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SPONTANEOUS MUTATIONS IN A CHROMOSOMALLY-LOCATED HSV-1 THYMIDINE KINASE GENE.

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Shortened version of thesis title:

Spontaneous mutations in a chromosomally-located HSV-1 *tk* gene

Avec affection et gratitude, j'aimerais dédier ce travail à mes parents, le vent qui souffle sous mes ailes...

ABSTRACT

Spontaneous mutations occur in all genomes as the result of normal cellular mechanisms and interactions with the environment. In addition to their important contribution to genetic diversity and evolution, spontaneous mutations are associated with several genetic disorders and certain cancers. Spontaneous mutational events comprise an impressive heterogeneity of DNA alterations which can be categorized into two major classes: "moderate" and "potent" mutations. Spontaneous moderate mutations are defined here as comprising mutations with a subtle (i.e. leaky, reversible) effect on the encoded phenotype of a gene. In contrast, potent mutations can severely and permanently alter a gene's phenotype and may result in more important DNA alterations. The selective enrichment for the isolation and characterization of potent mutations can be hindered by the frequent occurrence of more moderate mutations.

A selection procedure was thus developed to enrich for spontaneous potent mutations in the KT cell line, comprising a chromosomally-integrated, single-copy plasmid pSV2*neo*KT, which contains the *neo*^{R} and the HSV-1 thymidine kinase (*tk*) genes. Spontaneous *tk*$^-$ mutants were selected on the basis of their resistance to nucleoside analogues [acyclovir (ACV), trifluorothymidine (TFT), and ganciclovir (DHPG)]. In order to enrich for potent mutations bearing severe consequences for the function of the *tk* gene, over the "background" of more simple moderate mutations, an enrichment procedure was established and verified. Combinations of nucleoside analogues reduced the apparent mutation frequency in the *tk* gene and allowed the enrichment for potent mutations with a severe impact on the TK phenotype. The different potencies of the compounds and

their potential synergisms during the selection for *tk*⁻ mutants is also discussed.

Mutants resistant to ACV+TFT+DHPG demonstrated a stable TK⁻ phenotype, as they did not revert to wild type at a detectable frequency, and the severe effect on gene function was exemplified by the absence of a TK protein, discernible by Western blotting, in >85% of these multiple drug resistant mutants. In contrast, the moderate *tk*⁻ mutants resistant to TFT only, demonstrated phenotype reversal at a high frequency and a detectable TK polypeptide. Between 14.5% and 25% of the potent *tk*⁻ mutants resistant to combinations of drugs underwent a major DNA alteration, consisting mostly of partial, or complete, deletions of the *tk* gene. Complex rearrangements of the *tk* gene were also obtained, and an intragenic deletion/duplication event is presented and characterized. However, it was observed that more than 75% of the spontaneous potent mutational events implicated less than 5 bp of the *tk* gene, yet presented a null phenotype of stable complete alteration of the TK phenotype. The level of mutated *tk* transcripts was affected in 64% to 70% of the small potent *tk*⁻ mutants, as demonstrated by Northern blotting. Extensive DNA methylation and multidrug resistance (MDR) seemed not to be implicated in the generation of the TK⁻ phenotype observed in the small potent *tk*⁻ mutants.

Thus, the KT cell line, combined with the use of multiple nucleoside analogues, allows the enrichment for, and the characterization of, potent (and infrequent) spontaneous mutations in a human chromosomal context. The mutational events characterized in this study may then reflect the diversity, the complexity, and the dynamics of the human genome.

RÉSUMÉ

Les mutations spontanées se produisent dans tout génome, en réponse à des mécanismes cellulaires normaux et diverses interactions avec l'environnement. En plus de leur contribution à la diversité génétique et à l'évolution, les mutations spontanées sont associées à diverses maladies génétiques et certains cancers. Les mutations spontanées comprennent une variété d'altérations de l'ADN qui peuvent être catégorisées en deux classes principales: les mutations dites "modérées" et dites "fortes". Les mutations modérées, telles que définies dans ce travail, comprennent des mutations qui ont un effet subtil (reversible) sur le phénotype associé à un gène. Par contre, les mutations fortes peuvent altérer le phénotype de facon sévère et permanente, et résultent souvent d'altérations importantes au niveau de l'ADN.

Un protocole de sélection fût développé afin d'enrichir pour les mutations fortes spontanées dans la lignée cellulaire KT, qui comprend le plasmide pSV2*neo*KT intégré en une seule copie dans un des chromosomes. Ce plasmide possède le gène de résistance à la néomycine (*neo*^{R}), ainsi que le gène de la thymidine kinase (*tk*) de HSV-1. Les mutants spontanés *tk*$^-$ furent sélectionnés par leur résistance à des analogues de nucléosides [acyclovir (ACV), trifluorothymidine (TFT), et ganciclovir (DHPG)]. Afin d'enrichir pour des mutations fortes, par rapport au bruit de fond causé par les mutations modérées plus fréquentes, un protocol sélectif fût établi, puis vérifié. En effet, les analogues de nucléosides utilisés en combinaison réduirent la fréquence apparente de mutations dans le gène *tk* et permirent l'enrichissement pour des mutations fortes démontrant un impact considérable sur le phénotype TK. L'efficacité variable des

différents composés, et leur synergisme potentiel lors du protocol sélectifs pour les mutants *tk*$^-$, est aussi discuté dans ce travail. Les mutants résistants à ACV+TFT+DHPG démontrèrent un phénotype TK$^-$ stable, puisqu'ils ne retrournaient pas au type sauvage à une fréquence détectable, et la sévérité de leur effet sur la fonction du gène *tk* fut démontrée par l'absence de protéine TK, par analyses de Western, chez plus de 85% de ces mutants. Par contre, les mutants *tk*$^-$ modérés et résistants seulement à TFT purent retourner au type sauvage et démontrèrent la présence d'une protéine TK. Entre 14.5% et 25% des mutations fortes *tk*$^-$ résistantes à des combinaisons de composés subirent une altération majeure de l'ADN, consistant surtout en des délétions, partielles ou totales, du gène *tk*. Des réarrangements complexes du gène *tk* furent aussi obtenus, et une déletion/duplication intragénique est présentée et caractérisée. Toutefois, il fût noté que plus de 75% des mutations fortes spontanées impliquaient moins de 5 pb du gène *tk*, alors qu'elles présentaient néanmoins un phénotype "nul" d'altération complète du phénotype TK. Le taux d'ARN messager était toutefois affecté dans plus de 64 à 70% des petites (< 5 pb) mutations fortes, tel que démontré par des analyses de buvardage de Northern. La méthylation massive de l'ADN et un phénotype attribuable au phénomène de résistance multiple à des composés chimiques ("multidrug resistance", MDR), ne semblèrent pas impliqué dans la génération du phénotype TK$^-$ associé aux petites mutations fortes.

Ainsi, la lignée cellulaire KT, et l'utilisation de plusieurs analogues de nucléosides, permettent d'enrichir et de caractériser les mutations spontanées fortes dans un contexte chromosomique humain. Les mutations caractérisées au cours de cette étude peuvent ainsi refléter la diversité, la complexité, et la dynamique du génome humain.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. I developed an enrichment procedure which utilizes the nucleoside analogues acyclovir (ACV), trifluorothymidine (TFT), and ganciclovir (DHPG) in a combined fashion, in order to select for spontaneous potent mutations in a single-copy, chromosomally-integrated HSV-1 thymidine kinase (*tk*) gene. I selected and isolated ninety *tk*$^-$ mutants resistant to various combinations of nucleoside analogues.

2. I showed that ACV, TFT, and DHPG displayed different potencies for the selection of *tk*$^-$ mutations in the HSV-1 *tk* gene from the KT cell line, and that the drugs may demonstrate synergisms or antagonisms during the selection for *tk*$^-$ mutants in the KT cell line.

3. I categorized the *tk*$^-$ mutants isolated into two main classes. Those mutants resistant to a single nucleoside analogue (TFT) represent moderate mutations which demonstrate a detectable TK polypeptide and a revertible mutant phenotype. The mutants selected on the basis of their resistance to combinations of two (ACV+TFT) or three (ACV+TFT+DHPG) nucleoside analogues occur with a reduced frequency and constitute potent mutations in which a TK protein was not discernible, and the phenotype reversion to wild type was not discernible.

4. I demonstrated that less than 12% of the potent *tk*$^-$ mutants produced a TK polypeptide which could be detected by Western blotting analyses.

5. I showed that 14.5% to 25% of the spontaneous potent mutations underwent a major modification of the *tk* gene which involved more than 10-50 bp.

6. I demonstrated that the major class of large DNA alterations leading to spontaneous

potent *tk*$^-$ mutations in the KT cell line consisted of deletions involving either part of the HSV-1 *tk* gene, or the entire loss of the *tk* gene.

7. I also described a more complex rearrangement of the *tk* gene: an intragenic deletion/duplication event in the HSV-1 *tk* gene from potent *tk*$^-$ mutant ATD-1-19. I proposed two non-homologous (illegitimate) recombination models to account for the generation of the unusual deletion/duplication event observed in *tk*$^-$ mutant ATD-1-19.

8. I determined, using a fine-structure analysis, that a large proportion (> 75%) of the spontaneous potent mutations resulted from small DNA modifications implicating less than 5 bp of the *tk* gene.

9. I showed that the amount of mRNA transcript detected by Northern blotting analyses was altered in 64-70% of the small potent mutants, which suggests that transcription of the *tk* gene may be affected in these mutants.

10. I determined that, in contrast to previous studies on spontaneous mammalian mutagenesis, DNA methylation was not extensively involved in the generation of the potent *tk*$^-$ mutants in the KT cell line.

11. I demonstrated that resistance to a combination of nucleoside analogues in the KT cell line does not appear to involve the efflux of the drugs through a P-glycoprotein (multidrug resistance, MDR) phenotype.

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LIST OF ABBREVIATIONS

ACV:	acyclovir, 9-[(2-hydroethoxy)methyl]guanine
ACV-TP:	acyclovir triphosphate
aprt:	adenine phosphoribosyl transferase gene
bp:	base pairs
CMV:	cytomegalovirus
dATP:	deoxyadenosine triphosphate
dCTP:	deoxycytidine triphosphate
dGTP:	deoxyguanosine triphosphate
dhfr:	dihydrofolate reductase gene
DHPG:	ganciclovir, 9-[(1,3-dihydroxy-2-propoxy)methyl]-guanine
DHPG-TP:	ganciclovir triphosphate
DMEM:	Dulbecco's modified Eagle medium
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
dNTP:	deoxyribonucleotide triphosphate
dsDNA:	double-stranded deoxyribonucleic acid
dThd:	thymidine
EDTA:	ethylene diaminetetraacetic acid
HAT:	hypoxanthine/aminopterin/thymidine
HIV:	Human immunodeficiency virus
hprt:	hypoxanthine phosphoribosyl transferase gene
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
kb:	kilobase pairs
kDa:	kilodaltons
MDR:	multidrug resistance
MW:	molecular weight
neo:	neomycin resistance gene
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate bufferred saline
PCR:	polymerase chain reaction
RNA:	ribonucleic acid
SDS:	sodium dodecyl sulfate
ssDNA:	single-stranded deoxyribonucleic acid
TFT:	trifluorothymidine, trifluridine, 5-trifluoromethyl-2'-deoxyuridine
TFT-TP:	trifluorothymidine triphosphate
tk:	thymidine kinase gene
TK:	thymidine kinase enzyme
TTP:	thymidine triphosphate

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CHAPTER 1

GENERAL INTRODUCTION

I. THE NATURE OF SPONTANEOUS MUTATIONS

The genetic apparatus of any organism does not constitute a stable and static entity. All cellular genomes can spontaneously undergo a variety of DNA alterations as a consequence of normal cellular processes and purely random interactions with the environment. Spontaneous mutations represent crucial events for genetic evolution and genotypic diversity, but are also associated with the generation of diverse genetic diseases (Caskey, 1987), including Duchenne muscular dystrophy (Forrest *et al.*, 1987), sickle cell anemia (Caskey, 1987), cystic fibrosis (Tsui, 1992), and Huntington's disease (The Huntington's Disease Collaborative Research Group, 1993), to name a few. Certain forms of cancer, such as the eye cancer retinoblastoma (Cavenee *et al.*, 1983), and several types of leukemias, represent another unfortunate outcome of spontaneous mutations (Knudson, 1986; Rudiger, 1990; Standbridge, 1990).

Spontaneous mutations can result in an impressive heterogeneity of DNA alterations, ranging from simple DNA modifications, including base substitutions and frameshifts, to complex rearrangements such as large deletions or insertions, and chromosomal aberrations. Spontaneous mutagenesis represents a fascinating, but vast (and intricate) topic. This chapter will thus be devoted to the study of spontaneous mutations in eucaryotes, the cellular processes involved, and the various resulting molecular events.

I.1. DISTINCTION BETWEEN SPONTANEOUS AND INDUCED MUTATIONS

Because of their critical significance for mammalian cell mutagenesis, studies which focus strictly on the spontaneous aspect of mutations are very important. The concept of spontaneous mutations has evolved considerably since Hugo De Vries introduced the term "mutations" to describe permanent variant characteristics that arose in discrete steps (Allen, 1968). Luria and Delbrück provided, in 1943, the first convincing evidence that mutations could occur spontaneously in nature. Their elegant fluctuation test demonstrated that bacterial cells acquired a mutation conferring resistance to lysis by bacteriophage T1 prior to the addition of the phage, resulting in a "fluctuation" in the number of resistant cells, when cell populations were initially low. Spontaneous mutations can thus be defined as mutational events that occur without the influence of any external agents, and it rapidly became evident that eucaryotic organisms could be subjected to similar hereditary variations. The spontaneous inactivation of the function of a eucaryotic gene by genetic mutations occurs at a rate of approximately 10^{-6}-10^{-8} events per locus, per generation, but this rate varies considerably between genes, loci, cell types, individuals and species (Drake, 1992).

Alternatively, evidence accumulated showing that various external agents, referred to as mutagens, can cause mutations. Early studies by Muller (1927) showed that the number of mutations in *Drosophila* was increased by 15,000% upon treatment of the germ cells with X-rays. Demerec (1946) later provided direct evidence that mutations can also be induced by mutagens by demonstrating that heritable changes in bacteria could be provoked by X-rays and UV light. Characteristically, mutagens considerably increase

the rate at which mutations arise in a genome and comprise various agents, including numerous chemical compounds, X-rays, and UV light. Typically, a particular spectrum of mutations is generated by a given mutagen, and this spectrum differs from the spectrum of spontaneous mutations. Proflavine, a chemical mutagen which specifically causes frameshift mutations, and UV irradiation (254 nm), which induces the formation of cyclobutyl dimers between adjacent thymidines, represent two examples of the specificity of mutagens.

The fluctuation test of Luria and Delbrück (1943) and the impressive work on spontaneous mutations that it encouraged, did not completely eliminate speculations proposing that genetic variants may not always occur spontaneously, but can also represent a direct adaptive response to the environment. The potential contribution of "directed" mutations to genetic variability recently gained credibility with the work of Cairns *et al.* (1988) and Hall (1991). These two independent groups utilized bacterial systems to suggest that mutations can be "directed" to occur and be expressed when the outcome of such a mutation conveys a selective advantage for the microorganism. Different models have been proposed to account for such "directed" mutations. Cairns and co-workers (1988) suggested a reverse flow of information from proteins that sense the requirement for a particular mutation, back to the DNA sequence *per se*, in order to "direct" and select the proper mutation (Foster and Cairns, 1992). Recently, Hall (1993) reconsidered his original suggestion that a microorganism which requires a beneficial mutation in order to survive enters a "hypermutable" state (Hall, 1991). More evidence is thus required to support the above models, or others, like the "transcription-induced

mutations" theory of Davis (1989). Hence, although fascinating, this considerable challenge to the concept of spontaneous mutations has been strongly debated (Lenski *et al.*, 1989) and remains controversial.

I.2. CLASSIFICATION OF SPONTANEOUS MUTATIONS

Spontaneous mutations comprise a variety of different alterations of the DNA, and their consequence on the function of a gene also differs greatly. Consequently, an accurate description of spontaneous mutational events in mammalian cells necessitates a classification of mutations. However, distinguishing between a plethora of different types of spontaneous mutations proves very difficult, both conceptually and technically.

I.2.1. POINT MUTATIONS, CHROMOSOMAL MUTATIONS, AND GENOME MUTATIONS

DNA alterations can be separated on the basis of the structure of the mutational lesions, according to whether they produce small or large modifications of the DNA molecule. This molecular and structural description is widely employed to categorize spontaneous mutations under three broad classes: 1. point mutations (affecting small regions of DNA, such as base substitutions, frameshift mutations, and small deletions), 2. chromosomal mutations (involving large regions of chromosomes, such as inversions and duplications), and 3. genome mutations (altering the number of chromosomes). However, this separation of mutations fails to make important distinctions between certain types of events. For example, this classification would not discriminate between small

deletions, and frameshift mutations, both classified as point mutations. The incapacity of small deletions to revert to the wild type genotype, while frameshift mutations may revert more frequently, constitute an important characteristic since the stability of mutations can be crucially important.

I.2.2. LEAKY POINT MUTATIONS AND NULL MUTATIONS

Mutations are also occasionally differentiated on the basis of their outcome on the function of the gene. Point mutations may have only a subtle effect on gene function and are often referred to as "leaky" mutations, when the sequence modification can allow some residual activity of the gene product. In contrast, mutations which completely abolish or greatly reduce gene function are called "null" mutations. Again, a certain amount of overlap connects the two classes. Indeed, null mutations can include small point mutations, since a nonsense mutation, for example, could completely abolish the function of a gene. However, point mutations can be distinguished from null mutations on the basis of their ability to revert to the wild type genotype. In contrast, null mutations frequently consist of more extensive alterations of the DNA, such as large deletions, the insertion of a transposable element, and even chromosomal alterations (eg. non-disjunctions, translocations, etc.) and the complex nature of the mutations prevents genotype (and phenotype) reversal to wild type.

I.2.3. POTENT MUTATIONS AND MODERATE MUTATIONS

Alternatively, the variety of possible changes which constitute spontaneous mutations

can be divided into potent and moderate mutations, according to their effect on the mutated gene and the stability of the mutant phenotype. This classification, which does not directly depend on the type of DNA lesion, presents the significant advantage of discerning spontaneous mutations on the basis of the severity of their consequences on the phenotype of a gene, and consequently, on the organism. Potent mutations represent stable DNA alterations that do not readily revert to the wild type phenotype, which immediately suggests significant consequences for an organism. In contrast, moderate mutations often constitute more unstable events which frequently undergo genotype (and phenotype) reversal because wild type gene functions may be restored with more ease. In fact, potent mutational events, whose stability will have a severe impact on the function of a gene, can be subdivided into small DNA mutations (microlesions), and major DNA rearrangements (macrolesions). Moderate mutants, however, comprise simple DNA modifications, and the mutants phenotype can be often be readily restored to the wild type through another mutational event.

Potent spontaneous mutations may occur at a lower frequency than the moderate (and leaky) mutations, and often involve complex mutational events. The involvement of the potent and stable genetic changes with mammalian cell mutagenesis thus deserves particular consideration. However, despite their drastic impact on the phenotype, potent spontaneous mutations and the cellular mechanisms involved with their formation remain poorly understood, due to the difficulty to specifically isolate or characterize these events.

1.3. GENETIC AND EPIGENETIC MUTATIONS IN EUCARYOTIC CELLS

The classification of spontaneous mutations as potent and moderate reflected their impact on the genome, but a thorough comprehension of mammalian mutagenesis necessitates a description of the mutational events at the molecular level. Spontaneous mutations can be the result of classical genetic mutations which affect the gene product through a direct alteration of the sequence of the DNA encoding that gene (Ramel, 1989). Alternatively, spontaneous mutational events can consist of epimutations (Table 1), which characteristically influence the phenotype without directly modifying the genotypic constitution of an organism (Monk, 1990; Holliday, 1991).

1.3.1. GENETIC MUTATIONS

Small point mutations involving a single base pair change represent the simplest genetic mutations that can spontaneously mutate DNA. Single base pair substitutions can result in transitions (the change of one purine to the other or one pyrimidine to the other), or transversions (the change from a purine to a pyrimidine or from a pyrimidine to a purine). These simple alterations can modify a codon into one that specifies a new amino acid (a missense mutation), or generates a termination codon, producing an incomplete polypeptide chain with little or no biological activity (a nonsense mutation). Frameshift mutations, the addition or the deletion of one or more nucleotides (but not multiples of three), constitute an event that alters the translation reading frame of the protein-coding DNA sequence. Point mutations constitutes reversible mutational events since they can frequently revert to wild type either by restoring the original sequence or by gaining a

TABLE 1

GENETIC AND EPIGENETIC MUTATIONS

GENETIC MUTATIONS:

- -Point mutations
  - -base substitutions
  - -frameshifts
- -Deletions
- -Inversions
- -Insertion by transposable elements
  - -e.g. SINEs and LINEs

EPIGENETIC MUTATIONS:

- -DNA methylation
- -Chromatin structure
- -Position effect
- -Gene amplification
- -Variation in ploidy

compensating mutation at another location within the gene. Smith (1992) suggested that most base substitutions, both in *E. coli* and in the eucaryote *Saccharomyces cerevisiae*, arise from error-prone repair, while frameshift mutations and small deletions were a consequence of DNA replication and recombination processes.

Genetic mutations can also implicate more important DNA lesions which entails a molecular complexity that prevents genotype reversal. Deletions and insertions can also involve considerably long DNA segments. Large DNA sequences, consisting of hundreds or thousands of base pairs, can be deleted as the result of the action of the cellular recombination machinery (Albertini *et al.*, 1982). Even though transposition events do not appear to make a major contribution to the spectrum of spontaneous mutations in mammalian cells, extensive insertions of new DNA sequences can dramatically perturb the function of a gene. Eucaryotic genomes comprise highly repetitive sequences distributed on the genome (MacPhee, 1991; Nalbantoglu *et al.*, 1988), and in humans, the majority of such repetitive elements belongs to the *Alu* family. These repetitive sequences can be involved in homologous recombination in order to produce various chromosomal rearrangements. Although rare, insertion mutations have been observed in the *aprt* locus, but the spontaneous 285-bp insertion plus a γ-irradiation induced 52-bp insertion, did not show the duplication of sequences flanking the integration site suggestive of a transposable element (Breimer, 1990). In contrast, transposition accounts for more than 50% of the spontaneous mutations in *Drosophila* (Rubin, 1983). In addition, inversion of DNA sequences as a consequence of site-specific recombination, chromosome translocation, and non-disjunction also constitute gross chromosomal alterations that can

arise spontaneously. Furthermore, in diploid eucaryotes, the replacement of a wild type allele with a duplicated mutant allele (loss of heterozygosity, LOH) has been shown to constitute a major cause of spontaneous mutations (Ward *et al.*, 1990; Klinedinst and Drinkwater, 1991).

I.3.2. EPIGENETIC MUTATIONS

Epigenetic mutations do not alter the gene product, but rather affect the transcription of a gene, and occur at rates substantially higher than genetic mutations (Monk, 1990; Holliday, 1991). Epigenetic modifications of the DNA, however, confer an unstable phenotype, as the reversion to the wild type phenotype occurs at a higher frequency compared to that observed for genetic mutants (Holliday, 1991). Methylation represents a well characterized epigenetic event occuring at the level of the DNA sequence. (Dynan, 1989; Holliday, 1991). DNA methylation is symmetrical, perpetuated by the action of a maintenance DNA methylase, and clonally inherited (Monk, 1990). Cytosine residues are mainly methylated at the 5' position within dinucleotides (5'$_{m}$CpG3') at a frequency that varies between species and cell types, ranging from 40% to 80%. Interestingly, 45.5% of all methylated cytosine residues are in $_{m}$CpG dinucleotides, the remainder (54.5%) being in $_{m}$CpA, $_{m}$CpT, and $_{m}$CpC (Woodcock *et al.*, 1987). This underrepresentation of $_{m}$CpG in the mammalian genome, compared to cytosine residues methylated within other dinucleotides, may be the result of the high frequency of spontaneous deamination of $_{m}$C into thymidine (Bird, 1984).

In contrast, CpG islands, which comprise several copies of CpG dinucleotides,

remain distinctively non-methylated. This discrepancy may be explained by the loss of methylated CpGs by mutation following cytosine deamination, while the CpGs comprised in non-methylated regions of silent genes are retained to give rise to CpG islands (Antequera and Bird, 1993). The frequency of $_{m}$CpG is lowest in transcriptionally silent DNA (Beutler *et al.*, 1989). Indeed, non-methylated CpG islands are found near the promoter of several housekeeping (constitutively expressed) genes, and their abundance was estimated to approach 45,000 islands per haploid genome in humans (Antequera and Bird, 1993). Antequera *et al.* (1990) demonstrated that methylation of CpG islands was more frequent in cultured cell lines, compared to normal diploid somatic cells, and may correlate with the loss of certain tissue-specific characteristics.

One of the functions of DNA methylation in procaryotes is in mismatch repair and the protection of the DNA from restriction endonucleases. Since eucaryotic organisms lack the restriction-modification systems found in bacteria, the methylation of mammalian DNA serves a different purpose, and is believed to be involved in gene regulation and cellular differentiation (Bird, 1984). Several genes have been reported to be silenced by site-specific DNA methylation. Early work by Harris (1982) revealed this type of inheritance by showing that V79 cells deficient in thymidine kinase (TK) activity could be induced to revert to the TK^{+} phenotype by treatment with 5-azacytidine (5-azaC), a known inhibitor of cytosine methylation. In the cellular *tk* locus, methylation inversely correlated with gene expression (Christy and Scangos, 1984), and spontaneous *tk*$^{+}$ mutants resulting from methylation arose at a frequency of $6x10^{-5}$ (Holliday and Ho, 1990). Clough *et al.* (1982) also showed that 5-azaC could reactivate a previously silent,

chromosomally-integrated HSV-1 *tk* gene. Finally, in female mammalian cells, one of the two X chromosomes is silenced by extensive hypermethylation, mostly in the 5' regions of the inactive X-linked genes, and this inactivation can be reversed by 5-azaC (Pfeifer *et al.*, 1990). DNA methylation in vertebrate cells may be utilized during mismatch repair to direct strand choice for the cellular repair machinery (Hare and Taylor, 1985; Terleth *et al.*, 1991). Finally, another role for DNA methylation concerns genomic imprinting, the differences in gene expression observed between maternally and paternally derived alleles of mammalian genes (Moore and Haig, 1991).

An intriguing observation is the absence of DNA methylation in *Drosophila*. Hence, methylation may not necessarily be the most significant epigenetic event for gene regulation, but may act in concert with other events. For example, the inactivation of chromosome X is due to heterochromatinization by specific DNA binding proteins, and hypermethylation may only act as a secondary event for the stabilization of the inactivation (Piper *et al.* 1993). Hence, in addition to silencing promoters during transcription, DNA methylation in mammalian cells may indirectly cause epimutations through the formation of alternative chromatin structures (Sweet *et al.*, 1982). In HeLa cells, Tazin and Bird (1990) showed that CpG islands adopt a chromatin structure different from the bulk chromatin. Similarly, the presence of CpG islands in the α-globin gene facilitates the formation of a chromatin structure inaccessible to restriction endonucleases that recognize CpG, such as *Msp*I (Antequera *et al.*, 1990).

Other epigenetic events function at the level of the structure of the chromosomes, nuclear localization, and the location of a gene within a chromosome, to influence the

expression and the activity of a gene (position effect). The eucaryotic genome is compacted into chromatin and further condensed into chromosomes, yet these higher order structures must accomodate DNA replication and transcription. The structure of chromatin can influence mutagenesis by affecting the accessibility of sites to the cellular machinery, such as replication, repair and recombination (Robins *et al.*, 1981). Sites in the chromatin which are sensitive to digestion with the enzyme DNAase I were shown to correspond to regions of the chromosome where genes are being expressed, and dccondensation of the chromatin into DNAase I-sensitive regions has been associated with the activation of transcription (Sweet *et al.*, 1982; Hayashi *et al.*, 1991). DNA topology, the degree of supercoiling, the topoisomerase activity in the vicinity of the gene, the presence of proteins (such as transcription factors) prohibiting the formation of the usual nucleosomal conformation will all, to a certain extent, affect the structure of the DNA and consequently, the expression of a gene (Ben-Hattar *et al.*, 1989; Buschhausen *et al.*, 1985, 1987; Antequera *et al.*, 1990; Deobagkar *et al.*, 1990).

Position effect variegation is another *cis*-acting phenomenon that alters gene expression. Discovered in *Drosophila*, it represents the mosaic expression of a gene, associated with chromosomal or genomic rearrangements, caused by the juxtaposition of a segment of heterochromatin into euchromatin (Hayashi *et al.*, 1991). The effect of the localization of a gene at a particular region of the chromosome can be demonstrated when exogenous genes are transfected and integrated into the host genome. Genes introduced exogenously appear to be subjected to their sequence environment (Hardies *et al.*, 1983). Buschhausen *et al.* (1985, 1987) showed that a transfected herpes simplex virus type 1

(HSV-1) *tk* gene was methylated after integration into the genome of the mouse LTK$^-$ cell line, resulting in the transcriptional silencing of the *tk* gene through the formation of higher chromatin structure which hindered transcription. Hence, local chromatin structure represents yet another mechanism which can influence gene expression (Camerini-Otero and Zasloff, 1980; Deobagkar *et al.*, 1990).

I.4. SPONTANEOUS MUTATIONS AND GENETIC DISEASES

Despite their essential influence on genetic diversity and evolution, spontaneously occurring mutations can be detrimental since they are also involved with the predisposition and generation of various genetic disorders (reviewed by Caskey, 1987). Similarly, the generation of several forms of cancer has been associated with potent spontaneous mutations (Bishop, 1981; Cairns, 1981; Varmus, 1984).

Several common inherited genetic disorders are caused by simple point mutations. Single-base substitutions are the cause of a recessively-inherited liver disease, α-1-antitrypsin (AAT) deficiency (Kidd *et al.*, 1984) and 30% of the mutations causing phenylketonuria consist of a simple point mutation in the 5' splice donor of intron #12 (DiLella *et al.*, 1986). Similarly, base substitutions and small deletions in the β-globin gene result in severe thalassemia and in sickle-cell anemia as a consequence of the modification of a single amino acid from glutamic acid to valine. The Lesch-Nyhan syndrome, an X-linked recessive disease resulting in a deficiency in the enzyme hypoxanthine phosphoribosyl transferase (*hprt*) as a consequence of various mutations, consists mostly of point mutations, but can also comprise large deletions (Yang *et al.*,

1988; Yen *et al.*, 1990).

Gene deletions have also been associated with the generation of numerous human genetic disorders (Krawczak and Cooper, 1991). Duchenne's muscular dystrophy (DMD, on chromosome Xp21), the deficiency in the enzyme ornithine transcarbamylase (OTC) causing a defect in the urea cycle, and steroid sulfatase deficiency (SSD), are three other X-linked diseases resulting from spontaneous somatic mutations. Large deletions, or duplications, involving several exons account for 70% of the spontaneous mutations in DMD (Forrest *et al.*, 1987), more than 50% in the case of OTC (Caskey, 1987), and between 70 and 90% in SSD (Yen *et al.*, 1990). Reports on spontaneous transposition leading to genetic disorders are scarce, but the insertion of the non-viral retrotransposon Line 1 (L1) element was observed in two cases of factor VIII hemophilia A (Kazazian *et al.*, 1988; Dombroski *et al.*, 1991). Aging and age-related genetic disorders may also reflect irreversible DNA damage in somatic cells by free radicals and/or the failure to repair such DNA lesions (Harman, 1981). Another spontaneous mutational process is exemplified by the fragile X syndrome (Kremer *et al.*, 1991), the Kennedy disease (LaSprada *et al.*, 1992), myotonic dystrophy (Fu *et al.*, 1992), as well as Huntington's disease (The Huntington's Disease Collaborative Research Group, 1993), which were shown to result from the expansion of polymorphic trinucleotide repeats in their respective coding sequences.

Both gene mutations and chromosomal alterations have been implicated in carcinogenesis (reviewed by Varmus, 1984; Bishop, 1987; Bishop, 1991). A variety of spontaneous somatic mutations, ranging from small point mutations to chromosomal

abnormalities, occur naturally, accumulate, and persist, to upset the normal control of cell division resulting in the characteristic unrestricted growth observed for cancer cells (Ames, 1989; Rudiger, 1990).

Cellular proto-oncogenes, discovered through the study of oncogenic retroviruses, can be activated to their oncogenic form by a variety of mechanisms and lead to the development of cancer (Tabin *et al.*, 1982; Cairns, 1981; Bishop, 1991). The activity of the H-*ras* oncogene is altered by a single base change causing a single amino acid change from glycine to valine (Hollstein *et al.*, 1991). Other proto-oncogenes are activated by more complex mechanisms, such as the activation of c-*abl* by reciprocal chromosomal translocation of part of chromosome 9 into chromosome 22 (Adams, 1985; Bishop, 1987). The *c-abl* gene becomes fused to the breakpoint cluster region (*bcr*) gene, resulting in an aberrant *abl-bcr* oncogenic fusion protein (Adams, 1985). This abnormality, referred to as the "Philadelphia chromosome", results in various forms of chronic and acute leukemias. Burkitt's lymphoma is a cancerous disorder resulting from a 8:14 translocation that causes the newly translocated *myc* oncogene to be overexpressed by regulatory regions of the immunoglobulin heavy chain loci present on chromosome 14 (Hamlyn and Rabbitts, 1983). The enhanced transcription of N-*myc* was also observed following its translocation near promoter sequences from the immunoglobulin genes on chromosomes 2 and 22, as well as sequences coding for the T-cell receptor (chromosomes 11 and 14) in T-cell leukemias. The generation of cancer through the considerable amplification of the number of copies of a specific oncogene within regions of the chromosome that appear as homogenously staining regions (HSRs) (Varmus, 1984) was also observed in

tumor cell lines. Finally, the insertion of the long interspersed repetitive DNA L1 into the c-*myc* oncogene has been reported to occur in some human breast tumors (Morse *et al.*, 1988).

Conversely, cancer can result from the inactivation of an antioncogene whose product usually inhibits uncontrolled cell proliferation. The antioncogene retinoblastoma *Rb*-1, is associated with the heritable and sporadic form of the recessive eye cancer retinoblastoma as a consequence of the deletion of both copies of the *Rb*-1 gene located on chromosome 13, band q14 (Cavanee *et al.*, 1983). The *Rb* antioncogene has also been found to be inactivated in certain forms of osteosarcoma, breast cancer, and small-cell lung cancers. In Wilm's tumor, the antioncogene *Wg* represses a transformation gene *Tr* by cytosine methylation, and *Wg* inactivation through various mechanisms will induce cell proliferation. The disruption of the APC gene, another tumor supressor gene, by the somatic insertion of the mobile element LINE-1 has been observed in colon cancer (Miki *et al.*, 1992). Cancer cells were also shown to demonstrate an abnormal control of DNA methylation (Jones and Buckley, 1990; Holliday, 1991).

II. MECHANISMS OF SPONTANEOUS MUTAGENESIS

The prominent role of spontaneous mutations in several aspects of genetics renders essential the evaluation of the numerous cellular and molecular mechanisms involved. Normal cellular processes essential for cell survival, such as DNA replication and DNA repair, occasionally demonstrate a certain degree of inaccuracy which culminates in diverse spontaneous mutations (Ames 1989, and reviewed by Drake, 1989). Various

intrinsic attributes of cellular physiology, such as free radicals and alkylating agents inevitably generated during cellular metabolism, also predispose to (or influence) spontaneous mutations (Smith, 1992), as discussed in greater detail below.

II.1. INHERENT INSTABILITY OF THE GENOME

Spontaneous mutations usually occur at random positions throughout a given gene, yet particular sites are more prone to mutation than would be predicted by chance. During experiments in the rII locus of bacteriophage T4, Benzer (1961) discovered that some sites within a gene were more prone to mutations than others, and referred to these sites as "hot spots" for mutations. Subsequent studies on the *lac*I gene of *E. coli* revealed that redundant sequences constituted hot spots for frameshift mutations (Farabaugh *et al.*, 1978) and that methylated cytosines also considerably favored the generation of spontaneous mutations (Coulondre *et al.*, 1978). The inherent instability of DNA can also be exemplified by the spontaneous modification of bases by deamination, oxidation, and methylation. Such altered bases can pair with bases other than their usual complement, resulting in a mutation that will be perpetuated after DNA synthesis. Methylated cytosines represent hot spots for spontaneous mutation following their deamination into thymine, which, being a natural base, is not removed by glycosylases (Coulondre *et al.*, 1978; Brennan *et al.*, 1990). Thymine can pair with adenine in the next generation, generating a transition (C:G → T:A) mutation (Brennan *et al.*, 1990). The rate of cytosine deamination was calculated to occur at 15 residues/hour per mammalian cell for dsDNA (Smith, 1992). This frequency is even higher (266-fold) for single-stranded DNA,

presumably the state of the non-coding strand during transcription, making ssDNA a target for spontaneous mutagenesis. Spontaneous depurination or depyrimidination also occurs in mammalian cells, and the resulting apurinic and apyrimidic (AP) sites proved mutagenic during replication (Echols and Goodman, 1991). If these non-coding lesions are not corrected by DNA repair processes, adenine residues are generally inserted opposite the AP site, leading to a mutation if adenine was not the required base. Adenine was also found to be preferentially introduced to correct several mutagen-induced mutations (Strauss, 1991), a phenomenon known as the "A rule" of mutagen specificity.

Spontaneous mutations can also be influenced by particular DNA sequence motifs. The importance of DNA secondary structures in the generation of deletions and duplications has been reviewed by Ripley and Glickman (1983). Duplication of DNA segments is favored by the presence of sequences capable of forming stem-loop structures (Ohshima *et al.*, 1992). Eucaryotic chromosomes are compacted into euchromatin, containing regions of DNA being expressed, while transcriptionally inert regions of DNA are condensed into heterochromatin. Tandem blocks of highly repetitive satellite DNA found in heretochromatin, as well as minisatellite sequences (Jeffreys *et al.*, 1985), may be more vulnerable to genetic alterations caused by recombination and transposition events (Deininger *et al.*, 1992).

The presence of repetitive sequences suggestive of transposable elements implies a potential role for transposition mutagenesis (Deininger *et al.*, 1992). A class of highly repeated DNA sequences are found dispersed throughout the genome: the short (SINEs) and long (LINEs) interspersed repeated elements (Singer, 1982). SINEs are typically less

than 500 bp in length, occur on the order of 10^5 times per genome, and the primate *Alu* element represents the most abundant and best characterized family of short repeats (Singer, 1982). However, no tranpositionally-active Alu sequences have been isolated to date (Martignetti and Brosius, 1993). Recombination between Alu repeated sequences (Labuda and Striker, 1989) has been implicated in deletion mutations (Kornreich *et al.*, 1990). LINEs, over 5 kbp long, are present at ~ 10^4 copies per human genome (Singer, 1982), and the human L1 element represents the only transposable element known to be endogenous to the human genome (McMillan and Singer, 1993). Actively transposing L1 elements have been isolated (Dombroski *et al.*, 1991) and associated with the generation of colon cancer by the disruption of the APC gene (Miki *et al.*, 1992). To further illustrate the complex involvement of the instability of the genome in spontaneous mutagenesis, Ripley *et al.* (1988) reported that DNA topoisomerase II recognition sequences in bacteriophage T4 DNA represent hypermutable sites, and suggest that these results might be extrapolated to mammalian genomes.

The products of normal cellular processes, such as free radicals and alkylating agents, also play a role in mutagenesis through subsequent damage to the DNA. Oxidative stress, a consequence of normal cellular metabolism, has been shown to spontaneously damage DNA through oxyradicals which elude the cellular defense provided by oxygen-radical scavengers, and specific enzymes such as superoxide dismutase, catalase, and peroxidase (Breimer, 1990). Oxidative DNA damage causes mostly C:G→G:C transversions *in vitro* (Retèl *et al.*, 1993), but large deletions have also been observed at the *hprt* locus (Fuscoe *et al.*, 1986; Liber *et al.*, 1987).

II.2. DNA REPLICATION

To preserve the genetic integrity of an organism, DNA replication must be accurately conducted and monitored. Yet, some errors are tolerated and the resulting mutations can be maintained in the genetic pool, thus providing the basis for species evolution and genetic diversity. However, mistakes during DNA replication are also implicated in spontaneous mutagenesis, resulting in genetic diseases, cancer, and the process of aging. Mammalian cells possess four nuclear DNA polymerases: α, β, δ, and ε (Kornberg and Baker, 1992). DNA polymerases α and δ are both responsible for DNA replication: δ is highly processive and possesses a 3'→5' proofreading exonuclease activity, while α, with lesser processivity, is equipped with primase activity. DNA polymerase ε also demonstrates proofreading potential, and the distributive DNA polymerase β, with limited fidelity, is implicated in DNA repair. A fifth DNA polymerase (γ) serves for the replication of mitochondrial DNA. Both the specificity in the choice of the incoming base to be inserted at the growing point of the DNA chain by DNA polymerases, and the proofreading activity of the replicating enzymes (differential removal of uncorrectly inserted bases by a 3'→5' exonuclease), act to maintain genetic integrity. To illustrate the accuracy of DNA replication, Leob and Kunkel (1982) proposed that the average frequency of base substitutions ranges from 10^{-7} to 10^{-11} misincorporations per bp replicated *in vivo*, a rate considerably lower than the error rate for a purified DNA polymerase *in vitro* (10^{-3} to 10^{-6}). This discrepancy is ascribed to additional cellular mechanisms that ensure the integrity of the genome *in vivo*. The fidelity and processivity of the replicating enzymes depends on several accessory proteins required for DNA

synthesis (e.g. a single strand DNA binding protein (ssb) that enhances replication fidelity), as well as the sequence of the DNA template. In addition, since the intracellular pool of nucleotides (dNTPs) is small (< 0.2 mM), the size of this pool, as well as the relative ratio of each of the four dNTPs, must be optimized for high fidelity replication. An imbalance may lead to the misincorporation of the nucleotide present in excess (Phear and Meuth, 1989) and lead to a mutation. When mismatched base pairs elude the proofreading activity of the DNA polymerase, enzymes involved in DNA mismatch repair provide further accuracy (Holliday, 1964).

Particular DNA sequence motifs can also affect the fidelity of DNA replication. Stretches of identical bases favors the slippage of DNA polymerase during replication, resulting in frameshift mutations, due to small additions or deletions (Ripley, 1990). The misalignment of distant sequences during DNA replication constitutes another mechanism generating mutations (Kunkel, 1990). The presence of inverted repeats that can form hairpin or cruciform structures can interrupt the integrity of the duplex DNA, perturb DNA replication, and cause deletions (Glickman and Ripley, 1984). Direct repeats (two copies of a sequence repeated in the same orientation) can cause deletions, while stem-loop DNA structures can generate duplications of DNA segments (Ohshima *et al.*, 1992). The elongation of a trinucleotide repeat sequence represents, for example the expansion of the (CCG) repeat causing Huntington's disease, represent yet another mutational mechanism associated with genetic disorders (The Huntington's Disease Collaborative Research Group, 1993).

II.3. DNA REPAIR

DNA repair systems are crucial to maintain the authenticity of the DNA and mostly depend on the information present in the complementary DNA strand for efficient correction of the lesion. Any mistakes during the repair of DNA damage can be mutagenic (error-prone DNA repair). Many extraordinarily diverse and effective repair systems have been well characterized in bacteria, while considerably less is known about those in mammalian cells. Mismatch repair systems (Holliday, 1964; Holmes *et al.*, 1990) specifically replace an incorrect base inserted during DNA synthesis. Mammalian DNA polymerase δ demonstrates a high affinity for nicked duplex DNA molecules, and levels of this enzyme were shown to increase in the presence of DNA-damaging agents (Fornace *et al.*, 1989). Glycosylases are base-specific enzymes which detect and remove a damaged base from the deoxyribose sugar, without affecting the sugar-phosphate backbone, creating an AP site. AP sites are mutagenic during replication (Echols and Goodman, 1991) if they are not corrected by AP endonucleases. Since cytosine can spontaneously deaminate into uracil, the role of glycosylases for efficient removal of incorrect bases is very important. Additional accuracy is provided by excision repair systems that remove mispaired or damaged bases from DNA in a stretch of sequence that usually extends farther than the lesion itself (e.g. removal of UV-induced thymine dimers). Another type of repair process occurs post-replicatively. For example, genetic recombination associated with DNA repair retrieves a normal strand of DNA to replace a gap left on the other strand. Failure of certains steps during DNA repair can have dramatic consequences, as exemplified by Xeroderma pigmentosum (XP), a recessive

disease in which deficient excision-repair results in hypersensitivity to UV light, and consequently, an increased level of uncorrected mutations (Tanaka *et al.*, 1990).

II.4. GENETIC RECOMBINATION

Cellular loci can be spontaneously assorted by recombination to generate the deletion, duplication, or inversion of large segments of a gene. The outcome of such complex mutational events often consists of potent mutations with a severe impact on the phenotype associated with a given gene. In addition to the conventional excision-repair systems, the repair machinery of mammalian cells, described above, also utilizes the process of recombination. Recombination processes involve the breakage and reunion of DNA segments, and can be categorized into the following classes: homologous recombination, non-homologous recombination, and transposition. During homologous recombination, segments of a gene are replaced by a copy of the homologous segment found on the complementary strand. In diploid eucaryotic organisms, each locus comprises two alleles situated on homologous chromosomes, and genetic exchange between chromosomes has been asssociated with loss of heterozygosity in several genes (Barr *et al.*, 1990; Klinedinst and Drinkwater, 1991; Grovosky *et al.*, 1993). Large segments of DNA may potentially be deleted by the aberrant operation of the cellular recombination enzymes, favored by the presence of double strand breaks in the DNA (Sargentini and Smith, 1991). Futhermore, the reciprocal genetic recombination involved within the V(D)J regions of immunoglobulins represents a mutational event crucial for the generation of antibody diversity in mammals (Van Ness *et al.*, 1982). In addition,

spontaneous mutations caused by aberrant V(D)J recombinase activity have been observed at the *hprt* locus (Fuscoe *et al.*, 1991, 1992b).

Illegitimate recombination, in contrast, occurs without a requirement for extensive sequence homology, and represents the most frequent mechanism for chromosomal rearrangements in mammalian cells (Milot *et al.*, 1992). The molecular mechanisms involved in these complex events remain to be characterized, but the integration of exogenous vectors in the mammalian genome has been demonstrated to occur largely through non-homologous recombination. Recombination through unequal crossing-over can also result in the enormous amplification of DNA segments into homogenously staining regions (HSR) or double minute (DM) chromosomes (Varmus, 1984). Few studies have reported transposition, or site-specific recombination events in mammalian cells (Morse *et al.*, 1988; Miki *et al.*, 1992). However, recombination between repeat sequences, such as the eucaryotic *Alu* element, has been involved with the formation of deletions (Kornreich *et al.*, 1990).

III. ENDOGENOUS SYSTEMS TO STUDY SPONTANEOUS MUTAGENESIS IN MAMMALIAN CELLS

Because of their association with genotypic diversity and genetic disorders, the study of spontaneous mutations and the mechanisms through which they were generated deserves serious consideration. Mammalian cell mutagenesis can be explored by the analysis of spontaneous mutations occurring in endogenous chromosomal genes. Genetic markers employed in mutagenesis studies include the autosomal and highly polymorphic

human major histocompatibility (HLA) complex, in which more than 30% of the mutations represent large deletions caused by mitotic recombination (Nicklas *et al.*, 1985). However, because their size facilitates molecular analyses, and since they encode enzymes of metabolic pathways for which selection for both the active and the mutated forms are readily available, the *aprt*, *hprt*, *dhfr*, and *tk* genes represent the most commonly used loci for mammalian mutagenesis studies. The spectrum of mutations (spontaneous or induced) in mammalian cells reveals a broad range of genetic changes and reflects the extraordinary dynamics of the genome.

III.1. THE *aprt* LOCUS

The autosomal *aprt* locus from diploid mammalian cell lines represents an appealing mammalian gene system for mutational analyses (Drobetsky *et al.*, 1989). The *aprt* gene encodes the enzyme adenine phosphoribosyltransferase, involved in a purine salvage pathway, where it catalyzes the conversion of adenine to adenosine 5'-monophosphate. Mutations in a heterozygous (+/-) locus are selected on the basis of resistance of APRT deficient cells to toxic adenine analogues such as 8-azaadenine and 2,6-diaminopurine. Reverse mutations can also be selected for, using Hypoxanthine Aminopterin Thymidine (HAT) medium (Ouellette and Bradley, 1991). The molecular dissection of the mutants is greatly facilitated by the small size (2.5 kbp) of the *aprt* gene (Nalbantoglu *et al.*, 1986; de Boer and Glickman, 1991). However, both alleles of this autosomal locus from diploid eucaryotic cells must be altered for loss of enzyme function (Yandell *et al.*, 1986), which occurs with a spontaneous mutation frequency of approximately 5.9×10^{-5} mutants/cell

(Klinedinst and Drinkwater, 1991). The spontaneous mutational spectrum reveals that in heterozygous ($APRT^{+/-}$) lymphoblastoid cells, loss of heterozygosity (LOH), or the loss of the wild type *aprt* allele accompanied by the duplication of the mutant allele, takes place in more than 50% (Ward *et al.*, 1990; Klinedinst and Drinkwater, 1991) to 80% (Hakoda *et al.*, 1991) of the spontaneous $aprt^{-/-}$ clones. The deletion of the wild type allele from heterozygotes was observed in 19% of the *aprt* mutants, while small deletions of a few base pairs constituted 23% of the mutations (Klinedinst and Drinkwater, 1991).

To permit a single-step selection of mutations in the autosomal *aprt* locus from diploid cells, functionally hemizygous ($APRT^{+/0}$) cell lines have been developed (Simon *et al.*, 1982). In hemizygous situations, spontaneous mutations occur at a frequency ranging between 5×10^{-5} (Amundson and Liber, 1992) and 8.6×10^{-5} (Smith and Grosovsky, 1993). The spectrum of mutations at the hemizygous *aprt* locus differs from the types of mutations observed at the heterozygous locus, and uncovers the predominance of point mutations dispersed throughout the gene (Phear *et al.*, 1989). In a study by de Jong *et al.* (1988), 90% single base substitutions, among which 22/27 were G:C to A:T transitions, presumably caused by the spontaneous deamination of cytosine at mCpG dinucleotides, were reported. The splice donor at intron 4, as well as codon 87, may represent hot spots for spontaneous mutations (Belouchi and Bradley, 1992, Chen *et al.*, 1993). Similarly, the accumulation of transition and transversion mutations resulted in APRT deficiency in hemizygous ($APRT^{+/0}$) tumor cells (Harwood *et al.*, 1991). Few insertion mutations have been observed at the *aprt* locus: a single spontaneous insertion of 285 bp of exogenous DNA, and three γ-irradiation-induced insertions (Breimer *et al.*,

1986, Nalbantoglu *et al.*, 1988, Miles *et al.*, 1990) have been identified to date. Cooper *et al.* (1993) reported that DNA methylation maintained transcriptional inactivation of the *aprt* gene by imposing a nuclease-resistant chromatin structure forbiding proper RNA transcription. The spectrum of mutations induced by various mutagens unveiled, in addition to simple point mutations (Grosovsky *et al.*, 1988; de Boer and Glickman, 1989; Miles and Meuth, 1989), a higher occurrence of large deletions (Drobetsky *et al.*, 1987; Bradley *et al.*, 1988). Most deletion mutations extended upstream, but not downstream from the *aprt* gene, suggesting the presence of an essential gene near that locus (Grosovsky *et al.*, 1986). The localization of the *aprt* locus near a chromosomal fragile site may account for the generation of deletions and loss of heterozygosity events observed in several studies (Smith and Grosovsky, 1993).

III.2. THE *hprt* LOCUS

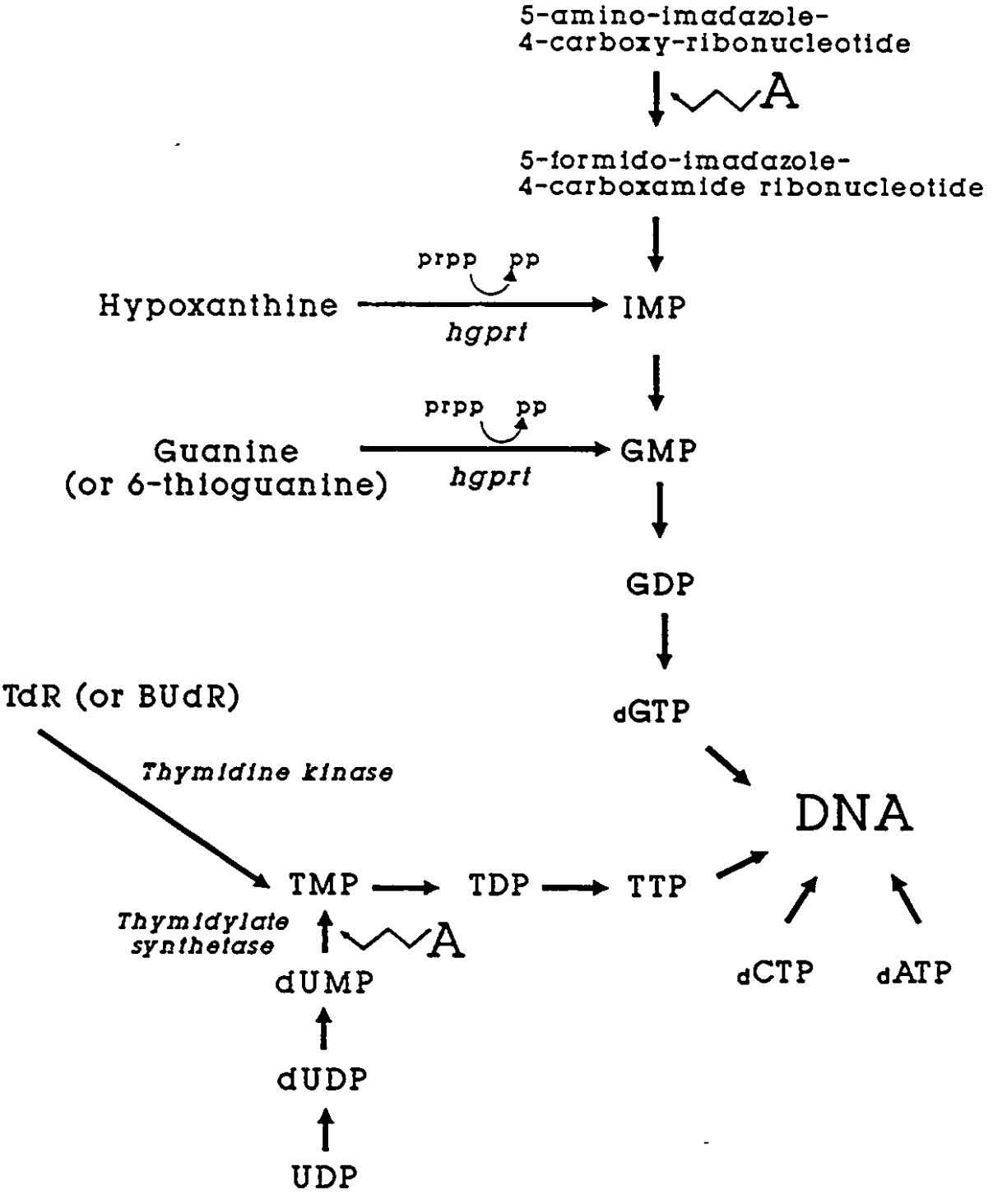
Another assay for mutagenesis exploits the physical hemizygosity provided by the X chromosome. The *hprt* gene codes for the hypoxanthine phosphoribosyltransferase enzyme, implicated in a purine salvage pathway to catalyze the phosphorylation of hypoxanthine or guanine to generate dGMP through a dIMP intermediate (Figure 1). The *hprt* gene is non-essential, constitutively expressed, and offers the possibility of both forward and reverse mutation analysis. Mutants deficient in the HPRT enzyme are readily selected using the guanine analogue 6-thioguanine (6-TG), and less frequently 8-azaguanine and 6-mercaptopurine, which are cytotoxic to wild type cells. Revertants to the wild type phenotype ($HPRT^- \rightarrow HPRT^+$) are selected in HAT medium because

FIGURE 1

Summary of the pyrimidine and purine salvage pathway for nucleoside biosynthesis.

The Hypoxanthine Aminopterin Thymidine (HAT) supplement provides aminopterin (A), which blocks the nucleoside biosynthesis pathways at steps indicated by an arrow. Hypoxanthine and thymidine are also provided in order to permit the utilization of the salvage pathways to generate dGTP and dTTP.

HAT METABOLISM



aminopterin interferes with folate metabolism, and thus the utilization of exogenous hypoxanthine through HPRT becomes essential for cell survival (Figure 1). In humans, a deficiency in the HPRT enzyme leads to the genetic disorder known as Lesch-Nyhan syndrome (extreme gout).

The 44-kb *hprt* housekeeping gene located on the X chromosome (Xq26), comprises 9 exons, but its coding region encompasses only 651 bp (Patel, *et al.*, 1990; Edwards *et al.*, 1990). The molecular analysis of mutations in the *hprt* locus has been performed in cultured cell lines and T lymphocytes *in vivo*, which permits the parallel identification of mutations in the T-cell receptor β gene. Hence, the differentiation of sibling colonies and the evaluation of the clonality of individual *hprt* mutants (Albertini *et al.*,1982) provides an accurate estimation of the mutation frequency *in vivo* in somatic cells in humans (Curry *et al.*, 1993). Spontaneous mutations at the hemizygous *hprt* locus occur at a frequency of 2.4×10^{-6} in cultured TK6 lymphoblast cells (Liber and Thilly, 1982) and $5\text{-}10 \times 10^{-6}$ in T cells *in vivo* (Fuscoe *et al.*, 1992a,b). However, a very limited number of spontaneous mutants at the *hprt* locus were analyzed, as most studies investigated induced mutational events. Simple point mutations caused more than 60% of spontaneous *hprt* inactivation (Fuscoe *et al.*, 1986; Thacker and Ganish, 1989; Davies *et al.*, 1993), frequently affecting proper splicing of the gene (Vrieling *et al.*, 1989; Zhang *et al.*, 1992). Similarly, Oller and Thilly (1992) reported that 61% of spontaneous mutations were caused by point mutations (transitions, transversions, and very small deletions of less than 4 bp) that were dispersed throughout the gene, with the exception of two potential hot spots in exon 3. Other studies confirmed that small deletions of less than 4 bp (Ikehata

et al., 1989) or from 1 to 6 bp (Oller and Thilly, 1992) dominated the spectrum of spontaneous mutations. However, the large size of the *hprt* gene, and the presence of numerous repeated sequences, confer a higher tolerance for large deletions (Fuscoe *et al.*, 1992a; Morris and Tacker, 1993) compared to the *aprt* locus. Among 6-TG resistant clones, 7% (Fuscoe *et al.*, 1992a) to 10% (Albertini *et al.*, 1990) underwent total *hprt* gene deletion involving up to 700 kb of DNA. According to Oller and Thilly (1992), the percentage of spontaneous large deletions in TK6 cells can be as high as 39%, among which 12% represent total gene deletions, and 27% complex rearrangements. In contrast, large deletions involving more than 250 base pairs constitute more than 73% of the ionizing radiation-induced mutations in *hprt* (Fuscoe *et al.*, 1992a). Unusual mutational events such as an insertion induced by X-rays (Fuscoe *et al.*, 1992a) and a deletion event mediated by V(D)J recombinase activity in T cells (Fuscoe *et al.*, 1992b) have also been reported at the *hprt* locus. When the spectra of spontaneous mutations at the *hprt* locus from adults and new-borns were compared, Cariello and Skopek (1993) discovered that while point mutations accounted for 85% of the total mutations in adults, they constituted only 25% of the events in infants. The high frequency of large deletions could be attributed to the V(D)J recombinase activity being higher during development, which coincides with the T-cell rearrangement period (Cariello and Skopek, 1993).

III.3. THE CELLULAR *tk* LOCUS

The cellular thymidine kinase (*tk*) locus provides another alternative for the biochemical and genetic analyses of mutagenesis in heterozygous mammalian cells (Flemington *et al.*, 1987). This marker, located on human chromosome 17 (chromosome 11 in the mouse), spans 12.9 kbp, is distributed into 7 exons, and consists of 702 bp of coding sequence (Flemington *et al.*, 1987). Thymidine kinase represents a key enzyme in the salvage pathway of pyrimidine biosynthesis, that phosphorylates thymidine into thymidine monophosphate for subsequent use in DNA synthesis (Figure 1). Thymidine kinase-positive cells can grow in HAT medium, since aminopterin forces the cells to use the salvage pathway to generate dTTP, and hence imposes a requirement for a functional *tk* gene (Figure 1). Cells deficient in thymidine kinase activity are readily selected on the basis of their resistance to trifluorothymidine (TFT), an analogue of thymidine. Following the phosphorylation of TFT by TK, the resulting TFT-monophosphates inhibit the cellular thymidylate synthetase activity, leading to the killing of tk^+ cells due to the inhibition of DNA synthesis (Otto *et al.*, 1982). The incorporation of TFT-triphosphates into the elongating DNA causes abnormal transcription and translation (Fujiwara and Heidelberger, 1970; Otto *et al.*, 1982) as well as DNA strand breakage (Fujiwara *et al*, 1970). Alternatively, tk^- cells can be selected according to their resistance to bromodeoxyuridine (BUdR), another nucleoside analogue. Mouse (Clive *et al.*, 1972) and human (Liber and Thilly, 1982) cell lines heterozygous for this autosomal gene were developed to permit the single-step analysis of mutants.

The frequency of spontaneous mutations at the heterozygous *tk* locus of human TK6

cells was calculated to be 2.6 x 10^{-6} (Liber and Thilly, 1982). Grovosky *et al.* (1993) reported that 58% of the spontaneous mutations in TK6 cells were attributable to loss of heterozygosity (LOH), 11% to intragenic deletions involving more than one exon, and 31% to point mutations, mostly G:C to A:T transitions. Compared with the well characterized *aprt* and *hprt* loci, *tk* seems to undergo the total loss of the gene with a higher frequency. Using various mutagens, Applegate and co-workers (1990) demonstrated a bimodal distribution of the resulting $tk^{-/-}$ mutants: rapidly growing mutants caused by intragenic deletions of the *tk* gene, and slow-growing mutants resulting from the entire loss of the gene as a result of extensive chromosomal deletions. More than 90% of the slow growing mutants arose from loss of heterozygosity (LOH) events (Liber *et al.*, 1989). Overall, loss of heterozygosity accounts for 51% to 70% of the spontaneous and induced mutations at this locus (Liber and Thilly, 1982; Yandell *et al.*, 1986; Barr *et al.*, 1993; Amundson and Liber, 1991, 1992). Homologous mitotic recombination between alleles generated most spontaneous LOH mutations, while induced LOH events may be the result of deletion mutations (Benjamin *et al.*, 1991; Benjamin and Little, 1992; Li *et al.*, 1992).

III.4. THE *dhfr* LOCUS

The dihydrofolate reductase (*dhfr*) gene represents another autosomal locus amenable to mutagenesis studies. The *dhfr* gene from CHO cells is 25 kb long and is distributed into 6 exons (Carothers *et al.*, 1983). DHFR, a housekeeping enzyme essential for the biosythesis of purines, converts folate and dihydrofolate into metabolically active

tetrahydrofolate. Mutant *dhfr*$^-$ cells can be easily detected by their resistance to methotrexate (mtx), a compound which blocks folate metabolism.

Spontaneous mutations in the *dhfr* gene occur at a rate of 1.3×10^{-7} (Mitchell *et al.*, 1986) and consist of both large deletions (>50%) implicating more than 100 bp (Turner *et al.*, 1985), and point mutations dispersed throughout the gene and often affecting splicing (Mitchell *et al.*, 1986). In contrast, mutations induced through chemicals or γ-irradiation comprise mostly large deletions (> 50-100 bp) and inversions, while UV-light causes mostly point mutations, in addition to large deletions (>100 bp) (Chasin *et al.*, 1987; Carothers *et al.*, 1989). In addition to genetic mutations resulting in DHFR enzyme deficiency, drug resistance (especially to methotrexate) can be conferred by enormous amplification of the *dhfr* gene into homogenously staining regions (HSR) on the chromosome or into double minute chromosomes. Spontaneous amplification occurs at the rate of 10^{-4} to 10^{-6}, and mtx^R cell lines can contain up to 400 copies of the *dhfr* gene (Schimke, 1984).

III.5. SPECTRUM OF SPONTANEOUS MUTATIONS AT CELLULAR LOCI

A consensus may be extrapolated from the mutational spectrum that emerged from the mammalian mutagenesis studies described above. Small point mutations involving one or a few base pairs, were frequently encountered. Consequently, the characterization of spontaneous potent mutations, as well as large or rare mutations, was hindered by the more frequent occurence of small point mutations.

A significant proportion of large mutational events were observed following

induction of mutations with a mutagen, and among the spontaneous major DNA alterations described, loss of heterozygosity was the most frequently isolated event. The diploid nature of mammalian cells may then limit the recovery of certain rare molecular recombinational events which can occur intragenically, for example.

IV. EXOGENOUS GENE SYSTEMS FOR THE STUDY OF SPONTANEOUS MUTATIONS IN EUCARYOTIC CELLS

Exogenous reporter genes can be introduced into new cellular chromosomal environments, providing a different approach to address questions about mutagenesis. The transfection of foreign DNA into mammalian cells can be performed by several methods, such as the calcium phosphate coprecipitation method (Graham and van der Erl, 1973), the DEAE dextran procedure (McCutchan and Pagano, 1968), by protoplast fusion (Schaffner, 1980), and more recently by electroporation (Chu *et al.*, 1987). Hence, various shuttle vectors have been developed to contain selectable markers for use in mammalian cells, and in bacterial cells, for efficient and facile screening in bacteria. One class of shuttle vectors can replicate autonomously in the nucleus of eucaryotic cells, and contain sequences from either SV40 virus (Lebkowski *et al.*, 1984; DuBridge and Calos, 1988), bovine papillomavirus (Ashman and Davidson, 1985), or Epstein-Barr virus (Drinkwater and Klinedinst, 1986). However, extrachromosomal replication of the vectors proved to be mutagenic, precluding the use of these vectors for spontaneous mutagenesis studies (Lebkowski *et al.*, 1984; Drinkwater and Klinedinst, 1986). The frequency of spontaneous mutations in shuttle vectors has been shown to vary from ~ 10^{-2} in a bovine

papillomavirus vector (Ashman and Davidson, 1984), to 6×10^{-5} in the HSV-1 *tk* gene from the autonomously replicating EBV-based vector (Drinkwater and Klinedinst, 1986).

To better understand the underlying mechanisms of spontaneous mutagenesis in a chromosomal context, vectors capable of integrating into the eucaryotic genome have been designed. Integrative vectors allow the study of mutagenesis in a target gene which is subjected to the normal environment encountered by the cellular genome. Mulligan and Berg (1980) reported that an exogenous reporter gene located on an SV40-based plasmid vector (pSV2gpt) could be efficiently introduced into and expressed in mammalian cells (Hartman and Mulligan, 1988). The integration process is frequently accompanied by deletions in the exogenously introduced DNA, or in host sequences, either because foreign DNA integrates at chromosomal breaks or because it induces rearrangements during its integration (Kato *et al.*, 1986; Murmane *et al.*, 1990). Repetitive sequences may also constitute preferred sites for integration of foreign DNA (Kato *et al.*, 1986). Vos and Hanawalt (1989) demonstrated that pSV2gpt plasmid vectors integrated into mammalian chromosomes through non-homologous recombination, and preferentially at sites demonstrating relaxed chromatin structure typical of decondensed DNAseI-sensitive regions of the chromosome. Recently developed retrovirus-based vectors are highly efficient for stable gene transfer and very versatile, being able to infect several types of mammalian cells (Ashman *et al.*, 1986). These retroviral vectors contain SV40 origins of replication, comprise two long terminal repeat (LTR) sequences, in addition to other viral sequences, and integrate as a single-copy in the mammalian genome. Upon induction of the SV40 origin of replication by fusion with COS cells (which provide the

required large T protein), the retroviral vectors can excise by homologous recombination through their LTRs (Ashman and Davidson, 1987).

V. THE ANALYSIS OF SPONTANEOUS POTENT MUTATIONS IN MAMMALIAN CELLS

To unveil the spectrum of spontaneous mutations and to consider the dynamic nature and the complexity of the mammalian genome, it is crucial to characterize rare mutational events. Furthermore, spontaneous potent mutations deserve particular consideration because their stable nature may play an important role in the severe inactivation of a particular phenotype. Most of the data concerning major DNA alterations was extrapolated from analyses devoted to mutagens as inducers of mutations, rather than from studies which focused on spontaneous mutations (Smith, 1992). Hence, a strategy which aimed at the specific investigation of spontaneous and potent mutational changes, as well as the characterization of major molecular alterations, is presented in this section.

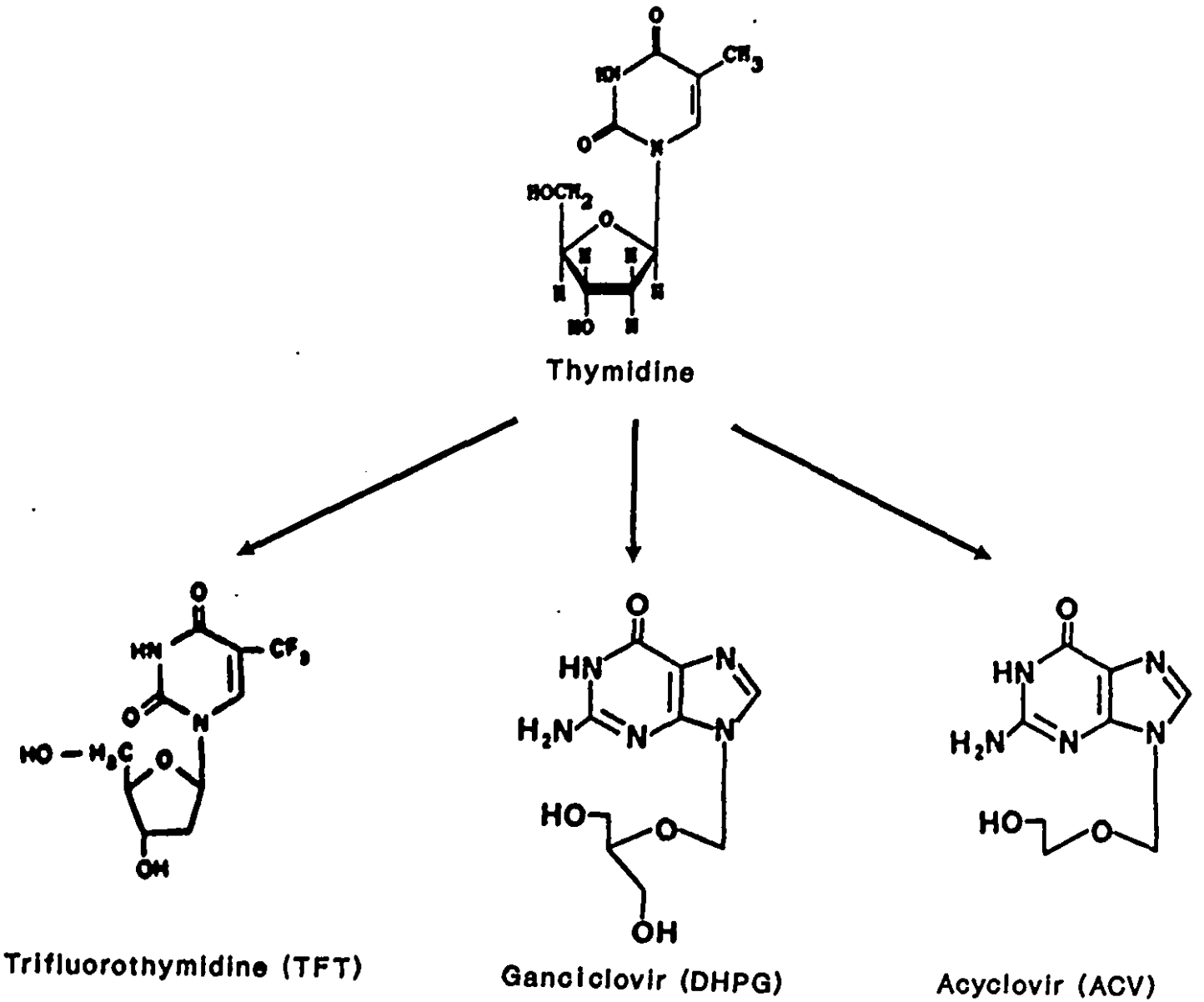
V.1. THE HSV-1 *tk* GENE AS A TARGET FOR SPONTANEOUS MUTATIONS

The HSV-1 thymidine kinase enzyme, an ATP-thymidine 5'-phosphotransferase, catalyses the transfer of the γ-phosphate from ATP to the 5'OH group of thymidine, and the usefulness of the *tk* gene as a reporter gene for mutagenesis studies stems from several advantageous characteristics (Davidson *et al.*, 1973). The well characterized HSV-1 *tk* gene has been sequenced (McKnight, 1980; Wagner *et al.*, 1981; McGeoch *et al.*, 1988) and the small size (3.4 kb) of this intronless viral gene permits the detection

of very small DNA changes to allow rapid characterization of the mutants. The *tk* gene encodes a protein of 41 kDa (Summers *et al.*, 1975), while the *tk* transcript is 1.4-kb in size (Zhang *et al.*, 1986). Moreover, the regulation of this gene has been studied in great detail (McKnight 1982; Jones *et al.*, 1985; Bandyopadhyay and Temin, 1984). Convenient selection for TK mutants is accomplished using nucleoside analogues that are cytotoxic for tk^+ cells, and selection for TK^+ revertants can be performed using HAT supplement. Unlike its cellular counterpart, the herpesvirus TK constitutes a general pyrimidine deoxynucleoside kinase, and its phosphorylating activity is not limited to thymidine but is extended to several structural analogues of nucleosides. The selection for tk^- mutants utilized nucleoside analogues that are not cytotoxic to the cells at the concentrations used in our system, and whose mechanism of action has also been characterized. Several different anti-herpes virus nucleoside analogues (Figure 2) are selectively and preferentially phosphorylated by the viral thymidine kinase to their monophosphate derivatives, which are subsequently converted by host cellular kinases to their triphosphate forms. These latter derivatives act as substrates for the DNA polymerase, disrupting DNA synthesis. The HSV-1 TK also shows significant thymidylate kinase activity (Chen *et al.*, 1979) and more promiscuity in its choice of phosphoryl donors, using either ATP or CTP. The absence of cell cycle regulation and the resistance to allosteric regulation by dTTP also distinguishes the viral TK from the cellular enzyme.

FIGURE 2

Molecular structure of thymidine and of the three nucleoside analogues used in this study, and preferentially phosphorylated by the HSV-1 thymidine kinase enzyme. Acyclovir and ganciclovir constitute analogues of guanine, while trifluorothymidine represents a structural analogue of thymidine.


Thymidine
Trifluorothymidine (TFT)
Ganciclovir (DHPG)
Acyclovir (ACV)

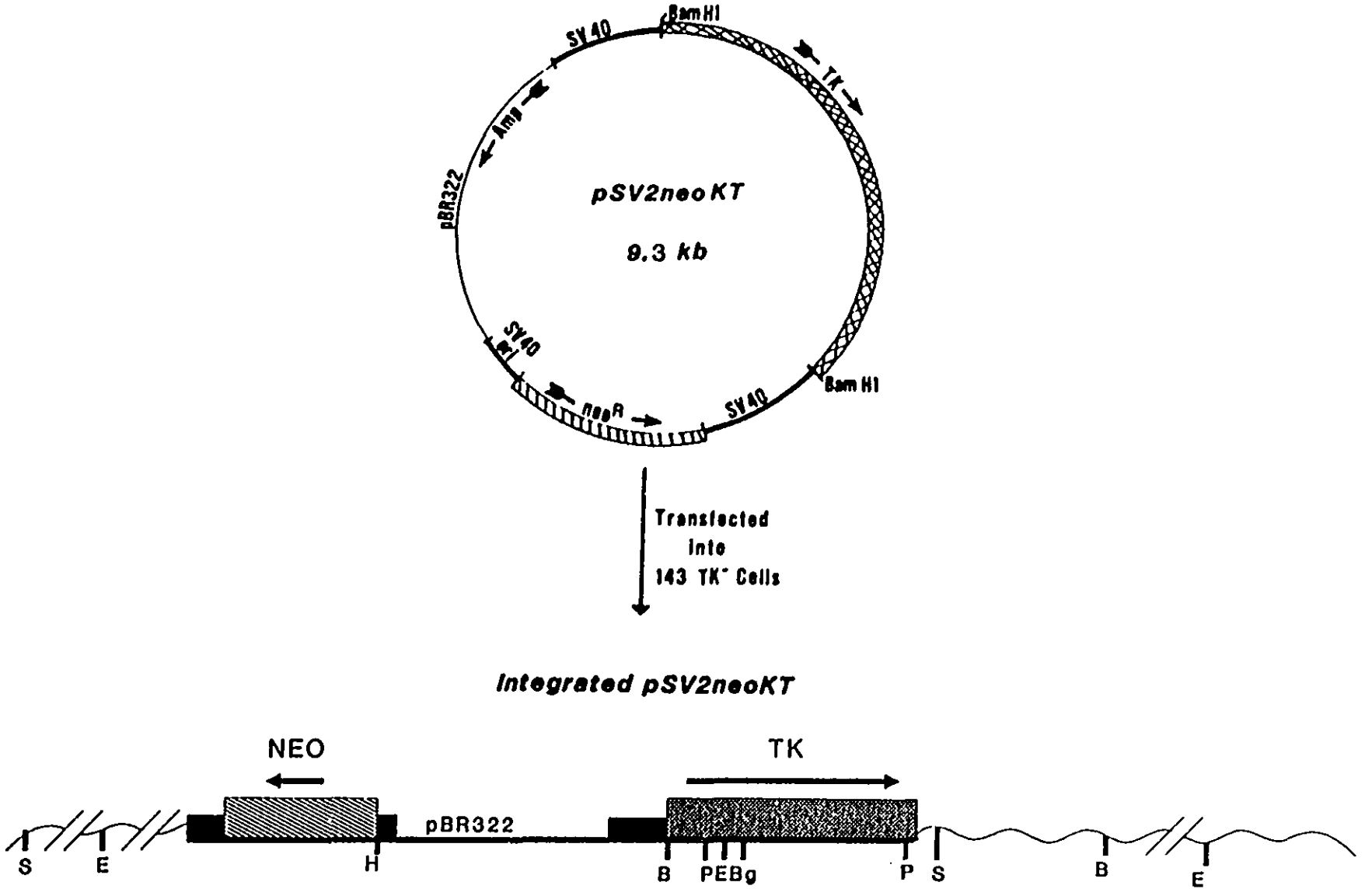
V.2. THE KT CELL LINE

To address spontaneous mutagenesis in the mammalian genome, this study exploits a hemizygous cell culture system, using a single chromosomal copy of the HSV-1 *tk* gene. The plasmid pSV2*neo*KT, previously constructed in our laboratory (Goring and DuBow, 1985) was transfected into 143B cells, a human cell line derived from the murine sarcoma virus transformed cell line R970-5 (Rhim *et al.*, 1975), which is deficient in thymidine kinase activity (Figure 3). The resultant neomycin-resistant (neo^R), thymidine kinase-positive (tk^+) cell line, referred to as the KT cell line, comprises a single copy of the chromosomally-integrated pSV2*neo*KT plasmid (Figure 3).

In addition to sequences allowing replication and ampicillin-resistance in bacteria, the plasmid pSV2*neo*KT contains the neomycin resistance gene (*neo*), which encodes an aminoglycoside 3' phosphotransferase (APH)II. This enzyme confers resistance to the aminoglycosides neomycin (or G418) in mammalian cells, and kanamycin in bacteria, through the phosphorylation of these protein synthesis-blocking compounds. In pSV2*neo*KT, the *neo* gene, under the control of the SV40 early promoter and adjacent to the *tk* gene, serves to screen out gross chromosomal alterations (such as non-disjunction of chromosomes or the loss of the entire plasmid) since the presence of a functional *neo* gene is ensured by the constant presence of G418 in the culture medium. The plasmid also possesses the thymidine kinase gene from HSV-1, which serves as the target for spontaneous mutation. Since transfection of the vector corrected the defect in thymidine kinase activity in 143B cells, the KT cell line can grow in HAT medium, which imposes an absolute requirement for a functional *tk* gene. Aminopterin blocks different steps in

FIGURE 3

Schematic representation of the plasmid pSV2*neo*KT and its integrated form into the 143B cell line to constitute the *neo*^{R}/*tk*$^{+}$ KT cell line. The arrows indicate the orientation of the *neo* and HSV-1 *tk* genes. The curved line represents cellular genomic DNA and certain restriction sites are also indicated: B=*Bam*HI, P=*Pvu*II, E=*Eco*RI, Bg=*Bgl*II, S=*Sac*I, and H=*Hind*III.


BamHI
SV40
TK
Amp
pBR322
pSV2neoKT
9.3 kb
SV40
neoR
SV40
BamHI
Transfected
into
143 TK⁻ Cells
Integrated pSV2neoKT
NEO
TK
pBR322
S
E
H
B
PEBg
P
S
B
E

the biosynthesis of purines and pyrimidines by interfering with folate metabolism. Consequently, cells can grow only if provided with exogenous hypoxanthine and thymidine to permit the utilization of the salvage pathways (Figure 1).

V.3. ANTIVIRAL COMPOUNDS UTILIZED TO SELECT FOR MUTATIONS IN THE HSV-1 *tk* GENE

Several viral functions are efficient targets for antiviral agents. Non-structural proteins such as kinases and polymerases are inhibited by antiviral agents, while their cellular counterparts are not significantly affected. The broad substrate specificity of the viral thymidine kinase (TK), a key enzyme in the pyrimidine salvage pathway (Figure 1), has been exploited to develop antiviral agents (Cheng *et al.*, 1981; Balfour, 1984; Elion, 1993; Coen, 1993).

V.3.1. ACYCLOVIR

Acyclovir (9-[(2-hydroxyethoxy)methyl]guanine; ACV) is a specific and effective antiviral agent against herpes simplex infections. This analogue of guanosine, with an acyclic side chain (Figure 3), is efficiently phosphorylated to ACV-monophosphate by the herpesvirus TK, but very poorly by the cellular TK (Elion *et al.* 1977, 1978; Fyfe *et al.*, 1978). The monophosphate derivative of ACV is then metabolized to the triphosphate (ACV-TP) through a diphosphate intermediate, by the host cellular GMP kinase [ATP:GMP phosphotransferase (Miller and Miller, 1980)]. The antiviral activity of ACV results from the incorporation of its triphosphate form into the elongating DNA. Since

the acyclic side chain is lacking the 3' hydroxyl group, the incorporation of the next nucleotide will be prevented, causing the termination of DNA synthesis. ACV-TP is also a potent inhibitor of the HSV-1 DNA polymerase (Elion *et al.*, 1977; Furman *et al.*, 1980) while the cellular α-DNA polymerase is not significantly affected. ACV is neither a substrate for, nor an inhibitor of, the cellular DNA polymerase β (Reardon, 1989). The cytotoxicity exhibited by ACV is not significant in human cells, which remain viable at concentrations up to 20 mM, the limit of solubility of ACV (Schaeffer *et al.*, 1978; Furman *et al.*, 1980; Davidson *et al.*, 1981). Because phosphorylation of ACV occurs almost strictly in virally infected cells, or in cells containing the HSV-1 *tk* gene, this guanine derivative proved to be a potent inhibitor of herpes simplex virus replication.

Resistance to ACV is conferred by two distinct viral loci: DNA polymerase and thymidine kinase (Coen and Schaffer, 1980; Coen *et al.*, 1982; Sanders *et al.*, 1982; Schnipper and Crumpacker, 1980; Crumpacker *et al.*, 1982a; Larder *et* al., 1983; Darby *et al.*, 1984;). Despite the important implication of drug resistance in HSV-1 and HSV-2 infections (Birch *et al.*, 1990; reviewed by Coen, 1993), especially with the emergence of resistant strains in immunocompromised patients (following organ transplantation or infection with the AIDS virus HIV-1), the genetic analyses of mutant viruses are scarce. Biochemical studies revealed that mutations of the viral *tk* gene fall into three classes: $TK^{altered}$, with modified substrate specificity which allows the phosphorylation of thymidine while the capacity to phosphorylate drugs has been severely impaired (Darby *et al.*, 1981; Larder *et al.*, 1983); $TK^{reduced}$, with a diminished TK activity (Furman *et al.*, 1981); and TK^{0} showing complete gene inactivation (Summers *et al.*, 1975; Crumpacker *et al.*,

1982a,b). Point mutations located near the catalytic site of the TK polypeptide and nonsense mutations causing a shorter polypeptide have been reported (Summers *et al.*, 1975). In clinical isolates of HSV-1, mutations to acyclovir resistance in the *tk* gene occured with a frequency of 10^{-4}, due to the richness of the *tk* gene in homonucleotide repeats of C and G residues (Parris and Harrington, 1982). ACV^R in the highly homologous HSV-2 virus (Swain and Galloway, 1983) was caused by point mutations resulting in the alteration of single amino acids, and frameshift mutations causing premature chain termination (Kit *et al.*, 1987). Inhibitors of ribonucleotide reductase potentiate the antiviral action of ACV by decreasing the intracellular pool of dGTP (Coen *et al.*, 1989a; Spector *et al.*, 1989; Reardon and Spector, 1991). In actively growing and virally infected cells, ACV increases the intracellular concentration of thymidine (Harmeuberg *et al.*, 1985) and thymidine-TP (Karlsson *et al.*, 1986). This imbalance in DNA precursor pools may be mutagenic, and may influence the relative antiviral efficacy of other nucleoside analogues. A discussion concerning potential consequences of a disequilibrium in the intracellular pool of nucleosides is presented in Chapter 6.

V.3.2. GANCICLOVIR

Since ACV proved to be a potent and safe antiviral compound (Whitley and Gnann, 1992), enormous work subsequently focused on the development of new nucleoside analogues with potentially unique effects towards virus-specified enzymes involved in DNA metabolism. Another analogue of guanosine was found to be a potent inhibitor of cytomegalovirus (CMV) and, to a lesser extent, of HSV-1 and HSV-2 DNA replication:

ganciclovir (Figure 3) (9-[(1,3-dihydroxy-2-propoxy)methyl]-guanine; DHPG) (Smith *et al.*, 1982; Cheng *et al.*, 1983; Smee *et al.*, 1983). Ganciclovir is metabolized to DHPG-monophosphate by the viral thymidine kinase (for HSV-1 and HSV-2) and by a deoxyguanosine kinase encoded by CMV, in which no TK activity is known (Matthews and Boehme, 1988). After subsequent phosphorylations by a cellular guanylate kinase followed by a phosphoglycerate kinase, the incorporation of DHPG-TP into the elongating DNA inhibits DNA replication (Smee *et al.*, 1985). DHPG also competitively inhibits the viral DNA polymerase and the very slow cellular catabolism of DHPG-TP amplifies its selective antiviral activity. The cellular DNA polymerase is only slightly inhibited by DHPG (St.Clair *et al.*, 1987). DHPG is phosphorylated more effectively than ACV (Smee *et al.*, 1985) but may not be an absolute DNA chain terminator because of its two available hydroxyl groups in the acyclic sugar moeity.

DHPG has mostly been used for the treatment of CMV retinitis and against CMV infections following organ transplant. The viral gene from CMV that contributes to DHPG-phosphorylation was identified to be gene product UL97 (Biron *et al.*, 1986). Thus, the genetic analysis of the mechanisms of resistance to DHPG in cytomegalovirus (Stanat *et al.*, 1991) or in herpes simplex viruses is still unclear.

V.3.3. TRIFLUOROTHYMIDINE

The fluorinated nucleoside analogue trifluorothymidine (5-trifluoromethyl-2'-deoxyuridine; trifluridine; TFT) is an effective anti-herpes virus compound (Heidelberger and Anderson, 1964) still in use for the topical treatment of herpes simplex keratitis (Kury

and Crosby, 1967; Birch *et al.*, 1992). TFT, whose molecular structure is shown in Figure 3, is phosphorylated both by viral and cellular kinases (Fujiwama *et al.*, 1970; Wigdahl and Parkurst, 1978). The incorporation of TFT-triphosphates into the DNA results in abnormal transcription and translation (Fujiwara and Heidelberger, 1970; Otto *et al.*, 1982) as well as strand breakage leading to incompletely joined fragments of DNA (Fujiwara *et al.*, 1970). The herpes simplex virus TK possesses thymidylate synthetase activity, which is strongly and non-competitively inhibited by the 5'-monophosphate of TFT. In contrast to the cellular thymidine kinase, the HSV-1 TK is not subject to feedback inhibition by dTTP since its activity is not controlled by the absence or presence of dTTP. The antiviral activity of TFT is also potentiated by the resistance of the viral TK to TFT-TP-mediated allosteric inhibition. Additionally, this fluorinated pyrimidine, through its direct inhibition of thymidylate synthase, leads to depletion of intracellular thymidine-TP, and this depletion in dTTP consequently exerts cytotoxic and mutagenic effects (Kunz, 1982). Even though TFT demonstrated very slight teratogenecity in chicken embryos (Kury and Crosby, 1967), its utilization in cancer chemotherapy is favored by its strong tumor-inhibitory properties (Dexter *et al.*, 1972; Armstrong *et al.*, 1992). As a consequence of its limited clinical use, the genetic basis of resistance to TFT has not been extensively evaluated.

V.4. COMBINATION OF DRUGS TO ENRICH FOR POTENT MUTATIONS

A strategy involving the enrichment for spontaneous potent mutations was developed in order to prioritize the analysis of those spontaneous mutational events leading to

serious consequences for the mammalian genome. The aforementioned antiviral compounds demonstrate slightly different modes of action, and the mechanisms of resistance to these drugs may differ. The complete termination of DNA chain elongation by ACV-TP, the specific inhibition of cellular thymidylate kinase by TFT-MP, the generation of nicks in the DNA by TFT-TP, and the incomplete block of DNA synthesis by DHPG-TP, represent only a few of the specificities that suggest a characteristic mode of action for each drug. Thus mutations in the HSV-1 *tk* gene that confer resistance to these nucleoside analogues may also vary.

Hence, it becomes theoretically possible that the use of various combinations of these drugs can cause the elimination of simple mutations conferring resistance to a single drug, and to selectively enrich for potent mutations that confer resistance to multiple compounds. The use of combinations of drugs may also limit the recovery of mutations that did not occur in the *tk* gene, yet conferred resistance to a single drug, such as a mutation that specifically inhibits the transport of a particular antiviral compound. Despite its structural similarity to the nucleoside guanine, ACV does not utilize the nucleoside transporter to traverse the cell membrane, but rather uses a more general purine nucleobase carrier (Davidson *et al.*, 1981; Mahony *et al.*, 1988). Without elaborating, Matthews and Boehme (1988) suggested that DHPG may also be subject to a different uptake mechanism. The transport of TFT through the cell membrane has not been deciphered, but the modification of the 3' group on thymidine alters the interaction of nucleoside analogues with the nucleoside transporter and permits the penetration, by non-facilitated diffusion, in the case of the anti-HIV nucleoside analogue AZT.

Consequently, the utilization of the hemizygous cell culture system provided by the KT cell line, combined with the possibility to use multiple nucleoside analogues to enrich for non-revertible *tk*$^-$ mutations, should allow the isolation and characterization of recessive mutations in a human chromosomal context.

OUTLINE OF THE THESIS

Spontaneous mutations constitute important events implicated in genetic diversity and evolution, and are also associated with the formation of genetic diseases and cancer. Spontaneous mutations can be classified as "moderate" and "potent" mutations. Moderate mutations often display an unstable and "leaky" mutant phenotype. In contrast, potent mutations severely affect the encoded phenotype of a gene and may comprise more important DNA alterations. The characterization of potent mutations in conventional genetic selection schemes may be hindered by the frequent isolation of moderate mutations. Thus a selection procedure was developed to enrich for potent mutations in a single-copy HSV-1 *tk* gene chromosomally-integrated in the human KT cell line.

This work describes the selective enrichment for spontaneous potent mutations in the *tk* gene, and the characterization of these mutational events. This thesis comprises a general introduction (Chapter 1), Material and Methods are described in Chapter 2, and experiments which addressed spontaneous mutagenesis are described in Chapters 3 through 5. Finally, the experimental data from this study is summarized and the implications of the results, as well as prospects for future research, are discussed.

In Chapter 3, spontaneous potent *tk* mutants were selected on the basis of their resistance to the nucleoside analogues: acyclovir (ACV), trifluorothymidine (TFT) and ganciclovir (DHPG). The enrichment procedure using combinations of ACV+TFT+DHPG may allow a selective enrichment for potent mutations conferring resistance to three drugs, over the "background" of moderate mutations is described in Chapter 3. The utilization

utilization of ACV+TFT+DHPG in a combined fashion resulted in a reduction of the apparent mutation frequency in the *tk* gene to 3×10^{-7}. The potent mutations also seemed enriched for mutations which severely affect the TK gene product, as demonstrated by Western blotting analyses. The interactions and potential synergisms between the nucleoside analogues used in the selection procedure is also presented in Chapter 3.

Chapter 4 describes the selection of ninety *tk*$^-$ mutants resistant to various combinations of nucleoside analogues. Southern blotting and PCR analyses revealed that less than 25% of the potent *tk*$^-$ mutants underwent DNA alterations implicating more than 25-50 bp, which consisted mostly of deletions of the 3' portion of the *tk* gene, or the entire loss of the *tk* gene. An unusual deletion/duplication event, as well as a complex rearrangement of the *tk* gene, are also presented and discussed.

The fine-structure analysis of the small, yet potent, mutations demonstrated that more than 75% of the potent *tk*$^-$ mutants resulted from DNA alterations involving less that 5 bp. The level of *tk* mRNA produced in the small potent *tk*$^-$ mutants was evaluated by Northern blotting, which revealed that wild type levels of *tk* transcripts were observed in only 30% to 36% of the potent mutants. The investigation of the methylation status and the possible involvement of the multiple drug resistance phenotype (MDR) in the generation of the small potent *tk*$^-$ mutants is also presented in Chapter 5.

CHAPTER 2

MATERIAL AND METHODS

I. CONSTRUCTION AND PROPAGATION OF THE KT CELL LINE

I.1. CONSTRUCTION OF THE KT CELL LINE

The construction of the plasmid vector pSV2*neo*KT and of the KT cell line has been previously described by Goring and DuBow (1985). Briefly, the vector pSV2*neo*KT was transfected, by the calcium phosphate coprecipitation method, into the human *tk*$^-$ cell line 143B derived from the murine sarcoma virus transformed line R970-5 (Rhim *et al.*, 1975). A *tk*$^-$ clone, containing a single copy of the chromosomally-integrated vector, was selected, and propagated in HAT medium [0.1 mM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine (Littlefield, 1964)] supplemented with G418, which selects for thymidine kinase activity and resistance to neomycin (G418). The resulting cell line constitutes the *neo*^{R}/*tk*$^-$ KT cell line. A schematic representation of the KT cell line with the single-copy, chromosomally-located vector is presented in Figure 3 of Chapter 1.

I.2. PROPAGATION AND STORAGE OF CELL LINES

The *tk*$^-$ human cell line 143B was grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Gibco Canada) containing 2.5 μg/ml amphotericin B (Fungizone), 100 units/ml penicillin, 100 μg/ml streptomycin (Pen-Strep, Gibco Canada), 10% (v/v) fetal bovine serum (FBS; Gibco Canada). This medium was supplemented with 400 μg/ml G418 (final concentration) (Geneticin, Sigma Canada) and HAT (Sigma

Canada), at the concentrations mentioned above, for the propagation of the *neo*^{R}/*tk*$^+$ KT cell line. The *tk*$^-$ mutant cell lines, selected using the appropriate combination of nucleoside analogues, were maintained and propagated in DMEM medium containing G418 and TFT at final concentrations of 400 μg/ml and 1.0 μg/ml, respectively.

The absence of contamination of the cell lines by mycoplasma was confirmed using the Mycoplasma Detection Kit (Boehringer Mannheim), an enzyme immunoassay for the detection of mycoplasmas/acholeplasmas in cell culture, according to the manufacturer's protocol.

To freeze down the cell lines for long term storage, the adhering cells were detached by adding 1.5 ml of trypsin [2.5 mg/ml in versene (0.2 g EDTA / L of phosphate buffered saline (PBS) [PBS is 8g/L NaCl, 0.2g/L KCl, 1.15g/L Na_2HPO_4 and 0.5g/L KH_2PO_4, pH 7.3] into a 250-ml tissue culture flask containing cells grown to 80% confluency, for approximately 3 minutes. The trypsin was neutralized by the addition of 8 ml of DMEM medium containing fetal bovine serum (FBS, Gibco Canada). The cells were recovered by centrifugation at 5000xg for 10 minutes at room temperature, resuspended in 1.25 ml of ice cold FBS, and kept on ice. Then, 0.25 ml of buffered DMSO [50% (v/v) DMSO, 3% (w/v) Trizma base, 1.25% (w/v) dextrose, 1.68% (w/v) citric acid, pH 6.7] was added, mixed rapidly but gently with the cells, and the cryogenic vial with the cell suspension was transferred to a -70°C freezer. After 30 minutes, the vials were stored in liquid nitrogen.

The cells were thawed out by briefly immersing the tube in a 37°C water bath. The thawed cells were rapidly and gently transferred into a 5-ml disposable Falcon tube

(Fisher Scientific) containing 2.0 ml of ice cold FBS, and immediately subjected to centrifugation at 5000xg at room temperature. The cell pellet was then gently resuspended in the appropriate cell culture medium and the cell suspension rapidly moved to a 37°C incubator with 5% CO_2.

II. SELECTION FOR *tk*⁻ MUTANTS AND RATIONALE FOR THE ENRICHMENT OF POTENT *tk*⁻ MUTANTS

II.1. NUCLEOSIDE ANALOGUES

The antiviral drug acyclovir (ACV) was kindly provided by Burroughs Wellcome Canada Inc. and was prepared as a 2 mg/ml stock solution in DMEM medium, whose pH had been precisely adjusted to 7.0 at 37°C with hydrochloric acid. Stock solutions of ganciclovir (150 µg/ml) (DHPG, Synthex Canada) were prepared in double distilled water. Trifluorothymidine (TFT), obtained from Sigma (Canada), was prepared as a 100 µg/ml solution in DMEM medium, pH 7.3. The stock solutions were stored at -20°C until use. The concentration of antiviral compound which was not cytotoxic to the *tk*⁻ parental cell line 143B, yet inhibited the growth of the *tk*⁺ KTcell line, was determined for each nucleoside analogue. The compounds were used in the selection procedure at the following final concentrations: ACV, 20 µg/ml (88.8 µM); TFT, 1.0 µg/ml (3.4 µM); and DHPG, 1.5 µg/ml (5.4 µM).

II.2. SELECTION PROCEDURE

The KT cells were removed from DMEM medium, supplemented with HAT and

G418, and seeded into medium containing only G418 in order to allow for a mutation in the *tk* gene to occur and be expressed (Goring *et al.*, 1985). After four days, this medium was removed, the cells were washed with phosphate-buffered saline (PBS) [0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.14% (w/v) Na_2HPO_4, 0.024% (w/v) KH_2PO_4, pH 7.3] and trypsinized by adding 1.5 ml of trypsin into a 250-ml tissue culture flask (Nunc, Gibco Canada). The cells were then washed with DMEM medium and replated, at a cell density of $1.2x10^6$ cells/150 mm tissue culture dish (Nunc, Gibco Canada), in medium containing G418 to select for the presence of the *neo* gene, as well as TFT to select for potential mutant tk^- clones. The medium was changed every three days until the appearance of tk^- colonies, for approximately 15 days. To select for mutants resistant to both ACV and TFT, the cells were plated, at the same cell density as for the TFT selection, in DMEM medium supplemented with G418, as well as ACV plus TFT. Again, the cells were fed with the selective medium for 15 days, until tk^- colonies emerged. A slightly modified selection procedure was utilized to select for tk^- mutants resistant to combinations of drugs that included DHPG (ACV+TFT+DHPG). Goring and DuBow (1985) reported that the selection for $DHPG^R$ mutants in the HSV-1 *tk* gene was precluded by the killing of the tk^- clones, most likely due to the release in the culture medium of toxic metabolites derived from DHPG. Thus, to circumvent this problem and to be able to select for $DHPG^R$ mutants, the KT cells were first grown in selective medium containing only TFT for 10 days. When most of the tk^- cells had been eliminated by the presence of TFT, the remaining cells were fed medium containing TFT plus DHPG or a combination of ACV, TFT, and DHPG for an additional 5 to 10 days.

After the selection period, the emerging colonies were isolated and propagated in selective medium containing the appropriate combination of antiviral compounds. Subsequently, they were maintained in selective medium containing G418 plus TFT. The *tk*$^-$ mutants were named according to the antiviral compound used in the selection procedure, followed by the clone number and the number of the selection experiment. For example, mutant ATD-1-19 was selected on the basis of its resistance to a combination of ACV, TFT and DHPG, and it was the first (1) clone isolated from selection number 19.

III. METHYLATION ASSAY

To determine if 5-azacytidine (5-azaC), an inhibitor of cytosine methylation, could induce the re-expression of the *tk* gene from the *tk*$^-$ mutants, the mutant cell lines were treated with medium containing G418 plus 2 μM 5-azaC (Sigma) for three days, and the medium was changed every day. Following this recovery period of three days, the cells were grown in medium supplemented with G418 and HAT until the emergence of revertant *tk*$^+$ colonies.

IV. MUTATION AND REVERSION FREQUENCIES

IV.1. STAINING AND COUNTING OF THE COLONIES

The *tk*$^-$ or *tk*$^+$ clones were enumerated by adding 12 ml of fixative solution [4% (v/v) formaldehyde, 0.5% (w/v) NaH_2PO_4, 0.65% (w/v) Na_2HPO_4, 1.5% (v/v) methanol] to 150 mm tissue culture plates containing the clones, for 2 hours. Once the cells were fixed,

the clones were stained with 12 ml of Giemsa stain [50% (v/v) glycerol, 50% (v/v) methanol, 0.5% (w/v) Giemsa, (Merchant *et al.*, 1964)] for 2 hours, and washed twice with PBS. The colonies were counted, and the mutation or reversion frequencies determined.

IV.2. CALCULATION OF THE MUTATION AND REVERSION FREQUENCIES

The mutation frequency corresponds to the average number of colonies obtained with a given selection experiment, divided by the number of tk^+ cells initially seeded in G418/TFT medium (1.2×10^6 cells).

To determine the reversion frequency of the tk^- mutants to the wild type TK^+ phenotype, the tk^- cells from a given mutant were propagated in selective medium containing G418 and TFT before they were seeded in medium supplemented with G418 only, in order to remove the selection pressure for the presence of a mutated tk^- gene. After 4 days, the cells were seeded at a cell density of 1.0×10^5 cells per 100 mm tissue culture plate (in 5 different plates) and fed every three days with fresh medium containing G418 plus HAT, to select for the presence of tk^+ revertants. After 15 days, the tk^+ colonies were fixed and stained as described above, and the colonies were enumerated. The reversion frequency corresponds to the number of tk^+ colonies divided by the number of tk^- cells originally seeded in G418/HAT medium.

V. DNA ISOLATION

V.1. ISOLATION OF TOTAL GENOMIC DNA FROM HUMAN CELLS

The monolayer of KT cells, *tk*⁻ mutants cell lines, or revertant cell lines contained in a 250-ml tissue culture flask was detached by trypsinization as described above. The cells were washed with PBS, transferred into a 12-ml disposable Falcon tube (Fisher Scientific Canada), and subjected to centrifugation at 5000xg for 10 minutes, at room temperature. The cell pellet was resuspended in 0.5 ml of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. Pronase (20 mg/ml in 10 mM Tris-HCl, pH 7.6) and 10% (w/v) sodium dodecyl sulfate (SDS) were added to a final concentration of 1.90 mg/ml and 0.53%, respectively, and the mixture was incubated at 37°C for 4 hours. The genomic DNA was then extracted by adding 1 volume (500 µl) of phenol and 1 volume (500 µl) of chloroform containing isoamyl alcohol (IAA) ($CHCl_3$:IAA; 24:1, v/v). This phenol-chloroform:IAA extraction was repeated, followed by two chloroform-IAA extractions, using 1 volume (500 µl) of chloroform-IAA. The DNA was precipitated by the addition of sodium acetate (pH 5.2) to a final concentration of 0.3 M, and ethanol (final concentration 70%). The flocculent DNA fibers were isolated with a U-shaped micropipette and transferred to a 1.5-ml conical centrifuge tube to be dried by vacuum dessication. After drying, the genomic DNA pellet was resuspended in 500 µl of 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) by gentle agitation on a rotary shaker at 4°C for 24 hours. The concentration of DNA was determined spectrophotometrically according to the following formula: 1 OD_{260} corresponds to approximately 50 µg/ml of double-stranded DNA. To estimate the purity of the DNA, readings were also performed at

OD_{280}, the ratio OD_{260}/OD_{280} was determined, and purified DNA preparations demonstrated a ratio of 1.8 to 2.0. The genomic DNA was then stored at 4°C until use.

V.2. LARGE SCALE PLASMID DNA ISOLATION

Bacterial strains containing the plasmid DNA to be isolated were grown in 1 L of LB broth (Miller, 1972). To perform chloramphenicol amplification of the plasmid content of the bacterial cells, 75 µg/ml of chloramphenicol were added to the cells when the culture reached a cell density of $4x10^8$ cells/ml, and the cells were further grown for 16 hours at 32°C. Plasmid DNA extraction was performed according to the alkali lysis method described in Sambrook *et al.* (1989), followed by purification of the plasmid solution by ultracentrifugation at 35 000 rpm, using a Beckman Ti-75 rotor, for 48 hours at 4°C in a cesium chloride/ethidium bromide gradient (Sambrook *et al.*, 1989). The bands from the gradients were visualized with UV light and the lower band corresponding to plasmid DNA was collected with a #21 gauge needle. Ethidium bromide was extracted from the DNA preparation by four extractions using equal volumes of isopropanol saturated with 40X SSC (6 M NaCl, 0.6 M sodium citrate, pH 7.0). The plasmid DNA preparation was finally dialyzed against 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

V.3. RAPID PLASMID DNA ISOLATION

Alternatively, plasmid DNA was isolated by a small scale rapid procedure. Briefly, bacterial cells were lysed in lysis buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10

mM EDTA, 4 mg/ml lysozyme). After addition of the alkaline solution [0.67% (w/v) SDS, and 0.13 N NaOH, final concentrations], the proteins and high molecular weight DNA were precipitated with ammonium acetate (final concentration 2.5 mM), and recovered by centrifugation at 10000xg for 15 minutes at 4°C. The plasmid DNA present in the supernatant fluid was precipitated with 1 volume of isopropanol at room temperature and the DNA was recovered by centrifugation for 10 min. at 10000xg at 4°C. The DNA pellet was dried by vacuum dessication and resuspended in 250 µl of 1X TE. The DNA was purified using a slight modification of the procedure described previously: with one phenol extraction, and two extractions with 1 volume of ether each. The DNA was finally precipitated with sodium acetate (pH 5.2) plus absolute ethanol (final concentrations of 0.3 M and 70%, respectively). After centrifugation and vacuum dessication, the DNA pellet was resuspended in 50 µl of 1x TE and stored at -20°C.

VI. DNA MANIPULATIONS AND MODIFICATIONS

VI.1. RESTRICTION ENDONUCLEASE HYDROLYSIS

All restriction enzymes were purchased from Bethesda Research Laboratories Inc. (BRL), New England Biolabs (NEB), Pharmacia, or Boehringer Mannheim Canada Ltd. Most of the DNA restriction endonuclease hydrolyses were performed at 37°C in 6 mM Tris-HCl (pH 7.5), 75 mM NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 125 µg/ml bovine serum albumin (BSA, Fraction V, Pentex). Reactions involving *Sma*I, *Taq*I, *Bst*NI and *Hae*III were performed according to the manufacturer's instructions.

DNA fragments were resolved by agarose gel electrophoresis through 0.75% to 1.0%

agarose gels in electrophoresis buffer (40 mM Trizma base, 20 mM acetic acid, 1 mM EDTA pH 8.0). Smaller fragments were separated by polyacrylamide gel electrophoresis in TBE buffer (45 mM Tris-borate, 1mM EDTA, pH 8.0). The acrylamide concentrations of the gels varied from 5% to 16%, depending on the size of the fragments to be resolved. After electrophoresis, both types of gels were submerged in a solution of ethidium bromide (0.5 µg/ml in water) for 15 min., then immersed in deionized water for 20 min. The DNA was visualized under UV illumination and the gels were photographed using a Polaroid apparatus and Polaroid 4x5 instant high speed films (type 57).

VI.2. DNA LIGATION REACTIONS

Approximately 0.5 µg of the DNA fragment(s) to be inserted into a plasmid vector were mixed with previously linearized plasmid DNA, using 2 units of T4 DNA ligase (BRL) in linker ligation buffer (0.06 M Tris-HCl pH 7.5, 10 mM $MgCl_2$, 15 mM DTT, 0.75 mM ATP, 1 mM spermidine, 50 µg/ml gelatin) in a total volume of 50 µl. The ratio of insert to vector varied, but the total amount of DNA per ligation reaction did not exceed one microgram. The mixture was incubated at 15°C for 16 hours, and then 10 min. at 65°C. The vectors used to generate recombinant plasmid constructs are listed in Table 1.

VI.3. RADIOACTIVE LABELLING OF DNA FRAGMENTS

The 3.4-kb *Bam*H1 fragment from pSV2*neo*KT, which comprises the HSV-1 *tk* gene, was purified from agarose gels by electroelution (Sambrook *et al.*, 1989). Alternatively,

DNA fragments were prepared using the Geneclean® procedure, following the manufacturer's protocol. Approximately 200 ng of DNA fragments were uniformly labelled with [α-^{32}P] dGTP or dCTP (3000 Ci/mmole, Amersham), using oligonucleotide primers from the Regional DNA synthesis Laboratory (Calgary, Canada), according to the random priming method according to Sambrook *et al.*, (1989). As a radioactive probe for Southern and Northern blotting experiments, approximately 1 x 10^8 to 1 x 10^9 cpm/μg DNA were added to each membrane.

For the multidrug resistance analysis, the MDR-1 probe (kindly provided by Dr. Philippe Gros, Department of Biochemistry, McGill University) consisted of the C-terminal portion of the mouse multidrug resistance (MDR-1) gene comprised within a 1.3-kb *Eco*RI fragment isolated from plasmid pUC19 by electroelution (Devault and Gros, 1990; Raymond *et al.*, 1990).

The actin DNA probe, used as an internal control for the Northern blotting analysis, was isolated as a 1.4 kb fragment from plasmid pUC119 and was a generous gift from Dr. Michael Ratcliffe (Department of Microbiology and Immunology, McGill University).

VII. BACTERIAL TRANSFORMATION OF PLASMID DNA

Bacterial cells were made competent for transformation by a modification of the $CaCl_2$ method (Mandel and Higa, 1970). Briefly, 0.5 ml of a culture of one of the bacterial *Escherichia coli* strains listed in Table 1 were grown overnight in LB broth, transferred into a flask containing 20 ml of LB broth, and the cells were grown to mid-log phase. The culture was subjected to centrifugation at 3500xg for 10 min. at 4°C and the

TABLE 1

Escherichia coli strains and plasmid vectors

BACTERIAL STRAINS

STRAIN	GENOTYPE
DH1:	F$^-$, *gyr*A96, *thi*, *hsd*R$^-$ M$^+$, *sup*E44, *rec*A1, *mcr*A$^+$, *mcr*B$^+$ Origin: Hanahan (1983)
HB101:	F$^-$, *hsd*S20 (r_B^-, m_B^-), *rec*A13, *leu*B6, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rpsL20* *xyl*-15, *mtl*-1, *sup*E44, λ^-, *mcr*A$^-$ *mcr*B$^-$ Origin: Boyer and Rolland-Dussoix (1969)
NM522:	*thi*, *rps*L, *hsd*S(r^-, m^+), *sup*E44, Δ(*lac*$^-$ *pro*AB) / F' *tra*D36, *pro*AB, *lac*I^{q}ZΔ M15 Origin: Gough and Murray (1983)

PLASMID VECTORS

PLASMID	CHARACTERISTICS
pSV2*neo*KT	Amp^R, *neo*^{R}, *tk*$^+$ Goring and DuBow (1985)
pUC118,pUC119	Amp^R, f1 origin, *lacZ*' (amino-terminal α portion) Vieira and Messing (1987)
pUC19	Amp^R, lacZ' (amino-terminal α portion) Yanisch-Perron *et al.* (1985)
pBluescript KS +/-	Amp^R, f1 origin, *lacZ*' (amino-terminal α portion) [Stratagene Cloning Systems, Short *et al.* (1988)]

cell pellet was resuspended in 12 ml of 10 mM $MgSO_4$, 2 mM Hepes (pH 7.5). Following an incubation period of 20 min., the centrifugation step was repeated and the cells were resuspended in 12 ml of 50 mM $CaCl_2$, 2 mM Hepes (pH 7.5). After 25 min. on ice, the cells were again subjected to centrifugation and resuspended in 2 ml of 50 mM $CaCl_2$, 2 mM Hepes (pH 7.5). The competent cells were kept on ice until used.

For bacterial transformation of plasmid constructs, approximately 0.5 µg of ligated plasmid DNA was added to 200 µl of 50 mM $CaCl_2$, 2 mM Hepes (pH 7.5), and 200 µl of competent *E. coli* cells. The mixture was chilled on ice for 25 min. and incubated at 37°C for 3 min. ("heat shock"), before adding 1.5 ml of LB broth, and the transformation mixture was incubated at 37°C, with gentle agitation, for 1.5 hours. Then, 200 µl of transformed cells were spread onto appropriate selective media [50 µg/ml of ampicillin to select for the presence of the *bla* (Amp^R) gene, or 50 µg/ml of kanamycin to select for a functional neo^R gene], and the plates were incubated at 37°C for 12 to 16 hours. When plasmid vectors pUC118, pUC119, or pBlueScript were used, the presence of an insert disrupting the β-galactosidase gene (*lac*Z-α complementation) was monitored by the white (as opposed to blue) appearance of the colonies on selective LB plates [1.5% (w/v) agar] supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, final concentration: 40 µg/ml) plus isopropyl-β-D-thiogalactoside (IPTG, Research Organics Inc.) at a final concentration of 1 mM.

Bacterial transformants were mastered as 1 cm^2 patches using a grid pattern, on selective plates (Miller, 1972). The presence of plasmids of the appropriate size was monitored by the "cracking" procedure. Half of a previously "mastered" patch was

transferred, using a sterile toothpick, into 50 µl of "cracking" buffer [50 mM Tris-HCl pH 8.0, 1.0% (w/v) SDS, 2 mM EDTA, 0.4 M sucrose, 0.01% (w/v) bromophenol blue]. Cell lysis was allowed to occur at room temperature for 15 minutes. Cellular debris was removed by centrifugation at 10,000xg for 15 minutes at room temperature, and the supernatant fluid, containing the DNA, was subjected to electrophoresis through a 0.75% (w/v) agarose gel in electrophoresis buffer (40 mM Trizma base, 20 mM acetic acid, 1 mM EDTA, pH 8.0).

VIII. ENZYMATIC DNA AMPLIFICATION

VIII.1. THE POLYMERASE CHAIN REACTION (PCR) PROCEDURE

Genomic DNA (200 ng) was mixed, in a 50-µl reaction, with PCR buffer (67 mM Tris-HCl (pH 8.8), 16.6 mM NH_4SO_4, 10 mM 2-mercaptoethanol), 10% (v/v) DMSO, 1.25 mM of each of the four dNTPs, and 25 pmoles of each oligonucleotide primer. Alternatively, DNA amplification was performed in a 100-µl reaction mixture containing 200 ng of genomic DNA, 0.125 mM of each dNTP (prepared as 250 mM stock solutions in 100 mM sodium phosphate buffer, pH 7.0) and 15 pmoles of each primer in the following PCR buffer: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$. In both situations, the mixture was incubated at 95°C for 5 min., prior to the addition of one unit of *Taq* DNA polymerase (Perkin Elmer-Cetus). A layer of mineral oil (20 µl) was added to the 1.5-ml conical centrifuge tube (Eppendorf) containing the PCR reaction to prevent evaporation, the mixture incubated at 80°C for 3 min., and the amplification reaction stimulated by the addition of 4 mM $MgCl_2$ (final concentration). The mixture was then

incubated for 3 min. at 94°C in order to denature the template DNA, and then the reaction was subjected to 30 cycles of primer annealing (1 min. at 56°C), primer extension (1.5 min. at 72°C), and denaturation (1.5 min. at 94°C). A final incubation of 1.5 min. at 56°C, followed by 5 min. at 72°C, ensured the complete extension of the amplified DNA products. Successful DNA amplification was monitored by removing a 10-µl aliquot directly from the PCR mixture and loading it onto a 0.75% (w/v) agarose gel in electrophoresis buffer. The presence of DNA was verified by photography of the ethidium bromide-stained agarose gel under UV light.

The oligonucleotide primers used in the PCR procedure were synthesized either by the Regional DNA Synthesis Laboratory (Calgary, Canada) or by the Sheldon Biotechnology Center of McGill University (Montréal, Canada). The sequence and the location of the primers used to amplify various segments from the coding region of the *tk* gene are depicted in Figure 1.

VIII.2. CLONING OF PCR-AMPLIFIED DNA SEGMENTS

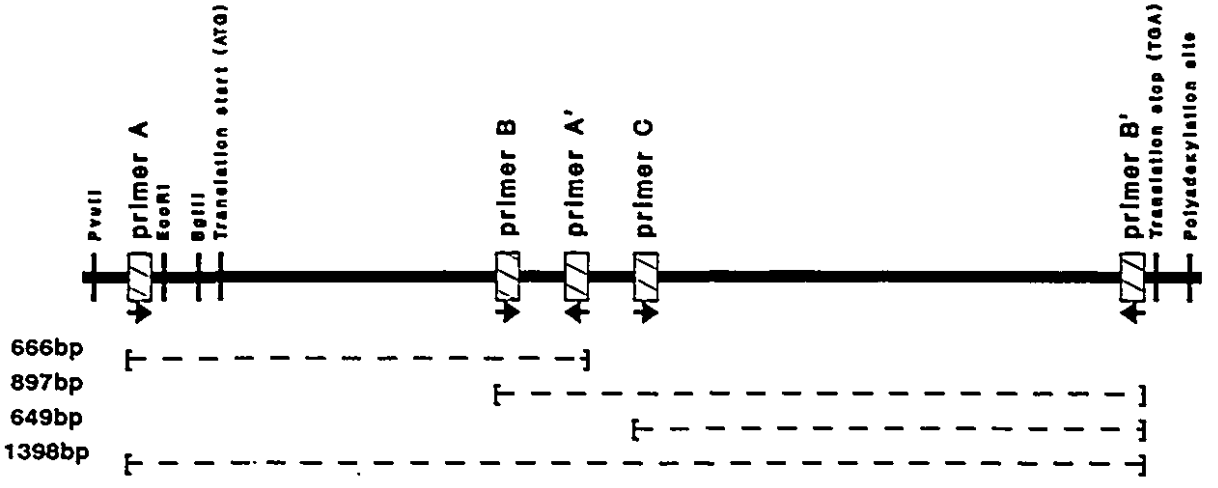
Following PCR, the mineral oil was removed by a chloroform extraction using 150 µl of chloroform for each 100-µl PCR mixture. The DNA was then purified and precipitated as described for the rapid plasmid DNA isolation (V.3). DNA segments from the *tk* gene to be sequenced were cloned into plasmid vectors.

To clone the *tk* segments comprised between primers C and B' in mutant ATD-1-19 (Figure 1), approximately 1 µg of amplified DNA was cleaved with *Sma*I plus *Sal*I restriction endonucleases. The hydrolysed fragments were ligated into plasmid pUC118

FIGURE 1

Schematic representation of the *tk* gene from the integrated plasmid pSV2*neo*KT. The position of the oligonucleotide primers (boxes) used in the PCR analyses and the size (in bp) of the *tk* gene segments amplified with different sets of primers are delineated. Relevant restriction sites, the *tk* translation start and stop codons, as well as the mRNA polyadenylation sites are shown.

Also depicted are the DNA sequences of the oligonucleotide primers. A single mismatch (boxed nt) was created in primers A and B in order to engineer *Sma*I and *Sal*I restriction sites, respectively (shown as underlined sequences), facilitating the cloning of the PCR amplified *tk* segments into plasmids. Primers A' and B' contain endogenous *Sal*I and *Sma*I sites, respectively.



oligonucleotide primer A: 5'TGGCGGTGTCCCGGGAAGAAATAT3'

oligonucleotide primer A': 5'AAGGCATGCCCATTGTTATCTGGG3'

oligonucleotide primer B: 5'TATCGTCGACGTACCCGAGCCGAT3'

oligonucleotide primer B': 5'TTAGCCTCCCCCATCTCCCGGGCAAA3'

oligonucleotide primer C: 5'ATCTTCGACCGCCATCCCATC3'

(Vieira and Messing, 1987) previously cleaved with *Sma*I plus *Sal*I. Alternatively, the fragments were cloned in the opposite orientation into *Sal*I plus *Sma*I-cleaved plasmid pBluescript (KS+) (Short *et al.*, 1988).

IX. DNA SEQUENCING

IX.1. SINGLE-STRANDED DNA SEQUENCING

Single-stranded DNA from the recombinant bacterial clones was prepared as described by Vieira and Messing (1987). One microgram of ssDNA, from the recombinant plasmids containing sequences amplified from the mutant *tk*⁻ genes, was used directly for dideoxy sequencing (Sanger *et al.*, 1977) with the Sequenase DNA Sequencing Kit (USB Biochemical Corp.), according to the manufacturer's instructions, and using [α-^{35}S] dATP (500 Ci/mmol; NEN DuPont Canada). Either the "-40" or the "reverse" primers provided with the Sequenase Kit, or one of the primers specific to the *tk* gene (Figure 1), were used in the sequencing reactions. The samples were subjected to electrophoresis through 8% polyacrylamide/ 7M urea denaturing gels, also according to the manufacturer's protocol. After electrophoresis in 0.6X TBE buffer (10X TBE represents 450 mM Tris-borate, 10 mM EDTA, pH 8.0), the sequencing gels were fixed in 12% (v/v) acetic acid, 10% (v/v) methanol, before being rinsed in deionized water, dried under vacuum and exposed to Kodak XAR-5 films under DuPont Cronex intensifying screens at -70°C for 16 hours to 3 days. Alternatively, the sequencing mixtures were subjected to electrophoresis through Long Ranger™ gels (J.T.Baker Inc.), according to the manufacturer's instructions.

IX.2. DOUBLE-STRANDED DNA SEQUENCING

Approximately 5 µg of plasmid DNA, prepared as described previously, were denatured by alkaline treatment, according to Sambrook *et al.*, 1989. The denatured DNA was sequenced by the dideoxy chain termination method using the Sequenase Kit (USB Corp.) and following the manufacturer's instructions for double-stranded DNA sequencing.

X. SOUTHERN BLOTTING ANALYSES

Genomic DNA (10 µg) from the KT cells, the 143B cell line or the *tk*⁻ cell lines was hydrolysed to completion with various restriction endonucleases, as described above. The cleaved DNA was subjected to electrophoresis through a 0.75% (w/v) agarose gel in electrophoresis buffer. The DNA was then transferred onto a Hybond-N nylon membrane (Amersham, Canada) using the method described by Southern (1975). Prehybridization was performed by immersing the membrane for 4 hours at room temperature in hybridization buffer (1.43X Denhardt's solution, 3X SSC, 20 mM $NaPO_4$ pH 7.0, 100 µg/ml denatured *E. coli* DNA). Denhardt's reagent at a concentration of 50x contains 1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (Pentex Fraction V, Sigma), while 20X SSC represents 3.0 M NaCl and 0.3 M sodium citrate. DNA hybridization was performed for 16 hours at 55°C in the same buffer supplemented with 50% (v/v) deionized formamide and 10% (w/v) dextran sulfate. Finally, approximately $1x10^9$ cpm/µg of the radioactively-labelled *tk* gene was added to the hybridization buffer as a DNA probe. When the MDR-1 probe was used in the multidrug resistance studies, the Southern blotting procedure was identical except that hybridization

was performed using 40% (v/v) formamide.

XI. NORTHERN BLOTTING ANALYSES

XI.1. ISOLATION OF TOTAL RNA

Eucaryotic cells, from which total RNA was to be isolated, were grown to approximately 75% confluency in 250-ml tissue culture flasks. Solutions were rendered RNAse-free by treatment with 0.1% (v/v) diethyl pyrocarbonate (DEPC, Sigma Canada) for 16 hours at room temperature and then autoclaved for 20 min. at 121°C, at 15 lb/sq. in. (liquid cycle). The cells were trypsinized, washed with RNAse-free ice-cold PBS, and subjected to centrifugation at at 5000xg, 4°C, for 10 minutes. Total RNA was isolated from the cellular pellet using the RNaid® PLUS Kit, according to the manufacturer's instructions. Briefly, total RNA was isolated using a guanidine thiocyanate solution, extracted with acid phenol, and purified on RNAMATRIX™. The concentration of RNA was determined spectrophotometrically according to the following formula: 1 OD_{260} corresponds to approximately 40 µg/ml of RNA. To estimate the purity of the total RNA preparation, readings were also performed at OD_{280}, the ratio OD_{260}/OD_{280} was determined, and purified RNA preparations demonstrated a ratio of 1.8 to 2.0. The RNA was finally stored at -70°C.

XI.2. NORTHERN BLOTTING PROCEDURE

Aliquots containing 20 µg of total RNA, in a volume of 4.5 µl, were mixed with 2 µl of 5X MOPS [5X MOPS is 0.2 M MOPS pH 7.0, 50 mM NaOAc, 5 mM EDTA],

3.5 µl of formaldehyde and 10 µl of formamide. These samples were heated at 55°C for 15 min, before 2 µl of loading buffer [50% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue and 0.4% (w/v) xylene cyanol] were added. The samples were finally loaded on a denaturing agarose gel consisting of 0.75% agarose, 2.2 M formaldehyde, and 1X MOPS. As molecular size standards, a 0.24 to 9.5 kb-RNA ladder (BRL Life Technologies Ltd) was utilized according to the manufacturer's protocol. Following electrophoresis, the gel was soaked in 20X SSC for 20 min., stained for 5 min. in a solution of ethidium bromide (0.5 µg/ml in water), rinsed for 5 min. in deionized H_2O, and photographed under UV illumination. The RNA was transferred onto a nylon membrane (Hybond-N, Amersham) according to the following procedure. The gel was put on three layers of Whatmann 3MM paper previously soaked in 10X SSC, then gently covered with the nylon membrane, followed by four layers of dry Whatmann 3MM paper, plus paper towels (thickness of 2 inches), a glass plate, and lead weights. After the overnight transfer, the filter was baked at 80°C for one hour in a vacuum oven, and kept at room temperature until used. The presence of a specific thymidine kinase mRNA transcript was detected using the 3.4-kb *Bam*HI fragment from pSV2*neo*KT, comprising the *tk* gene, as a DNA probe following the protocol described for Southern blotting hybridization.

As an internal control for Northern blotting, the presence of actin gene transcripts was also determined. The radioactivity was removed from the membranes, previously hybridized using the labelled *tk* gene, by immersion in 80% (v/v) formamide, 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 1% (W/V) SDS, for two hours at 65°C. The membranes were allowed to air dry and utilized for the Northern blotting procedure using

the radioactively-labelled actin gene as a probe, as described above.

XII. WESTERN BLOTTING ANALYSES

Protein extracts were prepared using the single-detergent lysis buffer procedure described in Sambrook *et al.* (1989). The proteins were subjected to SDS-polyacrylamide gel electrophoresis and electro-transferred onto a membrane, as follows. Approximately 200 μg of total protein from the crude extracts were boiled for 3 min. in 5X SDS-gel electrophoresis loading buffer [250 mM Tris-HCl pH 6.8, 500 mM dithiothreitol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 50% (v/v) glycerol] before they were loaded on an SDS-polyacrylamide gel [12% polyacrylamide for the resolving gel and 5% for the stacking gel, (Laemmli, 1970). Prestained molecular weight protein standards (high range) were purchased from BRL Life Technologies Ltd., and treated in the same way as the crude protein extracts. Electrophoresis was performed in Tris-glycine electrophoresis buffer [25 mM Tris-HCl pH 8.3, 250 mM glycine, 0.1% (w/v) SDS] at 8 V/cm until the dye front reached the resolving gel, followed by electrophoresis at 15 V/cm, until the dye front reached three quarters of the length of the gel.

When electrophoretic separation was complete, the proteins were electro-transferred in transfer buffer [12.5 mM Tris-HCl pH 8.3, 96 mM glycine, and 20% (v/v) methanol] to an Immobilon-P membrane (Millipore) according to Sambrook *et al.*, 1989. Detection of specific antigen, using rabbit anti-TK polyclonal antibodies (kindly provided by Dr. William C. Summers, Yale University), was performed using the horseradish peroxidase assay system of the Immuno-Blot Assay Kit (Bio-Rad), according to the manufacturer's

protocol. When the color reaction was complete, the Western blots were scanned with a Logitech ScanMan 256 scanner and image quality was adjusted for brightness and contrast using the Adobe Photoshop version 2.5 software. The images were then printed on a Hewlett Packard HP LaserJet 4 printer.

CHAPTER 3

THE ENRICHMENT FOR POTENT MUTATIONS IN A CHROMOSOMALLY-INTEGRATED, SINGLE-COPY, HSV-1 *tk* GENE

I. INTRODUCTION

Spontaneous mutations in mammalian cells comprise a variety of molecular changes ranging from simple point mutations implicating one or a few base pairs, to complex chromosomal rearrangements (Knudson, 1986; Rudiger, 1990). This heterogeneity of spontaneous mutational events can be arbitrarily categorized into two classes which would reflect their impact on the phenotype of a given organism: the moderate mutations, and the potent mutations.

Moderate mutations often demonstrate a subtle effect on the function of a gene. For example, a point mutation causing a single amino acid change may not result in a dramatic modification of the gene product. This "leakiness", or ability of the mutated gene product to display at least residual activity, as well as the capacity to readily revert to the wild type phenotype, and the high frequency at which these simple events occur, represent some characteristics of the moderate mutations (reviewed in Chapter 1).

In contrast, a second class of mutational events, the potent mutations, are implicated in the complete and stable alteration of the phenotype associated with a given gene and, consequently, may play a more prominent role in the phenotype, and the evolution, of an organism. Potent spontaneous mutations can comprise various DNA alterations, and may involve extensive alterations of the DNA sequence, such as large deletions or insertions,

and duplications of a DNA segment. It must be appreciated that a point mutation, such as a simple nonsense mutation causing the premature termination of the corresponding polypeptide chain, or a point mutation in a critical position within the catalytic site of a given enzyme, may also result in complete gene inactivation (a null phenotype). However, most moderate mutations could revert to the wild type phenotype at a detectable frequency. Indeed, the nature of the potent mutations, occurring at a much lower frequency compared to moderate mutations, greatly reduces the ability for phenotype reversal. Hence, spontaneous potent mutations could be defined as low frequency, rarely reversible mutational events which can completely, or greatly, alter the phenotype associated with a mutated gene.

Due to the frequent occurrence of moderate mutations in most genetic selection schemes, numerous studies on spontaneous mutagenesis in mammalian cells have been reported to lead to the characterization of few potent mutations at the *aprt* (De Jong *et al.*, 1988; de Boer and Glickman, 1989; Phear *et al.*, 1989) and the *hprt* (Fuscoe *et al*, 1986; Oller and Thilly, 1992) loci. When major DNA rearrangements involving genetic loci were investigated, large deletions associated with loss of heterozygosity were mostly recovered in the *aprt* (Ward *et al.*, 1990), *hprt* (Albertini *et al.*, 1990; Fuscoe *et al.*, 1992c) and *tk* (Amundson and Liber, 1992; Oller and Thilly, 1992) genes.

The frequent recovery of small point mutations, or large deletions through loss of heterozygosity, suggests that certain rare molecular events implicated in mutagenesis may not be readily characterized. The particularity of our selection scheme on spontaneous mutations lies on the design of a procedure to minimize the isolation of the

aforementioned events frequently encountered when conventional mammalian cell systems are utilized. Indeed, the study of a single-copy gene should preclude the selection of mutational events such as large deletions due to loss of heterozygosity. Moreover, as described in Chapter 1, the KT cell line utilized in this study comprises two selectable markers, the *tk* gene (the target for gene inactivation), and the neomycin resistance (*neo*) gene, which confers resistance to the aminoglycoside drug G418. The maintenance of a selection pressure for a functional neo^R gene should allow us to screen out gross chromosomal aberrations, such as non-disjunctions or total plasmid loss, in order to focus on mutations which specifically affect the single-copy, chromosomally-located HSV-1 *tk* gene. Hence, this study aims at the characterization of potent (and infrequent) mutational events in a hemizygous system, and may provide a different approach for the understanding of the types of mutations with potentially severe consequences on the phenotype of a gene and, consequently, on the organism.

As mentioned above, this mutagenesis study in human cells in culture, utilizes a hemizygous system in which the single-copy HSV-1 *tk* gene serves as the target for spontaneous mutational inactivation. An enrichment procedure has been developed with the aim to diminish the recovery of the moderate mutations, and to selectively enrich for more potent mutations which confer resistance to multiple nucleoside analogues. Briefly, the utilization of ACV, TFT, and DHPG, three nucleoside analogues that display slightly different structures, mechanisms of action, and modes of uptake by the cell, would permit the enrichment for potent mutations that completely inactivate the *tk* gene. Theoretically, a simple point mutation may confer resistance to a single antiviral drug, whereas

resistance to a combination of compounds may require a more important alteration of the *tk* gene, with respect to the resulting gene inactivation.

Hence, spontaneous mutations in the single-copy, chromosomally-integrated, HSV-1 *tk* gene were selected using different combinations of drugs, and the enrichment hypothesis was evaluated by the verification of the nature of the *tk*$^-$ mutants following three criteria: **1.** the frequency of occurrence of the mutations in the *tk* gene, **2.** the ability of the *tk*$^-$ mutants to revert to the wild type TK$^+$ phenotype, and **3.** the capacity of the mutant *tk* genes to generate a detectable TK gene product (Figure 1).

II. RESULTS

II.1. FREQUENCY OF MUTATIONS IN THE CHROMOSOMALLY-INTEGRATED HSV-1 *tk* GENE

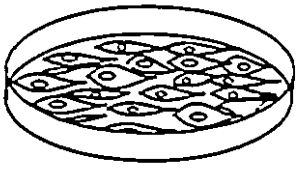
The cell culture system for the selection of spontaneous mutations in a single-copy and stably-integrated HSV-1 *tk* gene utilizes the KT cell line, hemizygous for the *neo* and *tk* genes, as developed by Goring and DuBow (1985). In order to provide an accurate interpretation of subsequent data, the stability of the chromosomally-located pSV2*neo*KT vector, and the number of mutants in the *tk* gene from the KT cell line population at the time of selection, were verified (Table 1). The KT cells (*neo*^{R}/*tk*$^+$), maintained in G418/HAT medium, were trypsinized and replated at low cell density (1.2×10^6 cells/150-mm tissue culture plate) in selective G418/TFT medium, immediately imposing upon the cells a selection pressure for the presence of a functional *neo* gene, but a mutated *tk* gene conferring resistance to TFT. In parallel experiments, the KT cells were

FIGURE 1

Schematic representation of the selection for *tk*$^-$ mutants in the KT cell line **(A)**, and the selective enrichment for potent mutations in the *tk* gene from the KT cell line **(B)**.

KT cells (neo^R / tk^+)

maintained in G418/HAT medium



Remove HAT for 4 days to allow the occurrence and expression of spontaneous mutations in the *tk* gene

Maintain the presence of G418 to screen out gross chromosomal alterations

A

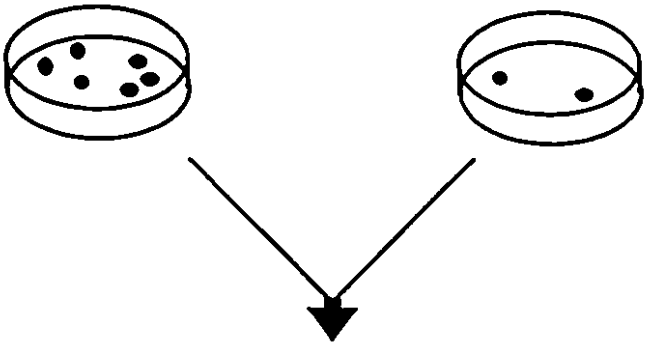
B

selection using ACV, or TFT individually

(+G418)

selection using combinations of ACV + TFT + DHPG or ACV + TFT

(+G418)



1. Determine frequency of occurrence of the mutations
2. Evaluate stability of the mutations
3. Determine the effect of the mutations on TK protein
4. PCR analysis of the mutated *tk* gene

TABLE 1

Mutation frequencies in the HSV-1 *tk* gene using different selection protocols

KT in G418/HAT → G418/TFT (15 days)	Mutation frequency = 1×10^{-7}
KT in G418/HAT → G418 (4 days) → G418/TFT (15 days)	Mutation frequency = 5×10^{-4}
KT in G418/HAT → G418 (9 days) → G418/TFT (15 days)	Mutation frequency = 5×10^{-4}

seeded in medium containing only G418 for a period of 4 or 9 days, to allow a mutation in the *tk* gene to occur and be expressed. These cells were subsequently fed selective G418/TFT medium for 15 days. Triplicate experiments were performed and the neo^R/tk^- colonies obtained through the different selection procedures were enumerated as described in Chapter 2. Table 1 reveals that the KT cell line population contained TFT^R tk^- mutants at a frequency of 1×10^{-7}.

Acyclovir (ACV), trifluorothymidine (TFT), and ganciclovir (DHPG) represent three antiviral compounds specifically targetted for the HSV-1 thymidine kinase enzyme (TK) (Coen, 1993; Elion, 1993) and utilized in our study to select for spontaneous mutations in a chromosomally-integrated HSV-1 *tk* gene. The use of nucleoside analogues, in a combined fashion, reduces the apparent frequency of mutation in the *tk* gene, which declines to 3×10^{-7} when ACV, TFT, and DHPG were combined in the selection procedure (Table 2). ACV and TFT exhibited different potencies in selecting for mutations in the *tk* gene, as shown by the 10-fold difference in their respective mutation frequency (5×10^{-3}, and 5×10^{-4}). As shown in Table 3, the different efficacies of the nucleoside analogues as selective agents were corroborated by the finding that 100% of the mutants resistant to TFT also demonstrated resistance to ACV, while only 14.3% were also $DHPG^R$. In contrast, 61.5% of the mutants selected with a combination of ACV and TFT displayed cross-resistance to DHPG (Table 3).

TABLE 2

Frequencies of mutation in the HSV-1 *tk* gene selected using different nucleoside analogues

Selective agent (including G418)	Mutation frequency (per cell per generation)
ACV	5×10^{-3}
TFT	5×10^{-4}
ACV+TFT	1×10^{-5}
TFT+DHPG	1×10^{-6}
ACV+TFT+DHPG	3×10^{-7}

TABLE 3

Cross-resistance of *tk*$^-$ mutants

Mutants	Resistance to		
	ACV	TFT	DHPG
ACV^R	NA	(43 %)[a]	ND
TFT^R	7/7 (100%)	NA	1/7 (14.3%)
ACV+TFT^R	NA	NA	8/13 (61.5%)

[a] From Goring and DuBow (1987)

ND: Not determined

NA: Non applicable

II.2. EVALUATION OF THE POTENTIAL INTERACTIONS BETWEEN ACV, TFT, AND DHPG DURING THE ENRICHMENT FOR POTENT MUTATIONS IN THE *tk* GENE

The rationale behind the enrichment for potent mutational events in the HSV-1 *tk* gene implies that a combination of nucleoside analogues with slightly different structures and mechanisms of action may reduce the selection of moderate mutations that would confer resistance to a single antiviral compound. To provide a coherent understanding of the effects of drug combinations during the selection for spontaneous *tk*$^-$ mutations, the combination strategy was investigated.

During the selection for spontaneous mutations in the *tk* gene from the KT cell line, a variation in the frequency at which *tk*$^-$ mutant clones emerged from the selection procedure was observed, depending on the selection protocols employed. The time required to isolate *tk*$^-$ clones (number of days in selective medium) was also found to depend on which nucleoside analogue was utilized. Hence, an evaluation of the interaction, and potential synergisms or antagonisms, between ACV, TFT, and DHPG was performed. The effectiveness of different combinations of drugs, as well as variations in the order of addition of the compounds during the selection for *tk*$^-$ mutants in the KT cell line, were evaluated.

To investigate the interaction between the antiviral compounds, the KT cells were treated with a single nucleoside analogue, prior to a selection procedure involving a combination of ACV+TFT+DHPG (Figure 2). The KT cells were maintained in DMEM medium, supplemented with G418 plus HAT, to ensure the continued presence of

FIGURE 2

Schematic representation of the selection procedure used to analyse the interaction between ACV, TFT and DHPG.

A.

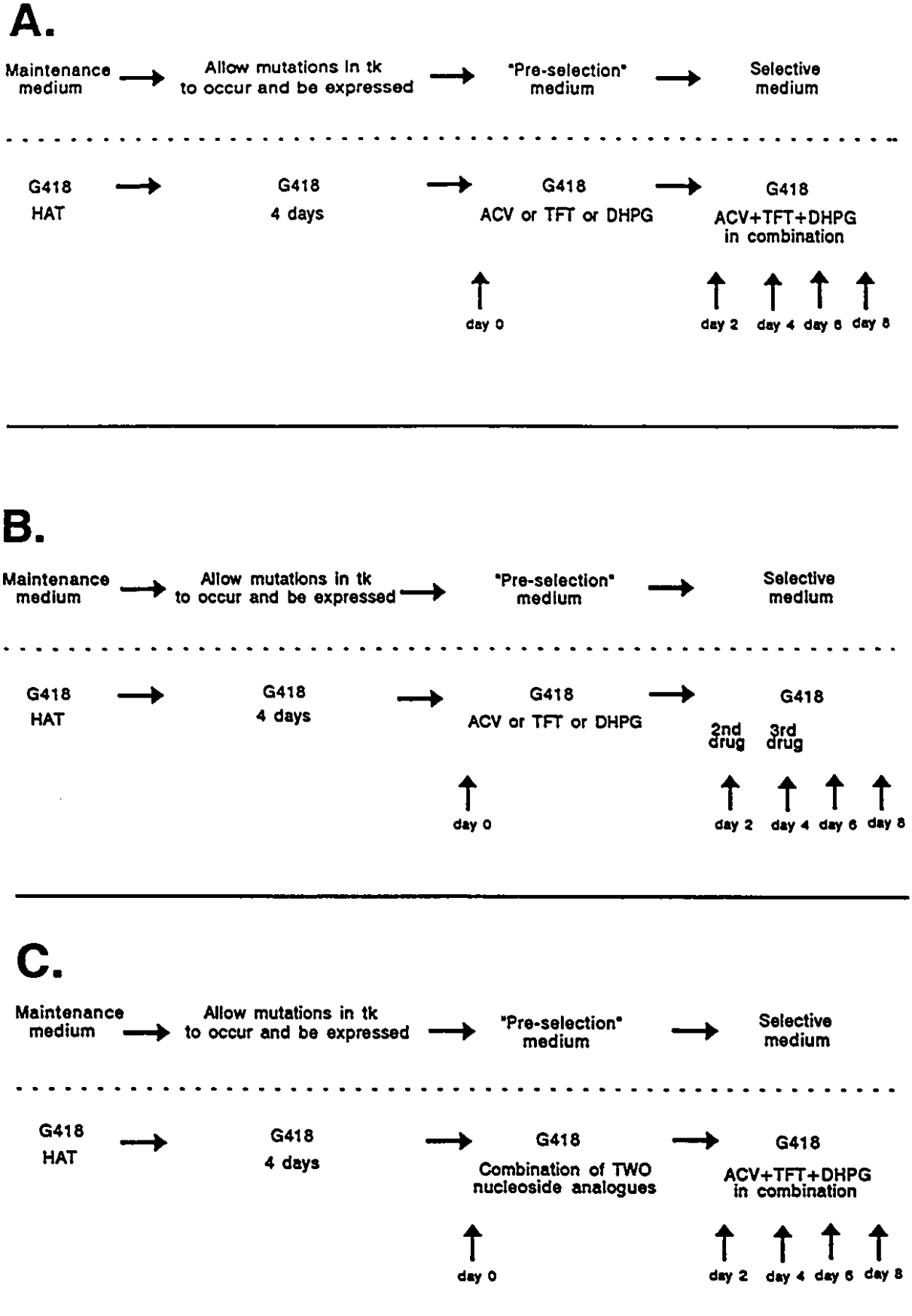
The KT cells (*neo*^{R}/*tk*$^+$) were maintained in DMEM medium supplemented with G418 and HAT. The cells were then trypsinized and replated ($1.5x10^5$ cells / 100-mm tissue culture plate) in medium containing only G418 for 4 days, and then with medium supplemented with G418 and one of the three nucleoside analogues (ACV or TFT or DHPG) for 2 days. This constituted the pre-selection step with a single antiviral drug. After 2 days, cell confluency had reached 100%, and the medium was replaced with one supplemented with all three drugs in combination (A+T+D medium). The cells were subsequently fed this multiple drug medium for 6 days.

B.

The KT cells (*neo*^{R}/*tk*$^+$) were maintained in DMEM medium supplemented with G418 and HAT. The cells were then trypsinized and replated ($1.5x10^5$ cells / 100-mm tissue culture plate) in medium containing only G418 for four days, and then with medium suplemented with G418, and one of the three nucleoside analogues (ACV or TFT or DHPG) for 2 days. This constituted the pre-selection step with a single antiviral drug. After 2 days, cell confluency had reached 100%, and the medium was replaced with one supplemented with a different nucleoside analogue for 2 days (from day 0 to day 2). At day 4, the medium was replaced with one containing the third and different drug for another four days.

C.

The KT cells (*neo*^{R}/*tk*$^+$) were maintained in DMEM medium supplemented with G418 and HAT. The cells were then trypsinized and replated ($1.5x10^5$ cells / 100-mm tissue culture plate) in medium containing only G418 for four days, and then with medium supplemented with G418, and two of the three nucleoside analogues (ACV or TFT or DHPG) for 2 days. This constituted the pre-selection step with two antiviral drugs. After 2 days, cell confluency had reached 100%, and the medium was replaced with one supplemented with all three drugs in combination (A+T+D medium). The cells were subsequently fed this multiple drug medium for 6 days.


A.
Maintenance medium
Allow mutations in tk to occur and be expressed
"Pre-selection" medium
Selective medium
G418 HAT
G418 4 days
G418 ACV or TFT or DHPG
G418 ACV+TFT+DHPG in combination
day 0
day 2
day 4
day 6
day 8
B.
Maintenance medium
Allow mutations in tk to occur and be expressed
"Pre-selection" medium
Selective medium
G418 HAT
G418 4 days
G418 ACV or TFT or DHPG
G418
2nd drug
3rd drug
day 0
day 2
day 4
day 6
day 8
C.
Maintenance medium
Allow mutations in tk to occur and be expressed
"Pre-selection" medium
Selective medium
G418 HAT
G418 4 days
G418 Combination of TWO nucleoside analogues
G418 ACV+TFT+DHPG in combination
day 0
day 2
day 4
day 6
day 8

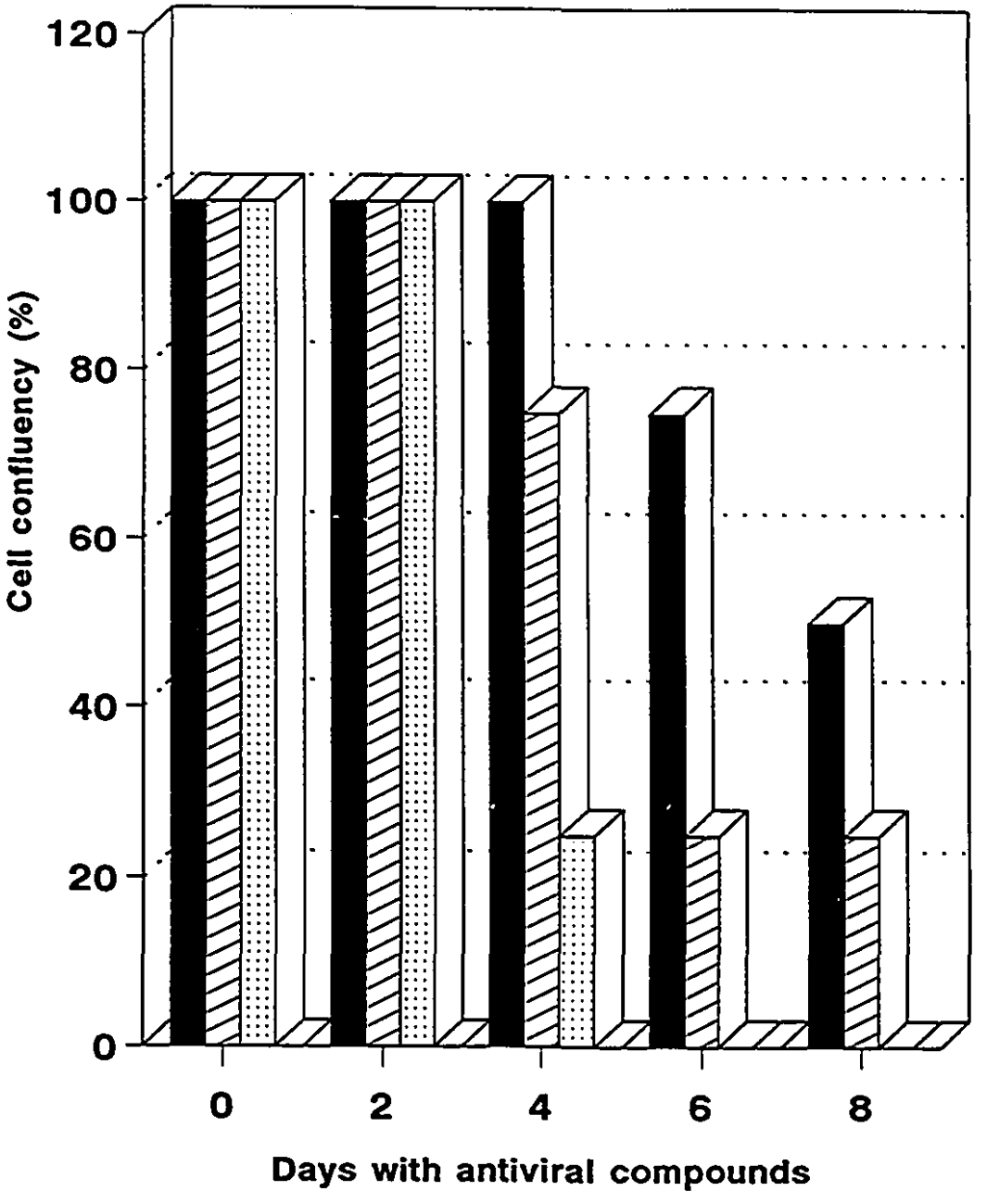
functional *neo* and *tk* genes, respectively. As described in Chapter 2, the cells were then fed with medium containing G418 only for 4 days, in order for a mutation in the *tk* gene to occur and be expressed. The cells were subsequently resuspended by trypsin treatment and grown in medium supplemented with a single nucleoside analogue for 2 days. This step is referred to as the "pre-selection" step. After a 2-day pre-selection period using a single selective agent, the cells were fed multiple-drug selective medium (ACV+TFT+DHPG or A+T+D medium) for an additional 6 days (Figure 2A). The effect of this pre-selection on the subsequent selection of *tk*$^-$ mutants using ACV, TFT, and DHPG in combination were evaluated as the relative cell survival at different times following drug treatment. The results were monitored as the percentage (%) of cell confluency at the following time intervals; day 0: upon the addition of a single nucleoside analogue (pre-selection step), day 2: upon the addition of multiple drug selective medium, as well as at day 4, 6, and 8: after 2, 4, and 6 days in multiple drug selective medium, respectively.

The results corresponding to the selection procedure described in Figure 2A are presented in Figure 3. When ACV was used as the only nucleoside analogue in the pre-selection period (solid bars), cell survival was still 100% after 2 days in A+T+D medium (day 4), and as high as 50% after 6 days (day 8). When TFT was the pre-selection drug utilized (hatched bars), different results were obtained. Two days following the addition of A+T+D medium (at day 4), cell survival was reduced to 75%, and to 25% after 6 days in multiple drug medium (day 8). The utilization of a third nucleoside analogue in the pre-selection period, DHPG, was also evaluated. However, the use of DHPG as the pre-

FIGURE 3

Bar graph representing the percentage of cell survival in A+T+D multiple drug selective medium, following a 2-day pre-selection in medium containing a single nucleoside as the selective agent.

Day 0: Addition of a single nucleoside analogue (pre-selection period)
Day 2: Addition of multiple drug selective medium (ACV+TFT+DHPG)
Day 4: After 2 days in multiple drug selective medium (ACV+TFT+DHPG)
Day 6: After 4 days in multiple drug selective medium (ACV+TFT+DHPG)
Day 8: After 6 days in multiple drug selective medium (ACV+TFT+DHPG)


120
100
80
60
40
20
0
Cell confluency (%)
0
2
4
6
8
Days with antiviral compounds

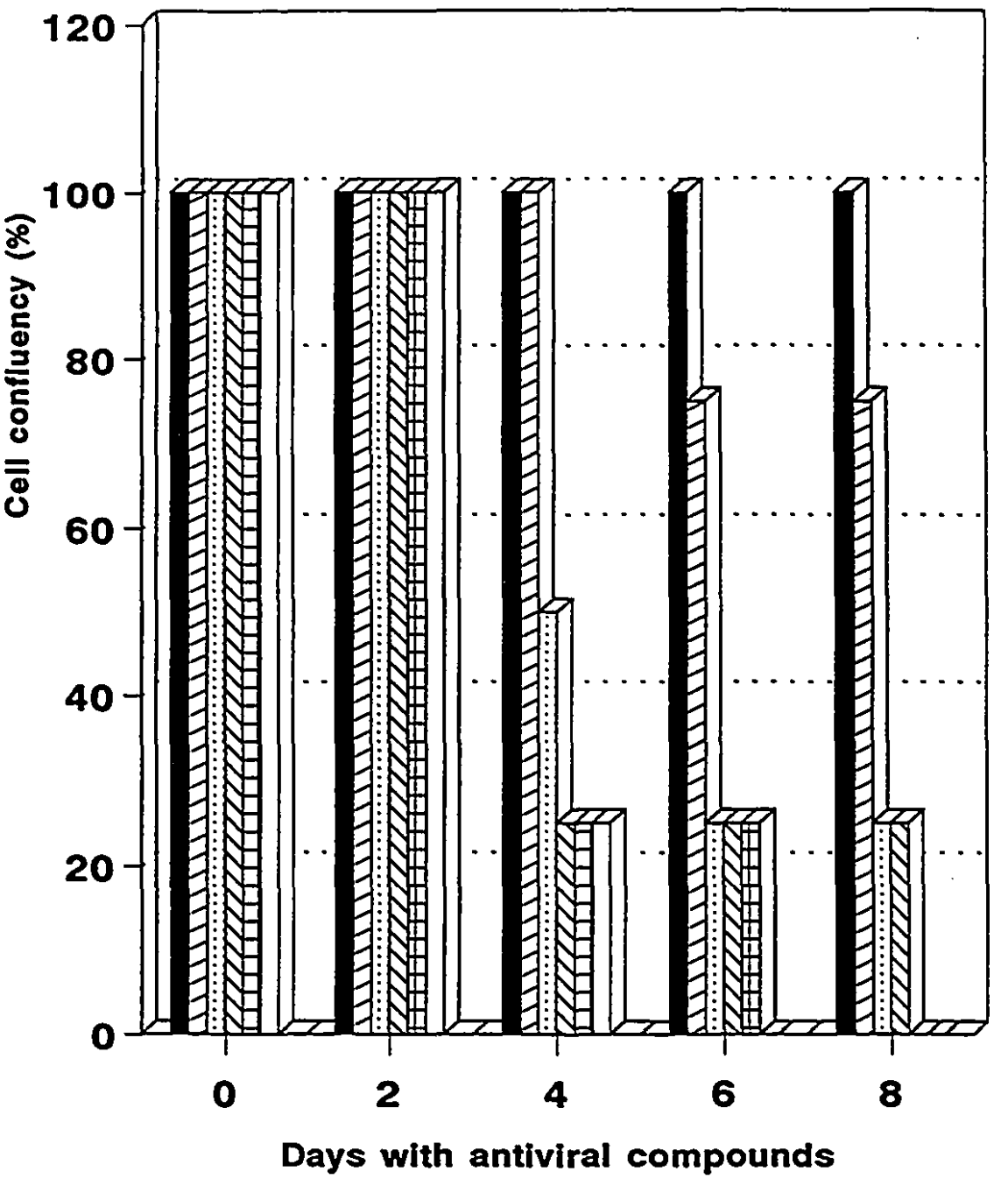
selection compound (dotted bars) was limited by the considerable reduction in cell survival observed as early as 2 days after growth of the cells in A+T+D medium (day 4). Following a DHPG-pre-selection, all the cells were killed after 4 days in multiple drug medium (day 6). The drastic effect on cell survival observed with DHPG could be due to toxic metabolites released into the medium by *tk*$^+$ cells, as observed by Goring and DuBow (1985).

The observation that the utilization of ACV as the pre-selection compound interferes with the elimination of the *tk*$^+$ cells through the subsequent use of multiple drug medium prompted us to further evaluate the interaction between ACV, TFT, and DHPG. The data presented in Figure 3 indicates that this effect was not observed when TFT was used as the pre-selection drug, and a pre-selection utilizing DHPG was hindered by its potential toxicity in cell culture, as discussed in Chapter 2, and below in this Chapter. The aim of the second part of this analysis was to determine if pre-selection with ACV demonstrates the same effects when single drugs (in various sequence of addition to the cells) are used during the 6-day selection period. This selection protocol is outlined in Figure 2B. Briefly, the KT cells were subjected to a 2-day pre-selection step using a single nucleoside analogue. Following this pre-selection period, the cells were fed medium containing a second and different compound. After two days, the same cells were treated with the third drug for the remainder of the selection procedure, and the results are presented in Figure 4. Following pre-selection with ACV, cell survival after 6 days in selective medium (day 8) is still 100%, when the sequence of addition was ACV, then TFT, then DHPG (solid bars). Similar results were obtained with the sequence of

FIGURE 4

Bar graph representing the percentage of cell survival in different types of selective medium following a 2-day pre-selection in medium containing a single nucleoside as the selective agent.

Day 0: Addition of a single nucleoside analogue (pre-selection period)
Day 2: Addition of a second and different nucleoside analogue as the selective medium
Day 4: Addition of a third and different nucleoside analogue as the selective medium
Day 6: Maintenance in the third selective medium
Day 8: Maintenance in the third selective medium


120
100
80
60
40
20
0
Cell confluency (%)
0
2
4
6
8
Days with antiviral compounds

addition ACV, then DHPG, then TFT, as cell survival was only slightly reduced at day 8. In contrast, when TFT was the initial selective agent, cell survival was reduced to 25% at day 8 with the sequence of addition: TFT, DHPG, ACV or TFT, ACV, DHPG. In agreement with the results presented in Figure 3, a pre-selection period using DHPG led to a dramatic reduction in cell survival at day 4 and to complete cell killing as early as at day 6, with two different orders of drug addition to the cells (Figure 4).

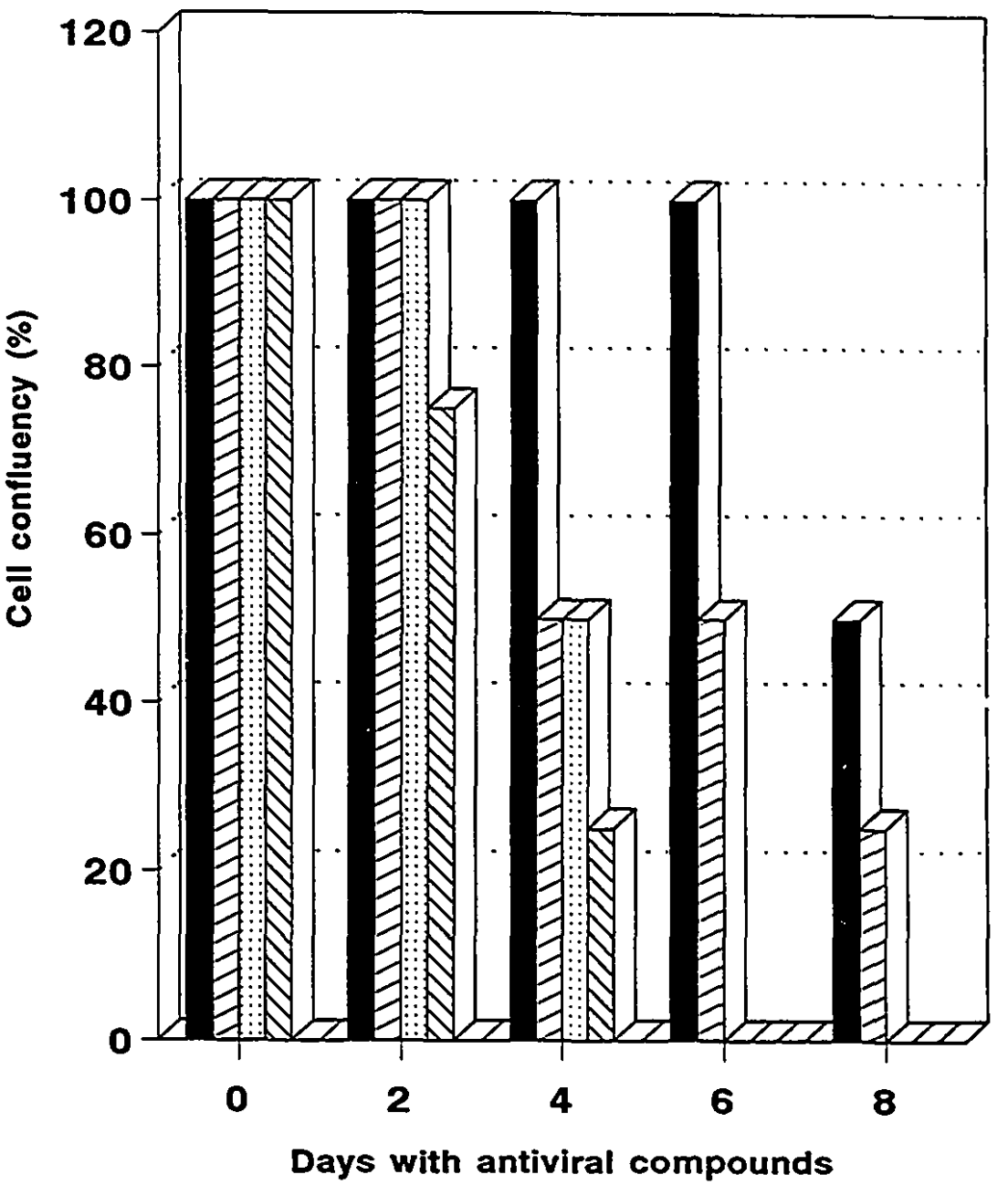
The observations from Figures 3 and 4 suggest that the interference with the killing of *tk*$^+$ cells by different nucleoside analogues is encountered following only the ACV pre-selection period. Hence, the presence of a second drug, along with ACV, during the pre-selection step may diminish, interfere with, or at least modify the effects of ACV on subsequent drug sensitivity. To examine this possibility, a third selection protocol was developed, and is presented in Figure 2C. In brief, ACV was utilized in combination with TFT or with DHPG, for the 2-day pre-selection step, in order to determine if the antagonism in the elimination of the *tk*$^+$ cells by TFT and DHPG was affected by the concomitant presence of TFT in the medium. Figure 5 demonstrates that when the initial drug selection is performed with ACV+TFT (hatched bars), the results differ from those obtained with the ACV-only pre-selection (solid bars). In fact, the cell survival results for the ACV+TFT pre-selection (Figure 5, hatched bars) are similar to those obtained when TFT was the only drug employed in the TFT pre-selection stage (Figure 3). Similarly, the results corresponding to an ACV+DHPG pre-selection (Figure 5, dotted bars) resemble those of a DHPG pre-selection (Figure 3, dotted bars), and differ from those obtained with the ACV pre-selection (solid bars). Moreover, the cell survival

FIGURE 5

Bar graph representing the percentage of cell survival in A+T+D multiple drug medium following a 2-day pre-selection in medium containing two nucleoside analogues as the selective agents.

A 2-day pre-selection with ACV alone was also included, as a control.

Day 0: Addition of two nucleoside analogues (pre-selection period)
Day 2: Addition of multiple drug selective medium (ACV+TFT+DHPG)
Day 4: After 2 days in multiple drug selective medium (ACV+TFT+DHPG)
Day 6: After 4 days in multiple drug selective medium (ACV+TFT+DHPG)
Day 8: After 6 days in multiple drug selective medium (ACV+TFT+DHPG)


120
100
80
60
40
20
0
Cell confluency (%)
0
2
4
6
8
Days with antiviral compounds

pattern corresponding to the TFT+DHPG pre-selection correlates with those observed following a DHPG treatment. However, the effects on cell survival of a pre-selection with DHPG are not as severe when DHPG is combined with ACV, compared to a TFT combination (Figure 5). Overall, the observations presented in Figure 5 suggest that when the KT cells are grown for two days in the presence of ACV only, the killing of *tk*$^+$ cells by other antiviral drugs is altered as a consequence of the ACV pre-treatment.

II.3. SELECTION FOR *tk*$^-$ MUTANTS IN THE KT CELL LINE

In light of the results from the drug interactions during the isolation of *tk*$^-$ mutants utilizing nucleoside analogues in a combined fashion, a selection procedure was developed in order to selectively enrich for potent mutational events which can severely affect the TK phenotype in the KT cell line.

A collection of 90 *tk*$^-$ mutants were isolated through three different selection protocols previously described in Chapter 2. Seven mutants were isolated on the basis of their resistance to TFT, 28 were isolated by the utilization of ACV+TFT as selective agents, and 55 were selected using ACV, TFT and DHPG in a combined fashion (Table 4).

II.4. PHENOTYPE REVERSAL OF THE SPONTANEOUS *tk*$^-$ MUTANTS

In order to verify that the enrichment procedure favored the selection of potent mutations in the *tk* gene, the stability of the mutant phenotype was investigated. The capability of the spontaneous *tk*$^-$ mutants, selected on the basis of their resistance to

TABLE 4

List of *tk*⁻ mutants selected on the basis of their resistance to various nucleoside analogues

TFT-resistant *tk*⁻ mutants

1.TFT-3	4.TFT-6	6.TFT-9
2.TFT-4	5.TFT-7	7.TFT-11
3.TFT-5		

ACV+TFT-resistant *tk*⁻ mutants

1.AT-1-1	10.AT-2-6	20.AT-4-13
2.AT-4-1	11.AT-6-6	21.AT-5-13
3.AT-6-1	12.AT-1-11	22.AT-6-13
4.AT-1-4	13.AT-2-11	23.AT-1-17
5.AT-3-4	14.AT-4-11	24.AT-4-17
6.AT-5-4	15.AT-5-11	25.AT-1-18
7.AT-6-4	16.AT-6-11	26.AT-2-18
8.AT-8-4	17.AT-8-11	27.AT-7-18
9.AT-1-6	18.AT-1-13	28.AT-12-18
	19.AT-2-13	

ACV+TFT+DHPG-resistant *tk*⁻ mutants

1.ATD-3-2	20.ATD-9-27	39.ATD-8-29
2.ATD-7-2	21.ATD-10-27	40.ATD-10-29
3.ATD-10-2	22.ATD-11-27	41.ATD-11-29
4.ATD-1-19	23.ATD-12-27	42.ATD-13-29
5.ATD-2-19	24.ATD-13-27	43.ATD-14-29
6.ATD-5-19	25.ATD-2-28	44.ATD-15-29
7.ATD-1-20	26.ATD-3-28	45.ATD-16-29
8.ATD-1-21	27.ATD-4-28	46.ATD-2-31
9.ATD-3-21	28.ATD-6-28	47.ATD-2-32
10.ATD-6-21	29.ATD-7-28	48.ATD-3-32
11.ATD-12-21	30.ATD-8-28	49.ATD-4-32
12.ATD-1-26	31.ATD-9-28	50.ATD-5-32
13.ATD-2-27	32.ATD-10-28	51.ATD-6-32
14.ATD-3-27	33.ATD-11-28	52.ATD-7-32
15.ATD-4-27	34.ATD-12-28	53.ATD-9-32
16.ATD-5-27	35.ATD-1-29	54.ATD-13-32
17.ATD-6-27	36.ATD-4-29	55.ATD-14-32
18.ATD-7-27	37.ATD-5-29	
19.ATD-8-27	38.ATD-7-29	

different combinations of antiviral drugs, to revert to the wild type TK^+ phenotype was evaluated by their ability to grow in the presence of HAT, which requires a functional thymidine kinase. The reversion frequency corresponding to each of the 45 *tk*⁻ mutants tested is listed in Table 5 and a summary of these results is presented in Table 6. The vast majority (86%) of the mutants resistant to a single drug (TFT) underwent phenotype reversal at a high frequency (Goring *et al.*, 1987) (Tables 5 and 6). In contrast, the mutant TK^- phenotype from the mutants resistant to multiple drugs proved to be more stable, since only 12% to 15% of the $ACV+TFT^R$ and $ACV+TFT+DHPG^R$, respectively, demonstrated the ability to revert to a TK^+ phenotype.

II.5. PRESENCE OF A DETECTABLE TK PROTEIN IN THE SPONTANEOUS *tk*⁻ MUTANTS

The hypothesis that the use of combinations of nucleoside analogues permits the enrichment for potent mutations with an important impact on the TK phenotype through the complete inactivation of the *tk* gene product was also evaluated. The effect of these mutations on the *tk* gene product, the presence, and the size, of the TK protein, was investigated by Western blotting analyses. Twenty-one *tk*⁻ mutants were examined for the detectable production of a TK polypeptide, and the results for seven TFT-resistant mutants and thirteen multiple drug-resistant mutants are presented in Figures 6 and 7, respectively. Figure 6 demonstrates that reduced levels of a TK protein with a molecular weight similar to the wild type TK polypeptide (41 kDa), were detected in 6/7 mutants resistant to TFT as a single selective agent. However, the presence of a TK polypeptide was observed

TABLE 5

Frequency of reversion to the wild type phenotype for *tk*⁻ mutants selected on the basis of their resistance to various nucleoside analogues

MODERATE MUTANTS:

	TFT-resistant *tk*⁻ mutants
TFT-3	$< 5.0 \times 10^{-5}$
TFT-4	8.0×10^{-3}
TFT-5	$> 10^{-2}$
TFT-6	$> 10^{-2}$
TFT-7	$> 10^{-2}$
TFT-9	$> 10^{-2}$
TFT-11	$> 10^{-2}$

POTENT MUTANTS:

	ACV+TFT-resistant *tk*⁻ mutants		
AT-1-6	$< 5.0 \times 10^{-5}$	AT-1-13	$< 5.0 \times 10^{-5}$
AT-2-6	$< 5.0 \times 10^{-5}$	AT-6-13	$< 5.0 \times 10^{-5}$
AT-6-6	$< 5.0 \times 10^{-5}$	AT-1-17	$< 5.0 \times 10^{-5}$
AT-1-4	$< 5.0 \times 10^{-5}$	AT-4-17	$< 5.0 \times 10^{-5}$
AT-8-4	$< 5.0 \times 10^{-5}$	AT-1-18	7.7×10^{-4}
AT-1-11	$< 5.0 \times 10^{-5}$	AT-12-18	$< 5.0 \times 10^{-5}$
AT-5-11	2.5×10^{-4}		

	ACV+TFT+DHPG-resistant *tk*⁻ mutants		
ATD-10-2	$< 5.0 \times 10^{-5}$	ATD-10-28	$< 5.0 \times 10^{-5}$
ATD-1-19	$< 5.0 \times 10^{-5}$	ATD-11-28	$< 5.0 \times 10^{-5}$
ATD-1-20	$< 5.0 \times 10^{-5}$	ATD-12-28	$< 5.0 \times 10^{-5}$
ATD-1-21	$< 5.0 \times 10^{-5}$	ATD-10-29	$< 5.0 \times 10^{-5}$
ATD-3-21	$< 5.0 \times 10^{-5}$	ATD-14-29	1.6×10^{-4}
ATD-1-26	$< 5.0 \times 10^{-5}$	ATD-15-29	$< 5.0 \times 10^{-5}$
ATD-2-27	$< 5.0 \times 10^{-5}$	ATD-2-32	$< 5.0 \times 10^{-5}$
ATD-3-27	$< 5.0 \times 10^{-5}$	ATD-3-32	$< 5.0 \times 10^{-5}$
ATD-5-27	$< 5.0 \times 10^{-5}$	ATD-4-32	$< 5.0 \times 10^{-5}$
ATD-6-27	2.9×10^{-2}	ATD-5-32	8.8×10^{-3}
ATD-8-27	$< 5.0 \times 10^{-5}$	ATD-6-32	$< 5.0 \times 10^{-5}$
ATD-2-28	$< 5.0 \times 10^{-5}$	ATD-7-32	$< 5.0 \times 10^{-5}$
ATD-8-28	$< 5.0 \times 10^{-5}$		

TABLE 6

Phenotype reversal of *tk*$^-$ mutants resistant to different nucleoside analogues

Mutants	Reversion-proficient ($tk^- \rightarrow tk^+$)
TFT^R	6/7 (86%)
$ACV+TFT^R$	2/13 (15%)
$ACV+TFT+DHPG^R$	3/25 (12%)

FIGURE 6

Western blotting analysis of moderate *tk*$^-$ mutants. Protein from crude extracts were subjected to electrophoresis through a 12% SDS-polyacrylamide gel and electro-transferred from the gel onto an Immobilon-P membrane. The TK polypeptide bound to anti-HSV-1 TK polyclonal antibodies was detected using a horseradish peroxidase immunoassay (Consult Chapter 2 for details).

Detection of the TK protein in TFT^R clones. Lane 1: KT (*tk*$^+$ cell line), lane 2: 143B (*tk*$^-$ parental cell line), lanes 3 to 9: mutants TFT-3, TFT-4, TFT-5, TFT-6, TFT-7, TFT-9,and TFT-11.

M= molecular mass markers: 68.0 kDa, and 43.0 kDa, 25.7 kDa.
Arrowheads indicate the 41-kDa TK polypeptide.

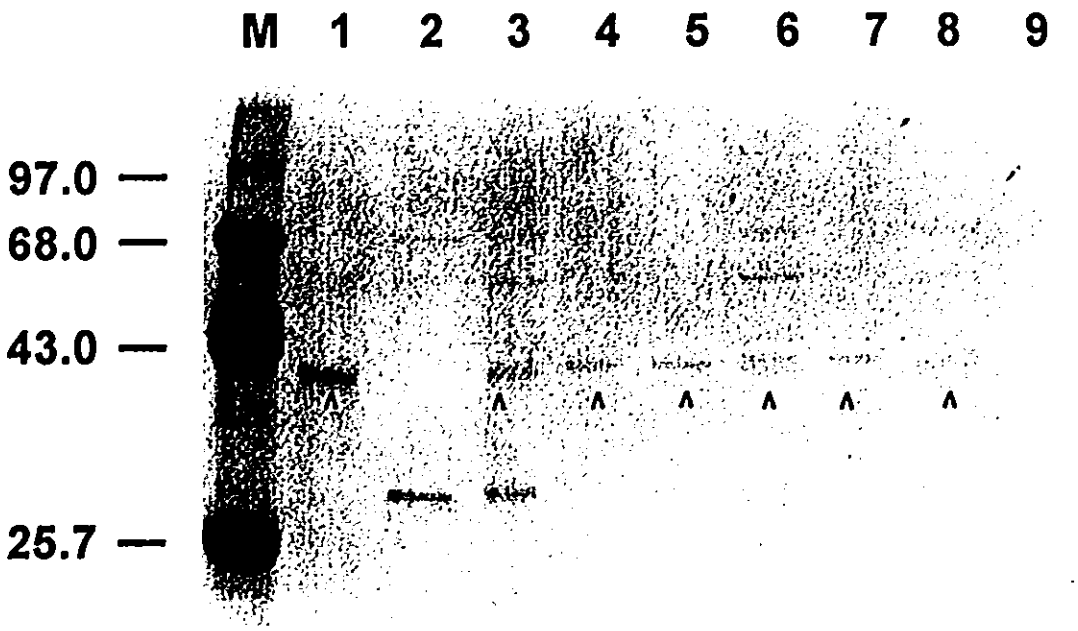
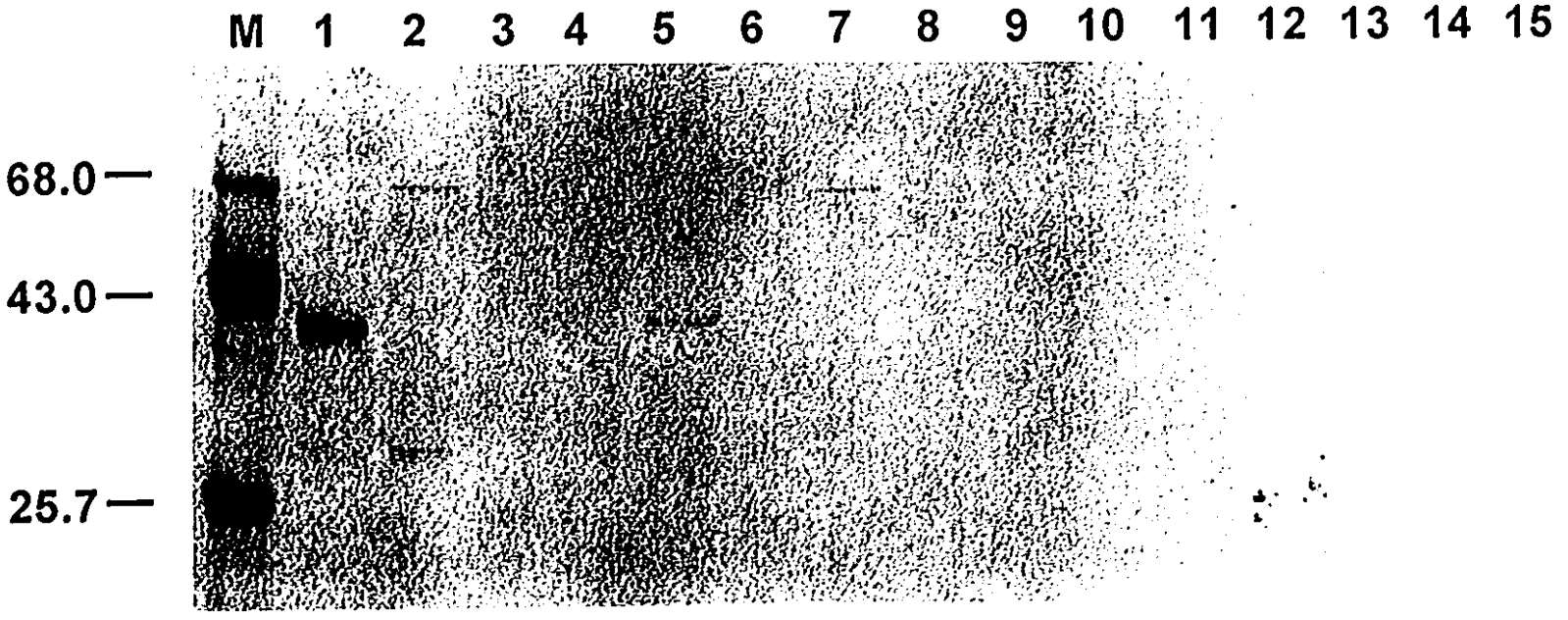

M 1 2 3 4 5 6 7 8 9
97.0
68.0
43.0
25.7

FIGURE 7

Western blotting analysis of potent *tk*⁻ mutants. Protein from crude extracts were subjected to electrophoresis through a 12% SDS-polyacrylamide gel and electro-transferred from the gel onto an Immobilon-P membrane. The TK polypeptide bound to anti-HSV-1 TK polyclonal antibodies was detected using a horseradish peroxidase immunoassay (Consult Chapter 2 for details).

Detection of the TK protein in potent mutants. Lane 1: KT (*tk*⁺ cell line), lane 2 143B (*tk*⁻ parental cell line), lanes 3 to 15: mutants AT-1-4, AT-2-6, AT-5-11, AT-1-13, AT-2-13, AT-6-13, AT-1-17, AT-1-18, AT-3-21, ATD-1-26, ATD-1-21, ATD-3-21, and ATD-1-20.

M= molecular mass markers: 68 kDa, 43 kDa, and 25.7 kDa.
Arrowheads indicate the 41-kDa TK polypeptide.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
68.0
43.0
25.7

only in one of the fourteen mutants resistant to combinations of antiviral drugs, *tk*⁻ mutant AT-5-11, as shown in Figure 7. The additional faint bands revealed by Western blotting represent non-HSV-1 TK polypeptides, which are also present in the *tk*⁻ parental cell line 143B (Lane 2 of Figures 6 and 7). A summary of the information gained from the Western blots (Table 7) reveals that a TK protein was detectable, albeit at reduced levels compared to the control wild type KT cell line, in 86% of the TFT^R mutants. In comparison, a TK polypeptide was discernable, by our assay, in only 7.5% of the spontaneous potent *tk*⁻ mutants resistant to ACV+TFT or ACV+TFT+DHPG.

II.6. STATISTICAL ANALYSIS OF THE DATA

In order to confirm that the *tk*⁻ mutants selected in this study fall into two classes, the moderate TFT^R mutants and the potent $ACV+TFT^R$ or $ACV+TFT+DHPG^R$ mutants, the results presented above were analyzed statistically. It is important to determine if the percentage of *tk*⁻ mutants able to revert to the wild type phenotype (Table 6) can be considered statistically different for the three groups of mutants: TFT^R, $ACV+TFT^R$, and $ACV+TFT+DHPG^R$. Hence, the data presented in Table 6 were subjected to a test for the comparison of percentages, more precisely to a modification of the Chi^2 test. In this study, the sample number (n=45) is large enough to perform a Chi^2 test. However, when $n \sim 40$, the data should be subjected to a strict "exact method test", also known as the $G_{adjusted}$ test, or the Chi^2_w test (Scherrer, 1984). In brief, this test will compare two groups of mutants (k=2) which were classified into three categories (r=3) in order to determine if the two groups should be considered statistically different, or statistically

TABLE 7

Proportion of *tk*$^-$ mutants showing the dectectable presence of a TK polypeptide

Mutants	Detectable TK protein
Moderate mutants:	
TFT^R	6/7 (86%)
Potent mutants:	
ACV+TFT^R	1/8 (12%)
ACV+TFT+DHPG^R	0/6 (0%)

similar. Table 8 was designed in order to demonstrate the utilization of the aforementioned test.

The first hypothesis (H_0) states that the proportion of *tk*$^-$ mutants that can revert to wild type TK$^+$ phenotype (reversion-proficient, or reversion$^+$) is the same for all three groups of mutants.

Alternatively, H_1 constitutes the opposite hypothesis, and implies that the proportion of reversion-proficient (reversion$^+$) mutants is different for the three groups of mutants.

Hypothesis H_0 was then submitted to the $G_{adjusted}$ test. If H_0 represents a true hypothesis, $G_{adjusted}$ will follow the Chi^2 rule with a degree of liberty of v = (k-1) (r-1). The degree of liberty corresponds to the number of independent factors involved in the statistical test and was calculated to be: v= (2-1) (3-1) = 2, in this experiment. The critical values of the Chi^2 test, which will ultimately determine which hypothesis should be accepted, can be derived from statistical distribution tables (Scherrer, 1984). The stringency of this statistical analysis was set to a 97.5% confidence level ($\alpha = 0.025$), thus the critical value is $Chi^2_{0.025} = 7.38$.

In summary, the first hypothesis (H_0) will be accepted if $G_{adjusted} < 7.38$, while the alternative hypothesis (H_1) will be accepted if $G_{adjusted} > 7.38$. Once these limits have been established, $G_{adjusted} = 10.186$, as calculated according to Scherrer (1984).(The calculations can be found in the appendix to Chapter 3)

When the sample number is small (in our case n~45), the calculation of the William's correction (q_w) provides additional accuracy to the $G_{calculated}$ test and the results is a better

TABLE 8

Reversion frequency results subjected to a $G_{adjusted}$ statistical test

	Selective agent			
	TFT	ACV+TFT	ACV+TFT +DHPG	TOTAL
Reversion^{+} Mutants	6	2	4	12
Reversion^{-} Mutants	1	8	14	23
TOTAL	7	10	18	35

approximation of the Chi^2 test, referred to as the $G_{adjusted}$ test.

William's correction (q_W) (according to Scherrer, 1984)= 1.154 (The calculations can be found in the appendix to Chapter 3).

The $G_{adjusted}$ value can finally be calculated: $G_{adjusted} = G_{calculated} / q_W$

$$G_{adjusted} = 10.186 / 1.154 = 8.827, \text{ and } 8.827 > Chi^2_{0.025} = 7.38$$

Thus, hypothesis H_1 can be accepted at the 97.5% confidence level. This demonstration confirms that the proportion of *tk*$^-$ mutants able to revert to the wild type TK$^+$ phenotype is statistically different for the three mutant groups analysed.

Once the $G_{adjusted}$ test confirmed the differences among the three classes of mutants, we identified which of the three groups was responsible for the heterogeneity demonstrated above, using a "multiple comparison" test, which also represents the application of a Chi^2 test, as described above.

Statistical decision:

The results revealed that the proportion of revertants among the TFT^R mutants (86%) is different from the proportion of ACV+TFT^R mutants (15%) and from ACV+TFT+DHPG^R mutants (12%), at the 95.0% confidence level (Table 6). In contrast, the percentage of revertants obtained for the ACV+TFT^R mutants and the ACV+TFT+DHPG^R mutants should be considered similar with a confidence level of 97.5% (Table 6). Hence, the TFT^R mutants constitute one class of mutants, the moderate

mutants, while the ACV+TFT^R and the ACV+TFT+DHPG^R mutants fall into the same class of mutants, the potent mutants.

III. DISCUSSION

III.1. THE ENRICHMENT PROCEDURE UTILIZING THREE NUCLEOSIDE ANALOGUES IN A COMBINED FASHION

To validate the hypothesis that combinations of nucleoside analogues permitted the enrichment for potent mutational events over the background of moderate mutations, the mutation frequencies corresponding to various selection protocols were determined and compared. In the KT cell line, the background frequency of mutation in the *tk* gene (TFT^R) was calculated to be 1.0×10^{-7}, when the selection pressure for the presence of the *neo* gene is maintained by the continuous presence of G418 in the medium (Table 1). This frequency approaches the stability expected for endogenous genes, $\sim 10^{-6}$ to 10^{-8} (Drake, 1992). Numerous genetic and experimental factors can influence the spontaneous mutation frequency (Ames, 1989; Drake, 1989), such as the growth medium utilized, the growth rate and the genetic background of the cell line employed (for example, the accuracy of its replication and repair systems), as well as the ploidy of the cells, since many cultured mammalian cells are not strictly diploid (Ouellette and Bradley, 1991). Mutation frequencies have also been shown to vary between organisms, genes, and even between intragenic sites (Drake, 1992).

When mutations in the *tk* gene were allowed to occur and be expressed, in the absence of HAT medium which selects for the presence of a functional *tk* gene, the

frequency of mutation varied according to the selective agents used (Table 2), as shown by the 10-fold difference in the mutation frequencies obtained when ACV or TFT were used as single selective agents. This diversity in antiviral drug effectiveness had been noted earlier (Collins and Bauer, 1977; Otto *et al.*, 1982; DeClercq *et al.*, 1980). The high efficacy of TFT in this assay has also been demonstrated by Goring *et al.* (1987) who showed that ~43% of ACV^R mutants were also sensitive to TFT. In this study, we show that all TFT^R mutants were also resistant to ACV, while only 14.3% of these TFT^R mutants showed cross-resistance to DHPG. The utilization of DHPG to select for *tk*⁻ mutants in the KT cell line originally presented certain impediments. As detailed in Chapter 2, the production of toxic metabolites by *tk*⁻ cells actively growing in the presence of DHPG prevented the isolation of $DHPG^R$ *tk*⁻ mutants (Goring and DuBow, 1985). This problem was circumvented by the use of DHPG in the second stage of the selection process. When most of the wild type *tk*⁺ cells have been eliminated by a previous treatment with TFT, the addition of DHPG to the remaining cells does not lead to the cytotoxic effect described above. Indeed, combined with other nucleoside analogues, DHPG proved efficient in reducing the apparent mutation frequency in the HSV-1 *tk* gene (Table 2). A selection procedure which utilized ACV, TFT, and DHPG in combination diminished the mutation frequency to a value as low as 3×10^{-7}, suggesting that the isolation of moderate mutants may have been substantially reduced.

The utilization of drugs in a combined fashion was found to enrich for mutational events occurring at low frequencies in the HSV-1 *tk* gene, in the KT cell line. The results obtained throughout the course of the enrichment procedure, suggest that the sequence of

addition of various drugs may affect the selection of *tk*⁻ mutants in our cell culture system. The treatment of a large number of actively growing *tk*⁺ cells with ACV for two days resulted in an interference with the killing of *tk*⁺ cells which normally follows the addition of other nucleoside analogues used either in combination (Figure 3), or alone (Figure 4). The presence of a second compound, along with ACV, during the 2-day pre-selection period, minimized the effects of ACV on subsequent sensitivity of the *tk*⁺ cells to other nucleoside analogues (Figure 5). These observations are particularly intriguing in light of the fact that ACV constitutes an efficient and potent antiviral drug targetted to the viral TK enzyme, and the most widely employed compound against HSV-1 and HSV-2 infections (Coen, 1993; Elion, 1993).

A better undestanding of the intracellular effects of nucleosides and their structural analogues may provide some insights into the observations presented above. Since the intracellular pool of nucleotides (the metabolically active triphosphate forms of the nucleosides) has to be maintained in order to ensure accuracy of DNA replication (< 0.2 mM), the ratio of each of the four dNTPs may be sensitive to even a subtle imbalance. One possible explanation for our results is that the utilization of ACV to select for spontaneous mutations in the HSV-1 *tk* gene generates an imbalance in the cellular nucleoside pool when used at a concentration of 20 μg/ml (88.8 μM) to treat actively growing *tk*⁺ cells. Indeed, in growing cells infected with HSV-1, treatment with ACV was shown to increase the concentration of thymidine (dThd) (Harmeuberg *et al.*, 1985) and thymidine triphosphate (dTTP) (Karlson *et al.*, 1986). This augmentation of the level of dTTP could, in turn, compete with the other drugs for the incorporation into elongating

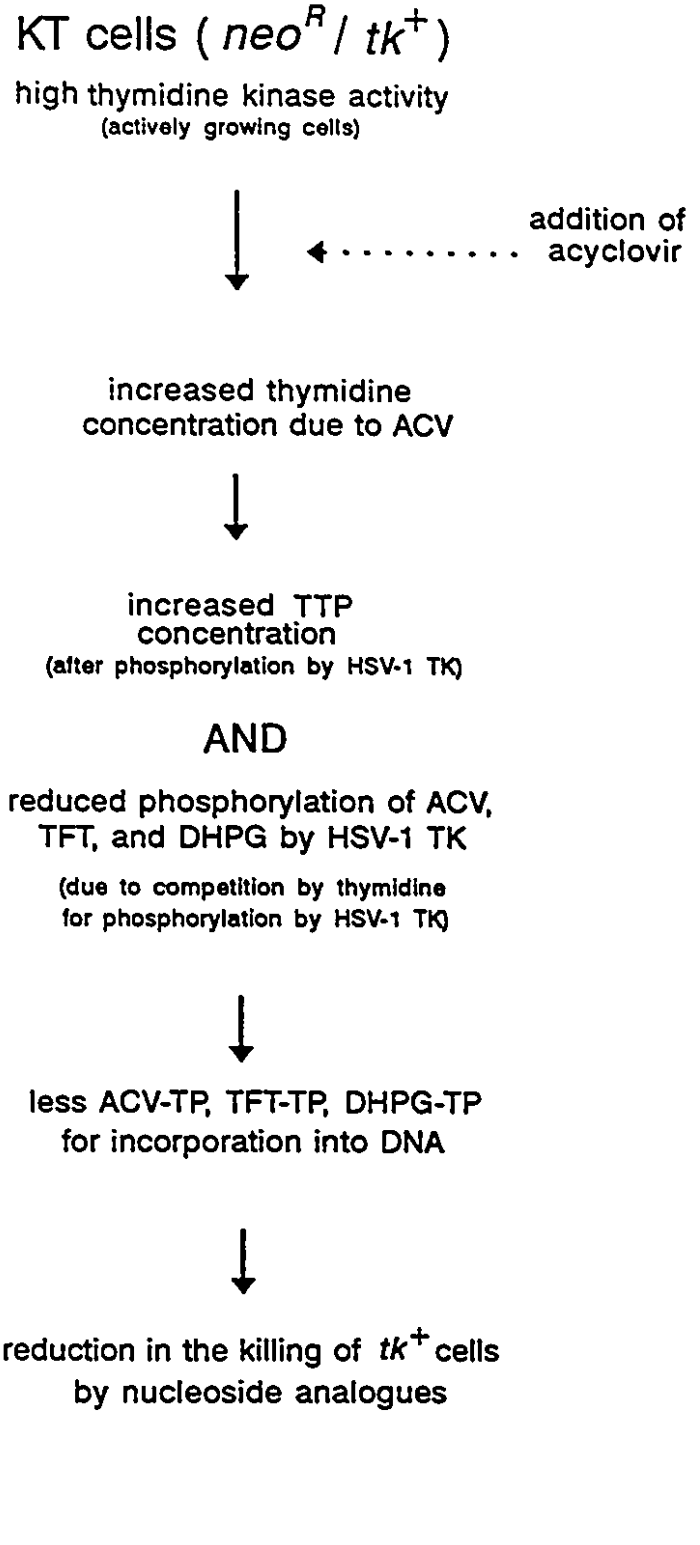
DNA. Following phosphorylation to the triphosphate form by cellular kinases, the resulting diminished levels of ACV-TP, and the other drug-triphosphates, available for incorporation into the DNA could lead to a reduced killing of the *tk*$^+$ cells by ACV (Figure 8). This competition between ACV-TP and dTTP for the viral TK has also been demonstrated by Larsson and co-workers (1983), who showed that the anti-herpes activity of several nucleoside analogues, such as ACV, could be reversed by increasing concentrations of thymidine.

The complex regulation of the intracellular concentration of dNTPs operates through various mechanisms such as substrate cycles and allosteric effects (Bianchi *et al.*, 1986). The HSV-1 TK enzyme possesses both thymidine and thymidylate kinase activities, and the latter activity plays an important role in the intracellular regulation of the pool of dTTPs (Chen *et al.*, 1979; Kauffman and Kelly, 1991). In contrast to its cellular counterpart, the viral TK is resistant to allosteric regulation by dTTP. Hence, dTTP can accumulate within the cell and hinder the antiviral activity of TFT by competing for incorporation into the elongating DNA chain. The accumulated dTTPs can, however, demonstrate an allosteric effect on the cellular ribonucleotide reductase, an enzyme which catalyses the reduction of ribonucleoside diphosphates into the corresponding deoxyribose counterparts. The inhibition of ribonucleotide reductase by dTTP has been shown to result in a depletion of the intracellular dCTP pool (Lockshin *et al.*, 1984).

Enzymatic affinity studies corroborate the intracellular consequences of an imbalance in the dNTP pool, which implicate nucleoside analogues. Elion *et al.* (1977), and more recently Hodge and Perkins (1989), showed that thymidine (K_m:0.4 μM) has a greater

FIGURE 8

Illustration of the potential mechanisms by which treatment of cells containing an integrated copy of the HSV-1 *tk* gene with acyclovir, may delay the sensitivity to subsequent treatments with other nucleoside analogues.


KT cells (neo^R / tk^+)
high thymidine kinase activity
(actively growing cells)
addition of
acyclovir
increased thymidine
concentration due to ACV
increased TTP
concentration
(after phosphorylation by HSV-1 TK)
AND
reduced phosphorylation of ACV,
TFT, and DHPG by HSV-1 TK
(due to competition by thymidine
for phosphorylation by HSV-1 TK)
less ACV-TP, TFT-TP, DHPG-TP
for incorporation into DNA
reduction in the killing of tk^+ cells
by nucleoside analogues

affinity for the HSV-1 thymidine kinase than ACV (K_m: 375-426 μM). Since TFT competes with thymidine for the viral thymidine kinase, the increased intracellular levels of thymidine, as a result of ACV treatment, could reduce *tk*$^+$ cell killing by a subsequent selection using TFT. However, when ACV and TFT are used in the initial selection stage (Figure 6), TFT-TP could be incorporated into the elongating DNA before the significant accumulation of dThd and dTTP resulting from the presence of ACV. Once incorporated into the DNA, TFT-TP will lead the the killing of *tk*$^+$ cells by mechanisms described earlier. In contrast to DHPG, ACV constitutes a relatively poor substrate for HSV-1 TK with K_m's of 11-66 μM and 375-426 μM, respectively. In addition, ACV shows a lower affinity for HSV-1 TK, with a V_{max}/K_m 30-244-fold lower than DHPG (Balzarini *et al.*, 1993). Therefore, ACV may be more sensitive than DHPG to competition by intracellular thymidine, as discussed earlier.

It is also important to note that the phosphorylation of ACV by HSV-1 TK does not obey classical Michaelis-Menten kinetics, and ACV-TP can act either as a substrate or an inhibitor of this enzyme, depending on the concentration at which it is used (Smee *et al.*, 1985). The relationship between the level of TK activity and sensitivity of *tk*$^+$ cells to antiviral drugs also differs. The sensitivity to ACV is proportional to enzyme activity only below 40% of wild type TK enzyme levels (Coen *et al.*, 1989a; Hill *et al.*, 1991). In contrast, Coen *et al.* (1989b) showed that the "cut-off" for DHPG is as low as 10%. Hence, ACV may not be the drug of choice in the first step of a selection procedure in cell culture, when TK activity is still high. This is even more important since, in the KT cell line, the HSV-1 TK from the chromosomally-located viral *tk* gene evades cell cycle-

regulation. The endogenous cellular TK is highly expressed early during the S-phase of the cell cycle and rapidly degraded after DNA synthesis (Roehl and Conrad, 1990; Kauffman and Kelly, 1991). In contrast, the HSV-1 *tk* gene lacks the regions involved in cell cycle control and, as a result, the presence of residual TK activity in the cells may be prolonged.

Further experiments are required to elucidate the effects of combinations of nucleoside analogues observed during our selection procedures. For example, the utilization of combination of ACV and the analogue of thymidine, zidovudine (AZT), has been reported by Pedersen and co-workers for the treatment of patients infected both with HSV-2 virus and the AIDS virus, HIV-I (1992). Moreover, DHPG has been shown to antagonize the antiviral activity of the thymidine analogue zidovudine (AZT) against HIV-I infections (Medina *et al.*, 1992). Hence, the observations presented in this Chapter may prove relevant for antiviral chemotherapy, and possibly for the use of combinations of drugs often encountered in cancer chemotherapy (Medina *et al.*, 1992; Field and Biron, 1994).

III.2. ANALYSIS OF THE POTENT MUTATIONS IN THE HSV-1 *tk* GENE

Once the combination procedure had been established, the selective enrichment for potent mutations was verified. The stability of the TK^- phenotype conferred by mutations selected through different protocols was thus investigated. Since as little as 5-10% of the wild type thymidine kinase activity is sufficient to permit growth in HAT medium (Dobrovic *et al.*, 1988), a valuable sensitivity can be attributed to the reversion analysis,

and a frequency of reversion < 5.0×10^{-5} constitutes the limit of detection of the reversion events in this study. Mutations conferring resistance to several drugs (ACV+TFT or ACV+TFT+DHPG) conferred a potent TK^- phenotype since the vast majority of these mutations rarely reverted to wild type at a detectable frequency. In contrast, most of the mutations allowing cell proliferation in the presence of TFT as a single selective agent reverted to wild type at a detectable frequency (Tables 4 and 5). Moreover, the very few multiple drug resistant *tk*$^-$ mutants capable of phenotype reversal (Table 4), displayed reversion frequencies significantly lower (ranging from 8.8×10^{-4} to 7.7×10^{-4}) than most TFT^R mutants ($>10^{-3}$). Mutants selected on the basis of their resistance to a combination of two of the three drugs displayed a similar efficiency for phenotype reversal (15% and 12%, respectively), as confirmed by a statistical analysis of these results which suggested that ACV+TFT^R and ACV+TFT+$DHPG^R$ mutants belong to the same class of potent mutations. This latter observation also correlates with the previous demonstration that 61.5% of the ACV+TFT^R mutants displayed cross-resistance to DHPG (Table3).

Phenotype reversal of epimutations, as described in Chapter 1, has been shown to occur at a characteristically high frequency, ranging between 10^{-4} and 10^{-3} (Holliday, 1991). In our study, reversal of phenotype occurred in only 12 to 15% of the potent mutants subjected to an enrichment procedure, implying that epigenetic events may not be implicated in the generation of the multiple drug-resistant *tk*$^-$ mutants. These findings are in contrast to those of Ostrander *et al.* (1982) who showed that up to 50% of their *tk*$^-$ mutants switched back to a TK^+ phenotype, and demonstrated that these mutations were due to extensive methylation of the *tk* gene. Other studies have shown that methylation

of cytosine residues frequently accounts for the inactivation of the cellular (Christy and Scangos, 1984) and HSV-1 (Clough *et al.*, 1992) *tk* genes. Contrasting results were also obtained by Ouellette and Bradley (1991) who demonstrated that *hprt* and *aprt* mutants induced by ethyl methylsulfonate rapidly undergo phenotype reversal. This group hypothesized that cell fusion conferred to the cells a tetraploid karyotype, along with reversion of the phenotype. However our reversion studies were performed on cells plated at low cell density, rendering this suggestion an unlikely possibility. An analysis of the methylation status of the *tk*$^-$ mutants from this study is presented in Chapter 5.

The low frequency of most *tk*$^-$ mutants resistant to combinations of antiviral compounds to revert to the wild type TK$^+$ phenotype, correlates with the hypothesis for the reduced recovery of moderate mutations. However, to establish if the use of multiple drugs permits an enrichment for permanent TK$^-$ phenotype resulting from complete gene inactivation events, the consequences on the *tk* gene product, of mutations selected through various combinations of nucleoside analogues, was also examined. A TK polypeptide of approximately 41 kDa (wild type TK protein, Summers *et al.*, 1975) was detected in 86% of the *tk*$^-$ mutants resistant to a single drug. However, the discernible presence of a TK protein was observed in only 7.5% of the multiple drug resistant mutants. It is thus permitted to propose that the mutations allowing resistance to combinations of drugs are enriched in events which severely affected the *tk* gene product and, consequently, the TK phenotype. The presence of a TK polypeptide in a single multiple drug resistant mutant (AT-5-11) will be discussed further in Chapter 5.

Surprisingly, despite its widespread use as a target for several anti-herpetic drugs,

little is known about the precise molecular nature of the HSV-1 TK protein, and X-ray crystallography revealed limited structural information (Sanderson *et al.* 1988). The amino acid alignment of twelve thymidine kinases revealed six highly conserved domains, identified as sites 1 through 6 (Balassubramanian *et al.*, 1990; Black and Hruby, 1992). The putative nucleotide binding site has been attributed to residues 49 to 66, a region highly conserved among the TK's of herpesvirus, poxvirus and cytosol TK's from human, mouse, and CHO cells (Darby *et al.*, 1986; Kit *et al.*, 1987; Liu and Summers, 1988; Robertson and Whalley, 1988; Black and Hruby, 1990). This region may form a nucleotide binding pocket homologous to several ATP-utilizing enzymes (Kit *et al.*, 1987; Liu and Summers, 1988; Black and Hruby, 1990).

The nucleoside binding site has yet to be precisely defined. The binding site for thymidine and structural analogues is believed to encompass two highly conserved regions: site 3 (residues 162-164) and site 4 (residues 171-173), acting either alone or in combination (Black and Loeb, 1993). The presence of essential hydrophobic residues near site 3 suggests their involvement with the formation of a catalytic pocket (Black and Loeb, 1993). Moreover, residue 161, flanking site 3, may be involved in a direct interaction with the nucleoside substrates (Black and Loeb, 1993). The region including residues 168 to 176 (comprising site 4) had been previously proposed to constitute the putative nucleoside binding site (Darby *et al.*, 1986). Kit and co-workers (1987) suggested the involvement of residue 168 for dTMP binding associated with the thymidylate kinase activity of the HSV-1 TK protein. A mutation at Arg^{176} conferred resistance to ACV and led Darby and Larder (1987) to propose a functional role for this

residue in nucleoside binding. Amino acid substitutions at residue 336 conferred very high resistance to ACV in the HSV-1 virus, and this residue was believed to be shared both by the ATP and the nucleoside binding sites (Darby *et al.*, 1986). However, the role played by this residue in the catalytic activity of TK was later strongly reconsidered. Halpert and Smiley (1984) showed that a deletion of the first 45 amino acids from the TK protein still permitted 40% of the wild type TK activity. It is thus expected that simple mutations in this region do not play an important role in the generation of the multiple drug resistant mutants selected in this study.

Mutations affecting the stability of the polypeptide may explain the observed reduction in the levels of TK protein. Dube *et al.* (1991) reported that amino acid substitutions at position Ile^{166} and Ala^{167} greatly reduced the stability of the HSV-1 TK protein. Similarly, no TK protein activity was observed when amino acids Tyr^{172} or Pro^{173}, located within the putative nucleoside binding site, were substituted. Unfortunately, the interpretation of the aforementioned data, without definite structural information, remains somewhat speculative. DNA modifications reducing the transcription of the *tk* gene, and consequently decreasing the amount of the TK protein present within the cell, constitute another alternative for the reduced levels of TK polypeptide observed in some of the mutants presented in this study.

The detectable presence of a TK polypeptide in 86% of the *tk*$^-$ mutants resistant to TFT (Table 6) may be explained by mutations in the putative thymidine binding (Darby *et al.*, 1986) or ATP-binding (Liu and Summers, 1988; Munir *et al.*, 1992) sites, hindering the phosphorylation of analogues of the nucleoside, and consequently allowing drug

resistance. Changes at these sites, associated with diminished phosphorylating activity (Furman *et al.*, 1981), or with altered substrate specificity of the TK enzyme (Darby *et al.*, 1981; Larder *et al.*, 1983), have been shown to confer resistance to antiviral drugs. Using the cellular *tk* locus, Ostrander and co-workers (1982) demonstrated yet another mechanism of resistance to nucleoside analogues by showing that certain *tk*$^-$ mutants were resistant to BUdR because of the transient expression of the TK protein.

In mutant HSV-1 viruses resistant to ACV, truncated forms of the TK protein were observed, as the consequence of a frameshift mutation that caused the premature termination of the polypeptide (Summers, 1975). A shorter TK protein resulting from a nonsense mutation has also been reported in thymidine kinase-deficient HSV-1 virus (Coen *et al.*, 1989b; Irmiere *et al.*, 1989) and the highly homologous HSV-2 virus (Palu *et al.*, 1988). However, no TK polypeptides differing in size from the wild type TK protein were detected in the *tk*$^-$ mutants selected in our study.

The results presented in this Chapter strongly suggest that the use of combinations of nucleoside analogues allows for enrichment of spontaneous potent mutations in a chromosomally-integrated HSV-1 *tk* gene. The limited genetic analyses that have been applied to the understanding of the important role of potent mutations in spontaneous mutagenesis render it appropriate to explore the nature of these potent mutations (Chapters 4 and 5).

APPENDIX TO CHAPTER 3

A.Calculation of $G_{calculated}$

$$G_{calculated} = 2[(6 \log_e 6 + 2 \log_e 2 + 4 \log_e 4 + 1 \log_e 1 + 8 \log_e 8 + 14 \log_e 14) - (12 \log_e 12 + 23 \log_e 23) - (7 \log_e 7 + 10 \log_e 10 + 18 \log_e 18) + (35 \log_e 35)]$$

$G_{calculated}$= 10.186

B.Calculation of William's correction:

Williams correction **qW**:

$$qW = \frac{1 + [35\,(1/7 + 1/10 + 1/18) - 1] \times [35\,(1/12 + 1/23) - 1]}{3 \times 35\,[(3-1)\,(2-1)]}$$

qW= 1.154

CHAPTER 4

CHARACTERIZATION OF MAJOR *tk* GENE ALTERATIONS IN POTENT MUTANTS

I. INTRODUCTION

Potent mutations resulting from major DNA alterations have not been studied extensively, since their isolation is often hampered by the high occurrence of simple point mutations in most genetic selections. In fact, major DNA rearrangements represent infrequent mutational events with potentially dramatic impacts on the function of a gene.

Heterozygous cellular gene systems from diploid eucaryotic cell lines were largely employed to address mutagenesis and analyses of small and frequent mutations at mammalian cellular loci, such as the *aprt* (Phear *et al.*, 1989; de Jong *et al.*, 1988) and *hprt* (Fuscoe *et al.*, 1986) genes. When major DNA modifications were reported, they consisted mostly of large deletions caused by loss of heterozygosity as the result of genetic recombination between the two alleles of an endogenous mammalian locus, such as the *aprt* gene (Ward *et al.*, 1990) and the cellular *tk* gene (Klinedinst and Drinkwater, 1991; Liber *et al.*, 1989; Amudson and Liber, 1992). Frequently, the analysis of induced, rather than spontaneous, mutations facilitated the recovery and characterization of large deletions (Grosovski *et al.*, 1986; Bradley *et al.*, 1988; Carothers *et al.*, 1989; Fuscoe *et al.*, 1992a) and genetic rearrangements (Bradley *et al.*, 1988; Fuscoe *et al.*, 1992a,b; Barr *et al.*, 1990, 1993), by increasing the frequency at which major gene modifications occur through induction by various mutagens.

This study aimed at the characterization of potent (and thus rare) mutations which can occur spontaneously in a human chromosomal context. A strategy to enrich for potent mutations in the *tk* gene through the use of nucleoside analogue combinations resulted in the subsequent reduction in the isolation of moderate *tk*$^-$ mutations (Chapter 3). The detection of a TK polypeptide in less than 12% of the potent *tk*$^-$ mutants revealed that the mutations selected on the basis of multiple drug resistance displayed severe consequences on the *tk* gene product.

In order to elucidate the nature of the mutational events observed in our system, the continuation of this analysis of spontaneous and potent recessive mutations in a human chromosomal context requires the further characterization of the molecular nature of the DNA alterations. Thus, ninety *tk*$^-$ mutants were subjected to a primary characterization of their *tk* gene, and the mutants presenting a major alteration discernible by Southern blotting and/or PCR analyses (≥ 10-25 bp) were investigated further. The molecular characterization of fifteen potent *tk*$^-$ mutants which underwent important DNA modifications is presented and discussed in this chapter.

II. RESULTS

Ninety *tk*$^-$ mutants, selected on the basis of their resistance to various combinations of nucleoside analogues, were subjected to Southern blotting and/or to polymerase chain reaction (PCR) analyses. The Southern blotting technique has been described in Chapter 2. Briefly, the DNA isolated from the different *tk*$^-$ mutant cell lines, as well as from KT (*tk*$^+$) and 143B (*tk*$^-$) cells was subjected to restriction endonuclease hydrolysis using the

following enzymes: *Sac*I, *Bam*H1, *Eco*RI, *Pvu*II, *Bgl*II. Figure 2 from Chapter1 shows the location of these restriction sites in the integrated *tk* gene, as well as within the surrounding genomic sequences (Goring and DuBow, 1985).

The polymerase chain reaction assay has also been detailed previously, and the position of the oligonucleotide primers utilized is presented in Chapter 2, Figure 1. In brief, different overlapping DNA segments comprised within the coding region of the *tk* gene from the *tk*⁻ mutants were amplified using the PCR procedure. The amplified DNA products were rapidly visualized following agarose gel electrophoresis and ethidium bromide staining (described in Chapter 2), and DNA changes involving 10 to 25 bp were readily detected by this assay.

Detectable DNA alterations were encountered in 18.1% of the potent *tk*⁻ mutants resistant to combinations of drugs. More precisely, 25.0% of the ACV+TFT-resistant mutants, and in 14.5% of the mutants selected by their resistance to a combination of all three nucleoside analogues, demonstrated detectable DNA alterations (Table 1). Table 1 also demonstrates that the moderate *tk*⁻ mutants resistant to a single selective agent (TFT) were all the result of mutations implicating less than 10-25 bp of the *tk* gene sequence. The molecular nature of the potent *tk*⁻ mutants which underwent important DNA modifications was established and different mutational events were unveiled.

II.1. DELETION MUTANTS

Southern blotting analysis revealed that deletion of a large portion of the 3' end of the HSV-1 *tk* gene, and the loss of the entire *tk* gene were the most frequent mutational

TABLE 1

Proportion of mutants presenting DNA alterations detectable by Southern blotting or PCR analyses

Mutants	Mutations >10-50 bp[a]
TFT^R	0/7 (0%)
$ACV+TFT^R$	7/28 (25.0%)
$ACV+TFT+DHPG^R$	8/55 (14.5%)

[a] Limit of resolution of the Southern blotting and PCR analyses

events leading to major DNA alterations in the potent *tk*⁻ mutants selected through the enrichment procedure. The twelve deletion mutants were characterized to a greater extent using different restriction enzymes in single- or double-restriction enzyme hydrolyses. Partial restriction maps were generated for each deletion mutants and are presented in Figure 1.

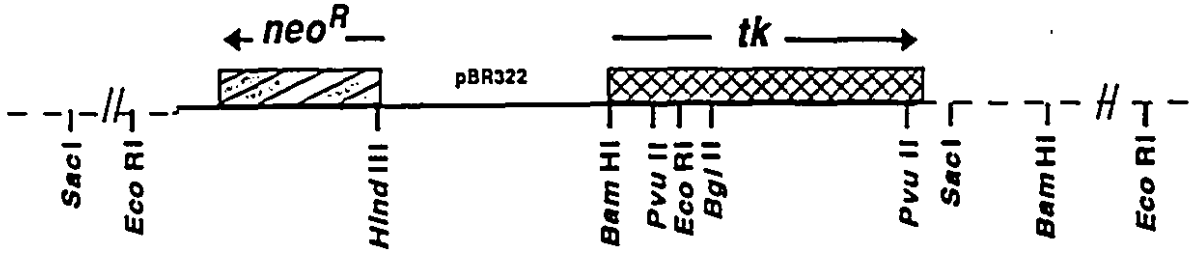
From the twelve deletion mutants analyzed, four underwent the complete loss of the *tk* gene (AT-1-4, AT-4-1, ATD-1-20, and ATD-8-28), while eight mutants retained a small portion of the 5' region of the gene (AT-1-1, AT-5-13, AT-7-18, ATD-2-19, ATD-1-21, ATD-3-21, ATD-11-28, ATD-12-28). Due to the continuous presence of G418 in the culture medium during the selection for *tk*⁻ mutants, the sequences encoding the *neo* gene, as well as some of the adjacent pBR322 sequences, have been retained in all the deletion mutants recovered. Mutant AT-1-1 conserved only the *tk* gene sequences located upstream of the promoter-proximal *Pvu*II site. Mutants ATD-1-21 and ATD-3-21 maintained approximately the first 800 bp of the 5' portion of the *tk* gene. A more detailed characterization by Southern blotting experiments, using the *neo* gene as a DNA probe, revealed that the deletion endpoint within the *tk* gene of mutants ATD-1-21 and ATD-3-21 was located downstream from the promoter-proximal *Bgl*II site. The extent of the deletion into the surrounding cellular DNA located downstream from the *tk* gene was also shown to differ for these two mutants, thus eliminating the possibility that ATD-1-21 and ATD-3-21 represent siblings which originate from the same parental mutant. Mutants ATD-11-28 and ATD-12-28 retained less than 350 bp of the 5' end of the *tk* gene, and Southern blotting analysis also demonstrated that the extent of

FIGURE 1

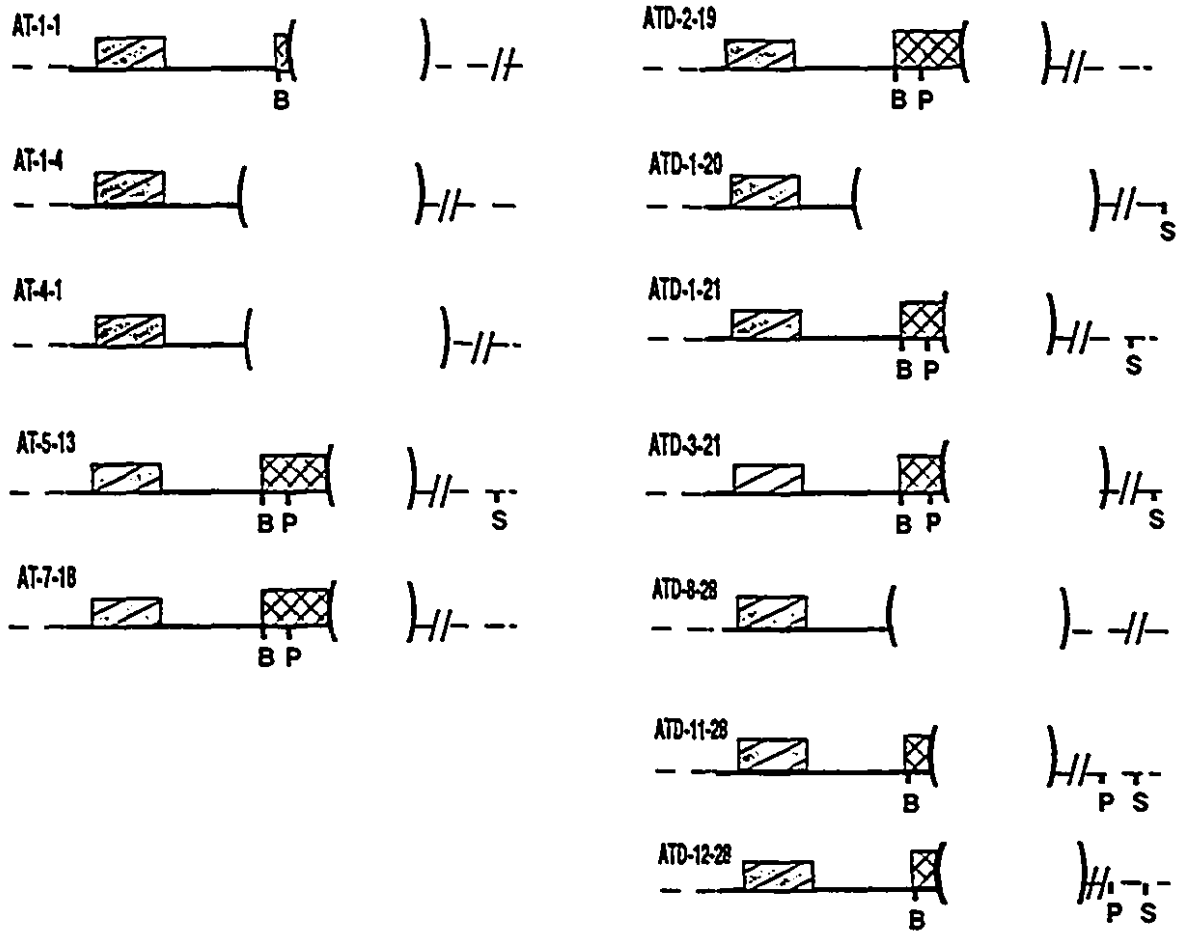
Maps of the single-copy integrated plasmid pSV2*neo*KT in the KT cell line and partial restriction endonuclease map of deletion mutants. The direction of transcription of the *tk* and *neo* genes is indicated by an arrow. The location of relevant restriction sites in the KT cell line as well as in the deletion mutant cell lines is also shown.

The *neo* gene is represented by hatched boxes, while the *tk* gene is shown as cross-hatched boxes. The dotted line represents genomic DNA and the deleted region in the *tk*$^-$ deletion mutants is comprised within parentheses.

KT CELL LINE



DELETION MUTANTS



deletion into the adjacent genomic sequences was different, as shown in Figure 1.

II.2. A MORE COMPLEX *tk* GENE REARRANGEMENT

A diversity of mutational events can cause major DNA alterations and result in potent *tk*⁻ mutants. Mutant AT-2-6 had been selected on the basis of its resistance to ACV and TFT. Further analysis revealed that AT-2-6 exhibited cross-resistance to DHPG, and that this mutant did not revert to the wild type TK phenotype at a detectable frequency (Chapter 3). A specific *tk* transcript could not be detected by Northern blotting analysis (Chapter 5), nor could a mutant TK polypeptide be detected, as shown by Western blotting studies (Chapter 3).

When PCR analyses were performed on mutant AT-2-6, a fragment of the expected size (666 bp) was obtained when primers A and A' were utilized, while it was impossible to amplify the *tk* gene region located at the 3' end and comprised within primers B and B' (Chapter 2, Figure 1). A PCR-amplified product corresponding to the entire coding region of the *tk* gene (primers A and B') was similarly impossible to obtain. Mutant AT-2-6 was then subjected to Southern blotting studies using the *tk* or *neo* gene as a DNA probe, and two putative restriction maps which may explain the restriction enzyme mapping results and justify the generation of this potent mutation are presented in Figure 2. Conceivably, a deletion may have occurred downstream from the location of primer B' in the *tk* gene (Figure 2A) and may have extended into the surrounding genomic sequences situated downstream from the integrated plasmid. This deletion would draw nearer a *Pvu*II site located in the cellular DNA to approximately the same distance as the

FIGURE 2

Maps of the single-copy integrated plasmid pSV2*neo*KT in the KT cell line and two plausible mutational events to account for the generation of *tk*$^-$ mutant AT-2-6.

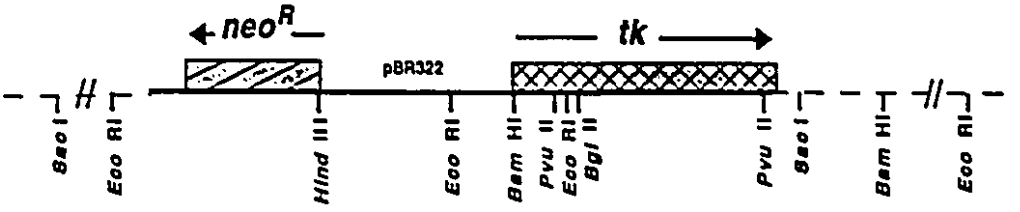
Shown are relevant restriction sites and the direction of transcription of the *tk* and *neo* genes (indicated by an arrow). The *neo* gene is represented as hatched boxes, while the *tk* gene is shown as cross-hatched boxes. The dotted line represents genomic DNA and the deleted region in the *tk*$^-$ deletion mutants is comprised within parentheses.

A. Large deletion occurring downstream from the location of primer A', and extending into the surrounding genomic DNA.

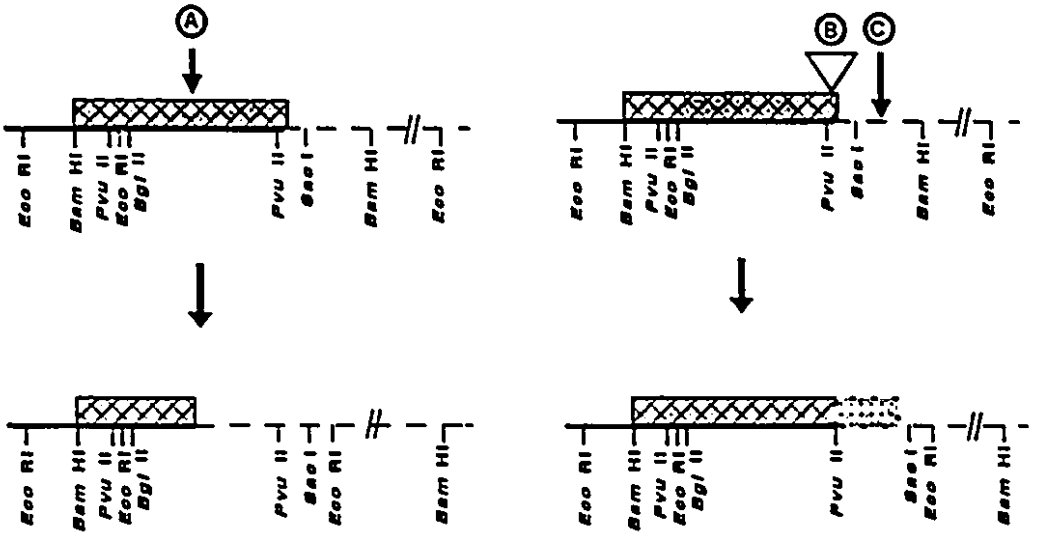
B. Insertion of approximately 500 bp of new sequences immediately downstream from the 3'-most *Pvu*II site in the HSV-1 *tk* gene.

C. Large deletion occurring in the cellular DNA adjacent to the chromosomally-integrated pSV2*neo*KT.

KT CELL LINE



MUTANT AT-2-6



original 3'-most *Pvu*II site in the *tk* gene from the KT cell line. Moreover, a restriction site pattern for *Sac*I, *Bam*HI, and *Eco*RI, different than the pattern observed in the KT (*tk*$^+$) cell line, was obtained in the surrounding host DNA. Alternatively, the Southern blotting and PCR results concerning mutant AT-2-6 may be explained by the gain of approximately 500-600 bp of DNA sequences immediately downstream from the 3'-most *Pvu*II site in the *tk* gene (Figure 2B). A second event, the large deletion of adjacent genomic DNA (Figure 2C), would then generate the restriction endonuclease motif observed in mutant AT-2-6.

II.3. MOLECULAR CHARACTERIZATION OF AN INTRAGENIC NON-HOMOLOGOUS RECOMBINATION EVENT

In addition to the deletion mutants presented above, an internal rearrangement of the *tk* gene was obtained in mutant ATD-1-19. The enzymatic amplification of the mutated *tk* gene from mutant ATD-1-19 revealed that the 3' end of the gene, comprised within primers B and B' (Chapter 2, Figure 1), was increased in size. To delineate the DNA segment involved in the mutation, the amplified DNA products corresponding to this region were subjected to a restriction endonuclease digestion using the enzymes *Bgl*I and *Alu*I, and the fragments were resolved by polyacrylamide gel electrophoresis. As shown in Figure 3, a third *Bgl*I site was observed in mutant ATD-1-19, leading to the generation of an additional *Bgl*I restriction fragment of approximately 70 bp (Figure 3, lane 4). The 5'-most *Alu*I segment was similarly increased in size by approximately 70 bp (Figure 3).

In order to determine the precise nature of this mutation, the DNA products resulting

FIGURE 3

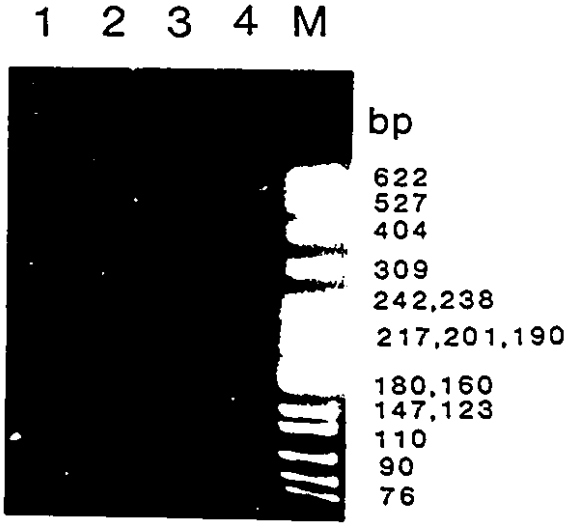
Characterization of the *tk*$^-$ clone ATD-1-19. Partial restriction enzyme hydrolysis of the *tk* gene segment amplified using primers B and B', from the KT cell line and from the *tk*$^-$ mutant cell line ATD-1-19.

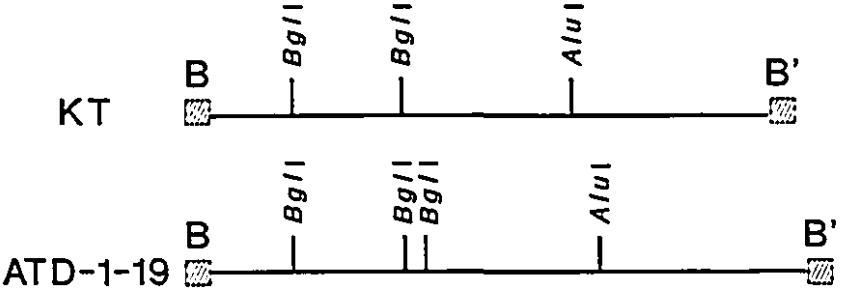
DNA from the KT (*tk*$^+$) and from mutant ATD-1-19 (*tk*$^-$) cells was amplified using primers B and B', hydrolysed with *Bgl*I and *Alu*I and subjected to electrophoresis through a 5% polyacrylamide gel, as described in Chapter 2. The gel was stained in ethidium bromide and photographed under UV light.

Lanes 1 and 3: Partial cleavage of PCR-amplified *tk* DNA from mutant ATD-1-19 with *Alu*I and *Bgl*I, respectively. The arrowheads point to the additional *Bgl*I fragment in the *tk* gene from mutant ATD-1-19.
Lanes 2 and 4: Partial cleavage of PCR-amplified *tk* DNA from the KT (*tk*$^+$) cell line with *Alu*I and *Bgl*I, respectively.
M= Size markers (622 bp to 76 bp), from plasmid pBR322 hydrolyzed with *Hpa*II.

Shown below the gel is a *Bgl*I and *Alu*I restriction map of the region of the *tk* gene amplified with primers B and B', from the wild type *tk*$^+$ KT cell line, and from the *tk*$^-$ mutant ATD-1-19 cell line.

A.


1 2 3 4 M
bp
622
527
404
309
242,238
217,201,190
180,160
147,123
110
90
76


KT
B
Bgl I
Bgl I
Alu I
B'
ATD-1-19
B
Bgl I
Bgl I
Bgl I
Alu I
B'

from PCR amplification using primers C and B' were cloned into pUC118 and pBluescript plasmid vectors, and sequenced by the dideoxy chain termination method, as described in Chapter 2. The DNA sequencing data revealed the direct duplication of a 106-bp segment (bp 46798 to 46693) into a deleted region of 43 bp (47174 to 47132) located further upstream (5') in the *tk* gene (Figure 4).

This mutant was also investigated by a reversion analysis, which showed that ATD-1-19 could not revert to wild type at a detectable frequency. Northern blotting studies showed that the mutated *tk* gene generates a corresponding mRNA transcript approximately 125 bp longer than the wild type *tk* transcript (1.4 kb) in our system (Figure 5A). DNA sequencing revealed that this intragenic rearrangement did not generate a stop codon, or alter the reading frame of the *tk* gene. Nevertheless, and despite the stable production of a *tk* transcript, a mutant TK polypeptide of an expected molecular weight of 42.9 kDa could not be detected by Western blotting analyses using anti-HSV-1 TK polyclonal antibodies (Figure 5B).

This unusual event involves an apparently non-reciprocal recombination process which resulted in the replacement of a region of the *tk* gene (the 43-bp deleted region) with a copy of a segment located downstream, while retaining an intact copy of the 106-bp duplicated segment at its original location. Two possible models to account for the generation of this deletion/duplication by a mechanism similar to gene conversion, but with the particularity that it occurred within a single-copy hemizygous gene, are presented below and further evaluated in the Discussion.

FIGURE 4

Schematic representation of the gene rearrangement in the *tk*$^-$ mutant ATD-1-19. Boxes represent oligonucleotide primers. The deleted and the duplicated regions are contained between parentheses and brackets, respectively. The enlarged region reveals the DNA sequences at the extremities of the rearrangement. Base pair numbering is from McGeoch *et al.* (1988).

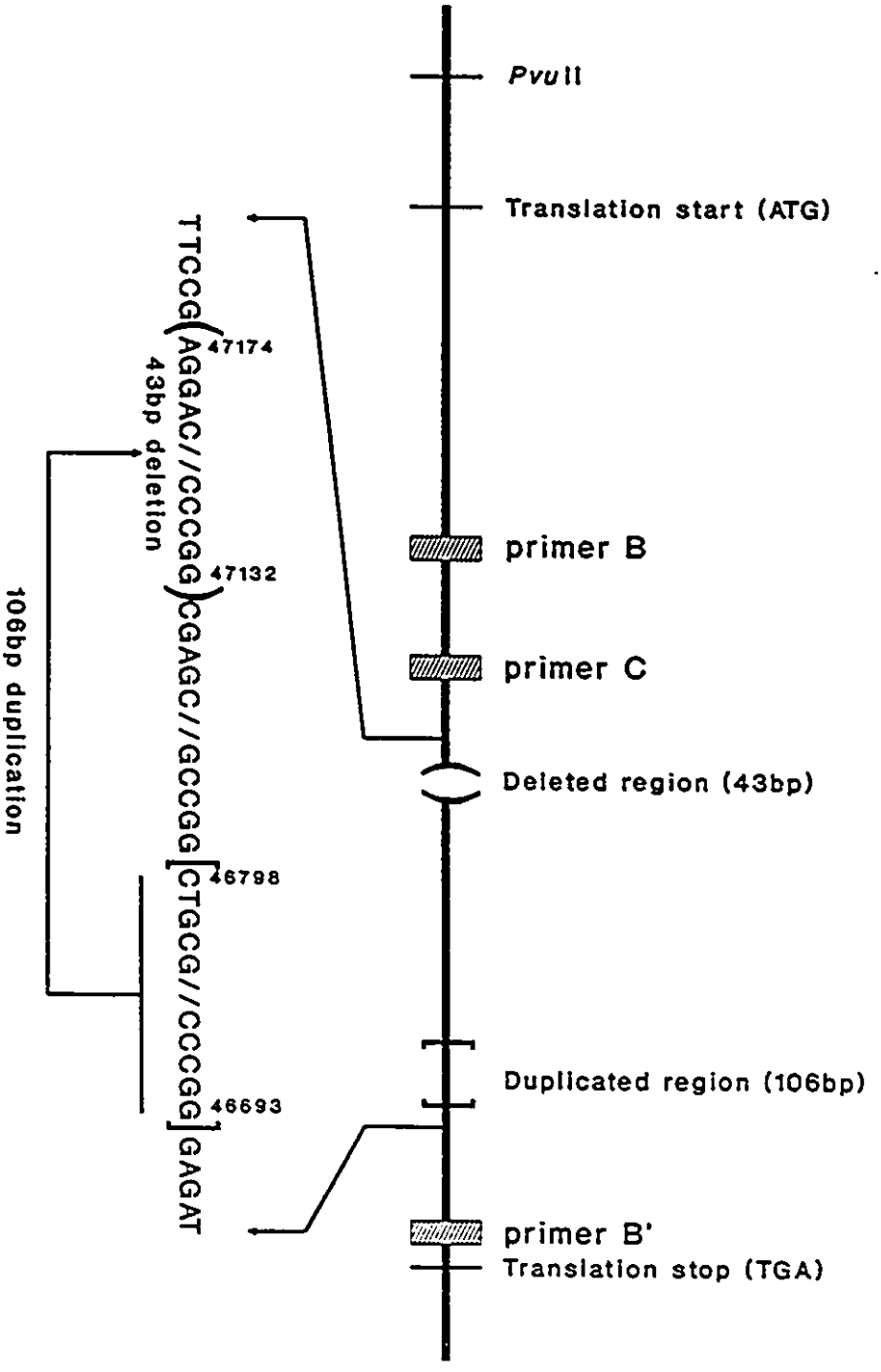

PvuII
Translation start (ATG)
TTCGG(AGGAC//CCGGG)CGAGC//GCCGG[CTGCG//CCCGG]GAGAT
47174
47132
46798
46693
43bp deletion
106bp duplication
primer B
primer C
Deleted region (43bp)
Duplicated region (106bp)
primer B'
Translation stop (TGA)

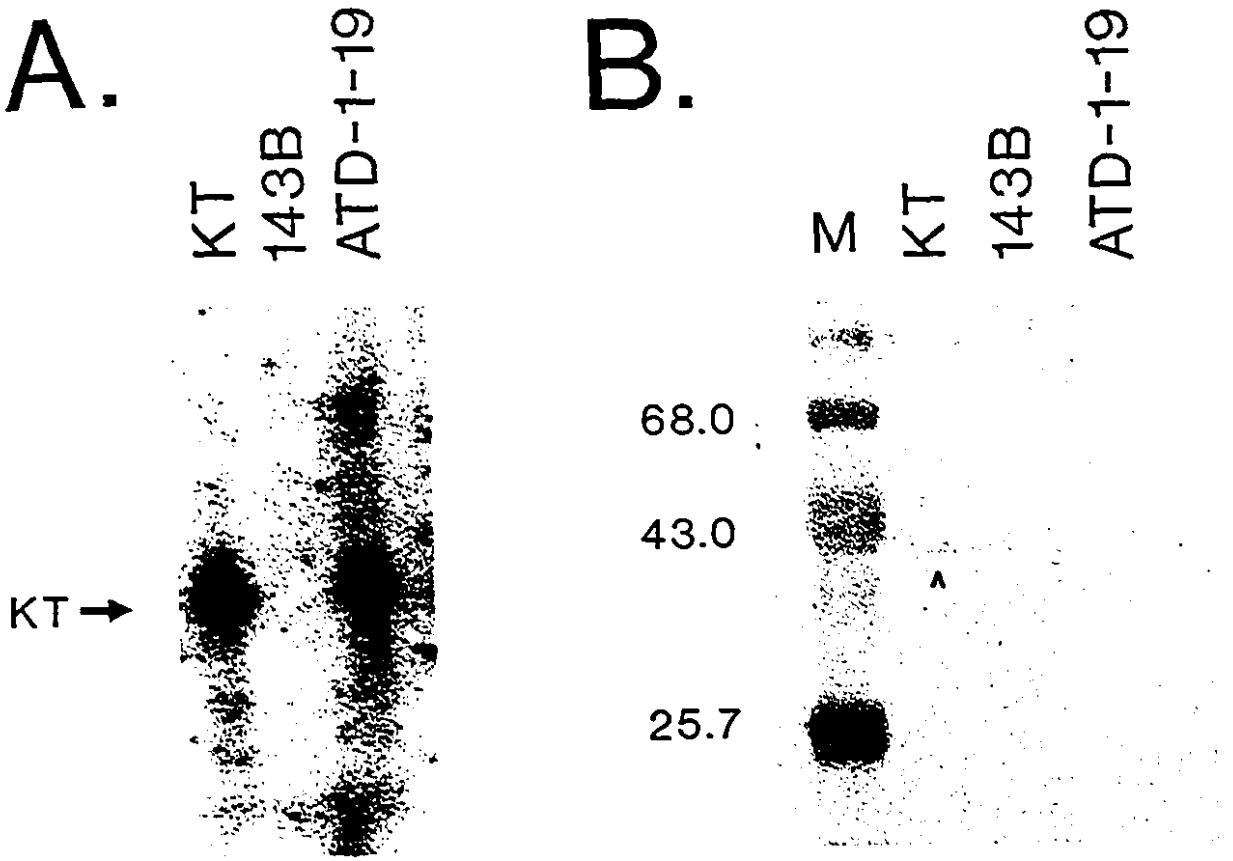
FIGURE 5

A.
Northern blotting analysis of *tk*⁻ mutant ATD-1-19. Total cellular RNA was isolated and subjected to electrophoresis through a denaturing agarose gel and transferred onto a Hybond-N nylon membrane. A specific *tk* transcript was detected using the ^{32}P-labelled *tk* gene as a probe, as described in Chapter 2.

Lane 1: KT (*tk*⁻ cell line), **lane 2:** 143B (*tk*⁻ parental cell line), and **lane 3:** ATD-1-19 (*tk*⁻ mutant cell line). The arrowhead indicates the wild type 1.4 kb *tk* transcript from the KT cell line.

B.
Western blotting analysis of *tk*⁻ mutant ATD-1-19. Protein from crude cellular extracts were subjected to electrophoresis through a 12% SDS-polyacrylamide gel and electro-transferred from the gel onto an Immobilon-P membrane. The TK polypeptide bound to anti-HSV-1 TK polyclonal antibodies was detected using a horseradish peroxidase immunoassay, as described in Chapter 2.

Lane 1: KT (*tk*⁻ cell line), **lane 2:** 143B (*tk*⁻ parental cell line), and **lane 3:** ATD-1-19 (*tk*⁻ mutant cell line). **M**= Molecular mass markers (97, 68, and 43 kDa). The arrowhead indicates the wild type 41 kDa HSV-1 TK polypeptide.


A.
KT
143B
ATD-1-19
KT
B.
M
KT
143B
ATD-1-19
68.0
43.0
25.7

II.4. RECOMBINATION MODELS TO ACCOUNT FOR THE GENERATION OF *tk*⁻ MUTANT ATD-1-19

Several possibilities were considered in order to account for the generation of the deletion/duplication event encountered in mutant ATD-1-19, and it was concluded that an intragenic illegitimate recombination process represents a likely molecular mutational event.

Sequencing results revealed that both the deleted and the duplicated regions are flanked by short regions of sequence homology, which could facilitate genetic recombination between these junctions (Figure 4). Following DNA replication, the misalignment of the two sister chromatids would result in the lining up of these short regions of junctional homology (Figures 6 and 7, A and B). Subsequently, the rearrangement observed in mutant ATD-1-19 may be explained by slight variations of a primary model.

Hypothetically, a double-strand break may have been created in the recipient molecule, immediately downstream from the deleted region (Figure 6C), which would generate two free 3' ends (C). The free 3' end from the recipient molecule could invade the donor molecule at a site of homology created by the initial misalignment, creating a D-loop structure (C). DNA synthesis, primed on the invading 3' end, would then extend the D-loop (D) until the 3' end encounters a new region of homology located just upstream from the deleted region. Unwinding of the recipient molecule would release the new strand (E) while the old strand would be slowly degraded by exonuclease attack on its free 3' end (E). DNA synthesis, primed on the remaining 3' end to fill the gap region,

FIGURE 6

Intragenic gene conversion model facilitated by short regions of junctional homology, to account for the generation of the deletion/duplication event in mutant ATD-1-19: misalignment of the two sister chromatids followed by the initiation of genetic recombination by a double-strand break.

The dashed line represents the 43-bp deleted region, while the dotted line shows the 106-bp duplicated segment and the solid line represents the remaining of the *tk* gene.

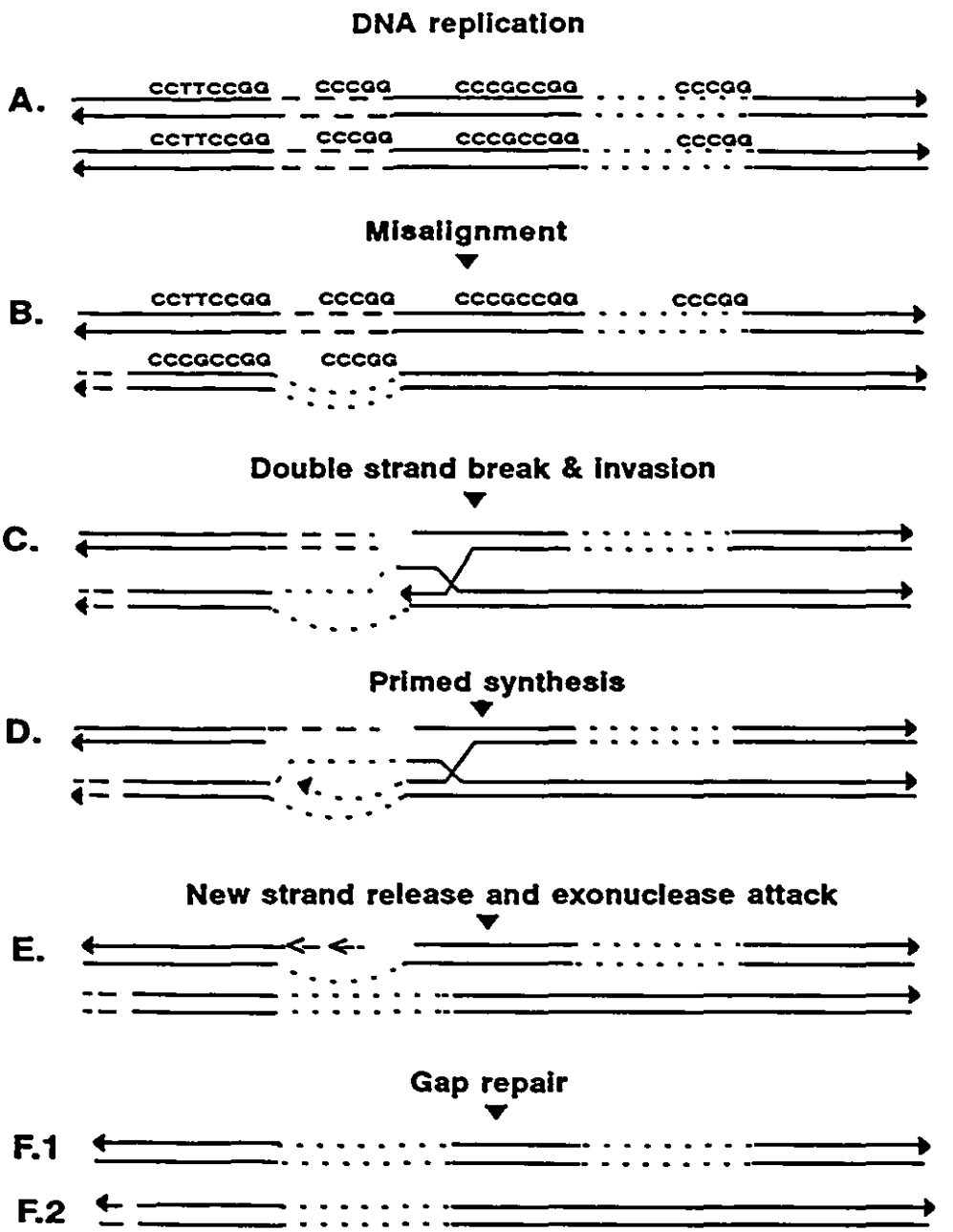
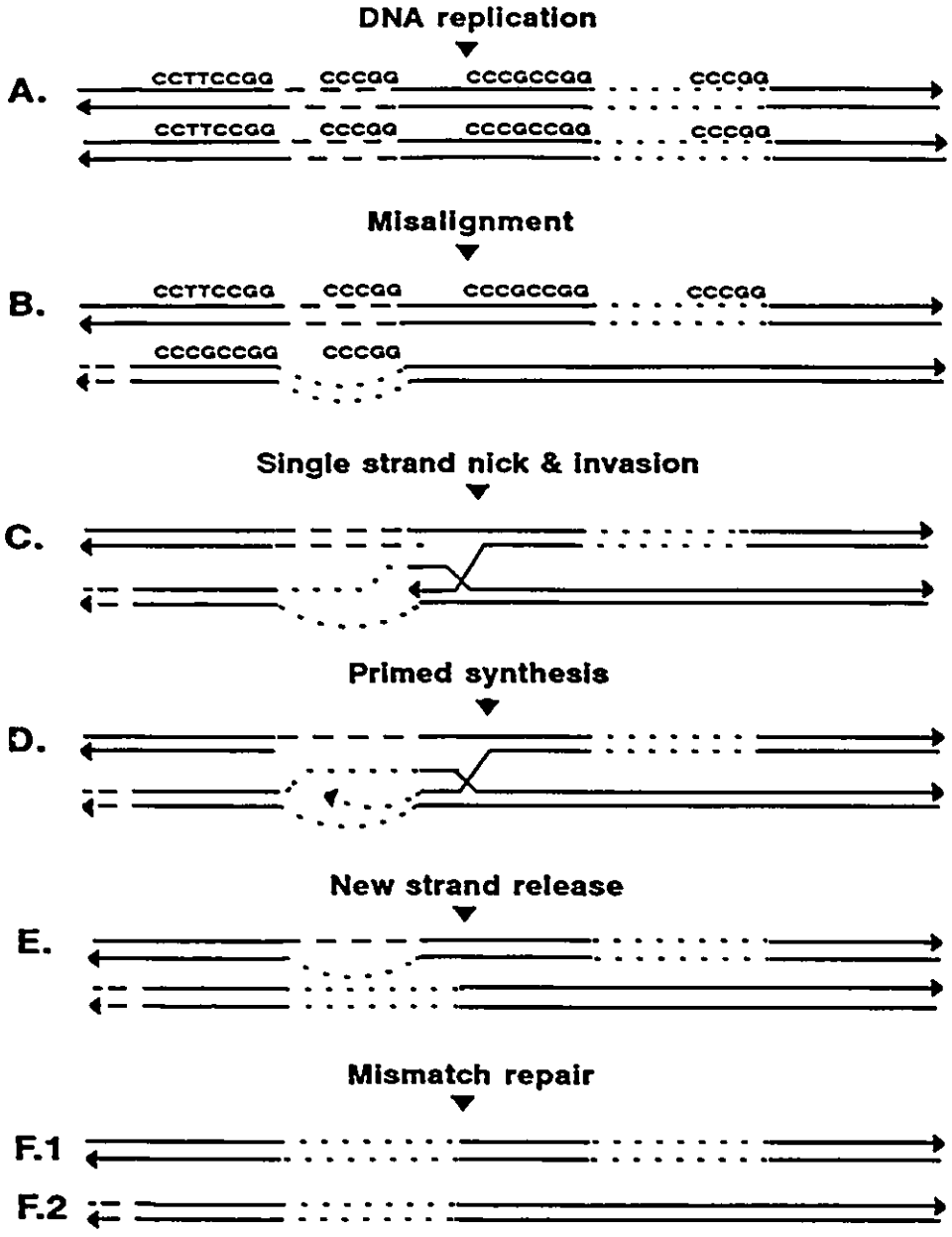

DNA replication
A.
CCTTCCAG
CCCAG
CCCGCCAG
CCCAG
CCTTCCAG
CCCAG
CCCGCCAG
CCCAG
Misalignment
B.
CCTTCCAG
CCCAG
CCCGCCAG
CCCAG
CCCGCCAG
CCCAG
Double strand break & invasion
C.
Primed synthesis
D.
New strand release and exonuclease attack
E.
Gap repair
F.1
F.2

FIGURE 7

Intragenic gene conversion model facilitated by short regions of junctional homology, to account for the generation of the deletion/duplication event in mutant ATD-1-19: misalignment of the two sister chromatids followed by the initiation of genetic recombination by a single strand nick.

The dashed line represents the 43-bp deleted region, while the dotted line shows the 106-bp duplicated segment and the solid line represents the remaining of the *tk* gene.


DNA replication
A.
CCTTCCGG CCCGG CCCGCCGG CCCGG
CCTTCCGG CCCGG CCCGCCGG CCCGG
Misalignment
B.
CCTTCCGG CCCGG CCCGCCGG CCCGG
CCCGCCGG CCCGG
Single strand nick & invasion
C.
Primed synthesis
D.
New strand release
E.
Mismatch repair
F.1
F.2

would generate, following cellular division, the molecule recovered in mutant ATD-1-19 (F.1).

Alternatively, a free 3' end may have been generated by a single-strand nick (Figure 7C) enabeling it to invade the donor molecule at a site of short junctional homology to create a D-loop structure (C) that would extend until the 3' end meets a second region of homology (D), followed again by strand release (E). If the newly-duplicated segment was placed on the original parental strand prior to DNA replication, a mismatch repair enzymatic process could repair the heteroduplex (F) to the 106-bp duplication, thus generating the molecule found in mutant ATD-1-19 (F.1).

III. DISCUSSION

The present study of mammalian cell spontaneous mutagenesis demonstrated the enrichment for potent mutational events, over the background of more frequent moderate mutations. Among the *tk*$^-$ mutants which demonstrated a potent phenotype, several DNA alterations involved changes in the *tk* gene implicating more than 10-25 bp (Table 1). Because of their severe consequences on gene function, the potent *tk*$^-$ mutants presenting discernible gene alterations were further characterized.

III.1. *tk* GENE REARRANGEMENTS

The loss of either the entire *tk* gene, or part of the sequences located downstream from the promoter-proximal *Bgl*II site, were the most frequent major DNA alterations encountered in the potent *tk*$^-$ mutants. The restriction endonuclease mapping of the

deletion mutants demonstrated the absence of specific breakpoints in the *tk* gene or in the surrounding genomic DNA, since the endpoints of all the deletion mutants characterized, as well as the restriction site patterns found in the surrounding cellular DNA, were found to vary. Moreover, these results suggest that these deletion mutants were not present in the original KT cell line, but were rather generated *de novo* and, subsequently, selected using nucleoside analogues.

The generation of spontaneous deletions in a single-copy and chromosomally-integrated gene can be the result of various cellular mechanisms (Ames, 1989). Errors during replication and repair of DNA lesions can cause deletions. DNA secondary structure has been associated with deletions and duplications (Albertini *et al.*, 1982; Ripley and Glickman, 1983). For example, direct repeats as short as 2-7 bp have been shown to generate spontaneous deletions in mammalian genes (Nalbantoglu *et al.*, 1986). Stretches of identical nucleotides favor the slippage of DNA polymerase during DNA replication, and have been shown to cause small deletions (Ripley, 1990). The misalignment of distant sequences during DNA replication has also been implicated with deletion mutations (Kunkel, 1990). Results from studies which also utilized a chromosomally-located HSV-1 *tk* gene implicated DNA methylation as the major inactivation event in the *tk* gene (Tasseron-de Jong *et al.*, 1989a,b). In this study by Tasseron-de Jong and co-workers, the integration site of the plasmid into the chromosomal DNA constituted the main factor allowing DNA methylation to occur. The methylation status of the potent *tk*$^-$ mutants from this study will be discussed in Chapter 5. Drinkwater and Klinedinst (1986) have shown that 30% of the chemically-induced

mutations in the HSV-1 *tk* gene from an autonomously-replicating vector contained deletions of more than 50 bp. However, this extrachromosomal HSV-1 *tk* gene might not have been exposed to an environment which resembles a chromosomal locus.

A different mechanism seems to be associated with deletion events at endogenous loci. In mammalian cellular loci such as the *aprt*, *hprt*, and *tk* genes, large deletions frequently resulted from homologous recombination between the two alleles of that gene (Liber *et al.*, 1989; Applegate *et al.*, 1990; Ward *et al.*, 1990; Klinedinst and Drinkwater, 1991). Notably, the cellular *tk* locus was shown to undergo the total loss of the gene at high frequency compared to the *aprt* and *hprt* loci (Amundson and Liber, 1991, 1992; Grosovsky *et al.*, 1993). The inactivation of the *tk* gene by deletions has also been reported in 90% of the spontaneous and γ-irradiated mutants in a human cell line heterozygous for the autosomal *tk* gene (Yandell *et al.*, 1986).

The important role of deletion mutations stems from their significant contribution to genetic disorders (Krawczak and Cooper, 1991; Morris and Tacker, 1993). Deletion mutants are characteristic of the Werner syndrome (Fukuchi *et al.*, 1989) and have been shown to represent more than 70% of the mutational events in the Duchenne muscular dystrophy (DMD) gene (Forrest *et al.*, 1987), up to 90% of the cases of steroid sulfatase deficency (Caskey, 1987) and represent a less frequent, yet important, event associated with cystic fibrosis (Tsui, 1992) as well as in many proto-oncogenes (Knudson, 1985, 1986; Hozier *et al.*, 1992).

III.2. INTRAGENIC GENE REARRANGEMENT

Mutant ATD-1-19 constitutes an unusual mutational event conferring resistance to multiple nucleoside analogues. This rearrangement maintained the reading frame of the gene without generating a stop codon, and a mutated *tk* transcript approximately 125 nt larger than its wild type counterpart was detected by Northern blotting. Neverthless, a mutated TK protein of an expected size of 42.9 kDa was not observed by Western blotting studies. The deleted region from mutant ATD-1-19 was found to comprise the putative binding site for ATP (Darby *et al.*, 1986; Liu and Summers, 1988; Robertson and Whalley, 1988). The duplicated segment comprises a cysteine residue which may be important for the folding of the protein (Lui and Summers, 1988). Hence, the mutational rearrangement may have altered the conformation of the TK polypeptide, resulting in reduced stability.

Several molecular models implicating DNA replication or recombination were considered in order to account for the generation of the deletion/duplication event recovered in mutant ATD-1-19, and the intragenic illegitimate recombination models presented in Figures 6 and 7 were retained as likely possibilities. Numerous examples of recombination and gene conversion exist in procaryotes (Dybvig, 1993), and in yeast (Szostak, 1986), two organisms which frequently utilize homologous recombination processes. The control of mating type switching in the yeast *Saccharomyces cerevisiae*, a gene conversion event initiated by endonuclease cleavage (Klar *et al.*, 1984), constitutes a well characterized homologous recombination event. Szostak (1986) elaborated a double strand break repair model to account for gene conversion in yeast. Homologous

recombination has also been reported as an interallelic mutational event induced by X-rays at the human cellular *tk* locus (Benjamin *et al.*, 1991; Benjamin and Little, 1992).

In mammalian cells, non-homologous recombination represents the most frequent process leading to chromosomal rearrangement (Milot *et al.*, 1992), yet very little is known concerning this sequence-independent mechanism. Non-homologous (illegitimate) recombination has been shown to occur efficiently in mammalian cells and to involve strand breakage, followed by end joining (Roth *et al.*, 1985; Wake *et al.*, 1985; Roth and Wilson, 1988). During genetic recombination, sequence homology promotes genetic exchanges, whereas lower homology prevents rearrangement with divergent sequences (Waldman and Linskay, 1987,1988; Deng and Capecchi, 1992). However, illegitimate recombination does not require the extensive homology required by homologous recombination (Roth and Wilson, 1988; Meuth, 1989; Desautels *et al.*, 1990). Thus, several reports demonstrated that recombination events can be facilitated by very short regions of homology (Mezard *et al*, 1992). Reynaud *et al.* (1987) showed that rearrangements in the chicken immunoglobulin Vl gene were associated with stretches of homology ranging from only 10 to 120 bp. Similarly, Morris and Tacker (1993) demonstrated that very short homologies of only 2 to 6 bp are present at the breakpoints of 40% of the large deletions associated with genetic disorders. Thus, even though relatively short regions of junctional homology (8/11 partial homology) were observed surrounding the deleted and duplicated region in mutant ATD-1-19, both models presented in this Chapter represent plausible alternatives.

How the intragenic recombinational event observed in mutant ATD-1-19 was

initiated remains unclear. Various enzymes involved with recombination (Salganik and Dianov, 1992) can be implicated in the formation of the initial strand breakage events shown in Figures 6 and 7 (Ganesh *et al.*, 1993). The model presented in Figure 6 suggests the formation of a double strand break. In addition to the possible action of endonucleases, it has been shown in *E. coli* that double strand breaks are formed in *dam* strains during mismatch repair (Glickman and Radman 1980; Wang and Smith, 1986). In mammalian cells, topoisomerase II has been implicated in the creation of double strand breaks (Wang, 1985), but sequences recognized by this enzyme were not detected in the vicinity of the mutations in ATD-1-19. However, Huang *et al.* (1992) suggested that the recognition specificity of topoisomerase II may not be critical, as this enzyme may select an energetically unstable region for its cleavage site. The illegitimate recombination model initiated by a double strand break (Figure 6) also necessitates the selective repair of the old DNA strand, and it has been suggested that damage in the transcribed strand can be selectively repaired (Terleth *et al.*, 1991). The initiation of illegitimate recombination by a single strand nick remains a plausible alternative, and various cellular enzymes may be involved. For example, the enzyme topoisomerase I can cause nicks in DNA but DNA sequences recognized by this enzyme (Bullock *et al.*, 1985), were not detected. Fujiwara and co-workers (1970) proposed that TFT, one of the nucleoside analogues employed in the selection procedure, may cause strand breakage. Thus, the possible involvement of TFT in creating the original single strand nick must also be considered.

The possibility that other events were involved in the rearrangement observed in

mutant ATD-1-19 cannot be eliminated. Misalignment mutagenesis during DNA replication has been reported to constitute a dislocation mechanism in which the misalignment of distant sequences during DNA synthesis generates mutations (Glickman and Ripley, 1984; Kunkel and Soni, 1988; Kunkel, 1990). However, the application of this model to mutant ATD-1-19 does not appear to be favorable. The dislocation mutagenesis model would require the initial generation of a large single-stranded region of 476 nt (located between the deleted and the duplicated regions), and a stem-loop structure required to delete the 43-bp region could not be formed following the misalignments implicated in this model. The simple misalignment of the two strands during DNA replication was similarly ruled out, since this mechanism would yield the inversion of the duplicated sequences within the deleted region (Kornberg and Baker, 1992), while the duplication in mutant ATD-1-19 preserved the orientation of the 106-bp duplicated segment.

The presence of other DNA sequence motifs known to facilitate genetic rearrangements (Ripley and Glickman, 1983) was also scrutinized. Inverted repeats, such as those associated with site-specific recombination in the generation of Ig gene diversity in vertebrates (Hesse *et al.*, 1989; Fuscoe *et al.*, 1992b) were not observed. Genetic recombination through repeated sequences from repetitive DNA, such as *Alu* elements (Labuda and Striker, 1989), has also been shown to be associated with deletions (MacPhee, 1991) and duplications (Kornreich *et al.*, 1990), but sequences indicative of such a process were not observed in mutant ATD-1-19.

The demonstration of this deletion/duplication event reveals that complex mechanisms

can generate a diversity of spontaneous rearrangements in a hemizygous gene subject to a chromosomal context. It also reinforces the notion that genetic recombination plays an important role in several types of mutagenic events in mammalian cells by producing extraordinary variability.

CHAPTER 5

ANALYSIS OF POTENT MUTATIONS IMPLICATING SMALL DNA ALTERATIONS

I. INTRODUCTION

The selection for mutations in the *tk* gene, using combinations of nucleoside analogues, resulted in the enrichment for potent mutational events which severely affected the TK phenotype (Chapter 3). Among the potent mutants resistant to combinations of drugs, 14.5% to 25% underwent major DNA alterations (≥ 10-25 bp), which consisted mostly of deletions involving either a portion of the HSV-1 *tk* gene, or the entire *tk* gene (Chapter 4).

However, the majority (75% to 85.5%) of the potent *tk*$^-$ mutations, did not display DNA alterations detectable by the Southern blotting or PCR analyses described in Chapter 4. Nevertheless, these potent mutants greatly altered the TK phenotype and resulted in the absence of a TK protein detectable by Western blotting (Chapter 3). Hence, this Chapter presents the analysis of the multiple-drug resistant mutants which did not undergo a major alteration in the *tk* gene (≥ 10-25 bp), yet demonstrate a TK null phenotype.

II. RESULTS

II.1. ANALYSIS OF THE *tk* GENE

The mutational changes associated with the potent *tk*$^-$ mutations were characterized

with more precision, in order to detect smaller alterations. The multiple drug resistant mutants, in which no DNA changes were discernible by Southern blotting and PCR analyses (Chapter 4), were submitted to a more refined investigation of their mutated *tk* genes (Figure 1). Two segments of the *tk* gene (comprised within primers A and A', or B and B', Chapter 4, Figure 2) were enzymatically amplified and hydrolyzed with the restriction endonuclease *Bst*NI (Figure 2). The resulting restriction fragments, ranging in sizes from 26 bp to 228 bp (Figure 2), were resolved by electrophoresis through 16% polyacrylamide gels. Plasmid pBR322, cleaved with the enzyme *Hpa*II, was used as a size marker, and DNA changes involving as little as 5 bp should be readily detectable by this assay. Seventy tk^- mutants were subjected to this restriction fragment analysis of their mutated *tk* gene: five moderate TFT^R mutants, and sixty-five potent $ACV+TFT^R$ and $ACV+TFT+DHPG^R$ mutants (Table 1). The restriction enzyme patterns obtained for the tk^- mutant cell lines were then compared to those of the KT (tk^+) and 143B (tk^-) cells. This study revealed that none of the potent tk^- mutants tested underwent a DNA modification involving more than 5 bp of the *tk* gene. (Table 2).

II.2. INVESTIGATIONS OF THE POSSIBLE INVOLVEMENT OF EPIGENETIC EVENTS

Since none of the seventy potent tk^- mutants listed in Table 1 presented a discernible DNA alteration ($\geq$ 5 bp), these mutants may thus result from point mutations which nevertheless revert at a very low frequency and do not permit the production of a stable TK polypeptide. Alternatively, DNA changes in the *tk* gene may not be the major cause

FIGURE 1

Schematic representation of the analysis of the nature of the small potent mutations on the chromosomally-located HSV-1 *tk* gene.

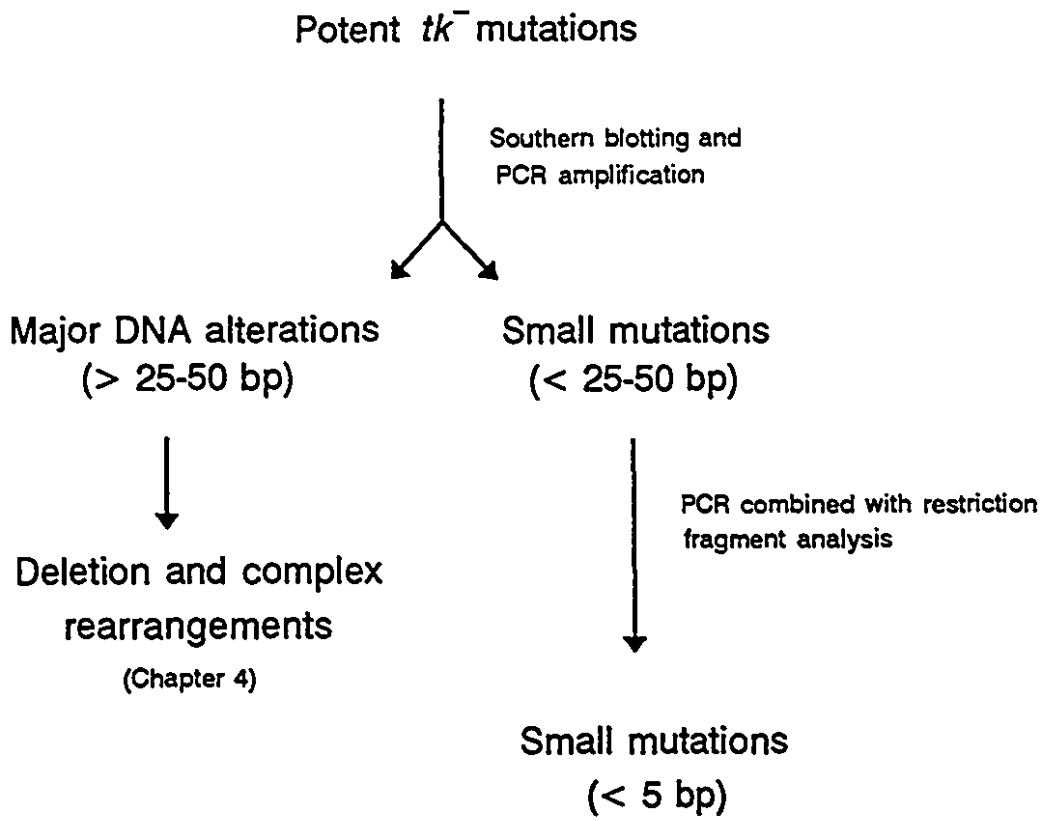

Potent tk⁻ mutations
Southern blotting and PCR amplification
Major DNA alterations (> 25-50 bp)
Small mutations (< 25-50 bp)
Deletion and complex rearrangements
(Chapter 4)
PCR combined with restriction fragment analysis
Small mutations (< 5 bp)

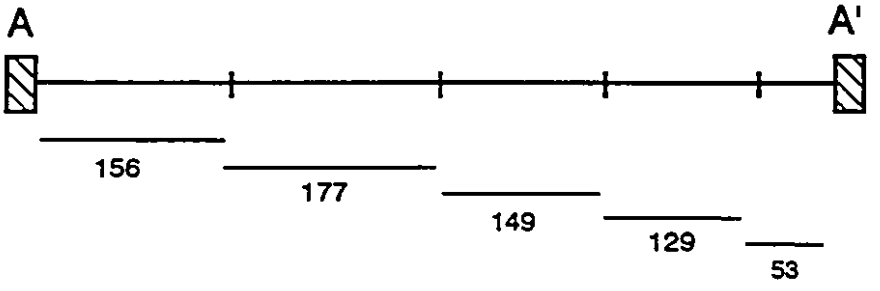
FIGURE 2

*Bst*NI restriction map of the HSV-1 *tk* gene segments enzymatically amplified using two different oligonucleotide primer sets. Shown are the *Bst*NI restriction sites as well as the size, in bp, of the restriction fragments.

A.
*Bst*NI restriction map of *tk* gene segment A-A'

B.
*Bst*NI restriction map of *tk* gene segment B-B'

A. *Bst*N1 restriction map of fragment A-A'



B. *Bst*N1 restriction map of fragment B-B'

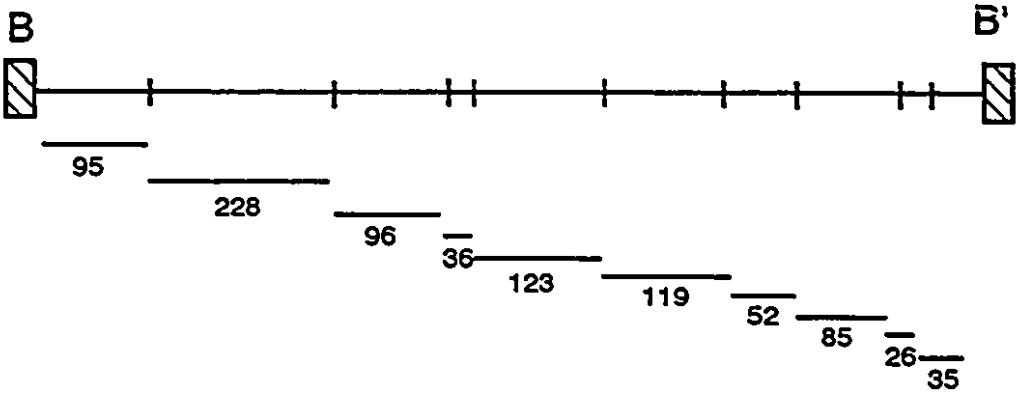


TABLE 1

List of *tk*$^-$ mutants subjected to restriction enzyme analyses to detect small mutational alterations

TFT-resistant *tk*$^-$ mutants

1.TFT-4	3.TFT-7	5.TFT-11
2.TFT-6	4.TFT-9	

ACV+TFT-resistant *tk*$^-$ mutants

1.AT-3-4	8.AT-4-11	15.AT-6-13
2.AT-5-4	9.AT-5-11	16.AT-1-17
3.AT-6-4	10.AT-6-11	17.AT-4-17
4.AT-8-4	11.AT-8-11	18.AT-1-18
5.AT-6-6	12.AT-1-13	19.AT-2-18
6.AT-1-11	13.AT-4-13	20.AT-7-18
7.AT-2-11	14.AT-5-13	21.AT-12-18

ACV+TFT+DHPG-resistant *tk*$^-$ mutants

1.ATD-10-2	16.ATD-11-27	31.ATD-7-29
3.ATD-2-19	17.ATD-12-27	32.ATD-8-29
4.ATD-15-19	18.ATD-13-27	33.ATD-10-29
5.ATD-6-21	19.ATD-2-28	34.ATD-11-29
6.ATD-12-21	20.ATD-3-28	35.ATD-13-29
7.ATD-1-26	21.ATD-4-28	36.ATD-14-29
8.ATD-2-27	22.ATD-6-28	37.ATD-16-29
9.ATD-3-27	23.ATD-7-28	38.ATD-2-31
10.ATD-4-27	24.ATD-8-28	39.ATD-2-32
11.ATD-5-27	25.ATD-9-28	40.ATD-4-32
12.ATD-7-27	26.ATD-10-28	41.ATD-6-32
13.ATD-8-27	27.ATD-11-28	42.ATD-7-32
14.ATD-9-27	28.ATD-12-28	43.ATD-9-32
15.ATD-10-27	29.ATD-4-29	44.ATD-13-32
	30.ATD-5-29	45.ATD-14-32

TABLE 2

Fine-structure analysis of the mutants

Mutants	Mutations ≥ 5 bp[a]
TFT^R	0/7 (0%)
$ACV+TFT^R$	7/28 (25.0%)
$ACV+TFT+DHPG^R$	8/55 (14.5%)

[a] Limit of resolution of the Southern blotting / PCR / restriction enzymes-polyacrylamide gel electrophoresis analyses

of drug resistance in these small potent mutants. In fact, the finding that a different restriction pattern was not observed in any of the seventy *tk*⁻ mutants tested could suggest that these mutants were the outcome of epigenetic events (Holliday, 1991), and this hypothesis was investigated.

DNA methylation has been frequently associated with the inactivation of different genes (Bird, 1984; Dynan, 1989), including the chromosomally-located HSV-1 *tk* gene (Ben-Hattar *et al.*, 1989; Antequera and Bird, 1993). To address that possibility, the methylation status of several of the potent *tk*⁻ mutants was first evaluated by Southern blotting. Genomic DNA was isolated from these small potent mutants, hydrolyzed with the following restriction enzymes: *Hpa*II, *Msp*I, *Sma*I, as well as *Eco*RI, and the restriction fragment patterns were compared to those of the KT (*tk*⁺) and 143B (*tk*⁻) cell lines. The enzymes *Hpa*II and *Msp*I recognize the same DNA sequence, but *Hpa*II cannot cleave methylated DNA, while *Msp*I can hydrolyze both methylated and non-methylated DNA. The recognition sequence for *Sma*I (5'CCCGGG3'), contains an internal CpG dinucleotide susceptible to methylation in the promoter/regulatory region of the HSV-1 *tk* gene, rich in cytosine and guanosine residues. Finally, *Eco*RI, which cannot cleave methylated DNA, was employed because of the previous demonstration by Tasseron-de Jong *et al.* (1989a,b) that the inhibition of the expression of the HSV-1 *tk* gene can be specifically accomplished through methylation of CpG residues at an *Eco*RI site found in the *tk* promoter. Table 3 lists the *tk*⁻ mutants which were analyzed at the level of DNA methylation, according to their resistance to different nucleoside analogues. The restriction fragment patterns from the KT (*tk*⁺), 143B (*tk*⁻ parental cell line), and the *tk*⁻

TABLE 3

List of *tk*$^-$ mutants subjected to the analysis of the methylation status of the *tk* gene and which did not demonstrate the involvement of extensive cytosine methylation at *Msp*I, *Hpa*II, *Sma*I and *Eco*RI sites.

TFT-resistant *tk*$^-$ mutants

1.TFT-4	3.TFT-7
2.TFT-6	4.TFT-11

ACV+TFT-resistant *tk*$^-$ mutants

1.AT-1-13	2.AT-1-17

ACV+TFT+DHPG-resistant *tk*$^-$ mutants

1.ATD-1-26	3.ATD-3-28
2.ATD-7-27	4.ATD-6-32

mutant cell lines, obtained by Southern blotting experiments, were then compared. This analysis revealed that none of the *tk*⁻ mutants investigated exhibited DNA methylation at these restriction sites.

Since the methylation of other residues or other sites within the *tk* gene may have gone undetected by the previous analysis, and since DNA methylation is not restricted to CpG residues (Woodcock *et al.*, 1987), a number of potent *tk*⁻ mutants were investigated with a different approach. The ability of the *tk*⁻ mutant cell lines to revert to the wild type TK⁺ phenotype, following treatment with the inhibitor of cytosine methylation 5-azacytidine (5-azaC), was evaluated (Table 4). As described in Chapter 2, 5-azaC can substitute for cytosine residues during DNA synthesis, but this analogue of cytosine resists methylation by the cellular methylase enzyme, causing hypomethylation of the newly replicated DNA. In the event that cytosine methylation played a role in the inactivation of the *tk* gene, hypomethylation, resulting from the presence of 5-azaC in the DNA, should permit the "reactivation" of the *tk* gene. The revertant TK⁺ phenotype can then be readily detected by the ability of the revertant cells to grow in medium supplemented with HAT, which imposes a requirement for a functional thymidine kinase (Chapter 1). In agreement with the preceeding methylation analysis, the results from the 5-azaC reversion studies revealed that none of the mutants tested were capable of reverting to a TK⁺ phenotype when methylation of cytosine residues was inhibited by 5-azaC (Table 4).

The involvement of a different epigenetic event occasionally associated with cellular resistance to combinations of structurally different compounds (West, 1990), a phenomenon known as multidrug resistance (MDR), was also investigated. Tumor cells

TABLE 4

List of *tk*$^-$ mutants subjected to the analysis of the methylation status of the *tk* gene and which did not demonstrate phenotype reversal following treatment with the demethylating agent 5-azacytidine (5-azaC)

ACV+TFT-resistant *tk*$^-$ mutants

1.AT-6-4	4.AT-1-17
2.AT-8-4	5.AT-4-17
3.AT-1-13	6.AT-12-18

ACV+TFT+DHPG-resistant *tk*$^-$ mutants

1.ATD-10-2	4.ATD-12-21
2.ATD-2-19	5.ATD-1-26
3.ATD-6-21	

have been shown to become resistant to multiple drugs by augmenting the efflux of drugs mediated by the increased production of a P-glycoprotein (West, 1990). In this study, the C-terminal portion of the mouse MDR-1 gene (kindly provided by Dr. Philippe Gros, Department of Biochemistry, McGill University), which is highly homologous to the human MDR-1 gene, was utilized as a radioactive DNA probe in order to detect the potential amplification of the gene encoding the P-glycoprotein (Devault and Gros, 1990; Raymond *et al.*, 1990). Table 5 enumerates the potent *tk*$^-$ mutants resistant to various combinations of nucleoside analogues that were submitted to the investigation of their MDR status. Dot blotting and Southern blotting results revealed that none of the *tk*$^-$ mutants tested displayed the amplification of the human MDR-1 gene.

II.3. NORTHERN BLOTTING ANALYSES

Most of the spontaneous potent *tk*$^-$ mutants selected on the basis of their resistance to multiple nucleoside analogues underwent DNA alterations implicating less than 5 bp, the limit of resolution of the restriction endonuclease hydrolysis/polyacrylamide gel electrophoresis assay (Figure 1 and Table 2). Nevertheless, these potent mutants were found to be deficient for the production of a stable TK polypeptide detectable by Western blotting (Chapter 3).

The possibility that the absence of a detectable TK protein was due to a reduction of transcription of the *tk* gene was thus investigated. Several *tk*$^-$ mutants, resistant to various combinations of drugs, were analyzed by Northern blotting (as described in Chapter 2), and Figure 3 presents the results from a relevant Northern blotting analysis

TABLE 5

List of *tk*$^-$ mutants subjected to a multidrug resistance (MDR) analysis and found not to contain amplified *mdr* DNA

TFT-resistant *tk*$^-$ mutants

1.TFT-4
2.TFT-6
3.TFT-7
4.TFT-11

ACV+TFT-resistant *tk*$^-$ mutants

1.AT-6-4
2.AT-1-5
3.AT-6-6
4.AT-4-13
5.AT-1-17
6.AT-4-17
7.AT-2-18
8.AT-12-18

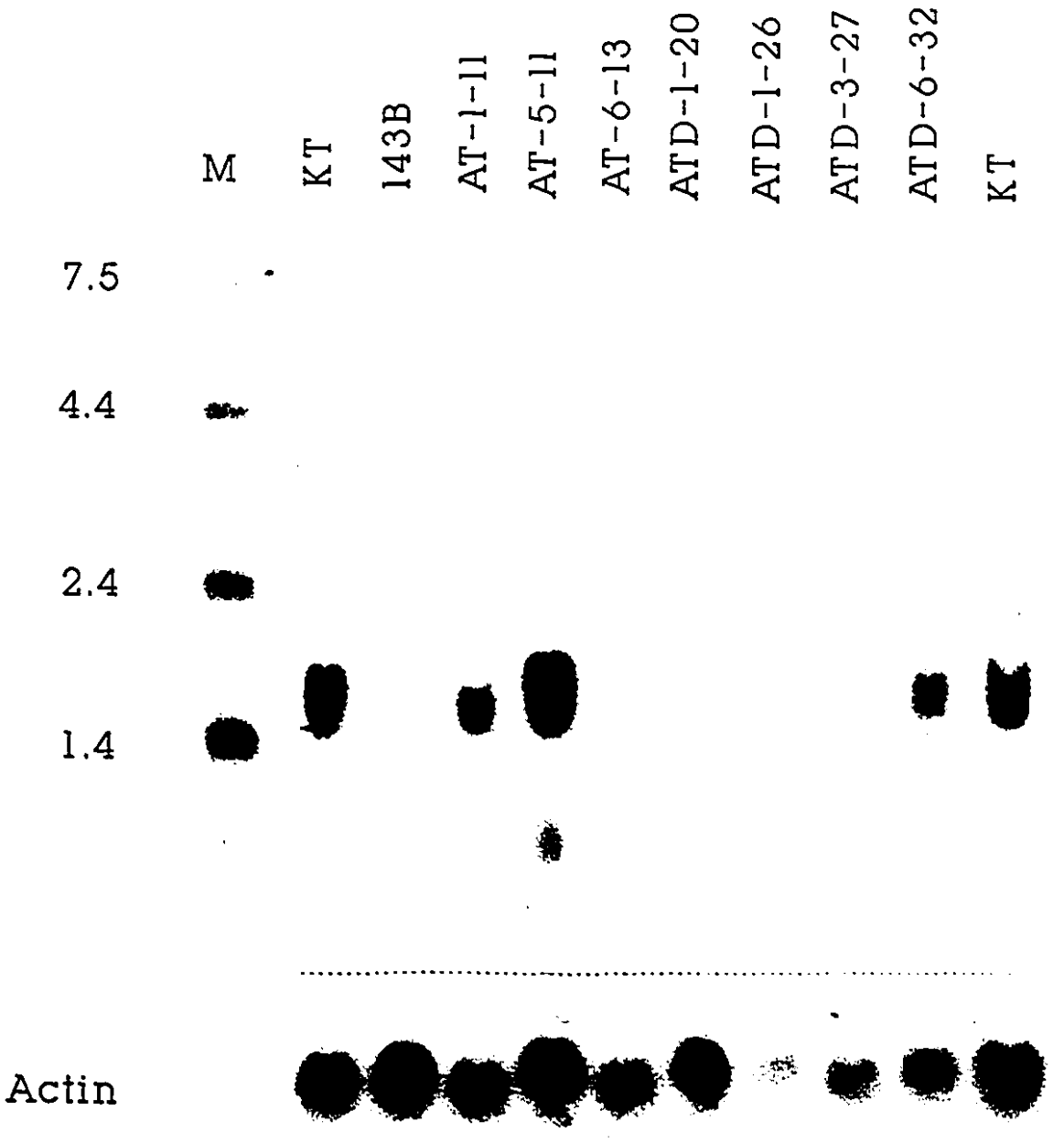
ACV+TFT+DHPG-resistant *tk*$^-$ mutants

1.ATD-10-2
2.ATD-2-19
3.ATD-6-21
4.ATD-1-26
5.ATD-7-27
6.ATD-4-28
7.ATD-1-29
8.ATD-7-29
9.ATD-13-29
10.ATD-4-32
11.ATD-9-32

FIGURE 3

Northern blotting analysis of the *tk*$^-$ mutants. Total RNA was isolated from the KT (*tk*$^+$) and 143B (*tk*$^-$) cell lines, as well as the *tk*$^-$ mutant cells, and subjected to denaturing gel electrophoresis, as described in Chapter 2. The RNA was then transferred on a nylon membrane, and the presence of a specific *tk* transcript was detected using the ^{32}P-labelled HSV-1 *tk* gene as a probe.

The bottom panel represents an internal control. The blots were stripped of any residual radioactivity and a transcript corresponding to the actin gene was detected using a radioactive actin gene segment as a ^{32}P-labelled DNA probe (described in detail in Chapter 2).


M
KT
143B
AT-1-11
AT-5-11
AT-6-13
ATD-1-20
ATD-1-26
ATD-3-27
ATD-6-32
KT
7.5
4.4
2.4
1.4
Actin

of selected potent *tk*⁻ mutants. To provide an accurate interpretation of the data, the levels of mRNA corresponding to the actin gene as detected by Northern blotting in the *tk*⁻ mutants, was evaluated and used as an internal control. The intensity of the bands, obtained after exposure of the radioactive blots to a Kodak X-ray film, was monitored by densitometry (as described in Chapter 2), and the results corresponding to Figure 3 are tabulated in Table 6. The ratio of *tk* mRNA/actin mRNA revealed that mRNA transcription was altered in several potent *tk*⁻ mutants (Table 6). A 29% increase in the level of *tk* mRNA for mutant AT-5-11, compared to the KT (*tk*⁺) cell line (Figure 3 and Table 6) was observed . Mutant AT-5-11 had been selected on the basis of its resistance to ACV plus TFT and demonstrated cross-resistance to DHPG. However, this mutant was shown to revert to the wild type TK+ phenotype at a frequency of 2.5×10^{-4} (Chapter 3). Interestingly, *tk*⁻ mutant AT-5-11 was the only mutant in which a TK polypeptide was detected by Western blotting. In contrast, no *tk* transcripts were detected in mutant ATD-1-20, while mutants ATD-1-26 and ATD-3-27 showed a reduction of 61% and 81%, respectively, in the amount of *tk* transcript (Figure 3 and Table 6). Mutants ATD-1-26 and ATD-3-27, resistant to ACV+TFT+DHPG, did not revert to wild type, and the presence of a TK polypeptide was not detected by Western blots (Chapter 3).

An estimation of the effect of the small potent mutations on the TK phenotype was provided by Table 7 which summarizes the results concerning the ability of the various *tk*⁻ mutants to produce a *tk* mRNA. The moderate mutants, resistant to TFT as a single selective agent, were associated with levels of *tk* mRNA similar to those of the wild type KT (*tk*⁺) cell line (not shown). In contrast, Table 7 shows that 30% to 36% of the potent

TABLE 6

Evaluation of the intensity of the bands corresponding to *tk* mRNA and actin mRNA by densitometry as detected by Northern blotting. *

CELL LINES	*tk* mRNA	mutant/ KT	actin mRNA	mutant/ KT	*tk*/actin mRNA	% difference
KT	11.559	---	9.162	---	---	100%
143B	0	0	12.213	1.333	0	0
AT-1-1	7.136	0.617	6.971	0.760	0.81	-19%
AT-5-11	20.898	1.808	12.898	1.407	1.29	+29%
AT-5-13	0.900	0.0789	5.725	0.625	0.12	-88%
ATD-1-20	0	0	10.699	1.168	0	0
ATD-1-26	1.125	0.0907	2.297	0.251	0.39	-61%
ATD-3-27	0.845	0.073	3.610	0.394	0.19	-81%
ATD-6-32	5.738	0.496	4.612	0.503	0.99	-1%

*Amount of mRNA (in percentages) in the *tk*$^-$ mutants cell lines, compared to the wild type KT (*tk*$^+$) cell line.

TABLE 7

Northern blotting analysis of the *tk*$^-$ mutants

Mutants	Normal *tk* transcript[a]
TFT^R	7/7 (100%)
ACV+TFT^R	3/10 (30%)
ACV+TFT+DHPG^R	5/14 (36%)

[a] size and amount of *tk* transcript similar to wild type, as revealed by Northern blotting analysis

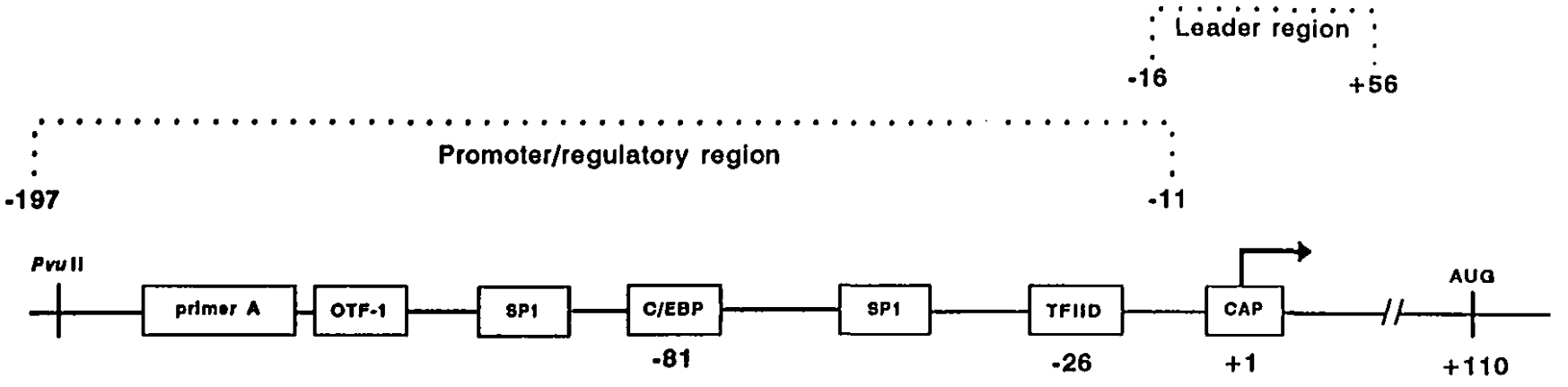
FIGURE 4

Regulatory region of the HSV-1 thymidine kinase (*tk*) gene.

Shown is the position of oligonucleotide primer A, utilized in the polymerase chain reaction (PCR) procedure. Also demonstrated are the binding sites and the recognition sequences for several transcription factors, as well as the mRNA cap site and the translation start (AUG).

Numbering is in base pairs. The +1 position corresponds to the cap site for the HSV-1 *tk* mRNA transcript.

HSV-1 *tk* REGULATORY REGION



OTF-1: ATTTGCAT
SP1: CCGCCC (2nd distal signal)
C/EBP: CGAAT (concensus=CCAAT)
SP1: GGGCGG (1st distal signal)
TFIID: TATTAA (concensus=TATAAA)
CAP: mRNA cap site
AUG: Translation start

mutants demonstrated either a significant reduction, or absence, of the *tk* transcript. Since the promoter/regulatory region of the *tk* gene has been shown to be important for the regulation of transcription, this region was sequenced in two small potent mutants: AT-5-11 and ATD-3-27. The region from the *tk* gene comprised within oligonucleotide primers A and A' contains the binding sites for several transcription factors (Figure 4), but was found not to be mutated in the small potent *tk*$^-$ mutants investigated.

III. DISCUSSION

Ninety *tk*$^-$ mutants were selected on the basis of their resistance to different nucleoside analogues used either alone or in a combined fashion (Chapter 3). The resistance to TFT as a single selective agent was proposed to result from moderate mutations in the *tk* gene. In contrast, mutants resistant to ACV+TFT and ACV+TFT+DHPG consisted mostly of potent mutations which severely modified the TK phenotype. The studies described in this chapter thus aimed at elucidating which mutational events involved in the formation of potent *tk*$^-$ mutants involving small DNA alterations, and which nevertheless demonstrate a characteristically null phenotype: the complete and permanent inactivation of the *tk* gene function.

A more precise analysis of the mutated *tk* gene was performed in order to detect the potential involvement of smaller DNA alterations in seventy *tk*$^-$ mutants (Figure 1). However, none of the multiple drug resistant *tk*$^-$ mutants were the result of a mutation implicating more than 5 bp (Table 2). It must be appreciated that this assay would not detect single point mutations, yet it can reasonably be anticipated that from such a large

number of *tk*⁻ mutants, a few would demonstrate a DNA alteration discernable by the combined PCR/restriction hydrolysis studies. The finding that potent mutations carry presumptive point mutations, yet displayed the stable and severe alteration of the TK phenotype, underscored the important association between small potent mutations and phenotypic alterations.

The role of DNA methylation in the silencing of the *tk* gene was also investigated by comparing the *tk*⁻ mutants with the KT (*tk*⁺) and the 143B (*tk*⁻) cells, with respect to the restriction endonuclease motifs corresponding to methyl-sensitive and -resistant enzymes, as well as by phenotype reversal following demethylation by 5-azaC. However, the involvement of DNA methylation as a major contributor in the formation of the *tk*⁻ mutants selected in this study seems remote. The absence of extensive methylation in the small potent *tk*⁻ mutant cell lines from this study corroborates preliminary results from our laboratory (Goring *et al.*, 1987). These findings also correlate with the observation that the potent *tk*⁻ mutations do not readily revert to the wild type, while mutations caused by DNA methylation typically undergo phenotype reversal at relatively high frequencies (Tasseron-de Jong *et al.*, 1989a; Holliday, 1991).

However, our results contrast with numerous studies concerning both a chromosomally-integrated HSV-1 *tk* gene, and the cellular autosomal *tk* locus. Tasseron-de Jong and co-workers (1989a) showed that cytosine methylation at an *Eco*RI site, located in the promoter region (Figure 4), constituted a major event in the inactivation of the HSV-1 *tk* gene. This group also demonstrated that *Hpa*II sites were methylated when multiple copies of the *tk* gene were integrated in the mammalian genome, while these

sites remained non-methylated in the promoter of a single-copy, chromosomally-located HSV-1 *tk* gene (Tasseron-de Jong *et al.*, 1989b). Similar results of methylation resulting in gene inactivation at clusters of *tk* genes were reported for the endogenous cellular *tk* locus (Hardies *et al.*, 1983; Christy and Scangos, 1985). Buschhausen and co-workers (1985, 1987) have also shown that the expression of the HSV-1 *tk* gene could result from DNA methylation which culminates in an altered chromatin structure hindering transcription (Deobagkar *et al.*, 1990). Moreover, the specific involvement of cytosine methylation at CTF and SP1 recognition sites present in the HSV-1 *tk* promoter (Figure 4) has been reported by Ben-Hattar *et al.* (1989). Previous work (Ostrander *et al.*, 1982; Dobrovic *et al.*, 1988) had shown that *tk*$^-$ mutants could result from methylation at the endogenous cellular *tk* locus. Similarly, Call and Thilly (1991) demonstrated that the mammalian *tk* gene acquired methylation when its expression was selected against using the nucleoside analogue BUdR, and that this gene inactivation effect could be reversed by the demethylating agent 5-azaC. Nevertheless, the implication of DNA methylation was ruled out as a major event causing the inactivation of the *tk* gene in the KT cell line from our study, which could be due to chromosomal location of the *tk* gene, where it is subject to different pressures brought about by the chromosomal context. In fact, difficulties were encountered in cloning the integration site of pSV2*neo*KT in the KT cell line (Pascali, 1990). The cellular DNA flanking the plasmid may thus have an influence on the generation of the small potent *tk*$^-$ mutants.

In certain tumor cell lines, resistance to several drugs is accomplished through the P-glycoprotein, a transmembrane pump which causes the efflux of the drugs from the cell

(Devault and Gros, 1990; Raymond *et al.*, 1990). Multidrug resistance (MDR) confers a phenotype of resistance to a wide range of structurally unrelated cytotoxic agents (Endicott and Ling, 1989; West 1990). In this study, none of the small potent *tk*$^-$ mutants resistant to combinations of drugs demonstrated the amplification of the gene encoding MDR efflux. Nevertheless, overexpression of the *mdr* gene (in single-copy) cannot be ruled out.

Even though mutational alterations were not discernible in the potent *tk*$^-$ mutants, Western blotting analyses had previously indicated that the function of the *tk* gene may be severely affected in the vast majority of the multiple drug resistant potent *tk*$^-$ mutants (Chapter 3). Subsequent investigations presented in this Chapter revealed that 30% to 36% of the potent mutants exhibited altered levels of *tk* mRNA, as observed on Northern blots. In contrast, the amount of *tk* mRNA was comparable to wild type in the moderate mutants, suggesting that moderate mutations may not significantly affect *tk* transcription.

The level of *tk* mRNA detected by Northern blotting was increased by 29% compared to the wild type level in mutant AT-5-11, which was the only multiple drug resistant mutant which demonstrated the detectable presence of a TK polypeptide by Western blotting (Chapter 3). The small mutation implicated in mutant ATD-3-27 ($\leq$ 5 bp), constitutes a potent mutation which led to reduced levels of *tk* transcripts and to the absence of a detectable TK polypeptide. The regulatory region of the *tk* gene from mutants AT-5-11 and ATD-3-27 was sequenced (Figure 4), and shown not to be mutated. This region is comprised within nucleotide -197 and -11 and contains binding sites for several transcription factors (McKnight *et al.*, 1981, 1984; Jones *et al.*, 1985; Pang and

Chen, 1993) (Figure 4). Mutations in the SP1 binding sites, in particular, have been shown to greatly affect the stablilty of the HSV-1 *tk* transcript (McKnight, 1982; McKnight *et al.*, 1984; Ben-Hattar *et al.*, 1989; Boni *et al.*, 1989). The leader region (-16 to +56, Figure 4) contains the mRNA CAP site and has also been shown to be important for efficient transcription of the HSV-1 *tk* gene (Pappavassiliou and Silverstein, 1990). Since mutations were not found in the promoter region of the few mutants investigated, other alternatives are conceivable. Mutations in the polyadenylation signals of the HSV-1 *tk* mRNA have been shown to dramatically alter the stability of the transcripts (Zhang *et al.*, 1986; Cleveland and Yen, 1989). Mutations in the 5' or 3' non-coding region of the *myc* oncogene, for example, have been associated with the increased the stability of the *myc* mRNA (Davies *et al.*, 1984). The characterization of the promoter/regulatory region, as well as the 3' non-coding region, from several small, yet potent, *tk*$^-$ mutants should provide information concerning the types of mutations which could modulate the transcription of the *tk* gene or affect the stability of the *tk* transcript.

Work by Plagermann and Wohlhuetter (1985) showed that mammalian cells resistant to purine analogues could still display wild type levels of HPRT enzyme activity, suggesting that resistance to structural analogues of nucleosides can be conferred by various, and yet unknown, cellular mechanisms. The inactivation of the HSV-1 *tk* gene from the KT cell line can thus be the result of numerous cellular events (Gebara *et al.*, 1989), and drug resistance can be similarly accomplished through various processes (Chatis and Crumpacker, 1992; Field and Biron, 1994).

Detoxification mechanisms such as the MDR efflux pump (Endicott and Ling, 1989;

West, 1990), or a more specific mechanism such as the defluorination of TFT into 5'carboxy 2' deoxyuridine (Fujiwara *et al.*, 1970; Dexter *et al.*, 1972), which is neither a substrate nor an inhibitor of the HSV-1 TK enzyme, could also be involved. However, the utilization of combinations of drugs should render this latter detoxification hypothesis an unlikely mechanism of resistance in the multiple drug resistant *tk*$^-$ mutants from this study. The inability to take up the drug also appears unlikely since the nucleoside analogues utilized are believed to be transported via different routes through the cell membrane (Davidson *et al*, 1981; Mahony *et al*, 1988; Matthews and Boehme, 1988; described in Chapter 1). Mutations in the cellular ribonucleotide reductase gene may favor a TK$^-$ phenotype by augmenting the sensitivity of the cells to nucleoside analogues (Karlsson and Harmenberg, 1988; Coen *et al.*, 1989a; Reardon and Spector, 1991; Yamada *et al.*, 1991). Alterations in the cellular DNA polymerase gene might also increase the affinity of this DNA replication enzyme for the metabolically active triphosphate forms of ACV, TFT and DHPG, leading to increased sensitivity of the *tk*$^+$ cells (Coen and Schaffer, 1980). Since the HSV-1 TK protein performs the initial phosphorylation of the nucleosides (and their structural analogues) to the monophosphate form, mutations in the cellular kinases involved in the phosphorylation to their corresponding di- and triphosphate forms could also affect the sensitivity of the cells to the antiviral drugs and confer a TK$^-$ phenotype. Finally, since the TK gene product was altered in the potent *tk*$^-$ mutants from this study, and knowing that the amount of *tk* mRNA was also affected, the involvement of a mutation in a gene which could specifically modulate the transcription and/or the translation of the *tk* gene constitutes yet

another a possibility. Alternatively, the spontaneous potent mutants may be enriched for point mutations which severely affect the *tk* mRNA, or the folding of the TK protein.

The overall implication of the findings presented in this Chapter is that a large proportion of the infrequent mutational events which occur in a chromosomally-located HSV-1 *tk* gene may carry small point mutations, and yet lead to potent, stable mutations which severely affect the TK phenotype. These results underscore the important role of small ($\leq$ 5 bp), yet potent, point mutations in the generation of a null phenotype.

SUMMARY, CONCLUSIONS, AND SPECULATIONS

Spontaneous mutations constitute essential events providing the basis for genetic diversity and evolution. These genetic events, which can occur as a result of normal cellular mechanisms and interactions with the environment (Smith, 1992), are also implicated in the generation of genetic disorders (Caskey, 1987) and cancer (Knudson, 1986; Rudiger, 1990; Bishop, 1991). Thus, because of their important role, the characterization of the various types of mutations, and the elucidation of the mechanisms by which they take place (Ames, 1989), should be thoroughly considered.

As discussed in Chapter 1, spontaneous mutations comprise various genetic events (Ramel, 1989), ranging from simple point mutations like transitions, transversions, and frameshift mutations, to major DNA alterations, such as large deletions or insertions, and chromosomal aberrations. It appears that no unambiguous criterion exists to classify spontaneous mutations, as a certain degree of overlap inevitably connects arbitrary categories of mutations. In this work, the mutations were classified into two classes according to the severity of their impact on the genome of an organism, in order to be able to focus on the analysis of mutations which have a considerable influence on the human genome. Spontaneous moderate mutations represent more frequent and unstable mutational events which may demonstrate "leaky" phenotypes. In contrast, spontaneous potent mutations constitute infrequent events, with more dramatic effects on the phenotype associated with a gene, because they completely abolish the function in a non-revertible fashion. The rarity and the important consequences of spontaneous potent mutations

render their characterization an important aspect of mammalian spontaneous mutagenesis.

Mammalian cells in culture (*in vitro*) offer an alternative for the systematic investigation of spontaneous mutations that can potentially occur *in vivo* (Oller and Thilly, 1992). Utilizing various genetic systems, spontaneous point mutations have been well characterized in several studies concerning mammalian mutagenesis (Skandalis and Glickman, 1990) at cellular loci such as the *aprt* (de Jong *et al.*, 1988; Phear *et al.*, 1989), *hprt* (Fuscoe *et al.*, 1986; Oller and Thilly, 1992) and the endogenous *tk* (Liber and Thilly, 1982) genes. However, because of the frequent occurrence of simple, and often reversible, point mutations, it is reasonable to assume that more rare genetic events might escape detection.

To circumvent this problem while addressing spontaneous mutagenesis in a human chromosomal context, the KT cell line was developed (Chapter 2) in order to select and analyse spontaneous mutations which arise by infrequent and, as yet, uncharacterized genetic events. The KT cell line comprises a single copy of a chromosomally-located pSV2*neo*KT plasmid which contains the neomycin resistance gene (*neo*) as well as the HSV-1 thymidine kinase gene (*tk*) (Goring and DuBow, 1985). The HSV-1 *tk* gene served as a target for spontaneous gene inactivation, while the continuous presence of a functional *neo* gene throughout the selection procedure allowed us to screen out gross chromosomal alterations, such as large chromosomal aberrations or total plasmid loss. Forward mutations ($tk^+ \rightarrow tk^-$), which occur spontaneously and result in TK deficiency, can be selected on the basis of the resistance of the tk^- mutants to three different analogues of nucleosides: acyclovir (ACV), trifluorothymidine (TFT), and ganciclovir (DHPG).

These three drugs require an initial phosphorylation to the monophosphate form by the HSV-1 TK, are subsequently phosphorylated to their metabolically-active triphosphate counterparts by cellular kinases, before they are incorporated into the elongating DNA chain. Reverse mutants (*tk*$^-$ → *tk*$^+$) can also be isolated by their ability to grow in the presence of HAT in the culture medium, which imposes upon the cells a requirement for a functional thymidine kinase (Chapter 2).

In order to characterize rare and potent mutations in the human genome, a selection procedure was developed to enrich for potent *tk*$^-$ mutations, over the background of the moderate mutations. This enrichment, described in Chapters 1 and 2, exploits the utilization of three different nucleoside analogues (ACV, TFT, DHPG) in a combined fashion to select for mutations in the chromosomally-located HSV-1 *tk* gene. The hypothesis for the enrichment of spontaneous potent mutations was presented and validated in Chapter 3. Briefly, it was hypothesized that the use of three drugs of different structures and mechanisms of action may allow a selective enrichment for potent mutations conferring resistance to combinations of drugs, while selectively reducing the recovery of moderate mutations which allow resistance to a single nucleoside analogue. It was demonstrated in Chapter 3 that the apparent mutation frequency in the *tk* gene is reduced through the use of combinations of drugs.

Since the aim of this study was to address spontaneous mutagenesis with an emphasis on the potent mutations, the stability of the mutated phenotype was evaluated, following proliferation of the mutant cell lines in the absence of the selection pressure for *tk*$^-$ cells imposed by nucleoside analogues. More than 86% of the mutations which came

through the enrichment procedure could not revert to the wild type genotype (or phenotype) at a detectable frequency, as shown by their inability to grow in HAT medium. Moreover, a mutated TK protein was not discernible, by Western blotting, in the vast majority (82.5%) of the potent *tk*$^-$ mutants resistant to combinations of drugs. It can thus be proposed that these spontaneous potent *tk*$^-$ mutants are also enriched for mutational events which greatly affected the protein product of the *tk* gene.

When the *tk*$^-$ mutants were analysed by Southern blotting and PCR analyses, it was observed that among ninety spontaneous *tk*$^-$ mutants investigated, fourteen underwent a DNA alteration involving more than 10-50 bp (Chapter 4). Deletions of the entire *tk* gene, or a large portion of this gene, were the main class of major DNA alterations selected in our system. In all deletion mutants isolated, the *neo* gene sequence and some of the adjacent plasmid DNA sequence were not affected, due to the continuous presence of G418 in the culture medium throughout the selection process. Thus, genotypic changes resulting from chromosomal loss or gross chromosomal aberrations may be avoided by the utilization of the *neo* gene as a second selectable marker. The deletion endpoints in the *tk* gene, as well as in the surrounding cellular DNA, varied in the deletion mutants. Thus, in the KT cell line, a hot spot for spontaneous deletions in the HSV-1 *tk* gene was not observed. The finding of deletion mutants with no specific endpoints within the *tk* gene corroborates other studies which reported deletions lacking extensive sequence homologies at the breakpoints (Morris and Tacker, 1993). These results contrast with previous reports of spontaneous deletions in eucaryotic genes performed in diploid cell lines in which the cellular genes are present in two copies. In these genetic systems, loss

of heterozygosity, as a result of homologous recombination between the two alleles of a gene, represented the vast majority of the deletion events (Ward *et al.*, 1990; Klinedinst and Drinkwater, 1991; Amundson and Liber, 1992). Because the KT cell line comprises a single-copy of the *tk* gene, the formation of deletion through such homologous recombination mechanisms was avoided. Moreover, because of their important role in the formation of several genetic diseases (Krawczak and Cooper, 1991), the characterization of deletion events in the human genome is very important. In fact, deletions have been implicated in more than 70% of the mutations causing Duchenne's muscular dystrophy (Forrest *et al.*, 1987), steroid sulfatase deficiency (Yen *et al.*, 1990), 50% of the ornithine transcarbamylase deficiency cases (Caskey, 1987), and 20% of the mutations in the Lesch-Nyhan syndrome (HPRT deficiency) (Yen *et al.*, 1990).

In addition to deletion mutations, a more complex intragenic rearrangement of the *tk* gene was isolated and characterized (mutant ATD-1-19). This spontaneous potent mutation resulted in a deletion/duplication event which may be the outcome of an illegitimate recombination event, facilitated by short regions of junctional homology. Two models of non-homologous recombination were presented in Chapter 4, in order to explain the structure of the mutational lesion observed in this potent *tk*$^-$ mutant, as well as the molecular events through which it was generated. Bacteria and yeast utilize homologous recombination frequently and efficiently (Szostak *et al.*, 1983; Dybvig, 1993), but in mammalian cells, non-homologous recombination constitutes the most frequent mutational event by which genes become rearranged (Desautels *et al.*, 1990; Roth and Wilson, 1988). Unfortunately, little is known about the cellular mechanisms implicated

in this process. Morris and Thacker (1993) reported that large deletions in the human *hprt* gene induced by X-rays, can result from non-homologous recombination. The characterization of another complex DNA alteration by Southern blotting was also presented in Chapter 4, and possible mechanisms implicated in the formation of the spontaneous potent mutant AT-2-6 were proposed. It must be appreciated that the formation of these latter two mutational events may have remained undetected in a cellular heterozygous system in which both copies of a gene have to be altered in order for a mutation to be detected. Thus, the characterization of this deletion/duplication event adds to the diversity of mutational events known to occur spontaneously in the human genome and may reflect the diversity and extraordinary dynamics of the human genome.

In addition to the characterization of important alterations of the *tk* gene (≥ 10-25 bp), we demonstrated that a large proportion (75% to 85%) of the potent mutations in the *tk* gene were the outcome of DNA alterations implicating less than 3-5 bp of DNA. Due to their stability and their severe effect on the genome, the role of small, yet potent, mutations was evaluated further. The possibility that the potent mutant phenotype (or genotype) was the outcome of epigenetic changes, such as extensive DNA methylation (Holliday, 1991), was evaluated. Our results suggested that cytosine methylation was not responsible for the generation of the mutants resistant to combinations of drugs. These findings contrast with those of several groups who demonstrated that DNA methylation was a major event resulting in the inactivation of the HSV-1 *tk* gene (Clough *et al.*, 1982; Ostrander *et al.*, 1982; Tasseron-de Jong, 1989a,b), as well as the cellular endogenous *tk* locus (Buschhausen *et al.*, 1987; Dobrovic *et al.*, 1988; Call and Thilly, 1991). The

involvement of the non-specific efflux of the nucleoside analogues through the multidrug resistance (MDR) glycoprotein (Ling *et al.*, 1988; Devault and Gros, 1990) similarly represents an unlikely event. It is thus possible that the selection pressure imposed by the presence of three nucleoside analogues also minimize the isolation of mutations resulting from (unstable) epigenetic events.

The analysis of the potent mutations implicating very small DNA changes was continued by evaluating the effects of the mutations on the levels of *tk* transcripts detectable by Northern blotting. While Western blotting studies (Chapter 3) suggested that the detectable presence of a TK polypeptide was greatly altered, Northern blotting studies revealed that the level of mutant *tk* mRNA was altered in most *tk*$^-$ mutants resistant to combinations of drugs. The promoter/regulatory regions of the *tk* gene (McKnight *et al.*, 1981; McKnight, 1982; McKnight *et al.*, 1984) from mutants demonstrating increased or reduced levels of *tk* mRNA was then sequenced. Even though this region is known to be important for the modulation of the transcription in the HSV-1 *tk* gene (Ben-Hattar *et al.*, 1989), a mutation was not detected. However, other regions within a gene, such as the polyadenylation signal (Cleveland and Yen, 1989), have been shown to alter the stability of mRNA transcripts, and may account for the various levels of *tk* mRNA detected in our study (Davies *et al.*, 1984). In contrast, moderate mutants resistant to TFT only showed wild type levels of *tk* mRNA.

The results presented in this thesis revealed that a large proportion of the potent mutations were the result of DNA changes implicating less than 5 bp. It is interesting to note that most potent mutants underwent small DNA alterations which severely affected

the production and/or the stability of the *tk* mRNA or the TK protein. In addition, the possibility that the potent mutations have a heterogenous nature should to be considered. As discussed in Chapter 5, mutations at various steps of complex and interrelated biochemical pathways of nucleoside biosynthesis may also influence the potent drug resistance phenotype reported in this study. Since combinations of nucleoside analogues which may be transported through the membrane via different routes were used (Davidson, 1981; Mahony *et al.*, 1988; Matthews and Boehme, 1988), the enrichment procedure presented in this study should limit the selection of *tk*$^-$ mutants resulting from a decreased permeability of the cells for the drugs. Another possibility is position effect variegation, the modulation of gene expression due to the location of a given gene within a region of heterochromatin, which has been shown to alter gene expression (Hayashi *et al*, 1991). However, the presence of a functional *neo* gene in the vicinity of the *tk* gene in the mutant *tk*$^-$ cell lines renders this hypothesis less plausible. The observation that the level of mRNA transcription may be affected raised the possibility that the mechanism of nucleoside analogue resistance may be through the modulation of the transcription of the *tk* gene. This possibility could be analyzed and the following experiment may provide an answer. The introduction, into the various *tk*$^-$ mutant cell lines, of a plasmid comprising the HSV-1 *tk* gene plus a second selectable marker [such as the histidinol resistance gene (Hartman and Berg, 1988)], different from G418^R. The selection for TK$^+$/*his*^{R} cell lines may indicate that the transcription of these two markers is similarly altered (i.e. a *trans*-acting mutation), or may suggest that the transcription of the resident HSV-1 *tk* gene seems specifically modulated.

In addition to contributing to the understanding of spontaneous mammalian mutagenesis, this study shows relevance to the analysis of the mechanisms of drug resistance. In fact, the study of the acquisition of resistance with a genetic basis, as well as the cellular mechanisms involved, were discussed throughout this thesis. For example, the immunocompromised state of patients suffering from the acquired immunodeficiency syndrome (AIDS) as a result of an infection with the human immunodeficiency virus (HIV-1), favored the emergence of resistant HSV-2 viruses in these patients (Hill *et al.*, 1991; Talarico *et al.*, 1993). Thus, the nature of resistance to nucleoside analogues, although complex, represents a problem of major clinical significance (Coen, 1991; Chatis and Crumpacker, 1992; Field and Biron, 1994). In addition, our results may prove pertinent for the design of antiviral chemotherapy exploiting different drugs in a combined fashion, as reported for the treatment of HSV-1 infections (Birch *et al.*, 1992) as well as HIV-1 and HSV-2 infections treated with a combination of zidovudine (AZT) and acyclovir (Pedersen *et al.*, 1992) or ganciclovir (Medina *et al.*, 1992).

When the results from this study, and from previous work on spontaneous mutagenesis are combined, it becomes clear that different *in vitro* genetic systems demonstrate distinct characteristics, as well as particular advantages and specific drawbacks. For example, the *aprt* gene system represents a cellular locus located on an autosome (Nalbantoglu *et al.*, 1986). Consequently, the selection of molecular events resulting in reduction to homozygosity of the mutant alleles through processes such as homologous mitotic recombination, or the complete loss of one copy of a chromosome, might be favored. In contrast, the utilization of a single-copy chromosomally-located *tk*

gene allows the detection of recessive mutations and the isolation of genetic events which can occur in an intragenic fashion. Thus the KT cell line provided a different and convenient approach to select and characterize rare and potent genetic events which occur spontaneously in a chromosomal context. The extraordinary diversity and variability of the human genome requires different genetic systems, such as the KT cell line presented in this study, in order to successfully reflect the types of mutations which can spontaneously occur in humans and potentially lead to genetic disorders.

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