in metaphase. Selective detachment of these cells by shake-off resulted in suspensions in which more than 98% of the cells were mitotic. These cells were washed twice in a balanced salt solution and resuspended in warm TM-buffer (20 m*M* Tris-HCl, 1 m*M* $MgCl_2$, 1 m*M* $ZnCl_2$, 1 m*M* $CaCl_2$, pH 7.4) with 0.1% Saponine at a concentration of 17–22 × 10^6 cells/ml. After 20 min at 37°C, the swollen cells were cooled and disrupted on ice in a Sorvall Omnimixer with micro-attachment. All further steps were carried out at 0–4°C. The cell homogenate was immediately diluted 20-fold in TM-buffer and large cellular debris was removed by centrifugation (5 min 100 × *g*). The supernatant, which contained the chromosomes and fine cellular debris, was centrifuged (15 min 1000 × *g*) and the resulting chromosome pellet was resuspended in TM-buffer. The chromosome suspension was checked microscopically for purity and, if necessary, the centrifugation steps were repeated. Finally, the chromosomes were pelleted by centrifugation for 15 min at 1000 × *g*.

Cellular DNA was isolated from HeLa S3 cells and purified using the method of Wigler et al. (1978), with modifications. Frozen pellets of washed cells were thawed and resuspended in 10 volumes of 10 m*M* Tris-HCl, pH 7.6. A solution of sodium dodecyl sulfate was added (2% final concentration) to lyse the cells and proteins were digested with Proteinase K (Boehringer, 1 h at 37°C). After extraction with phenol:chloroform:isoamyl alcohol 100:24:2 (two changes), the nucleic acids were precipitated from the aqueous phase (adjusted to 0.2 *M* sodium acetate, pH 5.7) with cold ethanol. The pellet was dissolved overnight in TE-buffer (1 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 7.6), treated with RNAse (Sigma, 1 h at 37°C) and Proteinase K, and reextracted with phenol/chloroform/isoamyl alcohol. After cold ethanol precipitation, the DNA pellet was redissolved in sterile TE-buffer at a final concentration of 300–800 µg/ml. Only DNA preparations with a $A_{260}:A_{280}$ ratio of above 1.8 were used for DMGT. The average molecular weight of the DNA preparations was approximately 10^5 DNA base pairs, as estimated by agarose gel electrophoresis.

For gene transfer, 10 m*M* Tris-HCl, 1 m*M* EDTA, 250 m*M* $CaCl_2$, pH 7.6 was used to resuspend pelleted chromosomes or dilute DNA solutions to the desired concentration, and calcium phosphate precipitates were produced as described before (de Jonge et al. 1982). CMGT was performed according to the method of Miller and Ruddle (1978), omitting the pretreatment of recipient cells with colchicine, Colcemid, and cytochalasin D. Routinely, approximately 10^6 chromosomes in 2 ml precipitate were added per 75 cm^2 culture flask with 4–6 × 10^6 recipient cells. DMGT was performed by adding 1 ml precipitate (approximately 20 µg DNA) per 10 cm cell culture dish with 2 × 10^6 cells (de Jonge et al. 1982). In a number of experiments, some of the CMGT or DMGT recipient cells were subjected to a posttreatment with dimethyl sulphoxide (DMSO) as described previously (de Jonge et al. 1982).

Selective medium was added 24 h later and changed initially every second or third day, later every fifth to seventh day. For A9, 3T3TK$^-$, and LTK$^-$ cells, HAT medium (Littlefield 1964) was used; for LTH-1 cells, HAS-medium (Graf et al. 1979). Clones, appearing 2–4 weeks later, were isolated using glass cloning cylinders and propagated in selective medium. The flasks or dishes were then fixed and stained to detect other clones. In all experiments controls were included in which an equal number of cells were subjected to identical treatments but omitting donor genetic material.

c. Electrophoresis

Preparations of cell extracts and procedures for Cellogel electrophoresis were carried out as described by Wullems et al. (1976, 1977). The following enzymes were assayed: TK1, GALK, HPRT, PGK, and G6PD.

d. Immunoadsorption assay for human acid alpha-glucosidase

An antiserum reactive with human acid alpha-glucosidase (GAA), but not with mouse GAA was prepared as follows. GAA was purified to homogeneity from human placentas with the method described by van Diggelen et al. (1982) and used for the immunization of Swiss mice. Blood for preimmune sera was collected from 10 mice by orbita puncture and each mouse was injected intraperitoneally with 100 µg GAA in 250 µl PBS (10 m*M* sodium phosphate pH 6.6 and 0.9% NaCl) emulsified with an equal volume of Freunds complete adjuvants. For intraperitoneal booster injections, given two and six weeks after priming, the same amount of enzyme was emulsified with incomplete Freunds adjuvants. The mice were bled nine days after the second booster and sera were prepared and pooled.

For the immunoadsorption assay, 10,000 × *g* supernatants of cell lysates prepared by sonication in PBS were used. Total GAA activity in the supernatants was determined as described by Galjaard (1980) and dilutions were made in PBS containing 1 mg/ml bovine serum albumin (PBS-A) to obtain an enzyme activity of 1–3 nmol 4-methylumbelliferone per 20 µl/h. From each diluted supernatant, 20 µl were incubated in duplicate at 4°C overnight with 20 µl of a 1:100 dilution of preimmune serum or antiserum in PBS-A. Subsequently 20 µl of a 1:1 suspension of Protein A-Sepharose 4B beads (Pharmacia) in PBS-A were added and the beads were intermittently resuspended during the following 1 h incubation at room temperature. The beads were then spun down (1 min 10,000 × *g*) and the activity of GAA in 10 µl aliquots of the supernatants was measured as described by Galjaard (1980), as a control. The beads were washed five times with PBS-A, the last wash fluid was carefully removed and 30 µl of 4-methylumbelliferyl-α-D-glucopyranoside substrate in 0.1 *M* sodium acetate pH 4.3 was added to determine the amount of GAA activity adsorbed onto the beads. After 1 h incubation at 37°C, the reaction was stopped by the addition of 500 µl sodium carbonate pH 10.7 and the amount of reaction product formed was measured as described by Galjaard (1980). Finally, the number of units of GAA specifically bound to the beads via the anti-GAA antiserum was calculated (1 unit = 1 pmol 4-methylumbelliferone per hour).

e. Stability analysis of TK expression

The stability of TK expression in 3T3 CMGT transformant cell lines was investigated as follows. After continuous propa-

gation in selective HAT medium, cell populations were transferred at day 0 to non-selective HT medium and at the same time samples of each culture were plated in triplicate culture dishes at two cell densities in both HAT- and HT medium. After 10–14 days growth, the culture dishes were fixed and stained with Giemsa or methylene blue and the ratio of macroscopically visible colonies arizen in selective HAT medium versus non-selective HT medium was determined. At regular time intervals after day 0, the cultures propagated in HT medium were assayed in the same way to determine the fraction of cells still able to clone in HAT medium.

f. Karyotypic analysis of transformants

Metaphase spreads were sequentially stained (Kozak et al. 1977) by standard alkaline Giemsa and Hoechst 33258 or trypsin-Giemsa and Hoechst staining methods. At least 25 metaphases of each cell line were analyzed.

Results

a. Generation of transformant cell lines

The transfer of human genes into cultured HPRT- or TK deficient mouse cells of various origin was performed using metaphase chromosomes or cellular DNA isolated from cultured HeLa cells. In most transfer experiments, some putative transformant cell lines were established from clones which had arisen in different culture flasks or dishes. Cell lysates prepared from such cell lines were assayed for the presence of human HPRT or TK by electrophoresis on Cellogel. Figure 1 shows a representative electrophoretic analysis of HPRT in a number of putative A9 transformants. HPRT activity is not detectable in mouse A9 recipient cells but all the lysates of putative transformants tested contained HPRT with human electrophoretic mobility. These data establish the transformant character of the clones generated. In control flasks or dishes, treated identically but for the omission of donor genetic material, no clones were observed. Figure 2 shows a representative electrophoretic analysis of TK in a number of putative 3T3 transformants. All the lysates of the putative transformants tested

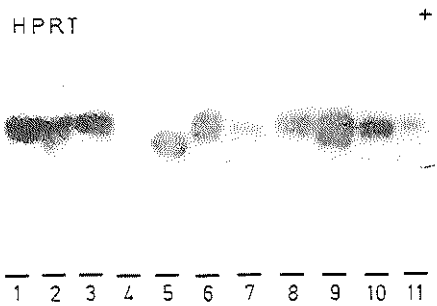

Fig. 1. The electrophoretic analysis of HPRT in lysates of putative transformant cells. Source of the lysates: channels 1–3 and 7–11, putative transformant cells; channel 4, A9 recipient cells; channel 5, 3T3TK$^-$ cells; channel 6, HeLa cells

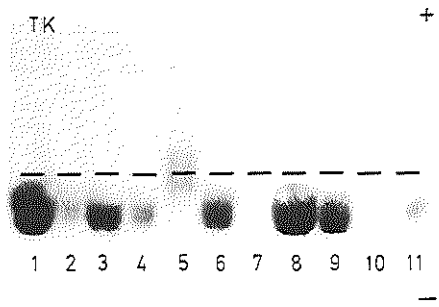

Fig. 2. The electrophoretic analysis of TK in lysates of putative transformant cells. Source of the lysates: channels 1–4 and 8–11, putative transformant cells; channel 5, A9 cells; channel 6, HeLa cells; channel 7, 3T3TK$^-$ recipient cells. The lysate run in channel 10 was positive for TK with human electrophoretic mobility in a subsequent assay

contained TK with the human electrophoretic mobility; mouse 3T3TK$^-$ recipient cells do not contain any detectable TK activity.

In repeat experiments, a considerable variability in the frequency of gene transfer was observed (not shown), but the summarized results, presented in Table 1, show that CMGT into A9 and 3T3TK$^-$ cells occurs at frequencies 10 and 14 times, respectively, higher than DMGT into these cells. Furthermore, a DMSO posttreatment reduces the frequency of CMGT into 3T3TK$^-$ cells by more than an order of magnitude. In contrast, CMGT into A9 cells and DMGT into LTK$^-$ cells are stimulated with factors of 7 and 2 respectively, by DMSO.

b. Cotransfer of syntenic genes

Various cell lines with human HPRT obtained through CMGT or DMGT were analysed for the presence of human PGK or G6PD by Cellogel electrophoresis. The results are summarized in Table 2. In A9 CMGT transformants, cotransfer of human PGK with HPRT was about 6% (2 out of 33); cotransfer of G6PD was not observed in the 33 CMGT and 7 DMGT transformants we have investigated. Cellogel electrophoresis was also used to assay for the expression of human GALK in CMGT- or DMGT transformant cell lines positive for human TK1. In 3T3 CMGT transformants, cotransfer of human GALK with TK1 was 29% (10 out of 35). Also, two out of the 18 L-cell DMGT transformants tested expressed human GALK (Table 2).

All 10 TK1$^+$ GALK$^+$ 3T3 CMGT transformants plus 10 randomly picked 3T3 CMGT transformants expressing only human TK1, as well as the 21 TK1$^+$ DMGT transformants isolated, were investigated for the expression of human GAA. The enzyme was assayed by immunoadsorption using a specific anti-human GAA antiserum, raised in Swiss mice, that did not crossreact with mouse GAA. Results of this analysis are shown in Fig. 3. Lysates of human HeLa cells yielded high levels of bound GAA activity, whereas no significant amounts were adsorbed from lysates of recipient 3T3TK$^-$ or LTK$^-$ cells. For comparison we also determined the amount of human GAA activity adsorbed from lysates of the human-mouse hybrid cell line 4CB7, obtained through fusion of

Table 1. Transformation of murine cells with human genetic material

Donor genetic material	Recipient cells	DMSO Post-treatment	Selected phenotype	No. of cells treated[a]	No. of clones observed	No. of clones isolated[b]	Transfer frequency
HeLa chromosomes	A9	no	$HPRT^+$	13.5×10^7 (4)	18	8	0.1×10^{-6}
		yes	$HPRT^+$	13.5×10^7 (4)	88	25	0.7×10^{-6}
	$3T3TK^-$	no	TK^+	3.0×10^7 (6)	84	29	2.8×10^{-6}
		yes	TK^+	4.5×10^7 (4)	7	6	0.2×10^{-6}
HeLa DNA	A9	no	$HPRT^+$	150×10^7 (6)	16	5	0.1×10^{-7}
	LTH-1	yes	$HPRT^{+c}$	3.0×10^7 (1)	7	2	0.2×10^{-6}
	$3T3TK^-$	no	TK^+	1.5×10^7 (2)	3	3	0.2×10^{-6}
	LTK^-	no	TK^+	7.0×10^7 (4)	99	18	1.4×10^{-6}
		yes	TK^+	6.0×10^7 (5)	157	0	2.8×10^{-6}

For experimental details, see Materials and methods
[a] The number of independent transfer experiments is shown in parenthesis
[b] All clones noted were confirmed as transformants by electrophoretic techniques
[c] Selection was performed in HAS-medium (Graf et al. 1979)

Table 2. Cotransfer of syntenic genes

Donor genetic material	Recipient cells	Selected human marker	No. of transformants with expression of human gene(s) $HPRT^+$	$HPRT^+PGK^+$	$HPRT^+G6PD^+$	$TK1^+$	$TK1^+GALK^+$	$TK1^+GAA^+$	$TK1^+GALK^+GAA^+$
HeLa chromosomes	A9	HPRT	33	2	0	–	–	–	–
	$3T3TK^-$	TK	–[a]	–	–	35	10	1[b]	4[c]
HeLa DNA	A9	HPRT	5	–	0	–	–	–	–
	LTH-1	HPRT	2	–	0	–	–	–	–
	$3T3TK^-$	TK	–	–	–	3	0	0	0
	LTK^-	TK	–	–	–	18	2	0	0[d]

[a] – Not determined
[b] Ten TK^+ CMGT transformants tested for expression of human GAA
[c] Ten TK^+GALK^+ CMGT transformants tested for expression of human GAA
[d] Two TK^+GALK^+ DMGT transformants tested for expression of human GAA

human lymphocytes with $3T3TK^-$ cells and containing a morphologically intact human chromosome 17 in more than 80% of the cells. In the immunoadsorption assay, lysates of 4CB7 cells yielded significant levels of bound GAA activity (about 30% of the amount adsorbed from HeLa cells). The majority of the transformant cell lines tested for expression of human GAA yielded no measurable amounts of bound enzyme (some of these transformants negative for human GAA are included in Fig. 3 as an illustration). Lysates of one $TK1^+$ CMGT transformant (designated 60C2) and four $TK1^+$ $GALK^+$CMGT transformants (designated 55C5, 55C10, 60C5, and 63CD3) yielded significant levels of bound GAA activity, some reaching the same level as found with lysates of 4CB7 cells. Expression of human GAA was not observed in any of the 21 $TK1^+$ DMGT transformant cell lines tested (Table 2).

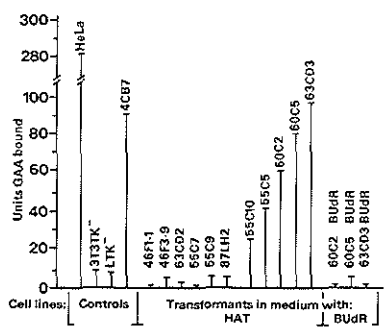

Fig. 3. Immunoadsorption assay of human GAA in cell extracts. For experimental details, see Materials and methods

c. Cytogenetic analysis and stability of transformants

The presence of human genetic material in the genome of 3T3 CMGT transformants 46F1-1 ($TK1^+GALK^+GAA^-$), 60C5 and 63CD3 (both $TK1^+GALK^+GAA^+$) was investigated using various procedures. After sequential alkaline Giemsa/centromere staining, a piece of human-staining chromosomal material with a human-staining centromeric region was observed only in metaphase plates of transformant 63CD3 (Figs. 4a and b). Some cells harbored two copies of this chromosome. However, it was not possible to discern from the trypsin-Giemsa banding pattern (Fig. 4c) which human chromosome is involved. The same metaphase as in Fig. 4c is illustrated in Fig. 4d, but poststained with Hoechst 33258. Therefore, linkage of a part of human chromosome 17 (carrying the syntenic group analysed) to the centromeric region of another human chromosome cannot be excluded.

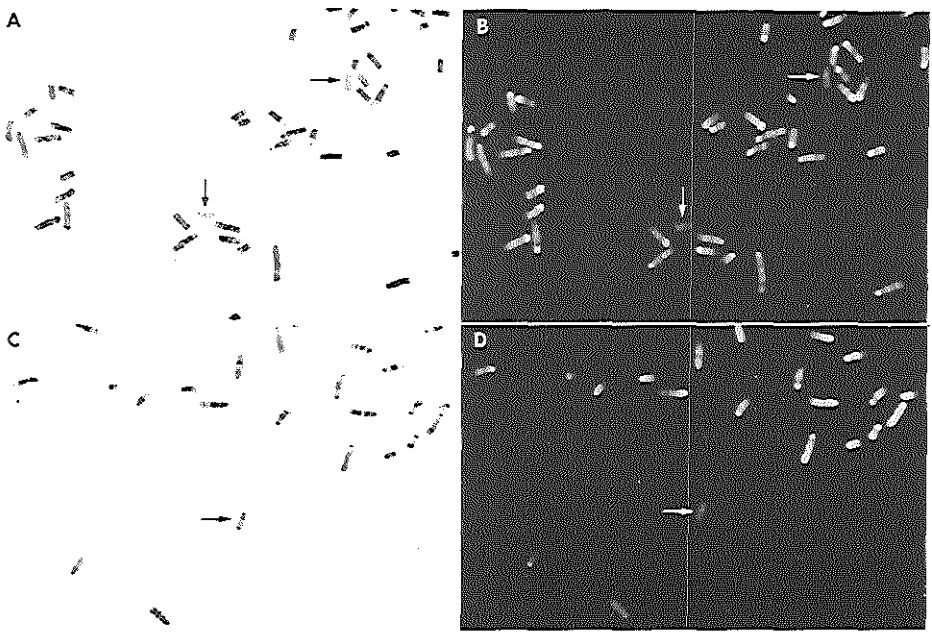

Fig. 4A–D. Visualization of human-staining chromosomal material in metaphase plates of transformant 63CD3. **A** Alkaline Giemsa staining (only part of the complement is shown). In this cell, two copies of a lightly stained putative human chromosome fragment were observed *(arrows)*. **B** Same metaphase as **A**, poststained with Hoechst 33258. The putative human fragments possess non-fluorescent (human-staining) centromeric regions *(arrows)*. **C** Trypsin-Giemsa staining of the putative human fragment. **D** Same metaphase as **C**, poststained with Hoechst 33258

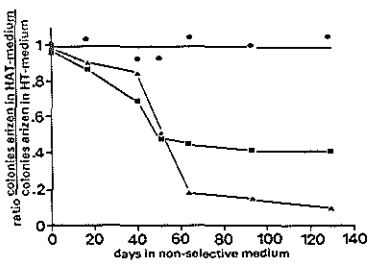

Fig. 5. Stability of the TK^+ phenotype in 3T3 CMGT transformant cells under non-selective conditions. For experimental details, see Materials and methods. ●——● transformant 46F1-1; ■——■ transformant 60C5; ▲——▲ transformant 63CD3

In situ hybridization of ^3H-labeled human repetitive DNA (Cot-1) to chromosomes in metaphase spreads has previously demonstrated the presence of a human chromosomal fragment in transformant 60C5 (de Jonge and Bootsma 1984). The same technique was used to visualize human genetic material associated with a host cell chromosome of transformant 46F1-1 and to verify that the human-staining chromosomal fragment observed in transformant 63CD3 was composed of human genetic material (not shown). In addition, the stability of TK expression under non-selective culture conditions was examined in cell populations of these three transformant cell lines. As shown in Fig. 5, a cell population of transformant 46F1-1 showed retention of TK expression in all cells during growth in non-selective medium for more than two months; cells expressing TK were gradually lost from the cell populations of transformants 60C5 and 63CD3.

Evidence was obtained for the genetic linkage of the cotransferred genes in 3T3 CMGT transformants 46F1-1, 60C2 ($TK1^+GALK^+GAA^+$), 60C5, and 63CD3. Mass cell populations of these four cell lines were selected for loss of TK-activity by prolonged culture in medium containing 50 µg/ml 5-bromodeoxyuridine (BUdR) and reanalysed. Concomitant with the loss of TK activity, both the cytologically detectable human genetic material and the expression of the cotransferred gene(s) were lost from these cell populations (see Fig. 3 for the activity of human GAA).

Discussion

In a comparative study of chromosome- and DNA-mediated transfer of Chinese hamster genes for TK and dihydrofolate reductase into LTK^- cells, Lewis et al. (1980) observed for both genes that CMGT occurred with a considerably higher frequency than DMGT. We have obtained similar results in two additional gene transfer systems: transfer of human TK1 into mouse $3T3TK^-$ cells and transfer of human HPRT into

mouse A9 cells were consistently found to occur at frequencies at least an order of magnitude higher when chromosomes were used as donor genetic material. These results may indicate that non-DNA chromosomal constituents (RNA or protein), or the packaging of the DNA in donor metaphase chromosomes, offer some protection against degradation in the recipient cell. Alternatively, if exonucleolytic breakdown predominates in the degradation of donor genetic material, a larger size of the DNA molecules in isolated chromosomes could result in a higher transfer frequency. It is also possible that damage introduced during isolation renders a fraction of the purified DNA inactive in gene transfer.

In our experiments, treating recipient cells with DMSO after the addition of donor genetic material had a marked positive effect in the CMGT of HPRT into A9 cells (a sevenfold increase in transfer frequency). With LTK^- cells as recipients of donor DNA, a DMSO post-treatment resulted in a doubling of the transfer frequency. DMSO stimulation of CMGT and DMGT into L-derived cells has been reported before (Gross et al. 1979; Miller and Ruddle 1978) but the mechanism of the stimulation is unknown. Furthermore, the effect of a DMSO post-treatment is not the same in all recipient cell lines.

For human cells, it has been reported (Gross et al. 1979; Gross Lugo and Baker 1983) that a relatively short treatment with a higher concentration enhances the transfer frequency. For cells of Chinese hamster origin no effect has been observed (Abraham et al. 1982; Srinivasan and Lewis 1980). We report here the posttreatment to be very disadvantageous for CMGT into $3T3TK^-$ cells, resulting in a 14-fold reduction in transfer frequency. The 3T3 cells proved to be very sensitive to DMSO; a considerable fraction of the cells did not survive the procedure. In view of these results it appears necessary to determine for every recipient cell line the optimal conditions for gene transfer.

Cotransfer of the human gene for GALK with the TK1 marker gene was observed in 30% of the 3T3 CMGT transformant cell lines we have analysed. In CMGT into mouse L-derived cells (B82, LTK^-), cotransfer frequencies of 20–25% have been reported (Klobutcher et al. 1980; McBride et al. 1978; Willecke et al. 1976). In CMGT into Chinese hamster cells or Chinese hamster-human hybrids, cotransfer has been observed in all five and four, respectively, transformants assayed for the expression of human TK1 and GALK (Wullems et al. 1977). It is not clear whether these differences in the cotransfer frequency of human TK1 and GALK after CMGT are due to differences in the recipient cells and gene transfer methods used, or mere coincidence.

Since the gene for GAA had been mapped to region q22–q25 of human chromosome 17 (Sandison et al. 1982; Weil et al. 1979), we investigated our 10 $TK1^+GALK^+3T3$ transformant cell lines plus 10 randomly picked $TK1^+3T3$ transformant cell lines for expression of human GAA. With an immunologic procedure using a murine antiserum against human GAA, one $TK1^+$ transformant (60C2) and four $TK1^+GALK^+$ transformants (55C5, 55C10, 60C5, and 63CD3) were found to express human GAA. Two $TK1^+GALK^+GAA^-$ transformants (60C5 and 63CD3) carried a free cytologically detectable human chromosome fragment. Back-selection for loss of TK expression resulted in concomitant loss of the visible human genetic material as well as loss of GALK- and GAA expression. These results constitute the first report, to our knowledge, of chromosome-mediated cotransfer of human GAA with TK1 and indicate a close linkage of the loci for TK1, GALK, and GAA on human chromosome 17.

Excluding chromosomal rearrangements or breakage-prone sites in the chromosomal region studied, our data allow a tentative regional localization of these three loci. Since only one of the randomly chosen $TK1^+$ transformants coexpressed human GAA, a reliable cotransfer frequency of GAA with TK1 cannot be calculated. However, only four of the 10 $TK1^+GALK^+$ cotransformants investigated also expressed GAA. Therefore, the GAA locus is most likely situated outside the TK1-GALK segment. The finding of one $TK1^+GAA^+$ cotransformant (60C2) suggests that the GAA locus is located closer to TK1 than to GALK. Thus a gene order of GALK-TK1-GAA is indicated. On the basis of this gene order, it would be expected that more $TK1^-GAA^+$ cotransformants would be generated than $TK1^+GALK^+GAA^+$ cotransformants. However, we have found one $TK1^+GAA^+$ versus four $TK1^+GALK^+GAA^+$ cotransformants. This discrepancy is probably due to the limited number of transformants we have analysed.

While this manuscript was in preparation, a localization of the GAA locus distal to the TK1-GALK segment on human chromosome 17 was deduced in our laboratory from in situ hybridization studies using a radiolabeled c-DNA probe for the GAA gene (Halley et al. 1984). This localization combined with our data suggests a gene ordering of CENTROMERE-GALK-TK1-GAA on human chromosome 17. With regard to the relative positions of TK1 and GALK, this ordering accords with two previous studies (Klobutcher et al. 1980; Klobutcher and Ruddle 1979) and conflicts with a third (Church et al. 1980).

Concerning X-linked genes, we have observed chromosome-mediated cotransfer of the human gene for PGK with the HPRT marker gene in two of the 33 A9 CMGT transformants analysed. Such chromosome-mediated cotransfer of a human gene syntenic with HPRT has also been reported by Miller and Ruddle (1978) who found one $HPRT^+PGK^+$, four $HPRT^+G6PD^+$, and two $HPRT^+PGK^+G6PD^+$ cotransformants out of 25 analysed. Wullems et al. (1976) reported transfer of a complete human X chromosome into Chinese hamster-human hybrid cells; we have been unable to generate clones using the same recipient cell lines.

In DNA-mediated gene transfer experiments, we have observed cotransfer of syntenic genes only for GALK and TK1 in two out of 18 L-cell transformants tested for the expression of human GALK and GAA. Peterson and McBride (1980) reported cotransfer of Chinese hamster GALK in one out of 87 TK^+ L-cell transformants. These results suggest that the TK-GALK intergenic distance is about 10^5 DNA basepairs (the length of isolated DNA molecules as estimated by agarose gel electrophoresis). Based on data obtained from gamma-irradiation-induced segregation of the two genes in hybrid cells (Goss 1979), the TK1-GALK intergenic distance in the human genome has previously been estimated at $12 \cdot 10^5$ DNA basepairs. Therefore, the genes coding for TK and GALK may be closer to each other than previously estimated. However, other explanations for the observed cotransfer cannot be excluded (for a discussion, see de Jonge and Bootsma 1984).

In conclusion, this report has shown in two gene transfer systems, not previously analysed in this way, that CMGT results in a higher gene transfer frequency and a higher incidence of cotransfer of closely linked syntenic genes than

DMGT using isolated cellular DNA. The utility of CMGT in the mapping of syntenic genes was demonstrated by the tentative ordering of the genes coding for TK1, GALK, and GAA on human chromosome 17.

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APPENDIX PAPER IV

Expression of human *hprt* gene on the inactive X chromosome after DNA-mediated gene transfer

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The paternal or maternal mammalian X chromosome is inactivated ('lyonization') at random early in the development of the female embryo[1,2]. Once established, the inactivation of the chromosome is maintained in the cell and in all its descendants. However, spontaneous local reactivation of the inactive X chromosome is found at low frequency in human–mouse hybrid cells[3,4]. So far only the X_g locus[5,6] and the locus for microsomal steroid sulphatase (*sts*)[7] (both assigned to the short arm of the X chromosome), have been shown to escape inactivation. The locus for hypoxanthine phosphoribosyltransferase (*hprt*) is susceptible to inactivation[8] and humans heterozygous for HPRT deficiency (the Lesch–Nyhan syndrome, L–N)[9], show a mosaic pattern of HPRT activity in their cultured fibroblasts[8]. To investigate the molecular basis of X inactivation, we have used DNA isolated from each of the two subpopulations of a L–N heterozygote, to transform cultured HPRT-deficient mouse fibroblasts. Transformation of the mouse cells with the inactivated human *hprt*$^+$ gene occurred with essentially the same efficiency as with the active human *hprt*$^+$ gene. Thus, reactivation of the lyonized human *hprt* locus is possible after interspecific DNA-mediated gene transfer.

To obtain sufficient cells of only the HPRT$^+$ or only the HPRT$^-$ subpopulation, primary fibroblasts of the L–N heterozygote (cell strain 79RD19) were transformed using the *ori*$^-$ simian virus 40 (SV40) mutant originally isolated by Gluzman *et al.*[10] and various of these transformed clones were tested for HPRT expression. One HPRT$^-$ clone, SV1 (the inactivated X chromosome carries the *hprt*$^+$ allele) and one HPRT$^+$ clone, SV3 (the active X chromosome carries the *hprt*$^+$ allele) were expanded for DNA isolation. Immediately before collection, the cells were checked again for HPRT$^+$ or HPRT$^-$ phenotype by measuring ^{3}H-hypoxanthine incorporation and also by a growth test in two selective media. Cells (10^7) of each clonal cell line were plated in medium selecting either for (hypoxanthine–aminopterin–thymidine (HAT) medium[11]) or against (medium containing 6 μg ml^{-1} 6-thioguanine, 6-TG) the expression of HPRT in the cells. Cells having a phenotype which differed from that expected were not observed in either test. Furthermore, the presence of an inactive X chromosome was verified in these cell lines by staining the Barr body in interphase nuclei (Fig. 1).

DNA isolated from SV1 or SV3 was used to transform mouse LTH1 cells. LTH1 is an HPRT-deficient subline of LTK$^-$ (ref. 12), which we have isolated after selection for a spontaneous mutant by culturing in 6-TG medium. Reversion of LTH1 to an HPRT$^+$ phenotype was $<2 \times 10^{-8}$ when tested by culturing cells in medium containing hypoxanthine as the exogenous purine source and azaserine to block the *de novo* biosynthesis of purines (HAS medium[13]). We have tested HPRT-deficient cell lines of different origins in transformation experiments with human HeLa DNA and found the highest transformation frequency of the HPRT$^+$ phenotype using LTH1 cells as DNA recipients (our unpublished results). The results of DNA-mediated gene transfer of the *hprt* locus into LTH1 cells are shown in Table 1. For comparison, we also selected for transfer of the thymidine kinase (*tk*) locus into LTK$^-$ cells using the same DNA–calcium phosphate precipitates.

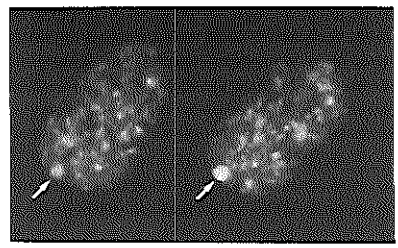

Fig. 1 Barr body in SV1 cells. After fixation in methanol/acetic acid (3 : 1) the cells, grown on coverslips, were air-dried and stained for 10 min in a 0.5% atebrin solution in methanol. After rinsing and mounting in 0.07 M phosphate buffer *p*H 6.5, the cells were examined by fluorescence microscopy and photographed. The Barr body is indicated by an arrow.

The *hprt* gene was transformed by SV1 DNA at a frequency intermediate between those obtained using HeLa and SV3 DNA. In addition, the transformation frequency of the *tk* locus in SV1 DNA was not significantly different from the frequencies obtained with the other DNAs used although for each DNA, transfer of the *hprt* locus into LTH1 cells occurred at a lower frequency than transfer of the *tk* locus into LTK$^-$ cells. Evidently there were no significant differences in transformation capacity between the different DNA preparations for either the *tk* or *hprt* locus. From LTH1 cells treated with HeLa, SV1 and SV3 DNA, respectively, 2, 9 and 15 transformed clones were isolated.

In a number of independent transformed cell lines isolated from different culture dishes, we identified the HPRT and glucose-6-phosphate dehydrogenase (G-6-PD) activities by electrophoresis on Cellogel[14,15] (Table 2). Figure 2 shows the electrophoretic mobility of human (SV3) and murine (LTK$^-$) HPRT. In extracts of SV1 and LTH1 cells, HPRT was not detectable, while in transformants isolated from LTH1 cells treated with SV1 DNA, human HPRT was synthesized. One cell line (clone 11) showed HPRT of murine electrophoretic mobility and was assumed to be a revertant. In all the transformants tested, only the murine G-6-PD was present.

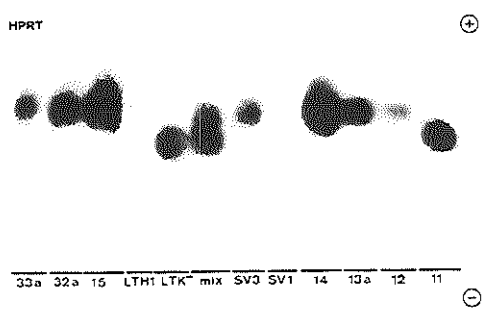

Fig. 2 Cellogel electrophoresis of HPRT. Cell lysates were produced by sonication and electrophoresis was carried out according to Meera Khan[14]. Enzyme activity was visualized by incubation with ^{14}C-hypoxanthine and autoradiography[15]. For an explanation of SV1, SV3, LTH1 and LTK$^-$, see text. Clones 12, 13a, 14 and 15 are independent transformant clones isolated after treatment of LTH1 cells with SV1 DNA. Clone 11 appears to be a revertant. Clones 32a and 33a are independent clones isolated after treatment of LTH1 cells with SV3 DNA. Mix is an artificial mixture of extract from LTK$^-$ and SV3 cells.

Table 1 Transformation of LTH1 and LTK$^-$ with HeLa, SV1 and SV3 DNA

DNA donor	LTH1 Selection for HPRT$^+$			LTK$^-$ Selection for TK$^+$		
	Plates with clones/total plates	No. of clones	Transfer frequency	Plates with clones/total plates	No. of clones	Transfer frequency
No DNA	0/25	0	0	0/7	0	0
HeLa	5/15	7	0.2×10^{-6}	4/4	18	2×10^{-6}
SV1	6/10	11	0.5×10^{-6}	3/3	26	4.5×10^{-6}
SV3	9/10	21	1×10^{-6}	3/3	17	3×10^{-6}

DNA was isolated using a modification of a previously described method[26]. DNA–calcium phosphate precipitate was produced by slowly adding DNA, dissolved at 40 μg ml^{-1} in 10 mM Tris-HCl *p*H 7.6, 1 mM EDTA, 250 mM $CaCl_2$ to an equal volume of HEBS buffer[27], with agitation. After 30–45 min, 1 ml of the fine precipitate was added to the medium over 2×10^6 cells in a 10-cm cell culture dish. Control dishes received calcium phosphate precipitate without DNA. Post-treatment with 10% dimethyl sulphoxide[28] was given 3.5–4 h after addition of precipitate. Selective medium (LTH1, HAS medium[13]; LTK$^-$, HAT medium[11]) was added 24 h later and changed initially every second or third day, later every fifth to seventh day. Clones appeared after 2–4 weeks and were isolated using glass cloning cylinders. The plates were then fixed and stained to detect other clones. The medium used in all cell culture procedures was a 1:1 mixture of Ham's F10 and Dulbecco's minimal essential medium (MEM; Flow) containing 5% fetal and 5% newborn calf serum and antibiotics, gassed with 5% CO_2.

The results are most readily explained by a reactivation of the lyonized *hprt* locus after interspecific DNA transformation. A simple reversion of the *hprt* mutation on the active X chromosome in the donor strain SV1 appears unlikely because of the equal transformation frequencies obtained with DNA isolated from SV1 and SV3, and from results of the tests performed on the cells immediately before DNA isolation. An alternative explanation is that in the cells of the particular L–N heterozygote used, the HPRT deficiency was not due to a mutation in the structural gene for HPRT, but due to a defect in some other function necessary for expression of the gene. This would render the SV1 cells phenotypically HPRT$^-$ despite their having an intact *hprt* gene on the active X chromosome. As SV1 and SV3 are two cell lines originally of identical genotype apart from the inactivated X chromosome, such a function would need to be susceptible to lyonization to account for the observed mosaic HPRT expression, and our results would then indicate that LTH1 cells can provide this function.

If SV1 cells carry an intact structural gene for HPRT on the active X chromosome and LTH1 cells provide a function necessary for expression of the gene, it should be possible to restore HPRT activity by fusion of the two cell lines. After pre-labelling the cells with latex beads of different sizes[16], we fused SV1 cells with LTH1 cells and looked for HPRT activity in heterokaryons by incubation in the presence of ^{3}H-hypoxanthine immediately, and 1,2 or 3 days after fusion. At each time point we examined at least 1,000 heterokaryons having 2–10 nuclei per cell, but found no restoration of HPRT activity. It is thus highly unlikely that SV1 cells have an active X chromosome carrying an intact structural gene for HPRT which can become expressed in LTH1 cells as a result of some murine function. These results agree with data reported elsewhere[17,18] which suggest that in cells obtained from L–N patients, the mutation has affected the structural gene for HPRT.

Table 2 Identification of HPRT and G-6-PD activities in cell lysates of independent transformant clones

DNA donor	Transformant	Electrophoretic mobility of HPRT	Electrophoretic mobility of G-6-PD
HeLa	95.1–1	H	M
	95.1–2	H	M
SV1	96.1–11	M	M
	96.1–12	H	M
	96.1–13a	H	M
	96.1–14	H	M
	96.1–15	H	M
SV3	96.1–31a	H	M
	96.1–32a	H	M
	96.1–33a	H	M
	96.1–34	H	M
	96.1–36	H	M

H represents human, M murine electrophoretic mobility.

Several mechanisms proposed for X inactivation agree with our results. (1) Taking the human X chromosome to be 2×10^5 kilobase pairs (kbp) (1/23 of the haploid genome) and the average length of the isolated DNA molecules to be 100 kbp (estimated by agarose gel electrophoresis), one isolated DNA molecule could represent, on average, only 0.05% of the X chromosome. If there are one or a few 'inactivation centres'[19] from which inactivation of the X chromosome is maintained, our procedure could have caused an uncoupling of such a centre from the *hprt* locus, resulting in expression of the gene. (2) Recently, evidence has been obtained that DNA methylation could be involved in X-chromosome inactivation[20]. Also, almost total, random methylation of a DNA sequence inhibits the expression of that sequence after DNA transformation[21]. We have shown here that the *hprt* locus, kept inactive in human cells, can be efficiently expressed in rodent cells. Taken together, these findings indicate that the inactive state of the X chromosome is maintained by a species-specific methylation pattern, the human pattern not being recognized as a lyonization signal in the rodent cells. In this respect, it is interesting that Liskay and Evans[22] have not found reactivation in experiments similar to ours, but they performed transfers between rodent cells only. (3) As purified DNA was used in our experiments it is possible that the inactivated state is maintained by non-DNA components of the X chromosome[23].

Our results do not support models based on differences in DNA base sequence being responsible for lyonization[24,25].

The system using the two L–N heterozygote subpopulations as a source of active and inactive X-chromosomal material should enable us to investigate further the possibilities of X-chromosome reactivation, for example after chromosome-mediated gene transfer, or in intraspecific transfer experiments.

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