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CARCINOGEN-INDUCED MUTAGENESIS IN THE SIMIAN VIRUS 40 GENOME

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Summary. - *Here are reviewed the most interesting results which have been obtained with a mutational assay based on the use of Simian Virus 40 (SV40) as a biological probe. This mutational assay allowed us first to study the mutation potency of some chemical and physical DNA damaging agents such as acetoxy-acetylaminofluorene and UV-light and of apurinic sites created by heat treatment under acidic conditions, and second to study at the molecular level the modifications induced by these treatments. A correlation between the location of the DNA adducts and the location of the hot spots of mutagenesis has tentatively been researched. No direct link has been found. Our results suggest that mutation hot spots are correlated with local DNA conformations which could be modified by the DNA damaging agents.*

Riassunto (Mutagenesi indotta da cancerogeni nel genoma di SV40). - *In questo articolo sono riassunti i risultati più interessanti ottenuti con un saggio di mutazione basato sull'uso di SV40 come sonda biologica. Questo saggio di mutazione ci ha permesso: a) di studiare la potenza mutagena di alcuni agenti chimici e fisici che danneggiano il DNA, quali l'acetossi-aminofluorene, la luce ultravioletta e i siti apurinici ottenuti dopo il trattamento con il calore in condizioni acide, e b) di analizzare a livello molecolare le modificazioni indotte da questi trattamenti. L'obiettivo della nostra ricerca è di stabilire la correlazione tra la posizione degli addotti al DNA e la localizzazione degli hot spots di mutazione. Non è stata trovata una correlazione diretta. I nostri risultati suggeriscono una correlazione tra gli hot spots di mutazione e la conformazione locale del DNA che potrebbe essere modificata dal trattamento con un agente danneggiante il DNA.*

Although studies of the mutagenic processes have been carried out for a long time, the discovery that some human cancers are related to point mutations in the *ras* gene family [1-3] has increased the interest in the analysis of the mechanisms by which mutations arise. However the size and complexity of the mammalian genome are important obstacles in the approach to the understanding of the molecular mechanism involved in mutagenesis. Moreover, in contrast to prokaryotes, no well characterized mutants exist in higher organisms. This is why viruses such Herpes simplex [4, 5], Simian Virus 40 (SV40) [6, 7] or parvovirus [8] have been important tools in the analysis of DNA repair and mutagenesis in eukaryotes. In our laboratory we have developed an experimental protocol using SV40 as a molecular probe to study the mutagenic properties of some physical or chemical carcinogens.

Simian Virus 40 as molecular probe

SV40 is a papovavirus whose genome is a supercoiled double stranded DNA of 5243 base pairs (Fig. 1). Its chromatin is organized as a minichromosome resembling eukaryotic chromosome [9] and the viral cycle is intranuclear for replication, transcription and maturation. Early transcription gives rise, after splicing, to two messenger RNAs coding for two proteins: small t antigen which is involved in transformation of non permissive cells and large T antigen which is necessary to initiate replication of the viral genome in permissive cells. Except for the T antigen protein which binds near the origin of replication, only cellular factors are implicated in viral replication. All DNA metabolism for the viral cycle, including repair of DNA damages, is processed by cellular enzymes. Late transcription leads to the synthesis of messenger RNAs coding for three capsid proteins VP1, VP2 and VP3 and for the agnoprotein whose function has not yet been well characterized.

SV40 is therefore very useful as a molecular probe since its DNA replication and DNA repair depend entirely on cellular enzymes. Moreover its nucleotidic se-

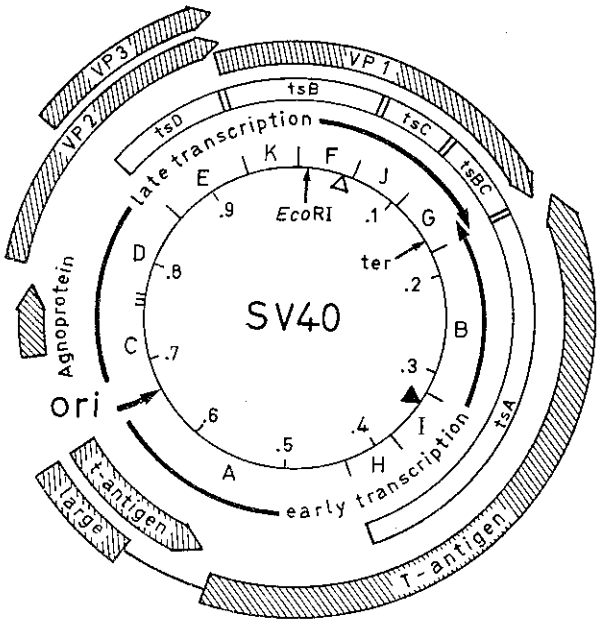

Fig. 1. - Genetic map of SV40. SV40 genome is represented by the inner circle, where letters refer to the restriction fragments obtained after digestion with the restriction endonucleases *Hin*cII + *Hin*dIII. ORI is the origin of the bidirectional DNA replication and ter its termination. Early and late transcriptions are indicated by the thin black arrows. Open bars show the domains where were mapped the different temperature-sensitive mutants. Close triangle and open triangle are respectively the location of the mutations tsA58 and tsB201. Open grey arrows indicate the genes coding for the viral proteins: small t and large T antigens, the three capsid proteins VP1, VP2, VP3 and the agnoprotein (modified from [30]).

quence was among the first known and various mutants have been isolated. In our experiments we used temperature sensitive mutants: the tsA58, unable to initiate DNA replication at the non permissive temperature of 41 °C [10] and the tsB201, unable to produce the VP1 viral capsid at the non permissive temperature. For the tsA58 mutant the temperature-sensitive phenotype is due to a base substitution in position 3505 (G→A) and for tsB201, to a base substitution in position 2303 (G→A) (unpublished results of our laboratory). In the experimental protocol we developed, DNA or virions of the temperature sensitive mutants of SV40 are treated *in vitro* with physical or chemical agents in order to induce DNA lesions. Permissive monkey kidney cells are then infected or transfected. After one lytic cycle at the permissive temperature of 33 °C, cells are scraped off the plates and stock virus were prepared from the viral progenies [6]. Titers of the viral progeny at permissive and non permissive temperatures allow us to quantify the mutation frequency from a temperature sensitive phenotype towards a wild type growth. Mutations are then located by the marker-rescue technique [11]. The DNA fragments able to complement the temperature sensitivity are sequenced in order to determine the mutations.

UV-light, apurinic site and acetoxy-acetylaminofluorene mutagenesis

The experimental protocol described has been used to study the mutagenic properties of various kinds of DNA damaging agents such as UV light, or acetoxy-acetylaminofluorene (AAAF) and of apurinic sites in mammalian cells. For example, as shown in Fig. 2, a 254 nm UV-irradiation of SV40 DNA before transfection into either permissive monkey kidney cells or into semi-permissive human KDN normal cells decreases survival of the viral progeny as a function of the UV dose. Survival is reduced by approximatively one order of magnitude after transfection of SV40 DNA irradiated with 2000 J/m² of UV light. No dramatic difference is seen between monkey and human cells. With this biological model the spontaneous mutation frequency is very low (10^{-6}) in both cell lines and this fact is a major advantage of this experimental protocol. Mutation frequency increases quickly with increasing doses of UV and reaches a plateau at 2000 J/m², when it is more than two orders of magnitude above the spontaneous mutation frequency level. With the same method, using *in vitro* treated virions or viral DNA followed by infection or transfection, we have been able to detect mutagenic properties of the ultimate carcinogen AAAF [12]. Survival of the viral progeny obtained from SV40 DNA treated *in vitro* with AAAF decreases with a lethal hit of approximately 85 acetylaminofluorene adducts per SV40 genome [12]. An increase of more than three orders of magnitude above the spontaneous mutation frequency was obtained with 400 adducts per SV40 genome. Similarly, we observed

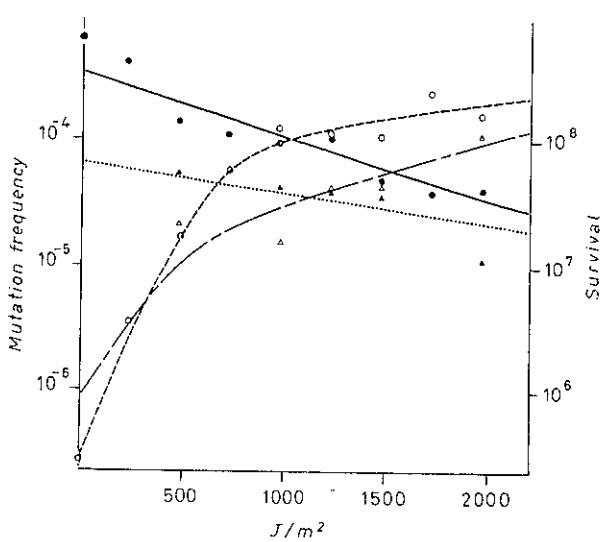

Fig. 2. - Survival (closed symbols) and mutation frequency (open symbols) of the viral progenies obtained after transfection of either CV1P monkey kidney cells (○,●) or KDN normal human cells (△, ▲) with SV40 DNA irradiated at different doses of UV at 254 nm.

mutagenicity of apurinic/apyrimidic sites (AP sites) obtained by heat treatment under acidic conditions of naked viral DNA [13]. The latter result is of prime importance since AP sites are one of the major lesions which occur in mammalian cell DNA, either by spontaneous hydrolysis of the phosphodiester bond (about one thousand per cell per hour) or after treatment with various carcinogen compounds [14-17]. In contrast to what has been shown in bacteria, where AP sites are mutagenic on the phage ϕX174 DNA only when the host bacteria have been "SOS induced" by UV-light before being transfected [18, 19], AP sites are mutagenic in our system without any UV pretreatment of the host cells. This indicates that AP sites are directly mutagenic in mammalian cells, unless transfection of damaged molecules induces the "SOS response" as has been shown under some conditions [20]. Table 1 shows the relative mutation potencies of UV, AP sites and AAF adducts after *in vitro* treatment of SV40 DNA mutants. There is no significant difference between the mutagenicity per lethal hit between UV-light and AP sites while this value is one hundred times higher after the AAAF treatment. The latter is less toxic and induces mutations at very low (or without) toxicity. It should be noticed that the number of adducts was determined before transfection. Whether these adducts are repaired by host cell repair enzymes at the same rate or not is not known.

Enhanced survival and enhanced mutagenesis

Enhanced survival of UV-irradiated viral probes infecting cells which have been previously treated by DNA damaging agents has been well established [21]. Whether there is enhanced mutagenesis under similar experimental conditions is still controversial. Indeed conflicting results have been reported using UV-irradiated SV40 [6, 8], Herpes virus [4, 5], or adenovirus [22]. In some cases, pretreatment of the host cells with UV-light or with various chemicals, increases mutagenesis of the viral progeny and in other cases it does not. We have shown that these discrepancies were in part due to an artefact linked to the experimental conditions [23]. Indeed, we showed that enhanced survival occurred in mitomycin-pretreated cells regardless of the multiplicity of infection used, while enhanced mutagenesis occurred only when a low multiplicity of infection of UV-irradiated virus was utilized. These results show therefore that the two phenomena may be dissociated under some experimental conditions. This dissociation has also been observed when damaged SV40 DNA was transfected into pretreated cells. In numerous cases, transfection of UV damaged DNA instead of infection of pretreated cells with UV-irradiated virus leads to enhanced survival, but did not lead to enhanced mutagenesis [24]. The explanation could be that using transfection, only a small number of cells are transfected with probably a high number of DNA molecules. Thus, the conditions of low multiplicity are not realized and consequently enhanced mutagenesis is not observed.

Table 1. - *Relative mutagenicity of UV, AP sites and AAAF*

	Adducts per lethal hit	Mutagenicity per lethal hit
UV	6	5×10^{-6}
AP sites	3	7×10^{-6}
AAAF	85	7×10^{-4}

Molecular analysis of carcinogen-induced mutations

Mutations induced by UV-light or AAAF on the tsA58 SV40 mutant were localized by the marker-rescue method, and DNA fragments able to complement the temperature sentivity were sequenced by the Maxam and Gilbert technique [25] or by the chain elongation terminator method [26]. All the mutations we found were single base substitutions [12, 27], using either treatment. UV-induced mutations were located opposite potential UV lesions, that is to say opposite putative pyrimidine dimers or pyrimidine (6-4) pyrimidone photoproducts. The result is quite different for AAAF induced mutations. Indeed AAAF binds to guanine in nucleic acids and gives rise chiefly to 2-N-(deoxyguanosin-8-yl)-N-acetylaminofluorene, leading to a local distorsion of the DNA structure [28]. Surprisingly the majority of the mutations obtained were not located opposite a putative guanine-AAF lesion. We found seven different sites for UV-induced mutations and eight for AAAF-induced mutations (Table 2). Only two sites were common for both DNA-damaging agents indicating, despite the fact that the AAAF mutations were not strictly targeted opposite AAF-adducts, the mutation sites were not random, but depended upon the carcinogen-induced lesions. In particular we found that 60 % of AAAF-induced mutants were true genotypic revertants, *i.e.* that the original wild type sequence (G instead of A at position 3505) was restored. This had never been the case after UV-irradiation. No mutation has been found at position 3505 although the probability to form UV-induced lesions is high on the complementary strand which sequence is TTTT. In contrast a hot-spot of UV-induced mutations was located in a sequence TAAT*TC (where * shows the mutated nucle-

Table 2. - *Location and types of mutations obtained with tsA58 SV40 after UV-irradiation or treatment with acetoxyacetylaminofluorene*

Position	Occurrence		Targeted (+) untargeted (-)		Transition		Transversion	
	UV	AAAF	UV	AAAF	UV	AAAF	UV	AAAF
3931	4	1	+	-	T →C	T →C		
3769		2		-		T →C		
3722		1		-				T →A
3700		1		-		T →C		
3609		1		-		T →C		
3596	1		+				T →G	
3505		11		-		T →C		
3495	6	1	+	-			T →A	T →A
3483		1		+		C →T		
3402	1		+				T →G	
3334	1		+		T →C			
3180	2		+				T →G	
2936	2		+		T →C			
			17+	1+	7	17	10	2
Sum of occurrences	17	19						
			0-	18-	41%	90%	59%	10%

All mutants are independent and position are given according to Tooze [9].

otide). 35% of UV-induced revertants were mutated at this site (nucleotide 3495) whereas only 5% of the AAAF mutants were found at this locus. These results indicate that mutation hot-spots are not strictly correlated with lesion hot-spots as we have directly demonstrated after AAAF treatment [12]. As far as the hot-spot of mutagenesis leading to a true wild type genotype is concerned, we have proposed two models to explain this result. The first is based upon mismatch repair of a quasi palindromic sequence which is found around the tsA58 mutation with a mismatch for the tsA58 position. This secondary structure could be stabilized by an AAF adduct. Repair of the mismatch at position 3505 leading to a fully base-paired structure will give rise to a wild type sequence. The second model involves the possibility of the DNA replication fork to continue synthesis at an AAF-adduct, by taking the complementary strand as template, where the nucleotidic sequence is identical to the wild type [12]. Indeed at a possible AAF adduct located at three base pairs of the tsA58 mutation a strand switch of the DNA polymerase may occur. The DNA polymerase could therefore copy the complementary strand for a few bases, before resuming normal replication. This complementary strand is identical to the normal template except for one base which restores the wild type sequence. At the second round of replication, the absence of repair will give rise to the wild type genotype [12]. The same kind of experiments carried out with the tsB201 SV40 mutant gave similar results. As for the tsA58, the local DNA structure seems to be of prime importance.

Detection of carcinogen-induced lesions on SV40 DNA

In order to correlate carcinogen-induced mutations with carcinogen-induced lesions we have located the latter on SV40 DNA restriction fragments on which most of the mutations had occurred. Fragments H and I resulting from digestion of SV40 DNA with *Hinc*II and *Hind* III restriction endonucleases (Fig. 1) were purified on polyacrylamide gels, then radioactively labelled at the 5' extremity with the polynucleotide kinase. We managed to obtain fragments with only one labelled extremity. These purified fragments were UV-irradiated or treated with AAAF at doses compatible with those used in mutagenesis experiments. The detection of UV-induced lesions was carried out either enzymatically using the endonuclease V of the T4 phage which detects pyrimidine dimers or chemically using 1M piperidine at 90 °C which cuts other lesions and in particular at the pyrimidine (6-4) pyrimidone. For AAAF lesions the associated 3'→5' exonuclease activity of bacteriophage T4 polymerase was utilized. This activity is indeed blocked at DNA-AAF adducts. Loaded on denaturing sequencing gels in

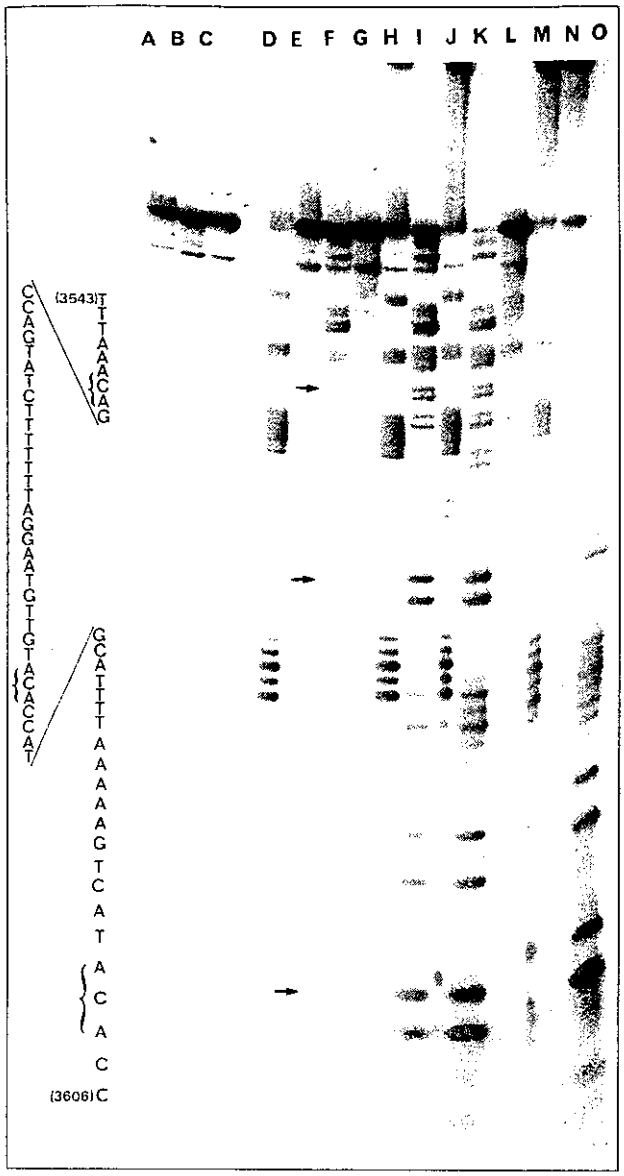

Fig. 3. - Determination of the location of DNA lesions on the SV40 DNA. Autoradiography of a 12 % polyacrylamide sequencing gel of 3' end labelled SV40 DNA fragment. Unirradiated DNA was loaded on lane A, lane B (treated with T4 endonuclease V), lane C (hot alkali treated), lane G (purine sequence ladder), lane L (pyrimidine sequence ladder). 2 kJ/m^2 UV-irradiated DNA was loaded on lane D (treated with T4 endonuclease V), lane E, lane F (hot alkali treated). 10 kJ/m^2 UV-irradiated DNA was loaded on lane H (treated with T4 endonuclease V) and lane I (hot alkali treated). 25 kJ/m^2 UV-irradiated DNA was loaded on lane J (treated with T4 endonuclease V), lane K (hot alkali treated). 50 kJ/m^2 UV-irradiated DNA was loaded on lane M (treated with T4 endonuclease V), lane N, and lane O (hot alkali treated). The three ACA sequences are labelled with braces. The arrows show the bands produced at ACA sequences with the hot alkali-treatment. (From Bourre *et al.* [29] with the kind permission of *Nucleic Acids Res.*)

parallel with DNA sequencing markers using the Maxam and Gilbert method, the treated DNAs give discret bands, on autoradiography, allowing detection of the lesions (Fig. 3). This experimental protocol allowed us to quantify the amount of lesions and therefore to directly compare lesions and mutations. No direct correlation between the two parameters was found for UV or AAAF. In an extensive study performed with UV-light [29] we have been able to quantify the frequency of induction of pyrimidine dimers depending on the two types of pyrimidines involved (TT, TC, CC, CT) and, for a given dimer (TT for example), we have determined the influence of the two adjacent nucleotides. A pyrimidine at the 5' of a dimer enhances the frequency of formation of this dimer. The frequency of pyrimidine (6-4) pyrimidone formation also depends upon the pyrimidine pairs involved. Although for a class of Py (6-4) Py this frequency varies greatly, the two adjacent nucleotides do not seem to play a major role. The heterogeneity of the results shows therefore the importance of the lesion environment and not only of the two adjacent nucleotides. On the other hand we have shown the induction of a UV lesion, not detected till now, in an ACA sequence, the lesion being detected after treatment with piperidine 1M (Fig. 3). This lesion is sensitive neither to the endonuclease V of the phage T4 nor to the photoreactivating enzyme of *E. coli*. This lesion could be involved in UV mutagenesis although no direct evidence has been yet given.

Conclusions

The use of SV40 as a probe in mammalian cells has allowed us to approach at the molecular level the effects of some DNA damaging agents. These interesting results in mammalian cells would not have been possible without the use of such a biological probe. Of course this system presents some limits, in particular due to the fact that the mutation assay is a reverse one which detects only base substitutions in a limited number of sites. Other probes have now been developed in our laboratory avoiding some of these disadvantages.

The use of these various systems and the analysis of the obtained results are aimed for a better comprehension of the mechanisms leading to mutagenesis in mammalian cells.

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