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MOLECULAR APPROACHES TO THE STUDY OF CHEMICAL MUTAGENESIS

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Summary. - *The use of shuttle vectors has been applied in recent years to develop a better understanding of the molecular mechanisms of mutagenesis in mammalian cells. These recombinant DNA molecules replicate together with the host eukaryotic cells and can be retrieved in bacteria for rapid detection and analysis of mutation. Two approaches based on the use of shuttle vectors for studying the mutagenic effects of DNA lesions induced by alkylating agents are presented.*

Riassunto (Studio dei meccanismi molecolari di mutagenesi). - *Negli ultimi anni i cosiddetti "shuttle vectors" sono stati usati con successo per elucidare i meccanismi molecolari di mutagenesi in cellule di mammifero. Queste molecole di DNA ricombinante sono in grado di replicare insieme al sistema eucariotico ospite e il rilevamento e l'analisi delle mutazioni possono essere effettuati negli idonei ospiti procariotici. In questo lavoro verranno descritti due sistemi sperimentali, basati sull'uso di "shuttle vectors", disegnati per studiare gli effetti mutageni delle lesioni del DNA indotte da agenti alchilanti.*

Introduction

The understanding of the mechanism by which mutations are fixed as a heritable change in mammalian cells is quite limited when compared to bacterial cells. In order to overcome this disparity, recombinant DNA molecules, so called shuttle vectors, have been constructed: a bacterial marker gene is placed on a vector which can be replicated and selected in both bacterial and mammalian cells (Fig. 1). The vector is then placed in the nucleus of a mammalian cell where it is subjected to many of the normal metabolic processes that the cell can perform. After some given lenght of time the vector can be re-isolated and transformed into *Escherichia coli (E. coli)* for analysis of the marker gene. This simple idea has proven to be a very powerful concept and has begun to allow the characterization of mammalian DNA metabolism using well defined bacterial and mammalian cells. The first attempts to use extracromosomal shuttle vectors were reported in 1983 [1, 2]. The vectors contained the SV40 origin of replication and SV40 T antigen that allowed extrachromosomal replication in permissive simian cells [3]. The spontaneus mutation frequencies (smf) in the target gene, lac I, was 1%, which is orders of magnitude greater than the smf usally reported for mammalian genes of 10^{-6} /10^{-7}. The DNA sequence analysis of these mutants suggested that the DNA molecules undergo depurination and deamination, possibly as a result of exposure to the low pH of the lisosomes during passage through the mammalian cells.

Another approach has been reported in 1982 [4] in which an SV40-derived shuttle vector was transfected into a cell line non-permissive for SV40 replication, Chinese hamster ovary (CHO) cells. The vector could then be liberated from the cell's chromosomes by fusion of the vector-containing cells to COS cells which provide SV40 T antigen and permissive factors [5]. This system had the unique advantage of allowing the investigators to clone cells containing vectors which had escaped damage associated with transfection. However there was a significant fraction of rearrangements. In 1986 Ashman *et al.* [6] modified the system by cloning the target gene in a retroviral vector. They isolated a cell line with a single copy of the target gene with a low smf of 2×10^{-5} and selected for mutants in mammalian cells. It was evident that all DNA transfected into mammalian cells encounters some mutational barriers upon entry into the nucleus but, once a DNA molecule has successfully passed throught this barrier it appears to be metabolizied with high fidelity much the same as normal chromosome DNA.

In 1984, Sudgen *et al.* reported the creation of a new group of shuttle vector based on the human herpes virus, Epstein-Barr [7, 8]. These vectors made use of EBV's latent origin of replication, ori-P, and could be stably propagated in EBV-transformed human lynphocytes. By 1985 these investigators had also cloned the EBNA-1 gene which acts in *trans* to allow replication from ori-P [9]. They demonstrated that vectors containing both EBNA-1 and ori-P were able to replicate in a variety of non EBV-transformed cell lines, as well as in cells from monkey and

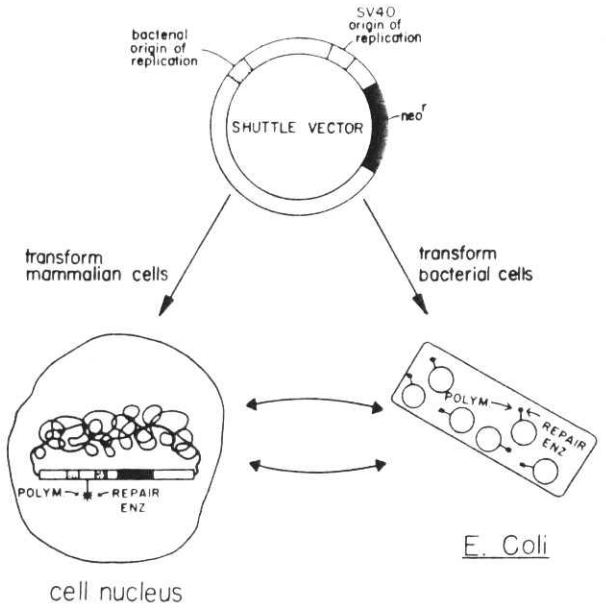

Fig. 1. - Scheme of a shuttle vector system.

dogs. In 1986 Drinkwater and Klinedinst [10] demonstrated that EBV-based vector systems could be designed to yield an extremely low spontaneous mutation frequency ($2.6 - 7.6 \times 10^{-5}$). The authors tested the ability of this system to detect mutations induced by treating the cells with the alkylating agent N-ethylnitrosourea. In related experiments, DuBridge *et al.* [11] had also explored the use of EBV-based vectors in human cells. They isolated a clonal cell line with a smf at the lac I gene of 6.4×10^{-6} which is also the smf of this target gene in *E. coli*. Treatment of this vector-containing clone with a concentration of 1 mM N-methylnitrosourea showed that the mutations increased to a level 300-fold above background. Analysis of the mutational pattern of the marker genes has provided information on the processing of DNA damage in mammalian cells.

Two approaches based on the use of shuttle vectors were undertaken in our laboratory. A brief description will follow.

Contruction of an EBV-derived shuttle vector for studying the influence of transcription on mutagenesis

We have constructed an EBV-derived shuttle vector to investigate the role of DNA transcription in DNA repair and mutagenesis. Preferential DNA repair of transcriptionally active genes appears to be a normal feature of rodent and human cells after UV damage [12, 13], but the relationship between transcriptional activity and efficiency of DNA repair is not understood. Furthermore the potential implications of this phenomenon in the processes of cell mutagenesis and carcinogenesis have not been clarified. In order to answer some of these questions we have constructed a shuttle vector which carries a gene whose transcriptional activity can be regulated experimentally. As shown in Fig. 2, the shuttle vector pFl-EBV contains the EBV ori-P and EBNA-1 sequences which allow replication of the vector as an episomic element in human cells, the pBR322 ampicillin resistance (amp^r) gene and origin of replication (ori), the bacterial *hph* gene under the control of initiation and termination sequences from the *Herpes simplex* virus which confers to mammalian cells the resistance to the antibiotic hygromycin (hyg^r) and the bacterial *gpt* gene encoding for the enzyme xanthine-guanine-phosphoribosyl-transferase located downstream of the mouse metallothionein-I (MT-I) promoter (Fig. 2). The structural sequences of the *gpt* gene have been fused to the promoter and presumptive control region of the mouse MT-I gene which have been used to confer metal, steroid and/or inflammatory response to a variety of genes that were transferred into cells or animals [14]. Human cells transformed with the recombinant plasmid synthesized *gpt* mRNA and the expression of the gene was inducible by zinc. By RNA dot-blot analysis (Fig. 3), we estimated a 4- and 26-fold increase in the *gpt* RNA levels in presence of 50 and 100 μM zinc acetate, respectively. Furthermore, the *gpt* gene offers a convenient system of selection for mutant plasmids by transformation into the appropriate *gpt⁻ E. coli* strain. *Gpt⁻* mutant plasmids arising after exposure of the host cells to mutagens can be easily selected by using selective media containing 6-thioguanine. The recombinant molecule that we have constructed should provide a tool for studying the role of gene expression in DNA repair and mutagenesis.

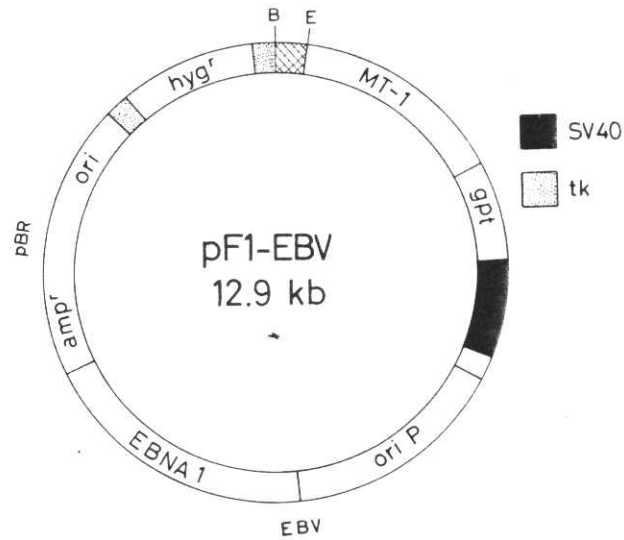

Fig. 2. - Construction of the EBV-derived shuttle vector, pFl-EBV. The regions of the *Herpes simplex* virus thymidine kinase (tk) gene and of SV40 that control initiation and termination of transcription are indicated by a stippled and a black filled box, respectively. The pBR322 0.3 kb *Eco*RI/*Bam*HI linker fragment is indicated by a cross-hatched box. Restriction enzyme cleavage sites are shown for *Eco*RI (E), BamHI (B) and *Bgl*II (BG).

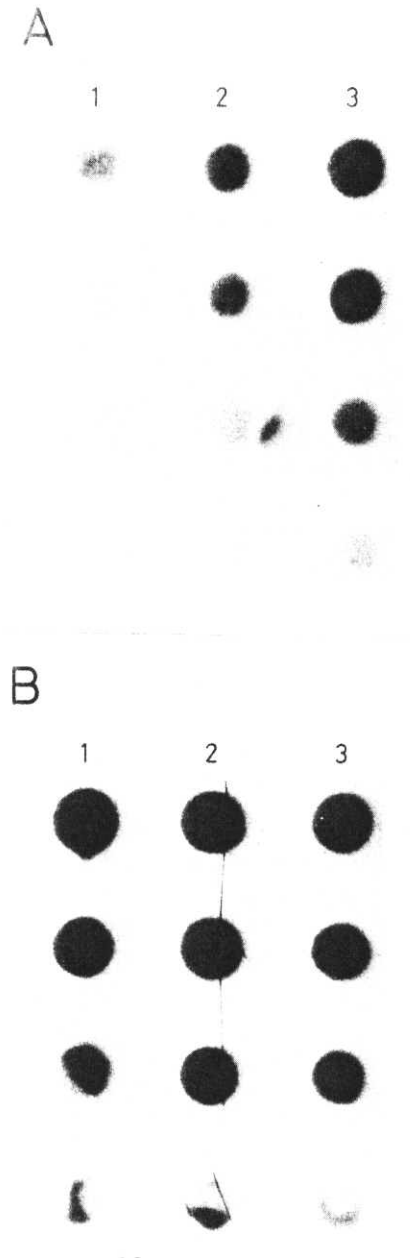

Fig. 3. - Dot-blot analysis of *gpt*-specific RNA (A) and actin-specific RNA (B). Total cytoplasmic RNA was serially diluted and immobilized on a nylon filter. Hybridization was done with a ^{32}P-labeled 1.8 kb *Hind*III/*Bam*HI *gpt* fragment (A) or a 2.3 kb PstI actin fragment (B). Lane 1: pFI-EBV transformed 293 cells; lane 2: after 3 h exposure to 50 μM zinc acetate; lane 3: after 3 h exposure to 100 μM zinc acetate.

Construction of an SV40-derived shuttle vector for studying the genetic effects of defined chemical carcinogen DNA-base adducts

Examination of a mutational spectrum can help in the formulation of an hypothesis that might implicate one or a few DNA modifications as the cause of a specific mutation. A more direct approach for testing the premutagenic potential of the DNA adducts is to construct a viral or plasmid genome containing a single DNA lesion at a known site. We have taken this approach to study the mutagenic effect of O^6-methylguanine (O^6-MeGua) in mammalian cells [15].

As shown in Fig. 4, the shuttle vector constructed for this study, pJ-*Pvu*I, contains the pML2 and SV40 origins of replication and the neomycin resistance (neo^r) gene, which is under the control of both bacterial and SV40 promoters (conferring kanamycin resistance in *E. coli* and G418 resistance in mammalian cells). The vector contains a unique *Pvu*I site located in a position unnecessary for vector viability. A single O^6-MeGua was inserted at the *Pvu*I site by techniques described below.

The strategy for fixation of mutations in mammalian cells and rescue of mutation progeny is summarized in Fig. 5. The adduct-containing vector is transfected into CHO cells, where it randomly integrates into the host chromosome. The host cell lines are isogenic strains that are either proficient (mex$^+$) or deficient (mex$^-$) in the

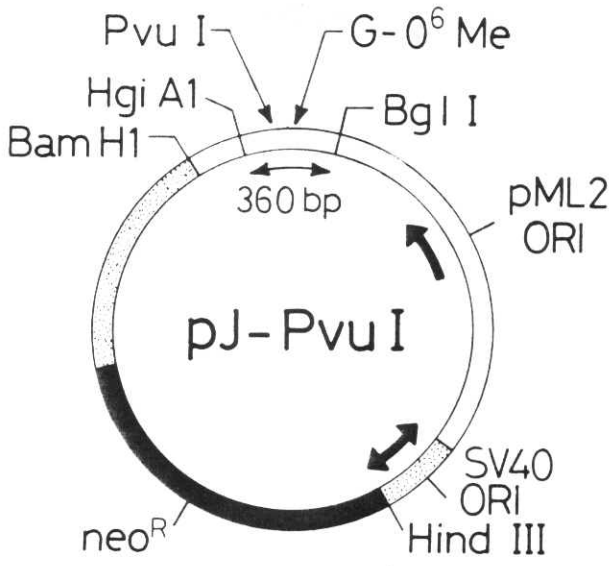

Fig. 4. - Construction of the SV40-derived shuttle vector, pJ-PvuI. The regions of the SV40 that control initiation and termination of transcription are indicated by a stippled box. The coding sequences of the neomycin resistance (neo^r) gene are indicated by a black filled box. The pML2 sequences are indicated by a white box.

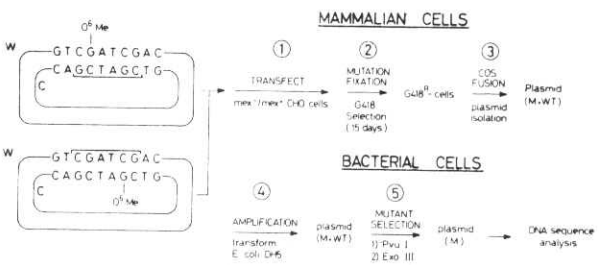

Fig. 5. - Scheme of the experimental system designed to study the mutagenic properties of O^6-MeGua in CHO cells by using an SV40-derived shuttle vector.

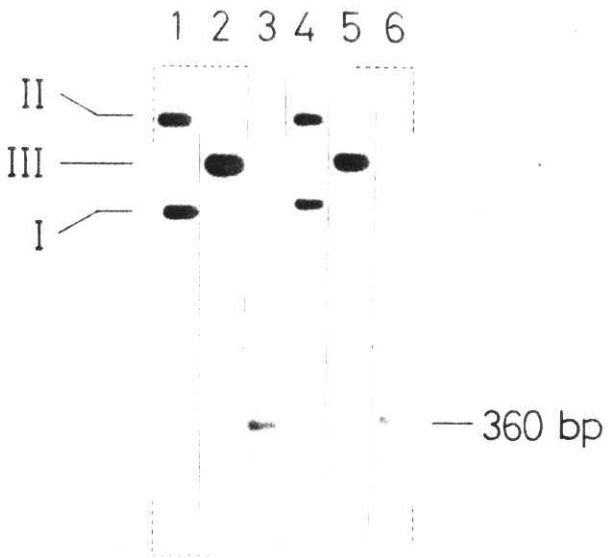

Fig. 6. - Analysis by agarose gel electrophoresis followed by autoradiography of gapped duplexes containing the radiolabeled tetranucleotide 5'-TCGA-3' (pJ-*Pvu*I) or 5' TCm^6 GA-3' (O^6-MeGua-pJ-*Pvu*I). Lane 1: pJ-*Pvu*I; lane 2: after *Pvu*I digestion; lane 3: after *Bgl*I-*Hgi*AI digestion; lane 4: O^6-MeGua-pJ-*Pvu*I; lane 5: after *Pvu*I digestion; lane 6: after *Bgl*I-*Hgi*AI digestion.

removal of O^6-MeGua. Cells harboring the shuttle vector are selected by resistance to G418. The vector is then rescued by fusion of these clones to COS cells. The progeny vector molecules are isolated and transformed into *E. coli* for amplification and analysis of possible mutations. Vectors with mutations that have occurred at the *Pvu*I site are selected by virtue of their resistance to this endonuclease.

The strategy for inserting an O^6-MeGua adduct at the *Pvu*I site was to construct an heteroduplex genome with a gap at the *Pvu*I site into which a chemically synthesized complementary tetranucleotide containing O^6-MeGua (5'-TpCpme^6 GpA-3') could be inserted. The 5' terminal deoxythimidine of this synthetic tetranucleotide and a control in which guanine replaced O^6-MeGua were phosphorylated in presence of ^{32}P-ATP prior to the ligation step. Electrophoresis followed by autoradiography of the ligation products (Fig. 6) revealed that incorporation of ^{32}P occurred in gapped duplex genomes and was precisely located in the 360 bp *Bgl*I - *Hgi*AI restriction fragment containing the *Pvu*I site. The modified shuttle vector was then transfected into mex$^+$ and mex$^-$ CHO cells. A higher frequency of mutation at the *Pvu*I site was observed when the vector replicated in the repair-deficient cell line [16]. The mutations occurring at the site where the adduct was originally were exclusively GC to AT transitions. The same results have been recently obtained by direct PCR amplification and DNA sequencing of the shuttle vector molecules present in the CHO genome (manuscript in preparation).

We have shown two examples where shuttle vectors have been used to explore the molecular mechanisms of mutagenesis in mammalian cells. The results indicate the great potential of vector systems as tools for studying a variety of metabolic pathways in eukaryotic cells.

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