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Mitotic recombination induced by the liver carcinogen aflatoxin B_1 in a human cell line

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Table of contents

Abbreviations

The following list contains the most often used abbreviations in the present thesis in alphabetical order:

AFB_1	aflatoxin B_1
AN mutant	aflatoxin B_1 induced normal growth mutant
AS mutant	aflatoxin B_1 induced slow growth mutant
BAC	bacterial artificial chromosome
DAPI	4,6-diamidino-2-phenyl-indol-dihydrochloride
FISH	fluorescence *in situ* hybridization
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
LOH	loss of heterozygosity
MN mutant	NMU induced normal growth mutant
MS mutant	NMU induced slow growth mutant
NG	normal growth
NMU	*N*-nitroso-methyl-urea
SG	slow growth
SN mutant	spontaneously occurring normal growth mutant
SS mutant	spontaneously occurring slow growth mutant
SSCP	single strand conformation polymorphism
TFT	trifluorothymidine
tk	thymidine kinase

Zusammenfassung

Die Kontamination von Nahrungsmitteln mit Schimmelpilzen der Art *Aspergillus flavus* und *Aspergillus parasiticus* stellt ein grosses Problem dar, vor allem im Süden Afrikas und in der Provinz Qidong in China. Diese Schimmelpilze produzieren verschiedene Mykotoxine, wovon Aflatoxin B_1 (AFB_1) das gefährlichste ist. In diesen Regionen ist die Inzidenz von Hepatozellulären Karzinomen (HCC) höher als in anderen Gegenden. Verschiedenste Studien bringen diese Tumoren mit AFB_1 und chronischer Hepatitis B Virus (HBV) Infektion in Verbindung.

AFB_1 ist ein sehr potentes Mutagen. Verschiedene *in vitro* Experimente haben gezeigt, dass AFB_1 vor allem zu G->T-Transversionen führt. In HCCs, die mit AFB_1 und HBV assoziiert wurden, enthielten etwa die Hälfte eine spezifische Mutation im Tumor Suppressor Gen *p53*, und zwar eine G->T-Transversion im Codon 249. In Tumoren, die nicht mit AFB_1 assoziiert waren, wurde diese Mutation dagegen nicht gefunden. Dies legt die Vermutung nahe, dass AFB_1 die Ursache für diese spezifische *p53* Mutation sein könnte. Ein weiterer Vergleich zwischen Tumoren aus der AFB_1 verseuchten Qidong Gegend und aus Peking, wo AFB_1 kein Problem darstellt, zeigte auch Unterschiede in der Häufigkeit von Heterozygositätsverlust (Loss of heterozygosity, LOH). Die Tumoren aus der Qidong Gegend zeigten häufiger Abschnitte mit LOH. Die Mechanismen, die zu diesen LOH-Abschnitten führten, sind jedoch nicht bekannt. Mitotische Rekombination wäre ein möglicher Mechanismus. Unsere Gruppe hat kürzlich die Wirkung von AFB_1 auf die Mitotische Rekombination im niederen Eukaryot *Saccharomyces cerevisiae* untersucht. In diesem Organismus wurden durch AFB_1 viel mehr Rekombinationen als Punktmutationen induziert.

Hätte AFB_1 eine vergleichbare Wirkung auf die Rekombinationsfrequenz in menschlichen Zellen, könnte diese Aktivität einen wichtigen Mechanismus darstellen, durch den heterozygote Gene während der Tumorentwicklung

inaktiviert würden. So könnte zum Beispiel ein durch Mutation verändertes *p53* Allel durch Mitotische Rekombination vom Wildtyp-Allel segregieren. Dadurch würde eine Zelle ohne *p53*-Aktivität entstehen, aus der sich dereinst eine Krebszelle entwickeln könnte.

Es war das Ziel der vorliegenden Arbeit, herauszufinden, ob der Effekt von AFB_1 auf die Rekombinationsfrequenz in einer menschlichen Zelllinie ähnlich stark ist wie in der Bäckerhefe *S. cerevisiae*. Ausserdem wollten wir die Mechanismen, die zum Verlust der Funktion eines heterozygoten Gens führen, unterscheiden und quantifizieren.

Die TK6 Zelllinie, die in dieser Arbeit eingesetzt wurde, ist heterozygot am Thymidin-Kinase (*tk*) Lokus auf Chromosom 17. Ein Allel produziert ein funktionelles tk Enzym, das andere Allel enthält eine inaktivierende Leseraster (frameshift) -Mutation in Exon 4.

Diese Mutation macht die TK6 Zelllinie zu einem idealen System, um Punktmutationen, Rekombinationen, grössere Deletionen und Chromosomenverlust am selben Locus zu untersuchen. In der vorliegenden Arbeit wurden Mutationsexperimente durchgeführt. Darauf wurden unabhängige Mutanten isoliert, die die *tk*-Aktivität verloren hatten. Auf diese Weise wurden 87 AFB_1-induzierte und 57 spontane Mutanten isoliert.

Um herauszufinden, welche genetischen Ereignisse zum Verlust der *tk*-Funktion in diesen Mutanten geführt hatte, wurden Methoden verwendet, mit denen man allelische Unterschiede an spezifischen Chromosomen-Loci entdecken kann. Dies wurde erreicht durch Einzelstrangkonformations-Analyse (SSCP-analysis) am *tk* Locus, sowie durch Mikrosatelliten-Analyse an verschiedenen Orten auf Chromosom 17. Mit diesen Methoden konnten LOH-Bereiche auf Chromosom 17 identifiziert werden. Zusätzlich wurde eine Fluoreszenz-*in situ*-Hybridisations (FISH) Analyse am *tk* locus durchgeführt. Damit konnte die absolute Anzahl von *tk* Allelen bestimmt werden. Durch diese molekularen Analysen konnten Punktmutationen, Rekombinationen, grosse Deletionen und Chromosom 17-Verluste unterschieden werden, die zum Ausfall der *tk*-Funktion in den Mutanten

geführt hatten. Anschliessend konnte die Mutanten-Fraktion zu den jeweiligen genetischen Ereignissen berechnet werden.

Unsere Resultate haben gezeigt, dass mitotische Rekombination die Hauptursache für den Verlust der tk-Funktion war. Chromosomenverlust und Deletionen waren vergleichsweise selten.

Parallel zu diesen Experimenten wurden 32 tk-defiziente Mutanten nach Behandlung mit NMU isoliert. NMU ist ein klassisches methylierendes Mutagen. Eine molekulare Analyse dieser Mutanten hat gezeigt, dass hier vor allem Punktmutationen zum Verlust der *tk*-Aktivität geführt hatten. Daraus liess sich schliessen, dass die Induktion von Rekombinationen eine spezifische Aktivität von AFB_1 darstellte und nicht ein genereller Effekt der Mutagen-Behandlung. Zusammengefasst zeigen unsere Resultate, dass AFB_1 nicht nur in der Hefe, sondern auch in menschlichen Zellen mitotische Rekombinationen induzieren konnte und dass dies ein fundamentaler Mechanismus sein könnte bei der Karzinogenese durch AFB_1.

Summary

Food contamination by the molds *aspergillus flavus* and *aspergillus parasiticus* is a major problem, mainly in southern Africa and in the Qidong area in China. These molds produce different mycotoxins of which aflatoxin B_1 (AFB_1) is the most potent. In these regions the incidence of hepatocellular carcinoma (HCC) is higher than elsewhere. Various investigations link these cancers to AFB_1 and chronic hepatitis B virus (HBV) exposure.

AFB_1 is known as a very potent mutagen. Various *in vitro* experiments suggested that exposure to AFB_1 leads mainly to G to T transversions. In AFB_1 and HBV related HCCs a specific mutation in the p53 tumor suppressor gene was present in half of the tumors, but was not found in AFB_1 unrelated cancers. This mutation is a G to T transversion in codon 249, and AFB_1 is the most likely candidate as cause

for this mutation. Differences in AFB_1 related and unrelated cancers were also found in the loss of heterozygosity (LOH) patterns. Higher incidence of LOH was found in the samples from the AFB_1 contaminated Qidong area compared to the samples from Beijing (low AFB_1). However, the mechanisms leading to LOH in these HCCs are not clear. Mitotic recombination is one possible explanation for such results.

Recent studies of our group found a relation between AFB_1 and mitotic recombination in the lower eukaryot *Saccharomyces cerevisiae*. AFB_1 induced much more recombinations compared to point mutations in this organism.

If AFB_1 had a comparable influence on the recombination frequency in human cells, this activity might provide an important mechanism for the loss of heterozygous genes in hepatic tumor development. Thus, a mutationally inactivated *p53* tumor suppressor allele might segregate from the corresponding wild type allele upon the induction of mitotic recombination and produce a cell devoid from p53 function that has the ability to develop into a cancerous cell. The aim of the present work was to determine if the effect of AFB_1 on the recombination frequency in a human cell line is as pronounced as in yeast. We wanted to discriminate and quantify the mechanisms that lead to the loss of function of a heterozygous gene.

The TK6 cell line used in this study is heterozygous at the thymidine kinase (*tk*) locus on chromosome 17. One allele produces an active tk enzyme, the other allele carries an inactivating frameshift mutation in exon 4. This mutation renders the TK6 cell line a suitable tool to examine point mutations, recombinational events, large deletions, and chromosome loss at the same locus. In the present work we performed mutation assays and collected independent mutants which had lost *tk* activity. In this manner we collected a representative number of AFB_1 induced mutants (87) and spontaneously occurring mutants (57).

To determine which genetic event had led to the loss of *tk* function in these mutants we used methods by which allelic differences at specific chromosomal loci could be detected. The methods we applied for this purpose were single strand conformation polymorphism (SSCP) analysis at the *tk* locus and microsatellite analysis at different loci on chromosome 17. This enabled us to detect loss of heterozygosity (LOH) tracts around the *tk* gene and on the other arm of chromosome 17. By fluorescence *in situ* hybridization (FISH) analysis the physical presence of one or two alleles of the *tk* region could be observed. With this information we could distinguish between point mutations, mitotic recombinations, large deletions, and chromosome loss that led to the loss of *tk* function, and we calculated mutant fractions for these events.

Our results revealed that mitotic recombination was the dominant cause for AFB_1 induced loss of tk function. Chromosome loss and deletions occurred rather infrequently.

In parallel, we collected 32 *tk* deficient mutants upon exposure to NMU, a classical methylating agent. Molecular analysis of these mutants suggested that point mutations were the prominent cause for loss of tk function, in contrast to the AFB_1 induced mutants. Thus, recombination induction was a specific activity of AFB_1, rather than a general effect of mutagen exposure.

I. Introduction

1. History of Aflatoxin B_1 (AFB$_1$)

Mycotoxins are toxic metabolites produced by molds which can contaminate food, especially in areas with a hot and moist climate. Several classes of these mycotoxins have been associated with highly lethal outbreaks of food poisoning in animals as well as in humans (reviewed in (McLean and Dutton 1995)). The diseases were classified and named after the symptoms resulting upon ingestion. During the 9th and 10th centuries there were numerous outbreaks of ergotism, the classical mycotoxicosis. Other examples of mycotoxicosis outbreaks include St Anthony's fire in the 11th century, yellow rice disease in Japan, sheep facial eczema in New Zealand, and alimentary toxic aleukia in the Ukraine in the 1950s. The aflatoxins, a class of specific mycotoxins, were detected in the early 1960s after the death of thousands of turkey poults, ducklings and chicks ("Turkey-X disease") in Britain. The aflatoxins were found to be produced by the fungi species *aspergillus flavus* and *aspergillus parasiticus*. These findings initiated the discovery of presently more than 300 mycotoxins, many of which are genotoxic in experimental systems. The aflatoxins, and other mycotoxins like sterigmatocystin, ochratoxin, zearalenone, some *Penicilium* toxins, and fumonisins, were shown to be carcinogenic in experimental animals (Ames 1989; Ueno and Kubota 1976). All of these carcinogenic mycotoxins except fumonisin are also genotoxic agents. The fumonisins are currently believed to induce cancer by impairing signal transduction pathways.

2. Activation and Detoxification of AFB$_1$

The AFB$_1$ molecule *per se* is chemically not very reactive. However, in humans and animals it can be oxidized by several enzymes to an extremely reactive epoxide (figure 1). This AFB$_1$-*exo*-8,9-epoxide is known to be the ultimate genotoxic product, and it can be formed by cytochrome P450s and microsomal monooxygenases, prostaglandin synthase, or lipoxygenases (Battista and Marnett 1985; Liu and Massey 1992; Raney *et al.* 1992).

From the cytochrome P450 enzyme family, P450 1A2 and the 3A4 seem to play the major role in AFB$_1$ activation. Both P450s have been shown to transform AFB$_1$ into the *exo*-epoxide. However P450 1A2 readily oxidizes AFB$_1$ to AFM$_1$, AFQ$_1$, and AFB$_1$ *endo*-8,9-epoxide, whereas P450 3A4 can transform AFB$_1$ to AFQ$_1$. These oxidation products are generally poor substrates for epoxidation or, after epoxidation, do not react with DNA and thus, P450s can also act as detoxification enzymes. Expression of different P450 isoforms depends on the species, the tissue, and also the individual which could explain some contradictory results of different groups. The 1A2 isoform seems to have the higher affinity to AFB$_1$ than the 3A4, but is expressed to a lower extent in human liver. Guengerich and his group have suggested that P450 3A4 was the dominant enzyme for AFB$_1$ bioactivation at all concentrations below 1µM (Guengerich *et al.* 1998; Shimada and Guengerich 1989). Reports which show a protective role of P450 1A2 in animals and humans support this opinion (Faletto *et al.* 1988; Gurtoo 1980; Lin *et al.* 1991). On the other hand, Crespi et al. expressed different P450 isoforms in human lymphoblastoid cell lines (Crespi *et al.* 1991; Crespi *et al.* 1997; Crespi *et al.* 1990). The cells expressing the 1A2 isoform were the most sensitive with respect to toxic and mutagenic effects of AFB$_1$, 3 to 6-fold more sensitive than cells expressing the 3A4 enzyme at a comparable level. Eaton and Gallagher used specific 1A2 and 3A inhibitors on human liver microsomes (Eaton and Gallagher 1994). They concluded that the 1A2 was the high affinity enzyme active at lower
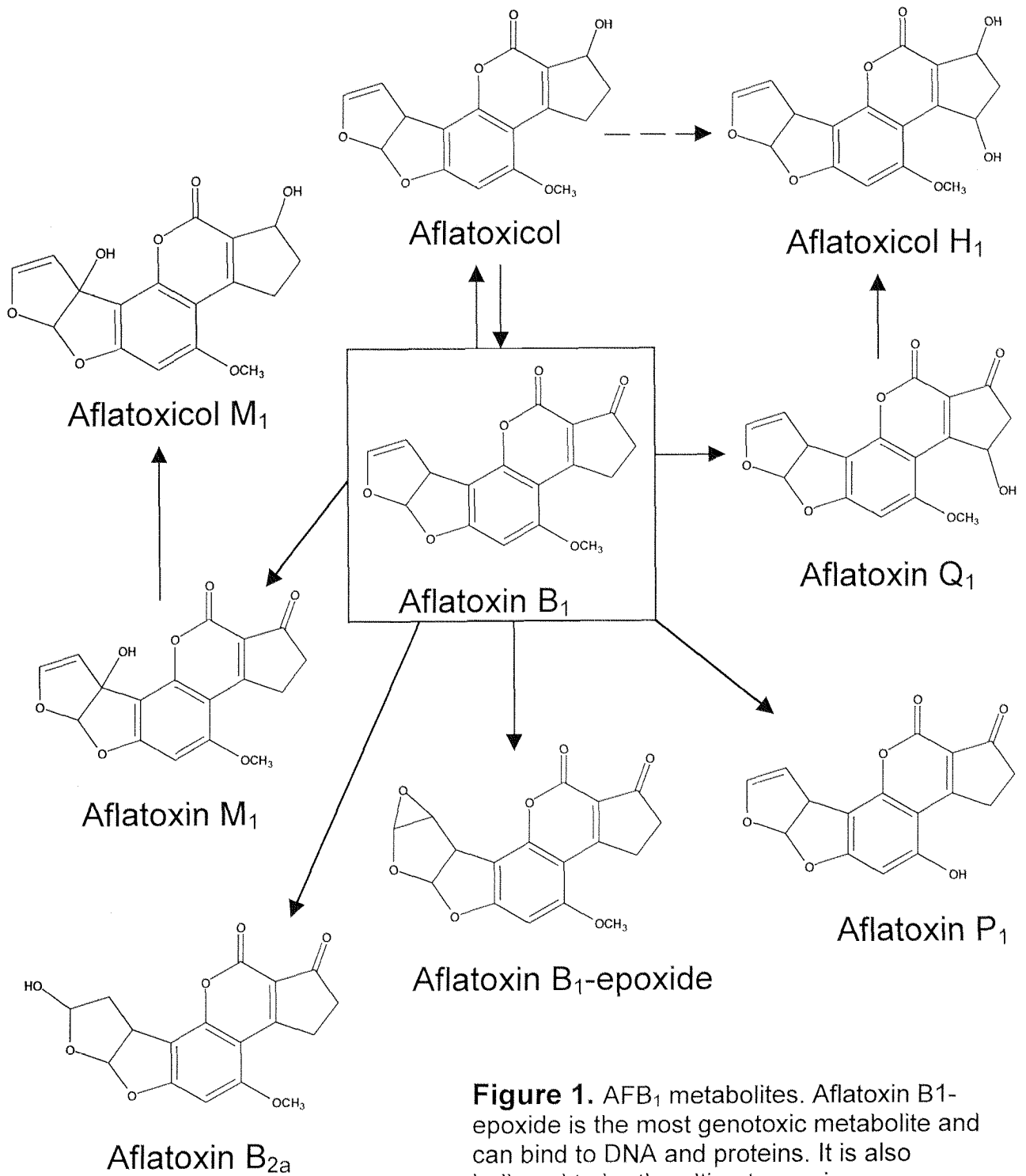
Figure 1. AFB$_1$ metabolites. Aflatoxin B1-epoxide is the most genotoxic metabolite and can bind to DNA and proteins. It is also believed to be the ultimate carcinogen.

substrate concentrations, whereas the 3A enzyme was capable of AFB_1 activation at relatively high substrate concentrations.

When encountering high doses of AFB_1 (usually by ingestion) cells of the small intestine, expressing high levels of P450 3A4, are the first cells with the capacity to activate AFB_1 to its mutagenic form. However, because the respective cells are readily sloughed, AFB_1-DNA adducts rarely lead to cancer. The AFB_1 molecules metabolized in liver cells may be detoxificated by several mechanisms. Apart from oxidation to AFQ_1, AFM_1, and AFB_1 *endo*-8,9-epoxide (discussed above), AFB_1 can be *O*-demethylated to form AFP_1. Once formed, the toxic AFB_1 *exo*-epoxide is quite unstable in aqueous solution ($t_{1/2}$=1s at 23°C, pH7)(Johnson *et al.* 1996). Non enzymatic hydrolysis leads to the harmless 8,9-dihydrodiol. The reaction of the AFB_1-epoxide with epoxide hydrolase has been discussed. However recent investigations suggest a negligible role of this detoxification pathway (reviewed in (Guengerich *et al.* 1998)). Other minor pathways include detoxification by the UDP-glucuronyl-transferase, and sulphotransferase.

Yet, the most important pathway for AFB_1 detoxification in mammals is the conjugation with glutathion (GSH) (reviewed in (Eaton and Gallagher 1994)), which is highlighted by the fact that species susceptibility to AFB_1 inversely correlates with the expression of certain glutathione S-transferases (GSTs) in the liver. The GSTs have been divided in 5 classes of isoenzymes and exert their function as homo- or heterodimeric enzymes. In one study liver cytosolic fractions from mouse had 50- to 100-fold greater AFB_1-*exo*-8,9-epoxide conjugating activity than did those from rat, although both fractions had a comparable conjugating activity towards 1-chloro-2,4-dinitrobenzene (Monroe and Eaton 1988). Mice contain at least three GSTs from the alpha class, which were shown to be mainly responsible for AFB_1-*exo*-8,9-epoxide- conjugation. The selectivity of the conjugation reaction to GSH also depends on the AFB_1 stereoisomer. The *exo*-epoxide was efficiently conjugated by mouse cytosolic GSTs, in contrast to the GSTs present in liver cytosolic fractions from rats and humans. The human GST mu form had significant conjugating activity toward the *endo*-epoxide, however this stereoisomer probably

has no mutagenic potential (see above). Human GSTs from the alpha class had only a very weak activity toward either epoxide. These studies can explain the relative resistance of mice against the effects of AFB_1 ingestion, compared to rats and humans.

Different chemicals have the potential to modify these detoxification activities, and thus, the susceptibility towards AFB_1 and other carcinogens may be altered. Modulation of detoxification can be reached simply by a change of diet. Cruciferous vegetables such as broccoli, cabbage, and brussel sprouts exhibited anticarcinogenic effects when incorporated into the diet of rats (Boyd et al. 1982; Godlewski et al. 1985; Ramsdell and Eaton 1988; Wattenberg 1978; Wattenberg 1979; Wattenberg 1985). This effect correlated with an induction of GSTs in rats, while epoxidation of AFB_1 was not significantly affected. Through mass education programs in affected regions in China, some individuals changed their nutrition from a maize-based diet to a rice-based diet, which is less affected by aspergillus contamination. Wild et al. observed that aflatoxin-albumin adduct levels were lower in inhabitants of such villages (Wild C.P., Chen J. and Montesano R., unpublished data). Apart from these natural food constituents, also synthetic chemicals had been used to modulate AFB_1 biotransformation. Early studies were performed using ethoxyquin and phenobarbital as potential chemoprotective agents in animal studies (Garner 1975; Mgbodile et al. 1975; Talalay et al. 1979). Both drugs were able to reduce binding to DNA, apparently via an induction of detoxification enzymes. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) also had the effect that treated rats exhibited an increased level of AFB_1-GSH conjugation and therefore a reduction of AFB_1-DNA binding (Williams et al. 1986). Currently, research has focussed on the dithiolthione oltipraz, a drug originally prescribed to treat schistosomiasis. It has been shown to induce rat GSTs (Kensler et al. 1992), and rats receiving a diet with 0.075% oltipraz for a 4-week period were completely protected against AFB_1-induced HCC and hyperplastic nodules in the liver (Roebuck et al. 1991). In human hepatocytes oltipraz induced alpha class GSTs and inhibited CYP450 3A4 and 1A2 (Langouet

et al. 1995; Morel *et al.* 1993). Next to these effects, oltipraz has been shown to block hepatitis B virus (HBV) replication, another risk factor for HCC (Chi *et al.* 1998). In an *in vivo* study oltipraz was administered to Chinese people exposed to AFB_1. A subsequent increase in the level of GST conjugation of activated AFB_1 as well as an inhibition of P450 1A2 was observed (Kensler *et al.* 1998; Wang *et al.* 1996a; Wang *et al.* 1999). Other, more potent GST-enzyme inducers are likely to become available in the next few years.

3. *AFB_1 as a carcinogen*

AFB_1 is the most potent carcinogen known at present and has been classified as a class 1 human carcinogen by the International Agency for Research on Cancer (Cancer 1993). It is mutagenic in many model systems and produces point mutations, chromosomal aberrations, micronuclei, sister chromatid exchange, mitotic recombination, unscheduled DNA synthesis, and chromosomal strand breaks. The reactive epoxide group of metabolically activated AFB_1 can react with proteins or with DNA. Main targets for AFB_1-epoxides on DNA strands are the N^7-positions of guanine bases. Such an electrophilic attack occurs mostly in GC-rich regions, presumably including an intercalated state. The resulting adduct can convert to a ring-opened formamidopyrimidine derivative (AFB_1-FAPY) which is relatively stable and resistant to DNA repair processes. Thus, AFB_1-FAPY-adducts can persist during several rounds of DNA replication. Mispairing at the sites of these bulky adducts often results in mutations. Mutations could also occur by depurination at the AFB_1-N^7-guanine site. However, *in vitro* experiments suggested that rather the AFB_1-FAPYs, and not the apurinic sites may be responsible for mutagenesis (reviewed in (Wang and Groopman 1999)). In addition, error prone repair of the bulky adducts can lead to base pair substitutions, frameshift mutations, or single strand breaks (Hsieh 1986).

The mutagenic spectrum resulting from AFB_1-DNA adducts is quite broad, ranging from frameshift mutations, base transitions and transversions to large scale genetic

damages such as recombinations or deletions. However, various *in vitro* experiments clearly suggested a predominance of G to T transversions upon AFB_1 exposure (Aguilar *et al.* 1993; Bailey *et al.* 1996; Foster *et al.* 1983; Levy *et al.* 1992). This tendency of AFB_1 to induce G to T transversions has its consequence *in vivo* (see below).

Today, food contamination by AFB_1 is still a major problem in the Qidong area in China and in southern Africa. The incidence of hepatocellular carcinomas (HCCs) is significantly higher in those regions than elsewhere. Various investigations link these cancers to AFB_1 and chronic HBV exposure (Fujimoto *et al.* 1994; Groopman *et al.* 1988; Groopman *et al.* 1996; Scorsone *et al.* 1992; Wang *et al.* 1996b; Wogan 1992; Yeh *et al.* 1989). A specific G to T transversion in codon 249 of the p53 tumor suppressor gene has often been observed in AFB_1 affected regions. In AFB_1 and HBV related HCCs from the Qidong area this specific mutation in the p53 tumor suppressor gene was present in 13/25 (52%) of the tumors, but was not found in 9 AFB_1 unrelated cancers from Beijing (Fujimoto *et al.* 1994). Scorsone et al. found the specific codon 249 mutation in 21/36 (58%) of AFB_1 and HBV related HCCs, 13 of which (61%) showed concomitant loss of the *p53* gene (Scorsone *et al.* 1992). In contrast, a study of *p53* mutations in HCCs from Japan and Western countries where AFB_1 exposure is negligible, found no mutations in codon 249 (Ozturk 1991). The investigation of 15 HCCs from Senegal, a country with high AFB_1 contamination, found mutations in codon 249 in 10 of the 15 HCCs. These epidemiological data, which suggest a causative relationship between AFB_1 exposure and this codon 249 mutation, find support in the *in vitro* experiments from Puisieux et al. and Aguilar et al. (Puisieux *et al.* 1991). Puisieux et al. showed that AFB_1 epoxide can bind to the *p53* codon 249 in a plasmid. Aguilar et al. mutated the codons 247-250 by rat liver microsome-activated AFB_1 in human HepG2 cells. They found the highest mutation frequency for the codon 249 G to T transversion, however they also induced G to T and C to A transversions in the adjacent codons.

Yeh et al. calculated the incidence of HCC in a heavily and lightly AFB_1 contaminated area and correlated the data with HBV infection of the individuals (Yeh *et al.* 1989). HCC incidence for individuals from a region with high AFB_1 contamination, who were also HBV surface antigen positive, was 649 cases per 100000, whereas only 66 cases per 100000 occurred in areas with light AFB_1 contamination. The HCC incidence for HBV surface antigen negative people was 99, and 0 cases per 100000, respectively. In several nested case-control studies in the 1990s, urine and blood samples were tested for the presence of urinary aflatoxin biomarkers and HBV surface antigen, respectively. Several years later some persons enrolled in the study developed HCC and thus, a relative risk for HCC in respect to AFB_1 contamination and HBV infection could be calculated (reviewed in (Wang and Groopman 1999)). For both risk factors an increase in the relative risk to develop HCC was calculated and even a much higher increase was calculated for people who were positive for both, AFB_1 exposure and HBV infection.

Regarding AFB_1 related carcinogenesis the alteration of proto-oncogenes had been investigated in several studies. In rats AFB_1 was capable of activating all three types of c-*ras* oncogenes (Ha-*ras*, Ki-*ras*, and N-*ras*) using AFB_1 transformed cell lines or AFB_1 induced primary liver tumors (reviewed in (Wang and Groopman 1999)). The main mutation found in these genes was a G to A transition and ,at lower frequency, a G to T transversion in one of the two first bases of codon 12. Two studies investigated AFB_1 induced liver tumors in two mouse strains susceptible to carcinogens. They also found activated c-*ras* oncogenes in AFB_1 induced liver tumors, however harboring a mutation in codon 61, not in codon 12 (Bauer Hofmann *et al.* 1990; Wiseman *et al.* 1987). Bailey investigated AFB_1 initiated hepatic tumors in trout and found mutations in the Ki-*ras* oncogene. The predominant mutation found in 58% of the tumors was a G to T transversion in codon 12, and one G to A transition in the same codon (Bailey 1994). So far, there is no evidence for a similar activation of proto-oncogenes in human HCCs related to AFB_1. Riley et al. investigated the *in vitro* activation of the human Ha-*ras* proto-

oncogene by AFB_1 and found G to T transversions in codon 12, suggesting that AFB_1 had the potential for Ha-*ras* activation (Riley *et al.* 1997). But as long as there is no evidence for such mutations from epidemiological studies, a possible relation remains elusive.

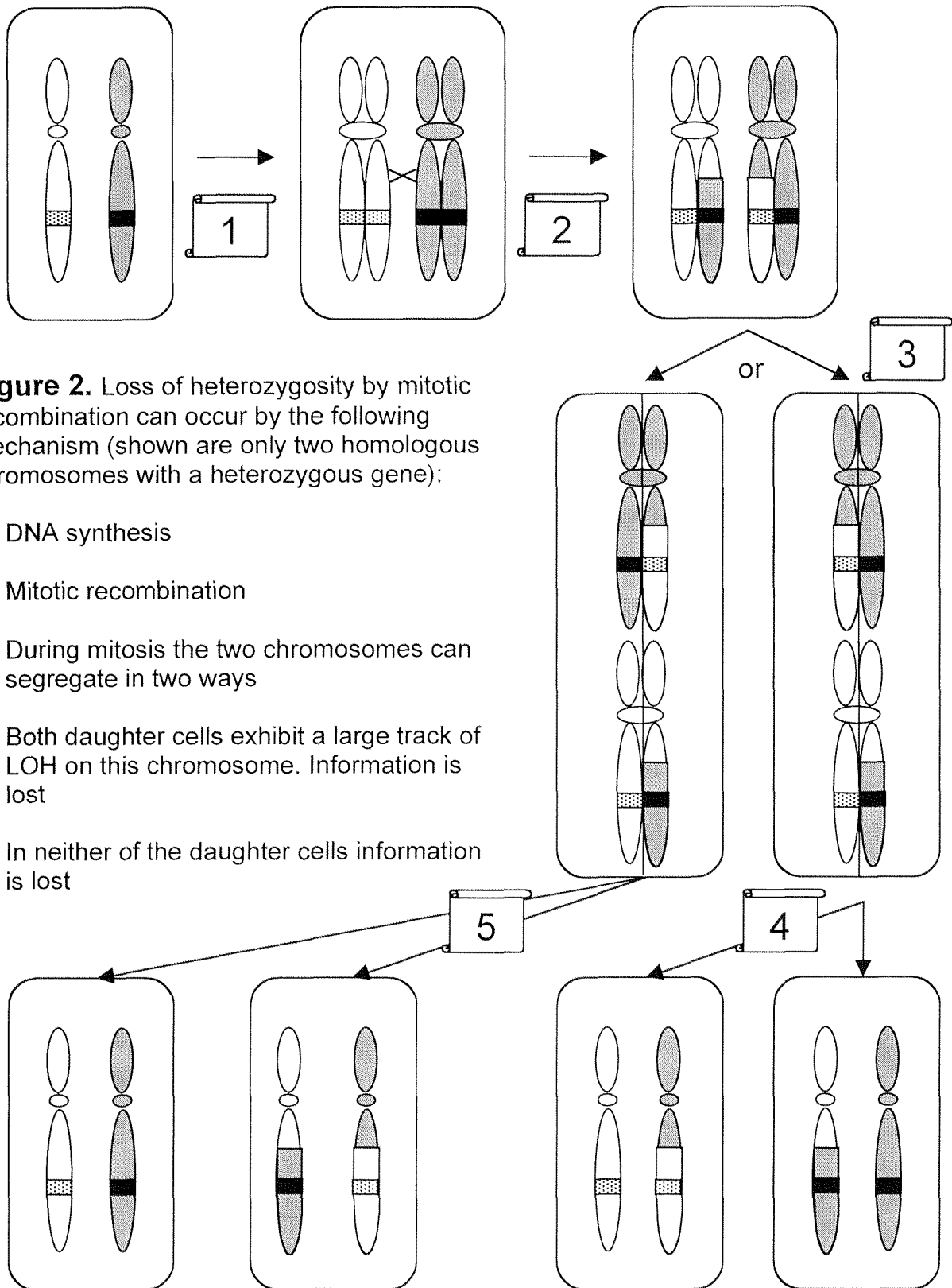## 4. The role of mitotic recombination in carcinogenesis

Meiotic recombination is a mechanism that contributes to the genetic variability in higher eukaryotes as well as in some microorganisms. This genomic flexibility renders a species the capacity to adapt to changing environmental conditions. It is also recombination which contributes mainly to the vast diversity of the immune system. However, what is beneficial for the survival of a population can have dramatic consequences for its individuals. The price for the genetic instability is often the development of cancer. The mechanisms how recombination contributes to carcinogenesis are discussed in this chapter.

Carcinogenesis is a multistep process. Models have been proposed by Fearon and Vogelstein for colon carcinogenesis and by Weinberg for the development of breast cancer (Fearon and Vogelstein 1990; Weinberg 1991). Both models include the activation of proto-oncogenes and the inactivation of tumor suppressor genes, events which probably also represent keysteps in the etiology of other cancer types. The activation of proto-oncogenes is a dominant event which can occur by a single incident such as a point mutation or a translocation. In contrast, the inactivation of tumor suppressor genes is a recessive event and usually requires two hits. The mutation of the first allele can occur by the same mechanisms as described for the activation of proto-oncogenes. The loss of the second allele can occur additionally by mitotic recombination.

Activation of a proto-oncogene by non-homologous mitotic recombination can occur by different mechanisms, depending on the DNA sequences which recombine (reviewed in (Sengstag 1994)). When recombination occurs between two non-homologous chromosomes, a cytogenetically observable translocation

results which can place a proto-oncogene into a new environment. The Philadelphia chromosome was the first karyotypic anomaly associated with a human hematologic neoplasm and is the result of a translocation between chromosomes 9 and 22 [t(9;22) (q34;q11)]. By this translocation a gene fusion between the two proto-oncogenes *bcr* and *abl* occurs. The resulting fusion protein has different biochemical activities from the original proteins and is interfering with cell cycle control in such cells. Another example where a translocation led to a fusion oncoprotein was found in almost 100% of investigated cases of acute promyelocytic leukemia. This translocation led to the fusion of the myeloid gene *pml* and the retinoic acid receptor α-gene *rar*. A further way to activate proto-oncogenes is their translocation into the proximity of enhancer elements of strongly expressed genes. In some Burkitt lymphomas the translocation of c-*myc* into the neighborhood of an immunoglobulin locus was observed. Such translocations into the vicinity of immunoglobulin or T-cell receptor genes may be the result of a malfunction of the V(D)J-recombinase, which usually recognizes specific signal sequences. Another mechanism how non-homologous recombination can activate proto-oncogenes is deletion or amplification of the gene.

Most tumor suppressor genes code for proteins which act as downregulators in the control of the cell cycle. Inactivation of a tumor suppressor gene often requires two steps. The loss of function of one tumor suppressor gene can occur by all the mechanisms discussed above, however the second copy usually produces sufficient protein for maintenance of normal cellular functions. Destruction or complete loss of the second allele may be required to produce a neoplastic phenotype. This again can occur by mutation or by non-homologous mitotic recombination, but additionally by homologous mitotic recombination (figure 2). This may happen by a crossing over event between homologous chromosomes followed by the swapping of information between them. During subsequent mitosis there is a 50% chance that one of the two daughter cells inherits the two non-functional alleles, while the other gets the two functional copies. The genetic

Figure 2. Loss of heterozygosity by mitotic recombination can occur by the following mechanism (shown are only two homologous chromosomes with a heterozygous gene):

1 DNA synthesis

2 Mitotic recombination

3 During mitosis the two chromosomes can segregate in two ways

4 Both daughter cells exhibit a large track of LOH on this chromosome. Information is lost

5 In neither of the daughter cells information is lost

endpoint of this mechanism is a tract of loss of heterozygosity (LOH) on the chromosome.

LOH can be investigated by molecular analysis at heteromorphisms near the locus of interest. In earlier studies often restriction fragment length polymorphisms (RFLPs) were used for this purpose, today PCR based methods such as microsatellite analysis are preferred. In studies, where the molecular mechanisms leading to LOH are of interest, this analysis should be supplemented by a quantitative method such as quantitative PCR or fluorescence *in situ* hybridization (FISH).

Various in vitro studies indicated that the inactivation of the second allele of a gene predominantly occurred by LOH rather than by point mutation. Acuña et al. found 82% LOH on chromosome 15 in the yeast *Saccharomyces cerevisiae* after mutagen exposure. Two studies investigated the spontaneous inactivation of a heterozygous *APRT* gene in human lymphoblastoid cell lines. In 25/32 (78%) and in 22/26 (85%) APRT-deficient clones this was due to LOH (Fujimori *et al.* 1992; Pongsaensook *et al.* 1997). Li et al. (1992) investigated the lymphoblastoid cell line TK6 for loss of function of the heterozygous *tk* gene (Li *et al.* 1992). Among spontaneous tk- mutants, 70% were of the "slow growing" phenotype (see below), and 35/36 of these slow-growth mutants exhibited LOH around the *tk* locus. Applegate et al. observed a similar fraction of LOH at the *tk* locus in the mouse lymphoma cell line L5178Y (Applegate *et al.* 1990). De Nooij-van Dalen et al. used the naturally heterozygous HLA locus in the human lymphoblastoid cell line ORI for LOH analysis. Clones from HLA-A2 deficient mutants were isolated by immunoselection and molecular analysis of surrounding loci revealed 80% LOH at the HLA-A2 locus (de Nooij-van Dalen *et al.* 1997). Gene dosage analysis implied that most of the mutants acquired LOH by mitotic recombination. The strongest evidence that the loss of heterozygous genes in humans mainly occurs by mitotic recombination comes from an *in vivo* study (Gupta *et al.* 1997). These authors investigated the *APRT* locus on chromosome 16 in human T lymphocytes from the peripheral blood of *APRT* heterozygous individuals. 61/80 (76%) *APRT* deficient

mutants exhibited LOH of linked microsatellite markers on 16q. Ten of these LOH mutants were investigated by FISH using an *APRT* cosmid probe and a deletion was found in only one mutant (10%). Thus, the other 90% of the LOH mutants acquired LOH by to mitotic recombination.

As the name implies, the retinoblastoma gene *RB-1* originally was identified in retinoblastoma, however mutated forms were also found in sarcomas, leukemias, and breast and bladder carcinomas. Children, who inherited one defective *RB*-1 allele, develop retinoblastomas at the high incidence of 85%. The close relationship between the *RB*-1 gene and retinoblastoma was underlined in numerous studies finding LOH tracts around the gene on chromosome 13 (reviewed in (Sengstag 1994)).

The most commonly altered gene in human tumors is the tumor suppressor gene *p53* located at the chromosomal locus 17p13.1. It has been discovered by investigation of the Li-Fraumeni syndrome, which describes an inherited susceptibility for sarcomas, breast carcinomas, and other neoplasms. In six Li-Fraumeni families, the presence of a heterozygous *p53* mutation was found, indicating a strong role of this gene in many different cancers (Malkin *et al.* 1990; Srivastava *et al.* 1990). As a consequence, studies of various different cancers focussed on the *p53* gene, looking for alterations. And indeed, LOH at this locus has been observed in various cancers. One study associated colorectal cancers with LOH at the *p53* locus (Baker *et al.* 1990). Niederacher et al. investigated LOH on chromosome 17 in 121 spontaneous invasive ductal breast carcinomas and 16 benign breast tumors (Niederacher *et al.* 1997). The authors found no evidence for LOH in any of the benign tumors, in contrast to sometimes complex LOH patterns in every of the invasive tumors. Two markers inside the *p53* gene showed LOH in 57% and 51%, respectively. Other regions with frequent LOH were identified, suggesting that other tumor suppressor genes might be located in those regions. The occurrence of LOH at the *p53* locus in HCCs will be discussed in the next chapter.

The role of the breast and ovarian susceptibility gene *BRCA1* in sporadic tumors is not very clear. This gene probably plays an important role in the inherited breast/ovarian cancer syndrome where 80% of investigated families exhibited a germline mutation in this tumor suppressor gene. In families with an inherited susceptibility towards breast cancer exclusively, 45% had germline mutations in *BRCA1* (Easton *et al.* 1993). In several studies investigating sporadic breast cancer the *BRCA1* region exhibited LOH in up to 70% of the tumors. However, extensive mutational analysis did not reveal any mutations in the coding region of this gene, which raises some doubts about the importance of *BRCA1* in sporadic breast cancers (Shattuck Eidens *et al.* 1995).

5. AFB₁ and recombination

Both *in vitro* and *in vivo* studies imply that recombination plays an important role in AFB_1 mediated carcinogenesis. Our group has previously shown that AflatoxinB₁ (AFB_1) is a potent inducer of mitotic recombination in the yeast *Saccharomyces cerevisiae* (Sengstag *et al.* 1996): Three metabolically competent tester strains were used to measure point mutations (GRF18), translocations (YB110), and gene conversions (YHE2). At $250\mu M$ AFB_1 a 139-fold increase in the mitotic recombination frequency was observed, compared to a 19-fold increase in the gene conversion frequency, and a 3-fold increase in the point mutation frequency. Recombination induction in yeast occurred also at much lower doses of AFB_1. At $15.6\mu M$ the mitotic recombination frequency was increased by 22-fold, the mutation frequency only by 1.2-fold (Keller Seitz and Sengstag, unpublished). Recombination induction after AFB_1 treatment was also observed in mammalian cells. An assay monitoring intrachromosomal recombination in SP5/V79 Chinese hamster cells showed an induction of recombination by 3-fold after treatment by $0.8\mu M$ S9-activated AFB_1 (Zhang and Jenssen 1994). In a recent study performed by Preisler et al., heterozygous gene inactivation after AFB_1 treatment was

investigated in L5178Y mouse lymphoma cells (Preisler *et al.* 2000). They found that 23/34 (68%) of AFB$_1$ induced mutants had lost *tk* activity by recombination.

No study investigated AFB$_1$ induced recombination in human cells so far. However, molecular analyses of AFB$_1$ related HCCs imply that recombination plays a role in liver carcinogenesis. Scorsone et al. investigated HCCs from an AFB$_1$ high risk area in China for the specific *p53* codon 249 mutation and LOH at three loci near the *p53* gene (Scorsone *et al.* 1992). A total of 22/36 (61%) tumors showed either direct or indirect evidence for LOH, thirteen (59%) of which contained the codon 249 mutation. Fujimoto et al. compared HCCs from a region with high (Qidong) and low (Beijing) AFB$_1$ contamination (Fujimoto *et al.* 1994). Among other alterations they investigated LOH at three tumor suppressor loci (p53, rb, APC), and of markers on chromosomes 4, 13, and 16, which are frequently lost in HCC. The incidence of LOH at the *p53* locus was nearly the same among the two subpopulations (58% LOH in Qidong, 57% in Beijing). However, at all other loci investigated, LOH differed strikingly. In 9 HCCs from Beijing only one single case of LOH at D13S1 was detected, whereas in the 26 HCCs from Qidong the incidence of LOH at the loci of interest was mostly between 28% and 58%. The peak incidence of LOH was observed at the TAT locus (16q22) with 9/10 (90%). In another study HCCs from Qidong and Shanghai were investigated (Rashid *et al.* 1999). 10/12 (83%) contained the specific codon 249 mutation, indicating that AFB$_1$ probably was involved. LOH was found on 17p (71%), 4q (50%), 1p (46%), 16q (42%), and 13q (38%). These data strongly suggest that mitotic recombination plays a major role in the etiology of AFB$_1$ related liver carcinogenesis.

In the present study it was aimed to discriminate and quantify the mechanisms that lead to the loss of function of a heterozygous gene. The TK6 cell line used in this study is heterozygous at the thymidine kinase (*tk*) locus on chromosome 17. One allele produces an active tk enzyme, the other allele carries an inactivating frameshift mutation in exon 4. This mutation renders the TK6 cell line a suitable

tool to examine point mutations, recombinational events, large deletions, and chromosome loss at the same locus. Upon selection for mutants which have lost thymidine kinase (*tk*) activity two phenotypically different types of mutants can be distinguished: Normal growth (NG) mutants with a similar growth rate like TK6 cells (10-18 hours) and Slow growth (SG) mutants with a growth rate of 30-40 hours. In the literature SG mutants were often associated with mostly large scale genetic events such as mitotic recombination, large deletions or chromosome loss (Li *et al.* 1992; Yandell *et al.* 1990). As will be shown below our data support these findings.

In this work we performed mutation assays and collected independent mutants which had lost *tk* activity. In this manner we collected a representative number of AFB_1 induced mutants, spontaneously occurring mutants, and NMU induced mutants. NMU is a methylating agent that is known to induce mainly point mutations.

To determine which genetic event had led to the loss of *tk* function in these mutants we used methods by which allelic differences at specific chromosomal loci could be detected. The methods we applied for this purpose were single strand conformation polymorphism (SSCP) analysis at the *tk* locus and microsatellite analysis at different loci on chromosome 17. This enabled us to detect loss of heterozygosity (LOH) tracts around the *tk* gene and on the other arm of chromosome 17. By FISH analysis the physical presence of one or two alleles of the *tk* region could be observed. With this information we could distinguish between point mutations, mitotic recombination, gene conversion, large deletions and chromosome loss that led to the loss of *tk* function and calculate mutant fractions for these events. Our study suggests that AFB_1 was capable to induce mitotic recombinations in a human cell line and that this might be a fundamental mechanism in AFB_1 related carcinogenesis.

II. Materials and methods

1. Materials

The following chemicals were obtained from Fluka Chemicals AG/Sigma (Buchs, Switzerland): AFB_1, NMU, TFT, aminopterin, deoxycytidine, hypoxanthine, DMSO, mineral oil, formamide, dextran sulfate, colcemid, Tween 20®, Tris-HCl, KCl, and Salmon sperm DNA. Ethanol, methanol, acetic acid, and $MgCl_2$ were purchased from Merck (Darmstadt, Germany). Gelatin for PCR buffer was obtained from Dr. Bender and Dr. Hobein AG (Zürich, Switzerland). RPMI 1640 medium, horse serum, penicillin-streptomycin, and human Cot-1 DNA were obtained from Life Technologies AG (Basel, Switzerland). Rat liver S9 extract was obtained from MolTox Inc (Boone, USA). Taq polymerase and dNTPs were purchased from Boehringer (Mannheim, Germany). PCR primers were synthesized by Microsynth GmbH (Balgach, Switzerland).

2. Cell culture

TK6 cells were obtained from Dr. P. Morgenthaler, University of Lausanne and handled as has been described previously (Liber and Thilly 1982). Cell suspensions were cultured at 37°C in a humidified 5% CO_2 atmosphere in complete medium (RPMI 1640, 10% heat-inactivated horse serum, 1% penicillin-streptomycin) and diluted daily to a density of $3*10^5$ cells/ml.

3. Mutation assays

Mutation assays have been basically performed as described by Li et al. (Li *et al.* 1992). A TK6 culture was pretreated for two days with CHAT medium (complete medium containing $10\mu M$ deoxycytidine, $200\mu M$ hypoxanthine, $0.2\mu M$ aminopterin, and $17.5\mu M$ thymidine) to eliminate most of the cells with a tk⁻ phenotype. The cells were then passaged to CHT medium (CHAT medium without aminopterin) for another two daysfollowed by exposure to the mutagen for 4 hours at a density of $3*10^5$ cells/ml.

AFB_1 mutagenesis was performed in the presence of 5% rat liver S9 extract and a NADPH-regenerating system (6mM $MgCl_2$, 4mM glucose-6-phosphate, and 3mM $NADP^+$). AFB_1 was dissolved in DMSO, negative controls included DMSO (1%) alone. NMU, dissolved in 1.5% acetate, was added directly to the cell culture in a volume/volume ratio of 0.1-0.4%.

After exposure the cells were washed, resuspended in complete medium and split up in subcultures. Cytotoxicity was determined from an extrapolation of the post mutagenesis growth curve. After a 3-day phenotypic expression period appropriate dilutions of each subculture were plated in medium containing $2\mu g/ml$ trifluorothymidine (TFT) in microtiter plates at a densitiy between 5000 and 40000 cells/well. In parallel, a small aliquot of each culture was plated at 2 cells/well in complete medium to determine plating efficiency.

The plates were scored for NG mutants after 14 days and refed with $2\mu g/ml$ TFT. Mutants which appeared on the plates after more than 21 days were classified as SG mutants. Mutants which became visible on the plates inbetween 14 and 21 days of incubation were replated in complete medium and the growth rate was measured. Those mutants with a doubling time < 22 hours were classified as NG mutants, the others as SG mutants. Some NG and SG mutants of every plate were randomly selected, recovered in complete medium, and replated in complete medium, in complete medium containing $2\mu g/ml$ TFT, and in CHAT medium. One NG and one SG mutant of every plate which showed comparable growth in

complete medium and TFT containing medium, but no growth in CHAT medium were selected for further chromosomal analysis. These selected mutants of independent origin were grown to 40ml cultures, samples were frozen in liquid nitrogen, and DNA was isolated from crude cell lysates (Sambrook *et al.* 1989) to serve as template for PCR-SSCP and microsatellite analysis.

3. PCR-SSCP analysis of tk exon 4

Amplification of exon 4 sequences was done by PCR using primers 5' GGTGGTCACGACAGTGCCATTCCC 3' and 5' GCAGGAAGAGTGATGCCAAGACAAGC 3'. PCR reactions contained 1μM of each primer, 0.2mM dNTPs, 10mM Tris-HCl (pH9), 50mM KCl, 2mM MgCl$_2$, 0.1mg gelatin, 1u Taq polymerase, 100-200 ng template DNA in a volume of 50μl that was overlaid with mineral oil. The PCR performed in a Hybaid Thermal Reactor (Hook & Tucker instruments Ltd., UK) included the following steps: initial denaturation 4.5 min at 95°C, 32 cycles of 30sec. at 95°C, 50sec. at 62°C, and 1min. at 72°C, followed by a final elongation 7min. at 72°C. Amplification products were either directly used or stored at -20°C.

For the SSCP analysis 2μl of PCR-products were mixed with 2μl of deionized formamide. The mixtures were boiled at 95°C for 5 minutes, put directly on ice, and 2μl samples were loaded on a native polyacrylamide mini-gel (PhastGel gradient 8-25, Pharmacia Biotech, Dübendorf, Switzerland). Electrophoresis and silver staining were performed using the Phast System (Pharmacia Biotech) according to the vendors instructions. The gel was prerun for 20Vh at 10mA, the samples were applicated at 1mA during 2Vh, and electrophoresis was performed at 10mA for 200Vh. As contact from the electrodes to the gel DNA buffer strips (Pharmacia Biotech) were used, and all steps were carried out at 4°C.

4. Microsatellite analysis

The following microsatellites on chromosome 17q were found to be informative in TK6 cells and were used for LOH mapping: (from centromere to telomere, distances in cM from top of the chromosome 17 linkage group) D17S794 (84.2cM), D17S937 (106.9cM), D17S802 (108.2cM), D17S784 (114.0cM), and D17S928 (128.7cM). D17S1289 (26.1cM) is located on the short arm of chromosome 17 and was investigated to detect chromosome loss. The *tk* gene has been mapped between D17S937 and D17S802.

Primer sequences are listed in table 1. The reaction mix was basically as described for PCR-SSCP exept for $MgCl_2$ concentration which was 1mM for D17S784 and D17S802, 1.4 mM for D17S928 and 2 mM for the others. PCR was performed in a Gene Amp 9600 thermocycler (Perkin Elmer AG, Basel, Switzerland) using the following protocol: initial denaturation 4:30min. at 95°C, 35 cycles of 40sec. at 95°C, 30sec. at 55°C, and 2min. at 72°C, followed by a final elongation 7min. at 72°C. Informativity of the microsatellites was tested by running heat denatured PCR samples in formamide buffer on a denaturing 6% polyacrylamide sequencing gel (Sambrook *et al.* 1989). Bands on the gel were visualized by silver staining.

For LOH analysis on native polyacrylamide mini-gels (PhastGel homogenous 12.5, Pharmacia Biotech), 2µl samples were electrophoresed at 4°C and 10mA current during 100-150Vh using the PhastSystem (PhastGel homogenous 12.5,

Table 1. Primer sequences for microsatellite analysis.

Microsatellite markers	forward primer reverse primer
D17S794	5' GGCACAGTCTGCCACCTTTA 3' 5' TGAGTTGCCACAGAGTGATG 3'
D17S937	5' CATGGAGGGACTTGCG 3' 5' TTCCCAGAACCCGGTTT 3'
D17S802	5' GCCACCTGCCCCTCAA 3' 5' CTGCCAGCAGAGGCCA 3'
D17S784	5' GAGTCTCCTAAATGCTGGGG 3' 5' AGCTCCTGCACAGTTCTTAAATA 3'
D17S928	5' TAAAACGGCTACAACACATACA 3' 5' ATTTCCCCACTGGCTG 3'
D17S1289	5' TGGTCTTTTTCCATTCCAAA 3' 5' TTCAGAACTTACTGCCTCTAAGC 3'

Pharmacia Biotech), followed by silver staining.

5. FISH analysis of the tk region

The probe for FISH analysis was prepared from the human BAC clone 526-7M
(Research genetics, Huntsville, USA). DNA was isolated from a chloramphenicol-
amplified bacterial culture using the Plasmid midi kit (Qiagen, Basel, Switzerland).
Verification of the clone was done by PCR amplification of marker Bda94c06 using
primer pair 5' ACACAATAGGTCGTTGACTCC 3' and 5'
AAGCCATGAGGAGTACATGAG 3'.

Probe labelling was performed by nick translation. A labelling reaction (50µl)
contained 1µg BAC DNA, 20 µM each dATP, dGTP, dTTP, and Cy3-dCTP (orange
fluorescence dye from Amersham Pharmacia Biotech, Buckinghamshire, UK), 5 µl
enzyme mix from the BioNick™ labeling system (Life Technologies, Basel,
Switzerland). After 1 hour incubation at 16 °C, the synthesized probe was ethanol
precipitated twice in presence of 10µg sonicated salmon sperm DNA and 10µg
human Cot-1 DNA. The probe was dissolved in 10µl sterile water and stored at -
20°C in the dark. Prior to a FISH experiment, 1µl of this labelled DNA was mixed
with 10µl hybridization mix (50% formamide, 2xSSC, 10% dextran sulfate). In some
cases, 1µl of TelVysion™ DNA probe (Vysis Inc, Downers Grove, USA), a 17p
telomeric probe, labelled with a green fluorescence dye, was added as an internal
control.

To prepare metaphase spreads, cells were treated with 0.1µg/ml colcemid for 2
hours (NG mutants) or 4 hours (SG mutants). Cells were harvested by
centrifugation at 100g and resuspended in 0.075 M KCl (37°C) for 20 minutes.
After this hypotonic treatment cells were fixed three times with methanol/acetate
(3:1). Each step consisted of 10 minutes centrifugation at 100g, followed by
carefully resuspending in fixing medium and a 12 minute incubation at room
temperature. The slides were washed with sterile water and the fixed cell nuclei

were dropped onto the moist slides from a height of 10 cm (3 drops per slide). Slides were stored in a N_2 atmosphere at -20°C.

Slides were thawed at 37 °C and put in 2xSSC for 30 minutes at 37 °C prior to hybridization. The slides were then dehydrated by an ethanol series (75%, 80%, 100%, 2 minutes each) at room temperature. Denaturation of the slides was performed for 2 minutes in 50% formamide/ 4xSSC at 72 °C, followed by an ice-cold ethanol series (75%, 80%, 100%, 2 minutes each). The slides were briefly prewarmed at 37 °C.

The labelled probe was denatured at 75 °C for 5 minutes, put briefly on ice, and preincubated at 37 °C for 30 minutes. Then the probe was placed on the denatured slide, covered with a 18x18 mm coverslip, sealed with rubber cement (Ross, Taylor, USA), and incubated in a moist chamber at 37 °C overnight.

After removal of the coverslips, slides were washed for 2 minutes at 72 °C in 1xSSC/ 0.05% Tween 20®, followed by a 2 minute wash at room temperature in 2xSSC/ 0.1% Tween 20®. The slides were counterstained by applying 30µl of antifade solution including 20ng/ml DAPI (Stratagene, La Jolla, USA) and sealed by nail polish.

Metaphases were observed using an Epi-fluorescence microscope axioplan II (Carl Zeiss AG, Feldbach, Switzerland) equipped with a 103 W mercury lamp and single bandpass filters (Chroma technology corp., Brattleboro, USA) for Cy3, Spectrum Green™ (17p telomere probe), and DAPI. At least 20 metaphases showing between 0 and 2 specific signals were scored on each slide. If more than 90% of the metaphases showed 1 or 2 specific signals, the mutant was considered hemizygous or homozygous, respectively. Image capture and processing (merging of single images) was performed using the Quips PathVysion Software (Vysis Inc, Downers Grove, USA).

6. Calculation of mutant fractions

To calculate the fractions of point mutations, recombinations, and deletions individually, the results from the LOH analysis were combined with those of the

mutant fractions upon mutagen treatment as follows. The mutant fractions of the NG and SG mutants were multiplied with the percentage of mutants in the corresponding category that resulted from either point mutation, recombination, or deletion. The spontaneous mutant fractions were multiplied with the percentages of the spontaneous mutants. Similarly, the mutant fractions upon mutagen treatment were multiplied with the percentages of the mutagen induced mutants. As an example the calculation of the point mutant fraction at $0.1\mu M$ AFB_1 is shown:

$$PMF = MF_{NG} * AN_{PM} + MF_{SG} * AS_{PM}$$

PMF is the point mutant fraction at $0.1\mu M$ AFB_1.

MF_{NG} is the determined mutant fraction at $0.1\mu M$ AFB_1 for the NG mutants.

AN_{PM} is the fraction of physically determined point mutants in the AN mutant group.

MF_{SG} is the measured mutant fraction at $0.1\mu M$ AFB_1 for the SG mutants.

AS_{PM} is the fraction of point mutants in the AS mutant group.

In this approximation we did not consider the fact that a few spontaneous mutants were among the AN and AS mutants. The AN mutants were collected at an AFB_1 concentration of $0.1\mu M$ or higher. At this concentration the NG mutant fraction was elevated 30-fold over the spontaneous NG mutant fraction which means that <3.3% (=1.7/51) of the AN mutants were spontaneous. Considering the distribution of genetic events in the SN mutant group, it could be estimated that of these 1.7 spontaneous mutants 0.97 were point mutants, 0.56 were recombination mutants, and 0.17 were deletion mutants. With this information we could calculate a better estimate for AN_{PM}: Identified AN point mutants-"spontaneous point mutants" divided by all AN mutants-"spontaneous AN mutants".

$$AN_{PM} \approx (50-0.97)/(51-1.7) = 99.5\%$$

Analogous calculations were performed for recombinations and deletions, and for the AS, MN, and MS mutants.

III. Results

1. Mutant collection

In order to gain information on the mechanism of AFB_1-induced genotoxicity in human cells, the molecular nature of AFB_1-induced thymidine kinase deficient mutants was investigated. To do so, aminopterin pretreated TK6 cultures (*tk+/-*), devoid of most pre-existing mutants, were exposed to AFB_1. The cultures were split up into individual sub-cultures and grown for 3 days for phenotypic expression. At this point, the cells were plated in TFT containing medium to select for mutants lacking tk activity. An aliquot was plated to determine plating efficiency. Cytotoxicity determination by growth curve extrapolation revealed a dose dependent killing (figure 3a). From these experiments a total of 87 independent TFT-resistant mutants were collected. Mutant fractions were calculated as described (Furth *et al.* 1981) and are shown in figure 3b for NG and SG mutants. For both types of mutants, a dose dependent increase of the mutant fraction was observed. The absolute mutant fractions upon AFB_1 treatment were in the same order. However, because the spontaneous mutant fraction of NG mutants was 7-fold lower than that of SG mutants, the induction of NG mutants upon AFB_1-treatment was higher than that of SG mutants. Further analysis of the mutant fractions will be discussed below.

36 mutants were of the SG-phenotype and were called AS mutants (Aflatoxin-related SG mutants), the other 51 were named AN mutants (Aflatoxin- related NG mutants).

In parallel to these experiments with AFB_1, spontaneously occurring mutants were also isolated from cultures exposed to the solvent alone. From these solvent controls we collected a total of 21 spontaneous NG mutants (SN mutants) and 36 SG mutants (SS mutants).
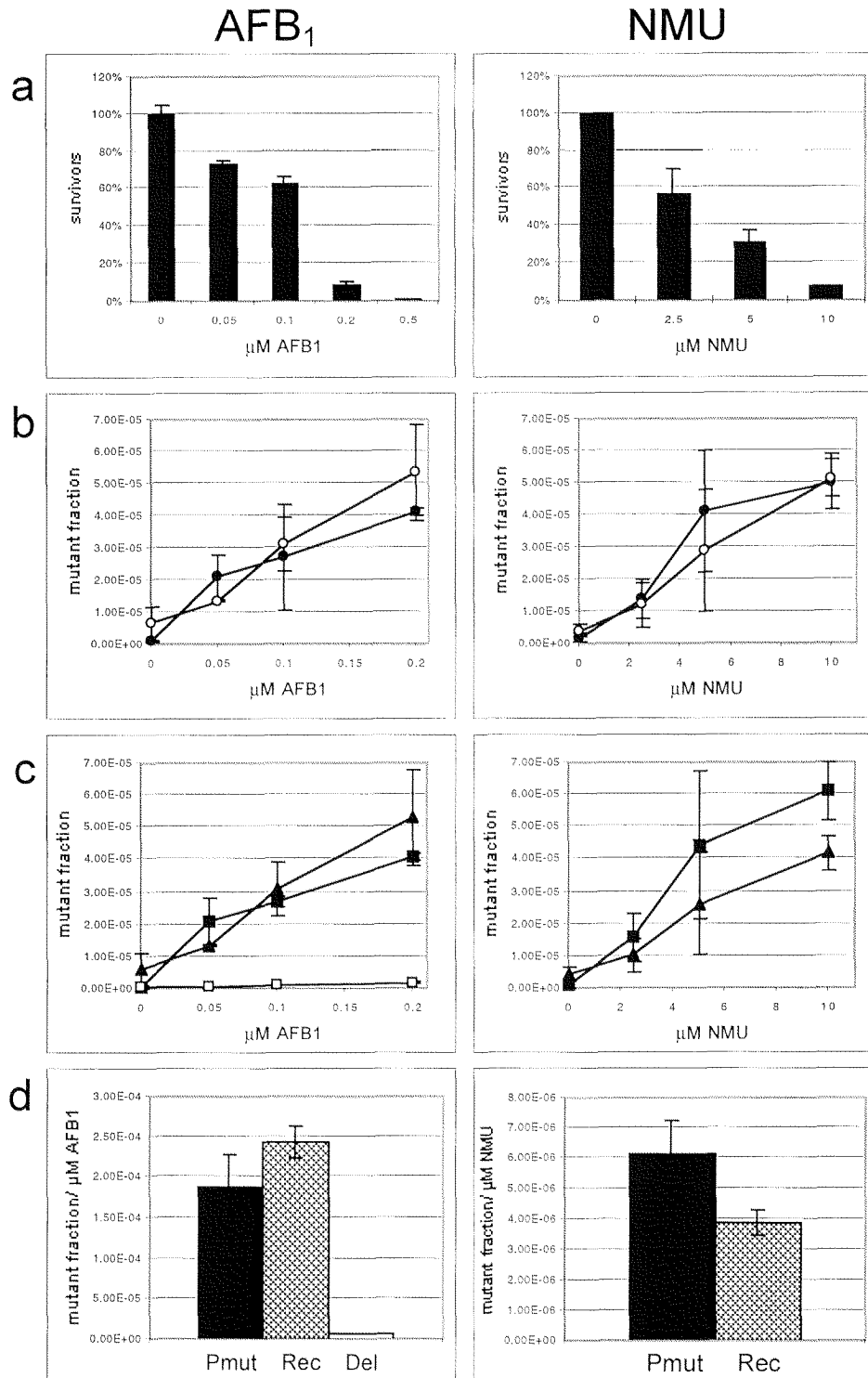
Figure 3. Mutagenesis experiments with TK6 cells exposed to AFB₁ (left) and NMU (right). Average values of at least two experiments are shown. Standard deviations are represented as error bars. **a.** Cytotoxicity upon mutagen treatment. **b.** Mutant fractions of NG mutants (filled circles) and SG mutants (open circles) upon mutagen treatment. **c.** Mutant fractions of point mutants (filled squares), recombinants (triangles), and deletion mutants (open squares) upon mutagen treatment. **d.** Mutant fractions per μM mutagen determined by linear regression of the data points of figure 1C (r ranged between 0.92 and 0.99). Shown are values for point mutants (Pmut), recombinants (Rec), and deletion mutants (Del).

Several possibilities exist for the arising of phenotypically thymidine kinase deficient mutants. Apart from a *de novo* mutation of the wild-type *tk+* allele, it was also possible that tk- mutants arose by a different mechanism, e.g. by loss of the heterozygous *tk+* allele. To determine whether individual mutants still carried two non-identical copies of *tk* alleles or whether loss of one allele had occurred, DNA of individual TFT resistant mutants was isolated and subjected to LOH analysis that focused on a genomic site within the *tk* gene on chromosome 17. This was performed by PCR-SSCP analysis taking advantage of a polymorphism present in exon 4.

2. Screening thymidine kinase deficient mutants for LOH at the tk locus

To test for the presence or absence of the *tk+* allele, the mutants were characterized by PCR-SSCP/ heteroduplex analysis. TK6 cells are heterozygous at the *tk* locus due to an inactivating one base pair insertion mutation in one allele of exon 4 (Grosovsky *et al.* 1993). We used this polymorphism to discriminate between heterozygosity and homo- or hemizygosity, respectively, at the *tk* locus. Following PCR amplification (see Materials and Methods) of a 216 bp fragment of *tk* exon 4, including the polymorphic site, the product was heat denatured and immediately put on ice, a procedure which favors the formation of characteristic secondary structures. The samples were then analyzed on a native polyacrylamide minigel (figure 4).

With this procedure, PCR products that derived from heterozygous cells produced at least four physically differently migrating species recognized as individual SSCP bands on the gel, each two of which was the result of a subtle difference in only one base pair. From a careful analysis of various mutants together with additional control experiments (not shown) it was concluded that exon 4 LOH could be diagnosed by the disappearance of one particular SSCP band, representing a secondary structure of the *tk+* allele and denoted SSCP1 in figure 4. Additionally, in heterozygous samples a heteroduplex band (denoted ds DNA) was present,

which exhibited a slightly retarded mobility compared to the homoduplex band and which served as an independent diagnostic marker. This heteroduplex band was not observed in mutants with LOH. Apart from the diagnostic bands, some other bands on the gel were present and these presumably represented either minor alternative secondary structures or PCR artifacts. The two diagnostic characteristics, i.e. the SSCP and heteroduplex bands, correlated in 140 of the 144 analyzed mutants. In the remaining four mutants the diagnostic SSCP band was absent, nevertheless a clear heteroduplex band was identified. Although the nature of these mutants was not characterized in more detail, a possible explanation was that they harbored a small lesion within the 216bp PCR product of the *tk+* allele. This would lead to a new SSCP band with simultaneous retention of the heteroduplex band. Supportive evidence for our assumption was gained from the fact that no additional LOH tracts were identified by subsequent microsatellite analyses performed on these mutants (see below).
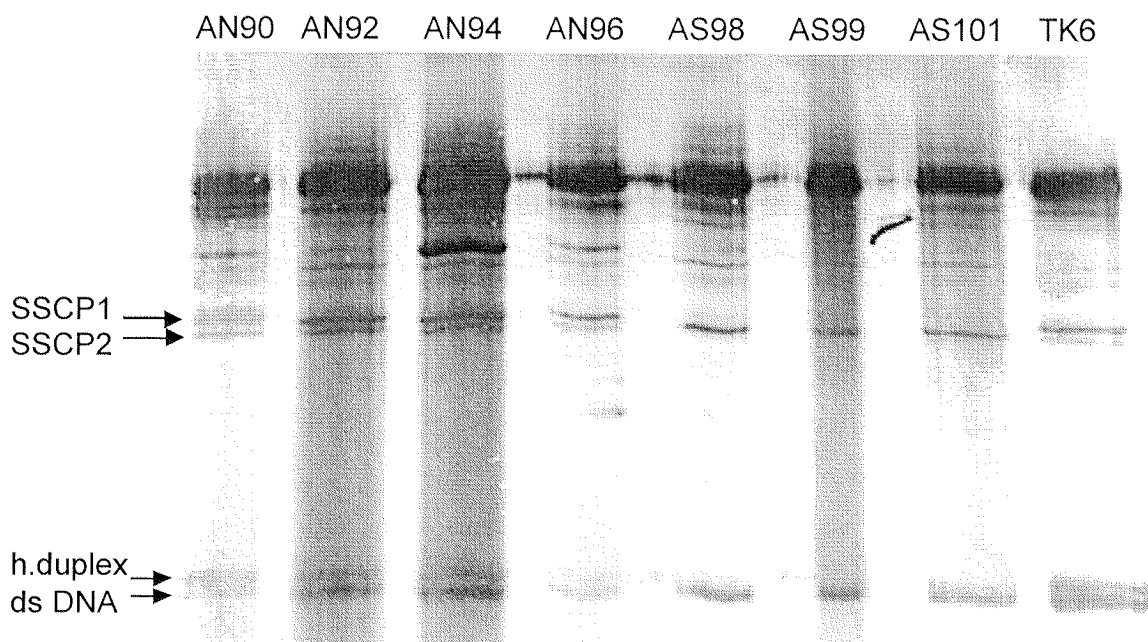

Figure 4. SSCP-/heteroduplex analysis of *tk* exon 4. DNA samples from 7 AFB$_1$-induced mutants and TK6 cells were analyzed on a 8-25% gradient Phast Gel. In heterozygous samples SSCP-band 1 was present as well as a heteroduplex DNA band (h.duplex) which ran slightly retarded compared to the double stranded (ds) DNA. In mutant samples with LOH (AS98, AS99, AS101) no SSCP-band 1 was present, however, SSCP band 2 had a stronger intensity compared to heterozygous samples. Moreover, no heteroduplex band was present in samples with LOH.

LOH at the *tk* locus strongly correlated with the SG phenotype. From AS and SS mutants only one mutant kept heterozygosity at the *tk* locus. In contrast, all but one AN mutants were heterozygous. The group of SN mutants showed more variability where about 50% exhibited LOH at the *tk* locus.

3. LOH analysis of flanking microsatellite markers

Having found that the majority of AFB_1-induced SG mutants exhibited LOH, we wondered whether this was the consequence of a recombinogenic activity exerted by AFB_1 similar to that previously identified in yeast (Sengstag *et al*. 1996). To gain more information we asked whether flanking loci on chromosome 17 suffered from simultaneous LOH or whether LOH was confined to the *tk* locus alone. To address this question several microsatellites located proximal as well as distal to the *tk* locus were investigated. Individual microsatellites were amplified by specific PCR primers (see Materials and Methods). A sample was then electrophoresed on a denaturing sequencing gel and visualized by silver staining. Figure 5a shows the result of such an analysis for two of the microsatellites, showing homo- and heterozygous mutants. The six microsatellites D17S794, D17S937, D17S802, D17S784, D17S928, and D17S1289 were informative based on the observation of two alleles of different size and were further investigated.

With the aim to analyze a large number of mutants for LOH at technical ease, efforts were made to perform the analysis on native rather than denaturing gels. For this purpose, PCR samples of the mutants were electrophoresed on native polyacrylamide gels followed by silver staining. A representative analysis is shown in figure 5b. Inspection of the band pattern revealed double stranded DNA as well as additional bands of lower mobility. The nature of the additional bands was investigated in a separate experiment which will be described elsewhere and involved the cloning of an individual microsatellite. From these experiments (not shown) it was concluded that some of the bands of lower mobility in fact represented secondary structures (SSCPs) of the microsatellite sequences, which
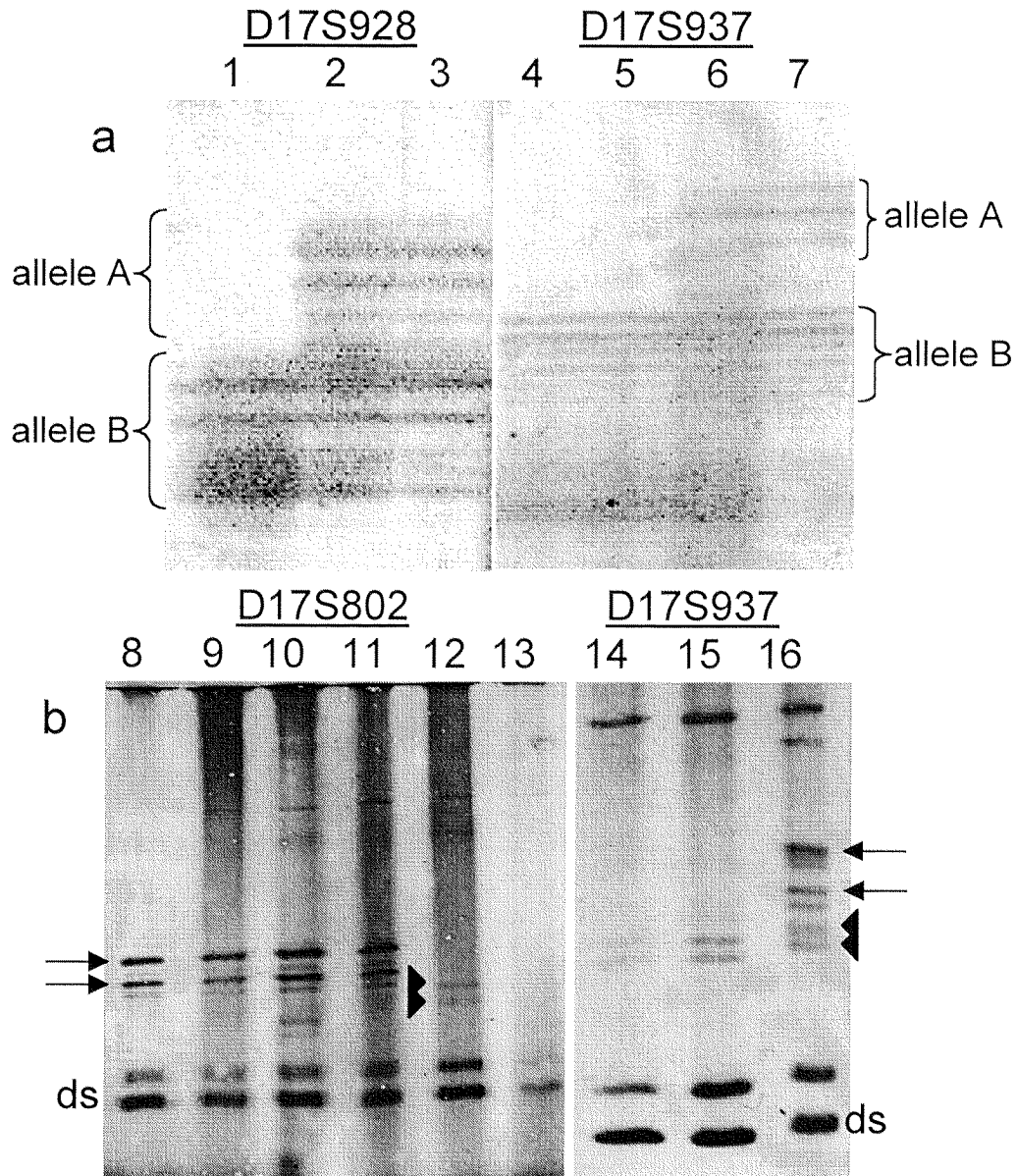
Figure 5. Microsatellite analysis of chromosome 17. **a.** Microsatellites D17S928 and D17S937 analyzed on a denaturing 6% polyacrylamide gel. Allele A was only present in heterozygous samples whereas allele B was present in all samples. Lanes 1, 4, and 5 represent mutant samples with LOH at the respective locus. **b.** Microsatellites D17S937 and D17S802 analyzed on native polyacrylamide mini-gels. Diagnostic SSCP bands which were not present in LOH mutants are indicated by arrows. Arrowheads indicate SSCP bands from the tk- allele. ds denotes double stranded DNA. Mutants 12-15 show LOH at the respective marker locus.

served us as further diagnostic tool to discriminate between LOH and heterozygosity of individual microsatellites. For each of the six microsatellites analyzed, a set of SSCP bands was identified whose presence or absence correlated with the status (LOH or heterozygous) of the respective microsatellite. In the case of microsatellite D17S928 the resolution of the gel was high enough to distinguish the two alleles as differently sized double stranded DNA molecules. However, for the other microsatellites we relied on the SSCP bands to distinguish between heterozygosity and LOH. The extent of LOH tracts deduced from the systematic LOH analysis at the *tk* locus as well as six microsatellites is depicted in figure 6. Mutants were identified retaining heterozygosity for all tested markers on chromosome 17q. Other mutants exhibited LOH for markers distal from a specific site on chromosome 17, whereas still other mutants exhibited LOH for markers flanking the *tk* locus but remained heterozygous at more distal markers. These latter mutants could have been the result of a double recombination event, alternatively they could have arisen by a deletion event eliminating internal sequences from chromosome 17. To discriminate between these two possibilities fluorescence in situ hybridization (FISH) experiments were performed on that subset of mutants which exhibited internal LOH.

4. FISH analysis of the tk region

A FISH analysis was performed on 21 mutants exhibiting internal LOH using a 140 kb probe prepared from BAC clone 526-7M (Research genetics, Huntsville, USA) which hybridizes to a region in the neighborhood of the *tk* gene. The identity of clone 526-7M was verified by demonstrating PCR amplimer Bda94c06 to be present in this clone and by co-hybridization with a 17p-telomere-specific control probe (results not shown). According to the NCBI GeneMap '98 (http://www.ncbi.nlm.nih.gov/genemap98/), Bda94c06 is located 0.71 cR3000 from the *tk* gene which corresponds to 177 kb according to Hudson et al. (Hudson *et al.* 1995).
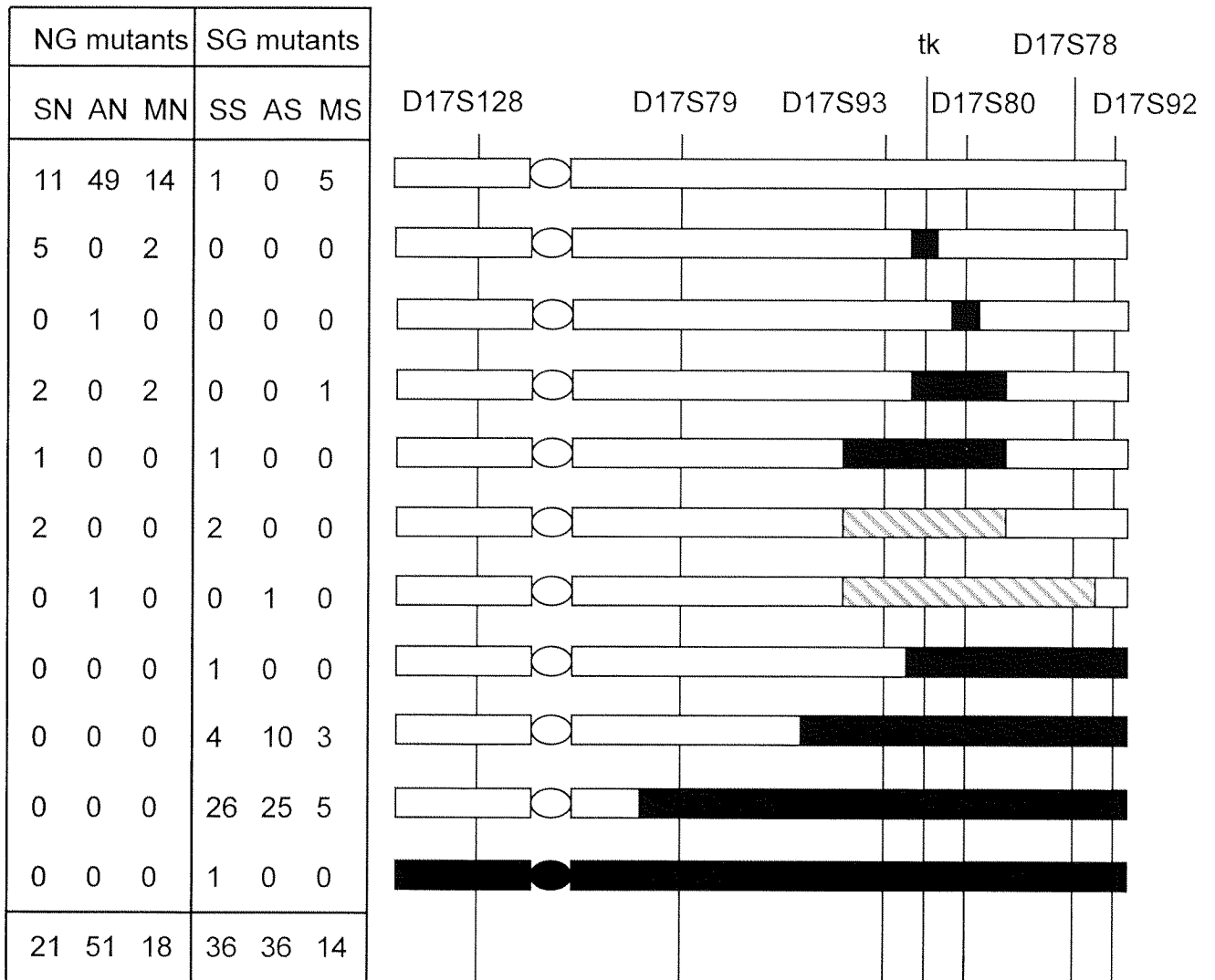
NG mutants			SG mutants		
SN	AN	MN	SS	AS	MS
11	49	14	1	0	5
5	0	2	0	0	0
0	1	0	0	0	0
2	0	2	0	0	1
1	0	0	1	0	0
2	0	0	2	0	0
0	1	0	0	1	0
0	0	0	1	0	0
0	0	0	4	10	3
0	0	0	26	25	5
0	0	0	1	0	0
21	51	18	36	36	14

Figure 6. Summary of the molecular analysis of TFT resistant mutants. Shown is a schematic picture (not to scale) of chromosome 17 with polymorphic sites that were investigated. Open boxes represent heterozygosity, black boxes represent tracts of LOH showing two alleles in FISH analysis, and striped boxes represent LOH tracts showing one allele in FISH analysis (deletions). The number of mutants of each mutant class are given at the left.

Metaphase spreads of the corresponding mutants were hybridized with this BAC probe alone, or together with the 17p-telomere control probe (figure 7). In case of a deletion mutant, only one specific hybridization signal was expected, whereas the presence of two signals would indicate the presence of two *tk* alleles and be diagnostic for LOH having occurred by a double recombination event. The limit of deletion detection depends on size of the clone and its distance from *tk*. Thus, with BAC clone 526-7M as probe only deletions larger than about 320 kb could be identified.

Deletion seemed to occur rather infrequently. Among 13 spontaneous mutants two NG and two SG mutants with deletions were detected. The two AFB_1-induced mutants with internal LOH both turned out to be deletion mutants.

Microsatellite analysis revealed one SS mutant with chromosome loss (see below). FISH analysis of this mutant showed that most of the cells were polyploid, with unstable chromosome numbers ranging from 70 to 90 chromosomes per cell, three to four of which were chromosomes 17. This suggests, that chromosome loss was possibly followed by reduplication of the remaining chromosome 17. We refrained from further investigation of this mutant, because it would not affect the outcome of this study.

5. Classification of mutants

Combining the results of *tk* exon 4 SSCP-, microsatellite-, and FISH- analysis allowed us to divide the mutants into four major classes (see below, figure 6). Mutants which showed heterozygosity at all loci investigated were classified as point mutants. This group included tk- mutants with base substitutions, small deletions, and insertions. Also mutations at other loci that could render the cells resistant to TFT would have been placed in this group. Mutants with LOH tracts and the presence of two copies of the *tk* region in the FISH analysis were considered recombinants. Mutants with LOH tracts and physical loss of one allele of the *tk* region were classified as deletion mutants. Only one mutant was detected

with LOH at all investigated loci including microsatellite D17S1289, which suggested that this mutant lost tk activity by loss of an entire chromosome 17. Our analysis revealed that the vast majority of the SG mutant population consisted of recombinants with extended LOH tracts around the *tk* gene. 32/36 (89%) SS mutants and 35/36 (97%) AS mutants fell in this category. Few deletion mutants were identified, 2/36 (6%) SS mutants, and 1/36 (3%) AS mutants. Only 1/36 (3%) SS mutants showed heterozygosity at all investigated loci and was thus classified as point mutant.

Figure 7. Metaphase FISH analysis of two LOH mutants. Chromosomes were counterstained with DAPI. Chromosomes 17 were identified using a telomere 17p specific probe, and Cy3-labelled BAC clone 526-7M hybridized near *tk* on chromosome 17q. **a**. The presence of two *tk* -specific signals suggest that LOH occurred by a recombinational event in this mutant. **b**. The presence of only one *tk* - signal suggests that LOH was due to a deletion in this mutant.
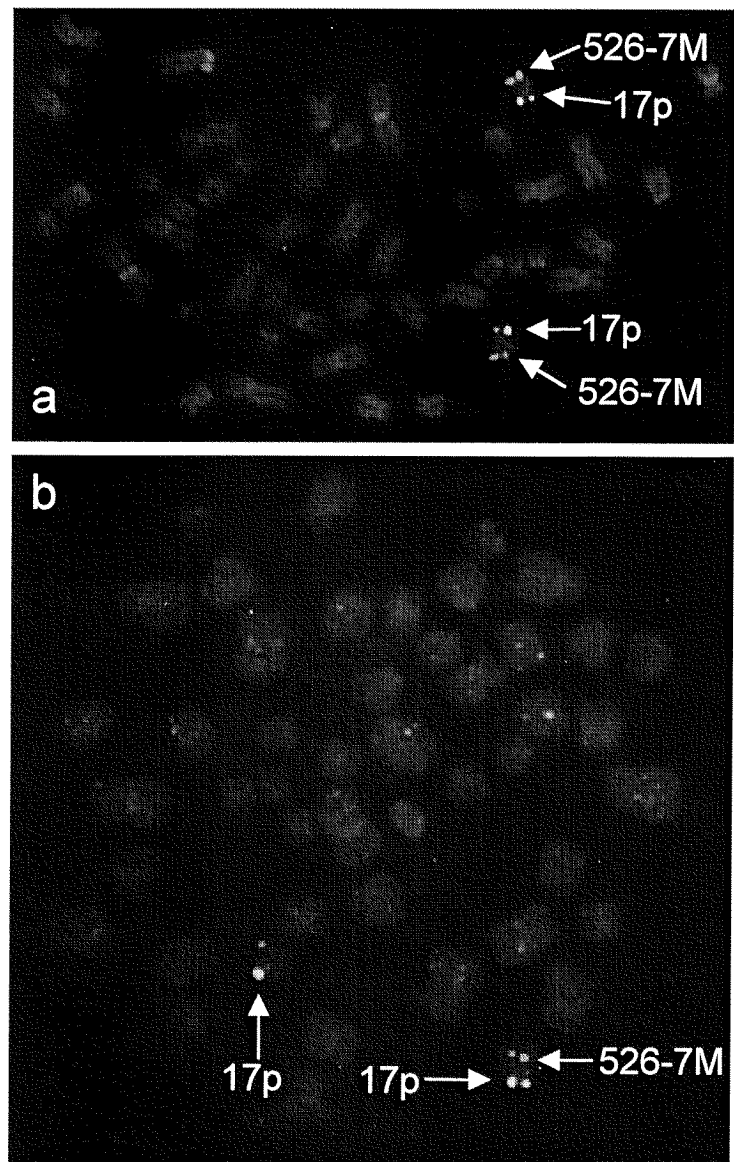
The populations of the spontaneous and the AFB_1 induced NG mutants remarkably differed. Whereas the AN mutant family consisted almost exclusively of point mutants, the SN mutants had a broader spectrum. 50/51 (98%) AN mutants were classified as point mutants, 1/51 (2%) had a deletion of the *tk* gene including surrounding markers. One AN mutant was heterozygous at the *tk* locus but showed LOH at D17S802. This mutant did not fit in any of the three categories. However, the fact that the mutant was heterozygous in *tk* exon 4 indicated that not loss of the *tk+* allele was responsible for the TFT resistance, but suggested the simultaneous occurrence of a *de novo* mutation in the *tk* gene together with a secondary event leading to the loss of microsatellite D17S802. Therefore this mutant was classified as point mutant.

From the SN mutants, 11/21 (52%) were point mutants, 2/21 (10%) were deletion mutants, and 8/21 (38%) were classified as recombinants. The LOH tracts in 5/8 SN recombinants were confined to the *tk* gene alone, three included D17S802, one included additionally D17S937. All of these 8 mutants retained heterozygosity at markers D17S784, D17S794 and D17S928, which represent the most centromeric and the most distal markers analyzed in our study.

6. Mutant fractions of AFB_1-related mutants

The physical analysis performed on individual mutants was then used to gain further information on the mutant fraction corresponding to point mutants, recombinants, and deletion mutants. The calculation of these parameters based on a combination of the NG and SG mutant fractions with the physical analysis of some mutants (for details see Materials and Methods). The calculated mutant fractions of AFB_1-related point mutants, recombinants, and deletion mutants are shown in figure 3c. Deletions occurred very infrequently; even at the highest AFB_1 dose (0.2 µM) the deletion mutant fraction was $1.7 * 10^{-6}$ which was only 3.6-fold higher than the spontaneous deletion mutant fraction. Because no mitotic recombinant was found among the AN mutants and no point mutant was identified

among AS mutants, the mutant fractions of the recombinants corresponded more or less to the mutant fractions calculated for the SG mutants, and vice versa, the point mutant fractions were about equal to the NG mutant fractions. The spontaneous mutant fraction was $6.6 * 10^{-7}$ for point mutations and $6.0 * 10^{-6}$ for recombinants. Upon treatment with 0.2 μM AFB_1, the point mutant fraction was increased by 64-fold, and the mutant fraction of recombinants by 9-fold. Thus, point mutations were more induced by AFB_1 than recombinations and deletions. However, at the highest two AFB_1 doses, the absolute mutant fraction for mitotic recombinants was higher than the mutant fractions for point mutants.

To characterize the mutagenic potency of AFB_1, with respect to the induction of the different classes of mutants, the individual induction curves were approximated by linear regression. The slope of this regression corresponded to the mutant fraction per μM AFB_1 and is shown in figure 3d. The linear increase of the mutant fraction per μM AFB_1 was higher for mitotic recombination than for point mutations, which was in stark contrast to the findings with another mutagen, NMU (see below). The potential of AFB_1 to induce deletions was comparably small.

7. Mutant fractions of NMU-related mutants

The results above suggest, that AFB_1 was able to induce mitotic recombination in the human TK6 cell line. To find out if this was a specific feature of AFB_1 or whether it was rather a general effect of mutagen exposure, we performed similar mutation experiments using NMU, a classical methylating agent. We treated TK6 cells with 0 μM, 2.5 μM, 5 μM, and 10μM NMU and performed mutation assays identically as described for AFB_1, except for the fact that no S9-mix was required with the directly acting mutagen NMU.

Cytotoxicity was determined by growth curve extrapolation. The results are shown in figure 3a. A dose dependent killing was observed.

A total of 32 mutants was collected for further molecular analysis from cultures exposed to 5 μM or 10 μM NMU. NMU-induced NG and SG mutants were named

MN and SG mutants, respectively. Molecular characterization was done by PCR-SSCP analysis, microsatellite analysis, and FISH analysis identically as described for AFB_1-induced mutants. The results are summarized in figure 6. In contrast to the spontaneous and AFB_1-induced mutants, no deletions were found among any NMU-induced mutant. In the MN mutant group point mutants (14/18, 78%) predominated over recombinants (4/18, 22%). Whereas recombinants clearly predominated among AS (97%) and SS (89%) mutants , only 9/14 (64%) MS mutants were recombinants. The other 5 (36%) were heterozygous at all loci investigated and thus were classified as point mutants.

The calculated fractions of MN and MS mutants are shown in figure 3b. The absolute mutant fractions of NG and SG mutants were in an equal range. The calculated mutant fractions for the point mutants and the recombinants clearly differed from those observed upon AFB_1 treatment (figure 3c). NMU had a stronger effect on point mutation than on recombination. The point mutations were not only stronger induced by NMU than recombinations, also the absolute mutant fractions for point mutations were higher.

Linear regression of the mutant fraction curves revealed the predominance of NMU-induced point mutations over recombination (figure 3d). The mutant fraction per μM NMU for point mutants was 50% higher than the value for mitotic recombinants. Thus, the two mutagens AFB_1 and MNU induced a different mutation spectrum in exposed human cells, the former inactivated the heterozygous *tk* allele predominantly by mitotic recombination, the latter by *de novo* mutation, a difference which may have its implication on our understanding of AFB_1-induced liver cancer in humans.

IV. Discussion

In this paper we have asked whether the powerful mutagen and liver carcinogen AFB_1 is able to induce similar recombination events in human cells as has

previously been observed in the yeast *S. cerevisiae (Sengstag et al. 1996)*. This question was addressed by exposing human TK6 cells to metabolically activated AFB_1 and, in parallel, to the classical point mutagen NMU, followed by molecular analysis of the heterozygous *tk* locus including flanking markers. Although both mutagens were found to induce point mutations within as well as LOH of the *tk* gene, our analysis revealed a fundamental difference in the frequencies at which the two genetic alterations were observed. In the case of NMU the induction of point mutations was stronger than that of LOH. At the highest dose observed, the fraction of NMU-related point mutants was 50% higher than the fraction of NMU-related LOH mutants. In contrast, at the highest two doses applied, AFB_1-exposure resulted in a higher proportion of LOH mutants than point mutants. Moreover, the molecular analysis performed on all the collected mutants strongly suggested that the majority of LOH mutants arose by a mechanism involving mitotic recombination rather than by deletion or chromosome loss. Thus, our previous observation of AFB_1 as a strong inducer of mitotic recombination in the lower eukaryote yeast was completely confirmed in the human lymphoblastoid cell line TK6.

Loss of heterozygosity represents a prominent event in carcinogenesis and may lead to the inactivation of heterozygous tumor suppressor genes. With the exception of dominant negative p53 mutants, the inactivation of tumor suppressor genes usually involves two individual steps. The first of those may represent a mutational inactivation of one tumor suppressor allele, leading to a heterozygous state, the second may then comprise recombination-mediated loss of the remaining wild-type tumor suppressor gene copy, a scenario which has been discussed in detail previously (Sengstag 1997).

Various *in vitro* studies have investigated the different mechanisms of inactivation of heterozygous genes. Studies were performed in yeast (Acuña *et al.* 1994), mouse lymphoma cells (Applegate *et al.* 1990), and human lymphoblast cell lines, focussing either at a heterozygous *URA3* gene (Acuña *et al.* 1994), at a heterozygous *APRT* gene (Fujimori *et al.* 1992; Pongsaensook *et al.* 1997), at the naturally heterozygous HLA locus (de Nooij-van Dalen *et al.* 1997), or the *tk* gene

(Li *et al.* 1992). Regardless of the different species and different gene loci investigated, the fraction of the mutants with LOH at the respective locus ranged from 70% to 85%.

Apart from deletions as one possible mechanism leading to LOH, mitotic recombinations seem to play a pivotal role, and the strongest evidence that LOH predominantly occurs by mitotic recombination in humans comes from an *in vivo* study (Gupta *et al.* 1997). These authors investigated the *APRT* locus on chromosome 16 in human T lymphocytes from the peripheral blood of *APRT* heterozygous individuals. 61/80 (76%) spontaneously arisen *APRT* deficient mutants exhibited LOH of linked microsatellite markers on 16q. Ten of these LOH mutants were further characterized by FISH using an *APRT*-specific cosmid probe and a deletion was found in only one mutant (10%). Thus, the other 90% of the LOH mutants acquired LOH by mitotic recombination, corroborating the importance of mitotic recombination for the inactivation of a heterozygous gene.

LOH is intrinsically linked to neoplastic transformation and is a common phenomenon observed in various tumor cells. For example, investigation of the tumor suppressor gene *p53* in colorectal (Baker *et al.* 1990) and breast cancers (Niederacher *et al.* 1997) revealed a high percentage (48-57%) of LOH at the p53 locus. It may thus be speculated that specific environmental factors, having the capacity to enhance the recombinational activity, could contribute to tumor development.

In the past few years there was growing evidence that AFB_1 induces mitotic recombination in specific systems. Apart from our group which has demonstrated a strong recombinogenic activity of AFB_1 in yeast (Sengstag *et al.* 1996; Sengstag and Würgler 1994), two groups reported a similar activity of AFB_1 in rodent cells. Zhang and coworkers used SP5, a mutant cell line derived from V79 Chinese hamster cells, harboring an inactivating tandem duplication in the *hprt* gene to measure intrachromosomal recombination in a reversion assay. Exposure of these cells to 0.6μM AFB_1 resulted in a 3.5-fold increase of the revertant frequency

(Zhang and Jenssen 1994). In another approach, Preisler et al. collected tk-deficient mutants from AFB$_1$ treated L5178Y mouse lymphoma cells and analyzed the mutants by LOH and FISH analysis. They suggested that at least 60% of the mutants lost tk activity by mitotic recombination (Preisler *et al.* 2000).

In the present study we asked whether we could directly demonstrate an induction of mitotic recombination by AFB$_1$ in human cells. With this aim we exposed TK6 human lymphoblasts to AFB$_1$ and collected independent TFT resistant mutants. Mutant fractions were calculated for NG and SG mutants and the mutants were subjected to LOH and FISH analysis of the *tk* gene including flanking regions. In previous studies, LOH at the *tk* locus was observed in 96% (115/120) (Yandell *et al.* 1990) and 97% (35/36) (Li *et al.* 1992) of the SS mutants. We found a similar high fraction not only among SS (35/36) but also among AS (36/36) mutants. The vast majority of these mutants acquired this loss by mitotic recombination (91% spontaneous, 97% AFB$_1$ induced). Recombination events were also a frequent cause in the generation of SN mutants (38%), whereas the AN mutants consisted almost exclusively of point mutants (50/51, 98%).

Chromosome loss and deletions occurred very infrequently in this cell line compared to point mutations and recombinations. To our knowledge, no other group performing similar experiments with TK6 reported any observation of spontaneous chromosomal non-disjunction (Amundson and Liber 1992; Li *et al.* 1992; Yandell *et al.* 1990). Our study supports this observation, since only one (spontaneous) mutant with chromosomal loss was identified. However, in contrast to the human TK6 cell line, chromosomal loss seems to occur more frequently in murine cells. In a similar study with mouse lymphoma cells, 2/41 (5%) analyzed tk-mutants had undergone chromosomal loss with duplication of the remaining chromosome 11 (Preisler *et al.* 2000). Also, chromosomal non-disjunction was reported to represent a common event for loss of a heterozygous APRT gene in a mouse cell line (Eves and Farber 1983). In our system, deletions were weakly induced by AFB$_1$ (3.6-fold at 0.2μM AFB$_1$), however the respective mutant fraction

was 30-fold lower at the same concentration compared to the mutant fraction of recombinants.

Indirect evidence for the involvement of mitotic recombination in AFB_1 related HCCs has been gained in various epidemiological studies. Although in most of these the actual exposure level was only ill-defined, HCCs isolated from patients living in AFB_1-problem areas exhibited a high extent (often more than 50%) of LOH (Fujimoto *et al.* 1994; Rashid *et al.* 1999; Scorsone *et al.* 1992). In the study of Fujimoto et al. HCCs from a AFB_1 high-risk area exhibited strikingly more LOH than HCCs from a AFB_1 low-risk area. Combining these epidemiological data with our results on the recombinogenicity of AFB_1 in TK6 cells as well as yeast (Sengstag *et al.* 1996), it may be suggested that this specific activity of AFB_1 substantially contributes to AFB_1 related liver carcinogenesis. Thus, the ability of AFB_1 to induce both, point mutations and recombinations, renders it a very powerful, complete carcinogen.

Identification of the cause of the SG phenotype would be of importance for the use of the TK6 cell line as a tool for mutagenesis studies. Because it is not known at what time after the mutagen treatment the NG phenotype turns into the SG phenotype, the resulting SG mutant fractions can only be regarded as approximations. If the phenotype would change sometime after the 3 day expression period, the calculated SG mutant fractions in this study would be correct. On the other extreme, if the phenotype would change right after the mutagen treatment, the SG mutants would be diluted during the 3 day expression period by the faster growing NG mutants. The dilution factor Q can be calculated as

$$Q = 2^{n_{NG}} / 2^{n_{SG}}$$

whereas n_{NG} is the number of generations of the NG mutants during the expression period, and n_{SG} is the number of generations of the SG mutants during the expression period. For the extreme of an immediate phenotype switch, calculating

with average doubling times of 15 hours for NG mutants and 34 hours for SG mutants, Q would be equal 6.4. In other words, the SG mutant fractions in this and previous studies using this cell line would be more than 6 times higher. This would lead to a gross underestimation of the SG mutant fractions, and thus, in our study, of the recombination frequency.

Liber et al. measured this dilution in an earlier study (Liber *et al.* 1989). They found a logarithmic decrease of the SG mutant fraction if the cells were plated 3 to 7 days after X-ray treatment. If the cells were plated earlier the SG mutant fraction was lower, because obviously significant amounts of mutants had not acquired the TFT resistance. At day 3 after irradiation the two ongoing processes, dilution of SG mutants and gain of TFT resistance, collided, resulting in a peak. However, the fact that the SG mutant dilution process was visible as a logarithmic curve after day 3, suggests that the mutants acquired the SG phenotype sometime during days 0 and 3 after exposure. Thus, the SG mutant fractions in our study were probably underestimated, and the recombination frequencies are in fact even higher than our results reveal.

A simplified model for AFB_1 related liver carcinogenesis could be proposed with AFB_1 as the initiator introducing point mutations in various genes. One of these could be the p53 gene, and mutations particularly in one specific codon (249) have been observed in a high percentage of AFB_1-related human liver tumors (reviewed in (Sengstag *et al.* 1999)). In a subsequent step, the second activity of AFB_1 could generally elevate the frequency of mitotic recombination in the affected cell. If this happens to occur during the G2 phase of the cell cycle, LOH will result (for details see (Sengstag 1997)) increasing the chance that the persisting functional copy of the tumor suppressor gene will be lost. Evidently, the frequently observed chronic infection by HBV in HCC patients would nicely fit into this model. Chronic infections are paralleled with regenerative growth of liver tissue, which in turn increases the proportion of cells present in G2 phase, the cell cycle phase where mitotic recombination events may lead to LOH. Further experiments will be necessary to provide evidence for or against this model of AFB_1-induced liver cancer.

Knowledge on the modulation of gene expression by AFB_1 in exposed cells will certainly help to understand the process in more detail, and such experiments are presently performed in our lab by applying the recently introduced gene-chip technology.

V. Additional experiments

1. Growth characteristics of TK6 cells, NG-, and SG-mutants

Growth of TK6 cells

Growth of TK6 cells under normal conditions (see materials and methods) was investigated by observation of two independent cell cultures. The cultures were seeded in 70 ml culture bottles (Falcon) in a volume of 8 ml and at a cell density of 10^4 cells/ml. During 8 days samples were taken from the culture and cell density was determined using a hemocytometer (Neubauer) (Figure 8).
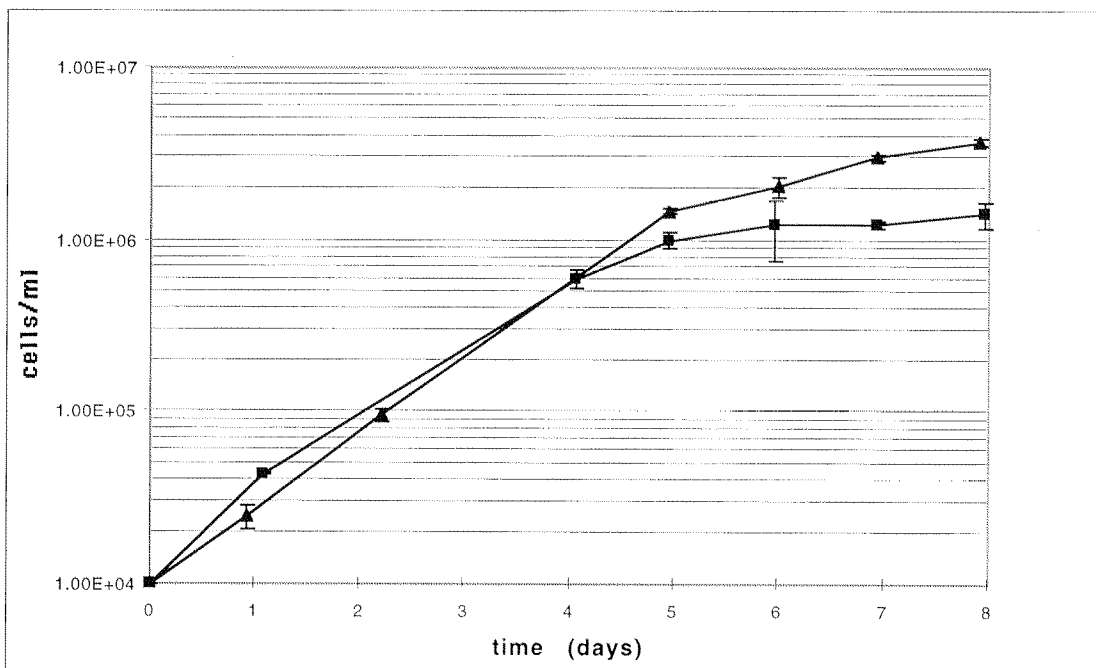
Figure 8. Growth of TK6 cells with (triangles) and without (squares) medium change. Cell density on the y-axis is plotted in a logarithmic scale.

The cells grew exponentially up to a cell density of 10^6/ml at day 5 where the growth curve started to reache a plateau. From day 5 to day 8 the cell number increased only marginally. To determine if reaching of the plateau was dependent on the cell density or simply on exhaustion of the medium, the same experiment was performed including a medium change at days 5 and 7. Again, the cells grew exponentially until day 5, and the growth curve flattened from that point. However, replacement of the medium had the effect that the plateau was reached at a higher cell density ($3.7*10^6$ cells/ml at day 8; without medium change: $1.6*10^6$ cells/ml at day 8). This suggests that exhaustion of the medium as well as high cell density limited exponential growth after 5 days of undiluted cell cultures.

Cell densities of TK6 cells in this study were kept - as also described in the literature (Furth *et al.* 1981; Liber and Thilly 1982) - in the range of 10^5 to 10^6 cells/ml which is a cell density where growth was exponential.

Population doubling times of NG- and SG-mutants

After one of the mutagenesis experiments described in "Materials and methods" some of the TFT resistant colonies were randomly selected and growth was observed during several days. Aliquots were taken at intervals of 48 to 72 hours and cell density was determined using a hemocytometer (Neubauer). Population doubling times (PDTs) were calculated using the following formula (assuming exponential growth):

$$\text{PDT}= \Delta t/(3.32*(\log(\text{cd2})-\log(\text{cd1})))$$

In this formula cd1 and cd2 are the cell densities at two different times, and Δt is the time interval between the two determinations. 75 PDTs of 5 different NG-mutants and 233 PDTs of 52 different SG-mutants were calculated this way. Figure 9 shows the relative distribution of these PDTs ranging from 11 to 59 hours. Clearly, the two distinct mutant populations can be distinguished. The majority of the NG-mutants had a PDT between 11 and 20 hours, peaking at 17 hours, which

is similar to that of the parent cell line TK6. The majority of the SG-mutants had a PDT between 30 and 40 hours, peaking at 34 hours. The distribution of the determined PDTs was similar to those previously observed by Liber et al. (Liber *et al.* 1989).

In this study, mutants with a PDT>22h were classified as SG-mutants, and mutants with a PDT<22h were classified as NG-mutants, a limit which was set arbitrarily.
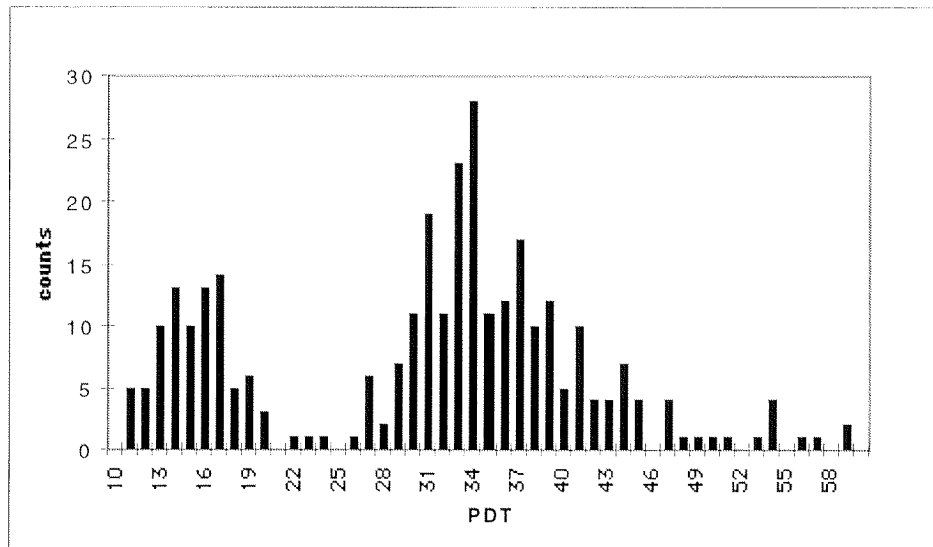

Figure 9. Population doubling times (PDTs) of 5 NG and 52 SG mutants. One count represents one PDT determination over a period of 1-3 days.

2. Cytotoxicity determined by two different methods

Cytotoxicity of AFB$_1$

To determine cytotoxicity of AFB$_1$ TK6 cultures were treated by different doses of AFB$_1$ in the presence of 5% rat liver S9 mix and a NADPH-regeneration mix (see above). Three independent cultures each, with and without S9-mix and containing DMSO (solvent) were used as negative controls. Two independent cultures each were treated with 0.05, 0.1, 0.2, and 0.5 µM AFB$_1$. After mutagen treatment the cultures were surveyed by daily cell counts during the next 3 to 6 days. The

corresponding growth curves are shown in figure 10a. It shows the average values

of counted cell densities (logarithmic scale on y-axis) at different times after

mutagen treatment (x-axis). Exponential growth would be seen as a straight line in

this graph. The growth curves flattened upon mutagen exposure or even dropped

first (at 0.5µM AFB$_1$). As reference slope for "normal exponential growth" the slope

of the culture with solvent and S9 mix was calculated by linear regression. To

determine cytotoxicity, a straight line with the reference slope was drawn through

the last determined data point. The extrapolated cell densitiy at the time of the

mutagen exposure (t=0) is an approximation for the cells in the starting culture

surviving the mutagen treatment. The survival in figure 10b shows the ratio of

these surviving cells and the initial number of cells. A dose dependent killing was

observed with only 0.6% survivors at 0.5 µM AFB$_1$. The negative control without

S9-mix had a survivor ratio of 124% relative to the negative control with S9-mix,

indicating that the S9-mix exhibited a weak cytotoxic effect.

Figure 10. Cytotoxicity of AFB1 determined by growth curve extrapolation. **a.** Growth curves after the mutagen treatment. Cultures were treated with DMSO (filled circles), with DMSO+S9-mix (filled squares), with 0.05 µM AFB1 (filled triangles), with 0.1 µM AFB1 (open triangles), with 0.2 µM AFB1 (open squares), and with 0.5 µM AFB1 (open circles). **b.** Survival compared to the control culture (DMSO+S9).
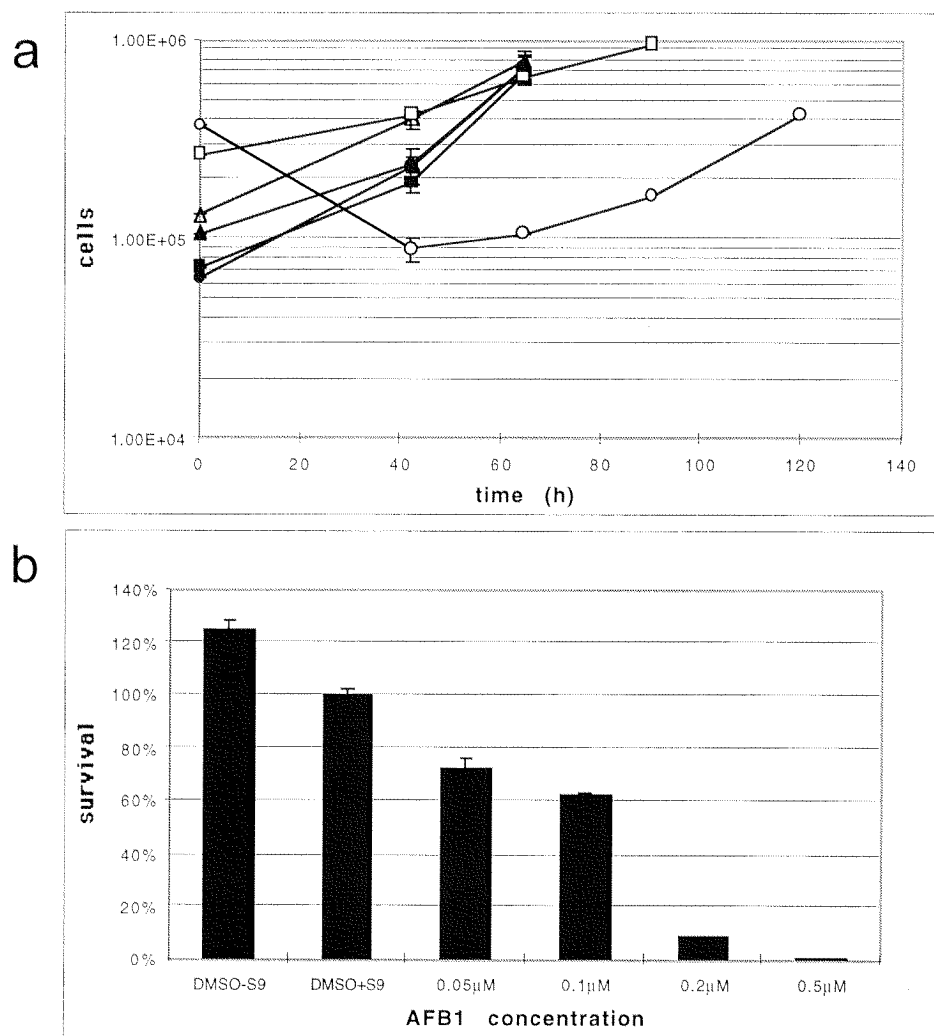
In two mutagenesis experiments using 0.1µM AFB$_1$, cytotoxicity was also determined by plating an aliquot of the cultures directly after the mutagen treatment at 2 cells/well in complete medium. The plates were scored after 28 days and the fractions of survivors were calculated by dividing the plating efficiency of the treated cultures by the plating efficiency of the untreated cultures (calculation of plating efficiencies were performed according to Furth et al. (Furth *et al.* 1981)). The survivor fractions were 44% and 61%, respectively, numbers which were in the same range as the survivor fractions calculated by growth curve extrapolation (62% at 0.1 µM AFB$_1$).

Figure 11. Cytotoxicity of NMU determined by growth curve extrapolation and by plating. **a.** Growth curves after the mutagen treatment. Cultures were treated with no NMU (filled squares), with 2.5 µM NMU (open squares), with 5 µM NMU (filled circles), and with 10 µM NMU (open circles). **b.** Survival after NMU treatment. Black boxes show survival calculated by growth curve extrapolation, white boxes represent results obtained by plating.
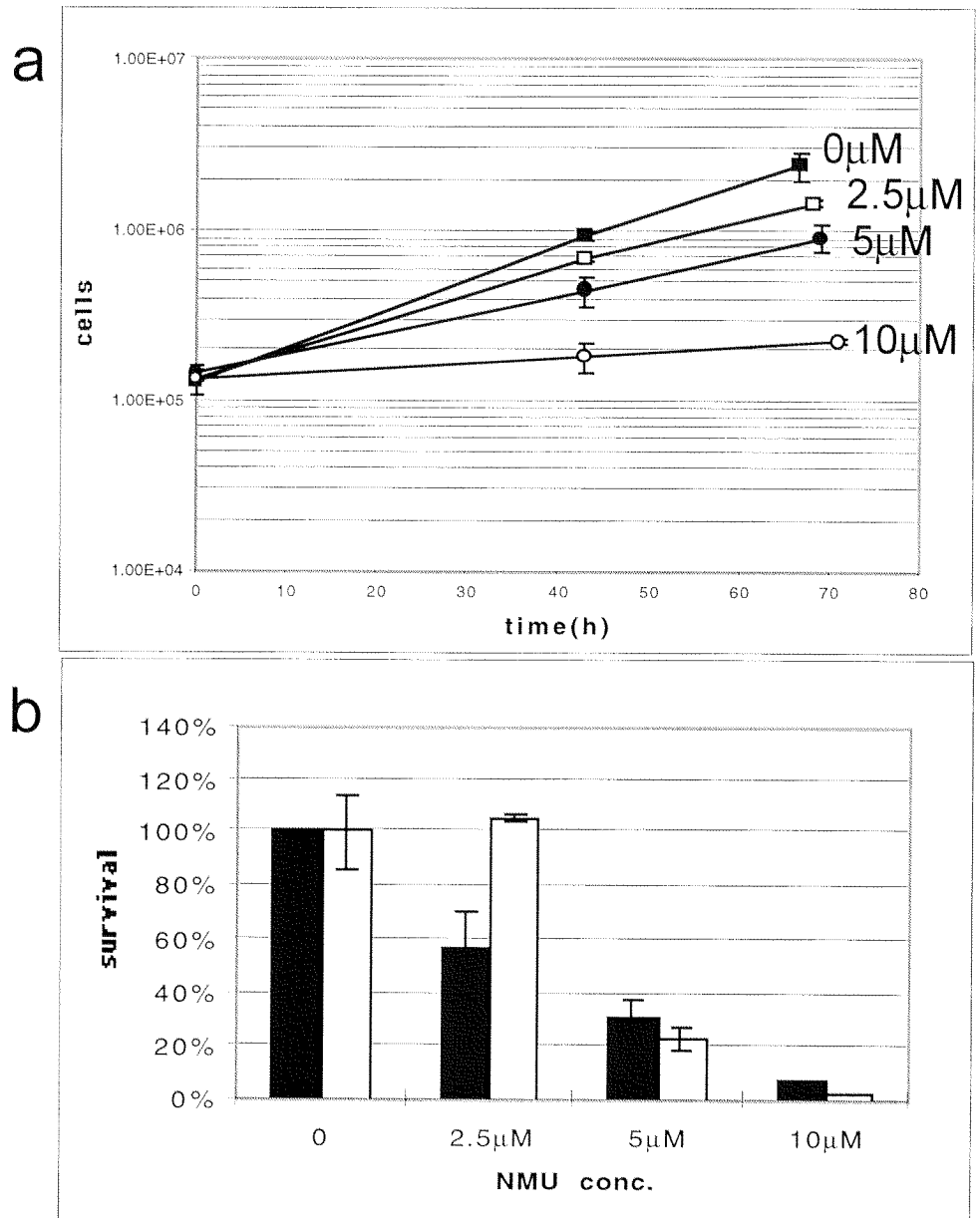
Cytotoxicity of NMU

Independent TK6 cultures were treated with 0, 2.5 µM, 5 µM, and 10 µM NMU. Cytotoxicity was determined by plating and by growth curve extrapolation as described above (figure 11). Each bar represents the average of at least two independent cultures. As for AFB_1 a dose dependent killing was observed. The obvious differences in results obtained by the two different methods could be explained by the different nature of the methods. At 2.5µM NMU growth curve extrapolation indicated a remarkable cytotoxic effect (57% survivors), whereas plating revealed none. This may result from a temporary cessation of cell division upon mutagen treatment, which allowed the cells to repair damages before they could result in mutations. This reversible cessation in growth would mimic cytotoxicity in the case of growth curve observation, even if the cells effectively survived. At higher concentrations one would therefore expect a similar outcome. However, the opposite was observed, a slightly higher cytotoxicity was seen by the plating method. An explanation for this result could be the form of the growth curves. Between day 2 and day 3 the slope of the cultures treated with 2.5 µM NMU was similar to the reference slope suggesting that the cells had resumed exponential growth. On the other hand the slopes of the cultures treated with 5 and 10 µM NMU were still lower, indicating that the cells had not resumed normal growth yet. Thus, the cytotoxic effect by growth curve extrapolation presumably had been higher if the culture would have been observed for a longer time.

For these reasons plating appears to be the better method to measure cytotoxicity, whereas growth curve extrapolation should be regarded as rough approximation.

3. Toxicity of aminopterin

The mutation assays performed in this work have been basically performed as described by Li et al. (Li *et al.* 1992). A TK6 culture was pretreated for two days with aminopterin containing medium to kill most of the cells with a tk⁻ phenotype. The mutation assays yielded tk- mutants which were rechecked for their ability to grow in complete medium, CHAT medium, and TFT containing medium. Virtually

all of the mutants stopped growing in CHAT medium, however, morphologically they often appeared normal during several days. This was especially the case with SG-mutants and we therefore wondered if CHAT treatment for two days was sufficient to kill most of the preexisting tk- mutants. To address this question the following experiment was performed.

TK6 cells, two NG-mutants (SN12, MN6), and one SG-mutant (AS82) were investigated for their ability to grow in complete medium, CHAT medium, and CH2AT medium which was CHAT medium containing twice as much aminopterin. The cells were placed in the appropriate medium for two days and morphologically normal looking cells were counted. According to this count the cultures were plated at a low cell density (2-20 cells/well) in an appropriate amount of CHT medium. The plates were scored after 28 days.
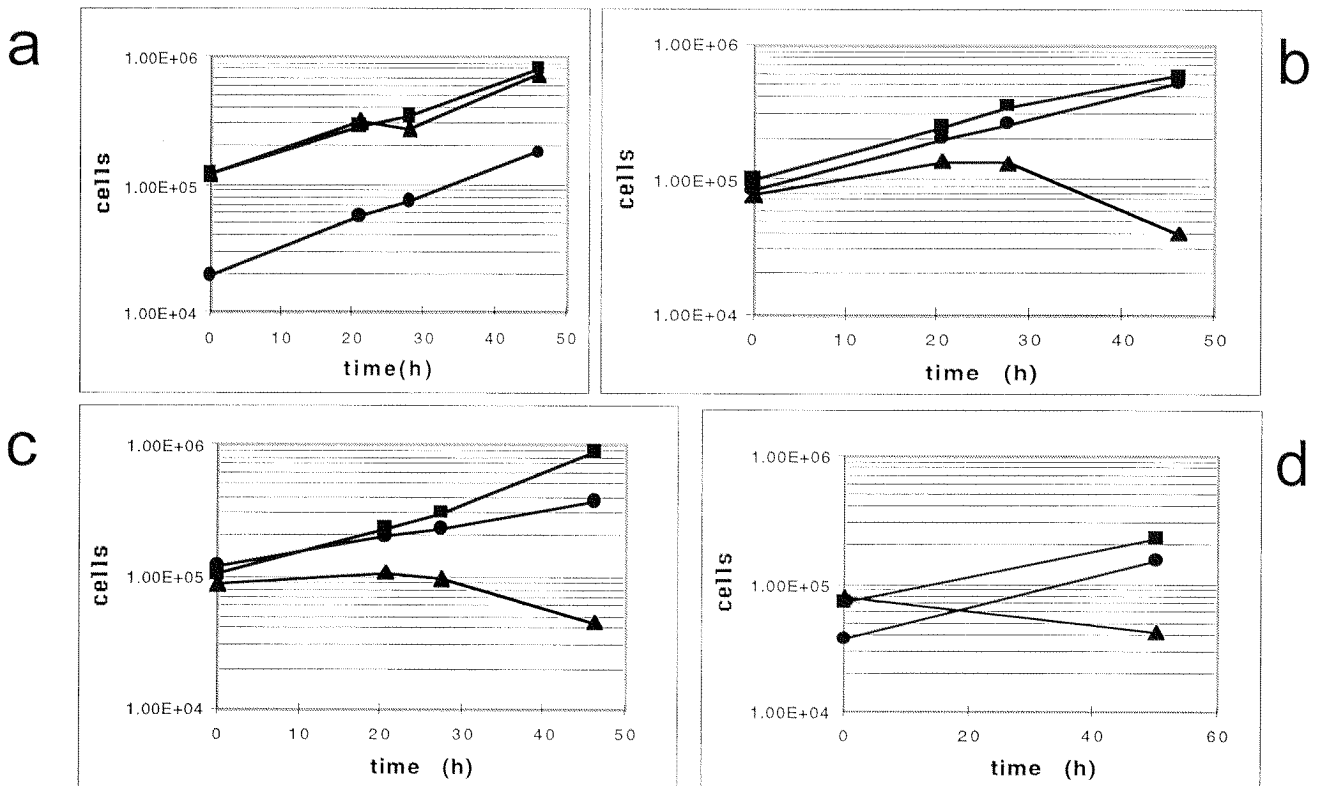

Figure 12. Growth curves after 2 days of aminopterin treatment. Squares represent untreated cultures, circles represent CHAT treated cultures, triangles represent CH2AT treated cultures. **a.** TK6 cells. **b.** NG mutant SN12. **c.** NG mutant MN6. **d.** SG mutant AS82.

To calculate a measure for the cytotoxicity of aminopterin, cell killing during the 2 day incubation period as well as cell death after plating must be considered. Some cells may have been killed during the aminopterin treatment, other cells may have looked normal after the treatment but were killed after plating. By plating of normal looking cells after the treatment, cell death on the plate was measured, however cytotoxic effects during the aminopterin treatment could not be considered by this method. Thus, cell killing during the treatment was calculated by growth curve extrapolation as described above (V.2. Cytotoxicity determined by two different methods) (figure 12). All results in this figure were single determinations. The reference slope was the slope from the culture in complete medium, and the straight line was laid through the cell count after two days. Death of the cells after plating was calculated by division of the plating efficiency of the treated cultures by the plating efficiency of the untreated culture. The calculation of plating efficiencies has been described by Furth et al. (Furth *et al.* 1981).

As value for the surviving fraction after aminopterin treatment, the surviving fractions of the growth curve extrapolation and the fractions of the survivors upon plating were multiplied. The results are summarized in figure 13. TK6 cells showed no cytotoxic effect in CHAT medium, the cells grew even somewhat better than in complete medium. A slight cytotoxic effect seemed to be apparent in CH2AT

Figure 13.
Cytotoxicity of aminopterin containing media. Black boxes represent cultures in complete medium (100%). White boxes represent survival after CHAT treatment, textured boxes represent survival after CH2AT treatment.
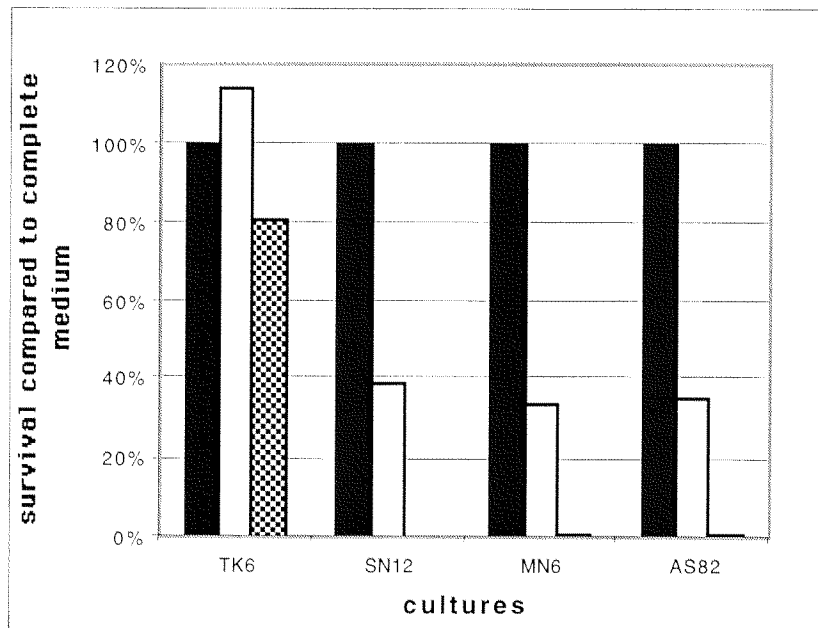
medium (81% survivors). This effect hardly can be explained by the removal of *tk*-mutants. The primary effect of aminopterin in human cells is inhibition of folic acid reductase and hence, blocking of *de novo* DNA synthesis. Cells with at least one functional tk gene should not be affected by this block. However, it would be possible that at higher concentrations of aminopterin such as in CH2AT medium, other cytotoxic effects occurred. The three investigated mutants lacking functional *tk*, showed a similar cytotoxicity towards aminopterin. The survivors in CHAT medium ranged from 34% to 39%, in CH2AT medium only 0.05% to 1% of the cells survived the treatment. This suggested that the CHAT treatment applied in the mutagenesis experiments of this work was sufficient to remove about two third of the pre-existing *tk*- mutants. However, for an efficient removal of preexisting mutants, a higher aminopterin concentration up to 0.4 µM as in CH2AT medium could be recommended.

4. Plating efficiency of SG-mutants

In the calculation of mutant fractions ((Furth *et al.* 1981), see above) a separately determined value for the plating efficiency was needed. This value was used for both, the calculation of the mutant fractions of the NG- and of the SG- mutants. However, the cells plated to determine plating efficiency mainly consisted of normally growing TK6 cells. Thus, the calculation of SG mutant fractions was based on the assumption that SG mutants had a similar plating efficiency as TK6 cells. In order to gain more insight into this problem, we determined the plating efficiency of SG mutants. For this purpose four different SG mutants were plated in complete medium at 10 cells/ml. Plating efficiencies were calculated after a 28 day incubation. The average plating efficiency for these SG mutants was 47.5±8.3%. In eight mutagenesis experiments a total of 21 independent plating efficiencies of solvent control cells were determined. The average plating efficiency was 51.5±24.7%. Thus, the plating efficiency of SG mutants was in the same range as

the plating efficiency of TK6 cells, and the plating efficiencies in the mutagenesis experiments could thus be used to calculate the SG mutant fraction.

5. Cloning of a 13kb-PCR fragment of the tk gene and usage as a probe for FISH

Cloning of a 13kb-PCR fragment

Molecular investigation of the mutants by microsatellite analysis and PCR-SSCP analysis revealed several mutants with LOH tracts at the *tk* locus. To distinguish if a large deletion or a gene conversion event led to this LOH tract, FISH analysis at this locus was performed.

To generate a *tk* specific probe for FISH, a 13.2 kb fragment of the *tk* gene was PCR amplified using the following primers:

5'GGAAACCCACACCAGACACATCC3', and

5'AAGTCCCAGCAAGGTTGGTGCC3'

The PCR reaction contained 600ng TK6 DNA (template), 300 nM primers, 500 μM dNTPs, 1/10 volume PCR-buffer 2, and 2.5 units enzyme mix (the latter two were provided in the Expand™ Long template PCR System (Boehringer, Mannheim)). Sterile H₂O was added to a final volume of 50 μl. PCR conditions were an initial denaturation at 94°C for 2 minutes, followed by 10 cycles of 10 seconds denaturation at 94°C, 30 seconds annealing at 60°C, and 10 minutes elongation at 68°C. During the following 20 cycles the elongation time was increased by 20 seconds with every cycle. After a final elongation of 7 minutes at 68°C the reaction was terminated by putting the reaction tube on ice.

The whole PCR product was electrophoresed in a 0.5% agarose gel. The DNA band at 13.2 kb was excised from the gel and purified using the QIAEX II Agarose Gel Extraction kit (Qiagen AG, Basel (Switzerland)).

It was tried to clone this 13.2 kb DNA fragment directly into vector pCR2.1 (Invitrogen, Groningen, Netherlands) by TA-cloning. The ligation product was

transformed by chemical transformation into *E.coli* DH5αF' and by electroporation into Sure™ (Stratagene, La Jolla, USA). However, none of the resulting transformants harbored a plasmid larger than 4 kb.

An alternative approach was therefore performed comprising digestion of the purified 13.2 kb PCR product by *Apa*I. The resulting digestion product was cloned into the *Apa*I site of the pBluescript II KS$^+$ (pBLKS+) (Stratagene, La Jolla, USA). For this purpose 1 µg of pBLKS+ was digested by 10 units *Apa*I during 70 minutes at 30°C (restriction enzyme and restriction buffer A by Boehringer). The product was phenol:chloroform (1:1) extracted and ethanol precipitated adding 1µg yeast tRNA as carrier. The DNA pellet was washed with 70% ethanol and resuspended in TE buffer. 200 ng *Apa*I digested pBLKS+ and 80ng *Apa*I digested 13.2 kb PCR product were ligated. The ligation was performed according to Sambrook et al. (Sambrook *et al.* 1989). The ligation product was chemically transformed in *E.coli* INVαF' cells (Invitrogen). One of the resulting transformants harbored a pBLKS+ vector with a 10.2 kb insert in the *Apa*I site. Restriction analysis, PCR analysis, and sequencing of a few hundred bp from both sides of the cloning site revealed that the insert was an *Apa*I fragment from the 13.2 kb PCR product containing genomic *tk* sequences. The clone was named pPS693 and was further used as a probe for FISH analysis of the *tk* gene.

FISH using pPS693 as probe

The following FISH experiments were performed in the laboratory of Dr. Markus Stumm, Uniklinikum Magdeburg, Germany. They were preceding the FISH experiments described above, using a BAC clone as probe.

Three probes with different labels were generated using the Nick translation kit (Boehringer). For each probe 2 µg of pPS693 DNA were labelled, one with fluorescein-12-dUTP (Stratagene), one with tetramethyl rhodamine-6-dUTP, and one with Cy3-dCTP (Pharmacia Biotech). The reaction mixes were incubated at 15°C for 90 minutes. The probes were ethanol precipitated upon addition of each 20 µg Cot-1 DNA (Life Technologies) and 20 µg salmon sperm DNA. The pellet
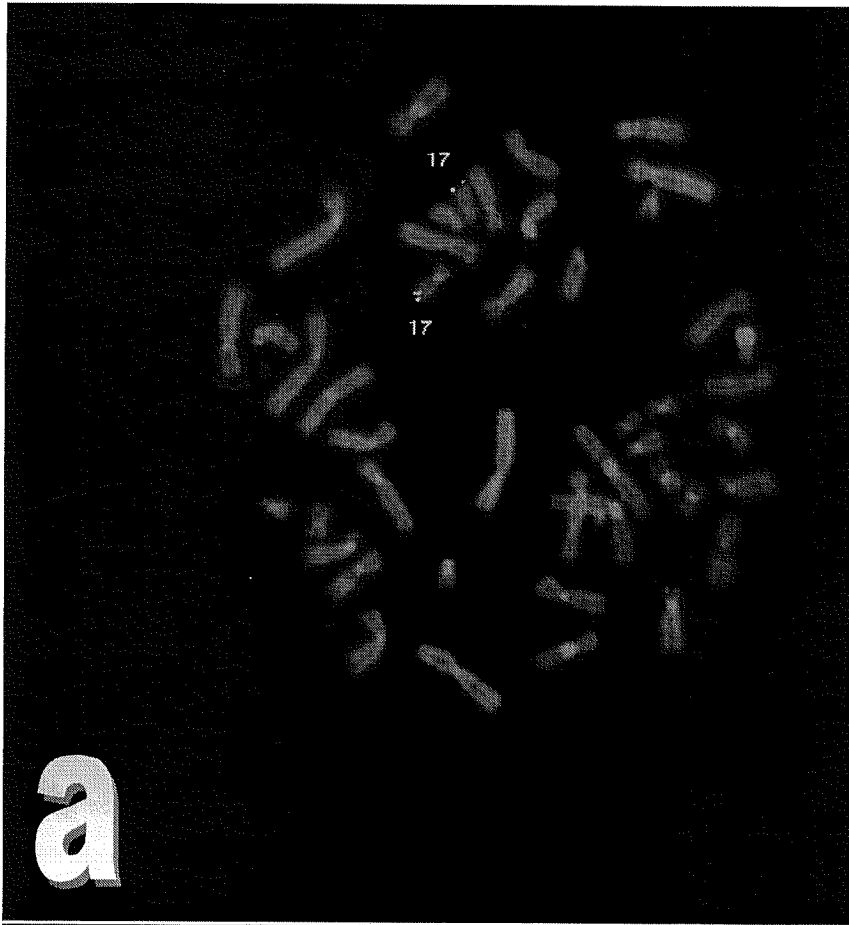
**Figure 14. a** and **b.** Metaphases of lymphocytes provided by Dr. Markus Stumm (Uni Klinikum Magdeburg) were hybridized with the *tk* specific probe pPS693. **b.** Cross hybridization to chromosomes 1, 19, and two other loci on chromosome 17 often occurred with this probe. **c.** TK6 metaphase hybridized with the SMS control probe. **d.** TK6 metaphase hybridized with pPS693. Discrimination of chromosomes 17 and 19 was not possible.

c

17

17

d

17/19

17/19

17/19

17/19

1

1
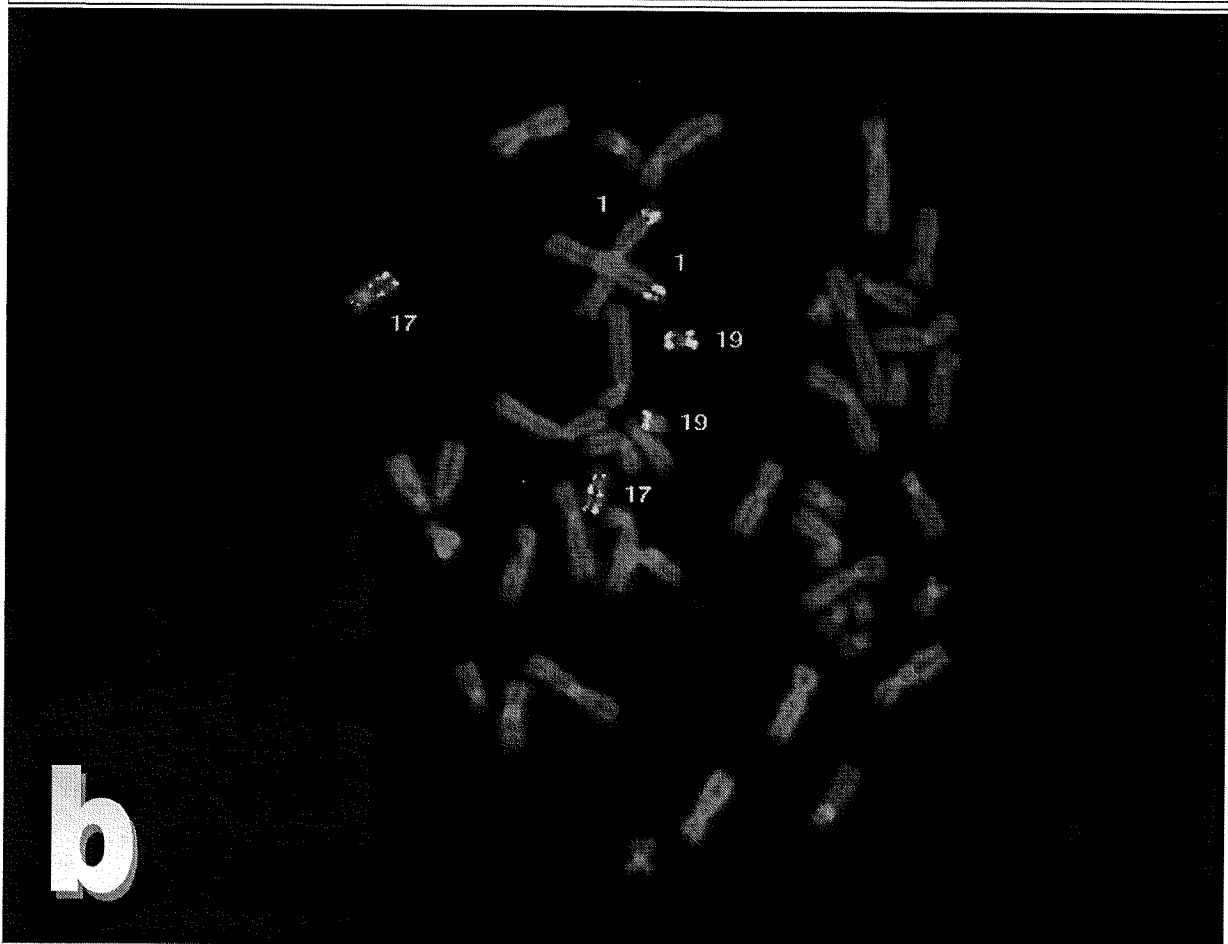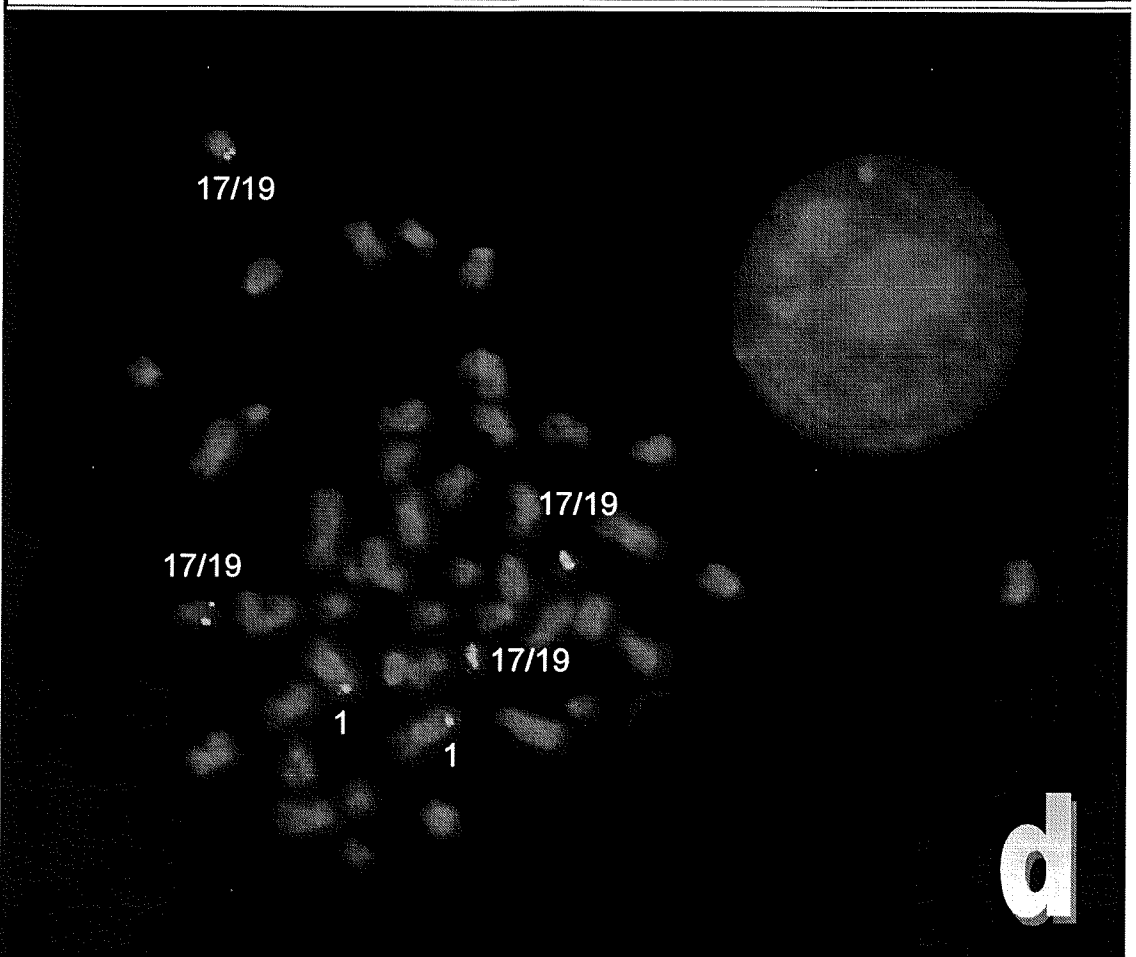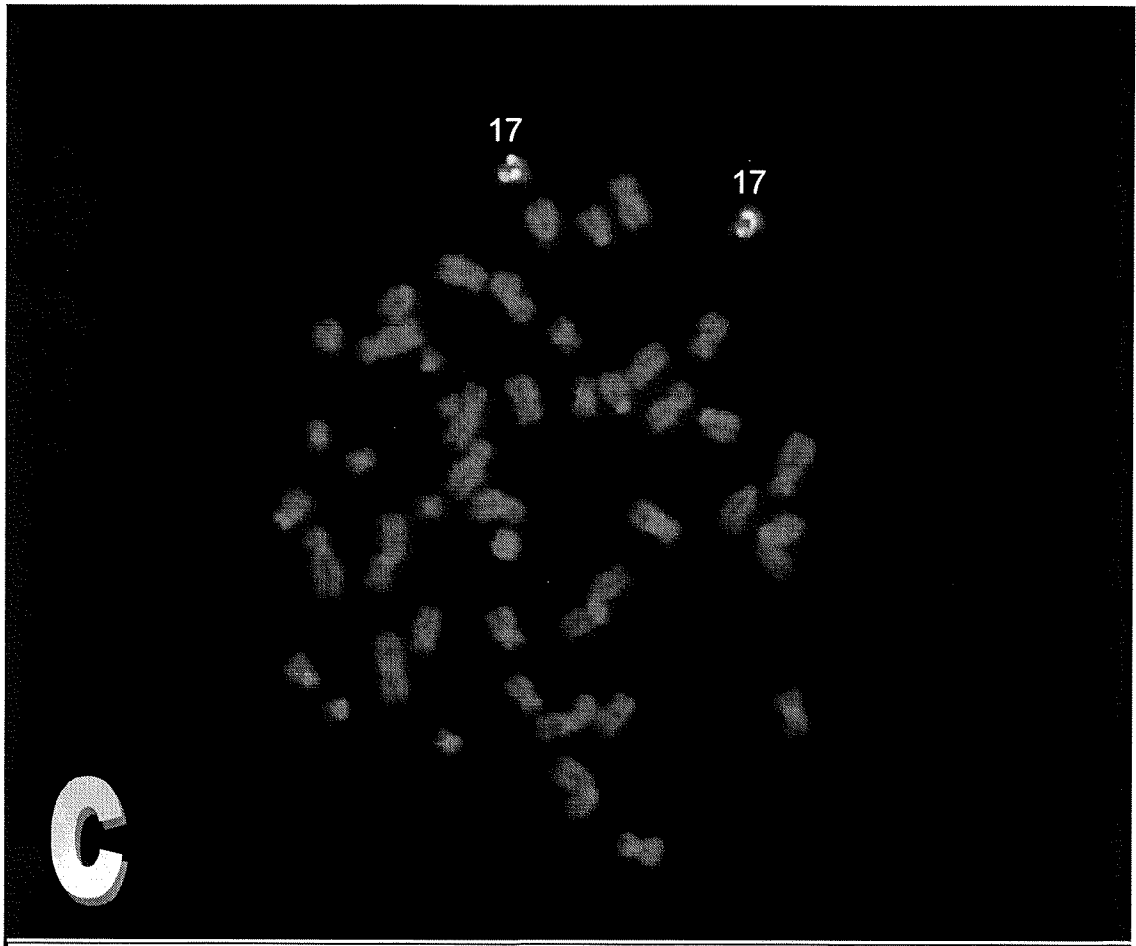
was washed with 70% ethanol and dried using a dessicator. The pellet was then resuspended in 10 μl TE and before usage as probe, 100 μl master mix (50% formamide, 20% dextran sulphate, 2XSSC) was added.

Metaphase spread preparation and FISH analysis were performed as described in materials and methods, except for the stringency wash, which was done 2 minutes at 72°C in 2XSSC, followed by a 2 minute wash at room temperature in 2XSSC/ 0.1% Nonidet P-40. The chromosomes were counterstained by Vectashield® mounting medium with DAPI (Vector Laboratories, Burlingame, USA).

The metaphase preparations used in this experiment were from TK6 cells and from lymphocytes provided by Dr. M. Stumm. As a positive control for the chromosome preparations, metaphases of a NG-mutant were hybridized with the commercially available SMS probe (Vysis Inc, Downers Grove, USA) which is known to give strong hybridization signals. The 2-colored SMS probe was designed to detect the Smith-Magenis-Syndrome and hybridizes to chromosome 17.

The results are shown in figure 14. Hybridization of pPS693-FITC and pPS693-Cy3 onTK6 metaphases resulted in hardly detectable signals which could not be distinguished from the background (results not shown). However pPS693 labelled with rhodamine resulted in weak signals which could not be seen through the microscope (magnification 100x) by eye, however electronic detection using a long enough exposure time resulted occasionally in satisfying signals (figure 14d). The same probe on lymphocytes produced also detectable signals on a few metaphases (figure 14a, b). Metaphases as in figure 14a were rare. In most of the metaphases, the signals were too weak, or cross hybridization occurred as in figure14b and 14d. Cross hybridization occurred on chromosome 1, on chromosome 19, and on two additional loci on chromosome 17. These cross hybridization signals made it very difficult to distinguish between chromosomes 17 and 19. Thus, a useful interpretation of the signals was not possible using pPS693. The cross hybridization signals might result from genes which have similar sequences as *tk*, for example from other kinases. As a consequence, FISH

experiments were performed using a BAC clone as probe, which provided stronger signals (see chapters I-IV).

The control probe SMS produced very clear and strong signals, which were detectable through the microscope by eye (figure 14c). This suggested that the quality of the metaphase preparation was sufficient to produce good signals with a large enough probe.

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