TRANSFER OF GENETIC INFORMATION VIA ISOLATED MAMMALIAN CHROMOSOMES

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. B. LEIJNSE EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN. DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP WOENSDAG 15 DECEMBER 1976, DES NAMIDDAGS TE 4.15 UUR

DOOR

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GEBOREN TE TER HOLE IN 1947

1976

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Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam met financiële steun van FUNGO, Stichting voor Medisch Wetenschappelijk Onderzoek.

Aan mijn ouders,

aan Loes, Bas en Iris.

CONTENTS

CHAPTER 1 GENERAL INTRODUCTION

Recombination of genetic information from different origin has provided insight in many aspects of the genetic mechanisms of the living cell. These aspects concern the location of genes on chromosomes, the regulation of gene expression and the interaction of different genes in the determination of a particular phenotype. The classic process to produce such new genetic combinations is the conjugation of a male and female gamete, resulting in the formation of a zygote.

Genetic studies at the molecular level with micro organisms, have lead to the discovery of other processes for the formation of new genetic combinations. One of these processes is genetic transformation, defined as the integration and expression of a small piece of deoxyribonucleic acid (DNA), extracted from donor cells and introduced into the genome of the recipient cells (1). A second process is transduction, which is defined as the bacteriophage mediated transfer of genetic information from one bacterium (donor) to another (recipient), (2). The bacteriophage involved, multiplies in the donor bacterium and, after lysis, it is able to transfer genetic information of the donor to recipient bacteria upon infection.

Many investigators have tried to establish comparable processes with cells of higher organisms. Although transformation in vivo, by injection of DNA into multicellular organisms, has been claimed (3), the results of the experiments of this type have, so far, not been convincing. Many complicating factors have to be taken into account : The long distance the DNA has to bridge before reaching its host genome, the existence of barriers on its way e.g. cellular and nuclear membranes, and the destructive action of DNA degrading enzymes. For this reason an unequivocal proof of genetic transformation in multicellular organisms has not yet been presented.

This scepticism also concerns experiments in which isolated exogenous DNA is used to tranform cells cultured in vitro (chapter 2.1). In many reports it has not been demonstrated, that the new phenotype of the host cells, following treatment with DNA, is actually coded by donor DNA.

Distinction between donor and host characteristics (for instance specific enzyme activities) can be achieved using host and donor cells from different species (interspecies system). Differences in aminoacid composition of homologous enzymes, as a result of evolutionary divergence, can be demonstrated by physico-chemical techniques, like electrophoresis. However, in the DNA transformation studies, using in vitro cultured cells, the donor and the host cells belonged, in most cases, to the same species (intraspecies system). These studies will be described more detailed in chapter 2 of this thesis.

The development of procedures for the isolation of metaphase chromosomes from eukaryotic cells (chapter 3.1) has facilitated another approach to achieve gene transfer. Chromosomes, isolated from mammalian cell lines, have been incubated with mutant cells from the same or from different origin. As presented in chapter 3.4 of this thesis, mouse and Chinese hamster cell lines, lacking the enzymes hypoxanthine phosphoribosyl transferase (HPRT) or thymidine kinase (TK), became HPRT or TK positive by incubating them with chromosomes from HPRT or TK positive cells. In our studies, using Chinese hamster recipient cells, evidence was obtained for the incorporation (appendix publication 1), the replication (appendix publication 1) and the expression, of incorporated chromosomal material (appendix publication 1-4).

In these systems in which the HPRT locus has been transferred, cotransfer of genes linked to the HPRT gene, has not been obtained. Moreover, chromosome analysis has never revealed the presence of recognizable donor chromosomal material in these systems. In the experiments in which the transfer of TK has been obtained. cotransfer of the gene for galactokinase (Gak), which is linked with TK, has also been accomplished. However, these experiments also failed in the detection of donor chromosomal material (appendix publication 4).

The only evidence for the transfer of a complete chromosome has been obtained in experiments in which human-Chinese hamster HPRT deficient cell hybrids were incubated with human chromosomes from HPRT positive cells. In the resulting HPRT positive cells, cotransfer of the other X-

linked genes coding for the enzymes glucose-6-phosphate dehydrogenase (G6PD), phosphoglycerate kinase (PGK) and α-galactosidase (α-Gal), has also been accomplished (appendix publication 3). The procedure of genetic transformation by isolated chromosomes might contribute to the knowledge of mechanisms of heredity, provided that many details of the processes involved in this genetic transformation become clarified. For instance, very little is known about the mechanism of chromosome uptake. In addition, very little is also known about the role which species specific factors seem to play concerning the uptake of chromosomes. However, with the development of more appropriate selective culture media, and with the use of cell systems in which the incorporation of complete donor chromosomes is a general event, the procedure of genetic transformation could turn out to be of major significance. There are, for instance, different approaches to investigate the mechanisms of chromosome action. In the first place the technique of chromosome isolation offers unique possibilities for manipulating chromosomes in vitro, for instance, by extraction of specific chromosomal proteins or chromosomal ribonucleic acids (RNA's) (4). Chromosomes, treated in this manner, can be introduced into the genome of appropriate recipient cells, to establish the significance of these chromosomal components in gene expression. Secondly, the procedure of gene transfer may contribute in the understanding of gene expression and gene regulation. A model for this research may be the lyonized X chromosome. Reports have presented indications for reactivation of the inactive lyonized X chromosome in human-mouse hybrid cells (5). It should be worthwhile to isolate lyonized X chromosomes and to introduce them in recipient cells, to investigate the possibility of reactivation of these chromosomes, or fragments of these chromosomes, in recipient cells.

Another aspect of the mechanism of heridity, in which genetic transformation may provide new tools, is gene mapping at the molecular level. The possibility of introducing chromosome fragments in recipient cells mediated by isolated chromosomes, may result in procedures for gene mapping, comparable with the use of DNA transformation to accomplish mapping of the chromosomes of some micro organisms, for instance Escherichia Coli.

CHAPTER 2 DNA MEDIATED TRANSFORMATION IN MAMMALIAN CELLS

2.1 UPTAKE OF DNA AND INCORPORATION IN THE HOST GENOME.

During the last 15 years experiments have been performed, analogous to experiments with micro organisms, to investigate the uptake of exogenous DNA by mammalian cells in vitro. In many cases, the donor DNA was radioactively labeled and the fate of the radioactivity was traced by different techniques. Autoradiography is used to determine the site of incorporation of the radioactive compounds and to follow the fate of the radioactivity (6-10). Another technique is the extraction of the DNA from the recipient cells, treated with radioactively labeled DNA, followed by the characterization of the radioactive components using density centrifugation and liquid scintillation counting (6,8,11-14).

With these methods, it is almost impossible to demonstrate that the radioactivity, detected in the recipient cells, is identical to the donor DNA. The radioactivity, which can be found in the cells, may also belong to break down products of the added DNA, incorporated in the DNA or RNA of the acceptor cells.

Experiments which have been carried out with radioactively labeled DNA, demonstrated that mammalian cells have the ability to take up exogenous DNA (6,8,11,14-16). Most of this DNA is presumably degraded in the cytoplasm of the recipient cells (8) and the degradation products can be reutilized for the synthesis of newly formed DNA (14,15). It has been shown, that a small fraction (about 1%) of the donor DNA is incorporated into the nuclei without degradation. This conclusion was based on the detection of the radioactivity in DNA with the same density as the donor DNA (6,14,16,17).

Experiments have also been performed to study the mechanism of the DNA uptake. Three different aspects of the uptake process are being considered : The permeability of the cellular membrane; the role of

the process of endocytosis, and the requirement of energy for the uptake of DNA. Affecting the cell membrane with polycations, such as DEAE-dextrane, protamine sulphate, polylysine and polyornithine, has in some experiments resulted in an enhancement (up to thirty fold) of the radioactivity in the cells (18). The importance of the process of endocytosis has been followed by electronmicroscopic observation of the uptake of DNA-gelatine (19,20) and DNA-gold complexes (20,21). It has been demonstrated that these particles enter the cytoplasm by endocytosis. These results, however, do not prove that the uptake of isolated DNA takes place in the same manner.

The results, obtained from experiments carried out to study the energy requirement of the uptake process are very contradictory. It is reported that inhibitors of the oxidative phosphorylation also inhibit the uptake of DNA (22). The inhibiting effect of these agents could not be confirmed by other investigators, using the same recipient cell lines (16,23).

The conclusion from these experiments is that, at this moment, the mechanism of the uptake of DNA by in vitro cultured cells, is largely unknown. The degradation of donor DNA in the cytoplasm of the recipient cells suggests that at least a fraction of the donor DNA is introduced into the cells by endocytosis, followed by degradation of the DNA in phagosomes by lysosomal deoxyribunocleases.

2.2 Expression of incorporated DNA.

The indications for the incorporation of donor DNA in the nuclei of recipient cells (chapter 2.1) do not prove the transfer of genetic information of the donor cells into the recipient cells. To prove such a transfer it is necessary that the presence of gene products, coded by the donor DNA, is demonstrated in the recipient cells following treatment with the donor DNA. This is only possible when assay systems are available to discriminate between the gene products coded by the donor DNA and by the host cell

DNA. In most of the experiments published, the donor and the recipient cells were derived from the same mammalian species. This hampers the distinction between homologous donor and host gene products, because they will in general have the same chemical composition. An exception to this rule would be the use of donor and recipient cells of the same species, which differ in one genetic trait by mutation, resulting in the expression of polymorphic enzymes. Such a system has been applied by Kraus (24) and Weisberger et al. (25) who used bone marrow cells from patients suffering from sickle-cell anaemia and from normal human individuals. In both reports, transfer of genetic information coding for haemoglobin, mediated by donor DNA, has been claimed.

In the classical experiments of Szybalski and Szybalska (26,27), human cells, deficient for the enzyme HPRT, were treated with DNA from HPRT positive human cells. Following incubation with DNA,HPRT positive cells were selected in medium containing hypoxanthine, aminopterin and thymidine (the H.A.T. selection medium) (26,28). Although the transfer of the HPRT gene into the recipient genome was claimed, alternative explanations for the restoration of the HPRT activity in the recipient cells are possible. The intraspecies system (human-human), used by Szybalski et al., prevents the identification of the HPRT positive phenotype as being from donor origin. The possibility exists, that the HAT resistant cells, isolated by Szybalski et al., are the result of reversion from HPRT deficient to HPRT positive by genetic or epigentic alterations. Transformation experiments, using an interspecies system, have been carried out by Shin et al.(29) using HPRT deficient mouse cells and DNA extracted from HPRT positive human cells. With this system they were able to isolate colonies in HAT medium. All isolated colonies contained the HPRT of the original mouse type, as determined by electrophoresis. These results indicate that the recovery of HAT resistant cells from HPRT deficient recipient cells cannot be taken as evidence for genetic transformation. The appearance of the colonies more likely indicates that reversion has taken

place.

The occurrence of reversion has also been reported in a number of cases following cell fusion of HPRT deficient rodent cells and HPRT positive human cells or chicken cells (30-32,74, see also chapter 3,4.3). In the hybrids, a rodent HPRT was observed instead of the human or chicken enzyme. Moreover, other investigators have repeated Szybalski's experiments and failed to reproduce these results (33).

Other claims of gene transfer by DNA, concerning resistance to a drug as a marker (13, 34-36), have the same weakness as Szybalski's claim, as these experiments, carried out in intraspecies systems, excluded the identification of the origin of the derived phenotype.

CHAPTER 3 GENETIC TRANSFORMATION IN MAMMALIAN CELLS MEDIATED BY ISOLATED CHROMOSOMES.

3.1 Isolation of metaphase chromosomes

The chromosomes in eukaryotic cells in interphase are dispersed and fragile. They cannot be identified as separate units. During the mitotic phase of the cell cycle, the chromosomes are condensed into well defined structures. In this stage it is possible to isolate the chromosomes from the cells.

Many reports have been published concerning the isolation of mammalian metaphase chromosomes from cultured cells of human and rodent origin. The procedure for the isolation of chromosomes from metaphase cells consists of the following steps: Firstly, synchronization of the cell cycle of the cultured cells, in order to enrich the cell population with cells in mitosis, followed by harvesting of the mitotic cells. Secondly, incubation of the cells in a hypotonic buffer, in order to spread the chromosomes inside the cells and to facilitate the breaking of the cells. Thirdly, breaking of the cells using homogenization procedures, resulting into a mixture of chromosomes, fine cellular debris, membranes, nuclei and intact cells. Fourthly, separation of the chromosomes from the cellular components, which is accomplished by differential centrifugation, zonal centrifugation or filtration through fine sintered porous filters. Within these steps, different approaches have been followed, all having their specific advantages and disadvantages.

Several synchronization techniques have been applied in these studies. They can be subdevided in two categories: Firstly accumulation of cells in mitosis by colchicine or vinblastine and selective detachment of the mitotic cells from the culture flask (37,38); secondly, inhibition of cells in S-phase by specific inhibitors of the DNA synthesis (39). In our experiments we have accumulated Chinese hamster cells and human HeLa S3 cells in mitosis by growing the cultures for 16 hours in the presence of colchicine. This relatively simple technique has resulted in high yields of mito-

tic cells (about 60-80% of the population). (appendix publication 1).

Buffers of different pH have been used for the swelling of the cells and the isolation of the chromosomes. Acid pH, generally between 2.1 and 5.7 (37,40,41-43) and near neutral pH (38,44-46) are most commonly used. In two publications, the isolation of chromosomes is described at alkaline pH of 9,6 (47) and 10.5 (48). Lowering the pH has the effect of increasing the contraction of the chromosomes (41). This contraction might increase the resistance of chromosomes to mechanical damage. However, the use of low pH buffers might result in denaturation of chromosomal proteins and DNA (41,49). This denaturation is not reflected in changes in the chemical composition of the isolated chromosomes. Chromosomes isolated at pH3 were found to contain 15-20% DNA, 14% RNA and 66-74% protein (37), whereas a similar distribution of these components was found in chromosomes isolated at neutral pH (44).

Degradation of DNA during hypotonic treatment at acid and neutral pH is described by Wray et al.(48). These investigators determined the molecular weight of the DNA, following extraction from chromosomes isolated at different pH. Molecular weights, observed in DNA from chromosomes isolated at acid or neutral pH, were lower than the molecular weights in DNA extracted from chromosomes isolated at alkaline pH. However, there is no evidence that these differences in molecular weight actually reflect differences in the DNA composition in these isolated chromosomes.

In general divalent cations, as Mg^{++}, Ca^{++} and Zn^{++} have always been added to the isolation buffer to maintain the metaphase structure of the chromosomes and to prevent clumping of the isolated chromosomes (41,44). In addition to these ions, nonionic detergents, such as Tween 80, Triton X-100 and Saponine, have been used to facilitate the disruption of the cells, the liberation of the chromosomes, and to avoid aggregation of the chromosomes.

In our studies of gene transfer, mediated by isolated chromo-

somes (appendix publications 1-4), we followed the method of Maio and Schildkraut (44) concerning the composition of the isolation buffer. It was expected that the biological activity of the chromosomes would be preserved best using neutral pH during swelling and isolation, as provided in this technique.

In order to liberate the chromosomes different homogenization techniques have been used. These techniques are vigorous shaking of the cells in the presence of glass beads (40,42), pressing the cells through a 22g needle (45,48,49), exposing the cells into a French pressure cell (49,50), homogenization with a Dounce homogenizer (41,44,47), or a Waring blendor (51). In our experiments we used a Sorvall Omnimixer (appendix publication 1-4).

In general two techniques are available to separate the chromosomes from the other cellular components. The most commonly used method is purification of the chromosomes by differential centrifugation through sucrose gradients (44). The other technique includes selective filtration of the homogenate through fine sintered filters (37,43). This technique results in a considerable loss of chromosomes (54). For this reason we have applied sucrose gradients in our experiments (appendix publications 1-4).

As shown in the appendix publication 1, our technique used for the isolation of chromosomes has yielded preparations of morphologically stable chromosomes. Staining of the isolated human chromosomes with the trypsin-Giemsa banding technique (52) resulted in the normal banding pattern of the individual human chromosomes (appendix publication 3). This indicates that the isolation procedure has not affected the chromosomal structures which are responsible for the banding pattern.

Several procedures have been devised for the fractionation of chromosomes and their isolation in separate classes. These procedures involve centrifugation of the purified chromosome suspension through lineair density gradients, mostly prepared with sucrose (42,50,53-55). In these expe-

riments, the chromosomes are found to be distributed through the whole gradient, according to their size (55). Some of these experiments resulted in isolation of three (42) or four (54) groups of chromosomes, which were enriched with chromosomes of particular size classes. The variation in contraction between homologous chromosomes, which is the result of differences in the duration of the metaphase arrest between the cells (appendix publication 3), hampers the fractionation of chromosomes into discrete classes.

Another method for the fractionation of chromosomes is based on their DNA content (56). The chromosomes are stained for DNA with the fluorescent dye ethidium bromide, analysed for the DNA content and separated with an electronic cell sorter. This method has resulted in a separation of nine distinct groups of Chinese hamster chromosomes. Variation in contraction does not influence these results. This novel technique allows separation of purified populations of individual chromosomes, suitable for biochemical and biological studies.

3.2 Incorporation of isolated chromosomes in cultured mammalian cells .

The first steps in the genetic transformation of mammalian cells, mediated by isolated chromosomes, consist of the uptake and incorporation of the chromosomes into the genome of the recipient cells.

By using radioactively labeled chromosomes, the uptake and incorporation can be demonstrated autoradiographically (59). We have obtained evidence for uptake of isolated chromosomes from autoradiographic studies, in which Chinese hamster cells were incubated with Chinese hamster chromosomes, labeled with ^{3}H-thymidine. Spots of silver grains over the cytoplasm and the nucleus have been observed (appendix publication 1).These spots were found in cells, fixed after a two hours incubation period with the chromosomes. The number of silver grain spots decreased when the cells were incubated for longer periods, followed by the appearance of radioactivity as a disperse label over all the chromosomes of the recipient cells. Similar

results were described by other investigators, all using rodent recipient cells and isolated chromosomes from rodent cells (39,43,58,59), or human cells (43,59). These results indicate degradation of the donor chromosomes and re-utilization of labeled DNA precursors during the S-phase of the recipient cells.

In experiments performed by Kato (58), the uptake of labeled chromosomes was found to be enhanced by a pre-treatment of the isolated chromosomes with protamine sulphate . Since protamine sulphate is known to interact with nucleic acids, resulting in the formation of an insoluble complex (9), the enhancement is probably due to decreased chromosomal degradation. It is expected that treatment with protamine sulphate has a destructive influence on the biological activity of the chromosomes, making it unattractive for application in gene transfer experiments.

The uptake of chromosomes has also been demonstrated by using isolated chromosomes which are morphologically distinguishable from the chromosomes of the recipient cells. Following incubation of diploid embryonic mouse cells, containing 40 chromosomes being telo- or acrocentric, with isolated HeLa S3 chromosomes being meta- or submetacentric, chromosomes of the HeLa type were observed in metaphase plates of the recipient cultures (37,52,60). These chromosomes were detected in cells fixed two days after incubation with the chromosomes. In preparations fixed four days after incubation, only denaturated chromosomes were detected in the mouse cells, in addition to the mouse complement (39).

These observations indicate that isolated chromosomes can penetrate into cultured cells and even reach the recipient genome. However, evidence for a stable incorporation of a complete chromosome in the genome of the recipient cells, has not been presented cytologically with these techniques.

3.3 Replication of isolated chromosomes following uptake in mammalian cells.

Stable incorporation of isolated chromosomes should include their participa-

tion in replication during the S-phase of the recipient cells. The experiments performed to study the replication of incorporated chromosomes involved the addition of predominantly (sub)-metacentric rat chromosomes to diploid mouse cells, containing 40 acrocentric chromosomes (39). Simultaneously ^{3}H-thymidine was added to the incubation medium. After two days, metaphases were harvested and in autoradiographic preparations cells were observed containing rat chromosomes. These chromosomes showed the same labeling pattern as the recipient mouse chromosomes. These results suggest integration of the donor chromosomes in the recipient genome and participation in the replication process.

In our studies on replication of incorporated chromosomes (appendix publication 1), a heavily labeled Chinese hamster chromosome suspension was prepared, in which the chromosomes were labeled in both chromatids. Twentyfour hours after the incubation of Chinese hamster cells with the labeled chromosomes, metaphases containing a chromosome with label over one of the two chromatids were observed in a frequency of $1x10^{-4}$. The observation of semi-conservative chromosome labeling suggests that the labeled chromosomes have been replicated in the recipient cells. Similar results were obtained by Sekiguchi et al.(39), using labeled rat chromosomes and Chinese hamster recipient cells. They found chromosomes in recipient cells with label over one chromatid, two days after the incubation, in a frequency of $1x10^{-6}$.

At least two explanations are possible for these different frequencies. Firstly in our studies we added about 10 chromosomes per recipient cell, whereas in Sekiguchi's experiments the ratio of chromosomes over recipient cells was approximately 1. The results indicate a positive correlation between the number of incorporated chromosomes and the ratio of isolated chromosomes over recipient cells in the incubation mixture. Secondly, the difference between the frequencies indicates that the uptake and incorporation is easier in intraspecies systems than in interspecies systems. Sekigu-

chi has used an interspecies system, whereas in our experiments (appendix paper 1) an intraspecies system was used.

3.4 Genetic transformation mediated by isolated chromosomes.

3.4.1 Isolation of transformed cells.

In order to prove transfer of genetic information by isolated chromosomes, the origin of the derived phenotype has to be established. This requires the selection of the cells that have been transformed. A selection system which is used for this purpose is based on the biochemical pathways of purine and pyrimidine nucleotide biosynthesis, which are present in cultured mammalian cells (61). In de novo pathways, purine and pyrimidine nucleotides are synthesized from small molecules in series of enzymatic reactions, which involve the conversion of ribose-5-phosphate to inosine monophosphate (IMP) and glutamine to thymidine monophosphate (TMP) respectively. One of the reactions from both pathways, the reduction of dihydrofolate to tetrahydrofolate by dihydrofolate reductase, can be blocked by the drug aminopterin (62). Purine and pyrimidine nucleotides can also be synthesized from preformed purine and pyrimidine bases by salvage pathways. The enzyme HPRT catalyses the reaction between phosphoribosyl pyrophosphate (PPRP) and hypoxanthine to yield IMP. The enzyme thymidine kinase (TK) catalyses the reaction between adenosine triphosphate (ATP) and (deoxy)thymidine, resulting in the formation of TMP.

The deficiency of the enzyme HPRT or TK has the potential of providing a basis for chemical selection, either for, or against, mutant cells which lack one of these enzymes. Selection against the mutant cells is obtained by the use of agents, such as aminopterin, converting thereby normal cells to hypoxanthine or thymidine requiring auxotroophs. If hypoxanthine is the only available source of purines, then conversion of hypoxanthine into IMP, catalyzed by HPRT, is required for survival. On the other hand, if thymidine is the source of pyrimidines, it has to be converted by TK for

survival of the cells. The mutant cells which lack one of the enzymes, cannot grow in medium supplemented with aminopterin, hypoxanthine and thymidine (HAT medium; 26,28).

All our experiments, performed to accomplish genetic transformation, mediated by isolated chromosomes, were based on this HAT selection system (appendix publication 1-4).

3.4.2 Genetic transformation in an intraspecies system.

Our first experiments concerning transfer of genetic information for HPRT activity, by means of isolated chromosomes, were performed in an intraspecies combination of donor and recipient cells. Chromosomes, isolated from HPRT positive Chinese hamster cells, were incubated with HPRT deficient Chinese hamster cells. Following selection in HAT medium, colonies of HPRT positive cells were isolated (appendix publication 1). The frequency of HAT resistant colonies depended on the number of chromosomes that was added (5×10^{-5} and 16.6×10^{-5} after incubation of 10^6 cells with 10^7 and 10^8 chromosomes respectively). Plating of the recipient HPRT deficient Chinese hamster cells in HAT medium, either without pretreatment with chromosomes from HPRT positive cells or following treatment with chromosomes from HPRT deficient cells, never yielded surviving colonies. Therefore, the occurrence of reversion of the HPRT deficient cells to HPRT positive cells may be excluded. Lethally irradiated Chinese hamster cells were used as recipients to exclude the possibility that intact HPRT positive cells were present in the chromosome suspension. These data indicate that the appearance of the HPRT positive cells is the result of the incubation with the isolated chromosomes. Due to the similar origin of the donor and recipient cells, it was not possible to prove the donor origin of the concerned HPRT activity of the recipient cells.

3.4.3 Genetic transformation in interspecies systems.

The transfer of genetic information coding for HPRT, in a system using human

chromosomes from HPRT positive cells and HPRT deficient Chinese hamster recipient cells, is indicated by the presence of HPRT positive cells 24 hours after the incubation (appendix publication 1). Recipient cells in mitosis seem to have a higher chance in being transformed to HPRT positive cells than have cells in interphase (appendix publication 1). Treatment of the recipient cells being in interphase, with inactivated Sendai virus according to the procedure used in somatic cell hybridization (63), resulted in higher yields of HPRT positive cells 24 hours after incubation with chromosomes. Similar enhancing effects caused by Sendai virus were found in the number of HPRT positive colonies observed 2-6 weeks after incubation. It is difficult to explain these observations, which indicate that the cellular and nuclear membranes play a role in the induction of HPRT in the recipient cells following incubation with the chromosomes. The similar morphologic state of donor chromosomes and the chromosomes of the recipient cells during mitosis, and the absence of the nuclear membrane might facilitate the uptake of chromosomes into the recipient genome during mitosis.

In a system using donor and recipient cells from different species one can discriminate between gene products coded by donor and host DNA. This distinction is based on differences in amino-acid composition of homologous enzymes originated during evolution (see also chapter 1). These differences can be demonstrated by physico-chemical characterization of the enzymes, e.g. by electrophoresis (51, 64-67), or by immunological characterization of the species specific proteins involved (65, 66).

The first evidence for genetic transformation, based on the expression of donor genes in recipient cells, was obtained by Mc Bride and Ozer in an interspecies system (64). HPRT deficient mouse cells were incubated with chromosomes from HPRT positive Chinese hamster cells. HPRT positive colonies were isolated in HAT medium. The electrophoretic characterization of the HPRT in these cells showed that the enzyme was coded by Chinese hamster genes. In our experiments we used Chinese hamster HPRT deficient

recipient cells and human chromosomes to accomplish genetic transformation (appendix publication 2). These experiments also resulted in the observation of HAT resistant colonies. By using electrophoresis, we were able to demonstrate that the HPRT in these cells was of human origin. Similar results were also obtained by Willecke et al. (66) and Burch et al. (51), both using mouse recipient cells and isolated human chromosomes. They could also demonstrate that the enzyme in the transformed cells, responsible for growth in HAT medium, was coded by human genes.

It is known from the HPRT deficiency manifested in the Lesch-Nyhan syndrome (68) and from cell hybridization studies (69), that the gene, coding for HPRT, is located on the human X chromosome and presumably also on the X chromosome of Chinese hamster cells. The gene coding for HPRT is linked with the genes coding for the enzymes phosphoglycerate kinae (PGK), α-galactosidase A (α-Gal A) and glucose-6-phosphate dehydrogenase (G6PD). The presence of HPRT in our experiments as well as in those of Willecke et al. (66) and Burch et al. (51) was never found to be accompanied by the transfer of the other X-linked genes. In these experiments the presence of donor chromosomal material could not be demonstrated. This indicates that only a small fraction of the human genome, estimated as less than 1% (66), is actually transferred into the recipient cells.

The stability of the transformed phenotype has been investigated by culturing the transformed cells under nonselective conditions (64,66, appendix publication 2). In our experiments, two different groups of transformed cells could be distinguished (appendix publication 2). One group of colonies lost the HPRT activity when they were cultured under nonselective conditions. In the other group, the HPRT activity was retained under nonselective conditions at the same level as found when they were cultured in HAT medium. These results, and the comparable data obtained by Mc Bride et al. (64) and by Willecke et al. (66), suggest that both unstable and stable integration of the donor genetic information for HPRT can occur in the rodent

cells. The occurrence of stable colonies may be due to incorporation of donor chromosomal material into the chromosomes of the recipient cells. The loss of the donor HPRT in the other group indicates the existence of a free chromosomal fragment, bearing the HPRT locus. This fragment can be lost during cell growth under nonselective conditions. This hypothesis is supported by the observation that the decrease of the HPRT activity in these cells is caused by a decrease in the number of cells, producing HPRT, as shown by autoradiography. The activity in the cells which retain enzyme activity seems to remain constant.

Most of the gene transfer data have, sofar, been obtained with the HPRT system. Its use is attractive because of the available selection systems, and its X-chromosomal localization in man and probably also in other mammals. However, data obtained in somatic cell hybridization experiments have suggested a rather complex regulation of the expression of the HPRT gene in mammalian cells. Fusion of HPRT deficient mouse cells and Chinese hamster cells with chicken erythrocytes resulted in the expression of chick-like HPRT activity in the absence of any recognizable chick chromosomal material (70-72). This indicates the incorporation of a small chick chromosomal fragment, bearing the HPRT locus. Similar results were obtained after fusion of HPRT deficient mouse cells with frog erythrocytes (73). However, re-expression of the rodent HPRT gene was indicated in other experiments in which HPRT deficient mouse and rat cells were fused with chicken or human fibroblasts (30-32, 74).

In view of these contradictory results, the proof for gene transfer mediated by isolated chromosomes, strongly requires the use of systems other than the induction of the HPRT positive phenotype.

Using the HAT selection medium it is also possible to select against mutant cells which lack the enzyme TK (see also chapter 3, 4.1). Based on this selection, we have been able to transfer the human gene for TK, using TK deficient Chinese hamster cells and isolated human chromosomes

(appendix publication 4). Following incubation, TK positive colonies were isolated in HAT medium. The electrophoretic characterization of the TK in these cells demonstrated the human origin of this enzyme.

It is known from cell hybridization studies that the gene, coding for TK, is located on the human chromosome 17 and is closely linked with the gene coding for galactokinase (Gak), (75). Following electrophoretic characterization of the TK positive cells we were also able to demonstrate the expression of the human gene coding for Gak. These results indicate cotransfer of the human TK and Gak genes. Cotransfer of these genes was also obtained by Willecke et al. (67), who used TK deficient mouse cells and human chromosomes. In our experiments, and in those of Willecke et al. the presence of donor chromosomal material could not be demonstrated, indicating that only a small fragment of the human chromosome 17, bearing the genes for TK and Gak, is transferred to the recipient cells.

The stability of the transformed phenotype was studied in our experiments, and by Willecke et al. (67), by culturing the HAT resistant cells under nonselective conditions. All colonies, isolated in HAT medium in our experiments, retained the TK activity under nonselective conditions. This indicates that donor chromosomal material is incorporated into the chromosomes of the recipient cells. The results obtained by Willecke et al. (67) suggested both a stable and an unstable integration of the human gene for TK in the recipient genome. By comparing the results, obtained with the HPRT and the TK system, it can be postulated that small fragments of the donor chromosomes, bearing the selectable enzymes, can be transferred to the recipient cells. The obtained HAT resistant phenotype may be due to either incorporation of the chromosomal fragment into the chromosomes of the recipient cells, or to the occurrence of a free donor chromosomal fragment in the genome of the recipient cells.

3.4.4 Genetic transformation by using human-Chinese hamster hybrids as recipient cells.

In order to investigate the influence of the presence of human chromosomes in recipient Chinese hamster cells on the uptake, integration and expression of donor human chromosomes, experiments have been carried out with HPRT deficient human-Chinese hamster cell hybrids (HRB28) as recipient cells (appendix publication 3). These hybrids contained a small number of human chromosomes. After incubation with isolated chromosomes, colonies were isolated in HAT medium. The electrophoretic characterization of the HPRT demonstrated that this enzyme, in all colonies isolated in HAT medium, was of human origin. In addition, the other X-chromosomal genes, coding for the enzymes G6PD, PGK and α-Gal A, were also expressed in these cells. Moreover, a cytogenetic analysis of these cells revealed the presence of a human X chromosome. With these recipient cells, transfer of only a fragment of the X chromosome, as observed using the interspecies system, has not been demonstrated. A strong argument in favour of uptake, incorporation and expression of a complete chromosome is the observation that in all colonies which have been isolated, G6PD had the A phenotype, corresponding with the G6PD in the donor cells, whereas the human leucocytes, used for the production of the hybrids, expressed the G6PD B phenotype. These results exclude the possibility that hybrid recipient cells could have retained the human X chromosome from the leucocytes in spite of selection in favour of HPRT deficient cells.

The transfer of a complete X chromosome in this system might indicate that the presence of human chromosomes in the recipient cells influences the incorporation and expression of intact donor chromosomes.

The transfer and the expression of this human chromosome was reproduced using another HPRT deficient human-Chinese hamster hybrid line, originating from another fusion between the same parental cells as used for the

production of the HRB 28 cell hybrids.

Hybrid cells have also been used in gene transfer experiments concerning the human TK-locus (appendix publication 4). Incubation of TK deficient hybrids with isolated HeLa chromosomes has resulted in the appearance of HAT resistant colonies. The presence of both the human enzymes TK and Gak was demonstrated by electrophoresis. This cotransfer has been observed in all isolated clonal cell lines. However, chromosome analysis of these isolated cell lines has revealed the absence of human chromosomes, indicating that, with the use of these recipient cells, only a fragment of chromosome 17, bearing the genes for TK and Gak, has been transferred. In the four TK positive colonies which were isolated, the TK positive phenotype was lost during growth in nonselective medium. This loss suggests that the transferred genes behave idependently from the recipient genome.

The unstable incorporation of small pieces of genetic information, observed in the TK deficient hybrids, clearly differs from the transfer of a complete chromosome in the HPRT deficient hybrids (appendix publication 3). Our explanation for this difference is found in the observation of a complete loss of the human chromosomes in the TK deficient hybrids prior to the incubation with the chromosomes. The transfer of fragments, observed in the experiments with Chinese hamster parental cells, as well as with the TK deficient hybrids, supports the hypothesis that the presence of human chromosomes is required to achieve the incorporation of a complete chromosome.

This hypothesis has to be proven in experiments in which TK or HPRT deficient hybrids, which have retained different sets of human chromosomes, are used as recipient cells. Moreover, these experiments might present evidence for the necessity of the presence of specific chromosomes in order to achieve transfer of a complete chromosome.

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SUMMARY

In this thesis the research is described concerning transfer of genetic information from mammalian cells into other mammalian cells, mediated by isolated DNA and isolated chromosomes. The purpose of this research is to contribute to the insight in the localization of genes on chromosomes, the regulation of gene expression and the interaction between different genes concerning the formation of a particular phenotype.

Data, obtained from the literature concerning the transfer of genetic information mediated by extracted deoxyribonucleic acid (DNA), indicated that donor DNA was incorporated into mammalian cells. However, most of this DNA was degraded by the recipient cells. A small fraction (about 1%) of the donor DNA was incorporated in the genome of the recipient cells without degradation (chapter 2, 1).

Incubation, in different experiments, of cultured mammalian cells with DNA from cells from the same species, resulted in the observation of alterations in the phenotype of the recipient cells. However, the coding of these altered phenotypes by incorporated DNA could not be demonstrated. It was not possible to distinguish between homologue gene products of the donor and recipient cells, because they had, in general, the same chemical composition.

In the research, which underlies this dissertation, we have accomplished transfer of genetic information via incorporation of isolated chromosomes (appendix publication 1-4). Uptake of isolated chromosomes was indicated following incubation of Chinese hamster cells with chromosomes, labeled with ^{3}H-thymidine (chapter 3, 2). In spite of the observation that degradation of the chromosomes took place, indications were also found for replication of incorporated chromosomes in the recipient cells. This was demonstrated with experiments in which chromosomes were observed, labeled in only one chromatide, 24 hours after incubation of Chinese hamster cells with

chromosomes labeled in both chromatids (chapter 3, 3).

After incubation of Chinese hamster cells, deficient for the enzyme hypoxanthine phosphoribosyltransferase (HPRT) with chromosomes from HPRT positive Chinese hamster cells, we have isolated colonies in selective (HAT) medium which were HPRT positive. The donor origin of the HPRT activity could not be demonstrated since both the donor and the recipient cells originated from the same species (chapter 3, 4.2).

HPRT positive colonies were also isolated following incubation of HPRT deficient Chinese hamster cells with human chromosomes. The human origin of the observed HPRT activity was demonstrated using electrophoretic techniques (chapter 3, 4.3). The transfer of the human X-linked gene for HPRT was never found to be accompanied by the transfer of other X-chromosomal genes. Chromosome analysis of the isolated colonies has revealed in the absence of human chromosomes. From these results it was concluded that a small fragment of the human X chromosome, bearing the HPRT locus, was transferred to the recipient cells. The experiments concerning the stability of the transformed phenotype resulted in the observation of colonies loosing HPRT activity under nonselective conditions, whereas in other colonies the HPRT activity retained under these circumstances. The occurrence of stable colonies might be due to incorporation of donor chromosomal material into the chromosomes of the recipient cells. The loss of the HPRT activity in the other colonies indicates that the transferred genes behaved independently from the recipient genome.

We were also able to isolate colonies in HAT medium following incubation of thymidine kinase (TK) deficient Chinese hamster cells with human chromosomes (chapter 3, 4.3). With electrophoresis it was demonstrated that the TK activity in these colonies was of human origin. In addition, the expression of the human gene for galactokinase (Gak), which is linked with the TK locus and located on the human chromosome 17, was also shown in these cells. This indicates cotransfer of the two genes, media-

ted by isolated chromosomes. However, the presence of chromosome 17 could not be demonstrated in these cells, indicating that in these experiments only a fragment of the chromosome was incorporated in the recipient cells, which was presumably integrated into a chromosome of the host cells.

In order to investigate the influence of the presence of human chromosomes in recipient Chinese hamster cells on the uptake and expression of human donor chromosomes, experiments were carried out with human-Chinese hamster hybrids as recipient cells (chapter 3, 4.4). These hybrids had retained a small number of human chromosomes and were HPRT deficient. Colonies were isolated in HAT medium following incubation of these cells with human chromosomes. In these colonies, it was demonstrated that the human gene for HPRT, and, in addition also other X-chromosomal genes were expressed. One of the products of the transferred genes (the enzyme G6PD) could be determined as originating from the donor of the isolated chromosomes. These results presented evidence for the uptake and expression of the human X chromosome into the HPRT deficient hybrids.

Hybrid cells were also used to accomplish the transfer of the human TK locus (chapter 3, 4.4). The hybrids which were used had lost all the human chromosomes at the time of incubation with chromosomes. After the incubation colonies were isolated in HAT medium. The presence of the human-like enzymes TK and Gak in these cells was demonstrated. Neither chromosome 17, nor any other human chromosomal material could be observed in these cells. With these recipient cells, transfer of only a fragment of chromosome 17 was accomplished.

The transfer of fragments of chromosomes, observed using the HPRT deficient and TK deficient Chinese hamster cells and the TK deficient hybrids, and the transfer of a complete X chromosome by using the HPRT deficient hybrids suggests that the presence of human chromosomes in the recipient cells at the time of incubation is required to accomplish the incorporation of a complete human chromosome.

SAMENVATTING

In dit proefschrift wordt een onderzoek beschreven naar de mogelijkheid om genetische informatie van de ene naar de andere zoogdiercel over te dragen d.m.v. geisoleerd DNA en geisoleerde chromosomen. Het onderzoek heeft als doel een bijdrage te leveren in het inzicht in de plaats van genen op de chromosomen, in de regulatie van genexpressie en in de interactie van verschillende genen bij het tot stand komen van een bepaald fenotype.

Uit de literatuur gegevens die betrekking hebben op de overdracht van genetische informatie met behulp van geextraheerd deoxyribonucleïne zuur (DNA), blijkt dat zoogdiercellen in staat zijn aangeboden DNA in de cel op te nemen. Het merendeel van dit opgenomen DNA wordt in de acceptor cellen afgebroken. Een fractie (ongeveer 1%) kan worden geincorporeerd in het genoom van de acceptor cellen zonder te worden afgebroken (hoofdstuk 2, 1).

Incubatie van gekweekte zoogdiercellen met DNA uit cellen van dezelfde diersoort heeft in verschillende onderzoekingen geresulteerd in fenotypische veranderingen in de acceptor cellen. Een overtuigend bewijs dat deze veranderingen zijn te herleiden tot de expressie van donor DNA is echter niet gegeven. De homologe genprodukten van de donor en de acceptor cellen konden in deze experimenten niet worden onderscheiden omdat ze in de meeste experimentele omstandigheden dezelfde chemische samenstelling hebben (hoofdstuk 2, 2).

In het experimentele werk dat ten grondslag ligt aan dit proefschrift (appendix publikaties 1 t/m 4) hebben we getracht overdracht van genetische informatie te bewerkstelligen d.m.v. incorporatie van geisoléerde chromosomen.

Na incubatie van Chinese hamster cellen met geisoleerde chromosomen, gemerkt met ^{3}H-thymidine, werden aanwijzingen verkregen voor de opname van deze chromosomen in acceptor cellen (hoofdstuk 3, 2). Wel

werden in het cytoplasma van deze cellen vaak verschijnselen waargenomen die wijzen op afbraak van de opgenomen chromosomen. Dat echter opgenomen chromosomen ook deel kunnen nemen aan de replicatie in de acceptor cellen is gebleken uit proeven waarbij chromosomen werden waargenomen, gemerkt in slechts één chromatide, 24 uur na incubatie van Chinese hamster cellen met chromosomen, die in beide chromatiden waren gemerkt met ^{3}H-thymidine (hoofdstuk 3, 3).

Na incubatie van Chinese hamster cellen, die deficient waren voor het enzym hypoxanthine phosphoribosyltransferase (HPRT), met chromosomen uit HPRT positieve Chinese hamster cellen, werden in selectief (HAT) medium HPRT positieve kolonies geisoleerd, (hoofdstuk 3, 4.2). Aangezien zowel de donor als acceptor cellen van Chinese hamster oorsprong waren, kon echter niet worden bewezen dat de HPRT activiteit in deze kolonies van donor origine was (hoofdstuk 3, 4.2).

In experimenten, uitgevoerd met HPRT deficiente Chinese hamster cellen en menselijke chromosomen werden eveneens HPRT positieve kolonies geisoleerd in HAT medium. Met behulp van electroforetische technieken werd aangetoond dat het HPRT van menselijke oorsprong was (hoofdstuk 3, 4.3). De overdracht van het bij de mens op het X-chromosoom gelegen locus voor HPRT gaat in geen van de geisoleerde kolonies gepaard met de expressie van andere X-chromosomale genen. Evenmin kon met cytologische technieken de aanwezigheid van menselijke chromosomen of gedeelten hiervan, in deze cellen worden vastgesteld. Hieruit werd geconcludeerd dat slechts een niet herkenbaar gedeelte van het X-chromosoom, waarop het HPRT gen ligt, opgenomen is in het genoom van de acceptor cellen. Uit onderzoek naar de stabiliteit van het HPRT positieve fenotype bleek dat in sommige kolonies HPRT activiteit verdween als ze gekweekt werden in niet selectief medium, terwijl in andere kolonies de HPRT activiteit onder deze omstandigheden aanwezig bleef. Deze resultaten suggereren dat het opgenomen fragment in sommige cellen geintegreerd kan zijn in een chromosoom van de

acceptor cellen, terwijl het zich in andere cellen onafhankelijk kan gedragen van de gastheer chromosomen.

Ook na incubatie van thymidine kinase (TK) deficiente Chinese hamster cellen met menselijke chromosomen werden in HAT medium kolonies geisoleerd (hoofdstuk 3, 4.3). Met electroforetische technieken werd aangetoond dat het TK in deze kolonies van menselijke oorsprong was. Tevens werd gevonden dat het menselijke gen, dat codeert voor het enzym galactokinase (Gak) en dat evenals het gen voor TK op chromosoom 17 is gelegen, in deze cellen tot expressie kwam. Dit wijst dus op de gelijktijdige overdracht van deze twee genen d.m.v. geisoleerde chromosomen. Chromosoom 17 werd echter in deze cellen niet teruggevonden. Dit wijst er op dat in deze experimenten slechts een klein deel van chromosoom 17 in de acceptor cellen is opgenomen, vermoedelijk geintegreerd in een chromosoom van de acceptor cellen.

Om na te gaan of de aanwezigheid van menselijke chromosomen in de acceptor cellen tijdens de incubatie met geisoleerde chromosomen invloed heeft op de opname en de expressie van menselijke donor chromosomen, werd gebruik gemaakt van mens-Chinese hamster hybride cellen als acceptor cellen (hoofdstuk 3, 4.4). Deze hybriden bevatten nog een klein aantal menselijke chromosomen en waren HPRT deficient. Na incubatie met geisoleerde menselijke chromosomen werden kolonies geisoleerd in HAT medium. In al deze kolonies werd aangetoond dat, naast het gen voor HPRT, ook andere genen, gelegen op het menselijke X-chromosoom, in deze cellen to expressie kwamen. Van één gen (het G6PD gen) kon worden vastgesteld dat dit van de menselijke donor van de chromosomen afkomstig was. Tevens kon de aanwezigheid van het X-chromosoom worden vastgesteld. Deze resultaten wijzen op de opname en de expressie van het menselijk X-chromosoom in de HPRT deficiente hybriden.

Hybride cellen werden ook gebruikt voor het onderzoek naar de overdracht men het menselijke TK gen (hoofdstuk 3, 4.4). De hier ge-

bruikte hybride cellen bleken geen herkenbare menselijke chromosomen meer te bevatten op het moment van incubatie met chromosomen. De incubatie van deze cellen met menselijke chromosomen resulteerde in de isolatie van kolonies in HAT medium waarin de aanwezigheid van de menselijke genen voor TK en Gak kon worden aangetoond. Chromosoom 17 van de mens, of andere menselijk chromosomaal materiaal werd in deze cellen niet teruggevonden. Deze resultaten wijzen erop dat in dit geval slechts een fragment van chromosoom 17 in deze cellen aanwezig was.

De overdracht van fragmenten van chromosomen welke gevonden werd in de systemen met de HPRT deficiente en TK deficiente Chinese hamster cellen en de TK deficiente hybriden en de overdracht van een compleet X-chromosoom in het systeem met de HPRT deficiente hybriden suggereert dat de aanwezigheid van menselijke chromosomen in de acceptor cellen nodig is om de opname van een compleet menselijk chromosoom te kunnen bewerkstelligen.

DANKWOORD

Bij het verschijnen van dit proefschrift wil ik mijn dank betuigen aan allen die direct of indirect hebben bijgedragen aan het tot stand komen van dit proefschrift. In de eerste plaats ben ik mijn promotor, Prof. Dr. D.Bootsma, zeer erkentelijk voor de continue steun die ik van hem heb gehad met betrekking tot het experimentele werk en de interpretatie van de resultaten hieruit. Zijn kritische beschouwing van deze resultaten en het meedenken over vervolg experimenten heeft mede geleid tot het uiteindelijke werk. Zeer veel heb ik geleerd van zijn nimmer aflatende kritische zin met betrekking tot het schrijven van de publikaties en dit proefschrift. Bovenal ben ik hem dankbaar voor de zeer plezierige wijze waarop ik met hem heb kunnen samenwerken.

Met name wil ik ook mejuffrouw J. van der Horst bedanken. Zij heeft een zeer belangrijk aandeel gehad in de uitvoering van de experimenten. Vooral bij het vaak moeizame werk, dat zo typerend is voor het ontwikkelen van nieuwe enzym scheidingsmethoden, bleek haar steun van essentieel belang. De prettige samenwerking met haar heb ik zeer op prijs gesteld.

Verder wil ik noemen de beide co-referenten, Dr.A.J. van der Eb en Prof. Dr. J.F. Jongkind, die dit proefschrift kritisch hebben doorgelezen. Hun waardevolle opmerkingen heb ik met dank aanvaard.

Met plezier denk ik terug aan de discussies die in wekelijkse werkbesprekingen, en ook daarbuiten, zijn gevoerd met de leden van de "linkage" groep van de afdeling Celbiologie en Genetica.

De heer T.M.van Os ben ik zeer erkentelijk voor de nauwgezette verzorging van het fotografische gedeelte van dit proefschrift. Dank ben ik ook verschuldigd aan mejuffrouw M. van Duuren en de heer P.Hartwijk voor het tekenen van de figuren en aan de heer J.G.M. Fengler voor de fotografische opnamen hiervan. Dank ben ik ook verschuldigd aan de heer A.H. Hammer voor de verzorging van de celkweek media.

Mevrouw A.M.Godijn en mejuffrouw J.A.Bolman dank ik voor het altijd schone en steriele glaswerk en voor de koffie tijdens momenten van ontspanning. Iedereen op het secretariaat van de afdeling, in het bijzonder mevrouw R.J.v.d.Hoek-Boucke en mevrouw C.M. van Vugt-van Vliet, wil ik bedanken voor het typewerk van de publicaties en van dit proefschrift. Mevrouw C.D. van de Berg-Haverman wil ik bedanken voor de kritische opmerkingen en adviezen over het in dit proefschrift geschreven Engels.

Prof. Dr. Ir. A.Rörsch, afdeling Biochemie van de Rijksuniversiteit te Leiden, ben ik bijzonder erkentelijk voor de geboden mogelijkheid om gedurende de afgelopen maanden mijn tijd geheel te besteden aan dit proefschrift.

Mijn ouders zal ik altijd dankbaar blijven voor de studie mogelijkheden die ze mij gegeven hebben.

Lieve Loes, ik wil jou bedanken voor het enthousiasme waarmee je in staat was mijn belangstelling voor andere zaken dan dit proefschrift hoog te houden.

CURRICULUM VITAE

In november 1947 ben ik te Ter Hole (Zeel.) geboren. Na hier een begin te hebben gemaakt met de lagere school ben ik in 1955 verhuisd naar Kraggenburg (N.O.P.) waar de lagere school werd voltooid. De middelbare school werd doorlopen in Bolsward waar in 1965 het eindexamen HBS-B werd afgelegd. In september van dat jaar ben ik begonnen aan de studie biologie aan de Katholieke Universiteit te Nijmegen. Het kandidaats examen werd afgelegd in januari 1969 en het doctoraal examen in september 1971 (hoofdvak biochemie, bijvakken zoologie en exobiologie). In october 1971 ben ik in dienst getreden binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Binnen deze vakgroep werd het onderzoek verricht dat in dit proefschrift is beschreven. Sinds september 1976 ben ik verbonden aan de afdeling biochemie van de Rijksuniversiteit te Leiden, waar ik zal gaan werken binnen de werkgroep moleculaire basis van celdifferentiatie en oncogenese bij planten en onderzoek zal gaan verrichten op het gebied van de somatische plantecelhybridisatie.

Appendic Publications 1-4

Somatic Cell Genetics, Vol. 1, No. 2, 1975, pp. 137–152

PUBLICATION 1

Incorporation of Isolated Chromosomes and Induction of Hypoxanthine Phosphoribosyltransferase in Chinese Hamster Cells

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Received 23 September 1974—Final 2 December 1974

Abstract—*Evidence is presented for the uptake of radioactive-labeled isolated Chinese hamster chromosomes following incubation with Chinese hamster cells. Metaphases were found which contained radioactive labeled chromosomes in a very low frequency, and in some of the labeled chromosomes only one chromatid was labeled. Incubation of hypoxanthine phosphoribosyltransferase (HPRT)-deficient Chinese hamster cells with chromosomes isolated from HPRT$^+$ Chinese hamster or human cells resulted in the appearance of HPRT$^+$ cells. Clones derived from these cells were isolated in HAT medium. Cells in mitosis during incubation with the chromosomes yielded three times more HPRT$^+$ clones than did cells in interphase. The intraspecies combination involving recipient cells and chromosomes from Chinese hamster origin yielded significantly higher numbers of HPRT$^+$ clones than did the interspecies system using human chromosomes and Chinese hamster recipient cells (5×10^{-5} and 6×10^{-6} respectively). Electrophoresis of HPRT from Chinese hamster cells treated with human chromosomes revealed the pattern of the human enzyme.*

INTRODUCTION

Evidence for transfer of genetic information in cultivated cells using isolated chromosomes has recently been reported by Mc Bride and Ozer (1). Hypoxanthine phosphoribosyltransferase (HPRT) activity of Chinese hamster origin was detected following incorporation of Chinese hamster chromosomes in mouse HPRT-deficient cells.

Genetic transfer by chromosome incorporation requires the introduction of chromosomes, or parts of chromosomes, into the cell, incorporation into the genome, and replication of its DNA. Finally, chromosomal genes should be expressed in recipient cells. Incorporation of chromosomes in the cytoplasm of cells is, in most cases, followed by degradation (2) and incor-

poration of degraded products into the host genome (3,4). Evidence for incorporation of intact chromosomes into the nucleus was found by Yosida and Sekiguchi (5). Replication of incorporated chromosomes in the genome of recipient cells has recently been indicated (6).

In the present study, the incorporation as well as the expression of isolated chromosomes in host cells was examined. These experiments were carried out using both an intra- (Chinese hamster) and an interspecies (human chromosomes in Chinese hamster cells) system. Differences in the uptake of chromosomes by cells in interphase and mitosis and the influence of treatment with inactivated Sendai virus were investigated. Evidence is presented that human chromosomal material is incorporated and is expressed in Chinese hamster cells treated with human chromosomes.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The mutant cell line Wg3-h was derived from the Chinese hamster cell line DON by stepwise selection in increasing concentrations of 8-azaguanine and propagated in the presence of 6 μg 8-azaguanine/ml of medium. HPRT activity is about 2% compared to normal DON cells. Details on isolation, propagation, and growth characteristics of this mutant line are published elsewhere (7).

The human HeLa S3-cell line, the human T cell line (8), and the Chinese hamster DON-cell-line were used to isolate chromosomes.

Cells were maintained in Ham's F10 medium supplemented with 10% calf serum and antibiotics. Cell cultures in petri dishes were incubated in a humidified 5% CO_2–95% air mixture at 37°C. Cells in metaphase were obtained by treating cultures of DON, Wg3-h, or HeLa S3 cells in F10 medium with colchicine (0.12 μg/ml) for 16 hours. A double, thymidine block (9), followed by treatment with colchicine, was used to synchronize the T cells. Metaphases were harvested by the selective detachment technique (10).

Isolation of Chromosomes. Chromosomes were isolated from metaphases, using a modification of Maio and Schildkraut's method (11). Approximately 10^8 metaphases were incubated for 15 minutes at 37°C in TMS buffer (0.02 M tris-HCl, pH 7.4; 0.003 M $CaCl_2$, and 0.1% Saponine). After this hypotonic treatment, the cells were homogenized with a Sorvall-Omni Mixer. This homogenization and all further steps were carried out at 4°C.

The homogenate was diluted and layered on top of a 1 M solution of sucrose in TMS buffer and centrifuged for 10 minutes at 100*g*. The upper layer, which contained the chromosomes and fine cellular debris, was poured off, diluted with buffer, and layered again on top of a 1 M solution of sucrose.

After centrifugation at 1000*g* for 20 minutes, the chromosome pellet

was suspended in TMS buffer and washed by centrifugation at 1000*g* for 10 minutes. This washing procedure was repeated until the chromosome suspension was free of microscopically visible contaminants.

Incubation of Cells with Chromosomes. Wg3-h cells were washed to remove the colchicine and were incubated with a variable number of chromosomes at 37°C in a concentration of 5×10^6 cells per milliliter of F10 medium. When Sendai virus was used, the cells were washed with Hank's solution, without glucose, at 37°C. The cells were then incubated with the chromosomes in 1 ml Hank's without glucose. One milliliter of inactivated Sendai virus (1000 HAU) was added, the mixture kept at 4°C for 5 minutes, and then incubated at 37°C for 20 minutes. Finally, the suspensions were diluted with F10 medium and seeded in petri dishes (4-cm, Falcon plastics) in a concentration of 10^6 cells per dish in F10 medium without hypoxanthine and thymidine.

Selection and Cloning Procedures. After the Wg3-h cells were incubated with the chromosomes for 24 hours, the medium was replaced by F10 medium containing hypoxanthine, aminopterin, thymidine, and glycine (HAT medium) (12). Wg3-h cells do not grow in this medium because they lack HPRT activity. They were refed at intervals of 3–4 days and cultured for about 6 weeks. Clones were isolated and propagated in F10-HAT medium.

Labeling Procedures and Autoradiography. Labeled chromosomes were isolated from Chinese hamster DON cells grown for 40 hours in the presence of [^{3}H]thymidine (0.1 μCi/ml; specific activity 2 Ci/mM). Uptake of labeled chromosomes was studied 24 hours after incubation with Wg3-h interphase cells. Cells in interphase and mitotic cells were collected 24 hours after seeding by treating the monolayers with 0.25% trypsin, followed by centrifugation and incubation with 0.075 M KCl. The cells were fixed with methanol-acetic acid (3:1), spread by air-drying, and stained with orcein. HPRT activity was determined autoradiographically by culturing the cells in the presence of [^{3}H]hypoxanthine (10 μCi/ml, specific activity 1 Ci/mM) for 16 hours between 24 and 40 hours after the chromosomes were added. The cells were fixed in Bouin's fluid. Autoradiographs were prepared using Ilfort K2 liquid emulsion and were exposed for 1 to 3 weeks. The autoradiographic preparations of the cells labeled with [^{3}H]hypoxanthine were stained with hematoxylin-floksine.

HPRT Assay. HPRT activities in cell extracts were measured using [^{3}H] hypoxanthine (50 μCi/ml, specific activity 1 Ci/mM) as substrate, as described by Harris and Cook (13), in an incubation volume of 100 μl. Cell extracts were prepared according to the method described by Meera Khan (14).

Electrophoresis of HPRT. Cell extracts were run on cellulose acetate gels for 3 hours in 0.02M phosphate buffer. The enzyme activity was

detected using [^{14}C]hypoxanthine as substrate and the inosine monophosphat was bound to DEAE-paper, followed by autoradiography (15).

RESULTS

Chromosome Isolation. The DON, T, and HeLa S3 cells were arrested in metaphase before chromosome isolation. After exposure to colchicine for 16 hours about 60% of the DON and T cells were in mitosis. For HeLa S3 cells, this percentage was 80. After the metaphase cells were selectively detached, cell fractions were obtained containing 90–95% mitotic and 5–10% cells in interphase.

In the final preparations, 20% of the chromosomes were recovered, as determined by hemocytometer counting. Less than 1% of the structures observed in these preparations were nuclei or fragments of nuclei. Purified chromosome preparations were morphologically stable in the isolation buffer and could be stored for prolonged periods of time in liquid nitrogen. Preparations of isolated Chinese hamster and HeLa chromosomes are presented in Fig. 1.

Uptake of Labeled Chromosomes. Wg3-h interphase cells were incubated with labeled Chinese hamster DON chromosomes and cultured for 24 hours, followed by fixation and autoradiography. These chromosomes were all heavily labeled in both chromatids (Fig. 2). Cells that incorporated labeled chromosomes are shown in Figs. 3 and 4. Almost all labeled chromosomes found in the metaphase plates were labeled in both chromatids (Fig. 3). A few metaphases were present, having a chromosome that showed label in only one of the two chromatids (Fig. 4 and Table 1). The percentage of interphases showing a distinct spot of silver grains above the nucleus was 0.7. This percentage was 0.3 for mitotic cells at the time of fixation. In a total of 100,000 metaphases, 10 were found to have a partially labeled chromosome. In these cases, one of the two chromatids showed a labeled region.

Expression of HPRT Activity. The appearance of HPRT-positive cells after HPRT-negative Chinese hamster cells were treated with chromosomes isolated from HPRT-positive cells is shown in Fig. 5. Cells able to incorporate [^{3}H]hypoxanthine were present 24 hours after incubation with the chromosomes. When mitotic cells were treated with chromosomes, HPRT-positive cells appeared in a frequency of 10^{-4} (Table 2). Because metabolic cooperation occurred, groups of labeled cells were counted as one labeled cell. Fewer HPRT-positive cells were present when recipient cells were in interphase during treatment with chromosomes (2×10^{-6}). Treatment with Sendal virus did not influence the number of HPRT-positive cells when mitotic cells were used. When recipient cells were in interphase, about 10

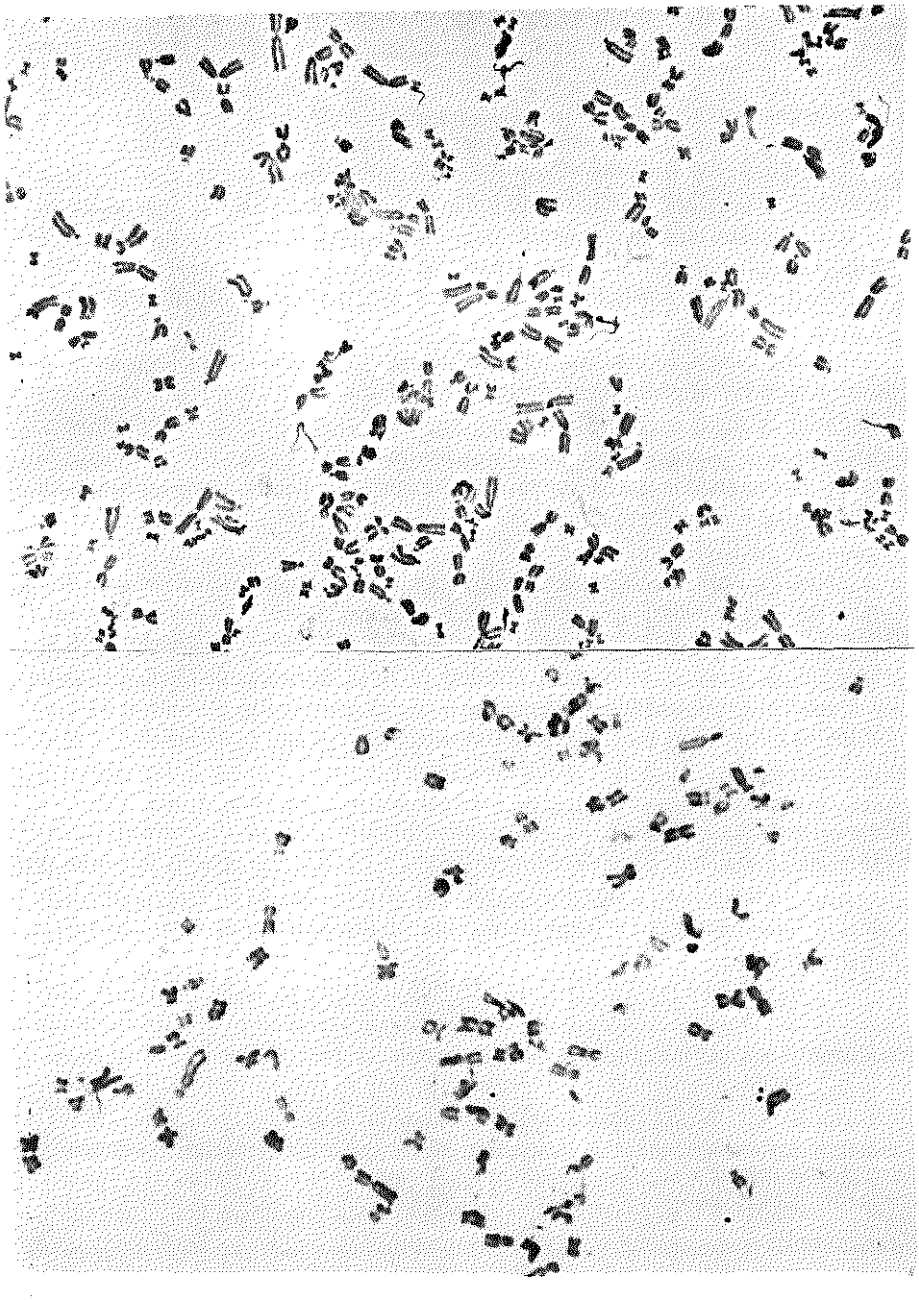

Fig. 1. Isolated metaphase chromosomes from cultured cells. Top, chromosomes from Chinese hamster DON cells. Bottom, chromosomes from human HeLa cells. Orcein stain × 400

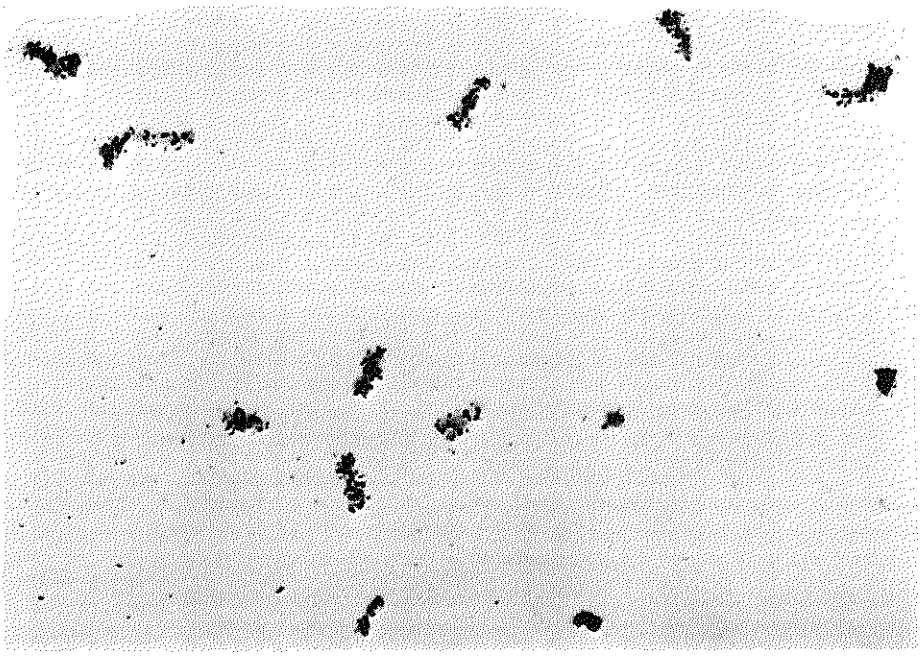

Fig. 2. Isolated metaphase chromosomes from Chinese hamster cells. The cells were labeled with [^{3}H]thymidine for 40 hours. (× 630)

times more HPRT-positive cells were found after treatment with Sendai virus than without the virus (Table 2).

Isolation of HPRT Positive Clones. Wg3-h cells were incubated with chromosomes from HPRT-positive Chinese hamster cells and seeded in normal F10 medium. Clones appeared after the addition of HAT medium, within a period of 2 to 4 weeks. The data presented in Table 3 show that HAT-resistant clones were found in all experiments in which Wg3-h recipient cells and chromosomes isolated from HPRT-positive cells were used. The frequency of resistant clones depended on the number of chromosomes that was added (5×10^{-5} and 16.6×10^{-5} after incubation with 10^7 and 10^8 chromosomes respectively). Moreover, the intraspecies Chinese hamster combination seems to be more efficient than the interspecies system, using Chinese hamster cells and human chromosomes (5×10^{-5}, compared to 6×10^{-6} under the same conditions). Lethally irradiated DON cells were used as recipient cells to exclude the possibility that intact HPRT-positive cells were present in the chromosome suspension. Wg3-h cells were incubated in another control experiment with chromosomes isolated from Wg3-h cells. The reversion rate of Wg3-h cells was tested in six separate experiments in which a total of 2.4×10^7 Wg3-h cells were incubated in HAT

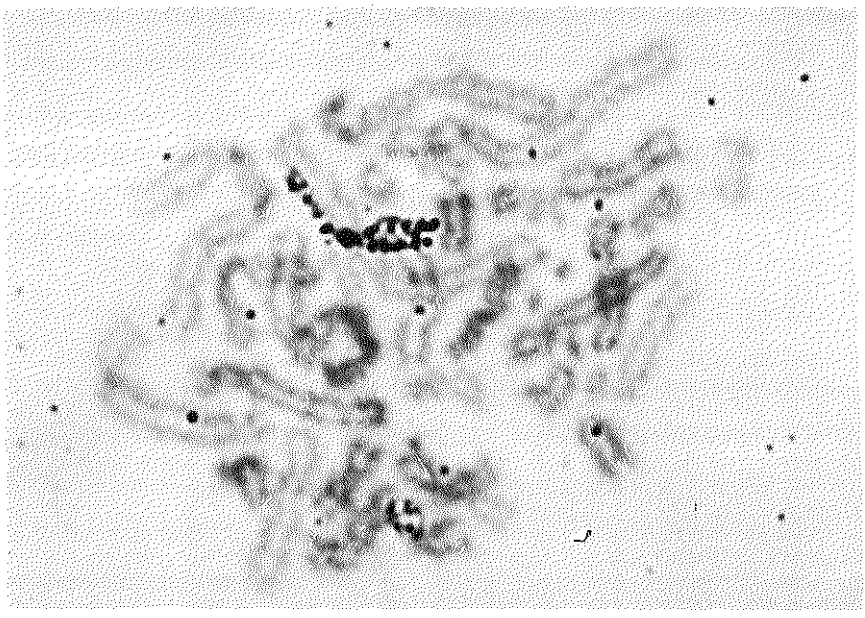

Fig. 3. A hamster cell in metaphase with one labeled chromosome. The chromosomes of the host cell are unlabeled. (× 630)

medium (Table 3). In addition, 8×10^7 cells were inoculated in HAT medium in bottles (not mentioned in the table). HPRT-positive clones were not observed in any of these experiments. The data presented in Table 3 clearly demonstrate that HAT-resistant clones were only observed after HPRT-deficient cells were treated with chromosomes originating from HPRT-positive cells. After Chinese hamster cells were treated with human chromosomes, 47 independent cell lines, all originating from different petri dishes, were isolated. Of these, 25 were isolated using T-cell chromosomes; the remaining 22 were isolated after treatment with HeLa chromosomes. In HAT medium, 26 of these cell lines gradually lost their capacity to grow and died within 6 weeks after isolation. The remaining 21 cell lines were cultured in HAT medium for more than 1 year. Three independent cell lines were isolated after Wg3-h cells were treated with Chinese hamster chromosomes. These cell lines did not lose HPRT activity.

The number of HAT-resistant clones was higher after chromosomes were incubated with cells in mitosis than with cells in interphase (Table 4). The plating efficiency of Wg3-h mitotic cells was examined after treatment with colchicine and found to be 30%. In the absence of colchicine, the plating efficiency of Wg3-h cells is 50–60%. The data presented in Tables 2 and 4 were obtained in the same experiment. A strong correlation was found

between the number of HAT-resistant clones (Table 4) and the number of [^{3}H]hypoxanthine labeled cells 24 hours after chromosome treatment (Table 2). Mitotic and interphase cells differed to the same extent under these distinct experimental conditions. Again pretreatment of cells in mitosis with Sendai virus did not change the frequency of HAT-resistant clones, whereas the number of clones yielded by virus-treated interphase cells was 10 times higher (Table 4).

Characterization of HPRT-Positive Cell Lines. HPRT activity was assayed in three Wg3-h clones isolated after treatment with DON chromosomes and five clones isolated after incubation with human chromosomes (Table 5). In the intraspecies combination, HPRT-activity closely approached the level found in DON wild-type cells. Treatment with human chromosomes resulted in 20–40% of the activity found in DON cells and about 15–25% of the activity in the human T-cell line. Fig. 6 shows the electrophoretic patterns of HPRT from 11 cell lines isolated after Wg3-h cells were treated with human chromosomes, and from human HeLa S3 cells as well as DON cells. Results of electrophoresis of an HPRT-positive hybrid cell line, isolated after Wg3-h cells fused with human lymphocytes, are also presented. HPRT-activity is not observed after the electrophoresis of extracts of Wg3-h cells. Gel electrophoresis shows that the HPRT products of

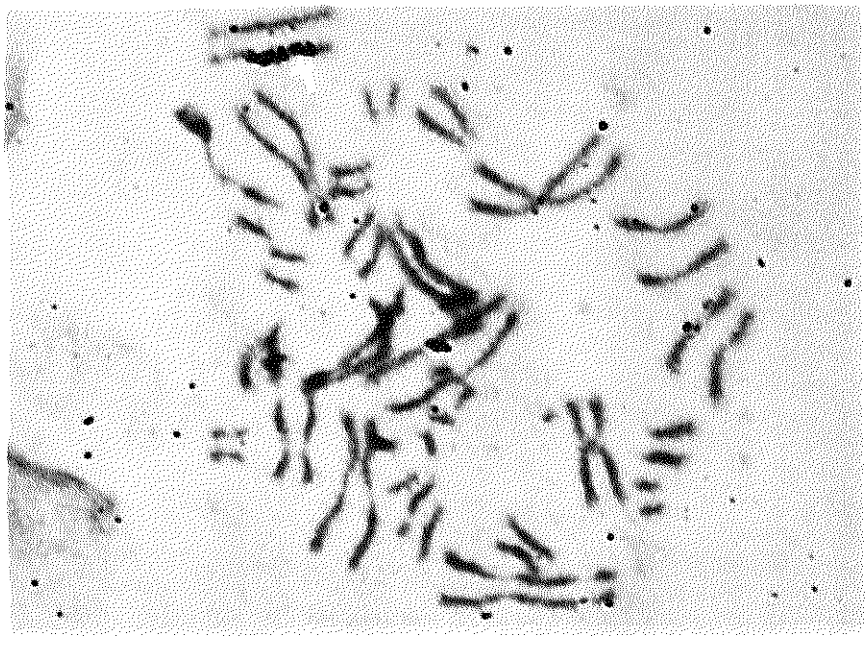

Fig. 4. A hamster cell in metaphase with one partially labeled chromosome. Note that the label is present in only one of the two chromatids (arrow). (× 630)

Table 1. Number of cells showing labeled chromosomes following the incubation of Wg3-h cells with labeled DON chromosomes[a]

Cell stage at fixation	Cells estimated (N)	Percent of cells with labeled chromosomes	Cells containing a chromosome with label in only one chromatid (N)
Mitosis	100,000	0.3	10
Interphase	6,000	0.7[b]	—

[a] Wg3-h cells, 10^6, were incubated with 10^7 labeled chromosomes and examined after 24 hours.
[b] Only cells showing a spot of silver grains over nuclei were counted.

clones H-Wg3-h 17, 30, 34, 36, 37, 40, 45, and 46 had the same electrophoretic mobility as human HPRT. No radioactivity was detected in cell lines H-Wg3-h 38, 42, and 47, despite their growth in HAT medium.

DISCUSSION

Uptake of radioactive labeled chromosomes by recipient cells is indicated by the presence of spots of silver grains over interphase nuclei in our autoradiographic preparations. Their location inside the nucleus is supported by the observation of labeled chromosomes in metaphase spreads.

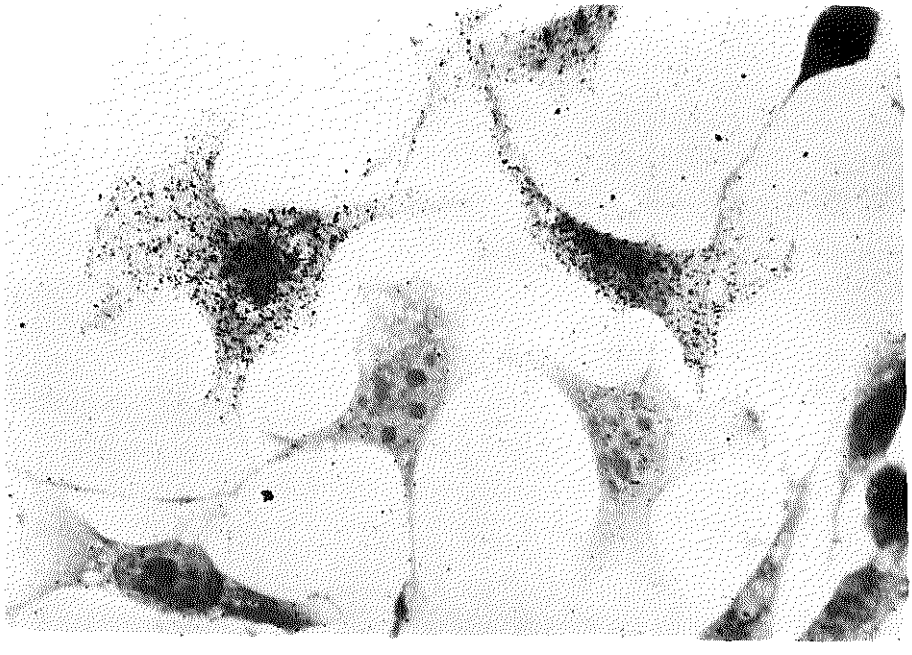

Fig. 5. Autoradiographic appearance of HPRT-positive cells after HPRT-deficient Chinese hamster cells were treated with chromosomes from HPRT-positive human cells. (× 400)

These findings confirm previous observations (6). The fate of these incorporated chromosomes is uncertain. Evidence has been presented that incorporated chromosomes are able to replicate (6). These observations were based on a very limited number of cells showing a chromosome labeled in only one of the chromatids. In a total of 100,000 mitoses, we found 10 cells showing label in a region of one of the two chromatids of a chromosome, whereas the other chromosomes in the same cell were unlabeled. Reuse of labeled DNA precursors from incorporated but degraded chromosomes would result in a sparse labeling of many chromosomes in the cell, which might escape detection by the autoradiographic technique. The nonreciprocal chromatid labeling most likely indicates the incorporation of a piece of a labeled chromosome in the host genome at a very low frequency.

The first evidence for gene transfer, using isolated chromosomes, was reported by Mc Bride and Ozer (1), using chromosomes isolated from HPRT-positive Chinese hamster cells and HPRT-negative mouse, recipient cells. HPRT was characterized as Chinese hamster HPRT. At 24 hours after HPRT-deficient Chinese hamster cells were treated with chromosomes obtained from HPRT-positive Chinese hamster or human cells, we found individual cells in the preparations that were able to incorporate [^{3}H]hypoxanthine. Those cells were only present when the chromosomes originated from HPRT-positive cells, indicating the specificity of HPRT induction in Chinese hamster cells. The frequency of these hypoxanthine-incorporating cells corresponded very well with the frequency of HAT-resistant clones isolated during a period of 6 weeks after treatment with chromosomes. Incubation of mitotic cells with the chromosomes seems to be slightly more effective than incubation with cells in interphase. In the latter case, pretreatment with Sendai virus results in a significantly higher number of HAT-resistant clones. It is difficult to explain these observations, which indicate that cellular and nuclear membrane play a role in the induction of HPRT activity in the treated cells. Moreover, the similar morphologic state of donor and host chromosomes during the incubation of cells might facilitate

Table 2. Frequency of [^{3}H]hypoxanthine-incorporating cells following incubation of Wg3-h cells with HeLa chromosomes

Cell stage at chromosome treatment	Frequency of cells incorporating [^{3}H]hypoxanthine[a] $\times 10^{-6}$		Plates with labeled cells per experiment (N)	
	+ Sendai virus	− Sendai virus	+ Sendai virus	−Sendai virus
Mitosis[b]	97 ± 14	111 ± 30	10	10
Interphase[b]	26 ± 20	2.2 ± 2.0	10	9

[a] Average of four experiments ± SD; in one experiment 10 plates were seeded. A group of labeled cells is counted as one HPRT-positive cell. The cells were examined 24 hours after incubation with chromosomes.

[b] Per plate 10^7 chromosomes were incubated with 10^6 cells.

Table 3. Frequency of HAT-resistant clones following incubation of Wg3-h cells with isolated chromosomes

Treated cell line	Origin of chromosomes	Chromosomes/ 10^6 cells (N)	Frequency of clones[a] in HAT medium ($\times 10^{-6}$)	Plates seeded[b] (N)	Plates with clones (N)
Wg3-h	$HPRT^+$ Chinese hamster	10^7	49 ± 6	8	8
Wg3-h	$HPRT^+$ Chinese hamster	10^8	166 ± 34	6	6
Wg3-h	$HPRT^+$ human	10^7	6 ± 2	12	12
Wg3-h	HPRT Chinese hamster	10^8	0	8	0
Wg3-h	———	0	0	24	0
DON (irradiated)	$HPRT^+$ Chinese hamster	10^8	0	6	0
DON (irradiated)	$HPRT^+$ Chinese hamster	10^7	0	8	0

[a] Average of 2–6 independent experiments ± *SD*.
[b] One plate was seeded with 10^6 cells.

Table 4. Frequency of HAT-resistant clones following incubation of Wg3-h cells with HeLa chromosomes

Cell stage at chromosome treatment	Frequency[a] of HAT-resistant clones × 10^{-6}		Plates with clones per experiment (N)	
	+ Sendai virus	− Sendai virus	+ Sendai virus	− Sendai virus
Mitosis[b]	81 ± 22	105 ± 10	10	10
Interphase[b]	26 ± 5	3 ± 2	10	8

[a] Average of four experiments ± *SD*; in one experiment 10 plates were seeded.
[b] Per plate, 10^7 chromosomes were incubated with 10^6 cells.

uptake in the host genome. The general validity of these observations has still to be proven, because in the present experiments only one cell type was used as the recipient cell (the Wg3-h).

The intra- (Chinese hamster) and interspecies (human chromosomes and Chinese hamster cells) systems differed in two aspects: (a) the intraspecies combination yielded a higher number of HAT-resistant clones and (b) HPRT activity after Chinese hamster Wg3-h cells were treated with human chromosomes was significantly lower than the activity in the intraspecies combination. These differences indicate that the induction of HPRT in Wg3-h cells is specifically determined by the cell type from which the chromosome suspension originates. Our cytologic data concerning the incorporation of labeled chromosomes and the specificity of HPRT induction in terms of chromosomal origin support the assumption that HPRT activity is the result of incorporation of donor genetic material. However, the instability of the enzyme in some of the clones and its apparent dependence

Table 5. HPRT activity in clones isolated after incubation of Wg3-h cells with chromosomes

Cell line	Specific activity[a] (nM/h/mg protein)	Percent activity in $HPRT^+$ Chinese hamster cells
DON	495	100.0
Wg3-h	12	2.4
T-cell	688	139.0
D-Wg3-h 1[b]	426	86.0
D-Wg3-h 2	389	78.5
D-Wg3-h 3	408	82.4
T-Wg3-h 1	127	25.6
T-Wg3-h 10	180	36.4
T-Wg3-h 17	200	40.4
T-Wg3-h 18	120	24.2
T-Wg3-h 20	116	23.4

[a] Mean values calculated from at least two independent assays.
[b] The D-Wg3-h lines were isolated from $HPRT^-$ Chinese hamster cells incubated with chromosomes from $HPRT^+$ Chinese hamster cells. The T-Wg3-h lines were isolated from $HPRT^-$ Chinese hamster cells incubated with chromosomes from $HPRT^+$ human cells.

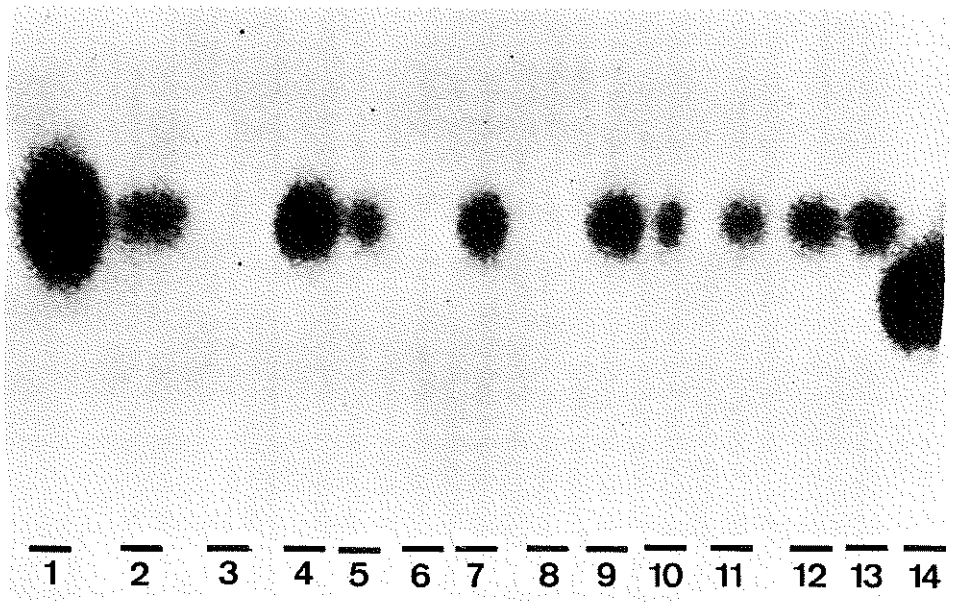

Fig. 6. The electrophoretic patterns of HPRT in clones isolated after treatment of Wg3-h cells with human chromosomes. Channel (1), human HeLa S3 cells; (2) hybrid cell line obtained by fusion of human lymphocytes with Wg3-h cells; (3–13) clones of Wg3-n cells treated with HeLa S3 chromosomes, the clones H-Wg3-h 47, 46, 45, 42, 40, 38, 37, 36, 34, 30, and 17 respectively; (14) DON wild-type cells.

for growth in HAT medium, which must be investigated further, does not exclude the occurrence of an epigenetic phenomenon. Experiments testing the stability of the HPRT activity by culturing the cells in nonselective medium are in progress.

A physicochemical and immunologic characterization of HPRT activity in isolated clones might answer the question as to whether HPRT is of human or hamster origin. A first series of electrophoretic analyses of HPRT activity in Wg3-h cells treated with human chromosomes has shown the presence of human-like HPRT. These results indicate that the human HPRT locus can be selectively incorporated by Chinese hamster cells, confirming previously published data by Mc Bride and Ozer (1) using mouse $HPRT^-$ cells and chromosomes from $HPRT^+$ Chinese hamster cells. However, our experiments have also shown that other genes, known to be located on the human X chromosome (glucose-6-phosphate dehydrogenase, phosphoglycerate kinase, and α-galactosidase), were not expressed in those clones. These results might indicate that only a small fragment of the chromosome is transferred into recipient cells. Karyotype analyses of HPRT clones carried out so far have not revealed the human chromosome in these cells.

Following fusion of mouse A9 cells with chick erythrocytes, HPRT activity, which showed chick-like electrophoretic behavior was observed (16–18). In other experiments using human-mouse and human-rat somatic cell hybrids, re-expression of HPRT of rodent origin occurred (19,20). It was suggested that a genetic factor from the human cell determined the expression of the rodent structural gene for HPRT. Transfer in chick erythrocyte-mouse cell fusions could not be reproduced using the thymidine kinase locus (17). These results indicate that HPRT activity in somatic cells is regulated in a special way, not yet understood. Therefore, it is necessary to repeat the experiments with isolated chromosomes, using marker genes other than HPRT. Experiments in which the transfer of thymidine kinase is being studied are in progress.

ACKNOWLEDGMENT

These studies were supported by FUNGO, Foundation of Medical Scientific Research in The Netherlands.

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Somatic Cell Genetics, Vol. 2, No. 2, 1976, pp. 155–164

PUBLICATION 2

Expression of Human Hypoxanthine Phosphoribosyl Transferase in Chinese Hamster Cells Treated with Isolated Human Chromosomes

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Received 14 October 1975—Final 12 December 1975

Abstract—*Chinese hamster cells deficient for the enzyme hypoxanthine phosphoribosyl transferase (HPRT) were incubated with isolated human metaphase chromosomes and 21 colonies were isolated in HAT medium. Three different types of cell lines were established from these clones. First, 4 cell lines had 10–30% of normal Chinese hamster HPRT activity with the same electrophoretic mobility as human HPRT. This HPRT activity remains detectable during at least 8 weeks of growth of the cells in nonselective medium. Second, 3 cell lines also had human-like HPRT with the same activity as the first type. This HPRT persists only if the cells are grown in HAT medium and disappears during 8 weeks of growth in nonselective medium. Third, other clones survived in HAT medium as well as in medium with 8-azaguanine. These cells had no detectable HPRT activity. Using differential chromosome staining techniques no recognizable human chromosome fragments were found in any of the cell lines.*

INTRODUCTION

Transfer of genetic information by means of isolated chromosomes was first described by McBride and Ozer in 1973 (1). They reported the appearance of hypoxanthine phosphoribosyltransferase (HPRT) positive mouse cells after incubation of $HPRT^-$ mouse cells with chromosomes isolated from $HPRT^+$ Chinese hamster cells. Chinese hamster HPRT was expressed in these cells. Recently, evidence was presented for expression of human-like HPRT in $HPRT^-$ Chinese hamster and mouse cells following incubation of these cells with isolated human metaphase chromosomes (2–4). In the present study growth characteristics of cell lines isolated from $HPRT^-$ Chinese hamster cells treated with human metaphase chromosomes and the stability of the human phenotype in these Chinese hamster cells were exam-

ined. Three different types of clonal cell lines could be distinguished, which differed in their resistance to HAT medium and medium with 8-azaguanine. The results suggested both a stable and an unstable integration of the genetic information for HPRT in Chinese hamster cells treated with human chromosomes.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. HPRT-deficient Chinese hamster cells, Wg3-h (5), were treated with human chromosomes isolated from HeLa S3 cells following a modified procedure of Maio and Schildkraut (6). Details on the isolation procedure were described elsewhere (4). Following incubation with chromosomes, colonies were selected in F10 medium containing hypoxanthine, aminopterine, thymidine, and glycine (HAT) (7). Colonies were isolated and cell lines were established from these clones. The derived H-Wg3-h lines were also cultivated in F10 HAT medium supplemented with 10% calf serum and antibiotics for periods of several months.

HPRT Assay. HPRT activities in cell extracts were measured using (^{14}C) hypoxanthine (100μCi/ml, spec. act. 62 mCi/mmol) as substrate as described by Harris and Cook (8), in an incubation volume of 100 μl. Cell extracts were prepared according to the method of Meera Khan (9).

Autoradiography. HPRT activity in cells was determined autoradiographically by culturing cells in the presence of (^{3}H)-hypoxanthine (10 μCi/ml, spec. act. 1 Ci/mmol) for 16 hours as described elsewhere (4).

Chromosome Analysis. Chromosome preparations were prepared following treatment of mitotic cells with an 0.075 M KCl solution for 15 minutes. Cells were fixed in a 3:1 mixture of methanol and acetic acid. Chromosome preparations were made by air drying. Slides were treated with trypsin (0.025%) and stained with Giemsa. The procedure of Bobrow et al. (10) for Giemsa-11 staining was followed.

RESULTS

Electrophoresis. 21 independent colonies were isolated in HAT medium after incubation of Wg3-h cells with human chromosomes. Electrophoresis of cell extracts from these lines revealed the presence of HPRT in cell lines H-Wg3-h 30, 34, 36, 40, 45, 46, and 47. These cell lines showed HPRT having the same electrophoretic mobility as human HPRT (4). HPRT activity was not detected in the other 14 cell lines in spite of their ability to grow in HAT medium. Chinese hamster HPRT was not detected in any of the 21 cell lines. The results of electrophoresis are shown in Fig. 1. In channels 4 and 5 extracts of cells obtained from the HPRT-positive cell lines H-Wg3-h 30 and 34 were added to the gel. Channel 3 contains an extract of

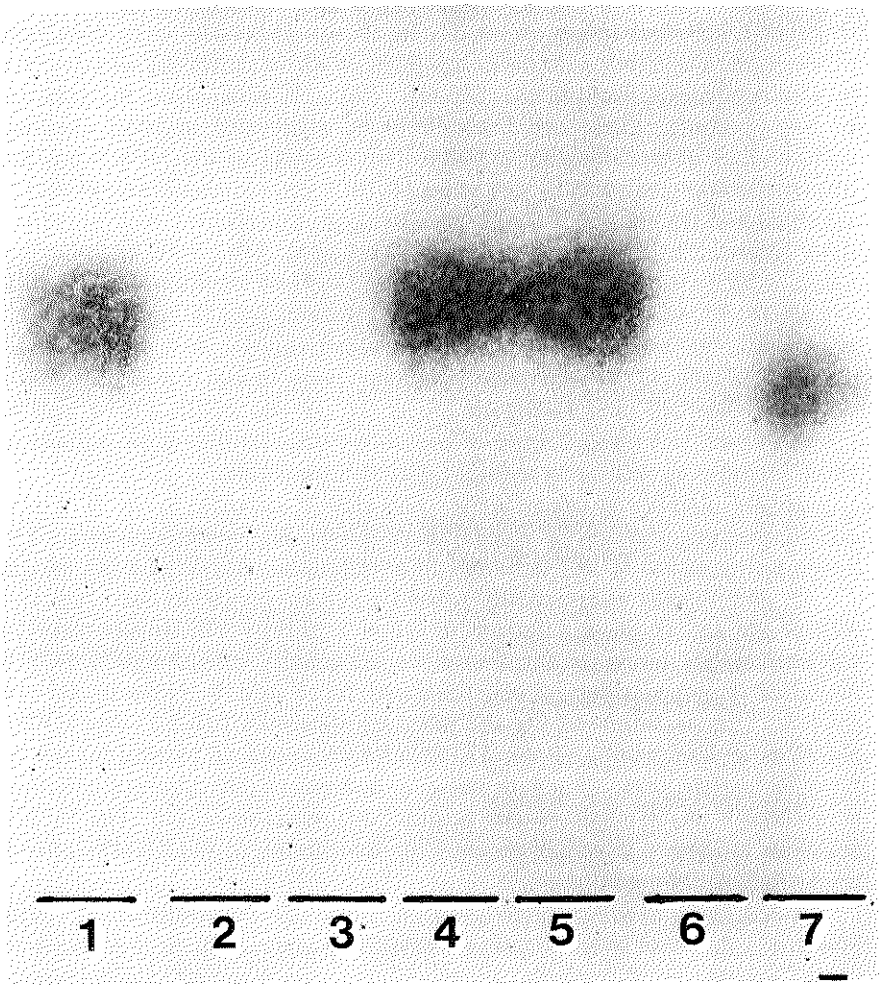

Fig. 1. The electrophoretic patterns of HPRT in clones isolated after treatment of Wg3-h cells with human chromosomes. Channel (1), human HeLa S3 cells; (2), H-Wg3-h 33, cultured in HAT medium; (3), H-Wg3-h 30, cultured in nonselective medium for 8 weeks; (4) idem cultured in HAT medium; (5) H-Wg3-h 34 cultured in nonselective medium for 8 weeks; (6) Wg3-h cells; (7), Chinese hamster DON cells.

H-Wg3-h 30 cells which were cultured in nonselective medium for a period of 8 weeks; HPRT activity has disappeared in this line. Channel 2 contains an extract of H-Wg3-h 25, which was isolated and cultured in HAT medium and did not show the presence of HPRT.

Survival of Clonal Lines in Selective Medium. The presence or absence of HPRT in the different lines was also studied by observing the ability of H-

Wg3-h lines to grow in medium containing 8-azaguanine (3γ/ml). As shown in Table 1, cell lines H-Wg3-h 30, 34, 36, 37, 40, 45, and 46 were eliminated in this medium, whereas the other cell lines, in which no HPRT was detected by electrophoresis, multiplied at the same rate as Wg3-h cells in the presence of 8-azaguanine. These cell lines could be recultured in HAT medium without loss of proliferating activity. The stability of the HPRT-positive phenotype was analyzed by culturing $HPRT^+$ cells in medium with hypoxanthine and thymidine in the absence of aminopterine (HT medium) for 3 days, followed by transfer to normal F10 medium. After 8 weeks in normal medium 10^7 cells of each line were recultured in HAT medium. Cell lines H-Wg3-h 34, 37, 40, and 46 (Type 1) showed normal growth and the lines H-Wg3-h 30, 36, and 45 (Type 2) degenerated rapidly and died in HAT medium (Table 1). In the latter case surviving clones have never been observed. The cell lines H-Wg3-h 25, 28, 31, 33, 35, 38, 39, 41–44, and 47 (Type 3) were not treated as described above because they grew initially in HAT medium as well as in medium with 8-azaguanine.

HPRT Activity in Clonal Lines. The HPRT activities in the H-Wg3-h cell populations and in wild-type DON cells were determined quantitatively. Table 1 shows that HPRT activities in populations of H-Wg3-h 30, 34, 36, 37, 40, and 46 cells after growth in HAT medium varied between 10% and 30% of the activity in populations of DON wild-type cells. In the other 14 cell lines HPRT activities did not exceed the activity in Wg3-h cells. HPRT activities in populations of cells cultured for 4 and 8 weeks in normal medium after isolation of the cell lines are shown in Table 1. The HPRT activities in lines H-Wg3-h 34, 37, 40, and 46 appeared to be constant in nonselective medium. After four weeks in nonselective medium the activities in H-Wg3-h 30, 36, and 45 cell populations were decreased. The HPRT activities in these 3 lines had reached the level found in Wg3-h cells after 8 weeks in normal medium.

A reduction in HPRT activity in these populations could be the result of either a decrease in the fraction of HPRT positive cells or a decrease in HPRT activity in all the cells. An experiment was designed to differentiate between these possibilities. The cells were cultured for 7 weeks in nonselective medium. At successive one-week intervals aliquots of the cells were cultured for 16 hours in medium containing (^{3}H)- hypoxanthine and the fraction of labeled cells was determined autoradiographically (Fig. 2). In the populations of H-Wg3-h 34, 37, 40, and 46 all cells remained labeled during the 7-week period of growth in nonselective medium. The percentage of labeled cells decreased in cultures of H-Wg3-h 30, 36, and 45. Although grain counts could not be performed, the high degree of labeling in the labeled cells indicated comparable levels of HPRT activity in positive cells in both groups of cell lines. After a period of 7 weeks almost all cells in the H-Wg3-h 30, 36, and 45 populations had HPRT levels comparable with the

Table 1. Growth Potential[a], HPRT Activities, and Chromosome Number of H-Wg3-h Cells in Selective and Nonselective Media

Cell line	Growth in medium containing			HPRT activity (% of DON cells)			Modal chromosome number (in HAT)
					nonselective medium		
	6γ/ml 8-azg.	HAT	HAT after 8 weeks in nonselective medium	HAT	4 weeks	8 weeks	
DON wild type	−	+	+	100	100	100	n.t.[b]
Wg3-h	+	−	−		3.3	1.9	38[c]
H-Wg3-h 34	−	+	+	30.4	28.4	29.0	34
H-Wg3-h 37	−	+	+	10.7	9.4	10.9	34
40	−	+	+	n.t.	9.5	9.2	39
46	−	+	+	19.5	17.8	19.2	34
30	−	+	−	29.1	11.2	1.2	19
36	−	+	−	17.1	6.8	1.6	35
45	−	+	−	23.3	10.0	2.2	38
25	+	+	n.t.	1.1	n.t.	n.t.	n.t.
26	+	+	n.t.	1.1	n.t.	n.t.	n.t.
27	+	+	n.t.	1.7	n.t.	n.t.	n.t.
28	+	+	n.t.	1.6	n.t.	n.t.	n.t.
31	+	+	n.t.	1.4	n.t.	n.t.	n.t.
33	+	+	n.t.	1.5	n.t.	n.t.	38
35	+	+	n.t.	0.9	n.t.	n.t.	n.t.
38	+	+	n.t.	3.0	n.t.	n.t.	n.t.
39	+	+	n.t.	0.8	n.t.	n.t.	36
41	+	+	n.t.	4.4	n.t.	n.t.	34
42	+	+	n.t.	1.2	n.t.	n.t.	n.t.
43	+	+	n.t.	1.4	n.t.	n.t.	38
44	+	+	n.t.	0.8	n.t.	n.t.	n.t.
47	+	+	n.t.	0.0	n.t.	n.t.	n.t.

[a] Cells were tested for their growth potential by plating them in high densities (10^7 cells/flask) in medium with 6μ/ml 8-azaguanine or HAT medium. No growth (−) means surviving clones have not been found. Normal growth (+) means that the proliferating activity was comparable with the proliferation of Wg3-h cells in 8-azaguanine and DON cells in HAT medium.

[b] n.t. means not tested.

[c] Wg3-h cells grown in normal medium treated with colchicine for 16 hours.

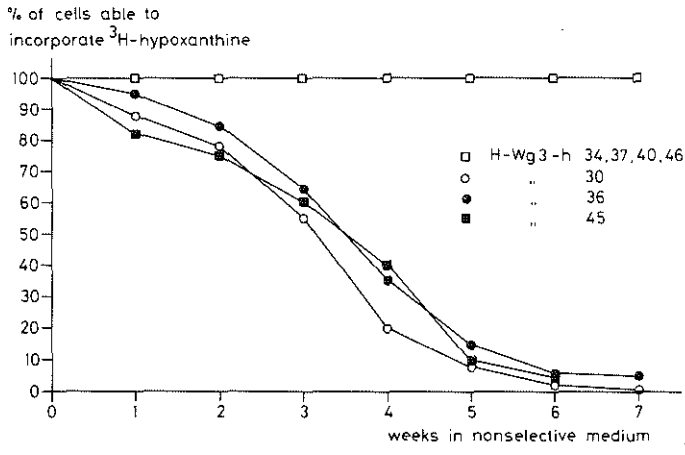

Fig. 2. Frequency of (^{3}H)-hypoxanthine labeled cells in H-Wg3-h cell populations during growth in nonselective medium. The cell lines were transferred from HAT medium (time zero) via HT medium to non-selective medium. At one-week intervals the incorporation of (^{3}H)-hypoxanthine was determined autoradiographically after incubation of the cells in medium containing 10 μCi/ml (^{3}H)-hypoxanthine (spec. act. 1 Ci/mmol) for 16 hours.

Wg3-h "parental" cells. In order to determine the influence of metabolic cooperation (11) on the number of labeled cells in these populations a plating experiment was carried out after a period of 5 weeks of growth in nonselective medium. Aliquots of the cells were obtained from the cultures, diluted, and cloned in normal medium and HAT medium. The results are presented in Table 2. As was expected, the plating efficiencies of H-Wg3-h 34, 37, 40, and 46 were the same in both media. The plating efficiencies of H-Wg3-h 30 and 36 cells in HAT medium were 11% and 16% of those in normal medium. The plating efficiency of H-Wg3-h 45 was not calculated. The values are in good agreement with the percentages of labeled cells found in the autoradiographic experiments after (^{3}H)-hypoxanthine labeling (Fig. 2).

Chromosomes Studies. 50 metaphases of the isolated cell lines were analysed for the presence of the human X chromosome. In the trypsin Giemsa-stained preparations human chromosomes were not observed. Since the Giemsa-11 technique is a differential staining procedure useful for the recognition of human chromosomes in man–Chinese hamster somatic cell hybrids (10), this technique was also applied. No human material could be recognized in any of the cell lines, even with Giemsa-11. All of the H-Wg3-h cell lines, except for H-Wg3-h 30, showed a modal chromosome number between 34 and 38 chromosomes per cell (Table 1, Fig. 3). H-Wg3-h 30 cells had a modal chromosome number of 19. The three groups of cell

lines (Type 1, 2, and 3) cannot be distinguished based on their chromosome content (Fig. 4). Even lines belonging to one type (e.g., H-Wg3-h 30, 36, and 45) might vary to a greater extent than cell lines belonging to different groups. The high chromosome numbers in these cells were most probably the result of the colchicine treatment of the Wg3-h cells before and during incubation with isolated chromosomes. A control Wg3-h "parental" cell line with a modal chromosome number of 22 chromosomes per cell was treated with colchicine for 16 hours. Cells in metaphase were collected and cultured for 4 weeks. Chromosome analysis of this line also revealed a modal chromosome number of 38. The cell line H-Wg3-h 30 most probably originated from a Wg3-h cell which was not affected by the colchicine treatment.

DISCUSSION

Transfer of genetic information after uptake of isolated chromosomes by recipient cells has now been demonstrated in several laboratories (1–4). Transfer of the gene coding for HPRT was accomplished in different cell systems, e.g., mouse A9 cells (1–3) and Chinese hamster Wg-3-h cells (4). The stability of some of the isolated clones has been investigated with variable results. McBride and Ozer (1) isolated 3 clones in HAT medium after treatment of mouse A9 cells with Chinese hamster chromosomes; 2 clones retained HPRT activity during growth in nonselective medium and 1 clone lost its HPRT activity. The three clones derived by Willecke and Ruddle (3) from A9 cells treated with HeLa chromosomes lost their HPRT activity during one month of growth in nonselective medium. In the present study three types of clones could be distinguished which were isolated in HAT medium after treatment of Chinese hamster Wg3-h cells with human chromosomes:

1. Clones having 10–30% of the HPRT activity found in wild-type DON cells. This HPRT had the same electrophoretic mobility as human

Table 2. Plating efficiencies of H-Wg3-h Cell Lines Cultured in Nonselective Medium for 5 Weeks

Cell line	Ratio of colonies in selective medium (HAT) to colonies in nonselective medium
H-Wg3-h 30	0.11
36	0.16
34	0.89
37	1.00
40	1.00
46	0.90

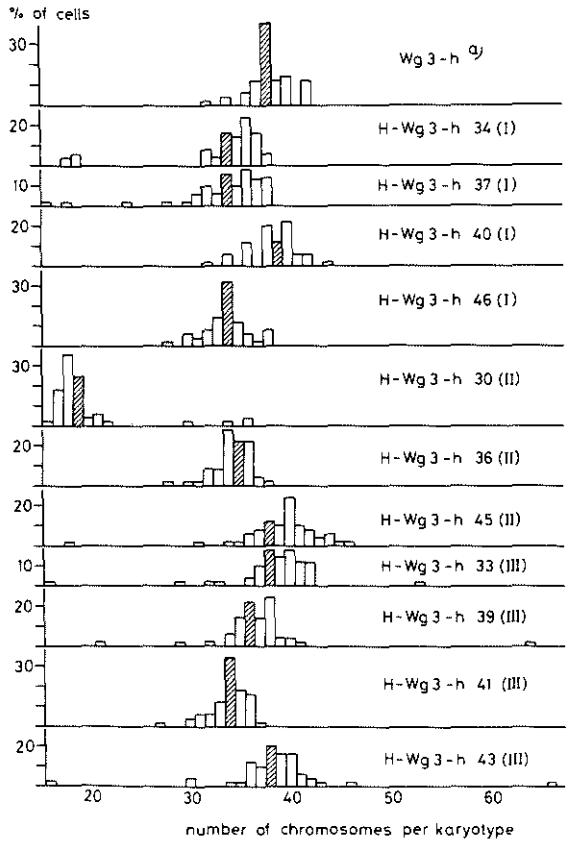

Fig. 3. Frequency distribution of chromosomal numbers in Chinese hamster Wg3-h cells treated with colchicine and in H-Wg3-h cells. The modal chromosome number is indicated by shading. 50 metaphases of every cell line were counted.

HPRT and was stable for at least 8 weeks of growth in nonselective medium (cell lines H-Wg3-h 34, 37, 40, and 46).

2. Clones with HPRT activity similar to that in Type I cells, but in contrast with Type I these clones lost their enzyme activity during 8 weeks of growth in nonselective medium (cell lines H-Wg3-h 30, 36, and 45).

3. Clones which survived in HAT medium as well as in medium containing 8-azaguanine. These cells did not have detectable HPRT activity (cell lines H-Wg3-h 25–28, 31, 33, 35, 39, 41–44, and 47).

From the gene transfer experiments in the literature a total of 6 clones with stable HPRT activity have been isolated (2 by McBride et al. and 4 in

our experiments). It is attractive to speculate on the nature of the difference between the stable and unstable HPRT positive clones. Stable clones may be due to incorporation of donor chromosome material into the recipient genome whereas unstable clones could be compatible with the existence for a free chromosome fragment bearing the HPRT genome. This fragment may be lost during cell growth, but cells which have lost the fragment can grow only when cultured in nonselective medium. This speculation is based on the observation that the decrease of the HPRT activity in these cells is caused by a decrease in the number of cells having HPRT activity as shown by autoradiography. The activity in the cells which retain enzyme activity seems to remain constant.

The application of new differential staining techniques for the identification of chromosomes and parts of chromosomes in our study did not reveal the presence of a human chromosome fragment either free or incorporated into the Chinese hamster complement. The failure to find a chromosome fragment and the absence of other X-chromosomal markers in these $HPRT^+$ cells (2–4) imply that the transferred HPRT-bearing chromosome fragment is very small and probably even smaller than 1% of the human genome as calculated by Willecke and Ruddle (3).

Most of our HAT-resistant clones were composed of cells with negligible levels of HPRT which were able to grow in HAT as well as in 8-azaguanine containing medium. It seems unlikely that the residual HPRT activity in these cells was responsible for growth in HAT medium because the Wg3-h "parental" line which does not survive in HAT medium has comparable levels of HPRT activity and (^{3}H)-hypoxanthine incorporation as seen by autoradiography (unpublished results). High levels of folate reductase, an enzyme which tightly binds to aminopterin, might be another cause for HAT resistance of these clones (12). The folate reductase activity in our clones has not yet been determined. So far clones of Type 3 have only been isolated from Wg3-h cells which were treated with chromosomes from HeLa cells. Type 3 clones did not appear following treatment of Wg3-h with chromosomes from DON wild-type cells. At the present time the nature of the chromosome fragment transferred with human chromosomes to deficient Chinese hamster cells remains unexplained.

ACKNOWLEDGMENTS

These studies were supported by FUNGO, Foundation of Medical Scientific Research in the Netherlands. The authors are very grateful to Dr. M. Heukels-Dully for the correction of the manuscript.

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Somatic Cell Genetics, Vol. 2, No. 4, 1976, pp. 359–371

PUBLICATION 3

Transfer of the Human X Chromosome to Human–Chinese Hamster Cell Hybrids via Isolated HeLa Metaphase Chromosomes

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Received 20 February 1976—Final 8 April 1976

Abstract—*Evidence is presented for the uptake of the human X chromosome by human–Chinese hamster cell hybrids which lack HPRT activity, following incubation with isolated human HeLa S3 chromosomes. Sixteen independent clonal cell lines were isolated in HAT medium, all of which contained a human X chromosome as determined by trypsin–Giemsa staining. The frequency of HAT-resistant clones was 32 × 10^{-6} when 10^{7} cells were incubated with 10^{8} HeLa chromosomes. Potential reversion of the hybrid cells in HAT medium was less than 5 × 10^{-7}. The 16 isolated cell lines all contained activity of the human X-linked marker enzymes HPRT, PGK, α-Gal A, and G6PD, as determined by electrophoresis. The phenotype of G6PD was G6PD A, corresponding to G6PD A in HeLa cells. The human parental cells used in the fusion to form the hybrids had the G6PD B phenotype. The recipient cells gave no evidence of containing human X chromosomes. These results indicate that incorporation and expression of HeLa X chromosomes is accomplished in human–Chinese hamster hybrids which lack a human X chromosome.*

INTRODUCTION

In several laboratories transfer of genetic information from isolated chromosomes into cultivated cells of rodent origin has been accomplished (1–5). In all experiments published so far, it has been the X-chromosomal gene, coding for hypoxanthine phosphoribosyl transferase (HPRT), which was transferred.

Evidence was obtained that transfer of the HPRT locus occurred at a higher frequency in intraspecies (Chinese hamster recipient cells and Chinese hamster chromosomes) than in the interspecies (Chinese hamster cells and human chromosomes) systems (4). These results might indicate that the uptake and/or the integration in the recipient genome is determined by species-specific factors.

The failure of cotransfer of the other X-chromosomal loci in these studies (2–4) indicated that in the interspecies combinations, only a small fragment of the X chromosome, carrying the HPRT gene, has been incorporated into the genome of the recipient cells. In intraspecies combinations the size of the transferred genome fragment cannot be determined, because of the similarity of the donor and recipient gene products and chromosomes.

In order to investigate the influence of the recipient genotype on the nature of the transferred genetic element, we have now studied the incorporation of human chromosomes into human–Chinese hamster hybrid cells. The same HPRT-deficient Chinese hamster cell line was taken as parental cell line for the cell fusion as was used as the recipient Chinese hamster line in our previous gene transfer experiments. Evidence was obtained for the incorporation of a complete human X chromosome and the expression of the X-chromosomal markers in these hybrid cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The hybrid cell lines HRB28 and HRB2 were derived from fusion experiments between Chinese hamster HPRT-deficient cells (Wg3-h)(6) and human lymphocytes from a male donor. The hybrids were selected in F10 medium containing hypoxanthine, aminopterin, thymidine, and glycine (HAT medium) (7). From these hybrids the HRB28 cell line was derived by culturing the cells in F10 medium without hypoxanthine in the presence of 8-azaguanine (6 μg/ml). The HRB 28 cells were HPRT deficient and were used as recipient cells in the chromosome transfer experiments. The HRB 2 cell line retained its human X chromosome from the lymphocytes and was used as control cell line for the electrophoresis of G6PD. The F10 media were supplemented with 6% fetal calf serum and antibiotics. HeLa S3 cells were cultured in F10 medium supplemented with 10% calf serum and antibiotics.

Chromosome Isolation. Chromosomes were isolated from human HeLa S3 cells according to a modified method (4) of Maio and Schildkraut (8). The chromosomes were isolated from cells in metaphase which were obtained by treating cultures of HeLa cells with colchicine (0.12 μg/ml) for 16 h. Metaphases were collected by the selective detachment technique (9).

Incubation of Cells with Chromosomes. HRB 28 mitotic and interphase cells were incubated with chromosomes in an incubation volume of 2 ml F10 medium containing 10^7 cells and about 10^8 chromosomes at 37°C. After 20 min the suspension was diluted with F10 medium and the cells were seeded in petri dishes in a concentration of 10^6 cells per dish, in F10 medium without hypoxanthine, and supplemented with 6% fetal calf serum.

Selection of HPRT Positive Cells. Twenty-four hours after incubation

of HRB 28 cells with HeLa chromosomes the medium was replaced by HAT medium. The cells were refed at intervals of 3–4 days and cultured for 6 weeks. Colonies were isolated and propagated in HAT medium.

Chromosome Identification. Identification of chromosomes in metaphase spreads was carried out after treatment of mitotic cells with a 0.075 M KCl solution for 15 min and fixation in a 3:1 mixture of methanol and acetic acid. Metaphase preparations were made by air drying. The slides were treated with trypsin and stained with Giemsa (10). From each clone at least 25 metaphases were analyzed. Preparations of aliquots of the isolated HeLa chromosomes were prepared by washing the suspension to remove the isolation buffer, followed by fixation in methanol–acetic acid (3:1) and stained with the trypsin–Giemsa technique.

Electrophoresis. Preparation of cell lysates and procedures for Cellogel electrophoresis were carried out as described elsewhere (11). The following enzymes were tested: hypoxanthine phosphoribosyltransferase (HPRT, EC 2.4.2.8.) (12), phosphoglycerate kinase (PGK, EC 2.7.2.3) (11), glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) (13) and α-galactosidase A (α-Gal A, EC 3.2.1.22) (14).

RESULTS

Chromosome Isolation. Chromosomes isolated from HeLa S3 cells, fixed, and stained with trypsin-Giemsa are shown in Fig. 1. A uniform staining pattern of these chromosomes cannot be obtained because of differences in mitotic phase of the donor cells at the time of isolation. However, the individual chromosomes are recognizable according to their G-banding, indicating that the isolation procedure does not influence the banding pattern of the chromosomes. X chromosomes, showing the characteristic banding pattern, are clearly visible (arrows).

Isolation of HAT-Resistant Cell Lines. The numbers of HAT-resistant clones following incubation of HRB 28 cells with HeLa chromosomes are shown in Table 1. When cells were incubated with chromosomes, clones appeared after addition of HAT medium within a period of 2–6 weeks in two separate experiments, whereas in control experiments a total of 5×10^7 cells inoculated in HAT medium did not yield any HAT-resistant clones. These observations indicate that the HRB 28 cell population did not contain cells having HPRT activity which had escaped the selection in 8-azaguanine prior to the HAT selection.

According to previously published results (4) a slight increase in the frequency of HAT-resistant clones was found if chromosomes were incubated with cells in mitosis (32×10^{-6}), instead of cells in interphase (13.5×10^{-6}). A total of 16 independent clones was isolated from separate plates in HAT medium from the two separate experiments in which HRB 28 cells

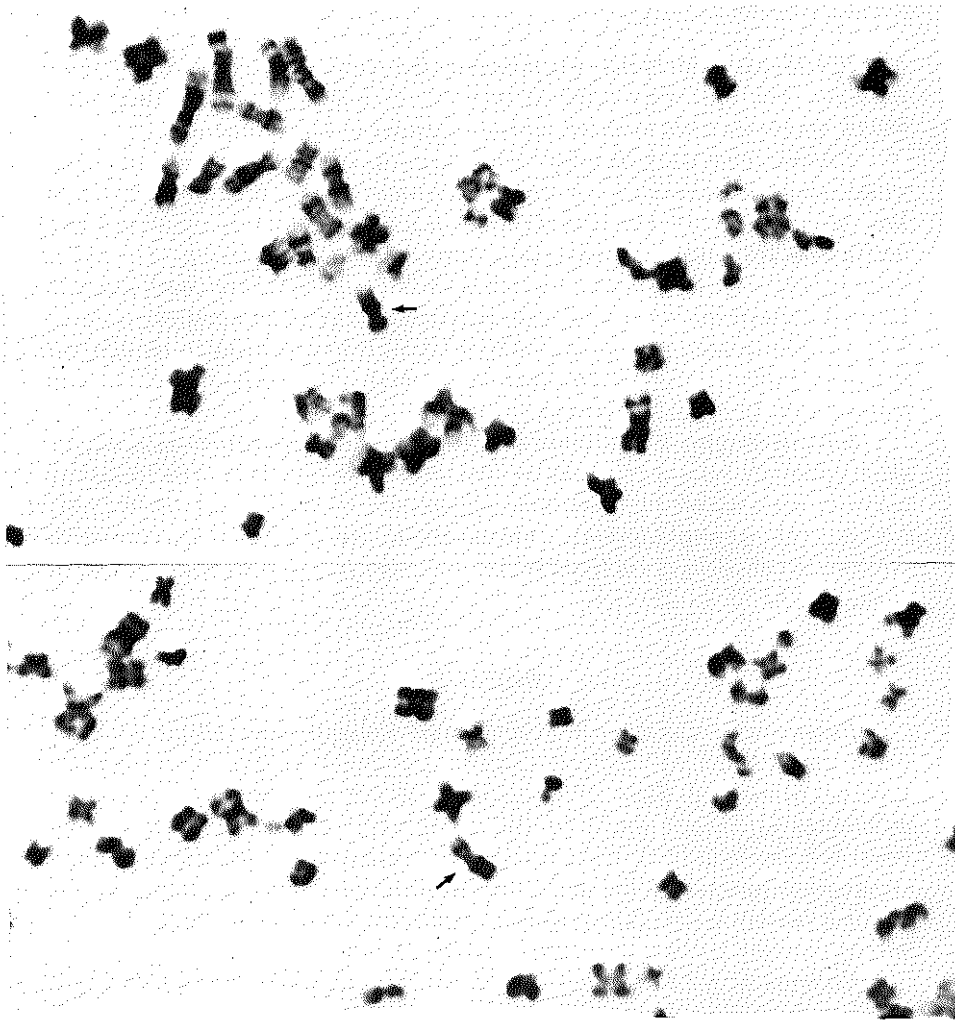

Fig. 1. A fixed preparation of isolated HeLa S3 chromosomes, stained with trypsin–Giemsa. X chromosomes are indicated by arrows (× 630).

were incubated with HeLa chromosomes. From these clones 16 H-HRB 28 cell lines were propagated in HAT medium. All cells of these lines died within a period of 2 weeks when the cells were plated in medium containing 6 μg/ml 8-azaguanine.

Chromosome Analysis of Hybrid Clones before and after Incubation with HeLa Chromosomes. Chromosome preparations were made after treatment of cells with colchicine for 3 hours. Figure 2 shows a karyogram of one of the HRB 28 recipient cells, containing the chromosome complement of the Chinese hamster parental cell line (Wg3-h), and in addition a few chromosomes from the human counterpart in the fusion (lymphocytes). 25 Meta-

Table 1. Frequency of HAT-resistant clones following incubation of HRB 28 cells with isolated HeLa chromosomes

Cell stage at chromosome treatment	Frequency of HAT-resistant clones ($\times 10^{-6}$)	Number of plates with clones[a]
Mitosis[b]	32 ± 7	10
Interphase[c]	13.5 ± 3.6	8

[a] 10^6 cells, incubated with about 10^7 chromosomes, were seeded per plate.
[b] Average of two experiments ± S.D. A total of 10 plates was seeded.
[c] Average of two experiments ± S.D. A total of 8 plates was seeded.

phases were analyzed, all containing the human chromosomes 11, 21, and 22, whereas chromosome 1 was found in 5 of the 25 cells. The human X chromosome was absent in the cells analyzed.

Figure 3 shows a karyogram of one of the cells of the clonal line H-HRB 28 5-1, which was propagated in HAT medium. These cells contained besides the human chromosomes 11, 21, and 22 also a human X chromosome which was found in all 16 clonal cell lines. This X chromosome was retained in all these lines during a growth period of at least 2 months in HAT medium.

The cell lines H-HRB 28 5-11 and 7-11 were also cultured for a 2-month period in nonselective medium. After this period 25 metaphases of each of these two cell lines were counted for the presence of the X chromosome, but no X chromosomes were observed.

Electrophoresis of the X-Chromosomal Markers. Cell lysates were prepared from H-HRB 28 cell lines which were cultured in HAT medium for 2 months. The electrophoretic pattern of HPRT from 5 H-HRB 28 cell lines is shown in Fig. 4. In channels 2, 3, 4, 5, and 6 lysates of H-HRB 28 cell populations are present, containing HPRT with the same electrophoretic mobility as HPRT in HeLa cells (channel 9). Channel 7 contains lysate of HRB 28 cells in which no HPRT was detected. Chinese hamster HPRT (channel 1) was not found in any of the H-HRB 28 cell populations. Zymogram patterns of the other X-chromosomal markers are shown in Figs. 5, 6 and 7. Figure 5 presents the electrophoresis of PGK. Lysates of 10 independent H-HRB 28 cell lines (channels 2–11) were electrophoresed together with a lysate of HRB 28 cells (channel 12). Human PGK is present in these H-HRB 28 cell lines and also in the other 6 cell lines, which are not presented here. Human PGK is absent in the HRB 28 lysate. The electrophoresis of α-Gal A is shown in Fig. 6, presenting lysates of 4 H-HRB 28 cell lines (channels 2–5). They all have the human α-Gal A band and an intermediate band. The human α-Gal A phenotype is also expressed in the

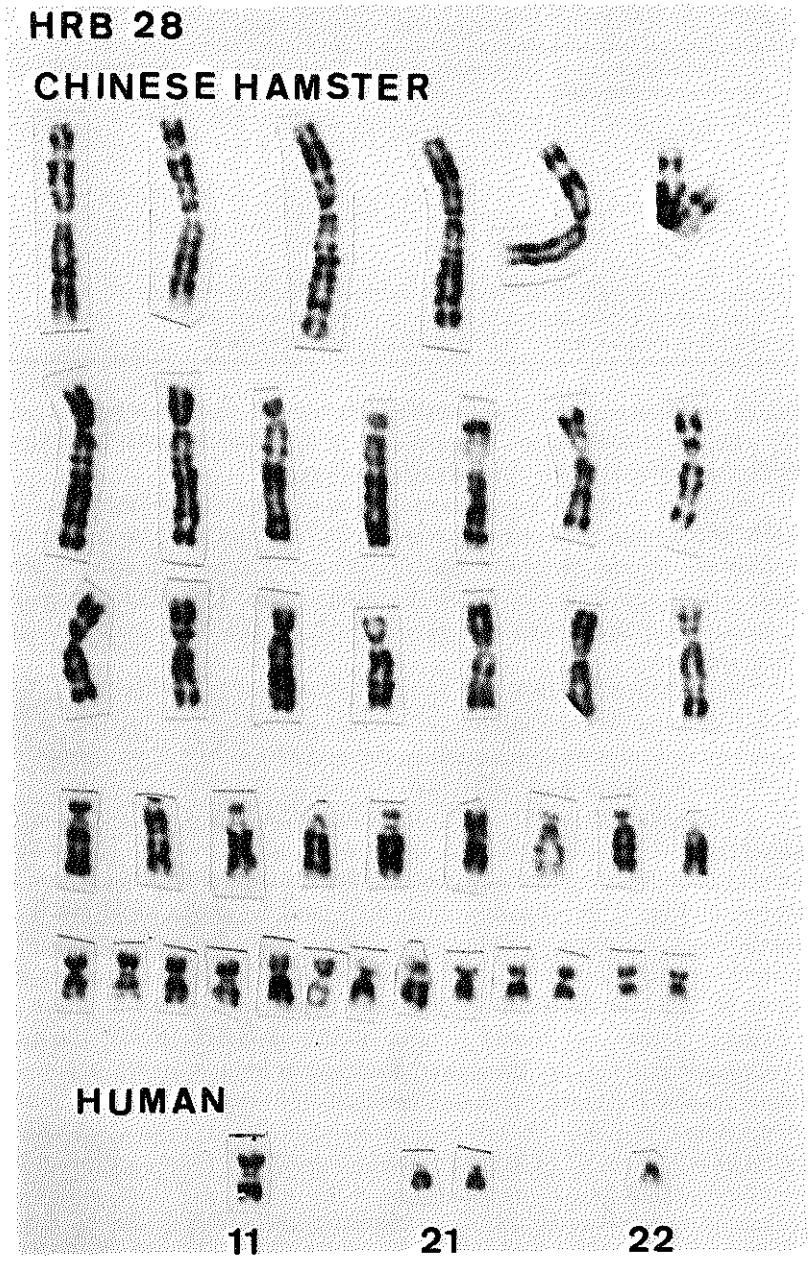

Fig. 2. A karyogram of the recipient hybrid cell line HRB 28. Besides the Chinese hamster complement the human chromosomes 11, 21, and 22 are also present in this cell.

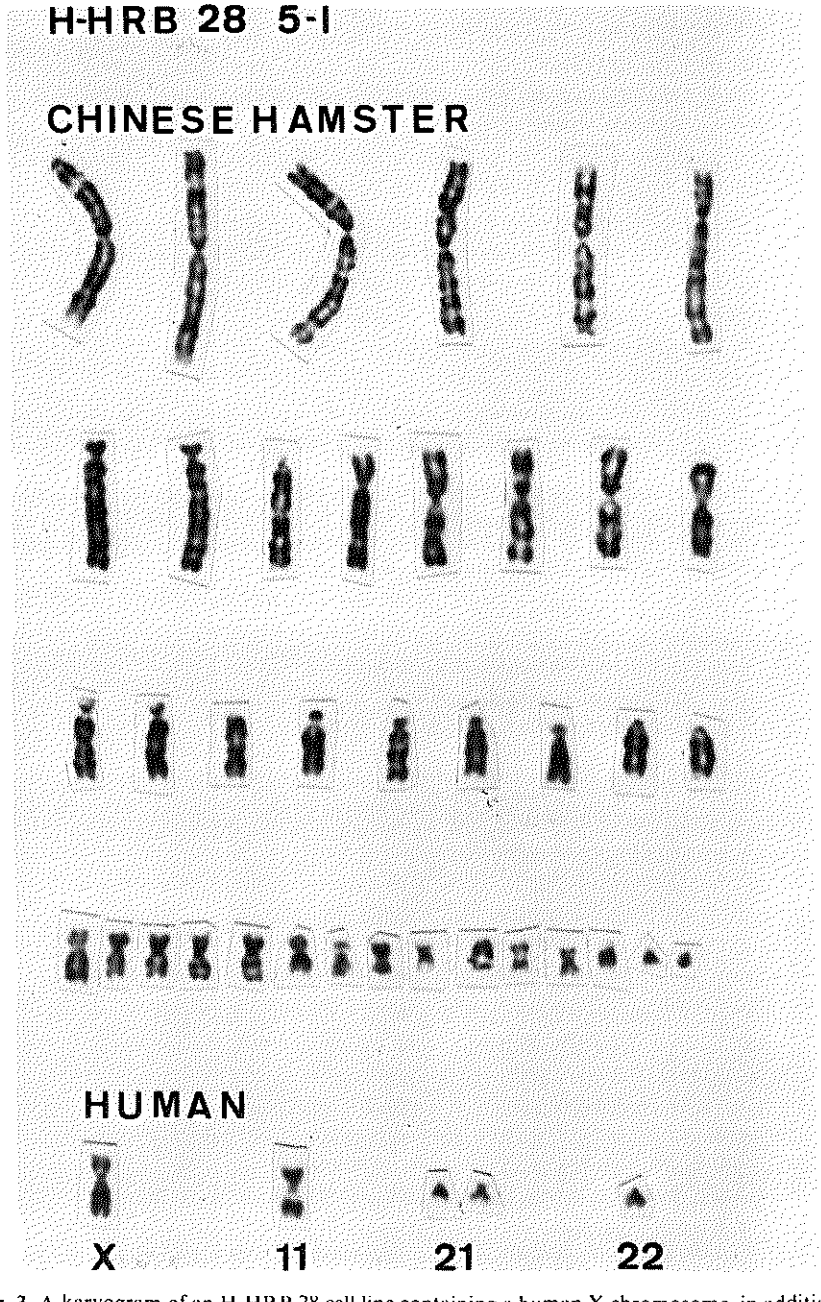

Fig. 3. A karyogram of an H-HRB 28 cell line containing a human X chromosome, in addition to the chromosome complement of the HRB 28 cells.

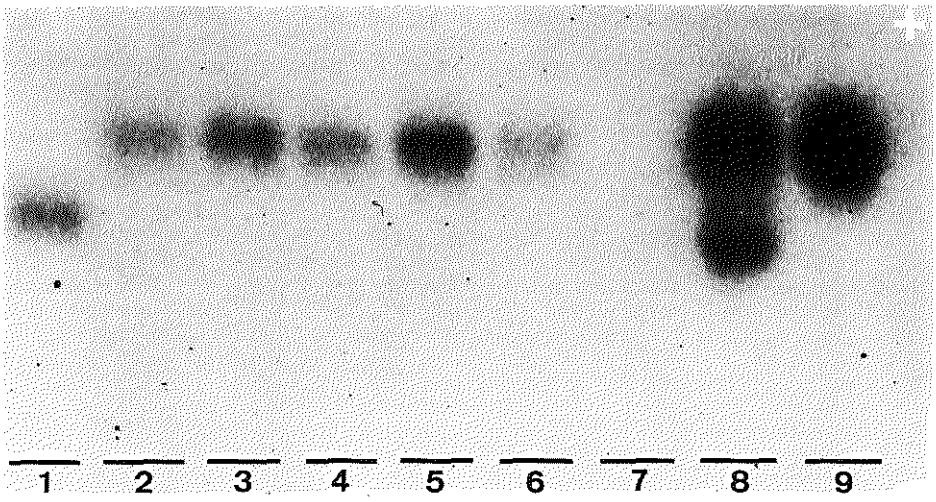

Fig. 4. The electrophoretic pattern of HPRT in hybrids incubated with HeLa chromosomes. Channel 1: Chinese hamster wild type DON cells; 2–6: hybrids incubated with HeLa chromosomes, the lines H-HRB 28 3-1, 4-1, 5-1, 7-1, and 9-11, respectively; 7: HRB 28 cells; 8: an artificial mixture of DON and HeLa cells; 9: HeLa cells.

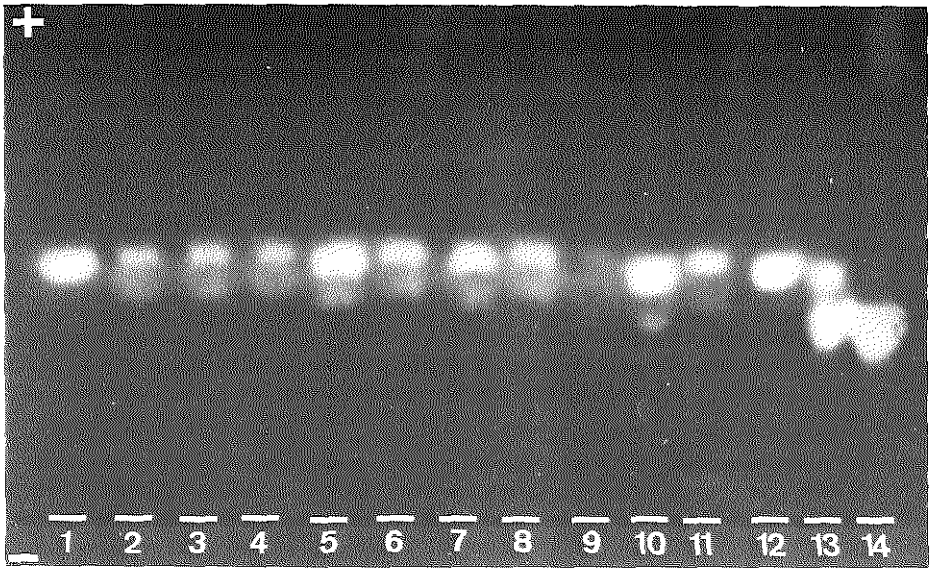

Fig. 5. The electrophoretic pattern of PGK in hybrids incubated with HeLa chromosomes. Channel 1, Chinese hamster DON cells; 2–11: H-HRB 28 1-11, 3-1, 4-1, 4-11, 5-1, 5-11, 7-1, 7-11, 9-1, and 9-11, respectively; 12: HRB 28 cells; 13: an artificial mixture of DON and HeLa cells; 14: HeLa cells.

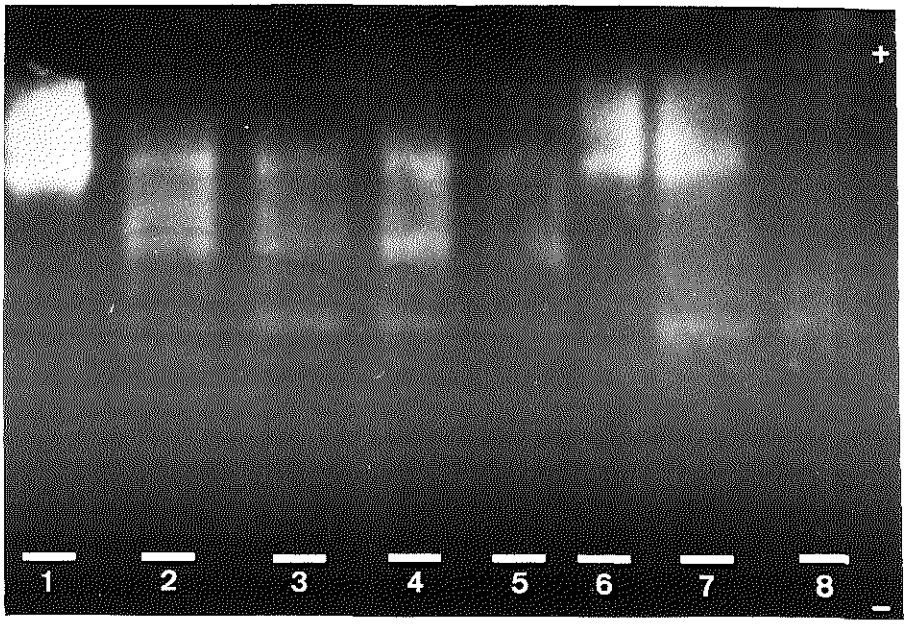

Fig. 6. The electrophoretic pattern of α-Gal A in hybrids incubated with HeLa chromosomes. Channel 1: Chinese hamster DON cells; 2–5: H-HRB 28, 3-1, 4-1, 5-1, and 7-1, respectively; 6: HRB 28 cells; 7: an artificial mixture of DON and HeLa cells; 8: HeLa cells.

other 12 H-HRB 28 cell lines, and is absent in the HRB 28 cell line (channel 6).

The electrophoretic pattern of G6PD of 4 H-HRB 28 cell lines is presented in Fig. 7 (channels 2–5), together with G6PD in HRB 28 cells (channel 6), and HeLa cells (channel 1). This zymogram also shows G6PD of extracts of the lymphocytes used for the formation of the HRB 28 hybrids (channel 7) and a mixture of Chinese hamster wild type DON cells and lymphocytes (channel 8).

G6PD in these lymphocytes has the G6PD B phenotype, whereas G6PD in HeLa cells has the A phenotype. The H-HRB 28 cells have a human G6PD band corresponding to G6PD A of the HeLa cells. In Fig. 8 (channel 2) the zymogram contains lysate of the original HRB 2 hybrid, which has retained its X chromosome from the lymphocytes. The human G6PD in this hybrid has the B phenotype, corresponding to the band in the lymphocytes (channel 9). The 5 H-HRB 28 lysates (channels 3–7) show G6PD A corresponding to G6PD A in HeLa cells (channel 1). This G6PD A is found in lysates of all 16 clonal lines isolated after the incubation of HRB 28 cells with HeLa chromosomes. The HRB 28 cell line in Fig. 7, channel 6 and in Fig. 8, channel 8, only contains Chinese hamster G6PD. The faint appearance of the Chinese hamster G6PD band in the zymogram

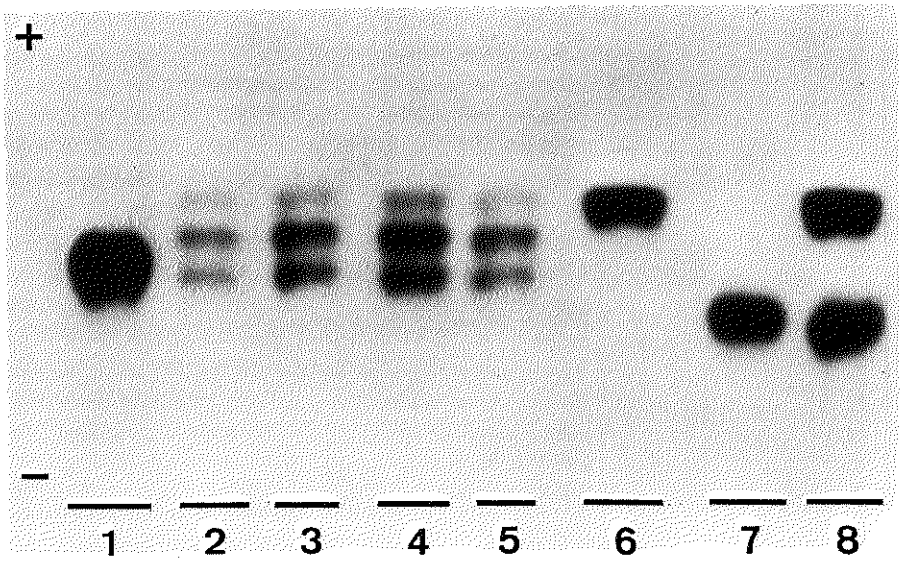

Fig. 7. The electrophoretic pattern of G6PD in hybrids incubated with HeLa chromosomes. Channel 1: HeLa cells; 2–5: H-HRB 28 1-11, 3-1, 4-1, and 5-1, respectively; 6: HRB 28 cells; 7: normal human lymphocytes used for the fusion as described in materials and methods; 8: an artificial mixture of DON cells and lymphocytes.

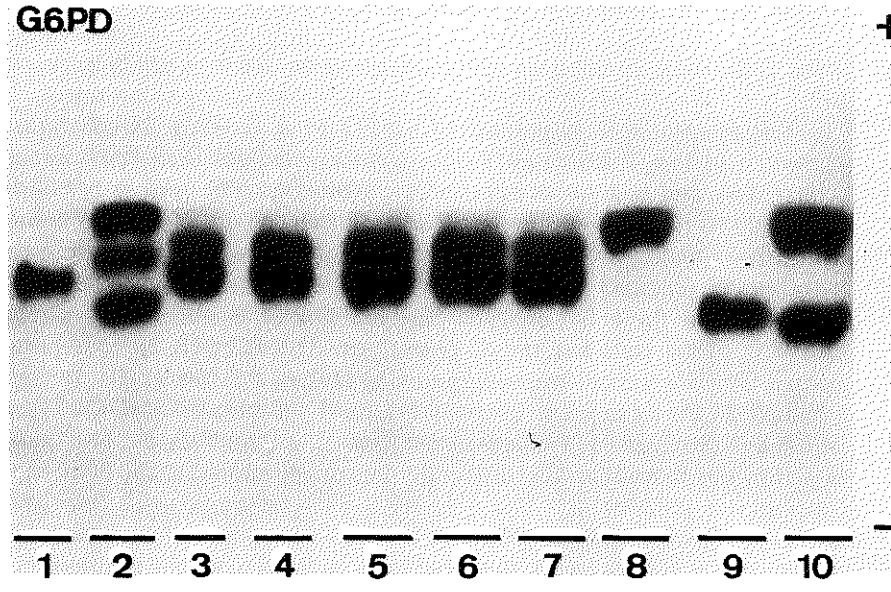

Fig. 8. The electrophoretic pattern of G6PD in hybrids incubated with HeLa chromosomes. Channel 1: HeLa cells; 2: HRB 2 cells (see materials and methods); 3–7: H-HRB 28 5-11, 7-1, 7-11, 9-1, and 9-11; 8: Chinese hamster DON cells; 9: human lymphocytes; 10: an artificial mixture of DON cells and lymphocytes.

of Fig. 8 was not found reproducably in the H-HRB 28 cell lines and was only seen in this particular electrophoretic run.

Lysates of the cell lines H-HRB 28 5-11 and 7-11, which were cultured in nonselective medium for 2 months, were electrophoresed for the enzymes PGK, α-Gal A, and G6PD. These lines, which had lost the human X chromosome, were also found to be negative for these human X-chromosomal markers.

DISCUSSION

Very little is known about the mechanism of gene transfer in mammalian cell system mediated by isolated chromosomes. It has been accomplished in only a few systems either using mouse A9 cells (2,3) or Chinese hamster Wg3-h cells (4), both being HPRT deficient. Chromosomes were derived from Chinese hamster (1,4) or human cells (2,3,4). By comparing the frequency of gene transfer, the intraspecies system (Chinese hamster recipient cells and Chinese hamster chromosomes) seems to be more efficient than the interspecies system (Chinese hamster recipient cells and human chromosomes) (4), indicating that species specific factors might be involved by the uptake or integration of chromosomes or fragments of chromosomes.

Since the origin of the derived phenotype in intraspecies systems cannot be identified, we decided to study gene transfer by using interspecific somatic cell hybrids as recipient cells. We were able to compare the transfer of the human HPRT gene to cells having only the Chinese hamster Wg3-h genome as well as to cells having several human chromosomes in addition to the Wg3-h genome. In principle this system might also be helpful in elucidating the genetic basis of species-specific factors, if they are involved in gene transfer.

It is difficult to draw conclusions regarding the frequency of gene transfer in the different experimental systems. The variable frequencies found in different experiments with the same system makes comparison difficult. By using cells in interphase as recipient cells, the highest frequency of transfer of the HPRT locus has been found with recipient Wg3-h cells and Chinese hamster chromosomes ($49 \pm 6 \times 10^{-6}$) (4). With the same interphase cells as recipients and isolated HeLa chromosomes, the frequency was about 6×10^{-6} (4), which is only slightly lower than the frequency found with HRB 28 hybrid cells as recipient cells for human chromosomes (13×10^{-6}). More experiments are required to demonstrate that these differences are significant.

A rather unexpected finding was the transfer of the other X-chromosomal markers as well as the demonstration of a complete X chromosome in the H-HRB 28 cells. This X chromosome was present in all the 16 clones

that have been isolated after incubation of HRB 28 cells with HeLa chromosomes. Using the hybrid recipient cells, transfer of a fragment of the X chromosome, as was demonstrated in previous experiments with mouse A9 (2,3) and Chinese hamster Wg-3-h (4) recipient cells, has not been found. These observations, at first sight, might indicate that we have selected an X-chromosome-containing hybrid cell from the original HRB 28 cell population, instead of having accomplished the transfer of a complete X chromosome. However, several observations support the identification of the X chromosome as being of HeLa origin. Firstly, in control experiments, 5×10^7 HRB 28 cells all died in HAT medium following a 1-month growth period in medium containing 8-azaguanine. Only after incubation with HeLa chromosomes did these cells yield surviving human HPRT positive clones. A second strong argument in favor of uptake of a complete chromosome is our observation that in all 16 clones that have been isolated G6PD had the A phenotype. The G6PD of the original HRB 2 hybrid and of the donor of the human parental cells in the hybridization had the B phenotype. It seems very unlikely that a change from G6PD B to G6PD A had occurred in the original hybrid cell population. The use of human cells of male origin in the production of the HRB 28 hybrids rules out the possibility of reactivation of an inactivated X chromosome coding for the G6PD A phenotype. The most likely explanation of our data is that we have accomplished the transfer of a complete human X chromosome. Elimination of this X chromosome, which occurs at a rather high rate as seen in the H-HRB 28 5–11 and 7–11 lines under nonselective conditions, is accompanied by the loss of the X-chromosomal markers including the G6PD A phenotype. These results show that these markers have not been incorporated elsewhere in the genome, for instance as a result of fragmentation of incorporated donor genetic material.

The reason why under these circumstances fragmentation of the incorporated material does not occur is a matter of speculation. It seems likely that the presence of several human chromosomes in the recipient cells protects the incorporated chromosomes from degradation. If this is true, then we might expect that the transfer of HPRT, observed in the intraspecies system (4), is also the result of the incorporation of a complete chromosome. This hypothesis can be tested by using a chromosome-donor cell line which has an HPRT-bearing marker chromosome.

The general validity of these observations has to be proven by using other hybrid cell lines as recipient cells, as well as by testing the transfer of markers other than HPRT. Experiments are in progress in which the transfer of the human chromosome 17, bearing the thymidine kinase and galactokinase loci, to Chinese hamster cells and to human–Chinese hamster hybrids is investigated.

ACKNOWLEDGMENTS

The authors are most grateful to Dr. W. Burgerhout for supplying the HRB 28 cell line. We also want to thank Drs. W. Burgerhout, A. P. M. Jongsma, A. Hagemeijer-Hausman, P. L. Pearson, P. Meera Khan, and A. Westerveld for valuable discussions and Mrs. C. D. van de Berg-Haverman for correction of the English usage. This work was supported by FUNGO, Foundation of Medical Scientific Research in The Netherlands.

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PUBLICATION 4

TRANSFER OF THE HUMAN GENES CODING FOR THYMIDINE KINASE AND GALACTOKINASE TO CHINESE HAMSTER CELLS AND HUMAN-CHINESE HAMSTER CELL HYBRIDS.

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ABSTRACT

Cotransfer of two linked human genes, coding for the enzymes thymidine kinase (TK) and galactokinase (Gak) was demonstrated following incubation of Chinese hamster TK deficient cells with isolated chromosomes. 5 Colonies were isolated which all expressed a stable TK positive phenotype.

Cotransfer of the human genes coding for TK and Gak has also been observed in experiments in which isolated human chromosomes were incubated with TK deficient human-Chinese hamster cell hybrids. These recipient hybrids had lost all human chromosomes at the time of incubation. From these experiments, four colonies were isolated, all expressing an unstable TK positive phenotype. Using chromosome staining techniques, the presence of human chromosomes could not be demonstrated in either of the transformed clonal lines, obtained with the Chinese hamster and the hybrid recipient cells. This indicates that incorporation of only the fragment of the human chromosomes 17, bearing the genes for TK and Gak, has occurred in the recipient cells.

INTRODUCTION

In several reports, it has been shown that cultured mammalian cells are able to incorporate isolated mataphase chromosomes, presented to the medium. Expression of genes originating from the donor of the chromosomes in the recipient cells, was demonstrated (1 - 6). By incubating rodent cells, deficient for the enzyme hypoxanthine phosphoribosyl transferase (HPRT), as recipient cells and chromosomes from HPRT positive human cells, the X-chromosomal human gene for HPRT was found to be expressed in the recipient cells. Cotransfer of the other X-linked genes could not be demonstrated (2 - 5). These experiments suggest that incorporated chromosomal material is fragmented in the recipient cells. Small pieces of the donor chromosomes are then integrated in the genome of the recipient cells and can be expressed.

Cotransfer of two closely linked human genes in a mouse-human interspecies system, has recently been reported by Willecke et al (6). These genes code for the expression of the enzyme thymidine kinase (TK) and galactokinase (Gak), and are located on chromosome 17 (7).

In neither of these gene transfer experiments, the presence of human chromosomal material could be demonstrated cytologically.

Cotransfer of linked genes has also been reported in a system involving the incubation of HPRT deficient human-Chinese hamster hybrid cells with isolated human chromosomes. The cotransfer of four X-linked genes could be demonstrated (5). With this system evidence was obtained for the incorporation of a complete human X chromosome.

In the present report, the transfer of the TK locus is investigated in our system using Chinese hamster TK deficient recipient cells and human chromosomes. Our results confirm the observations of Willecke et al., indicating the cotransfer of the genes for TK and Gak. Similar experiments have also been carried out by using human-Chinese hamster hybrid cells as

recipients for human chromosomes. Again cotransfer of the loci for TK and Gak has been observed. However, neither chromosome 17, nor other human chromosomes, could be detected in the isolated TK positive colonies.

MATERIALS AND METHODS

Cell lines and culture conditions

The mutant cell line A3 is derived from the Chinese hamster cell line DON by selection in 5-bromodeoxyuridine (5-BUdR) and propagated in the presence of 5-BUdR (50 μgr/ml). The A3 cell line is deficient for the enzyme thymidine kinase (TK) (8). The hybrid cell lines AW1A (TK^+) and AW1B (TK^+) were derived by fusion of A3 cells with human leucocytes from a female donor. The hybrids were selected in Ham's F10 medium containing hypoxanthine, aminopterin, thymidine and glycine (HAT medium), (9, 10). Shortly after isolation, electrophoretic characterization of these hybrid lines revealed the presence of the human genes coding for the enzymes lactate dehydrogenase-A (LDH-A), superoxide dismutase (SOD-1) and phosphohexose isomerase (PHI), (unpublished results).

The AW1A (TK^-) and AW1B (TK^-) cell hybrids were derived from the AW1A (TK^+) and AW1B (TK^+) cells respectively, after selection in F10 medium containing 5-BUdR (50 μgr/ml). The A3, AW1A (TK^-) and AW1B (TK^-) cells were cultured for a minimal period of two weeks in the presence of gentamycine (50 μgr/ml) to prevent the growth of PPLO.

Human HeLa S3 cells were cultured in F10 medium and were used for the isolation of metaphase chromosomes. Chinese hamster wild type DON cells and human-Chinese hamster (E36) cell hybrids (11) were used as control cells for electrophoresis. The F10 medium was supplemented with 6% fetal calf serum and the antibiotics penicelline and streptomycine.

Chromosome Isolation

Metaphase chromosomes were isolated from human HeLa S3 cells

according to a modified method (12) of Maio and Schildkraut (13). The chromosomes were isolated at pH 7.2 from cells in metaphase which were obtained by treating cultures of HeLa S3 cells with colchicine (0, 12μgr/ml) for 16 hours. The metaphases were collected by a selective detachment technique (14).

Incubation of Cells With Chromosomes

A3, AW1A (TK^-) and AW1B (TK^-) cells, growing in monolayer, were treated with colchicine (0, 12 μgr/ml) for 16 hours to obtain recipient cells in mitosis. The mitotic cells were harvested, washed to remove the colchicine, and incubated at 37°C with the isolated chromosomes in an incubation volume of 2 ml F10 medium. The incubation mixture contained approximately 10^7 cells and 10^8 chromosomes. After shaking for 20 minutes the suspension was diluted with F10 medium and seeded in petri dishes in a concentration 10^6 cells per dish, in F10 medium without thymidine and supplemented with 10% fetal calf serum and antibiotics. The dishes were then placed in an incubator with a gas mixture of 95% air and 5% CO_2. Control suspensions of the same recipient cell populations were incubated and seeded without the addition of the chromosomes.

Selection of HAT Resistant Cells

Twenty four hours after the incubation of the TK deficient cells with and without the HeLa chromosomes, the medium was replaced by HAT medium. The cells were refed at intervals of three or four days, and cultured for at least six weeks. Colonies, which appeared within this period, were counted, isolated and propagated in HAT medium. From each dish, one colony was isolated.

Estimation of Plating Efficiency

Colonies isolated and cultured in HAT medium, were transferred to

nonselective medium (F10 medium supplemented with hypoxanthine and thymidine). After intervals of two or three weeks, aliquots of the cells were counted and plated both in HAT medium and in nonselective medium in petri dishes. One to two weeks later the colonies were fixed, stained with haematoxaline and the ratio of colonies in HAT medium over colonies in nonselective medium was calculated.

Chromosome Identification

Identification of chromosomes in metaphase spreads was carried out after treatment of cells with a 0.075M KCl solution for 15 minutes, followed by fixation in a 3 : 1 mixture of methanol and acetic acid. Metaphase spreads were made by air drying. The slides were treated with trypsine and stained with Giemsa (15). Other slides were stained following the Giemsa 11 technique as described elsewhere (16). From each clonal cell line, at least 25 metaphases were analyzed.

Autoradiography

TK activity in clonal cell lines was determined autoradiographically following culture of the cells in F10 medium without thymidine and supplemented with ^{3}H-thymidine (0,05 μCi/ml, spec. act. 2 Ci/mM) for 48 hours, followed by fixation of the cells in Bouin's fluid. Autoradiographs were prepared using Ilford K2 liquid emulsion. The preparations were exposed for one to three weeks, developed and stained with haematoxalin-floksine.

Electrophoresis

The electrophoretic technique, used for the characterization of the enzymes TK (E.C. 2.7.1.75) and galactokinase (Gak, E.C.2.7.1.6) was analogous to the electrophoretic technique for the enzyme hypoxanthine phosphoribosyl transferase (HPRT), (17).

Preparation of cell lysates and procedures for Cellogel electrophoresis were carried out as described elsewhere (18). The electrophoretic

buffer system was prepared according to Stephan Goss of the University of Oxford (personal communication). It contained 25 mM tris-HCl, pH 8,2; 1mM Mg Cl2; thymidine (1mg/l) and 10 mM mercapto-ethanol. In addition ATP (800 mg/l) was added to the buffer in the negative electrode room, to stabilize the TK during electrophoresis. Electrophoresis was carried out on Cellogel for two and a half hours at 4°C, keeping the current constant at a level which was obtained with an initial potential difference of 300V. After the run, the gel was used immediately for enzym assay. For each assay the reaction mixture was prepared immediately before use, and contained the following components : 10 mM tris-HCl, pH 8,0; 5 mM Mg Cl2 ATP (2mg/ml) and ^{14}C-thymidine (2,5μCi/ml) (19). The gel was placed in a moisty chamber with a peace of Whatman DE81 paper, wetted with the reaction mixture and incubated at 37°C for two hours. Autoradiograms of the gel were obtained following the procedure published before (17).

The electrophoresis of Gak was carried out on Cellogel, similar to the method described for TK. The electrophoresis buffer and the reaction mixture were described elsewhere (20). The electrophoresis was carried out for two and a half hours at room temperature with an initial potential difference of 250 V. After the run, the gel sheet, applied with DE81 paper, was included for two hours in a moisty chamber at 37°C. The reaction mixture contained 0,2M tris-HCl, pH 7,2;3,61 mM ATP; 7,84 mM MgCl2 and ^{14}C-galactose (2 μCi/ml; spec.act. 60 μCi/mM). After the incubation the DE81 paper was applied to X-ray film as described elsewhere (17).

RESULTS

Characterization of TK Deficient Recipient Cell Lines

The A3 cells and the AW1A (TK$^-$) and AW1B (TK$^-$) cells were cultured in the presence of ^{3}H-thymidine for 48 hours. In the autoradiographic preparations radioactive labeled cells have not been observed. For each cell line

a total 5×10^5 cells was analyzed. Following incubation of 2×10^7 cells of each line in HAT medium, surviving colonies have not been found. These results indicate that the A3, AW1A (TK^-) and AW1B (TK^-) cell populations did not contain cells, having TK activity, which could have escaped the selection in 5-BUdR. This conclusion was confirmed by electrophoresis of lysates of these cells (figures 1 and 2).

Isolation of HAT Resistant Cell Lines

A3, AW1A (TK^-) and AW1B (TK^-) cells were incubated with chromosomes isolated from TK positive human HeLa S3 cells. After addition of HAT medium, colonies appeared in all plates within a period of two to six weeks (Table 1). In control experiments, in which the cells were incubated in the absence of chromosomes, no surviving colonies were observed (Table 1).

TABLE 1 FREQUENCY OF HAT RESISTANT COLONIES FOLLOWING INCUBATION OF TK DEFICIENT CELLS WITH ISOLATED HUMAN CHROMOSOMES.

CELL LINE	CHROMOSOMES/ 10^6 CELLS	FREQUENCY OF COLONIES ($\times 10^{-6}$)	PLATES SEEDED [c]	PLATES WITH COLONIES
A3	10^7	10.7 ± 4.5 [a]	7	7
AW1A (TK^-)	10^7	66 ± 3 [b]	6	6
AW1B (TK^-)	10^7	32.2 ± 2.9 [b]	4	6
A3	0	0	4	0
AW1A (TK^-)	0	0	2	0
AW1B (TK^-)	0	0	2	0

[a] Average of two experiments $\pm$ S.D.
[b] Result of one experiment $\pm$ S.D.
[c] 10^6 Cells were seeded per plate

Following incubation of A3 cells with the chromosomes, seven independent H-A3 colonies were isolated. From the AW1A (TK^-) and AW1B (TK^-) cell populations, after incubation with chromosomes, six independent H-AW1A and six H-AW1B colonies were isolated respectively. The H-A3, H-AW1A and

H-AW1B clonal lines were cultured in HAT medium supplemented with gentamycine (50 gr/ml). Aliquots of each line were cultured for 48 hours in F10 medium containing ^{3}H-thymidine (0,05μCi/ml; spec.act. 2Ci/mM). As shown by autoradiography, all cells were found to be radioactively labeled in their nuclei, which indicates the presence of the enzyme TK in these cells.

Chromosome Identification

At least 25 metaphases of the isolated cell lines were analyzed for the presence of human chromosomes. Neither in the trypsin-Giemsa stained preparations nor in preparations stained with the Giemsa 11 technique, human chromosomes could be recognized.

Electrophoresis of the Enzymes TK and Gak

Cell lysates were prepared from H-A3, H-AW1A and H-AW1B cell populations cultured in HAT medium in the presence of 50 μgr/ml gentamycine. The electrophoretic pattern of TK of lysates of the H-A3 cells is presented in firgure 1. The TK enzyme in the H-A3 cells (channels 4-8) has the same electrophoretic mobility as the human TK (channel 2). The A3 cells (channel 3) do not contain any detectable TK activity (compare channel 1).

Figure 2a shows the electropherograms of TK in H-AW1A cell lysates and lysates of control cells. The TK activity in the H-AW1A 1 and 2 (channels 2 and 3) corresponds with the TK in HeLa cells (channel 6) and with the human TK in the human-Chinese hamster control hybrid (channel 4). The original AW1A (TK^{-}) cells (channel 5) do not contain any TK activity, whereas in the lysate of the control hybrid, applied in channel 7, only the Chinese hamster isoenzyme is present, comparable with the TK in the wild type Chinese hamster DON cells (channel 1).

Figure 2b presents the electrophoretic pattern of TK in the cell lines H-AW1B (channel 2-6). Also these lines have TK activity with the electrophoretic mobility of the HeLa cells (channel 8). The original AW1B (TK^{-})

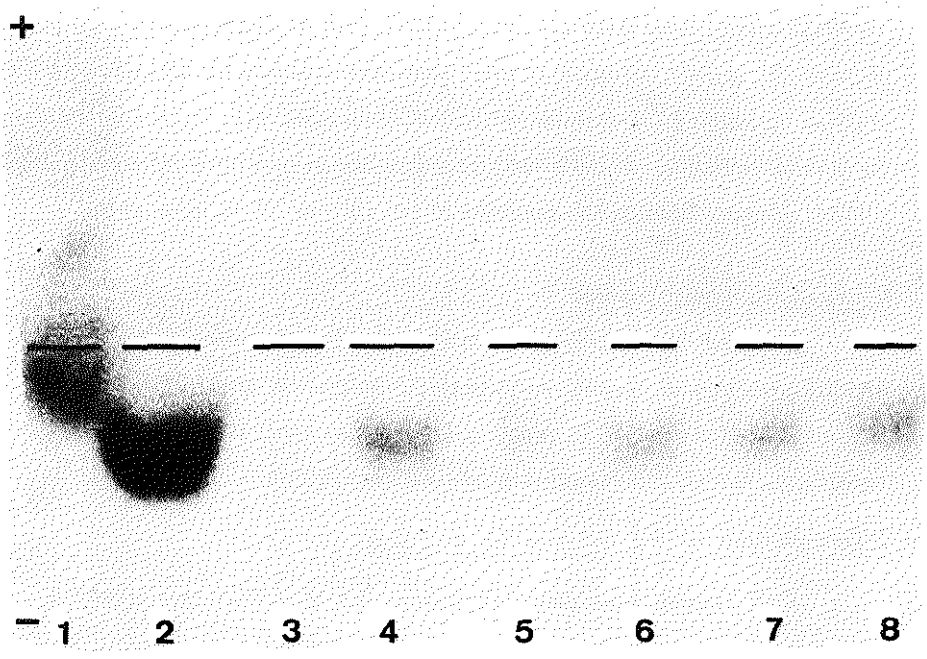

Figure 1. The electrophoretic pattern of TK in H-A3 cells. Channel (1), Chinese hamster DON cells;(2) HeLa cells;(3) A3 cells; (4-8) the cell lines H-A3-2,3,4,5 and 6 respectively.

cells do not express detectable TK activity (channel 7). The TK activity present in Chinese hamster DON cells is presented in channel 1.

The electrophoretic pattern of Gak is presented in the figures 3 and 4. Figure 3 shows the electrophoretic mobility of the Gak activities in the cell lines H-A3 (channels 2-7). All these lines contain a Chinese hamster isoenzyme which is also found in the Chinese hamster A3 cells (channel 8). The H-A3 cells also show Gak activity with a similar mobility as the Gak in HeLa cells (channel 1).

Figure 4a shows, that the human-like Gak enzyme is also expressed in the H-AW1A cell lines (channels 2 and 3). In a control experiment the same human-Chinese hamster hybrids, which were tested

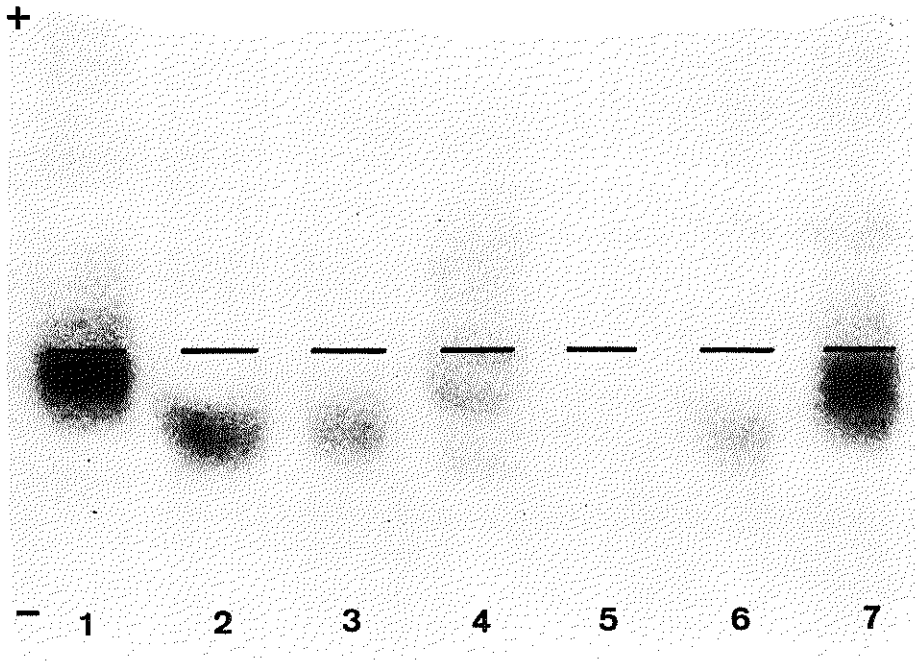

Figure 2a. The electrophoretic pattern of TK in H-AW1A cells. Channel (1), Chinese hamster DON cells; (2 and 3), H-AW1A-1 and H-AW1A-2 cells respectively; (4) human-Chinese hamster (E36) hybrid cells, positive for human TK; (5) AW1A (TK^-) recipient cells; (6) HeLa cells; (7) human-Chinese hamster (E36) hybrid cells, negative for human TK.

for TK activity, were also applied to this gel. The hybrid, which was positive for the human TK enzyme, also contains the human Gak (channel 5), whereas the human isoenzyme for Gak is absent in the TK deficient hybrid (channel 6). The original AW1A (TK^-) cells (channel 4) only contains the Chinese hamster isoenzyme (see also channel 1).

Figure 4b presents the electrophoretic pattern of Gak in the H-AW1B cell lines (channel 2-6). These cell lines all possess the human-like Gak together with the Chinese hamster isoenzyme (compared with channels 8 and 1). The original AW1B (TK^-) cells only have the Chinese hamster isoenzyme (channel 7).

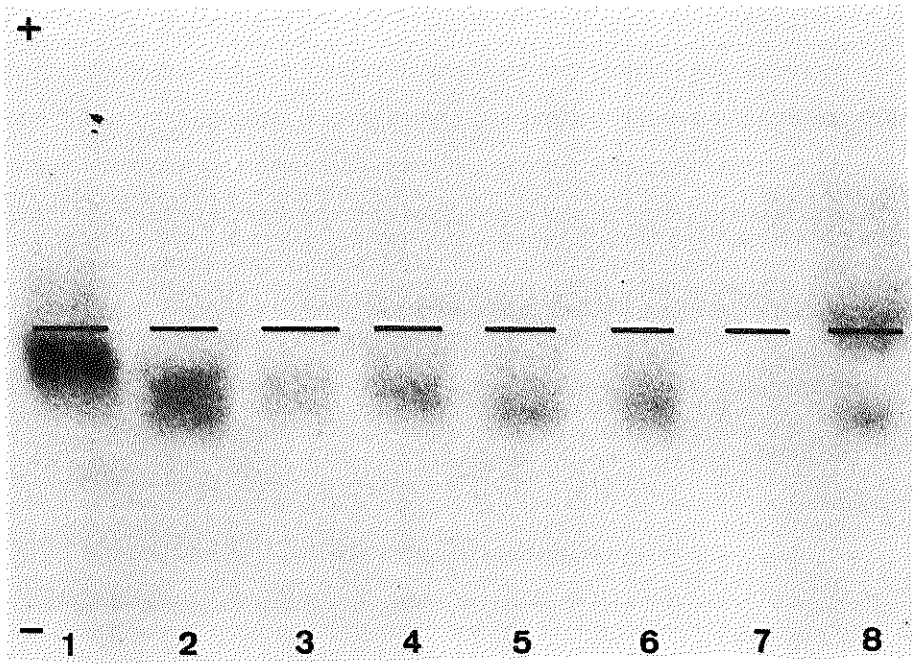

Figure 2b. The electrophoretic pattern of TK in H-AW1B cells. Channel (1), Chinese hamster DON cells; (2-6), H-AW1B-2,3,4,5 and 6 respectively; (7) AW1B (TK^-) recipient cells; (8) an artificial mixture of DON and HeLa cells.

Expression of the Transformed Phenotype

The stability of the TK activity in the H-A3, H-AW1A and H-AW1B cell populations has been investigated by growing the cells under nonselective conditions (see Materials and Methods). The results, expressed as ratio of colonies in HAT medium over colonies in nonselective medium, are presented in figure 5. The constant ratio of the H-A3 cells found during a growth period of ten weeks, indicates that the HAT resistancy is retained in these cells, even in the absence of the selective pressure. The gradual decrease in these ratios, observed with the H-AW1A

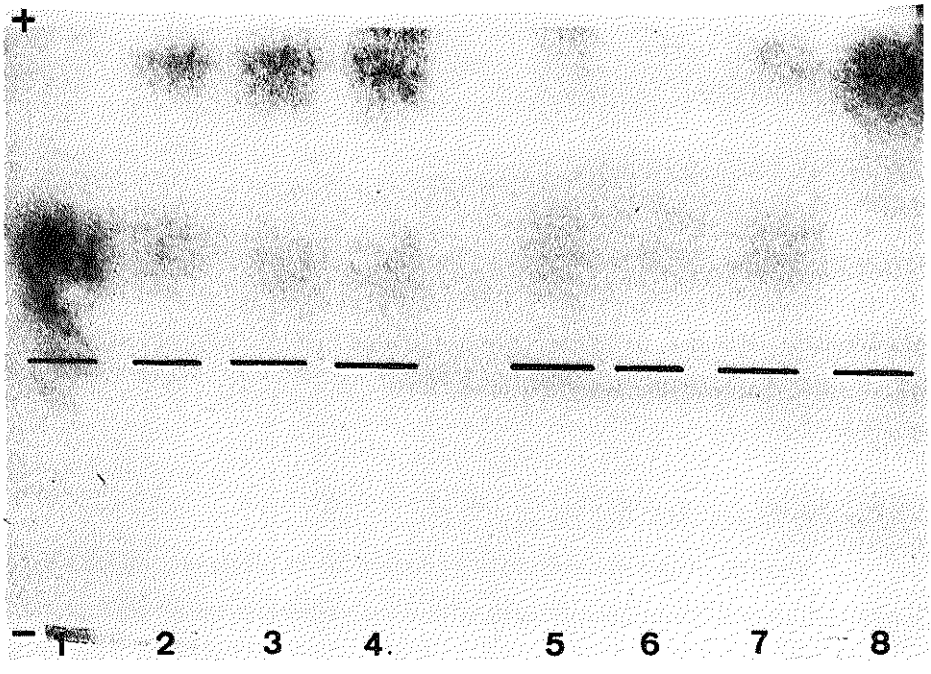

Figure 3. The electrophoretic pattern of Gak in H-A3 cells. Channel (1), HeLa cells; (2-7) H-A3-1,2,3,4,5 and 7 respectively; (8) A3 recipient cells.

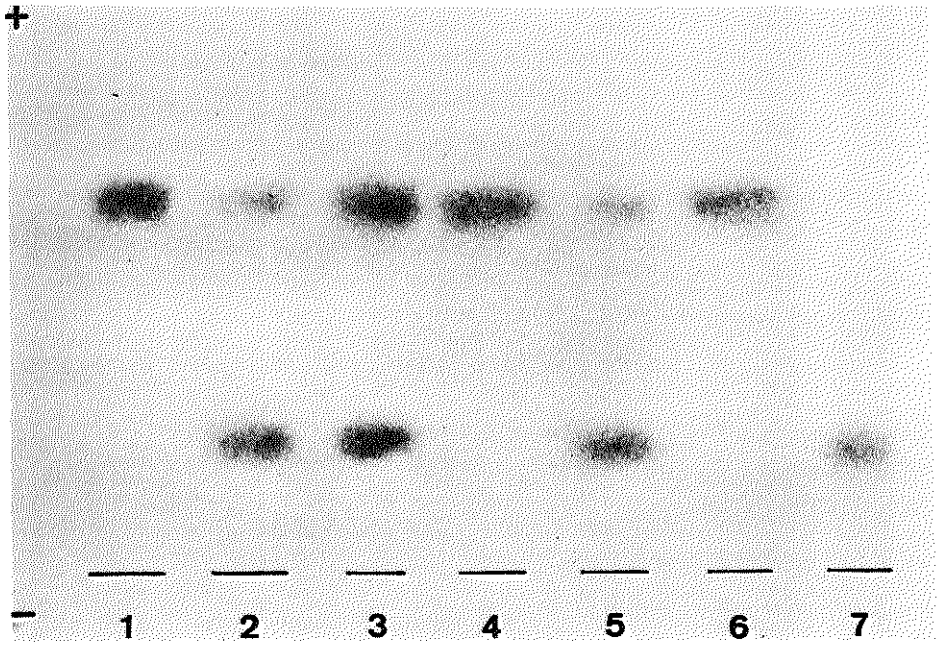

Figure 4a. The electrophoretic pattern of Gak in H-AW1A cells. Channel (1), Chinese hamster DON cells; (2 and 3), H-AW1A-1 and H-AW1A-2 cells; (4) AW1A (TK^-) recipient cells; (5 and 6) human-Chinese hamster (E36) hybrid cells, positive and negative for human TK, respectively; (7) human HeLa cells.

and H-AW1B cell lines, indicates that these cells lose the capacity of growth in HAT medium.

Transfer of these cell populations, after the ten weeks growth period in nonselective medium to medium containing 5-BUdR (50 μg/ml) yielded no survivors with the H-A3 cell lines, whereas 5-BUdR resistant populations could be obtained with the other lines.

DISCUSSION

Transfer of genetic information, coding for TK, following incubation of TK deficient Chinese hamster cells with human chromosomes from TK positive cells, is indicated by the appearance of colonies in HAT medium. The absence of revertants in our control experiments, and the human-like electro-

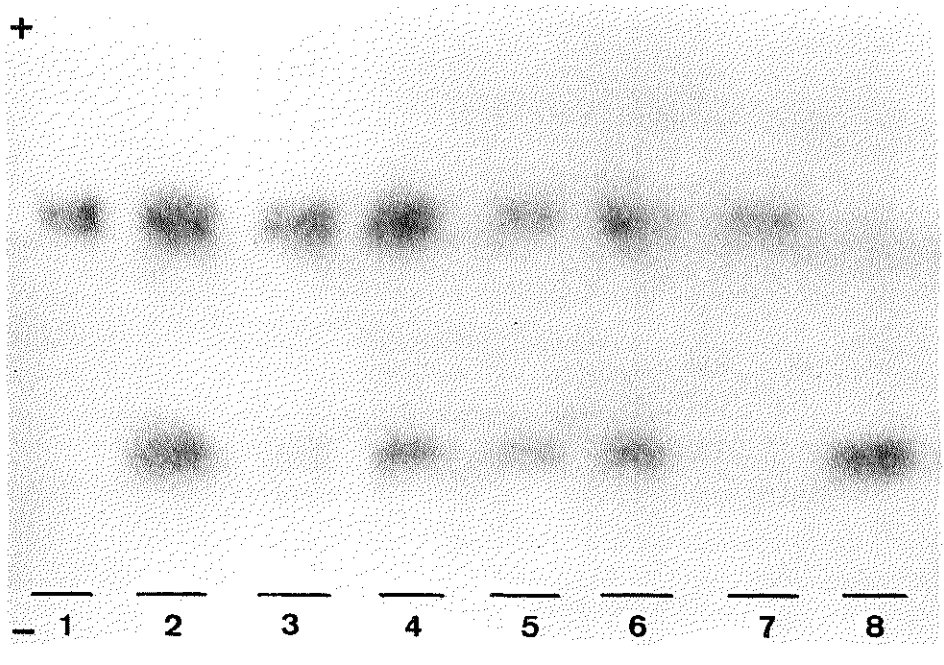

Figure 4b. The electrophoretic pattern of Gak in AW1B cells, incubated with human chromosomes. Channel (1), Chinese hamster DON cells; (2-6) H-AW1B-2,3,4,5, and 6 respectively; (7) AW1B (TK^-) recipient cells; (8) human HeLa cells.

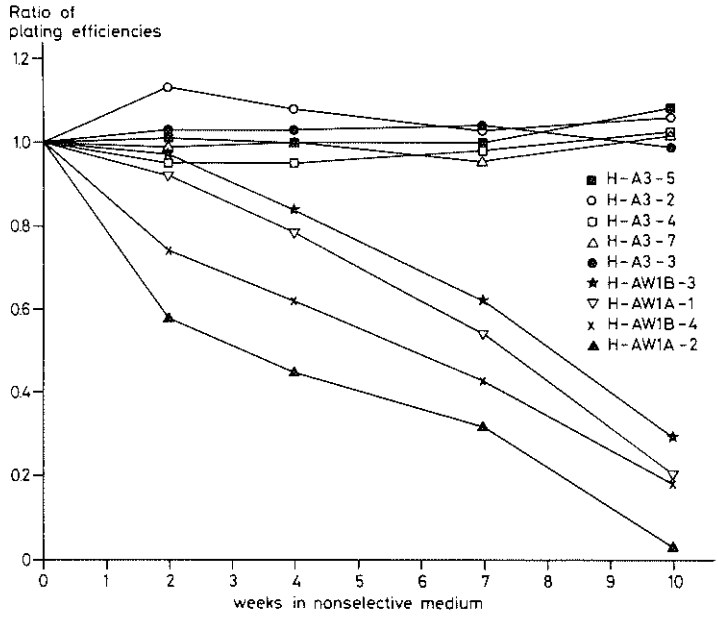

Figure 5. Stability of the TK positive phenotype in transformed cells. The cell lines were transferred at time O from selective (HAT) medium to nonselective medium, containing hypoxanthine and thymidine, but not aminopterin. Aliquots of about 100 cells were removed at indicated times and tested for plating efficiencies in selective and nonselective media. The ordinate gives the ratio of colonies in selective medium over the colonies in nonselective medium (see also Materials and Methods).

phoretic pattern of the obtained TK positive phenotype, provides strong arguments in favour of gene transfer.

Cotransfer of the human genes, coding for TK and Gak, in the five colonies isolated in HAT medium, was demonstrated following electrophoresis. Cytological analysis of these colonies has not revealed the presence of human chromosomes or chromosome fragments. This indicates, that only a small fraction of the human chromosome 17, bearing the genes for TK and Gak, is transferred to the recipient cells. In comparable experiments, using TK deficient mouse cells and isolated human chromosomes, Willecke et al.(6) have isolated eight HAT resistant colonies. Cotransfer of TK and Gak was demonstrated in

two of these colonies, whereas the other six had only obtained the TK positive phenotype. Therefore, our finding of cotransfer in all the five isolated colonies might be a coincidence.

In our experiments, performed to investigate the stability of the transformed phenotype, it was demonstrated that the HAT resistancy is retained in the cells of the five colonies, even in the absence of the selective pressure. This suggests that the transferred genes are incorporated in the chromosomes of the recipient cells. Experiments performed by Willecke et al. (6), using TK deficient mouse cells, have resulted in the isolation of two colonies with a stable transformed phenotype, whereas in five other colonies an unstable TK positive phenotype was observed. These results also indicate the incorporation of the transferred genes in the chromosomes of the recipient cells and, in other recipient cells, the occurrence of the transferred genes in free fragments in the genome of the host cells. The absence of the unstable phenotype in our experiments might be due to the relatively low number of isolated colonies.

The results, obtained from our experiments and from those of Willecke et al. (6), correspond with the results concerning the stability of the transformed HPRT positive phenotype, acquired by incubation of HPRT deficient Chinese hamster cells (4) and mouse cells (3) with human chromosomes. In those systems, the obtained HPRT positive phenotype of the recipient cells was also found to be either stable of unstable.

Transfer of the human gene coding for TK was also demonstrated after incubation of the AW1A (TK^-) and AW1B (TK^-) cell populations with human chromosomes. Gene transfer was indicated by the appearance of colonies in HAT medium. Cotransfer of the human genes, coding for TK and Gak was shown by electrophoresis, and was demonstrated in the four colonies which were isolated. However, chromosome analysis has shown a complete absence of human chromosomes in these cells, indicating that again only that fragment of the chromosome 17, bearing the genes for TK and Gak, has been transferred. These results are similar to those obtained by using the Chinese hamster A3 cells as recipients. Results, different from those obtained with the A3 cells

were found concerning the stability of the transformed phenotype. In the H-AW1A and H-AW1B cell lines, the TK activity disappears under nonselective conditions, indicating that the transferred chromosome fragment is behaving independently in the genome of the recipient cells. The demonstration of the transfer of a chromosome fragment, using these hybrid cells, clearly differs from the data obtained in gene transfer experiments concerning incorporation of the gene for HPRT in hybrid cells (5). In the HPRT system we found the transfer of a complete chromosome. The crucial difference between these two sets of experiments might be the presence of the human chromosomes in the HPRT deficient hybrid cells. Cytogenetic analysis of parallel cultures of the recipient TK deficient hybrid cells at the time of incubation with chromosomes, has revealed a loss of all the human chromosomes. This complete loss of human chromosomes might be the result of the growth period in 5-BUdR containing medium, previous to the incubation. Cytogenetically, these cells seem to be similar to the A3 parental cells. However, the presence of nonrecognizable pieces of human chromosomes cannot be excluded.

A difference in behaviour in chromosome uptake experiments between these cells and the parental A3 cells is indicated by the differences in stability of the conceived phenotype between these cell types (by using A3 parental cells five stable colonies were isolated, whereas in the hybrid system four unstable colonies were observed). In view of the relatively small number of isolated colonies, and in view of the results obtained by Willecke et al.(6), the absence of unstable colonies, using the A3 recipient cells, might have been a coincidence.

Considering the comparison of the different gene transfer systems, our data, obtained with the TK deficient hybrids, support the hypothesis that the presence of human chromosomes in the recipient cells is required for the incorporation of a complete human chromosome. However, these results have not confirmed the transfer of a complete chromosome by using hybrid recipient cells. Experiments are in progress in which gene transfer in other TK deficient hybrids is being investigated. In these hybrids, the presence of human chromosomes at the time of incubation has to be demonstrated.

ACKNOWLEDGEMENTS

The authors are most grateful to Stephen Goss from the Oxford University for sending us data concerning the electrophoresis of the enzyme TK. Mrs. A.Hagemeijer-Hausman is thanked for supplying the TK deficient hybrid cell lines. We also want to thank all the members of the working group on human gene linkage from the Genetic Institute, Erasmus University Rotterdam and from the Department of Anthropogenetics, State University Leiden, for valuable discussions. We thank also Mrs. C.D. van de Berg-Haverman for correction of the English usage.

This work was supported by FUNGO, Foundation of Medical Scientific Research in The Netherlands.

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