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HARD

STUDIES ON THE ~~STRUCTURE AND~~ REPAIR ~~OF~~ AND *CONFORMATION OF DNA* ~~OLIGONUCLEOTIDES~~ CONTAINING O^6-ALKYLGUANINE AND

O^4-ALKYLTHYMINE

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TO MY PARENTS

" We are searching for the essence that lies behind the fortuitous"

Paul Klee

ABSTRACT

The carcinogenic N-nitroso compounds alkylate DNA. Among the different products of alkylation, O^6-alkylguanine and O^4-alkylthymine have attracted most of the attention since they are highly mutagenic and a correlation exists between their formation and persistence, and oncogenesis in animal model systems. Both DNA adducts in E. coli and at least O^6-alkylguanine in mammalian cells are repaired by enzymes, the so-called O^6-alkylguanine-DNA-alkyltransferases.

Based on the HPLC separation of short, self-complementary oligonucleotides containing O^6-methylguanine, O^6-ethylguanine, or O^4-methylthymine from the respective parent non-alkylated oligomers, the rate constants for their repair by the E. coli *ada* and *ogt* and the human alkyltransferases were determined. Although all alkyltransferases were able to repair O^6-methylguanine, O^6-ethylguanine and O^4-methylthymine, the relative efficiencies were found to differ significantly.

Using an immunoprecipitation assay, the rates of repair of an O^6-methylguanine residue in various positions in 15 base-pair DNA duplexes were measured. The sequence of the oligomers was that of the rat H-ras sequence around codon 12 and the rates of repair were found to vary up to 25-fold depending on the sequence flanking the methylguanine. An O^6-methylguanine in the second position of the GGA codon 12 was the least well repaired. The combination of this slow repair and sequence selectivity in alkylation appears to be the explanation of the selective mutation of this position observed in rat mammary tumours. The avidity constants between antibody and O^6-methylguanine were also dependent on the sequence flanking the adduct, with the most rapidly repaired being those most easily bound to the antibody. It is suggested that the rate of repair is a reflection of the conformation of the oligomers containing O^6-methylguanine.

An unusual feature of DNA which is often associated with protein-DNA interactions is DNA curvature. A characteristic of curved DNA is that it has less

electrophoretic mobility than normal DNA. In order to assess if alkylated adducts in DNA induce DNA curvature or flexibility, DNA duplexes containing O^4-alkylthymine or O^6-methylguanine were synthesized and self-ligated to form multimers with the alkylated bases out of phase (16 base-pairs apart) or in phase (21 base-pairs apart) with the helical repeat of DNA. All the sequences containing O^4-alklylthymine migrated more slowly than expected in a non-denaturing polyacrylamide gel. In general the effect was seen when the alkylated base was out of phase or in phase with the helical repeat suggesting that the altered base-pair confers flexibility which is largely isotropic, i.e has no preferred direction, rather than anisotropic flexibility or bending. The effect of O^4-methylthymine in the mobility of the oligonucleotides was much greater than that of O^6-methylguanine and the effect of O^4-ethylthymine slightly greater than that of O^4-methylthymine. DNA duplexes containing O^4-alkylT:A base-pairs were more retarded, and had lower thermal point (Tm) than DNA duplexes containing O^4-alkylT:G base-pairs.

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ABBREVIATIONS

2-D NMR	two-dimentional nuclear magnetic resonance
5me-C	5-methylcytosine
alkG, O^6-alkG	O^6-alkylguanine
alkT, O^4-alkT	O^4-alkylthymine
ALL	Acute lymphoblastic leukaemia
Arg	arginine
Asn	asparagine
Asp	aspartic acid
CHO	chinese hamster ovary
CLL	chronic leucocyte leukaemias
CPG	control-pore glass
Cys	cysteine
DBU	1,8 -diazabicyclo-[5.4.0]-undec-7-ene
DMBA	7,12-dimethyl-benz[a]anthracene
DMT	dimethoxy-triphenylmethyl
DTT	dithiothreitol
EDTA	diaminoethanetetra-acetic acid, disodium salt
ENU	N-ethyl-N-nitrosourea
ethylT, O^4-ethylT	O^4-ethylthymine
Gln	glutamine
Glu	glutamic acid
His	histidine
HPLC	high-performance liquid chromatography
M W	molecular weight
meG, O^6-methylG	O^6-methylguanine
methylT, O^4-methylT	O^4-methylthymine

MMS	methyl methanesulphonate
MMTV	mouse mammary tumour virus
MNNG	N-methyl-N-nitroso-N'-nitroguanidine
MNU	N-methyl-N-nitrosourea
NBMA	N-benzyl-N-methyl-nitrosamine
NDEA	N-diethylnitrosamine
NDMA	N-dimethylnitrosamine
NHL	non-Hodgkins lymphomas
NNK	N'-4-(methylnitroso)-1-(3-pyridyl)-butanone
oxime	(E)-2 nitrobenzaldoxime
PBS	phosphate-buffered saline
PEG	polyethylene glycol
Pro	proline
sp. gr.	specific gravity
$t_{1/2}$	half-life
TEAA	tetraethyl-ammonium acetate
Tm	melting point temperature
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet light
Val	valine

CHAPTER 1

INTRODUCTION

1.1. N-NITROSO COMPOUNDS OCCURRENCE AND SIGNIFICANCE

1.1.1. Why study N-nitroso compounds?

Following the discovery of the carcinogenicity of dimethylnitrosamine (NDMA) (Magee and Barnes, 1956), it soon became apparent that this compound was just one member of a very large family of chemical carcinogens with common structural features, the so called N-nitroso compounds (Recent review by Magee, 1989). As the name implies, all members have an N-nitroso group combined with alkyl or other groups (figure 1.1). They can be divided in two major sub-families, nitrosamines and nitrosamides. Today more than 300 N-nitroso compounds are known to be carcinogenic in animals, and tumours have been induced by one or more family members in every animal species tested (currently more than forty). These compounds, nitrosamines in particular, have been used extensively for studies on the pathology, biochemistry and molecular biology of carcinogenesis since:

1) In sharp contrast with other chemical carcinogens, nitrosamines can induce virtually any type of tumour, and these tumours are closely similar to their human counterparts in their morphological and biological properties.

2) Some nitrosamines show remarkable organ specificity, for example, asymmetric compounds such as N-benzyl-N-methylnitrosamine (NBMA) are highly specific for the oesophagus in the rat, while n-dibutyl-N-nitrosamine induces almost exclusively bladder tumours (Druckrey, 1967).

3) Unlike most carcinogens, nitrosamides do not require metabolic activation and thus tumour induction can be dissociated from tissue specific activation, and the response of different tissues to the carcinogenic challenge can be addressed directly.

4) N-nitroso compounds are complete carcinogens. With some of them only a single dose

Perhaps the most convincing correlation of human cancer induction and

enviromental exposure to N-nitroso compounds, is the oral cavity tumours associated with

tobacco chewing. Tobacco contains various N-Nitroso compounds, the so called tobacco

specific nitrosamines, the most important of which are the N'-4-(methylnitroso)-1-(3-

pyridyl)-butanone [NNK] (see figure 1.1) and N-nitrosonornicotine (NNN). NNK is

carcinogenic in rats, and exhibits, regardless of the route of administration, a remarkable

specificity for the lungs (IARC,1985), suggesting that lung cancer associated with

cigarette smoke, might also be caused by this carcinogen. However, cigarette smoke is a

very complex chemical mixture, containing a number of other chemical carcinogens, some

of them more potent and in higher concentrations than nitrosamines, and hence the

assessment of which compounds are responsible for human lung cancer is difficult. Other

human tumours in which exposure to nitrosamines have been causally implicated are gastric

cancer, oesophageal cancer in Northern China, nasopharyngeal cancer in Southern China

and bladder cancer associated with bilharzia in Egypt (Review by Preston-Martin and

Correa, 1989). In all these cases indications exist supporting the correlation but the

evidence is not conclusive.

1.1.3. Endogenous formation of N-nitroso compounds

Among the different chemical carcinogens, N-nitroso compounds are probably the

only carcinogens that can be formed endogenously from precursor molecules.

Following the discovery that carcinogenic nitrosamines can be formed from amines and

nitrite under acidic conditions resembling those in the mammalian stomach, a large amount

of effort and work from many investigators was devoted to prove that intragastric

nitrosation does occur in vivo (Review by Leaf *et al.* 1989). The intragastric biosynthesis

of N-nitroso compounds involves: a) reduction of the abundant nitrate in saliva, to nitrite

by bacteria in the mouth and b) nitrosation of secondary and tertiary amines or of amides,

in the acidic environment of the stomach. Demonstration that ingested L-proline is

nitrosated, confirmed that this process occurs in humans (Ohshima and Bartch, 1981). It

also provided evidence that nitrosation probably takes place and in other compartments of

the body (Wagner *et al.*, 1985). The location of extragastric nitrosation of amines was found to be the macrophages, and occurs under physiological conditions. Macrophages are capable of nitrate biosynthesis from arginine. However, the compound involved in nitrosation is not nitrite but nitric oxide, an intermediate of the nitrite/nitrate biosynthesis (Leaf *et al.*, 1989).The as yet unproved hypothesis that endogenous biosynthesis of N-nitroso compounds consistutes a lifelong risk factor for tumour induction, is intriguing and attractive for further studies.

1.1.4. Metabolism of N-nitrosamines

From the early studies on nitrosamine-induced carcinogenesis it was clear that these compounds are highly selective in their carcinogenic effect, producing tumours in some organs but not in others, independently of the route of administration. In contrast, nitrosamides have a large spectrum of target tissues and also tend to produce tumours at or near the site of their administration. These results are consistent with the concept that metabolic activation is required for nitrosamines, but not for nitrosamides.

Early experimental evidence on the in vivo and in vitro metabolism of dimethylnitrosamine suggested the metabolic pathway shown in figure 1.2. Recent experimentation has confirmed this activation pathway for other aliphatic and cyclic nitrosamines, although some differences and alternative pathways exist for high molecular weight and more complex nitrosamines (reviewed by Archer, 1989). The first step in the metabolic pathway involves hydroxylation in one of the two carbons adjacent to the N-nitroso group (α-hydroxylation). This reaction is catalysed by the cytochrome P-450 enzyme system. This membrane-bound system consists of a family of isoenzymes which hydroxylate a number of chemicals foreign to the organism, including drugs and carcinogens, as part of a detoxification process. The identification of the P-450 which is responsible for the metabolism of nitrosamines in vivo, was and still is, a difficult task since these enzymes exhibit overlapping specificity and quite a few are able to metabolize nitrosamines, though with differing efficiency (Kadlubar and Hammons, 1987). It now seems reasonably certain that the P-450 system responsible for the metabolism of at least
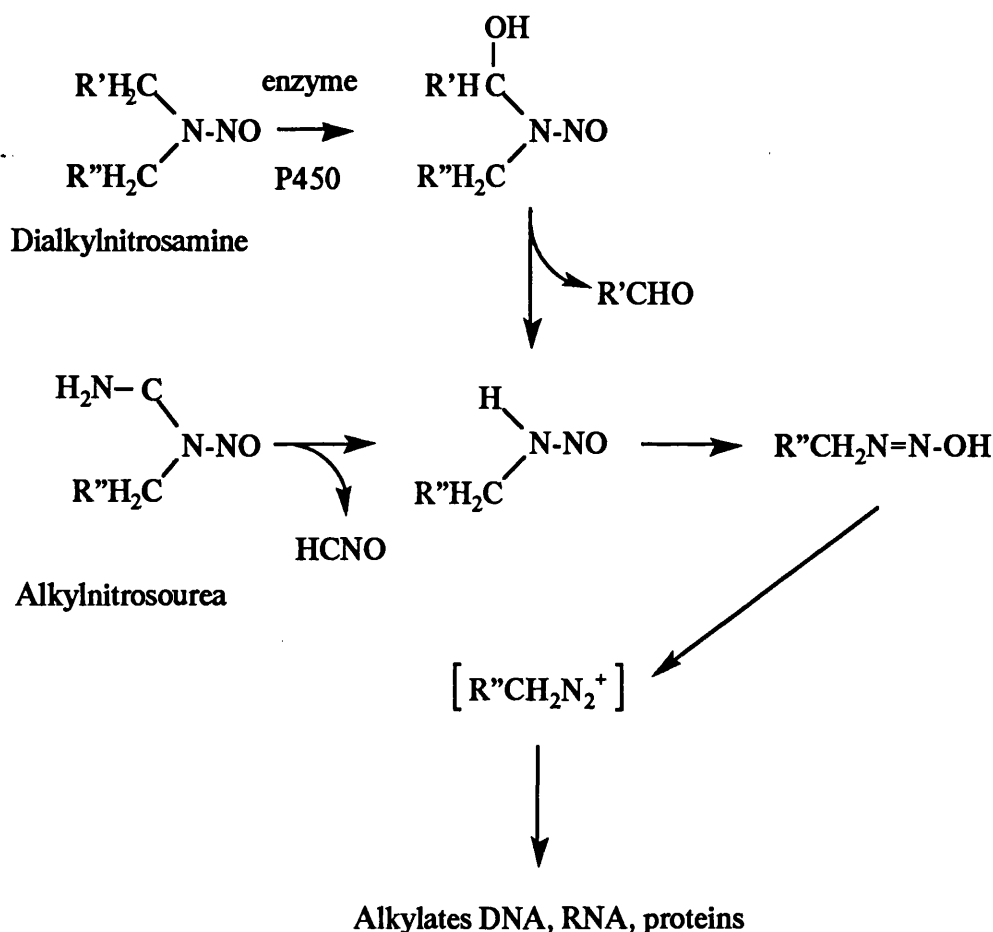
Figure 1.2 : Bioactivation of dialkylnitrosamines and alkylnitrosoureas

dimethylnitrosamine, belongs to the II E subfamily of ethanol inducible enzymes (Yang *et al.*, 1985 a,b). This first step in the activation of nitrosamines, is the only one where enzymes are involved, and a strong correlation exist between the ability of each organ to metabolize nitrosamines and tissue susceptibility for tumour induction. (Montesano and Magee, 1974). The α-hydroxy derivative is unstable and is easily hydrolyzed, to yield

aldehyde and alkyldiazohydroxide. The latter finally produces diazonium cation which is the potent electrophile which alkylates nucleophilic atoms of biomolecules such as the N and O atoms of DNA and RNA, the N, O, and S atoms of proteins, and of course water. Since water is the major component of the cell, it is to be expected that the major part of the

alkylating metabolite reacts with it, and is thus destroyed. Although alkylation of all cellular macromolecules can be a potential hazardous damage for the cell, adducts within DNA have special significance in view of their potential to force replication errors, and thus be the chemical progenitors of heritable genetic alterations (Miller, 1978).
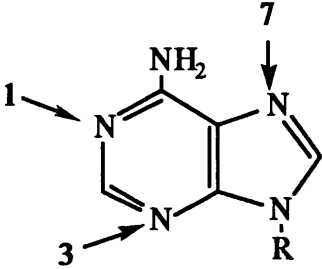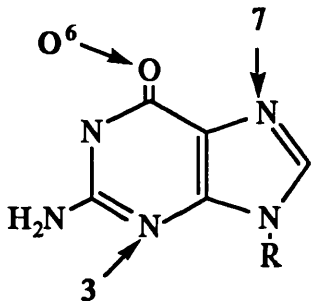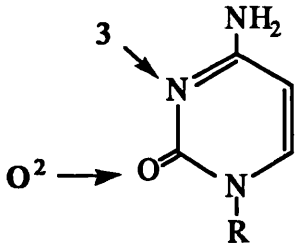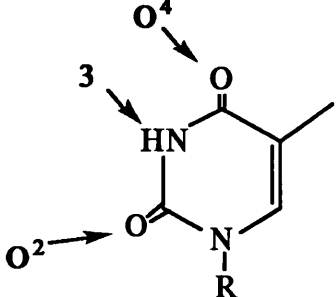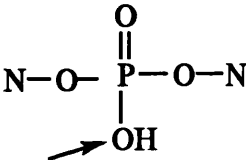
1.2. O⁶-ALKYLGUANINE AND O⁴-ALKYLTHYMINE FORMATION AND PERSISTENCE : RELEVANCE TO CARCINOGENESIS AND MUTAGENESIS BY ALKYLATING AGENTS

1.2.1. O⁶-alkylguanine

N-nitroso compounds are a sub-family of a wider group of chemical compounds (alkylating agents), which alkylate cellular macromolecules. At least 12 sites in DNA are targets for alkylation under physiological conditions (table 1.1). The proportion of each alkyl derivative depends on the type of alkylating agent used, but not on whether the alkylation takes place in vivo or at neutral pH in aqueous buffer in vitro (Singer, 1979). On the assumption that one of these adducts is the crucial one that leads to neoplastic growth, investigators initially focused their attention on N7-alkylguanine, the most abundant product of the reaction between alkylating agents and DNA. Swann and Magee (1969) studied the induction of kidney tumours in the rat after a single dose of dimethylnitrosamine, N-methyl-N-nitrosourea, and methyl methanesulphonate. The two former agents gave a high incidence of kidney tumours, whereas methyl methanesulphonate did not produce any tumours under the same experimental conditions. However, the levels of N7-methylguanine produced, were the same for all three agents. Therefore, it was suggested that if a single lesion is responsible for the carcinogenesis induced by alkylating agents, this could not be N7-alkylguanine.

At the same time, Loveless (1969) working on mutations of T2 bacteriophage, found a parallel between the ability of these alkylating agents to act as mutagens in this system, and their ability to induce cancer in animals. He re-examined the chemistry of alkylation, discovered O⁶-alkylguanine, and showed that there was a general correlation between the production of this alkylated product, carcinogenesis and mutagenesis. He already knew that the mutations were mainly G:C to A:T transitions and suggested that cancer in animals might result from mutations caused by O⁶-alkylguanine mispairing with T, instead of C, during replication. The differences in carcinogenicity of alkyl sulphates like methyl methanesulphonate, and N-nitroso compounds like dimethylnitrosamine or N

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Table 1.1: Reaction of N-methyl-N-nitrosourea (MNU), N-ethyl-N-nitrosourea (ENU), and methyl methanesulphonate (MMS) with DNA in vitro. The data is from Singer and Grunberger (1983) and Pegg (1984).

		% total alkylation		
		MNU	ENU	MMS
Adenine	N1-	1.3	0.2	3.8
	N3-	9.0	4	10.4
	N7-	1.7	0.3	1.9
Guanine	O6-	6.3	7.8	0.25
	N3-	0.8	0.6	0.6
	N7-	67	11.5	85
Cytosine	O2-	0.1	3.5	-
	N3-	0.6	0.22	-
Thymine	O2-	0.11	7.4	-
	O4-	0.4	2.5	-
	N3-	0.3	0.8	0.08
Phosphate		16	52	0.8

-methyl-N-nitrosourea can be explained by the relatively greater production of

O^6-alkylguanine by the latter agents. O^6-alkylguanine is produced in significant amounts by

alkylating agents like N-methyl-N-nitrosourea (MNU), N-ethyl-N-nitrosourea (ENU),

N-methyl-N-nitroso-N'-nitroguanidine (MNNG) and dimethylnitrosamine (NDMA) in

vitro, but is only a minor product in the reaction of DNA with the weak carcinogenic agents

like methyl methanesulphonate and ethyl methanesulphonate (Lawley and Orr, 1970;

Lawley, 1974; Lawley, 1980). A dose of dimethylnitrosamine, sufficient to induce liver

tumours in rats, produces much more O^6-alkylguanine than a similar but non-carcinogenic

dose of methyl methanesulphonate (O'Connor et al., 1973). A correlation between

O^6-alkylguanine production by N-nitrosoureas and yield of mouse thymic lymphomas, has

been clearly indicated in a number of studies by Lawley and colleagues (reviewed by

Lawley, 1980).

The correlation however, is not exact and universal and there is evidence that cell

replication is an important factor. For example, a single dose of dimethylnitrosamine or

N-methyl-N-nitrosourea rarely induces hepatocellular carcinoma in rats, unless the dose is

given during the period of restorative cell proliferation following partial hepatectomy

(Craddock, 1971). Similarly, adult rat brain is less susceptible to ENU-induced tumours

than foetal brain (Craddock, 1984) and the induced tumours are derived from glial cells

which divide, rather than neuronal cells that do not (Kleihues et al., 1973). It appears that

alkylation of DNA can lead to the initiation of tumours only if DNA synthesis occurs whilst

the alkylation products are still present in the DNA. These observations strengthen

Loveless' hypothesis that O^6-alkylguanine initiates oncogenesis via a mutational

mechanism, although alteration in gene expression caused by this adduct, might have an

enhanced effect in rapidly dividing cells and thus, cannot be totally excluded. The different

susceptibility of tissues in a different proliferation state, is also indicative of a cell defensive

mechanism which excises certain products of DNA alkylation. In a slowly dividing tissue,

the elapsed time between DNA alkylation and the following DNA synthesis, could be

sufficient for the repair mechanism to excise crucial DNA adducts and protect the cells

whereas, a rapidly dividing tissue cannot be protected as efficiently and effectively.

The general correlation between the carcinogenic activity of any alkylating agent and its ability to alkylate the O^6 of guanine is not sufficient to prove cause and effect, because the amount of other alkylated products, for example the alkylation of the internucleotide phosphodiesters, is similarly correlated with carcinogenic potency. The particular importance of O^6-alkylguanine and its active removal from the cells was first shown by Goth and Rajewsky (1974). They showed that in rats the O^6-ethylguanine produced by N-ethyl-N-nitrosourea is rapidly removed from liver DNA ($t_{1/2} \approx 36$ hours) while it is very slowly removed from brain DNA ($t_{1/2} \approx 229$ hours). Under these conditions none of the rats develop liver tumours but 95% develop brain tumours. Margison and Kleihues (1975) extended this observation by showing that induction of nervous system tumours in rats by repetitive low doses of N-methyl-N-nitrosourea was consistent with the slow removal of O^6-methylguanine from brain DNA, compared to kidney (intermediate removal), and liver DNA (fastest removal). The removal in liver was so fast that after 5 weeks liver DNA contained only 1% of the O^6-methylguanine present in the brain DNA. The enzyme responsible for the removal of O^6-alkylguanine (O^6-alkylguanine-DNA-alkyltransferase) was later identified, and is a protein which catalyses the transfer of the alkyl group to a cysteine-residue within its own sequence and thus restoring the normal DNA sequence (reviewed by Pegg, 1990). The number of O^6-alkylguanine-DNA-alkyltransferase molecules per cell is both species and organ specific, and in general terms there is an inverse correlation between the alkyltransferase cellular content and tissue susceptibility for oncogenesis in the above mentioned examples (see also chapter 1.3).

The association of O^6-alkylguanine formation and persistence with carcinogenesis, raised the optimism of investigators, but the correlation soon proved to be weaker than initially believed. O^6-methylguanine is highly persistent in the cerebral DNA of mongolian gerbil treated with N-methyl-N-nitrosourea but no brain tumours are induced as a result of this treatment (Kleihues *et al.*, 1980). A/J mice and CH HeB/FeJ mice both remove O^6-methylguanine from brain DNA at a similar rate but differ in their response to the neuro-oncogenic effects of N-methyl-N-nitrosourea. These species differ in the removal of

O^6-methylguanine from liver DNA but are equally susceptible to

N-methyl-N-nitrosourea-induced hepatic carcinomas (Buecheler and Kleihues, 1977).

Equimolar doses of N-benzyl-N-methyl-nitrosamine or dimethylnitrosamine in rats

following a partial hepatectomy produce similar amounts of O^6-methylguanine but only

dimethylnitrosamine produces pre-neoplastic foci and hepatocellular tumours (Silinskas *et al.*, 1984). However, these observations do not necessarily imply that O^6-alkylguanine is

not responsible for tumour induction by alkylating agents. What they merely suggest is,

that the concept of carcinogenesis by alkylating agent is more complex than a simple

correlation between O^6-alkylguanine production and tumour induction, and other

parameters might be involved. Possibly, it is not the overall O^6- alkylation of guanine

residues which is the determining factor for carcinogenesis, but the alkylation of specific

genes, for example oncogenes, that might be inaccessible for alkylation in certain type of

cells in a given state of differentiation. Furthermore, O^6-alkylguanine formation is just

one, although presumably the first, in a series of steps that would additively lead to

neoplastic growth. Cells where O^6-alkylguanine is persistent, might be at a higher risk for

tumour initiation than cells where it is not, but in the subsequent steps of carcinogenesis

their relative susceptibility may well be altered as a consequence of the involvement of other

factors. Finally other DNA adducts might be equally or even more important than

O^6-alkylguanine in predisposing cells in high risk for neoplastic transformation.

1.2.2. Possible role of O^4-alkylthymine in carcinogenesis

The second best candidate as critical DNA adduct for the oncogenesis induced by

alkylating agents, is O^4-alkylthymine. This adduct, first identified by Lawley *et al.* (1973)

is a potent pro-mutagenic adduct, both in vitro (reviewed by Singer and Kusmiereck, 1982)

and in vivo (Preston *et al.*, 1986; Preston *et al.*, 1987). The amount of O^4-methylthymine

produced by N-methyl-N-nitrosourea accounts only for 0.5% of the total alkylation sites

and 5% of the amount of O^6-methylguanine (table 1.1). Thus, for many years the potential

role of O^4-alkylthymine in the mutagenesis and carcinogenesis has been underestimated. It

was not till it was shown that O^4-ethylthymine produced by N-ethyl-N-nitrosourea in vitro, constituted a significantly higher proportion of the total alkylation sites [2.5%] (Singer, 1976), that its contributing role has been reconsidered.

Formation and persistence of O^4-alkylthymine has been correlated with tumour induction in a number of situations. Continual daily administration of diethylnitrosamine to rats in drinking water gives a 100% incidence of hepatocellular tumours. Although approximately 4-times more O^6-ethylguanine than O^4-ethylthymine is initially formed in DNA, there is 7.5 times more O^4-ethylthymine than O^6-ethylguanine present in hepatocyte DNA two days after the initial treatment. The amount of O^4-ethylthymine is steadily increasing whereas, the quantity of O^6-ethylguanine is declining, until 28 days after the initial treatment, 50-fold less O^6-ethylguanine than O^4-ethylthymine is present in hepatocyte DNA (Swenberg et al., 1984). Dyroff et al. (1986) measured induction of preneoplastic foci of diethylnitrosamine- altered hepatocytes as a function of O^4-ethylthymine production by immuno-histochemical staining for gamma-glutamyl transpeptidase (GGT+). There was a positive correlation between O^4-ethylthymine accumulation, production of GGT+ foci, and hepatocellular tumour induction after exposure of rats to diethylnitrosamine. In a more detailed study of the distribution of GGT+ foci and O^4-ethylthymine within three lobes of the liver, both were twice as numerous in the hepatocyte population of the left and right median lobes compared to the right anterior lobe (Richardson et al., 1986). This correlated well with the 100% incidence of hepatocellular carcinoma in the left and right compared to the 30% incidence in the right anterior lobe.

While all the above experiments were performed using ethylating agents, analogous conclusions were reached for tumours induced by methylating agents. Richardson et al. (1985) observed that despite the very low initial quantities of O^4-methylthymine compared to O^6-methylguanine in rat hepatocytes DNA during continuous exposure to dimethylhydrazine, there was only 1.5 times more O^6-methylguanine after 4 weeks, suggesting that O^4-methylthymine might well be equally important in the induction of the

hepatocellular carcinoma. O^4-methylthymine is lost fairly rapidly from rat liver ($t_{1/2} \approx 20$ h) whereas, O^4-ethylthymine has a much longer half life [$t_{1/2} \approx 11$ days] (Richardson *et al.*, 1985). The great differences in the half-life between ethyl and methyl derivatives suggest that cell turnover is unlikely to play a major role in establishing the rates of removal and that an enzymic repair of these adducts probably takes place. Although enzymic repair was also indicated in a number of kinetic studies of in vivo removal of O^4-alkylthymine (Den Engelse *et al.*, 1986; Dyroff *et al.*, 1986; Den Engelse *et al.*, 1987), the enzyme responsible for this repair remains unidentified.

The E. coli alkyltransferase which removes the alkyl group from O^6-alkylguanine can also act on O^4-alkylthymine and dealkylation involves the same transfer mechanism. However, it is still unclear if mammalian alkyltransferases can repair O^4-alkylthymine, and if so, whether the rate of repair is consistent with the half-life observed in vivo (Brent *et al.*, 1988; Koike *et al.*, 1990; Sassanfar *et al.*, 1991).

1.2.3. O^6-alkylguanine: A pro-mutagenic adduct

Loveless' hypothesis on the mutagenic properties of O^6-alkylguanine (Loveless, 1969) has been substantiated with assays of in vitro replication. These experiments showed that homopolymers or heteropolymers containing O^6-methylguanine when copied by bacterial or mammalian RNA or DNA polymerases direct the incorporation of T (or U) opposite O^6-methylguanine to a much greater extent than the incorporation of C (reviewed by Saffhill *et al.*, 1985). Figure 1.3 shows schematically the process of O^6-methylguanine-induced mutagenesis. Alkylating agents alkylate DNA producing among other adducts, O^6-alkylguanine. At this point the cell has a chance to repair O^6-alkylguanine by the action of alkyltransferases, restoring the normal G:C base pair in the DNA sequence. If the cell does not remove O^6-alkylguanine before DNA synthesis (e.g. because of depleted alkyltransferase or rapid cell proliferation), the DNA polymerase when it comes to an O^6-alkylguanine in the template DNA strand, would misincorporate T

1st replication   2nd replication

CH₃
|
—— G ——   ——— A ———
—— T ——   ——— T ———
+
—— G ——
—— C ——

CH₃
|
—— G ——
—— C ——

AT-SH

AT-S-CH₃ +   —— G ——
                —— C ——

1st replication   2nd replication

CH₃
|
—— T ——   ——— C ———
—— G ——   ——— G ———
+
—— T ——
—— A ——

CH₃
|
—— T ——
—— A ——

AT-SH

AT-S-CH₃ +   —— T ——
                —— A ——
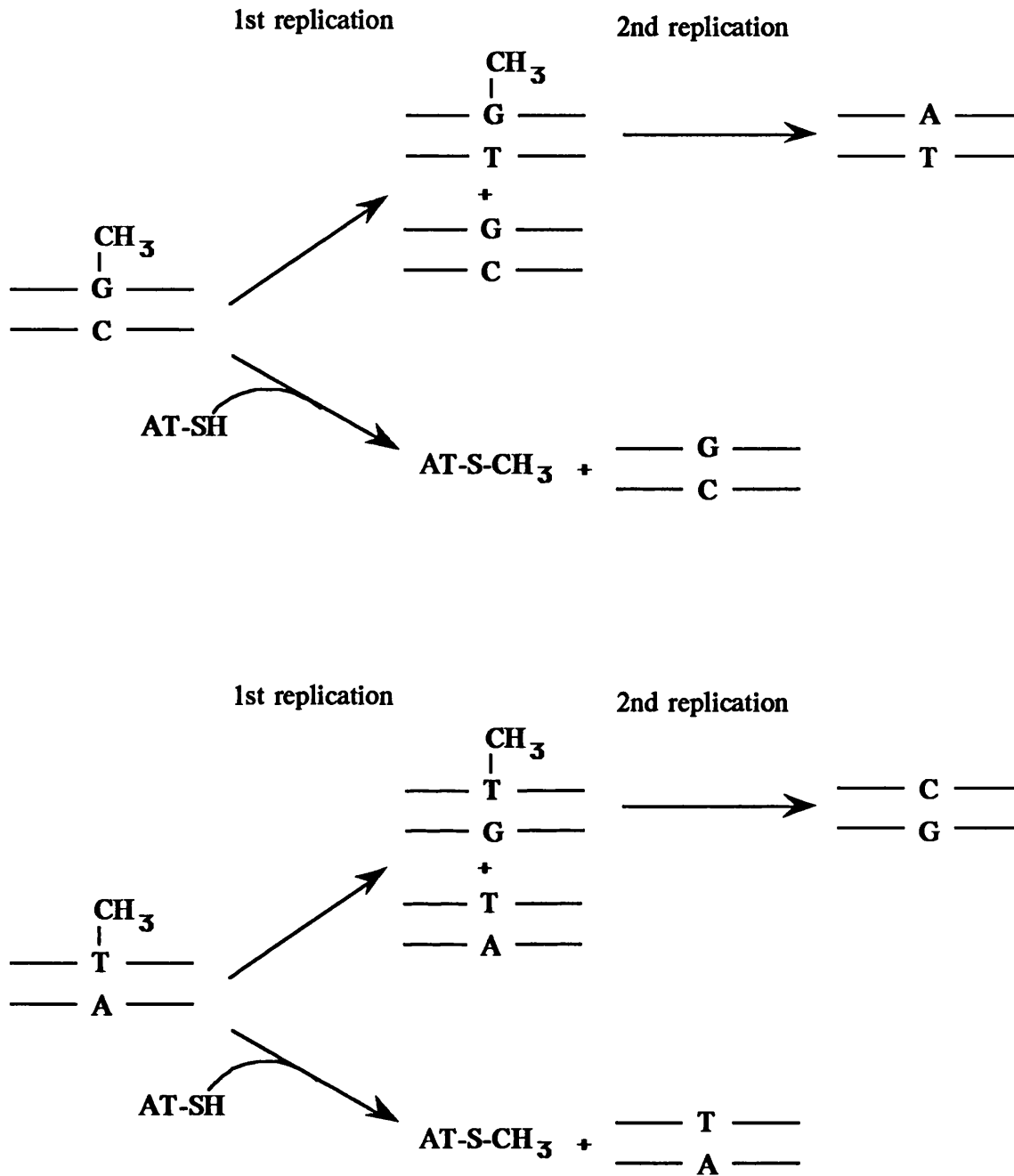
Figure 1.3: Mechanism of mutagenesis caused by O^6-methylguanine and O^4
-methylthymine; AT-SH: O^6-methylguanine-DNA-alkyltransferase.

instead of C, forming an O⁶-alkylG:T mismatch. During the second round of replication the polymerase incorporates an A opposite to T forming an A:T base-pair, and thus establishing the G:C to A:T transition mutation. The alkyltransferase can also act to the O⁶-alkylG:T base-pair between the two rounds of replication producing a G:T mismatch. However, if this base-pair is not repaired by the G:T specific mismatch repair mechanisms, it would also lead to the same transition mutation.

The in vitro replication assays can only give some evidence for the mispairing properties of O⁶-alkylguanine. The only definitive way to establish that O⁶-alkylguanine can cause G:C to A:T mutations in vivo as well as in vitro, is to situate a unique O⁶-alkylguanine adduct in a vector DNA molecule, and after transfection of host cells and replication, to recover progeny and examine the mutations in the site where the adduct has been originally introduced. Loechler *et al.* (1984) following this strategy transfected E. coli with a modified M13 phage containing a single O⁶-methylguanine. After in vivo replication, the only mutations detected at the site of the modified base were G:C to A:T transition mutations. The very low frequency of mutations (0.4%) was attributed to the alkyltransferase mediated repair of O⁶-methylguanine. To overcome repair, the endogenous alkyltransferase was depleted by treating the cells prior to transfection with MNNG, and this treatment was paralleled with an increase in the mutation frequency to approximately 20%. Similar results have been obtained in other studies (Chambers *et al.*, 1985; Hill-Perkins *et al.*, 1986).

The mutagenic potential of O⁶-alkylguanine in eukaryotes has long been a matter of specu-lation but only recently confirmed. A shuttle vector was constructed containing a single O⁶-methylguanine or O⁶-ethylguanine at a preselected position (Ellison *et al.*, 1989a). The vector was introduced into CHO cells and after intrachromosomal replication, the vector DNA was amplified by Polymerase-Chain-Reaction (PCR) and analyzed for mutations. Consistent with all other available data on O⁶-alkylguanine, the predominant mutations were once again G:C to A:T transitions (Ellison *et al.*, 1989b). The observation that mutations in both E. coli and human cell lines induced by methylating agents, are

exclusively G:C to A:T transitions (Coulondre and Miller, 1977; Dubridge *et al.*, 1987)

suggests that at least for the methylating agents, O^6-methylguanine is the major

pro-mutagenic adduct. Ethylating agents also induce transitions which are mainly G:C to

A:T mutations, but in that case a significant amount of A:T to G:C mutations was observed

as well, and they were attributed to the miscoding properties of O^4-alkylthymine

(Richardson *et al.*, 1987; Richardson, 1988).

1.2.4. O^4-alkylthymine is also a pre-mutagenic adduct

The observation that O^6-alkylguanine miscodes in assays of in vitro replication was

followed by attempts to prove the same for O^4-alkylthymine using very similar approach. It

was shown that O^4-alkylthymine directs the incorporation of G rather than A by the RNA

or DNA polymerases (Singer *et al.*, 1986). If this applies in vivo the so formed

O^4-alkylT:G mismatch would give rise to a T:A to C:G transition mutation after the second

round of replication (figure 1.3). However synthetic oligonucleotides containing

O^4-alkylthymine were not available till very recently (Li *et al.*, 1987) and thus, site-directed

mutagenesis experiments were very difficult to perform. Preston *et al.* (1986) used an

alternative method to introduce a unique O^4-alkylthymine into a plasmid DNA. O^4-alkyl-

dTTP is substrate for the E. coli DNA polymerase I and can be incorporated in vitro

opposite A, if there is no dTTP present to compete for the active site of the enzyme. Using

this approach and single stranded phi X174 am3 DNA as template they incorporated

O^4-alkylthymine opposite the adenine of the amber codon. After further extension of the

primer with unmodified nucleotides, and transfection of *ada*- E. coli, amber revertant

mutants were scored. The mutation frequency was markedly above background and

sequencing revealed that all mutants had the expected T:A to C:G mutations at the original

site of O^4-alkylT:A base pair. When synthesis of oligonucleotides containing

O^4-alkylthymine became possible, the mutations caused by this adduct in mammalian cells

were examined, and were found to be the expected T:A to C:G transition mutations (Klein

et al., 1990).

1.2.5. Alkylation of the DNA precursors pool and mutagenesis

Modified O^6-methyl-dGTP and O^4-methyl-dTTP are acceptable substrates for DNA synthesis by both bacterial and mammalian polymerases and are mainly incorporated opposite T and G respectively (Hall *et al.*, 1983; Toorchen and Topal, 1983). Based on this finding and the high reactivity of dNTP with alkylating agents, it has been proposed that alkylation of the dNTP pool can be a major source of modified nucleotides in DNA (Saffhill, 1985; Saffhill, *et al.*, 1985). According to this hypothesis, the polymerase when copying opposite T can use either dATP or O^6-alkyl-dGTP. Once incorporated into DNA, O^6-alkylguanine can be repaired by the alkyltransferase forming a G:T mismatch. In the second round of replication G is paired with C, and hence, incorporation of O^6-alkylguanine in DNA by the polymerase would overall lead to A:T to G:C transition mutations (Eadie *et al.*, 1984). Similarly incorporation of O^4-alkylthymine would lead to G:C to A:T transitions. However, most experimental evidence argues against such a model for mutagenesis.

a) O^6-methyl-dGTP and O^4-methyl-dTTP are poor substrates for the DNA polymerase in vitro (Hall *et al.*, 1983).

b) Attempts to incorporate O^6-methylguanine into cellular DNA by treatment of cells with the free base, nucleoside or nucleotide have been totally unsuccessful (reviewed by Saffhill *et al.*, 1985).

c) Treatment of cells with methylating agents produces mutations which are in the vast majority G:C to A:T mutations instead of the A:T to G:C mutations which would have been predicted from this model for mutagenesis (Coulondre and Miller, 1977; Dubridge *et al.*, 1987).

1.2.6. Properties of other alkylated adducts

Among the other adducts only O^2-alkylthymine has miscoding properties in in vitro

replication assays . It directs the misincorporation of guanine by DNA polymerase but with a much reduced frequency compared to that of O^4-alkylthymine (reviewed Saffhill *et al.*, 1985). The relevance of O^2-alkylthymine in mutagenesis and lethality caused by alkylating agents is yet unknown. (see Singer *et al.*, 1989a).

N3- and N7- alkylpurines may be a source of mutation because they depurinate at a rate some 10^6, 10^7 times faster than normal bases, as a consequence of the instability of their glycosidic bonds and the action of DNA glycosylases. The so formed apurinic sites (AP) are mutagenic intermediates yielding mainly G:C to T:A transversions (for review Laval *et al.*, 1990). N1-alkylpurines and N3-alkylpyrimidines are inhibitory for DNA synthesis and thus they might be involved in causing the cytotoxic effects of the alkylating agents (Shaffhill *et al.*, 1985).

1.2.7. Why do O^6-alkylguanine and O^4-alkylthymine miscode?

Although the role of O^6-alkylguanine and O^4-alkylthymine as pro-mutagenic adduct was established more than a decade ago, the reason for their miscoding behaviour remains unclear. It is widely believed that base selection during DNA synthesis, is template directed by the formation of stable hydrogen-bonded base-pairs with the incoming nucleotide triphosphates. On that basis it would be supposed that O^6-alkylguanine interacts preferably with thymine in the polymerase complex, because it forms a more stable base-pair with this base, rather than any other base including cytosine. Thus, the structures for the O^6-alkylG:T and O^6-alkylG:C base-pairs were suggested and widely accepted (Loveless, 1969) (Figure 1.4 B, C). A similar scheme was conceived for base pairs involving O^4-alkylthymine [Figure 1.5 B, C] (Lawley, 1984).

However, recent theoretical and physicochemical studies put in doubt these straightforward mispairing schemes. A crucial aspect in the pairing properties of the alkylated bases, is the conformation of the alkyl group. The O-alkyl group which preferentially lies in the plane of the base ring, could be either *syn* (proximal) or *anti* (distal) to the N1 of alkylguanine (or to the N3 of alkylthymine). An alkyl group in the *syn*
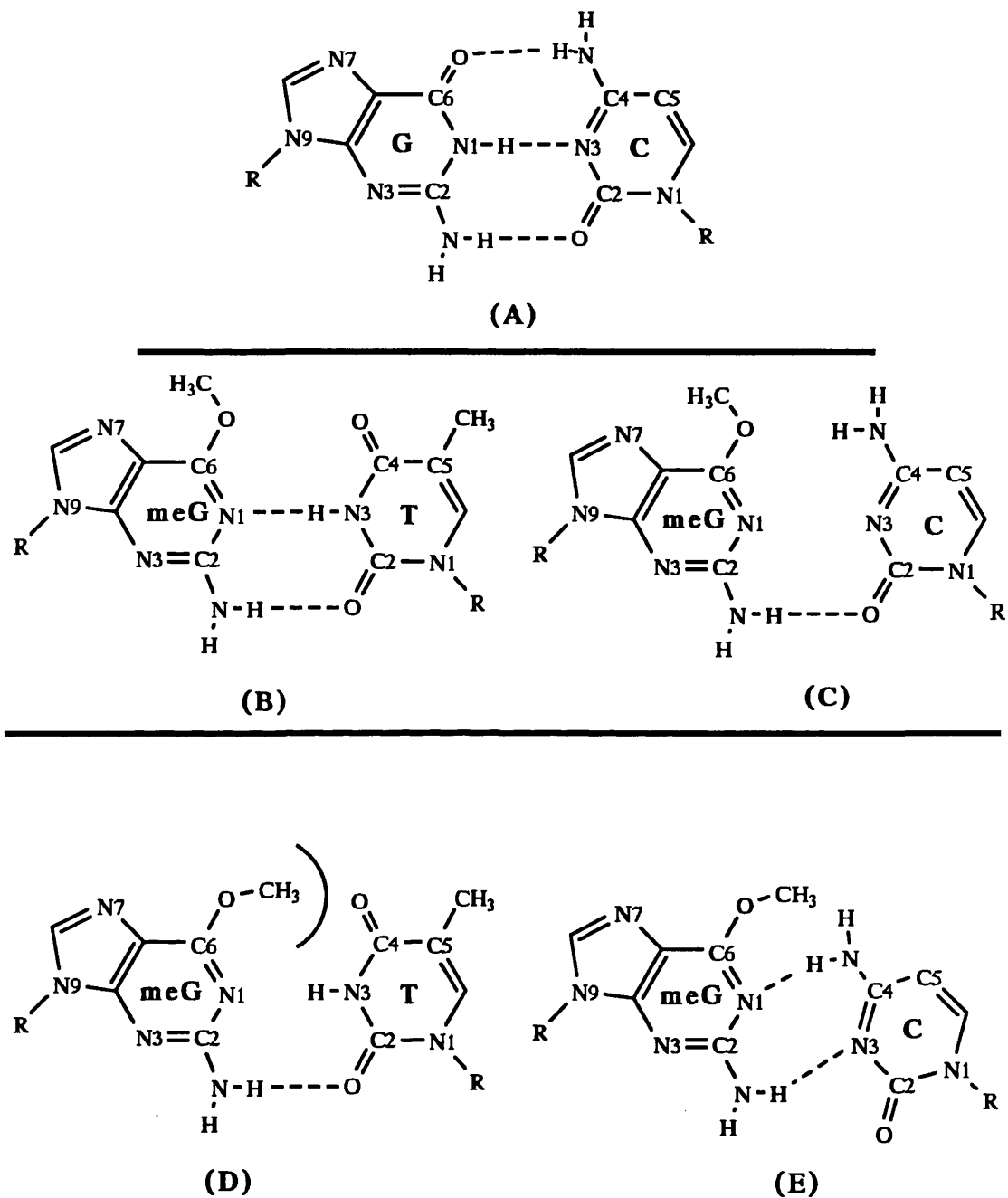
Figure 1.4: Postulated structures for the base-pairs between O^6-methylgulanine and thymine or cytosine. (A) normal G:C Watson-Crick base-pair; (B) and (C) base-pairing modes of O^6-methylgulanine as proposed by Loveless, (1969); (D) and (E) base pairing as predicted based on 2-D NMR data (Kalnik et al. 1989a,b). Only hydrogen atoms of interest are shown.
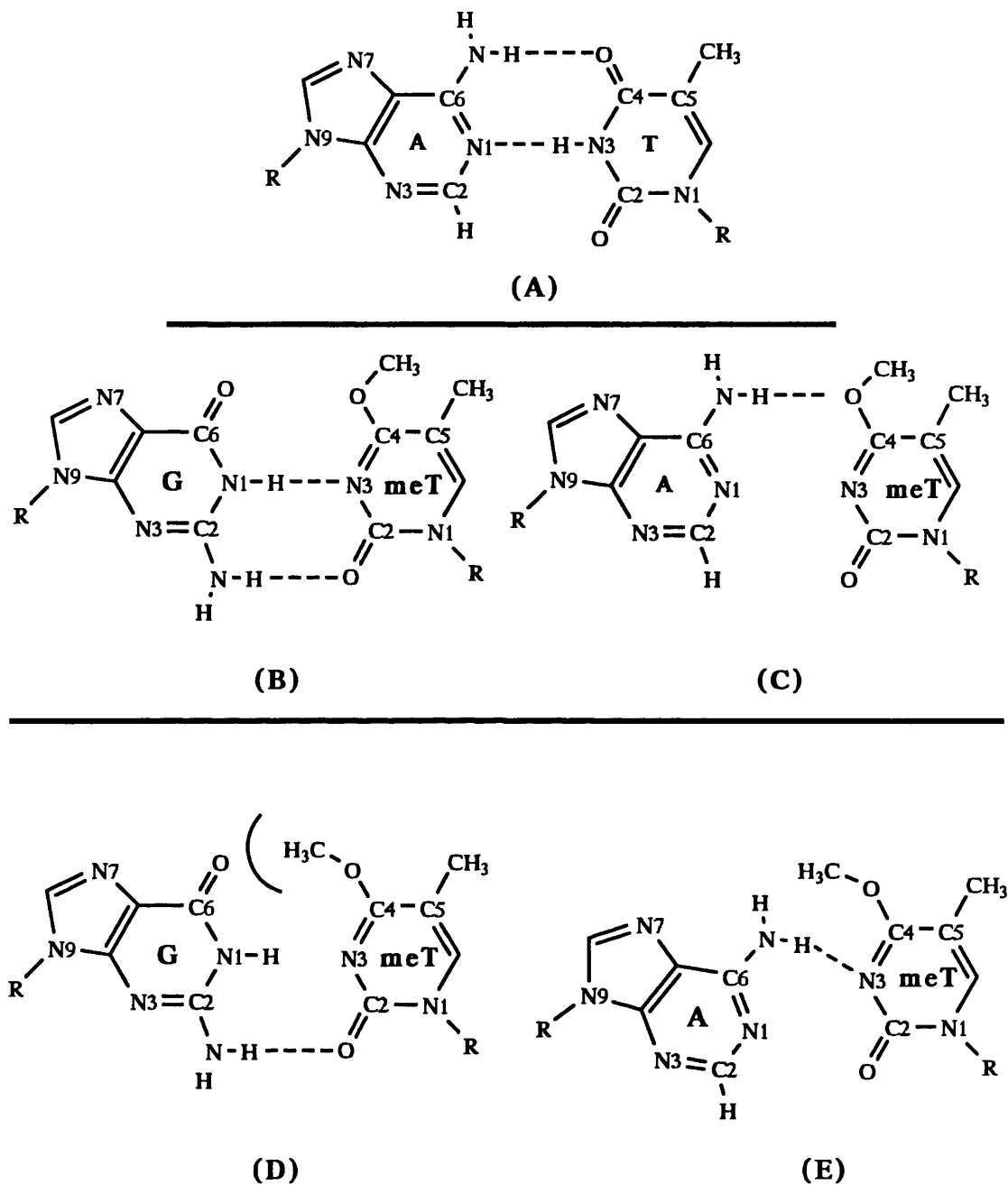
Figure 1.5: Postulated structures for the base-pairs between O^4-methylthymine and

guanine or adenineine. (A) normal A:T Watson-Crick base-pair; (B) and (C) base-pairing

modes of O^4-methylthymine as proposed by Lowley, (1984); (D) and (E) base pairing as

predicted based on 2-D NMR data (Kalnik et al. 1988a,b). Only hydrogen atoms of

interest are shown.

periplanar orientation would block the classic Watson-Crick hydrogen bonding sites because of the imposed steric constraints between the two bases (see figures 1.4 and 1.5 D, E). In contrast, an alkyl group in the *anti* orientation, faces away from the interior of the DNA helix and thus, would not interfere with the formation of hydrogen bonds between the normal donor and acceptor sites of the bases (see figures 1.4 and 1.5 B, C). X-ray crystallography studies of O^6-methylguanine, O^4-methylthymine and O^4-ethylthymine, at the nucleoside level, showed that the O-alkyl groups in crystals adopt the *syn* periplanar orientation (Birnbaum *et al.*, 1986; Brennan *et al.*, 1986; Parthasarathy and Fridey, 1986). Based on the X-ray structures of O^6-methylguanosine, molecular orbital calculations showed that the energy of the *anti* conformer is about 2.7 Kcal/mol higher than that of the *syn* conformer (Yamagata *et al.*, 1988). Thus, either the commonly used base-pair structures of figure 1.4 and 1.5 B, C are wrong, or the orientation of the alkyl group in the unpaired alkylated nucleotides is not the same as in the DNA helix. If the first explanation is true, then the preference for O^6-alkylG:T and O^4-alkylT:G as seen in the biological studies cannot be explained in terms of stability and hydrogen bonding. Brennan *et al.* (1986) preferred the second explanation and suggested that the alkoxy group of O^4-alkylT rotates from the *syn* to the *anti* conformation when the alkylated base is inserted into a double-stranded DNA helix. The energy required for the rotation in the unfavoured *syn* configuration is compensated by the favourable interactions which lead to a more stable DNA helix (i.e. hydrogen bonds, better stacking). This hypothesis was supported by recent findings based on quantum-mechanical calculations on O^6-methyl-9-methylguanine (Pedersen *et al.*, 1988). This theoretical study showed that the *anti* orientation of the O^6-methyl group, while energetically less favoured in the isolated molecule, is more stable and less disruptive to the DNA double helix, when present in a pentamer duplex (= 5 Kcal /mol energy binding difference). While many investigators were reluctant to accept that O^6-alkylG:T base-pair is less stable than O^6-alkylG:C base-pair, Gaffney *et al.* (1984) and Gaffney and Jones (1989) reported that this in fact was the case. Using a set of oligonucleotide duplexes in which O^6-methylG was situated opposite any of the four

bases, they studied the stability of each set of duplexes as reflected in the Tm (melting point) temperature. Indeed on a purely thermodynamic basis, O^6-methylG:C base-pair is more stable than the O^6-methylG:T base-pair .

2-D proton NMR spectra of a set of self-complementary oligonucleotides containing O^6-alkylguanine were obtained in a collaboration study between our laboratory and that of Dr. D. Patel. In summary the NMR data showed:

1) Duplexes containing either O^6-alkylG:C or O^6-alkylG:T base-pairs form right handed B-form helixes, with all the bases stacked into the helix having the normal *anti* glycosidic torsion angle.

2) There is a very short distance between the O-alkyl group and the hydrogen bonded protons of the neighbouring bases, which is indicative of a *syn* (proximal) orientation of the alkyl group in both O^6-alkylG:C and O^6-alkylG:T base-pairs.

3) The N3 imino proton of thymine in the O^6-alkylG:C base-pair is not involved in a short hydrogen bond while one of the two N^4-amino protons of C in the O^6-alkylG:C base-pair is evidently hydrogen-bonded.

4) ^{31}P-NMR spectrum showed no distortion of the phosphate backbone in the oligomer containing O^6-ethylG:T, while a significant distortion was evident in the the oligomer containing O^6-ethylG:C base-pair.

Based on these findings Kalnik *et al.* (1989 a,b) suggested the base-pair structures of figure 1.4 D, E (reviewed by Swann, 1990). The O^6-alkyl group is in the *syn* orientation forcing the bases in the O^6-alkylG:T base-pair apart , excluding the possibility of a hydrogen bond between the N1 of meG and the N3 imino proton of T . This base-pair is stabilized with only one hydrogen bond but maintains a geometry consistent with an unperturbed Watson-Crick base-pair. In contrast, O^6-alkylG:C is stabilized with two hydrogen bonds but the base-pair adopts a wobble conformation with cytosine forced towards the major groove.

Likewise Kalnik *et al.* (1988a,b) reached similar conclusions for the O^4-alkylT:G

and O^4-alkylT:A base-pairs (figure 1.5. D, E). In figure 1.5 D only one hydrogen bond is shown in the O^4-methylT:A base-pair. However, it is possible a second hydrogen bond to be formed at lower pH, between the protonated N1 of adenine and the O^{2-} atom of O^4-alkylthymine. As a consequence of these results the mechanism of alkylation-induced point mutation must be questioned and probably revised.

A hypothesis which is in accordance with both the holding model for recognition of the correct base-pair by the polylmerases, and the relative stability of the base-pairs as observed by Gaffney and Jones (1989), was put forward by Williams and Shaw (1987), in an attempt to explain their results. They obtained the NMR spectra of base-pairs between O^6-methylG and C or T derivatives in a chloroform solution, which is an environment which they thought might mimic the hydrophobic core of duplex DNA. Their studies suggested the formation of 2 hydrogen bonds in the O^6-methylG:T base-pair while the O^6-methylG:C base-pair could be stabilized only after protonation of N3 of C by three hydrogen bonds.Thus, they suggested that in the polymerase complex T is inserted opposite O^6-methylG because this base-pair is more stable than the O^6-methylG:C base-pair. However, in the double stranded DNA cytosine is protonated and the order of stability is inverted. Since protonation promotes deamination, it was also suggested that in addition to mispairing during replication, G to A mutations could be also caused be the deamination of the protonated C to U. This model, although in agreement with the observed stability of the base-pairs, requires an *anti* orientation of the alkyl group and thus, contradicts with the NMR data.

The NMR and the denaturation studies can only suggest that the architecturally uniform helix as occurs in base-pairs which adopt a Watson-Crick geometry could be the most important requirement for the polymerase during replication, and not the stability of the base-pair.

1.3 REPAIR OF O^6-ALKYLGUANINE AND O^4-ALKYLTHYMINE

1.3.1. Adaptive response of E. coli

Detailed understanding of the repair mechanism for O^6-alkylguanine in DNA

followed from the identification of the adaptive response in E. coli. The adaptive response

refers to the observation that exposure of cells to low levels of N-methyl-N-nitroso-N'-

nitroguanidine (MNNG) results in increased resistance of these cells to the mutagenic and

cytotoxic effect of the carcinogen (Samson and Cairns, 1977). This response could not be

associated with the recA-lexA controlled SOS repair system because SOS repair produces

increased survival and increased mutagenesis. Furthermore, adaptive response is

specifically induced by alkylating agents (N-methyl-N-nitrosourea, N-methyl-N-nitroso-N'-

nitroguanidine, methyl methanesulphonate) but not by UV irradiation, a common inducer

of the SOS response in E. coli (Jeggo *et al.*, 1977). The identification of the adaptive

response as a mechanism distinct from the SOS response was confirmed by the isolation of

mutants defective in one, but not the other, defence mechanism (Jeggo, *et al.*, 1977; Jeggo

et al., 1978; Jeggo, 1979).

Three genes, *ada*, *alkA*, and *alkB* were originally found to be induced as part of the

adaptive response. They were isolated through a genetic screen for mutants with increased

sensitivity to mutagenesis or killing by methylating agents (Yamamoto *et al.*, 1978; Jeggo,

1979; Kataoka *et al.*, 1983). A fourth gene aidB was latter identified to be overexpressed

after exposure to methylating agents (Volkert and Nguyen, 1984).

1.3.2. The *ada* gene

O^6-methylguanine-DNA-methyltransferase was the first activity to be identified as

part of the adaptive response. The first indication of this activity was that adapted E.coli

cells accumulate less O^6-methylguanine in their DNA than unadapted cells (Schendel *et al.*,

1978). Experiments with extracts revealed a repair process involving transfer of the methyl

group from O^6-methylguanine to a cysteine residue on an acceptor protein [termed

O^6-alkylguanine-DNA-alkyltransferase] (Olsson and Lindahl, 1980) leaving an unmodified

guanine in DNA (Foote *et al.*, 1980). Unadapted E. coli contains 2-3 alkyltransferase

molecules per cell and this amount increases several hundred fold after adaptation (Robins

and Cairns,1979; Rebeck *et al.*, 1989). [the numbers refer to the *ada* alkyltransferase]

$$H_2N - \boxed{\begin{array}{c|c} 20\ K & 19K \end{array}} - COOH$$

Cys69
(mePT)

Lys178-glu179
(in vitro cleavage
site)

Cys321
(O^6methylG)
(O^4methylT)
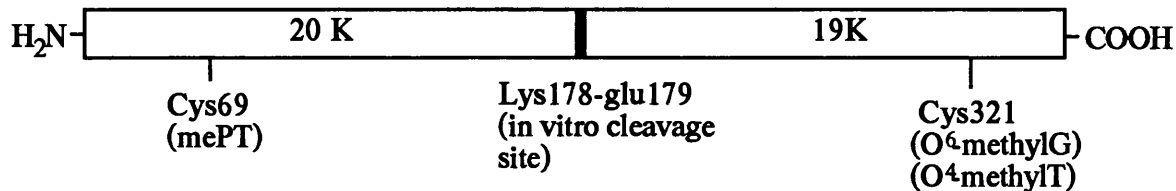
Figure 1.6: Schematic representation of the 39 K *ada* protein The methyl-accepting cysteine

residues along with the DNA adducts repaired by each residue are indicated; mePT,

methylphosphotriesters;

Following purification to apparent homogeneity of a 19,000 daltons protein (19

K), associated with the alkyltransferase activity, the active cysteine residue was located in

the carboxy-terminal domain of the molecule (Demple *et al.*, 1982). Unexpectedly,

antibodies raised against this protein were found to cross-react with the 39,000 dalton

protein (39 K) encoded by the *ada* gene (Teo *et al.*, 1984). The 19 K protein has been

subsequently identified as a fragment (the carboxy-terminal) of the intact 354 amino acids

long 39 K *ada* protein and arises, after cell lysis, from in vitro proteolysis of the

lys178 -gln179 bond of the *ada* protein [figure 1.6] (Demple *et al.*, 1985). The methyl

acceptor cysteine is Cys321 of the 39 K protein (figure 1.6), and no mechanism appears to

exist to demethylate the resulting S-methylcysteine (Demple *et al.*, 1982). Thus, unlike the

vast majority of enzymes, O^6-alkylguanine-DNA-alkyltransferase reacts stoichiometrically

with O^6-methylguanine and the protein is not regenerated after the reaction but accumulates

in the methylated form as a dead-end product of the reaction (Lindahl and Robins, 1982).

An important consequence of this inactivating stoichiometric reaction with

O^6-methylguanine is that the protein is rapidly depleted when E. coli is treated with

methylating agents, normal amounts being restored only after RNA and protein synthesis

(Robins and Cairns, 1979). The inactivating reaction can also explain why the capacity of

the adaptive response to protect against mutagenesis is limited and inefficient when high

doses of alkylating agents are used (Schendel *et al.*, 1978).

The *ada* protein requires no cofactors, is strikingly resistant to heat inactivation

under reducing conditions and has an optimum pH of about 7.8-8.5 (Lindahl and Robins,

1982). The repair activity is strongly inhibited by several metal ions but not when high

concentration of DTT is present (Scicchitano and Pegg, 1987). The *ada* alkyltransferase has

a wide spectrum of substrate activity. It is active in repairing O^6-methylguanine, O^6-

-ethylguanine, O^6-n-propylylguanine, O^6-butylguanine, O^6-chloroethylguanine although

with different efficiencies (Robins *et al.*, 1983; Sedgwick and Lindahl, 1982; Pegg *et al.*,

1984; Morimoto *et al.*,1985). It can also dealkylate O^4-alkylthymine with the same

irreversible repair process (McCarthy *et al.*, 1984). The substrate specificity of this and

other alkyltransferases will be discussed in detail in a following chapter.

Extracts of E. coli were also active in the repair of methylphosphotriesters formed

by methylation of the phosphate backbone of DNA. This repair activity was also inducible

(McCarthy *et al.*, 1983). It has been demonstrated that the 39 K *ada* protein but not the 19

K proteolytic fragment has this activity. It exhibits an apparently absolute stereospecificity

in this reaction and only the S stereoisomer (methyl group protruding perpendicular to the

DNA axis) but not the R stereoisomer (methyl group accommodated within the major

groove) is repaired (McCarthy and Lindahl, 1985). The methyl group from the

methylphosphotriesters is transferred not to Cys 321 but to Cys69 residue of the

amino-terminal domain of the protein [figure 1.6] (Sedgwick *et al.*, 1988). This

methylation at Cys69 converts the *ada* protein into an efficient transcriptional activator of

the adaptive response (to be discussed in detail below).

The other genes of the adaptive response are:

A. The *alkA* gene which codes for 3-methyladenine glycosylase II (MW 31,400 daltons).

Mutations in this gene make the cell more sensitive to killing by methylating agents

(Yamamoto *et al.*, 1978) and the protein is known to repair N3-methyladenine,

N3-methylguanine O^2-methylcytosine, and O^2-methylthymine (Karran *et al.*, 1982;

McCarthy *et al.*, 1984).

Thus, this particular enzyme probably recognizes a specific structural feature, rather than particular DNA adduct, absent in native DNA such as a methyl group protruding into the minor groove of the DNA double helix (McCarthy *et al.*, 1984).

B. The *alkB* gene which is downstream of the *ada* gene in an operon. It encodes a 24,000 daltons protein with unknown function.

C. The *aidB* gene whose encoded protein's size and function is still unknown. Surprisingly, aidB mutants become more resistant, rather than more sensitive to exposure to alkylating agents (Volkert and Nguyen, 1984).

1.3.3. Transcriptional activation

The adaptive response to alkylating agents was found to be positively regulated by the *ada* gene. Overproduction of the *ada* protein (39,000 daltons) by cloning the gene into an expression vector, leads to elevated expression of the activities associated with the response such as *alkA*-encoded glycosylase, even in the absence of alkylating agents (Sedgwick, 1983). In vitro experiments have shown that the *ada* protein methylated at Cys69 is a powerful transcriptional activator of the adaptive response genes (Nakabeppu and Sekiguchi, 1986; Teo *et al.*, 1986). Teo *et al.* (1986) used a plasmid containing the complete *ada* gene and its promoter to direct protein synthesis in a cell-free system. Various forms of the *ada* protein were added in attempts to stimulate expression of the *ada* gene. Addition of the 39 K or the 19 K carboxy-terminal fragment of the protein did not induce synthesis of new *ada* protein. However, the 39 K protein methylated by preincubation with NMU-treated DNA was an efficient activator of *ada* gene expression. The *ada* protein
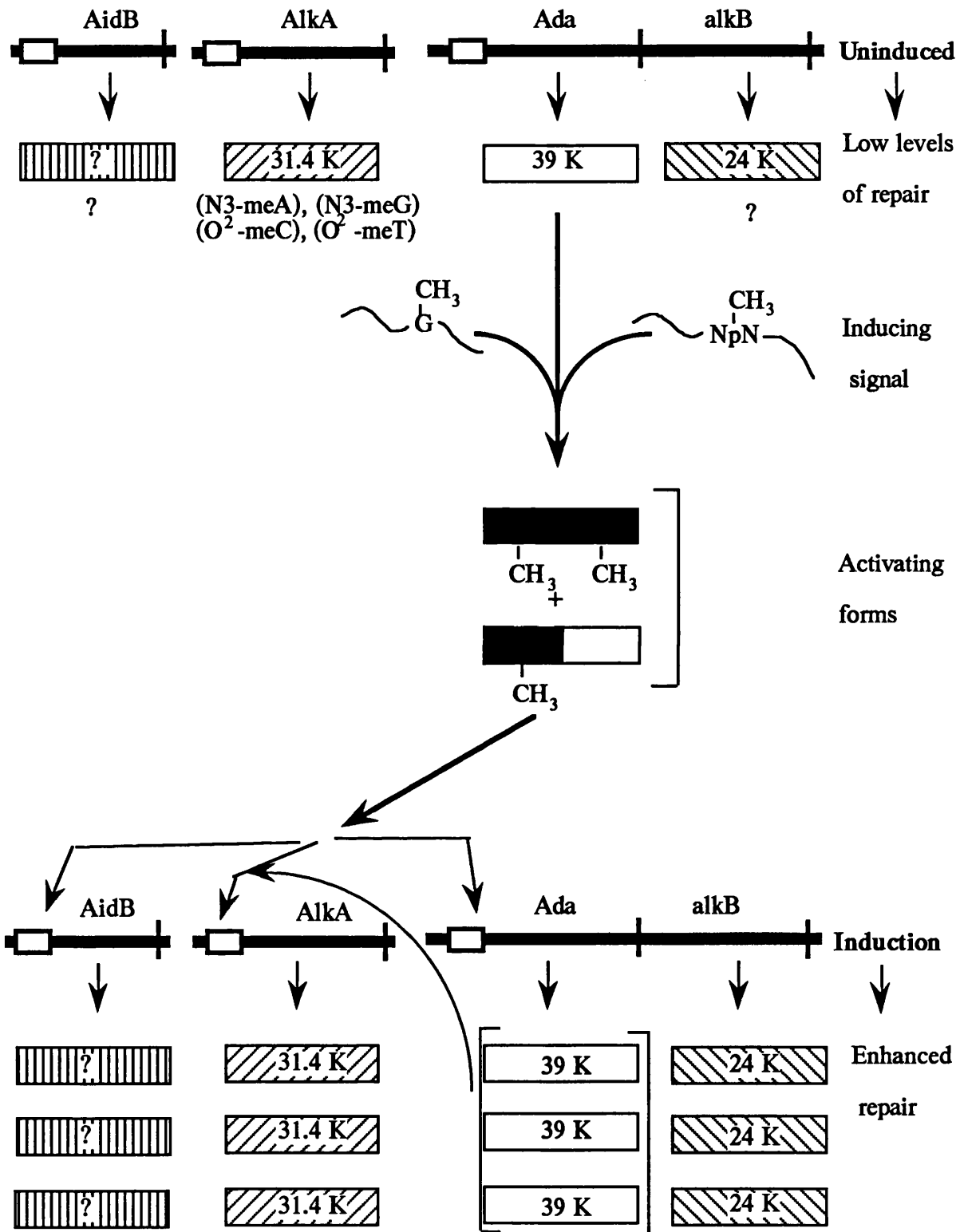
Figure 1.7: Model for regulation of the adaptive response genes by the ada protein

single methylated by repair of O^6-methylguanine (Cys 321) did not stimulate new

synthesis while single methylated by repair of methylphosphotriesters (Cys 69) was an

efficient activator of *ada* gene expression. The model for the adaptive response regulation as

suggested from these results is shown in figure 1.7. Agents such as

N-methyl-N-nitroso-N'-nitroguanidine and methyl methanesulphonate methylate the E. coli

DNA, forming many lesions including methylphosphotriesters, O^6-methylguanine and

O^4-methylthymine. The *ada* alkyltransferase molecules present in an uninduced cell can

each repair one phosphotriester adduct and activate, in the methylated form, the

transcription from the *ada* promoter, producing large amounts of alkyltransferase. Once the

ada protein is induced, either the methylated alone or both methylated and unmethylated

protein (Nakabeppu and Sekiguchi, 1986) can promote the transcription of the other

adaptive response genes, and thus protect the cell from the mutagenic and cytotoxic effects

of alkylating agents.

1.3.4. The ada and alkA promoters

DNA footprinting studies revealed that the *ada* protein methylated at Cys 69 or fully

methylated (at Cys321 and Cys69), but not the unmethylated protein, can bind strongly to a

sequence within the *ada* and alkA promoter, and protected it from DNAase I digestion. The

sequence is 5' -AAANNAAAGCGCA- 3' and is called the "*ada* box" (Teo *et al.*, 1986;

Sakumi and Sekiguchi, 1989). Methylation at Cys69 of *ada* is thought to cause a

conformational change in the protein that converts *ada* from a weak to a strong

DNA-binding protein. In support of this hypothesis, Sedgwick (1988) has observed that

the methylated *ada* protein gave a different trypsin digestion pattern than the unmethylated

protein. The mechanism that facilitated the DNA binding of the *ada* protein is still

unknown. The protein does not have the acidic or basic domains observed in many

eukaryotic transcriptional activators. Also there is no obvious homology to helix-turn-helix,

leucine zipper, or zinc finger motifs. However, many metal cations interfere with the *ada*

gene expression and the alkyltransferase activity, and the possibility that the *ada* protein

contains a novel metal binding pattern, should not be excluded (Scicchitano and Pegg,

1987; Takahashi *et al.*, 1988).

The mechanism by which *ada* activates transcription is also not known and a lot of research is being done towards this direction. DNA footprinting studies have shown that methylated *ada* protein must be present for RNA polymerase to bind to the *ada* promoter (Teo *et al.*, 1986), while the unmethylated *ada* can also bind to and facilitate RNA polymerase binding to the alkA promoter (Nakabeppu and Sekiguchi, 1986). However, *ada* protein does not function as a sigma subunit and the sigma subunit of the RNA polymerase was required for efficient in vitro transcription from the *ada* and *alkA* promoters (Sakumi and Sekiguchi, 1989).

In vitro and in vivo studies showed that the methylated amino terminal domain is able to bind in both *ada* and alkA promoter but it can promote transcription only from the alkA promoter (Sedgwick, *et al.*, 1988; Shevell *et al.*, 1988a; Yoshikai *et al.*, 1988). The importance of the carboxy-terminal domain in the transcriptional activation of *ada* and *alkA* has also been shown in experiments where part of it has been deleted or altered. Almost always the mutant protein was a constitutive activator of the *ada* protein while it had a diverse effect on the alkA protein (Shevell *et al.*, 1988a).

1.3.5. The E. coli *ogt* gene

Margison *et al* (1985) in an attempt to clone the *ada* gene, constructed an E. coli genomic library in an expression vector, transformed bacteria, and selected clones which had elevated alkyltransferase activity. Among the different clones, one showed increased alkyltransferase activity, but the inserted sequence gave a different restriction endonuclease pattern to that of the *ada* gene. Also the insert's encoded protein reacted very weakly with antibodies for the *ada* protein. Thus, it was concluded that a second alkyltransferase gene exists in E. coli. This was named the *ogt* gene (Potter *et al.*, 1987). The existance of the *ogt* gene was confirmed when E. coli *ada*- mutants were found to contain detectable levels of alkyltransferase activity (Shevell *et al.*, 1988b; Rebeck *et al.*, 1988). In wild type cells the *ogt* gene is expressed in higher levels than the *ada* gene (about 10-20 times). Unlike the *ada* alkyltransferase it does not possess alkylphosphotriester alkyltransferase activity

(Margison, 1990; Rebeck, *et al.*, 1989).

The *ogt* coding region spans 515 nucleotides and the encoded protein has a molecular weight of 19,000 daltons. The predicted amino acid sequence contains regions of considerable homology with the 19,000 daltons fragment of the *ada* protein and also of the alkyltransferase encoded by the constitutively expressed *Dat 1* gene of B. subtilis (Morohoshi *et al.*, 1989). In particular a pentapeptide region that contains the cysteine residue known to be the acceptor site for the alkyl group for the *ada* protein (Cys321) is common for all three proteins (figure 1.8).

There is a number of evidence indicating that the *ogt* gene is not inducible upon treatment with methylating agents but is expressed in a constitutive level.

a) The characteristic *ada* box does not exist in the *ogt* promoter (Potter, *et al.*, 1987).

b) Northern analysis of E. coli RNA did not show overexpression of the *ogt* gene under conditions that induce the *ada* gene (Potter *et al.*, 1989).

c) Pretreatment of wild type E. coli cells with N-methyl-N-nitroso-N'-nitroguanidine induces both O^6-alkylguanine and alkylphosphotriester repair activities to a similar level. In *ogt* ˉ mutants the levels of the two activities before and after induction are the same and within the range found for the *ada* and *ogt* wild type strains while in *ada* ˉ cells pretreatment with N-methyl-N-nitroso-N'-nitroguanidine exhausts, rather than induce, the preexisting pool of alkyltransferase activity (mainly *ogt* protein), to undetectable levels (Margison *et al.*, 1990).

The existence of two parallel systems for the repair of O^6-alkylguanine is not limited to E. coli., for B. subtilis also possesses one inducible and one constitutive repair system for O^6-alkylguanine (Morohoshi *et al.*, 1989; Morohoshi *et al.*, 1990). In addition other adducts can be repaired by more than one pathways (e.g. in E. coli both the *alkA* and *tag* E. coli gene products repair 3-methyladenine). The presence of two enzymes for the repair of O^6-alkylguanine possibly reflects the importance of this adduct and its repair for the survival of the cell under different conditions.

```
1.human ATase:        M D K D C E M K R T T L D S│P L G│K   18
2.Ada(19KD) :Q F R H G G E N L A V R Y A L A D C│E│L G│R  200
3.    Ogt   :         M L R L L E E K I A T│P L G│P   15
4.    dat 1 :         M N Y Y T T A E T│P L G│E   13


1.│L│E L S G C│E│Q G L H E I K│L│L G K G T S A A D A V E   45
2. C L V A E S│E│R G I C A I L│L│G D D D A T L I S E L Q  227
3.│L│W V I C D│E│Q F R L R A V│E│W E E Y S E A M V Q L L   42
4.│L│I I A E E│E│D R I T R L F│L│S Q E D W V D W K E T V   40


1. V P A P A A V L G G P E P L M│Q│C T A - - - W│L│N A│Y│  69
2. Q M F P A A D N A P A D L M F│Q│Q H V - - - R E V I A  251
3. D I H Y R K E G Y E R I S A T│N│P G G L S D K│L│R D│Y│  69
4. G N T E H K E T P N L A E A K│Q│Q - - - - - -│L│Q E│Y│  61


1.│F│H Q P E A I E E F P V P A L H H P V│F Q│Q│E S F T R   96
2.│S│L N Q R D T P L T L P L D I R│G T│A│F Q│Q│- - - - -  273
3.│F│A G N L S I I D T L P T A T G│G T│P│F Q│R│E - - - -   92
4.│F│A G E R K T F - S L P L S Q K│G T│P│F Q│Q│K - - - -   83


1. Q│V│L│W│K L│L│K V V K F│G│E│V I│S│Y│Q Q L│A│A L A│G│N  123
2. Q│V│-│W│Q A│L│R T I P C│G│E│T V│S│Y│Q Q L│A│N A I│G│k  299
3. -│V│-│W│K T│L│R T I P C│G│Q│V M│H│Y│G Q L│A│E Q L│G│R  117
4. -│V│-│W│Q A│L│E R I P Y│G│E│S R│S│Y│A D I│A│A A V│G│S  108


1.│P K A│T│R A V│G│G│A│M R G│N│P V P│I│L I│P C H R V│V│C  150
2.│P K A│V│R A V│A│S│A│C A A│N│K L A│I│V I│P C H R V│V│R  326
3.│P│G│A│A│R A V│G│A│A│N G S│N│P I S│I│V V│P C H R V│I│G  144
4.│P K A│V│R A V│G│Q│A│N K R│N│D L P│I│F V│P C H R V│I│G  135


1. S S│G│A V G│N│Y│S│G│G│- L A V│K│E W│L L│A H│E│G H R L  175
2  G D│G│S L S│G│Y│P│W│G│- V S R│K│A Q│L L│R R│E│A E N E  352
3. R N│G│T M T│G│Y│A│G│G│- V Q R│K│E W│L L│R H│E│G Y L L  170
4. K N│S│A I T│G│Y│A│G│S│K T E I│K│A F│L L│N I│E│R I S Y  162


1. G K P  GLGGSSGLAGAWLKGAGATSGSPPAGRN            207
2. E R                                            354
3. L                                              171
4. K E K                                          165
```

Figure 1.8 : Comparison of the amino acid sequence of the 1. human alkyltransferase;

2.E.coli ada protein; 3. E.coli ogt protein; and 4. the B.subtilis dat 1 protein. Amino

acids identical in at least three proteins are shown boxed. The active site of the enzymes is

shown in the double framed box.

1.3.6. Isolation and characterization of the mammalian alkyltransferase

Rapid removal of O^6-alkylguanine from rat liver DNA compared to other organs (e.g. brain, lung) contrasts with the general persistence of N7-alkylguanine, and indicates that O^6-alkylguanine is removed enzymatically and not by dilution through cell turnover. This view, and an analogy with the E. coli repair system, is supported by experiments which show that efficient removal of O^6-methylguanine, can be inhibited by pretreatment with alkylating agents, and is restored to the normal levels if a sufficient time-period is allowed between doses (Kleihues and Margison, 1976; Pegg, 1978). The ratio O^6-methylguanine / N7-methylguanine produced in rat liver after exposure to dimethylnitrosamine, is similar to the 0.11 value obtained in vitro, only for doses above 5 mg / kg of body weight. As the dose decreases the O^6-methylguanine amounts decline significantly while the N-7 methylguanine production is at all times proportional to the dose (Pegg and Hui, 1978).

The protein responsible for the removal of O^6-methylguanine in mammalian tissues has been identified in tissue extracts (Mehta et al., 1981), and cultured cells (Myrnes et al., 1982; Pegg et al., 1982) and has been subsequently characterized. The repair enzyme is an alkyltransferase which transfers the methyl group from O^6-methylguanine to a cysteine residue of the mammalian enzyme in a stoichiometric reaction in exactly the same fashion as the E. coli alkyltransferases (Mehta et al., 1981; Pegg et al., 1982; Foote et al., 1982). Despite considerable efforts in a number of laboratories, mammalian alkyltransferases have proved difficult to purify, mainly due to their thermo-lability. Only very recently has high yield purification been achieved (Gonzaga and Brent, 1989; Wilkinson et al., 1990), and the active site sequence determined (Rydberg et al., 1990a). Finally, the human gene has been cloned (Hayakawa et al., 1990; Rydberg et al., 1990b; Tano et al., 1990). The purified human alkyltransferase has a molecular weight of 22,000 daltons. A region of the human protein (residues 106-169) shows substantial similarity to regions of ogt (residues 103-163) and ada proteins (residues 285-345) which contain the acceptor cysteine molecule (figure 1.8). In fact the sequence -Pro-Cys-His-Arg-Val- is the same for all the

proteins, suggesting very strongly that cysteine 145 in the human enzyme is the methyl acceptor residue. The biochemical properties of the the mammalian alkyltransferase are very similar to these of the *ada* alkyltransferase although certain differences exist, that will be discussed in a following chapter.

The inducibility of the mammalian alkyltransferase, upon treatment with methylating agents, has been tested extensively. With the exception of rat liver and rat hepatoma cells where a 3-4 fold increase of the activity was observed, studies in other species and types of cells failed to demonstrate induction of the mammalian alkyltransferase (review by Pegg, 1990). Thus, this gene should be considered to be expressed constitutively like the *ogt* and not like the inducible *ada* gene.

Interferon inducers enhance the levels of alkyltransferase activity in rat liver (Bertini *et al.*, 1990), and Karran *et al.* (1990 a,b) have observed that the loss of alkyltransferase activity appears to be correlated with the loss of two other gene products (thymidine kinase and galactokinase) in human lymphoblastoid cell lines, indicating a coregulation of the three genes. It was shown by northern blotting that the thymidine kinase mRNA was still present in cells with low thymidine kinase activity. This suggests post-transcriptional regulation for this, and by extension, probably for the alkyltransferase and galactokinase genes. However, the mechanism underlying the regulation of the alkyltransferase gene expression and / or translation is not known and the availability of cDNA should assist further studies to be conducted towards this direction.

1.3.7.Variation in the expression-relevance in carcinogenesis

Alkyltransferase activity has been found in all mammalian species and in a wide variety of other vertebrates. However, the content of alklyltransferase varied considerably between different species, and cell type. Human tissues contain considerably higher amounts than the comparable rodent tissues (about 10 fold). Liver and spleen contain relatively the highest levels of alkyltransferase activity while the brain and the mammary gland the lowest (review by Montesano *et al.*, 1985).

It was mentioned in the previous section (chapter 1.2) that a good correlation exists

between alkyltransferase activity and the site of tumour formation in various animal model systems, but the association is not exact. The correlation is stronger when the variations of alkyltransferase activity within different cells of the same tissue are taken into consideration. For example, during chronic exposure of rats to low oral doses of dimethyl-hydrazine, hepatic tumours are induced, although this tissue has the highest levels of alkyltransferase and removes O^6-methylguanine even faster than the unexposed liver (Bedell *et al.*, 1982). However, the alklyltransferase activity is not distributed homogeneously in rat liver. There is 3-4 times lower activity in the nonparenchymal cells than in the hepatocytes, and this difference is even greater (12-fold) when the alkyltransferase is induced in the parenchymal cells [no induction is observed in the nonparenchymal cells] (Planche-Martel *et al*, 1985; Swenberg *et al.*, 1982). Using the regimen described above the hepatic tumours are mainly hemangioendotheliomas and angiosarcomas which are derived from nonparenchymal cells and not hepatomas derived from the parenchymal cells. Consequently, the alkyltransferase content of the non-parenchymal cells, rather than that of the whole tissue, is correlated with the hepatic tumour induction. Variations in alkyltransferase expression within different cell types have also been observed in other tissues. Belinsky *et al.* (1988) for example, have shown that there are significant differences in the levels of alkyltransferase present in different cell types isolated from the lung, with the Clara cells having particular low activities. Therefore, the levels of alkyltransferase activity as well as the amount of alkylated bases should be determined in samples of individual cell type and not in tissue homogenate. Separation of different cell population is not always possible with the conventional fractionation techniques, and studies on the heterogeneities in repair of complex tissues became possible, only after the development of immunocytochemical analysis (reviewed by den Engelse *et al.*, 1990). Fan *et al.* (1989) using antibodies against O^6-methylguanine, demonstrated that after a single high dose of dimethylnitrosamine, O^6-methylguanine accumulates in the renal cortex, particularly in the proximal tubules and mesenchymal cells which are the cell-targets for neoplastic transformation by this treatment regimen.

Immunocytochemical analysis is the most recent of the applications of antibodies

against O^6-alkylguanine or O^4-alkylthymine. Antibodies against these adducts have been previously used in various situations. Perhaps the most important of them is the detection of alkylated bases in human tissues (reviewed by Wild, 1990). Umbenhauer *et al.,* (1985) used Radio-Immuno-Assay (RIA) to detect O^6-methylguanine in the oesophagus DNA of individuals from the Linxian county of China. The levels of O^6-methylguanine in this high risk for oesophagus cancer group was found to be higher than the levels in a low risk group. Antibodies against O^4-ethylthymine were also used to demonstrate the presence of this adduct in human liver (Huh *et al*, 1989). Antibodies can also be applied in highly sensitive immunoassays for the detection of alkyltransferase activity in human biopsy samples even when very limited tissue sample is available (Souliotis and Kyrtopoulos, 1989). Recently, antibodies were raised against the alkyltransferase itself and used successfully for the determination of alkyltransferase present in very low amounts (Brent *et al.,* 1990). These applications are of significant importance since there are significant intraindividual variations of alkyltransferase activity in humans (Myrnes *et al.,* 1984; Montesano *et al*, 1985), and it is of interest to see if lower alkyltransferase activity and susceptibility to cancer are linked. Recently, Sagher *et al.* (1988) have measured the alkyltransferase activity in peripheral blood lymphocytes of patients that developed acute non-lymphocytic leukaemia after a procarbazine-based therapy for Hodgkin's disease. It is known that procarbazine is metabolized in different derivatives including active DNA methylating agents. Sagher *et al.* (1988) have observed decreased alkyltransferase activity in these patients relative to controls and suggested that O^6-methylguanine may accumulate to an increased extent during procarbazine treatment in individuals with low alkyltransferase levels. Such accumulation may increase the susceptibility of these individuals to the secondary therapy-related leukaemia. Souliotis *et al.* (1990) in support of this hypothesis have reported an increase of the O^6-methylguanine levels in patients treated with procarbazine which was also negatively correlated with the amount of alkyltransferase in the leucocytes. Fibroblasts from patients with lung cancer, considered to be prone to that disease, had lower alkyltransferase activity (6.64 pmols / 8×10^6 cell) than healthy controls

(10.35 pmols / 8 x 10^6 cells). It was therefore suggested that reduced capacity to repair O^6-alkylguanine may be an endogenously predisposing factor for lung cancer (Rudiger *et al.*, 1989).

1.3.8. Absence of alkyltransferase in some cultured cell lines

Mammalian cells deficient in alkyltransferase or with their levels experimentally depleted, are useful in studying the biological significance of O^6-alkylguanine and the role of the alkyltransferase in moderating the effects of alkylating agents. For example, by comparing cells with high levels of alkyltransferase with cells that are totally devoid of such activity for the mutation frequency, genetic recombination, or lethality induced by alkylating agents, one can determine if O^6-alkylguanine plays a significant role in inducing such events in the cells. A high proportion of human cell lines derived by viral transformation, and many human tumour cell lines, lack alkyltransferase activity and are extremely sensitive to the mutagenic and cytotoxic effects of the alkylating agents (reviewed by Day *et al.*, 1987). This phenotype which has been termed mer⁻ or mex⁻ was discovered independently in two laboratories (Day *et al.*, 1980; Sklar *et al.*, 1981). In contrast to the tumour cell lines which are commonly mer⁻ the human tumour material as a whole is mer⁺ (Wiestler *et al.*, 1984), suggesting that while the mer⁻ phenotype is associated with transformation, mer⁺ cells may became mer⁻ during propagation in vitro. The possibility that the mer⁻ cell lines are derived from a small subpopulation of mer⁻ cells of the tumour with a particular proliferating advantage in tissue culture, is less likely since a sample of lymphoblastoid cells derived from the same patient may show either mer⁺ or mer⁻ phenotypes when established in culture (Sklar and Strauss, 1983). Northern blotting failed to detect alkyltransferase mRNA in various mer⁻ cells indicating that the alkyltransferase activity is absent in these cells probably because of a silent gene, rather than any other posttranscriptional cause (Tano *et al.*, 1990).

1.3.9. Role of O⁶-alkylguanine in the cytotoxicity by alkylating agents

One method of identifying mer⁻ cells is based on their extreme sensitivity to the cytotoxic effects N-methyl-N-nitroso-N'-nitroguanidine (MNNG). This correlation between absence of O^6-methylguanine repair and cytotoxic response, strongly suggests that O^6-alkylguanine in addition to being a mutagenic base, is also a lethal lesion induced by alkylating agents. Extensive research had been done on that direction with often conflicting results. Domoradzki *et al.* (1984) compared the sensitivity to the toxic effects of N-methyl-N-nitroso-N'-nitroguanidine of 17 cell lines that differ significantly in the level of alkyltransferase activity. They observed an inverse correlation between the degree of cell killing, mutation frequency, and alkyltransferase activity. However, Ikenaga *et al.* (1987) in similar experiments failed to detect a quantitave relationship between N-methyl-N-nitroso-N'-nitroguanidine sensitivity and alkyltransferase cell context. The lethality of O^6-alkylguanine has been challenged even more by a number of studies on the basis of two distinct types of experiments.

1) The amount of alkyltransferase in mer⁺ cells can be depleted by growing the cells in the presence of free base O^6-methylguanine (Karran, 1985; Dolan *et al.*, 1985a). The free base is not incorporated into DNA but acts as a weak substrate for alkyltransferase. Depletion of the alkyltransferase by the free O^6-methylguanine dramatically increases the frequency of mutations induced N-methyl-N-nitroso-N'-nitroguanidine in mer⁺ cells confirming the major role of O^6-methylguanine in the mutagenesis caused by this agent (Domoradzki *et al.*, 1985). However, similar treatment with the free base either failed to sensitize mer⁺ cells to N-methyl-N-nitroso-N'-nitroguanidine (Karran and Williams, 1985), or the degree of sensitization was very small and incompatible with the almost complete depletion of the alkyltransferase (Dolan *et al.*, 1985b). These results could suggest that unrepaired O^6-methylguanine residues induced by N-methyl-N-nitroso-N'-nitroguanidine, are not particularly toxic. However, cell killing by alkylating agents such as N-methyl-N-nitrosourea and N-methyl-N-nitroso-N'-nitroguanidine (MNNG) is more likely to occur only after a certain period has elapsed after exposure and after a second round of DNA

replication (Maher *et al.*, 1990; Roberts and Basham, 1990). This lag time could be enough for cells with depleted alkyltransferase to resynthesize the enzyme and survive the potential lethal effects of O^6-methylguanine.

2) Another line of arguments against the contribution of O^6-alkylguanine in the cytotoxic effects of alkylating agents cames from the observation that MNNG-sensitive, alkyltransferase deficient cells could give rise to MNNG-resistant cells without simultaneously acquiring alkyltransferase activity (Scudiero *et al.*, 1984; Ishida and Takahashi, 1987; Samson and Linn, 1987). These mer - revertants are still hypermutable and produce a higher number of sister chromatid exchanges (SCE) upon treatment with alkylating agents. The very existence of these cell lines could be considered to be the proof of the dissociation of the resistance to alkylating agents and the repair of O^6-alkylguanine. However, mechanisms other than repair of a potential lethal lesion can permit the cells to survive.

The cytotoxic effects of O^6-alkylguanine and the importance of the alkyltransferase activity in the defence of mammalian cells have been elucidated in experiments where the whole or truncated *ada* gene was inserted into vectors driven by mammalian or viral promoters and expressed in mer - cells. Reduced cytotoxicity, mutagenicity, and sister chromatid exchange (SCE) inducing ability of alkylating agents was observed when the whole or the carboxy terminal coding domain of the *ada* gene was expressed (Brennand and Margison, 1986b; Samson *et al.*, 1986; White *et al.*, 1986). The alkylphosphotriester amino terminal coding domain did not have any significant effect in the cellular responses to alkylating agents (Brennand and Margison, 1986a; Hall *et al.*, 1988). Similarly *ada* gene expression in yeast confers increased resistance to the mutagenic and cytotoxic effects of N-methyl-N-nitroso-N'-nitroguanidine (Brozmanova *et al.*, 1990). Studies in which the *ada* gene product was placed in a mammalian expression vector under the control of glucocorticoid-inducible MMTV promoter, and transfected into HeLa cells, have been used to illustrate the importance of the expression of this activity in permitting survival from the toxic effects of N-methyl-N-nitroso-N'-nitroguanidine (Waldstein, 1990).

Following this evidence, O^6-alkylguanine has to be considered as a major cytotoxic lesion induced by alkylating agents. What is not known is the mechanism by which O^6-alkylguanine residues exert their cytotoxic effects and studying the MNNG-resistant, alkyltransferase deficient cell lines might possibly provide some answers. Roberts and Basham, (1990) proposed a mechanism for O^6-alkylguanine cytotoxicity that could explain the mer - revertant phenotype. According to Roberts and Basham's hypothesis base mispairing opposite O^6-alkylguanine adducts could be recognized by a mismatch repair system (Karran and Marinus, 1982), which removes the aberrant base in the newly replicated DNA leaving single-strand-breaks (Kalamegham et al., 1988). Persistence of such gaps would then be a block to DNA synthesis which would lead to mitotic arrest and cell killing. Absence of such a system, or presence of another system that could "repair" or tolerate the gaps would give rise to resistant cells.

1.3.10. Chloroethylating agents

While for many years there was a dispute over the cytotoxic effects of O^6-alkylguanine and the protective role of alkyltransferase against the lethal effects of monofunctional alkylating agents, there was never any doubt for similar protection against the action of the bifunctional chloroethylating agents. Chloroethylnitrosoureas are decomposed in aqueous solution into reactive species which can react with all four bases at the oxygen or nitrogen atoms producing chloroethyl- or hydroxymethyl- derivatives. After alkylation of DNA, the monoadducts are still reactive, and in a slow subsequent process heterocyclic rings and cross-linked bases are usually formed. A lot of the cross-links arise from O^6-chloroethylguanine, which first undergoes an intramolecular rearrangement to form O^6, N1 ethano-guanine (figure 1.9). This then reacts with the complementary cytosine on the opposite strand to form the ethano cross-linked N1-guanine-N3 cytosine. Cross-linked bases are especially important since it has been shown that formation of cross-linked DNA is directly correlated with the chloroethylnitrosoureas cell cytotoxicity (reviewed by Lundlum, 1990). Erickson et al. (1980) showed that in cells with high
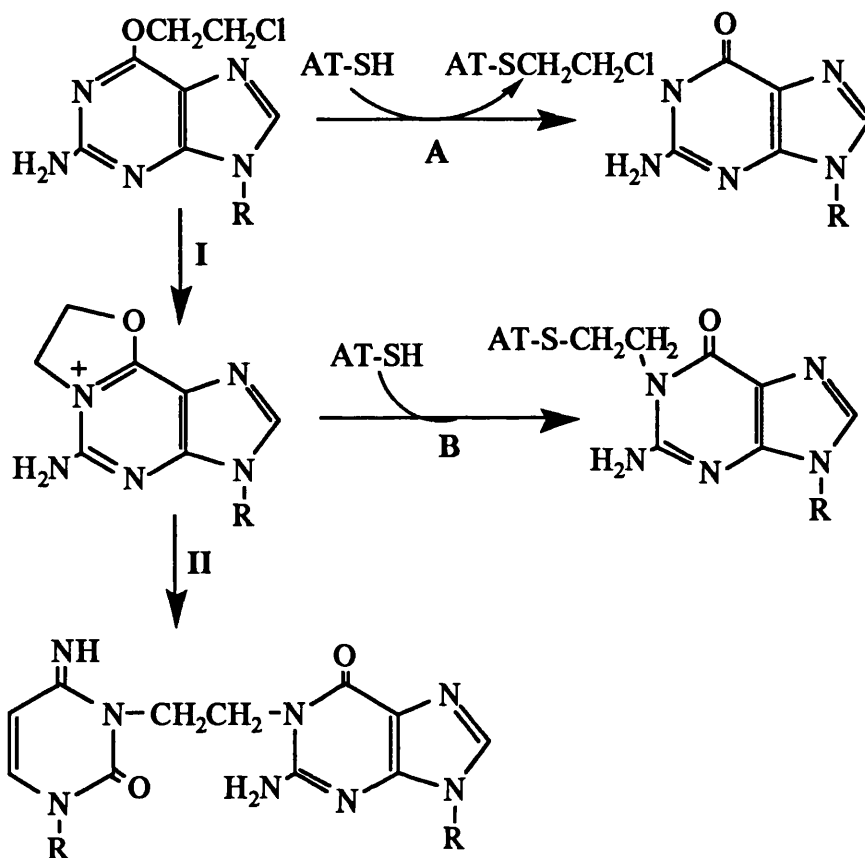
Figure 1.9: Mechanism for the formation of the dC-dG ethano cross-link, and reaction of

the O^6-alkylguanine-DNA-alkyltransferase (AT-SH) with O^6-chloroethylguanine (A),

and the derivative $N1,O^6$-ethanoguanine (B).

alkyltransferase activity (mer +) a challenge with chloroethylnitrosourea produce less

cross-links than cells deficient in this repair process (mer -). There is a quantitive correla-

tion between levels of alkyltransferase activity in different cells, cell killing, and sister

chromatide exchange induced by chloroethylating agents (Ikenaga et al., 1987; Schwartz et

al., 1989). Depletion of alkyltransferase by growing the cells in the presence of free base

O^6-methylguanine sensitize the cells significantly (Yarosh et al., 1986) and increases the

amount of the lethal cross-links (Dolan et al., 1989). The mechanism underlining this

protection involves the repair of O^6-chloroethylguanine by the alkyltransferase (Robins *et al.*, 1983) before the formation of the O^6, N1-ethanoguanine (figure 1.9). Recently it was shown that the enzyme can also react with O^6, N1-ethano-guanine in DNA. This reaction leads to the alkyltransferase becoming covalently linked to the DNA, presumably by the ethano group attached at the other end to the N1 position of guanine (Brent *et al.*, 1987a; Brent *et al.*, 1987b; Brent and Remack, 1988). However the biological significance of this reaction is unknown.

The cytotoxicity of chloroethylating agents is of clinical interest since these agents are used in chemotherapy, although with low effectiveness. Brent *et al.* (1985) showed that the sensitivity of tumours to chloroethylnitrosourea, grown as xenographs in mice, is directly correlated to the levels of alkyltransferase activity of the tumour cells. This observation suggests that the clinical effectiveness of the chloroethylating agents could be improved by administration of appropriate drugs that will aim at the reduction of the alkyltransferase. A combined treatment of methylating agent (to deplete the alkyltransferase) and chloroethylnitrosoureas could be effective but the long term hazardous and carcinogenic effects of the methylating agents is a significant drawback.The limited solubility and uptake of O^6-methylguanine, combined with its low potency prevent the use of this compound to reduce alkyltransferase in intact animals.   Recently, Dolan *et al.* (1990) found that O^6-benzylguanine is a vastly better inactivator of the alkyltransferase than O^6-methylguanine. Exposure of alkyltransferase in vitro with O^6-benzylguanine decreased the alkyltransferase activity faster, and in a concentration 100 times lower than O^6-methylguanine. Similar differences were observed in cultured cells and treated mice. The inability of the alkyltransferase to repair S^6-thiomethylguanine (Yarosh *et al.*, 1986) may provide an alternative approach to enhance the effectiveness of chloroethylnitrosoureas. Pretreatment of growing cells with 6-thio-guanine prior to exposure to these agents increases their cytotoxicity (Bodell, 1986).

1.3.11. Excision repair of O⁶-alkylguanine

Bulky DNA adducts (T-T dimers, 2-acetyl-aminofluorene-guanine) are repaired in both eukaryotes and prokaryotes by an excision repair mechanism. For example in E. coli the uvrABC excision repair system cuts the DNA strand containing the DNA adduct both upstream and downstream of the adduct, and the small fragment of DNA containing the DNA lesion is excised. The remaining intact strand acts as a template for synthesis of new DNA refilling the gap, and subsequently ligated to complete the repair process. (reviewed by Van Houten, 1990). Although O⁶-alkylguanine in DNA is generally considered to be repaired mainly by the alkyltransferase, this adduct is also recognized by the excision repair mechanism. Warren and Lawley (1980) first demonstrated that the uvr system of E. coli is responsible for O⁶- ethylguanine repair in vivo. Different human cell lines show a good correlation between sensitivity and endogenous levels of alkyltransferase activity after treatment with methylating but not ethylating agents. However, in the latter situation the relationship is restored when both repair activities (alkyltransferase and excision repair capacity) are taken into consideration, indicating that O⁶- ethylguanine is repaired by both systems (reviewed by Maher *et al*, 1990). Similarly the removal, as monitored by the use of specific antibodies, of other O⁶- alkylguanine adducts from the DNA of both E.coli and human cell lines is dependent upon the capacity of the cells for excision repair. The relative contribution of this repair system increases with the size of the alkyl group. (Boyle *et al.*, 1986; Boyle *et al.*, 1987; Samson *et al.*, 1988). Recently, DNA footprint studies have shown that the uvrABC proteins also recognize O⁶-methylguanine residues and incise DNA in the same way as they do for bulkier adducts, although with reduced efficiency (Van Houten and Sancar, 1987; Voigt *et al.*, 1989).

Under condition of low or moderate DNA alkylation it seems rather unlikely that excision repair of O⁶-methylguanine plays an important role in the protection of the cells. However as the level of DNA methylation increases and the alkyltransferase repair system becomes saturated, or when higher alkylating agents are used, excision repair might play a major role in the repair of this highly mutagenic and carcinogenic DNA lesion.

1.4. RAS ONCOGENES AND THEIR IMPLICATION TO CARCINOGENESIS

1.4.1. Structure, properties and function of *ras* proteins

It was long believed that genesis of cancer is associated with alterations or damage of the genetic material of the cell. Evidence that gave substance to this hypothesis came from diverse roots but only recently the genes, targets for oncogenic alterations, have been identified. These genes are cellular genes (proto-oncogenes), highly conserved during evolution, and vital for some essential functions of the cell, possibly proliferation and/or differentiation. However several events could convert (activate) these otherwise normal genes to potential tumorigenic genes (c-oncogenes), either by affecting their expression or the functions of the encoded proteins. (Recent reviews by Bishop, 1987; Freeman *et al.*, 1989).

Among the oncogenes, *ras* genes have been the focus of intense research since, a variety of human tumours (reviewed by Bos, 1989) and most of the carcinogen induced animal tumours (reviewed by Balmain and Brown, 1988) contain activated *ras* genes. These oncogenes have been highly conserved in both sequence and function, in species as disparate in the evolution ladder as man, fruit flies, plants, and even yeasts. Three *ras* genes have been identified in the mammalian genome designated as H-*ras*-1, K-*ras*-2, and N-*ras* and they code for highly related proteins generically known as p21 (reviewed by Barbacid, 1987). *Ras* genes are invariably expressed in all cells, although significant differences exist in the levels of their expression between different tissues, and in the same tissue at different times during development (Leon et al, 1987).

Mammalian *ras* genes acquire transforming properties in NIH 3T3 cells by single point mutations within their coding sequence. The locations of the mutations have been identified either in naturally occurring oncogenes or using in vitro mutagenesis techniques. Changes in codons 12, 13, 59, 61, 63, 116, and 119 give transforming properties to the *ras* oncogenes. In particular substitution of Gly[12] and Gln [61] by any other amino acid (except Pro[12] , Pro[61] and Glu [61]) results in the oncogenic activation of these molecules (Seeburg *et al.*, 1984; Der *et al.*, 1986).

All *ras* proteins have been shown to bind guanine nucleotides (GTP and GDP) and

possess intrinsic GTPase activity. The indegrity of these activities is essential for the biological function of the p21 proteins, and reduced GTPase activity is a common characteristic of mutated proteins encoded by activated *ras* oncogenes. X-ray crystallography of the purified p21 protein revealed the regions involved in nucleotide binding (Pai *et al.*, 1990). The amino acids Gly[12], Glu[61], Asn[116], Asp[119], are essential for the interaction of the protein with the nucleotide, the GTP hydrolysis, and the proper folding of the protein. Thus, it is not surprising that substitution or deletion of these amino acids confers the otherwise normal proteins with transforming properties.

The *ras* proteins, as all the guanine nucleotide binding proteins, are believed to be involved in signal transduction and they act as molecular switches. In the OFF or inactive state, they are complexed to guanosine diphosphate (GDP). In the ON or active state they are complexed to guanosine triphosphate (GTP). Most of the molecules in a cell are in the inactive state until they receive a stimulus from another protein which results in the exchange of GDP for GTP followed by a conformational change of the protein in its active form. The activated protein subsequently stimulates the action of another downstream element of the signal transduction pathway (effector molecule). The lifetime of this interaction is regulated by either the effector mediated or the intrinsic GTPase activity, which would catalyse the GTP hydrolysis and inactivate the protein. The activating mutations by inhibiting the GTPase activity, stabilize the active state of the protein. How a shift of the equilibrium in favour of the active form alters different cell functions, and subsequently leads to cell transformation, is not known. Despite extensive research, the signals that induce activation of the *ras* proteins and the downstream elements of the putative pathway remain unclear. The location of the *ras* proteins in the inner surface of the membrane and their structural and functional similarity to the G proteins (Hurley *et al.*, 1984), have raise the possibility that *ras* proteins may participate in the adenylate cyclase signal transduction pathway (like the Gs protein) or the phosphatidylinositol pathway (like G_p protein). Experiments have shown that *ras* p21 proteins, with the unique exemption of the S. cerevisiae protein *RAS2*, are not linked functionally with the adenylate cyclase system (Beckner *et al.*, 1985). A glucocorticoid inducible N-*ras* proto-oncogene however, was found to stimulate, when expressed, the accumulation of the inositol

triphosphate (IP_3), an intermediate in the phosphatidylinositol pathway, indicating that the p21

proteins might be involved in the latter pathway (Wakelam *et al.,* 1986).

Activation of the *ras* oncoprotein leads to transformation-specific phenotypes which are

characterized by uncontrolled cell division.Thus, it was suggested that *ras* proteins participate

in the transduction of a signal for cell proliferation. The first indications for a role of *ras*

proteins in cell proliferation came from microinjection studies using the Y13-259 monoclonal

antibody, capable of recognizing all known *ras* proteins. Microinjection of this antibody into

the cytoplasm of *ras* transformed NIH 3T3 cells prevent them from entering the S phase

(Macalhy *et al.,* 1985). Although more evidence is accumulating suggesting this hypothesis

(Barbacid, 1987), the level of *ras* expression is not always correlated with the state of

proliferation of different tissues and some terminally differentiated cells often express high

levels of p21 proteins (Chesa *et al.,* 1987).

1.4.2. *Ras* activation in chemical carcinogenesis

The first direct implication of *ras* activation in chemical carcinogenesis came in 1983

from Barbacid and colleagues (Sukumar *et al.,* 1983). N-methyl-N-nitrosourea induces

exclusively mammary carcinomas in female rats, when injected with a single dose of this

carcinogen during puberty. Sucumar *et al.* (1983) showed that 90% of these tumours contain

activated H-*ras* genes capable of transforming NIH 3T3 cells, and in all cases, the activation

occurs via a single point mutation G to A of the second G of the -GGA- codon 12. The same

type of rat tumours are also induced by another carcinogen 7,12-dimethyl-benz[a]anthracene

(DMBA) and 20% of these tumours were found to contain activated H-*ras* genes. However,

in this case activation does not involve codon 12 but point mutations in either of the two As in

codon 61 (Zarbl *et al.,* 1985). Because the only difference between the two groups of tumours

was the initiating carcinogen used, the activating mutations in the H-*ras* gene must be the result

of the mutagenic effects of the carcinogen and not preexisting in a subpopulation of cells that

acquired overgrowth properties after the carcinogen treatment. Further analyses of activated

ras oncogenes in various chemically induced tumours showed that the activating mutation

always reflects the known DNA-binding, and mutagenic characteristics of each carcinogen and

Table 1.2: Some chemically induced animal tumours. (Data is from Balmain and Brown, 1988; Topal, 1988, Belinsky *et al.*, 1989, Wang *et al.*, 1990).

Carcinogen	tumour	gene	codon	mutation	occurrence
	RAT				
MNU	mammary	H-ras	codon 12	GGA to GAA	61/61
NBMA	oesophagus	H-ras	codon 12	GGA to GAA	18/18
DMBA	mammary	H-ras	codon 61	CAA to CNA	
	MOUSE				
MNU	thymus	K-ras	codon 12	GGT to GAT	5/18
			codon 13	GGC to GAC	1/18
	T-lymphoma	K, N-ras	codon 12	GGA to GAA	85%
MNNG	skin papilloma	H-ras	codon 12	GGA to GAA	11/11
NDEA	hepatoma	H-ras	codon 61	CAA to AAA	6/16
				CAA to CTA	4/16
				CAA to CGA	3/16
NNK	A/J lung	K-ras	codon 12	GGT to GAA	7/11
			codon 61	CAA to CGA	2/11
NDMA	A/J lung	K-ras	codon 12	GGT to GAA	7/10
			codon 61	CAA to CGA	3/10
DMBA	skin carcinoma	H-ras	codon 61	CAA to CTA	45/45
γ –rays	thymic	K-ras	codon 61	CAA to AAA	3/4

thus, is caused by and not preexisting the carcinogen treatment (table 1.2). In particular, the vast majority of *ras* oncogenes isolated from N-nitroso compounds-induced tumours, are activated by the same G^{35} to A^{35} transition mutation. This type of mutation would be expected to arise if O^6-alkylguanine was produced at this position and misreplicated by DNA polymerase. Thus, O^6-alkylguanine formation might be the crucial initiating step in *ras* activation and carcinogenesis in these animal model systems.

In A/J mouse lung tumours induced by NNK and dimethylnitrosamine a minor activation pathway of the K-*ras* oncogene involves A:T to G:C transitions in codon 61. This result could implicate O^4-alkylthymine formation in activation of the *ras* oncogenes, since such transitions would be expected from the miscoding properties of this DNA adduct (Belinsky *et al.*, 1989).

1.4.3. Tissue specific gene activation

It can be seen from table 1.2 that various animal model systems differ not only in the activating mutation, but also in the type of *ras* gene which is activated. The latter might reflect a different susceptibility of the genes to the electrophilic attack of the carcinogens depending upon the chromosomal structure surrounding each family member. Alternatively, mutation in one *ras* gene could be silent due to luck of external stimulus, while mutation in an other family member that is active in signal transduction could lead to cell transformation. When rats are treated during sexual development with N-methyl-N-nitrosourea, they develop mammary carcinomas with a high proportion of activated H-*ras* genes (Sukumar *et al.*, 1983). When the treatment is carried out during fetal development, adult rats develop gliomas and schwannomas that have an activated *neu* gene (Barbacid *et al.*, 1986). When the treatment is carried out at 2 weeks old rats, the mammary carcinomas contain activated H- *ras* oncogene as well as a significant proportion of activated N-*ras* oncogene (Kumar *et al.*, 1990). The variation in tumour type and pattern of oncogene activation as a function of the time of carcinogen treatment, clearly indicates that other factors, hormonal status or the differentiation state of the target tissue, are important in determining the specificity of *ras* activation.

1.4.4. *Ras* activation in multistage carcinogenesis

The hypothesis that *ras* gene activation is involved in the very early stages of carcinogenesis was initially suggested by the finding that such activated genes are present in preneopastic skin papillomas produced by DMBA and phorbol ester treatment (Quintanilla *et al.*, 1986). Similar observations were made in the rat mammary system. Injection of N-methyl-N-nitrosourea into 2 day old female rats results in the development of mammary tumours in 80% of the animals with a mean latency of 3.7 months. Activated H- and N-*ras* genes were found in both the tumour cells, and in mammary glands isolated two weeks after N-methyl-N-nitrosourea injection, which is long before the histological manifestation of preneoplasia or cancerous growth (Kumar *et al.*, 1990). Mouse mammary epithelial cells, treated in vitro with N-methyl-N-nitrosourea and transplanted back into mice, give rise to hyperplastic alveolar nodules and subsequently mammary tumours both of which contain activated K-*ras* genes [G^{35} to A^{35}] (Miyamoto *et al.* 1990). All these studies strongly support the causative role of *ras* activation in tumourigenesis.

Ras activation is not by itself sufficient for the development of the malignant phenotype and additional events are needed that probably stimulate the cellular proliferation and/or differentiation. The stimulus for these events could be an exogenously applied compound (12-O-tetradecanoylphorbol 13-acetate [TPA] promoter in the mouse skin system) or an endogenous growth promoter such as a steroid hormone. A good example for the latter situation has been observed in the rat mammary system, where the tumour development is directly correlated with sexual maturity. When rats were treated neonatally with N-methyl-N-nitrosourea and ovariectomized before reaching adulthood the incidence of mammary tumours is diminished (from 80% to 4%). However, when ovariectomized rats are treated with oestrogen after they reached adulthood, they develop tumours in high frequency (Sukumar and Barbacid, 1990).

Two step carcinogenesis with one step involving *ras* activation, and the other one being also a genetic event has been suggested by transformation experiments of primary rodent fibroblasts. Transformation of these cells cannot be accomplished by transfection with *ras* oncogenes alone and normally a second oncogene (*myc, myb*) has to be supplied (Land *et al.*,

1983). In some cases a second epigenetic or genetic event is not necessary for tumour induction. For example, transgenic mice which harbour an activated H-*ras* oncogene under the control of the pancreas specific elastase I promoter, develop pancreatic tumours directly after the onset of elastase gene expression (Quaife *et al.*, 1987). However, in these experiments all pancreatic cells express the H-*ras* oncogenes, while under physiological conditions, the initiated cells are surrounded by normal cells. It is known that normal cells inhibit the growth of tumorigenic cells, probably through gap junction communication, and it was suggested that promoters may act as inhibitors of the gap junction communication of normal cells allowing the growth of the neoplastic cells. Such a promoter function would not be required if all the cells of a tissue were abnormal and potentially tumorigenic, which is the case in the pancreatic tumour model described above.

1.4.5. *Ras* activation and human cancer

Since most of the chemically induced tumours in animals contain activated *ras* oncogenes, investigators focused their attempts to correlate human tumour induction and *ras* activation (reviewed by Bos, 1989). Activating mutations in *ras* oncogenes are present in a variety of human tumours, although the frequency and the type of the *ras* gene activated varies strongly among different tumours (table 1.3). The highest incidence is found in the exocrine pancreas (90%) and all activating mutations occur in codon 12 of the K-*ras* gene. K-*ras* oncogenes are also present in a lot of the colon tumours (47%) and mutations are mainly G to A transitions at the 12th and 13th codon. In lymphoid malignancies N-*ras* is activated by G to A mutations in codons 12 and 13 only in acute lymphoblastic leukaemias (18%), but no activation of K-, N-, and H-*ras* is seen in non-Hodgkins lymphomas or chronic leucocyte leukaemias (Neri *et al.*, 1988). Similar differences in *ras* activation are observed in non-small cell lung carcinomas. Activation of K-*ras* oncogenes occurs in adenomas but not in epidermoid carcinomas and large cells carcinomas. Finally, no activated *ras* genes were observed in stomach or oesophagus tumours. Overall about 15% of the humans tumours examined contained *ras* oncogenes, suggesting an important role of these genes in the development of human cancer. The type of mutations found might even provide information about the type of

Table 1.3: Incidence of ras activation in human cancer. data is from Boss, 1989 and Neri *et al*, 1988.

Tumour	ras activation	incidence	mutation
Pancreas : adenocarcinoma	K ras codon 12	28/30	18/28 G to A
			9/28 G to T
Colon: adenocarcinoma	K ras codon 12 and 13	60/119	30/60 G to A
			29/60 G to T
Lung : adenocarcinoma	K ras codon 12	14/45	9/14 G to A
			4/14 G to T
melanomas	N-ras	7/37	
Lymphoid disorders			
ALL	N-ras	6/33	
NHL		0/78	
CLL		0/51	
Oesophagus		0/25	
stomach		0/26	

mutagen involved in the induction of the mutations and consequently in the induction of the tumour.

However, in cases where no *ras* oncogene is detected other genetic events of unknown origin might be involved, which ultimately have the same effect with *ras* activation. Such events could be alterations in the tumour suppressor genes (see Freeman *et al*, 1990). Oesophageal cancer for example, in which exposure to nitrosamines has been suggested as a causative factor, appears to involve in some cases alterations in the P53 gene. One third of the oesophageal tumours examined, have point mutations, mainly G to A at dispersed sites in the 5th-8th exons, which probably inactivate the tumour suppressor function of that gene (Hollstein *et al.*, 1990).

CHAPTER   2

SYNTHESIS AND PURIFICATION OF OLIGONUCLEOTIDES

2.1. INTRODUCTION

Advances of the past decade in molecular biology and biochemistry would not be so impressive without the parallel advances in nucleic acid chemistry that made the automatic chemical synthesis of oligonucleotides a routine procedure. Following the developments of methods for the synthesis of normal DNA, reliable methods for synthesis of modified oligonucleotides are now emerging.

Modified bases are often generated as a result of physiological post-replication modifications (e.g. 5me-C), or the action of chemicals and radiation. Chemical carcinogens attack DNA in various positions and N-nitroso-compounds in particular react with DNA in no less than 12 positions (table 1.1). Thus, assessment of the genetic and structural effects of an individual adduct becomes a very difficult task. Ingenious techniques had been evolved to study these effects, either by treatment of a normal oligonucleotide with a carcinogen, or by forcing a modified base into a growing DNA strand by polymerases (see chapter 1.2.4). There is however, a great advantage in total synthesis of oligonucleotides containing a single modified base in a pre-selected position. Using such synthetic oligomers, the impact of an individual adduct on the DNA structure, the contribution of each adduct to the spectrum of mutations induced by chemicals, the relative ability of DNA adducts to act as cytotoxic lesions, and the relative efficiency with which different adducts are removed by repair enzymes can be directly examined. None of the studies in this thesis could have been possible without the availability of synthetic oligonucleotides containing O^6-alkylguanine or O^4-alkylthymine.

The process of synthesis and purification of the oligomers used in this thesis is summarized in figure 2.1.
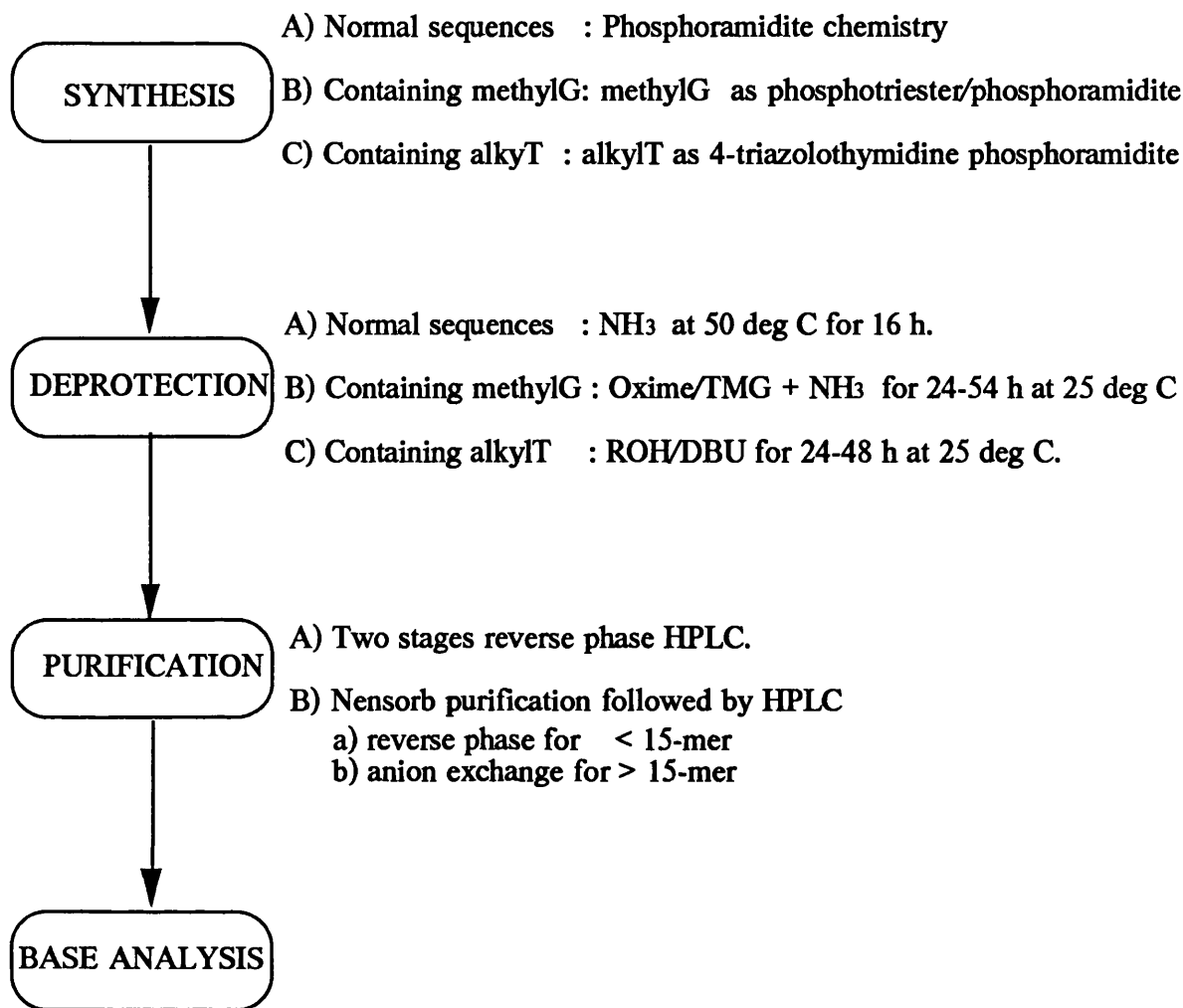
Figure 2.1: Schematic representation of the synthesis and purification of normal and

modified oligomers; TMG : N1, N1, N3, N3 tetramethylguanidine; ROH : methanol

or ethanol; DBU : 1, 8-diazabicyclo-[5.4.0]-undec-7-ene

2.2. MATERIALS AND METHODS

2.2.1. Chemicals and enzymes

Oligonucleotides were synthesized on a Cruachem PS 200 automatic DNA synthesizer using 2-cyanoethyl phosphoramidite chemistry. The CPG-linked monomers and the chemicals used on the synthesizer were obtained from Cruachem (Glasgow, Scotland). The 2-cyanoethyl phosphoramidites were either from Cruachem or Pharmacia (figure 2.2). Anhydrous methanol (99+% Gold label) was from Aldrich. Absolute ethanol (99.7 % Analar) and aqueous ammonia (sp. gr. 0.88 Aristar) were from BDH. Acetonitrile (HPLC grade) was from Rathburn. All other chemicals were obtained from Aldrich or Sigma. Phosphodiesterase and alkaline phosphate were both supplied from Boehringer-Mannheim.

Particular care was taken to ensure that chemicals used in the DNA synthesis were free of water. Water reacts with the activated monomers and hence, the water content of reagents and solvents should be kept as low as possible in order to optimize the overall yield of DNA synthesis. Thus, anhydrous solvents were used, molecular sieve 4 Å was added to them at least one day before use, and the water content was checked by Karl-Fischer titration. The acetonitrile used for the monomers and tetrazole solution had < 50 ppm water and the other solvents < 200 ppm water. The monomer and tetrazole solutions were prepared under N_2 by injecting (dried syringe and needle were used) the appropriate amount of acetonitrile in the sealed manufacturers' vials containing the reagents.

2.2.2. Synthesis of oligonucleotides containing only normal bases

DNA synthesis starts from the 3' nucleotide which is attached to controlled-pore glass (CPG) with a long spacer chain, and prepacked in small disposable column. The CPG-nucleotide and the other monomers have all their nucleophilic centres, not involved in the formation of the 3'-5' phosphodiester bond, protected with groups (permanent protecting groups), which remain stable throughout the synthesis. The 5'- OH is protected
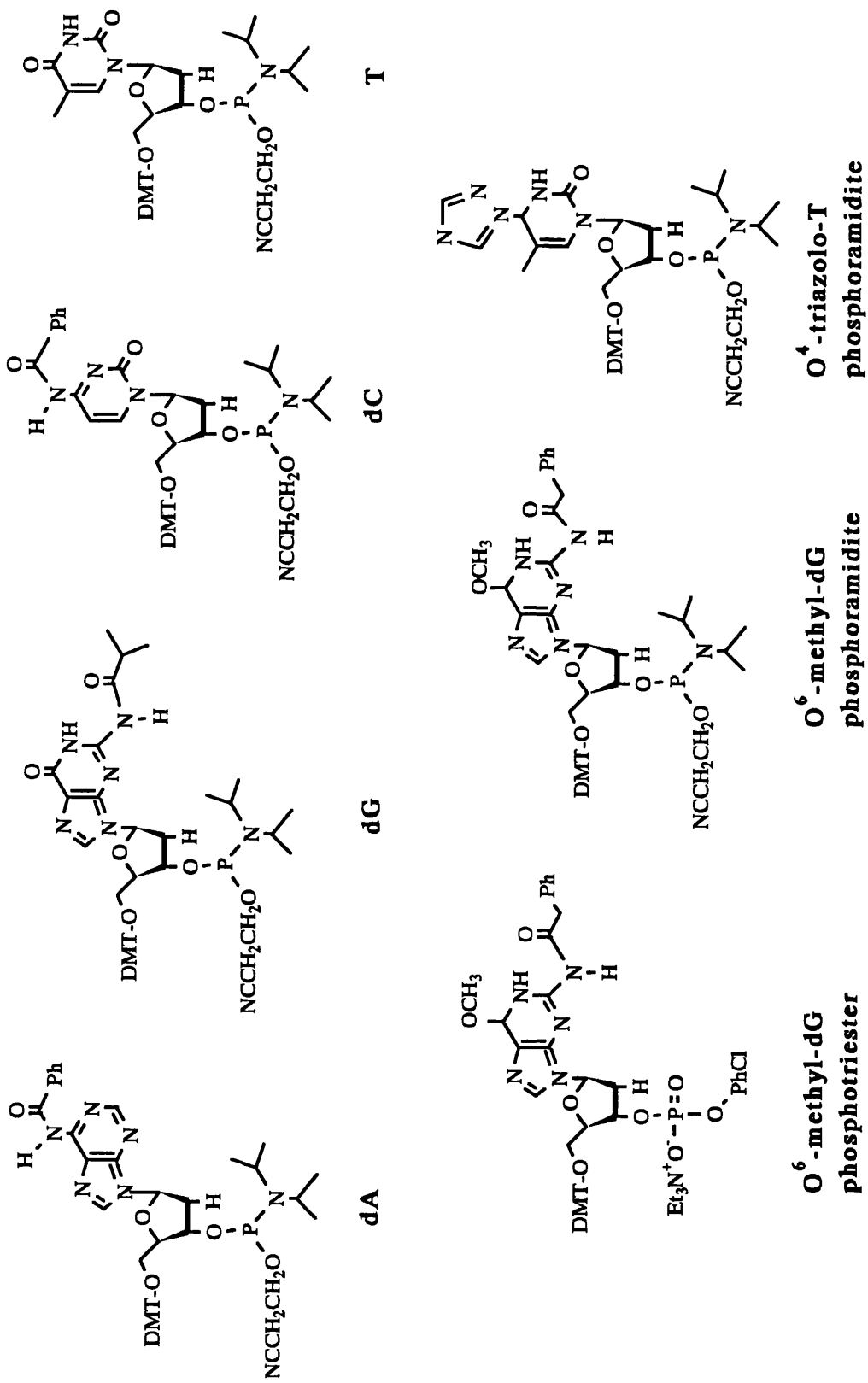
Figure 2.2 : Protected natural and modified monomers used in DNA synthesis.

by dimethoxy-triphenylmethyl group [DMT] which is removed after each coupling step (temporary protecting group) to enable the formation of the next phosphodiester bond. The basic steps involved in each cycle of nucleotide addition with the phosphoramidite chemistry are:

1) Removal of the dimethoxytrityl group (DMT) from the last nucleotide in the growing DNA chain. This is accomplished with dichloroacetic acid in dichloroethane (3% v/v).

2) Activation of the phosphoramidite monomer with tetrazole, and its addition to the growing chain.

3) Esterification of the unreacted 5'- OH by acetic anhydride (acetic anhydride: 2,6 lutidine: tetrahydrofuran [1:1:9]), and N- methylimidazole [4.4% v/v in tetrahydrofuran] (capping reaction). This step is designed to block chains that have not reacted in the last coupling reaction.

4) Oxidation of the phosphite to the phosphotriester with 0.1 M I_2 in tetrahydrofuran: pyridine: water [40:9:1].

The yield of each coupling reaction was assessed by measuring the amount of the 5' DMT protecting group released by dichloroacetic acid. The dichloroacetic acid wash containing the released dimethoxytrityl cation was collected, a sample mixed with 0.1 M p-toluenesulphonic acid in acetonitrile, and the absorbance read at 495 nm. The amount of the cation released was calculated assuming a molar extinction coefficient of 70,000.

After synthesis was completed the final DMT was left on the oligomer in order to assist in separation of it from the failure sequences. The column was removed from the synthesizer, the glass support was dried under vacuum and transferred to a screw-capped micro-vial. One ml of ammonium hydroxide (specific gravity 0.88) was added, the vial was tightly sealed and incubated at 50 °C for 16 hours. The ammonia treatment cleaves the oligonucleotide from the solid support, and removes the protecting groups from both the phosphate and nucleosides.

2.2.3. Synthesis of oligonucleotides containing O^6-methylguanine

The O^6-methylguanine phosphotriester monomer (figure 2.2) was prepared by Dr

C.A. Smith according to published procedure (Li and Swann 1989). The

O^6-methylguanine phosphoramidite (figure 2.2) was prepared by Dr Y-Z Xu (Smith *et al.*,

1990). The portion of the oligonucleotide 3' to the O^6-alkylguanine was automatically

synthesized in the DNA synthesizer as described above. The final DMT was removed by

dichloroacetic acid and, from its absorbance at 495 nm, the quantity of the free 5' hydroxyl

groups available for the reaction with O^6-alkylguanine monomer was calculated. Initially

the O^6-alkylguanine phosphotriester monomer was used, because it is more stable than the

phosphoramidite under storage conditions, but subsequently the phosphoramidite monomer

was made, and preferred over the phosphotriester because of the speed and simplicity of

the process of its incorporation to the oligonucleotide.

A. Addition of O^6-alkylguanine by phosphotriester chemistry

After synthesis of the 3' end of the sequence, the column was removed from the

DNA synthesizer and attached to a valve connected to four reagent bottles pressurized (2

p.s.i) with helium. The CPG-glass support was first washed with double-distilled pyridine

(over 2-mesitylenesulphonyl chloride and potassium hydroxide). The O^6-alkylguanine

monomer (15 equivalents to the amount of DMT released from the previous step) was

placed in a conical glass vial with septum top (Wheaton reacti-vial). In another septum top

vial a solution of 1-(mesitylene-sulphonyl)-3-nitro-1,2,4,triazole (MSNT) [75 equivalents]

in pyridine (0.1 ml) was made. Using a gas-tight syringe with a side-hole needle, and

under N_2, the pyridine solution was injected to the vial containing the O^6-alkylguanine

monomer and mixed thoroughly for 5 min. The solution was injected slowly into the

column using a dry gas-tight syringe and drawn in and out of the column several times.

The reaction was allowed to proceed for 1 hour. The column was then washed with

pyridine (10 ml), reconnected to the synthesizer and run through the capping cycle to block

any DNA chains that had not reacted with O^6-alkylguanine. The column was returned to the

manual solvent delivery system and washed first with 15 ml of anhydrous CH_2Cl_2/

propan-2-ol (85:15 v/v) and then with anhydrous $ZnBr_2$ (1M in CH_2Cl_2/propan-2-ol,

85:15 v/v) to remove the DMT protecting group of the O^6-alkylguanine. The CH_2Cl_2/

propan-2-ol and $ZnBr_2$ wash was repeated several times (each time the column was left in

the $ZnBr_2$ solution for 3-7 minutes) until no more colour was formed in the column during

the $ZnBr_2$ wash. In early experiments the Lewis acid $ZnBr_2$ was preferred over the

commonly used protic acids (dichloroacetic acid, trichloroacetic acid) because in strong

acidic environment the O^6-alkylguanine with free 5' OH might be depurinated (Li and

Swann, 1989). However, subsequent experiments have shown that this precaution was

unnecessary since O^6-alkylguanine is sufficiently stable in acidic solution, in the short-lived

exposure conditions used in the DNA synthesizer (Smith *et al.*,1990).

The column was then washed with 10 ml of CH_2Cl_2/propan-2-ol and 10 ml of 1

M aqueous ammonium acetate and reconnected to the synthesizer. The remaining sequence

was synthesized automatically. The final DMT protecting group was left on the oligomer at

the end of the synthesis to aid the subsequent purification of the oligomer.

B. Addition of O^6-alkylguanine by phosphoramidite chemistry

The phosphoramidite (10 equivalents to the amount of DMT released after the previous

coupling) was dissolved under N_2 in 0.1 ml of anhydrous acetonitrile and 0.1 ml tetrazole

(0.5 M) in acetonitrile in a septum top glass vial. The column containing the 3' end of the

sequence was dried by passing helium through it for a couple of minutes. The bottom end

of the column was disconnected from the synthesizer and the O^6-alkylguanine/tetrazole

mixture was injected slowly into the column using a gas-tight syringe. The mixture was

drawn very slowly in and out of the column over a period of 3 minutes and the column was

reconnected to the synthesizer to complete the synthesis.

2.2.4. Deprotection of oligonucleotides containing O^6-methylguanine

A. O^6-alkylguanine added as phosphotriester

The CPG support was dried in vacuo, removed from the column and transferred in

a screw-capped micro-vial. A solution of 0.3M (E)-2 nitrobenzaldoxime and 0.27 M N1,

N1, N3, N3, -tetramethylguanidine (TMG) in water/acetonitrile (70/30 v/v) was added (1 ml), and left at room temperature for 24 hours. The oxime/TMG mixture removes the 2-chlorophenyl groups protecting the phosphate of O^6-alkylguanine. In addition, this treatment cleaves the oligonucleotide from the solid support and removes the 2-cyanoethyl groups protecting the other phosphates. The solvent was then removed in vacuo, and ammonium hydroxide (2 ml, sp. gr. 0.88) was added and left at room temperature for 30 hours. During this treatment all the remaining protecting groups, including the N^2 -phenylacetyl group of O^6-alkylguanine are removed. The ammonia was then evaporated in vacuo. Water (10 ml) was added to the remaining oily material and the pH adjusted to 6.5 with a few drops of acetic acid. After this treatment the solution was discoloured and a white precipitate of oxime appeared. The aqueous solution was placed in a separating funnel and extracted 4-6 times with 10 ml diethylether to remove the remaining oxime and TMG. The aqueous solution was collected in a round-bottom flask and evaporated to dryness in vacuo.

B. O^6-alkylguanine added as phosphoramidite

The CPG support was removed from the column, put in a screw-capped micro-vial and 0.075 M (E) -2- nitrobenzaldoxime and 0.0625 M TMG dissolved in 2 ml aqueous ammonia solution (sp. gr. 0.88) was added and left at room temperature for 30 hours. The ammonia was then evaporated under reduced pressure.

The necessity of ether extraction, either when O^6-alkylguanine was added as a phosphotriester or phosphoramidite, was dependent on the method of purification. If a two stage HPLC purification was to be used (i.e. first isolation of the oligomer still containing the DMT by reverse-phase HPLC, followed by removal of the DMT and a second purification by HPLC), complete removal of the oxime/TMG mixture before purification was essential. This is because the oxime/TMG mixture gave an A_{260} absorbing peak, very close to that of the DMT-protected oligomer. However, extraction was not necessary when a Nensorb cartridge was to be used since the oxime/TMG could be removed from the

cartridge together with the failure sequences (see below in purification procedure).

2.2.5. Synthesis and deprotection of oligonucleotides containing
O^4-alkylthymine

The method for DNA synthesis was similar to that when O^6-alkylguanine phosphoramidite was introduced to oligomers. In brief, the sequence 3' to the modified base was synthesized automatically, the bottom end of the column was disconnected and the activated modified monomer was injected manually into the column. The column was then reconnected to the synthesizer to complete the synthesis.

The characteristic however, of this synthesis was that the monomer was not an O^4-alkylthymine phosphoramidite but an O^4-triazolothymine phosphoramidite which, after DNA synthesis, was converted to a number of O^4-substituted nucleotides, using different deprotection reagents. Another difference from previous synthesis was that the monomers of the normal bases were "PAC" amidites from Pharmacia. The "PAC" monomers are protected with phenoxyacetyl on the amino groups of adenine and guanine and isobutyryl on the amino group of cytosine. These groups are very easily removed and the oligomer is released from the support and deprotected using very mild deprotection conditions (i.e. alkoxide ions at room temperature, for a relative short time).

After synthesis, the CPG-support bearing the O^4-triazolothymine oligomer was divided into two equal fractions, and placed in screw-capped micro-vials. In the vials either 1 ml of methanol / 1,8 -diazabicyclo-[5.4.0]-undec-7-ene (DBU) [9:1 v/v] or ethanol / DBU [9:1 v/v] were added. In both vials 10 mg of cetyltrimethylammonium bromide (counter-ion) were added to keep the partially deprotected oligomer in solution, and the samples were left at room temperature for 2 days. The methanol / DBU mixture fully deprotects the oligomer and the methoxy group replaces the O^4-triazol group forming O^4-methylthymine. Similarly treatment with ethanol / DBU gives O^4-ethylthymine.

The solution was neutralized by adding approximately 100 µl of 50% aqueous acetic acid (1.5 equivalent to DBU). The DBU and the counter-ion were removed by

passing the solution through a Dowex ion exchange column (Na$^+$ form, 50 x 8 mm, 400

mesh, 10ml wet volume) and the oligomer was eluted with 10 ml of water and collected in

1 ml fractions. The oligomer was usually found in fractions 4 to 6 by measuring the UV

absorbtion at 260 nm.

2.2.6. Purification of synthesized oligonucleotides

In early experiments, oligonucleotides were purified using a two-step

reverse-phase HPLC purification procedure. The separation of the desired oligonucleotide

from impurities, is based in the hydrophobicity of the DMT group which is present only on

the 5' end of the full length oligomer and not on the shorter failure sequences. The very late

eluted peak (most hydrophobic peak) is the desired product. A 4µ Nova-pack C18 [or C18

phenyl] column (Waters) in a Waters Z-module radial compression system was used

connected to a Gilson HPLC apparatus. DNA was eluted at room temperature and flow of

3 ml / min with buffer A and increasing gradient of buffer B (the buffers and elution

gradient are shown in table 2.1). Buffers were made using water purified by a milli RO

-milli Q water purification system (Millipore), and filtered through 0.4 µ nylon membrane

filters (Millipore).

In the first step of purification the oligonucleotide sample was eluted using a 2% /

minute increasing gradient of buffer B (an example is shown in figure 2.3 A). The very last

eluted peak (in about 60 % buffer B) was collected in a round-bottom flask and evaporated

to dryness under reduced pressure. Two ml of 80% glacial acetic acid were then added to

remove the 5' DMT group, and left for 20 minutes at room temperature. The acetic acid

solution was evaporated to dryness in vacuo, 2 ml of water were added and the pH was

adjusted to pH 7 using 10 M KOH.

The DNA was desalted using Waters sep-pak C18 cartridges attached to a peristaltic

pump. Before application of the samples, cartridges were activated and equilibrated by

passing through the cartridge 10 ml of CH$_3$CN followed by 10 ml of water. Following

addition of the samples, cartridges were washed with a further 20 ml of water and eluted

with 10 ml of 70% v/v CH$_3$CN / water. The majority (90-95%) of the DNA was eluted in

the first 3 ml of the eluent. All 10 ml of the eluent were collected in a round-bottom flask and evaporated to dryness. The DNA was redissolved in 1 ml of water and reinjected to the HPLC using the same column , buffers, flow rate, as before. This time a 1% / minute gradient of buffer B was used. The fully deprotected oligomer was eluted as as single

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Table 2.1: Buffers and elution gradients for the purification of oligonucleotides, and the separation of their constituent nucleosides by HPLC.

	Buffers	Elution Gradient
Reverse-phase	A: 0.33 M KH_2PO_4 pH 6.3	1% or 2% per min
HPLC	B: 0.33 M KH_2PO_4, 33% CH_3CN pH 6.3	increasing gradient
purification		of buffer B
anion exchange	C: 0.4 M NaCl, 0.01 M NaOH (pH 12)	0-2 min 0% buffer D
chromatography	D: 1.2 M NaCl, 0.01 M NaOH (pH 12)	2-5 min 0 - 25% buffer D
		5-25 min 25 - 50% buffer D
Base analysis	E: 50 mM KH_2PO_4 pH 4.5	0-8 min 4% buffer F
	F: 50 mM KH_2PO_4, 40% CH_3CN pH 4.5	8-12 min 4 - 50% buffer F

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sharp peak at about 15-25 % of buffer B (figure 2.3 B). The peak was collected and desalted as described above.

The tedious and time consuming two-step HPLC purification procedure was replaced by a faster and more simple one when purification cartridges for oligonucleotides
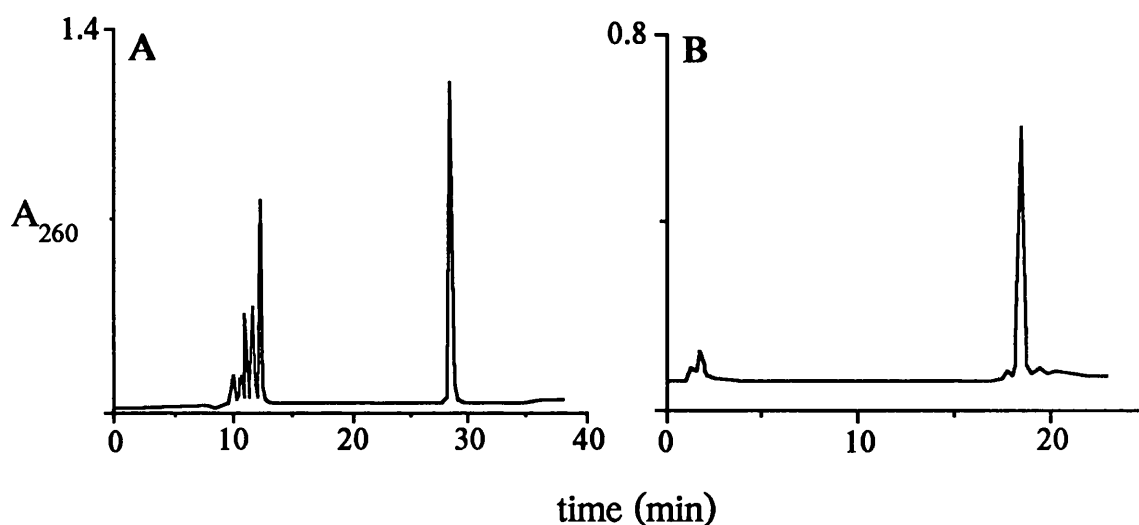
Figure 2.3 : Two-stage reverse-phase HPLC purification of the GGCGCTTGAGGCGTC oligomer using a nova-pack C18 phenyl cartridge (4 micron). A, isolation of the 5' DMT-protected oligomer and B, purification of the oligomer after the 5' DMT has been removed by 80% acetic acid. The chromatographic conditions are described in section 2.2.6 and table 2.1.
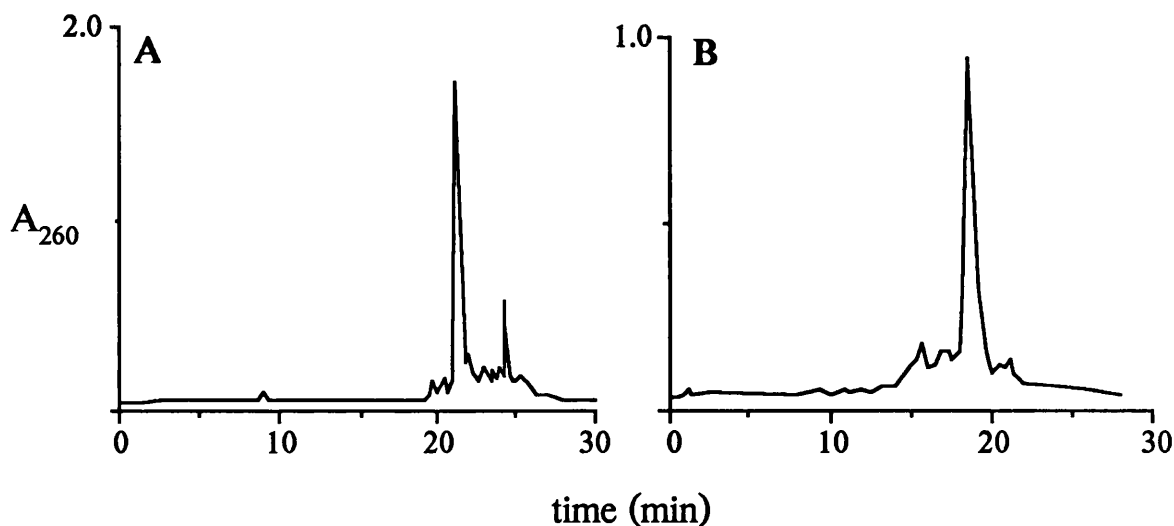
Figure 2.4 : Chromatographic analysis of crude oligomers after deprotection / purification using Nensorb cartridges. A, reverse-phase of the GGCGCTTmeGAGGCGTC oligomer; and B, anion exchange chromatography of the GGACCTCCCTCmeTTGACCTGCT oligomer. The conditions are described in section 2.2.6 and table 2.1.

became available commercially. The one used throughout this study was the Nensorb prep cartridge of Du Pont (NEN Research Products, Du Pont Co, Boston, USA). The purification using Nensorb cartridges is based on the elution of failure non-DMT containing sequences with 10% v/v acetonitrile in triethyl ammonium acetate (TEAA) [0.1 M pH 7], but not of the DMT-protected oligonucleotide of interest which is retained in the hydrophobic material of the column. The DMT is subsequently removed by 3% trifluoroacetic acid in TEAA (0.1 M) and finally the fully deprotected oligonucleotide is eluted by 35% methanol in water. It was found that following the manufacturer's protocol, both the yield and purity of the eluted oligomer were quite satisfactory (figure 2.4). Oligonucleotides containing O^6-alkylguanine were passed through the cartridge without previously extracting the oxime/TMG mixture. This mixture was removed, as monitored visually by the migration of the orange coloured oximate anions, together with the failure sequences in the 10 % acetonitrile/ TEAA wash (20 ml were used instead of the 10 ml used for normal oligomers).

All oligonucleotides used in the present study were further purified using HPLC. Oligonucleotides up to 15 residues in length were purified by reverse-phase HPLC, using the same conditions as described above and a 1% / minute gradient increase in buffer B. Oligomers longer than 15 nucleotides were eluted as very broad peaks, and thus reverse-phase HPLC could not be used for their purification. For these oligomers anion exchange chromatography was used instead. The chromatography was carried out on a Dionex BIOLC system with a Dionex variable wavelength detector using a Pharmacia monoQ HR5-5 column (figure 2.4 B). Gradients were formed from buffers C and D at a flow rate of 1 ml/min (the buffers and elution gradient are shown in table 2.1). The oligonucleotide peak was collected, immediately neutralized with 0.1 M HCl, and desalted as previously described.

2.2.7. Base analysis

The purity of the oligomers was assessed by base analysis. In general 0.3- 0.5 A_{260} units of an oligomer were completely digested to nucleosides by phosphodiesterase

and alkaline phosphatase. Snake venom phosphodiesterase I solution (10 μl, 10 μg protein, 0.05 units) was added to the oligomer (120 μl, in 60 mM Tris-HCl, 6 mM MgCl$_2$ pH 8.5) and incubated at 37°C for 40 min. Then calf intestine alkaline phosphatase solution (10 μl, 5 μg protein, 5 units) were added and the incubation continued for 20 min. The deoxyribonucleosides were separated by HPLC using a 4μ Nova-pak C18 cartridge. Duplicate samples (60 μl) were injected onto the column and eluted at 3 ml/minute with a gradient of buffers E and F (the buffers and elution gradient are shown in table 2.1). The eluate was monitored at 260 nm and the peaks corresponding to the nucleosides were integrated with a Gilson 620 data module. The amount of each nucleoside was then measured by comparison of the integrated areas with those obtained from injection of a standard mixture of nucleosides. From this data, the proportion of each base in the oligomer could be calculated and thus, the accuracy of the synthesized sequence could be checked. In addition the concentration of the oligomer in the original sample could be calculated.

2.3 RESULTS AND DISCUSSION

2.3.1 Oligonucleotides containing O^6-methylguanine

All oligonucleotides, after purification, gave single peaks when chromatographed by either reverse-phase or anion exchange HPLC. The chromatographic profiles of some oligomers containing O^6-methylguanine, are shown in figure 2.5 A. The enzymic digest chromatographs showed a small amount of inosine in the nucleoside mixture (figure 2.5 B). This is produced from deamination of adenine by contaminating adenosine deaminase in the alkaline phosphatase solution. The results of the enzyme digest (table 2.2) show that the empirical nucleoside composition is in excellent agreement with the expected values.

The main problem encountered by other investigators in the synthesis of oligonucleotides containing O^6-alkylguanine has been the side-products produced during the deprotection of the oligomers, mainly the production of 2,6 diaminopurine. This compound is formed by the nucleophilic attack of ammonia on the C6 of O^6-alkylguanine. 2,6 diaminopurine pairs with thymine during replication and its presence even in minute quantities might not be tolerable, in particular when genetic studies are involved. The group used for protection of N^2 of alkylguanine has a critical influence on the formation of this impurity. The commonly used isobutyryl group is difficult to remove with ammonia so that the deblocking conditions, time and temperature have to be severe [72 hours at 65 °C has been found to be necessary (Borowy-Borowsky and Chambers, 1987)]. Alternatively, deblocking agents other than ammonia can be used [methanol / DBU for 6 days at room temperature has been reported (Pauly et al., 1988)], or N^2-protecting groups more labile than the isobutyryl group should be tried. In our laboratory Dr B.F.L. Li used the phenylacetyl group which is easily removed without the need of severe conditions. The N^2-phenylacetyl protecting group has been reported to have, at the nucleoside level, a half-life of only 48 minutes in ammonia at room temperature (Li and Swann, 1989). Using the procedures described under Materials and Methods no 2,6 diaminopurine was detected in the enzyme digest chromatogram (figure 2.5 B), and only trace quantities (< 0.2%) can be seen in a high detector sensitivity (Smith et al., 1990).
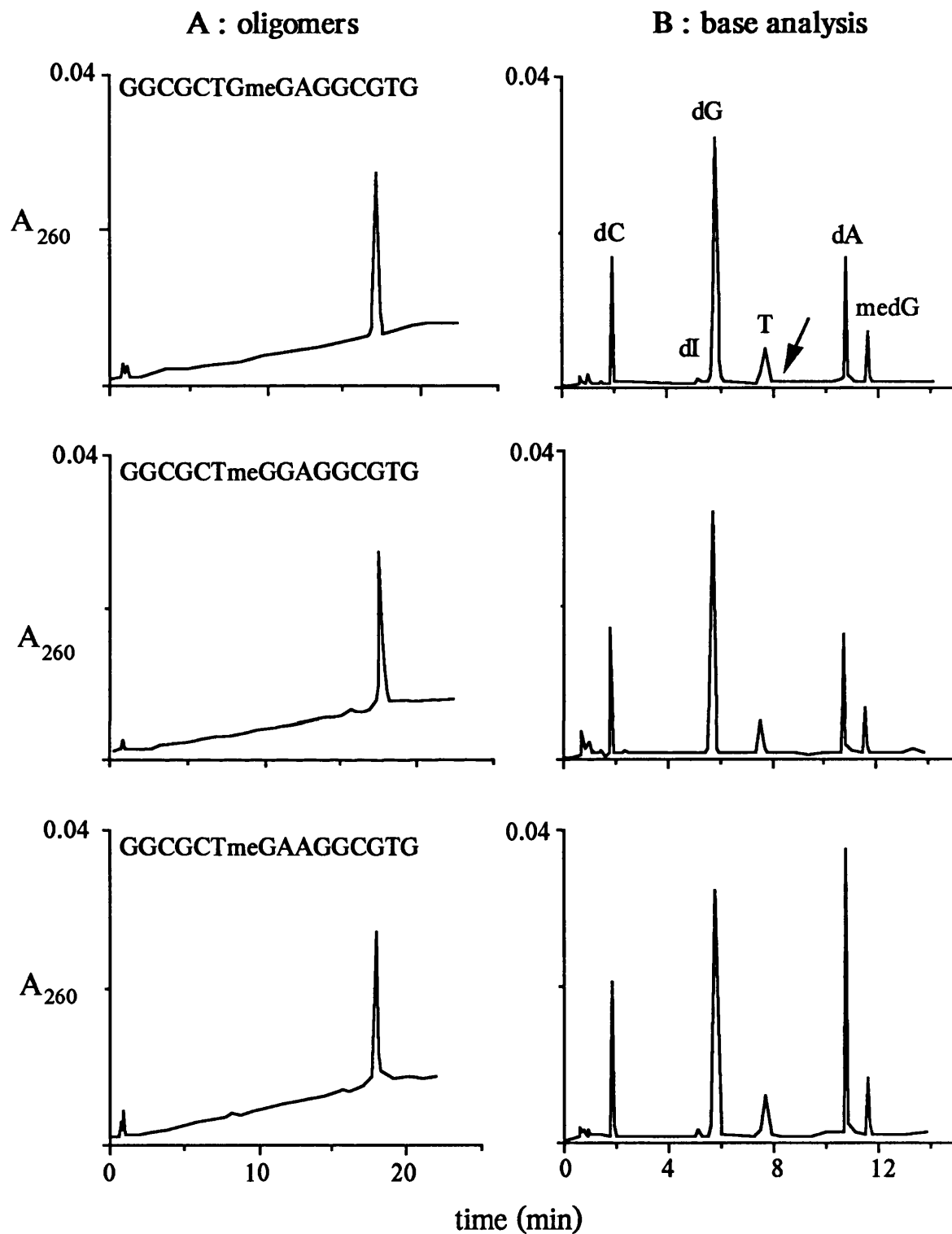
Figure 2.5 : A, reverse-phase HPLC of purified 15-mers containing O^6-methylguanine and

B, analysis of their constituent nucleosides. The conditions are described in table 2.1. The

arrow indicates the position of elution of 2, 6 diaminopurine. dC: deoxycyditine;

dI: deoxyinosine dG: deoxyguanosine; T: thymidine; dA: deoxyadenosine;

medG: O^6-methyl- deoxyguanosine.

Table 2.2 : Base analysis of oligonucleotides containing O6-methylguanine, O4- methylthymine and O4-ethylthymine.

oligonucleotide		dC	dG	T	dA	medG	meT	etT
GGCGCTGmeGAGGCGTG	found	2.90	8.23	2.09	0.98	0.97		
	expected	3.0	8.0	2.0	1.0	1.0		
GGCGCTmeGGAGGCGTG	found	2.86	8.3	2.07	1.07	0.91		
	expected	3.0	8.0	2.0	1.0	1.0		
GGCGCTmeGAAGGCGTG	found	3.09	6.87	2.06	1.98	1.00		
	expected	3.0	7.0	2.0	2.0	1.0		
GGACCTCCCTCTmeTGACCTGCT	found	9.03	4.19	4.80	1.90		1.07	
	expected	9.0	4.0	5.0	2.0		1.0	
GGACCTCCCTCTetTGACCTGCT	found	9.20	4.19	4.77	1.76			1.12
	expected	9.0	4.0	5.0	2.0			1.0
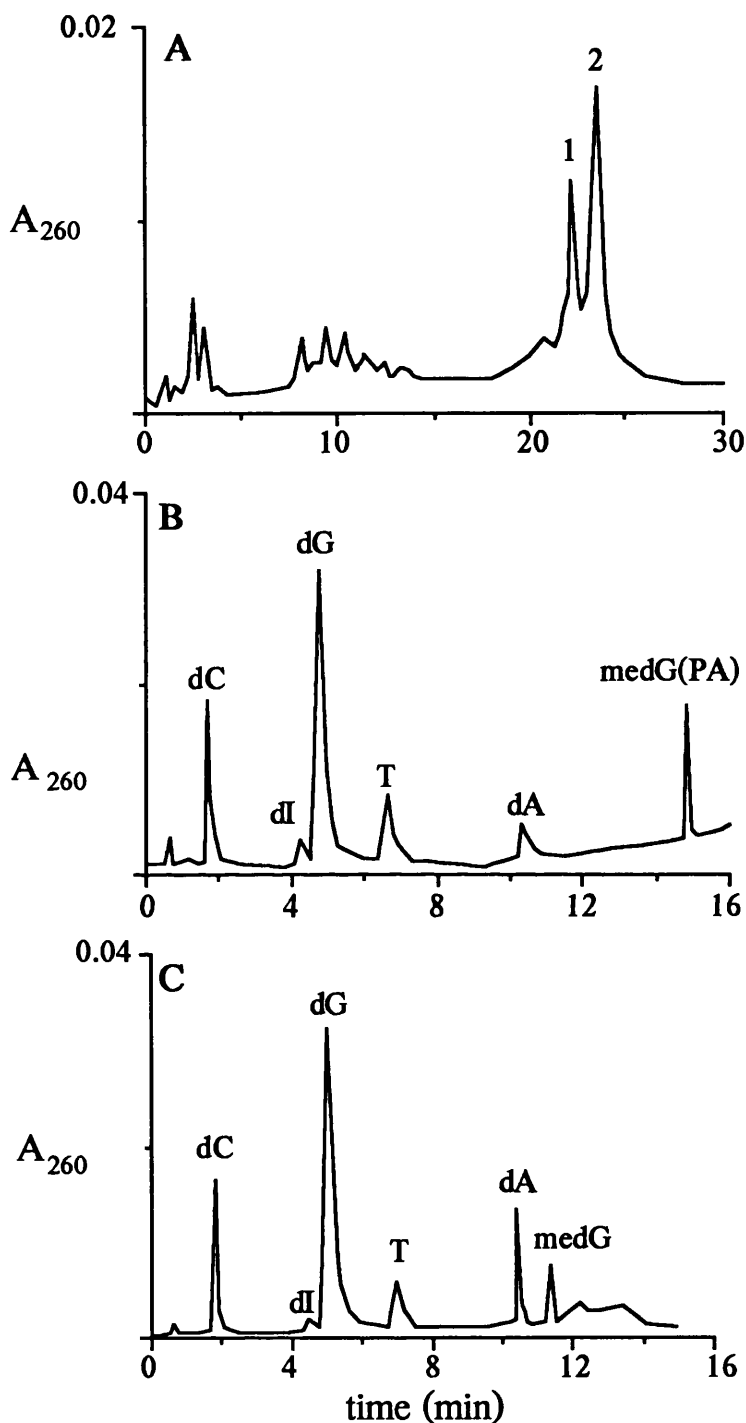
Figure 2.6 : A, Anion exchange chromatographic analysis of the crude oligomer

GGACGGGCGCTGmeGAGGCGTGT, after deprotection by NH_3 only. B, analysis of

the nucleoside composition of peak 2 showing that this oligomer contains N^2

-phenylacetyl- O^6-deoxyguanosine. Peak 1 is the fully deprotected oligomer;

C, Analysis of the nucleoside composition of the major product after treatment with

oxime/TMG and NH_3. medG(PA) : N^2-phenylacetyl- O^6-deoxyguanosine; medG: O^6

-methyldeoxyguanosine. The chromatographic conditions are described in table 2.1.

Oxime / TMG treatment is an essential step of the deprotection procedure when O^6-alkylguanine is added as a phosphotriester, since oximate anions remove the chlorophenyl groups protecting the phosphate of the O^6-alkylguanine residues. On the contrary this step seemed unnecessary when O^6-alkylguanine was added as a phosphoramidite, since one might expect that all the groups, the N^2-phenylacetyl group included, would be easily removed by ammonia. However, when oligonucleotides synthesized with the phosphoramidite chemistry were treated by ammonia alone (room temperature, 30 hours), the deprotection was incomplete. Anion exchange chromatography showed that two major products were formed in this reaction (figure 2.6 A). The first was the fully deprotected oligomer (peak 1 in figure 2.6 A), whereas the second (peak 2 in figure 2.6 A) contained N^2-phenylacetyl- O^6-methylguanine instead of O^6-methylguanine (figure 2.6 B). Thus, the N^2-phenylacetyl group is far more stable in ammonia at the oligonucleotide level than at the nucleoside level. However, when ammonia was replaced by a mixture of oxime/TMG in ammonia, a single major product was formed, and its base analysis confirmed that deprotection was complete (Figure 2.6 C). The (E)-2-nitrobenzaldoxime / TMG mixture apparently increases the rate of removal of the phenylacetyl group by ammonia, as it was previously observed for the rate of removal of the N^6- benzoyl protecting group of adenine (Li and Swann, 1989).

2.3.2 Oligonucleotides containing O^4-alkylthymine

The method for the synthesis of O^4-alkylthymine has been developed in our laboratory by Dr Y-Z Xu. An oligonucleotide is synthesized containing O^4-triazolothymine instead of O^4-alkylthymine. O^4-triazolothymine is stable to the normal procedures of DNA synthesis, but has sufficient chemical reactivity to allow one to convert it into a number of products, after synthesis of the oligomer. O^4-methylthymine and O^4-ethylthymine were made using methanol/DBU (methoxide ions) and ethanol/DBU (ethoxide ions) respectively to substitute the 4-O position of thymine, and deprotect the oligonucleotide. Chromatograms of oligonucleotides containing O^4-methylthymine and O^4-ethylthymine and the respective enzyme digest chromatograms are shown in figure 2.7. The results from the enzymic

A: oligomers   **B: base analysis**

GGACCTCCCTCTmeTG.ACCTGCT

GGACCTCCCTCTetTG.ACCTGCT

time (min)
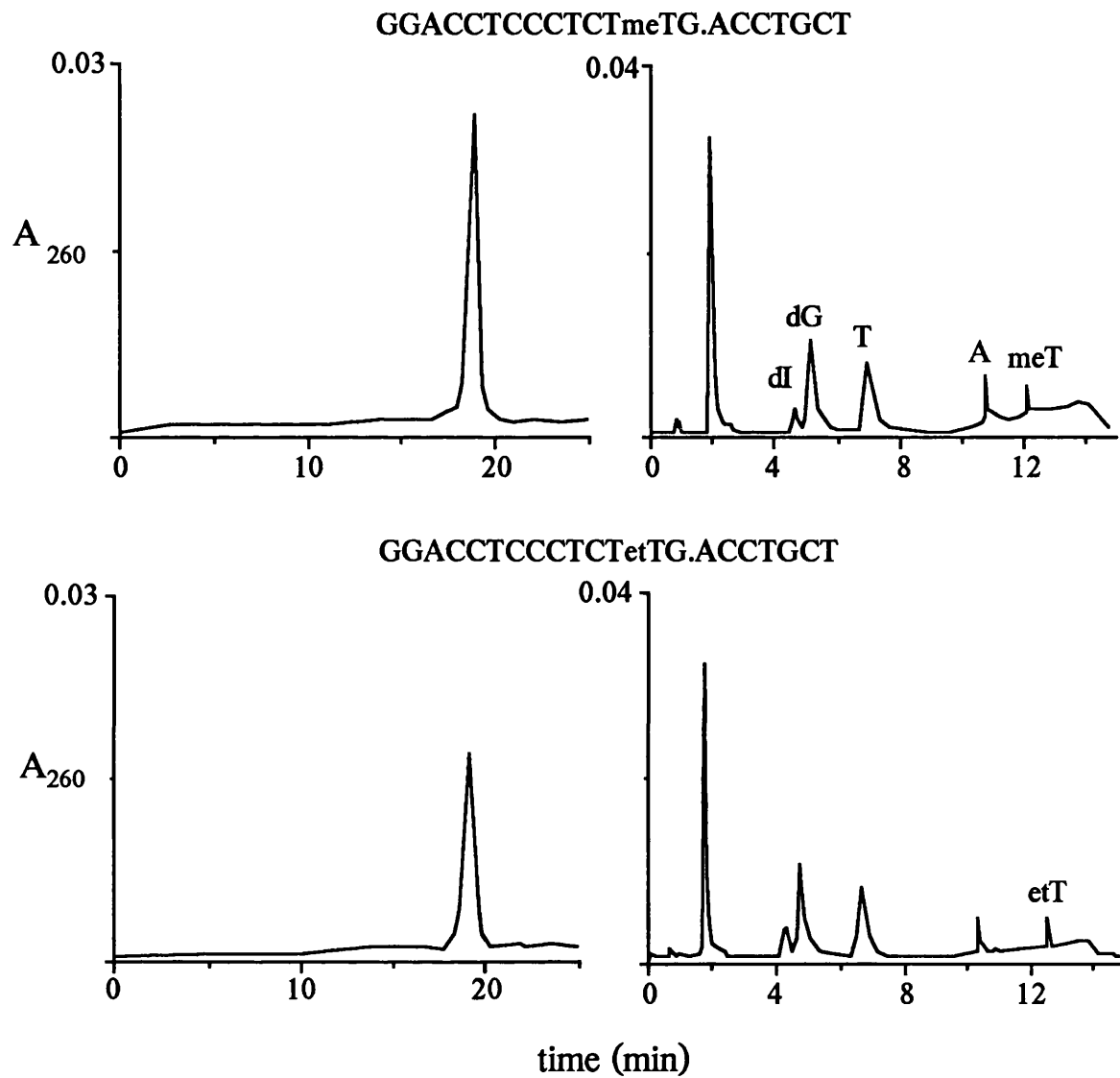
Figure 2.7 : A, anion exchange chromatography of purified 21-mers containing O^4-alkyl-thymine and B, analysis of their constituent nucleosides. The conditions are described in table 2.1. dC: deoxycyditine; dI: deoxyinosine; dG: deoxyguanosine; T: thymidine; dA: deoxyadenosine; meT: O^4-methylthymidine; etT: O^4-ethylthymidine
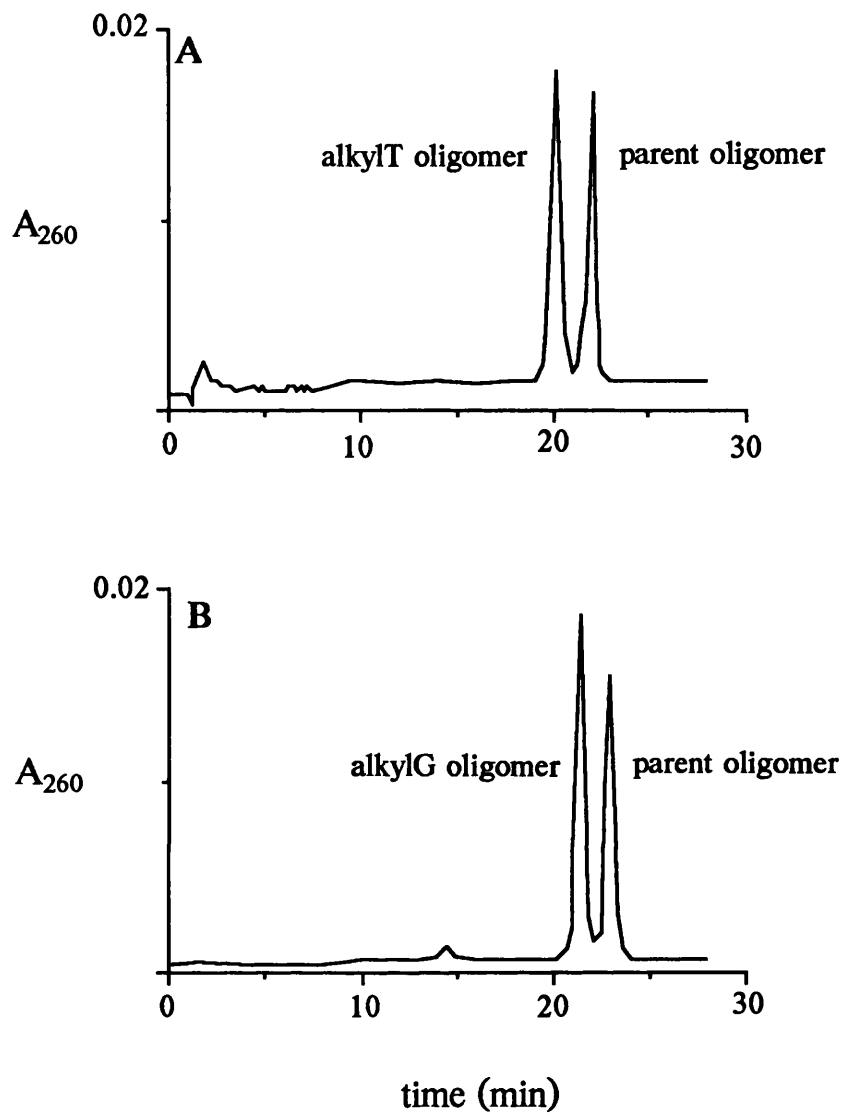
Figure 2.8 : Separation of A, GGACCTCCCTCTmeTG.ACCTGCT and B,

GGACGGGCGCTGmeGAGGCGTGT from the respective parent oligomers using

anion exchange chromatography. The conditions are as described in table 2.1.

digestion are shown in table 2.2 along with the expected base composition.

Under the basic conditions (pH 12) of the anion exchange chromatography, an oligomer containing O^4-alkylthymine was eluted earlier than the parent non-alkylated oligomer (figure 2.8 A). At pH 12, the N-3 imino proton of thymine is probably lost leaving a negative charge (pKa =10). The absence of this imino proton in O^4-alkylthymine would thus, reduce the net charge of the alkylated oligomer. Similarly, at pH 12, guanine residues would be negatively charged at the N1 position (pKa = 9.5) but as O^6-methylguanine does not have this imino proton, not surprisingly a 21-mer containing O^6-methylguanine was also eluted earlier than the respective parent oligomer (2.8 B).
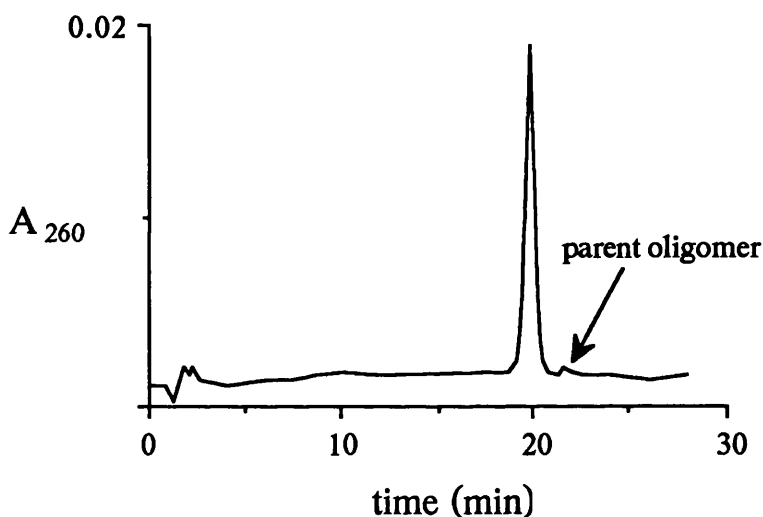
Figure 2.9 : Conversion of the GGACCTCCCTCTmeTG.ACCTGCT to the parent oligonucleotide in the alkaline conditions of the anion exchange chromatography (pH 12). The conditions are as described in table 2.1.

It is known that O^4-alkylthymine is converted to thymine in alkaline conditions (Li et al., 1987). If anion exchange chromatography at pH 12 was to be used for purification, the extent of the conversion had to be assessed. A fraction of an oligomer containing O^4-alkylthymine was purified by anion exchange chromatography and immediately reinjected to the column. As figure 2.9 shows only traces of the oligomer were converted to

the parent, non-alkylated oligomer, during this second pass through the column. The apparent resistance of O^4-alkylthymine to destruction by alkali, caused probably by steric hindrance by the neighbouring nucleotides, enabled us to use anion exchange chromatography for the purification of these oligomers as well as for the O^6-alkylguanine containing oligomers.

CHAPTER 3

SUBSTRATE SPECIFICITY OF THE E. COLI AND HUMAN O^6-ALKYLGUANINE-DNA ALKYLTRANSFERASES

3.1. INTRODUCTION

The structure, the biochemistry, and molecular biology of the alkyltransferases have been summarised in the general introduction. The substrate specificity of these enzymes needs to be discussed in detail, since it is the background knowledge for the experiments described in this chapter.

The E. coli *ada* protein can repair O^6-methylguanine, O^6-ethylguanine (Sedgwick and Lindahl, 1982), and higher alkylguanine derivatives like n-propyl-, n-butyl-, isopropyl, isobutyl-guanine (Morimoto *et al.*, 1985). However as the alkyl group increases in size, the efficiency of repair declines markedly. It has been reported that the repair of O^6-ethylguanine is 10-100 fold slower than O^6-methylguanine (Pegg *et al.*, 1984), and branched alkylguanine adducts (e.g. isopropylguanine) are poorer substrates than the respective linear alkyl- derivatives [e.g. n-propylguanine] (Morimoto *et al.*, 1985). On the other hand, the alkyltransferase activity found in mammalian tissue extracts was less efficient than the *ada* alkyltransferase in removing O^6-methyl- groups, but more efficient in removing bulkier adducts (Pegg *et al.*, 1984; Morimoto *et al.*, 1985). It was thus, suggested that steric constraints have a considerable effect on the activity of both bacterial and mammalian alkyltransferases, and that the mammalian enzyme is less sterically hindered, and possibly has a more hydrophobic binding cleft, than its bacterial counterpart. In these experiments natural DNA was used as substrate, randomly alkylated with the appropriate agent. However, different agents give different DNA alkylation patterns and this inevitably means that the DNA substrates used in these comparative studies were not identical. The E. coli *ada* and the human alkyltransferases are also active in the repair of O^6-chloroethylguanine residues (Robins *et al.*, 1983; Pegg *et al.*, 1984). This repair

process is of particular interest since it might explain the poor cytotoxic effects of chloroethylating agents in cells with fully functional repair activities [see chapter 1.3] (Ludlum, 1990).

O^4-alkylthymine is also substrate for the *ada* alkyltransferase. Repair of O^4-alkylthymine is more difficult to measure in natural DNA because this modified base is formed in very low quantities in DNA (table 1.1). However, using poly (dT) methylated with N-methyl-N-nitrosourea and annealed to poly (dA), as a substrate for the alkyltransferase, McCarthy *et al.* (1983), Ahmmed and Laval (1984), Dolan *et al.* (1984) and McCarthy *et al.* (1984) have demonstrated demethylation of O^4-methylthymine involving the same protein, with the same stoichiometric repair mechanism, and probably the same cysteine receptor residue as in the repair of O^6-alkylguanine. In contrast, many investigators failed to detect repair of O^4-methylthymine by mammalian tissue extracts, or partially purified mammalian alkyltransferases (Dolan *et al.*, 1984; Dolan *et al.*, 1985; Yarosh *et al.*, 1985; reviewed by Brent *et al.*, 1988). The only report of repair of O^4-methylthymine by mammalian extracts is that of Becker and Montesano (1985), who used as substrate poly (dA:dT) methylated with N-methyl-N-nitrosourea, and found that the amount of O^4-methylthymine was reduced by crude extracts of human, monkey and rat liver. No free O^4-methylthymine was produced, showing that this was not the result of glycosylase activity, and loss of O^4-methylthymine was proportional to the amount of protein added. These mostly negative results for the repair of O^4-alkylthymine in vitro, do not parallel observations in vivo. There is strong evidence that the loss of O^4-alkylthymine observed in various tissues is caused by enzymic reaction and not by cell turnover, although this loss was not always related with the O^6-alkylguanine-DNA- alkyltransferase activity. The rate of the removal of O^4-ethylthymine from different rat tissues after a single dose of N-ethyl-N-nitrosourea, parallels the rate of the removal of O^6-ethylguanine and the alkyltransferase content of the different tissues (den Engelse *et al.*, 1987), but repeated exposure of rats to NNK results in slow removal of O^6-methylguanine, but more rapid

removal of O^4-methylthymine from lung DNA (Belinsky *et al.*, 1986). The controversy over the nature of the repair of O^4-alkylthymine in mammalian cells is still open and highlights the technical difficulties encountered in previous studies mainly caused by the unavailability of suitable DNA substrates and the lack of purified enzymes.

Very little is known about the mechanism by which the alkyltransferases recognize and repair alkylated DNA. The enzymes bind non-specifically to natural DNA (Demple *et al.*, 1982; Pegg *et al.*, 1982) and the affinity of this interaction is probably similar for single or double stranded DNA (Bhatacharayya *et al.*, 1990). However, all known alkyltransferases repair O^6-methylguanine significantly faster when the adduct is present in double stranded than in a single stranded DNA (Lindahl and Robins, 1982; Rebeck *et al.*, 1989; Bhatacharayya *et al.*, 1990). Furthermore, the B configuration of DNA seems to be essential for the enzyme action since O^6-alkylguanine residues present in Z-DNA cannot be repaired by the E. coli alkyltransferase (Boiteux *et al.*, 1985). It is possible that alkyltansferases recognize alterations of the B-DNA helix caused by the alkylated base, but although NMR showed that the distortions of the DNA helix caused by the O^6-methylG:C and O^6-methylG:T base-pairs are dissimilar, the rate of repair of O^6-methylG:C is not significantly different to that of the O^6-methylG:T base-pair (Toorchen *et al.*, 1984; Scicchitano *et al.*, 1986; Graves *et al.*, 1989).

Recent advances in synthetic nucleotide chemistry has made possible the synthesis of oligonucleotides containing O^6-alkylguanine or O^4-alkylthymine (Gaffney *et al.*, 1984; Li *et al.*, 1987; Li and Swann, 1989). The application of such oligonucleotides in repair assays overcomes the major disadvantages of the previously used methods, the variety and the different proportions of the alkylation products, and the difficulty of monitoring the repair of minor adducts like O^4-alkylthymine.

In the study described in this chapter the efficiencies of the bacterial *ogt* and the human alkyltransferases for the repair of O^6-methylguanine, O^6-ethylguanine, and O^4-methylthymine were determined and compared with these previously obtained for the *ada* alkyltransferase (Graves *et al.*, 1989). Self-complementary oligonucleotides of defined

sequence containing O^6-methylguanine, O^6-ethylguanine, or O^4-methylthymine were used

as substrates for the repair by the homogeneous, *ada, ogt* and human alkyltransferases.

Based on the chromatographic separation of the alkylated from the repaired, non-alkylated

oligomer, the repair process was followed and the rate constant for the reaction was

determined.

3.2 MATERIALS AND METHODS

3.2.1. 5'- 32P- labelling of oligonucleotides

Self-complementary dodecanucleotides of the general type CGCalkGAGCTCGCG

and CGCAAGCTmeTGCG   were synthesized by Dr B.F.L. Li

using the phosphotriester method in solution (Li and Swann, 1987, 1989).

Oligonucleotides (0.2 A_{260} units) were 32P-labelled at 37 °C in 70 mM Tris-HCl,

10 mM $MgCl_2$, and 1 mM spermidine pH 7,6 by 10-20 units T4 polynucleotide kinase

(Amersham) and 200 μCi gamma-[32P- ATP] in a volume of 30-50 μl. After 30 min

incubation, 5 μl of 10 mM unlabelled ATP was added and the reaction allowed to continue

for a further 30 min. Under these conditions, virtually complete phosphorylation of the

alkylated oligomers was obtained, giving approximately 200,000 cpm per pmol of 5' ends.

3.2.2. HPLC purification of labelled oligonucleotides

Following the 5'-32P- labelling of the oligonucleotide, excess ATP was removed

by passing the reaction mixture through a Biospin P6 column (Bio-rad) following the

manufacturers' procedure. The labelled oligomers were further purified by HPLC, using a

4 μ Nova-pack C18 cartridge (Waters) in a Waters Z-module radial compression system.

DNA was eluted at room temperature and flow of 3 ml/min with buffer A (0.33 M

KH_2PO_4 pH 6.3) and 1% / min gradient of buffer B (0.33 M KH_2PO_4 , 33% CH_3CN

pH 6.3). Buffers were made using water purified by a milli RO -milli Q water purification

system (Millipore) and filtered using 0.4μ nylon membrane filters (Millipore). The

phosphorylated oligomers was then collected in micro-test tubes and freeze-dried using a

speedvac concentrator (Savant). After redissolving in 2 ml water the DNA was desalted

using Waters sep-pak C18 cartridges attached to a peristaltic pump as described previously

(section 2.2.6). The majority (90-95%) of the DNA was eluted in the first 3 ml of the

eluent which was collected and freeze-dried.

The amount of the labelled oligomer, retrieved after purification, can be accurately

determined by simply measuring the absorbance of the solution at 260 nm assuming that the molar extinction coefficient of the oligomer is the sum of the molar extinction coefficients of each nucleoside in the oligomer sequence. The values used were E(dA) 15,200; E(dG) 11,800; E(dC) 7,400; E(T) 8,800; E(medG) 5,300; E(etdG) 5,100; E(meT) 2,800.

3.2.3. Assay for the repair of alkylated oligonucleotides with O^6-alkylguanine-DNA-alkyltransferases.

Purified E.coli *ada* alkyltransferase (19,000 daltons) was provided by Dr D. Yarosh (Applied genetics Inc, Freeport, N.Y.). Purified E. coli *ogt* and human alkyltransferases were generous gifts from Dr J. Margison. The concentration of the enzymes was calculated from their reaction with a known excess of ^{32}P-labelled alkylated oligomers. For the enzyme titration the oligomers were usually in a two fold excess over the enzyme and the time of incubation was such that it would ensure the completion of the reaction.

The kinetics for the reaction between the oligonucleotides and the E. coli alkyltransferases (*ada* ,*ogt*) was followed at 37 °C in a reaction mixture containing 50 mM tris-HCl pH 7.6, 10 mM DTT, 1 mM EDTA and 200 μg / ml bovine serum albumin (Sigma). The kinetics for the repair by the human alkyltransferase was followed at room temperature (25 °C) using the same reaction buffer as above with the addition of 5% glycerol. The concentrations of both oligomer and enzyme in the assay varied depending on the predicted speed of the repair reaction. The amount of oligomer in the assay was always in excess over the enzyme, and the amount of enzyme added was confirmed from the end-point of the reaction. At intervals, a fraction (usually 1/10) of the total volume was taken out of the reaction mixture and quenched with approximately 0.05 A_{260} units (450 pmol) of the phosphorylated but non-radioactive alkylated oligonucleotide, which also served as an HPLC marker. The same amount of phosphorylated but non-radioactive parent oligonucleotide was also added as a HPLC marker. Quenched samples were injected

without further treatment onto a Nova-pak C18 column, eluted at 3 ml/minute at room temperature with buffer A (see section 3.2.2) and 1% per min increasing gradient of buffer B. This separated the alkylated from the repaired, non-alkylated oligomer. The eluate was collected in 15 -30 seconds fractions and the radioactivity was measured by scintillation counting.

3.2.4. Calculation of rate constants

The kinetics of repair by the alkyltransferase can be described as follows:

$$A + B \underset{K_2}{\overset{K_1}{\rightleftharpoons}} AB \overset{K_3}{\longrightarrow} A^* + B^* \tag{1}$$
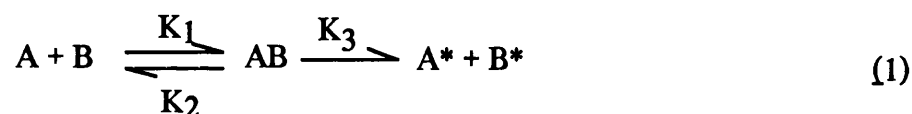
The enzyme B reacts with the alkylated DNA (A) to give the active intermediate AB. The alkyl group is then transfered from DNA to the enzyme generating the alkylated enzyme (B*) and the dealkylated DNA (A*). The chemical reaction (1) can be simplified to:

$$A + B \overset{k}{\longrightarrow} A^* + B^* \tag{2}$$

For this second order reaction we have:

$$d[A]/dt = -k[A][B] \tag{3}$$

Let the concentrations of A and B at time t = 0 be A_0 and B_0 and at time t $[A_0 - x]$ and $[B_0 - x]$ respectively. Thus, from equation (3) we obtain :

$$- dx/([A_0 - x][B_0 - x]) = -k\,dt \tag{4}$$

which can be transformed and then intergrated to give the equation

$$\ln([B_0-x][A_0]/[A_0-x][B_0]) = ([B_0] - [A_0])\,k\,t \tag{5}$$

The graph of $\ln([B_0-x][A_0]/[A_0-x][B_0])$ as a function of time should be linear with a slope $([B_0] - [A_0])$ k. Thus the rate constant k of the repair process can be determined knowing the initial concentrations of oligomer (A_0) and enzyme (B_0), and also the amount of oligomer repaired (x).

3.3. RESULTS

During the repair process of DNA by alkyltransferases, the alkyl group is removed from the alkylated base, and hence the natural base and DNA sequence is restored. The 5'-^{32}P-labelled dodecanucleotides containing O^6-alklylguanine or O^4-alkylthymine could be separated from the respective parent oligomers by reverse-phase HPLC. Thus, the progress of their repair by the alkyltransferases was followed by the decrease of the radioactive alkylated oligomer and the concurrent appearance of the parent oligomer (figure 3.1). The alkyltransferase is not regenerated after the reaction and the process of repair cannot be described by Michaelis-Menten enzymic kinetics but should be considered as a second order chemical reaction. The rate constant can then be obtained from the equation

$\ln ([B_0\text{-}x] [A_0] / [A_0\text{-}x] [B_0]) = ([B_0] - [A_0]) \, k \, t$ as shown in "Materials and Methods".

It has been previously shown, using the same method and oligonucleotides, that O^6-methylguanine is repaired 1,000 times faster than O^6-ethylguanine, and 10,000 times faster than O^4-methylthymine by the *ada* alkyltransferase. These results are now extended with the rates of repair of the same substrates by the bacterial *ogt* and human alklytransferases.

The progress of repair of O^6-methylguanine, O^6-ethylguanine, and O^4-methylthymine by the *ogt* protein are shown in figure 3.2. From the curved lines of this figure, the linear graphs of figure 3.3 were derived, and from the slopes of these lines the rate constants were calculated as described in "Materials and Methods". The rate constants shown in table 3.1 are the mean values from two independent determinations. O^6-methylguanine is the preferred substrate for the *ogt* protein followed by O^6-ethylguanine and O^4-methylthymine as it has been previously observed for the ada alklyltransferase. The rate constant for the repair of the O^6-methylguanine containing oligomer by the *ogt* protein is 2.9×10^7 M^{-1}s^{-1}, while the rate constants for the repair of O^6-ethylguanine and O^4-methylthymine are 4.5×10^6 M^{-1}s^{-1} and 2.1×10^5 M^{-1}s^{-1} respectively (table 3.1).
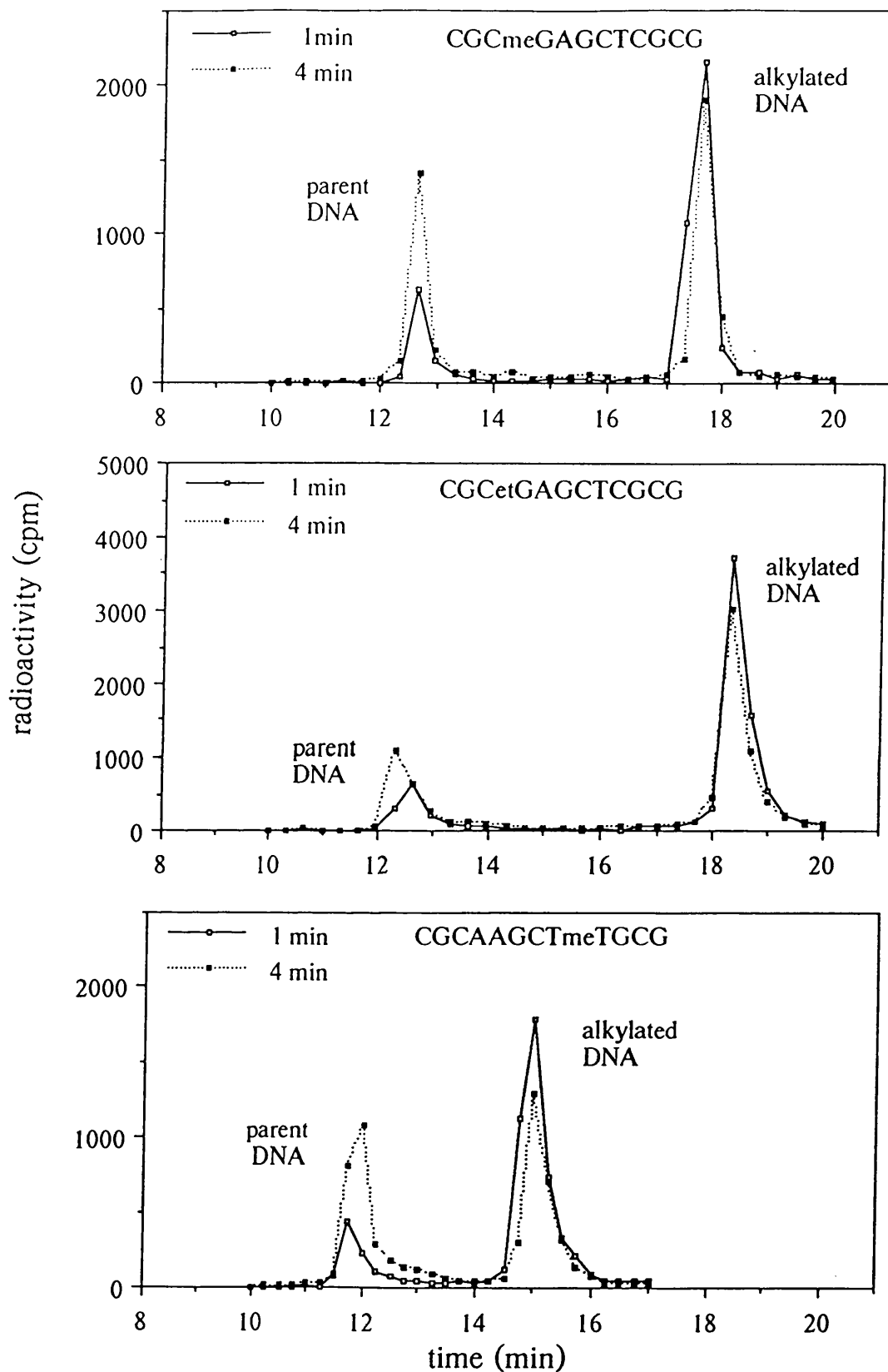
Figure 3.1 : Sequential HPLC traces showing the convertion of the O^6-methylguanine, O^6-ethylguanine and O^4-methylthymine containing oligomers to the respective parent oligomers by the action of the *ogt* alkyltransferase.
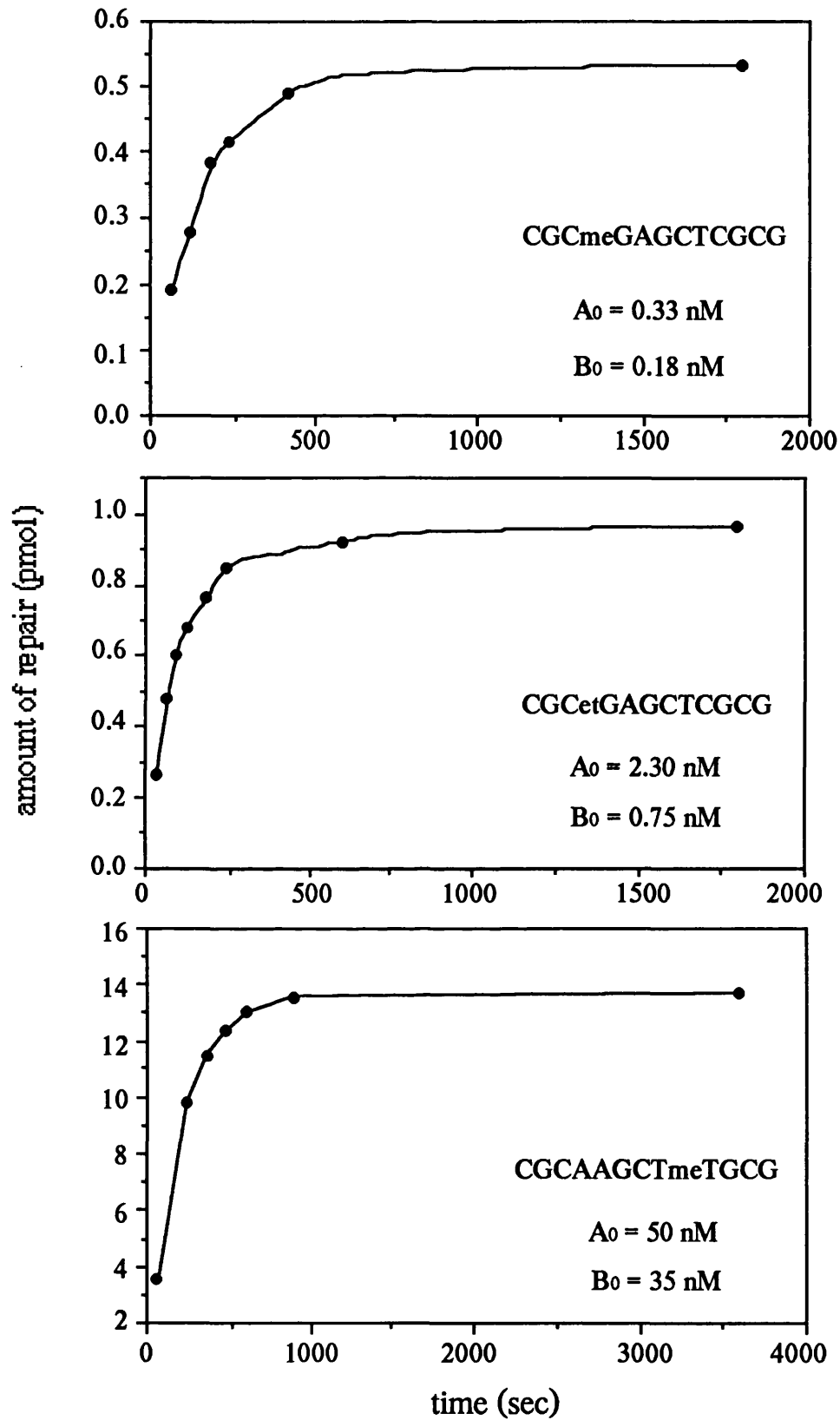
Figure 3.2 : Rate of formation of the parent oligomer by repair of the alkylated oligomers by the *ogt* alkyltransferase. Ao and Bo are the initial concentrations of oligomer and enzyme respectively.
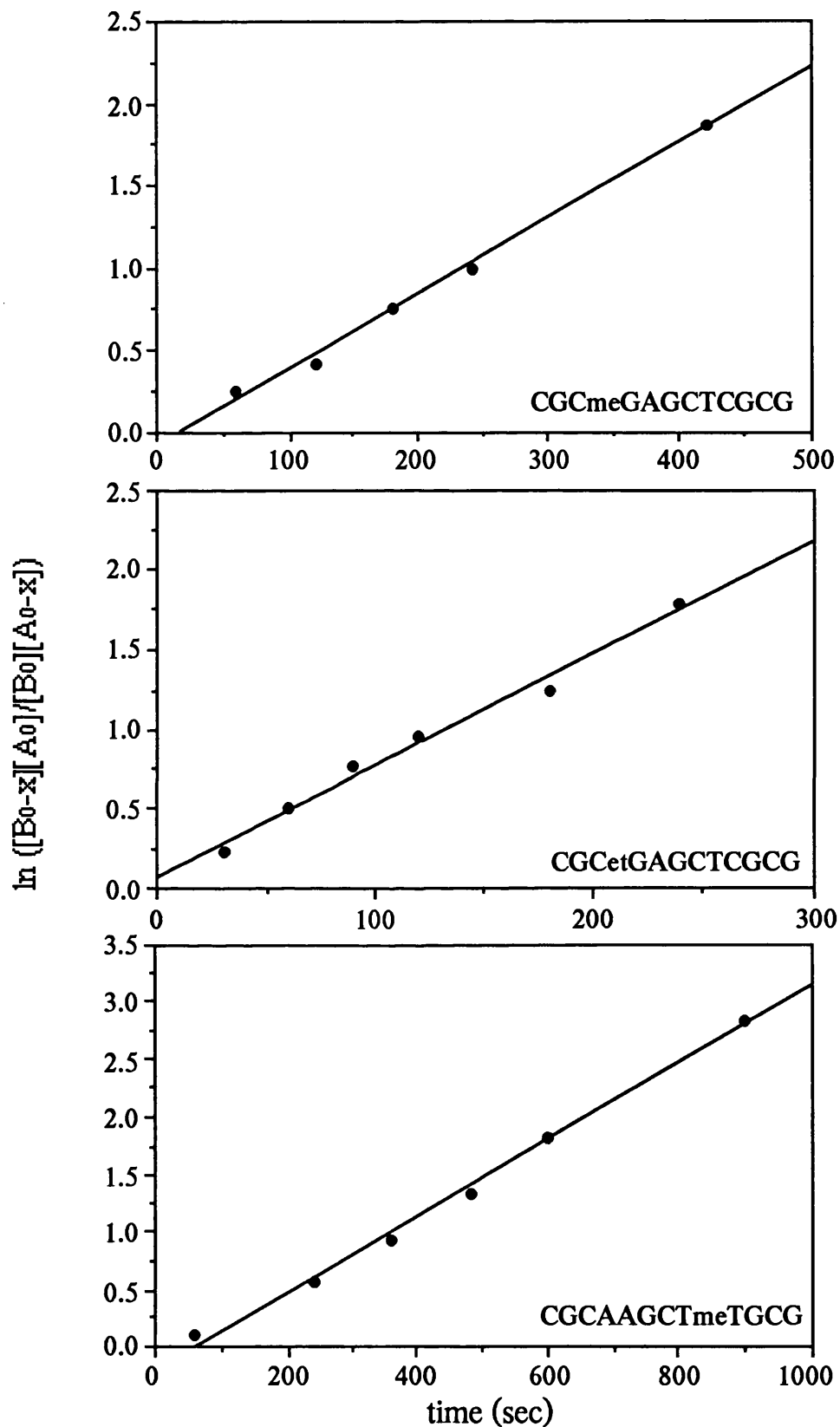
Figure 3.3 : Graphs of ln ([Bo-x][Ao]/[Bo][Ao-x]) as a function of time, derived from the

graphs of figure 3.2. Ao and Bo are the initial concentrations of oligomer and enzyme

respectively, x is the amount reacted after time t. The slope of each line is equal to k(Bo-Ao).

The human enzyme was found to be labile. At 37 °C more than 60% of the alkyltransferase was inactivated within 3 hours, whereas at room temperature about 20% was inactivated over the same incubation period. Therefore, the rate constants were not determined at 37 °C but at room temperature (25 °C). In the assays for the repair of O^6-methylguanine and O^6-ethylguanine, the concentrations of both oligomer and enzyme were such, that the reaction was completed in less than 1 h ($\approx$ 6% inactivation). The rate constants for the repair of methyl and ethylguanine by the human alkyltransferase were 1.4 x 10^6 $M^{-1}s^{-1}$ and 2.3 x 10^4 $M^{-1}s^{-1}$ respectively (table 3.1).
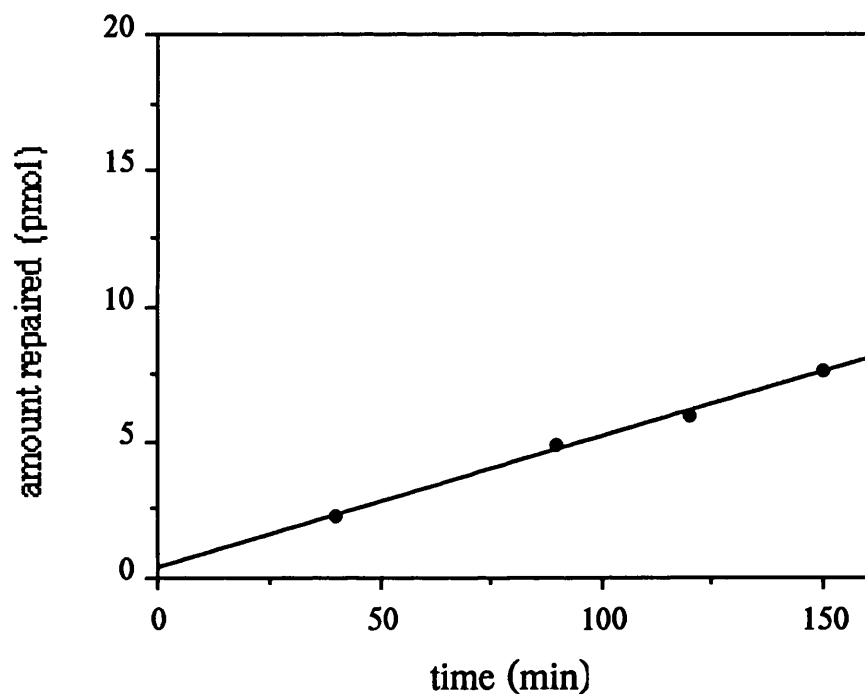
Figure 3.4 : Rate of demethylation of the CGCAAGCTmeTGCG oligomer by the human alkyltransferase at 25 °C. The initial concentrations in the assay were: 1 μM oligomer and 0.9 μM enzyme in 100 μl reaction mixture.

Surprisingly, and in contrast with previous observations, the human alkyltransferase was also able to repair O^4-methylthymine (figure 3.4). The demethylation process was extremely slow and detectable only when both substrate and enzyme were in

μM concentrations. Parent oligonucleotide was not detected after incubation of the alkylated oligomer in the absence of the alkyltransferase, indicating that demethylation was caused by the enzyme and not by degradation of the alkylated oligomer. The slowness of the repair reaction and the lability of the enzyme did not allow very accurate determination of the rate constant ($K = 9\text{-}13\ M^{-1}s^{-1}$). The lower value in table 3.1 represents the repair process assuming that no heat denaturation of the enzyme occurred within the 2.5 hours period that the reaction was followed. The higher value was obtained assuming that the initial amount of alkyltransferase added, was less by a fraction equal to the amount which was inactivated over the 2.5 hours incubation period.

3.4. DISCUSSION

The results in this chapter report, for the first time, rates of repair of

oligonucleotides containing O^6-methylguanine, O^6-ethylguanine and O^4-methylthymine by

the E. coli *ogt* and human alkyltransferases. The same, or similar, oligonucleotides and the

ly used to measure the rates of repair of single alkylated bases

ferase. The rate of repair of O^6-methylguanine by the *ogt*

rate obtained previously for the repair by the *ada* protein and

maximum (10^9 $M^{-1}s^{-1}$) for a diffusion limited second order

ests that almost every collision between the oligonucleotide

rect orientation of the active centre and O^6-methylguanine leads

oup from the oligomer to the enzyme. Unexpectedly, the

for the repair of O^6-ethylguanine and O^4-methylthymine was

of the *ada* protein. The *ogt* protein repairs O^6-ethylguanine

mine 83 times faster than the ada alkyltransferase (figure 3.5).

e about 10-20 fold more *ogt* molecules per cell than *ada*

1990; Rebeck *et al.*, 1989). Taking this into consideration and

the high efficiency of the *ogt* protein for the repair of O^6-methylguanine, O^6-ethylguanine

and O^4-methylthymine, it seems very likely that in uninduced conditions this protein rather

than the *ada* protein is responsible for the protection of the E. coli cells against the

hazardous effects of these alkylated bases.

It was recently shown that the same cysteine of the *ogt* protein accepts the alkyl

group from both O^6-alkylguanine and O^4-alkylthymine (Rebeck *et al.*, 1989), as it was

previously shown for the *ada* protein (Demple *et al.*, 1985). Considering the extensive

similarities of the *ada* and *ogt* proteins in the amino acid sequence around the alkyl-

accepting cysteine residue, it is surprising that the proteins repair O^6-alkylguanine and

O^4-alkylthymine with such different efficiency. It is possible that amino acid sequences

away from the alkyl accepting cysteine, do participate in the formation of the active centre
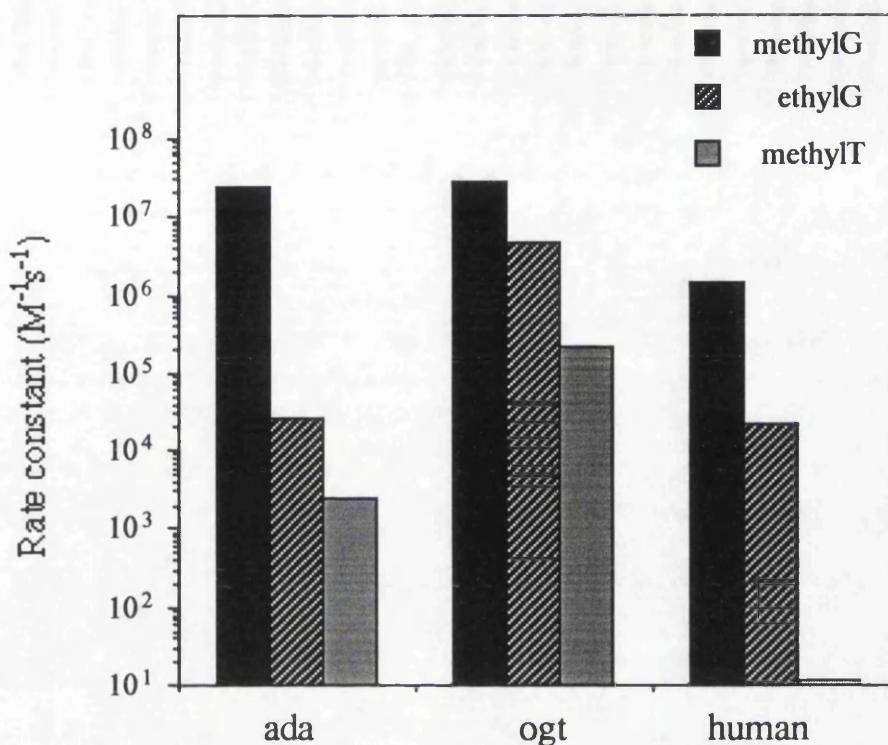
Figure 3.5 : Comparison of the efficiencies of the *ada, ogt,* and human alkyltransferases

for the repair of O^6-methylguanine (methylG), O^6-ethylguanine (ethylG), and

O^4-methylthymine (methylT).

Table 3.1 : Repair of synthetic oligonucleotides by O^6-alkylguanine-DNA-alkyltransferases

The oligonucleotides were CGCalkylGAGCTCGCG and CGCAAGCTmeTGCG. The rate

constants for the repair by the the *ada* protein are from Graves *et al.* (1989).

Substrate	*ada*	*ogt*	human (25 °C)
O^6-methylG:C	2.5×10^7 $M^{-1}s^{-1}$	2.9×10^7 $M^{-1}s^{-1}$	1.4×10^6 $M^{-1}s^{-1}$
O^6-ethylG:C	2.6×10^4 $M^{-1}s^{-1}$	4.5×10^6 $M^{-1}s^{-1}$	2.3×10^4 $M^{-1}s^{-1}$
O^4-methylT:A	2.5×10^3 $M^{-1}s^{-1}$	2.1×10^5 $M^{-1}s^{-1}$	9-13 $M^{-1}s^{-1}$

of these enzymes. However structural studies are needed to elucidate the amino acid regions involved in the function of these enzymes.

Very recently Sassanfar *et al.* (1991) measured the relative efficiencies of the *ada*, *ogt*, and yeast and human alkyltransferases for the repair of O^6-methylguanine and O^4-methylthymine. Their results with the *ada* and *ogt* proteins agree in principle with the results presented in this chapter and published previously (Wilkinson *et al.*, 1989). They too found that the *ogt* is significantly more efficient than the *ada* protein in repairing O^4-methylthymine. However, they observed that the *ogt* protein repairs O^4-methylthymine more efficiently than O^6-methylguanine, whereas the results reported here show the opposite (figure 3.5). The differences between the two studies are probably the result of the different methodology used. The assay used in this chapter is specific for the alkyltransferase and measures directly the formation of repaired non-alkylated oligomer. Sassanfar *et al.* (1991) measured the amount of alkyltransferase available to react with high molecular weight alkylated DNA, after preincubation of the enzyme with oligomers containing O^6-methylguanine or O^4-methylthymine. Thus, their results (as stated in their discussion) do not reflect the efficiency of the methyl transfer but the efficiency with which the alkyltransferase recognises (bound to and repair) the methylated DNA substrates.

The mutagenicity of O^6-methylguanine, O^6-ethylguanine, and other higher alkylguanine derivatives in E. coli have been examined in several laboratories using site-directed mutagenesis techniques (Rossi and Topal, 1991; Champers, 1991; Pauly *et al.*, 1991). It was generally observed that O^6-ethylguanine is more mutagenic than O^6-methylguanine. This order of mutagenicity could reflect the efficiency of repair of these adducts by the *ogt* protein but not the efficiency of repair by the *ada* protein, in which case differences larger than those observed would be expected. However, other factors (i.e. miscoding frequency, other repair systems like the uvrABC system) might contribute to the mutation frequency of O^6-alkylguanine. O^6-alkylguanine residues with bulkier alkyl groups are significantly less mutagenic than both O^6-methylguanine and O^6-ethylguanine, which is an indication for the involvement in their mutagenicity of parameters other than the

efficiency of repair by alkyltransferases.

Evidence that O^4-methylthymine is a substrate, albeit a poorer one than O^6-methylguanine, for the *ogt* protein in vivo, came also from site-directed mutagenesis experiments in E. coli. Dosanjh *et al.* (1991) transfected E. coli with M13 containing a unique O^6-methylguanine or O^4-methylthymine. While both adducts were miscoded in vitro (Singer *et al.*, 1989; Dosanjh *et al.*, 1990) only O^4-methylthymine was highly mutagenic, in cells with fully functional *ogt* and *ada* proteins. In *ada*- mutants the mutagenicity of O^6-methylguanine was increased, whereas that of O^4-methylthymine was not altered. However the O^4-methylthymine mutagenesis was more than doubled when the *ogt* protein was depleted by pretreatment of the cells with MNNG. These findings are in agreement with the relative efficiencies for the repair of O^6-methylguanine or O^4-methylthymine by the E. coli *ada* and *ogt* proteins as presented in this chapter.

The kinetics of repair by the human akyltransferase could not be followed at 37 °C because of the enzyme's instability in solution. The incubation temperature was thus reduced to 25 °C and the reaction was followed for 1 h, over which period no significant inactivation of the enzyme occurs ($\approx$ 6%). Thermodynamically a temperature decrease of 12 °C should reduce the rate of repair by about 2 fold. The human alkyltransferase seems to be less efficient than the ada protein in repairing O^6-methylguanine, while at the same time more efficient in repairing O^6-ethylguanine (figure 3.5, table 3.1). As a result of this, the human alkyltransferase repairs O^6-ethylguanine only 60 times slower than O^6-methylguanine, whereas the respective difference in repair by the *ada* protein is about 1,000 fold. This is in agreement with previous observations made using rat liver extracts and DNA alkylated by N-methyl- or N-ethyl-N-nitrosourea, and could suggest that the active centre of the human protein can accommodate bulkier adducts more easily than can the *ada* protein.

The human alkyltransferase does repair O^4-methylthymine (figure 3.4 and table 3.1). Relevant work led to the same conclusion (Koike *et al.*, 1990; Sassanfar *et al.*, 1991). However, Koike *et al.* (1990) provided evidence only for the dealkylation of O^4-

methylthymine, without any reference to the rate of reaction, and Sassanfar *et al.* (1991)

measured the extent of alkyltransferase inhibition by an oligomer containing O^4-

methylthymine   , rather than the extent of DNA dealkylation. The method used in

this chapter measures directly the rate of dealkylation and the results show that under the

assay conditions, the transfer of the alkyl group from the DNA to the enzyme proceeds at

an extremely slow rate. If we were to extrapolate the in vitro kinetic parameters for the

repair of O^6-methylguanine and O^4-methylthymine to the in vivo situation, the contribution

of the akyltransferase in the removal of O^4-methylthymine from DNA must be extremely

low if not negligible. However, O^4-alkylthymine is enzymatically removed in vivo, and the

rate of its removal does not always reflect the level of the alkyltransferase activity. When

hamsters are treated with a single high dose of dimethylnitrosamine, the alkyltransferase in

the liver is totally depleted, without being regenerated for at least 4 days after treatment.

However, over that period, O^4-methylthymine is still being actively removed, whereas no

reduction in the levels of O^6-methylguanine is observed (Hall *et al.*, 1990). The likelihood

that the mammalian alkyltransferase is not responsible for the repair of O^4-alkylthymine in

vivo, is also supported by a recent study by Wani *et al.* (1991). They used a very sensitive

immunoslot-blot assay and showed that similar quantities of O^4-ethylthymine are removed

from ethyl-DNA by human brain, kidney and liver tissue extracts which is not compatible

with the significant differences of these tissues in their alkyltransferase content.

The oligomers used in the present study are self-complementary and can adopt

either a duplex or a hairpin-loop structure (figure 3.6). It initially seemed reasonably safe to

assume that the oligomers were linear B-DNA duplexes under the assay conditions both

because 2-D NMR spectra showed that they form normal B-DNA helixes in solution

(Kalnik *et al.*, 1988 a,b; Kalnik *et al.*, 1989 a,b), and because the O^6-methylguanine

containing oligomer was repaired extremely fast by all three alkyltransferases while it is

known that single stranded DNA is a poor substrate for these enzymes (Lindahl *et al.*,

1982). The DNA melting studies though, showed quite irregular denaturation profiles

(figure 3.7 A). Oligomers containing O^6-alkylguanine or O^4-alkylthymine were much less

2

```
        C:G
        G:C
        C:G
       *G:C                6 G—C 7
      5 A:T 8              5 A   T 8
      6 G:C 7   ←——→        *G:C
      7 C:G 6                C:G
      8 T:A 5                G:C
        C:G*                 C:G
        G:C                hairpin
        C:G
        G:C
     B-DNA duplex
```
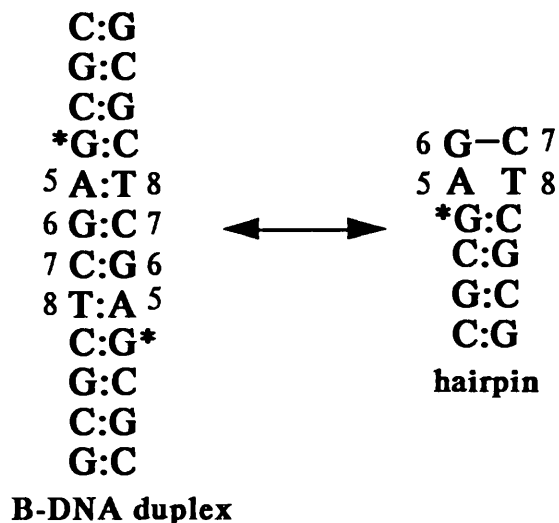
Figure 3.6: Possible conformations for the CGCalkGAGCTCGCG oligomer. The hairpin could have a two member loop (G6C7) or a four member loop (A5G6C7T8).

stable than the respective parent sequences, they melt over an extended temperature and the melting curves were either biphasic or had no obvious transition point. These findings are not in agreement with a unique duplex to coil transition. In contrast, a non-self-complementary oligonucleotide containing O^6-methylguanine, with the same sequence around the modified base as the self-complementary oligomer, gave the expected sigmoidal melting curve of figure 3.7 B.

It has been shown that perfect self-complementary oligonucleotides exist as a mixture of two slowly interconverting species which were identified as the dimeric duplex and the monomeric hairpin-loop structures (Hilbers et al., 1985; Wemmer et al., 1985; Xodo et al., 1988; Pieters et al., 1989). The equilibrium between the two conformations is shifted in favour of the one or the other form depending on the oligomer concentration, the ionic strength and the temperature. At high oligomer and salt concentrations the dimeric duplex is the predominant structure whereas at low concentrations formation of hairpins is strongly favoured. Thus, it is not surprising that the NMR studies, where very high concentration of oligomers was used, showed exclusively the presence of B-DNA helixes.
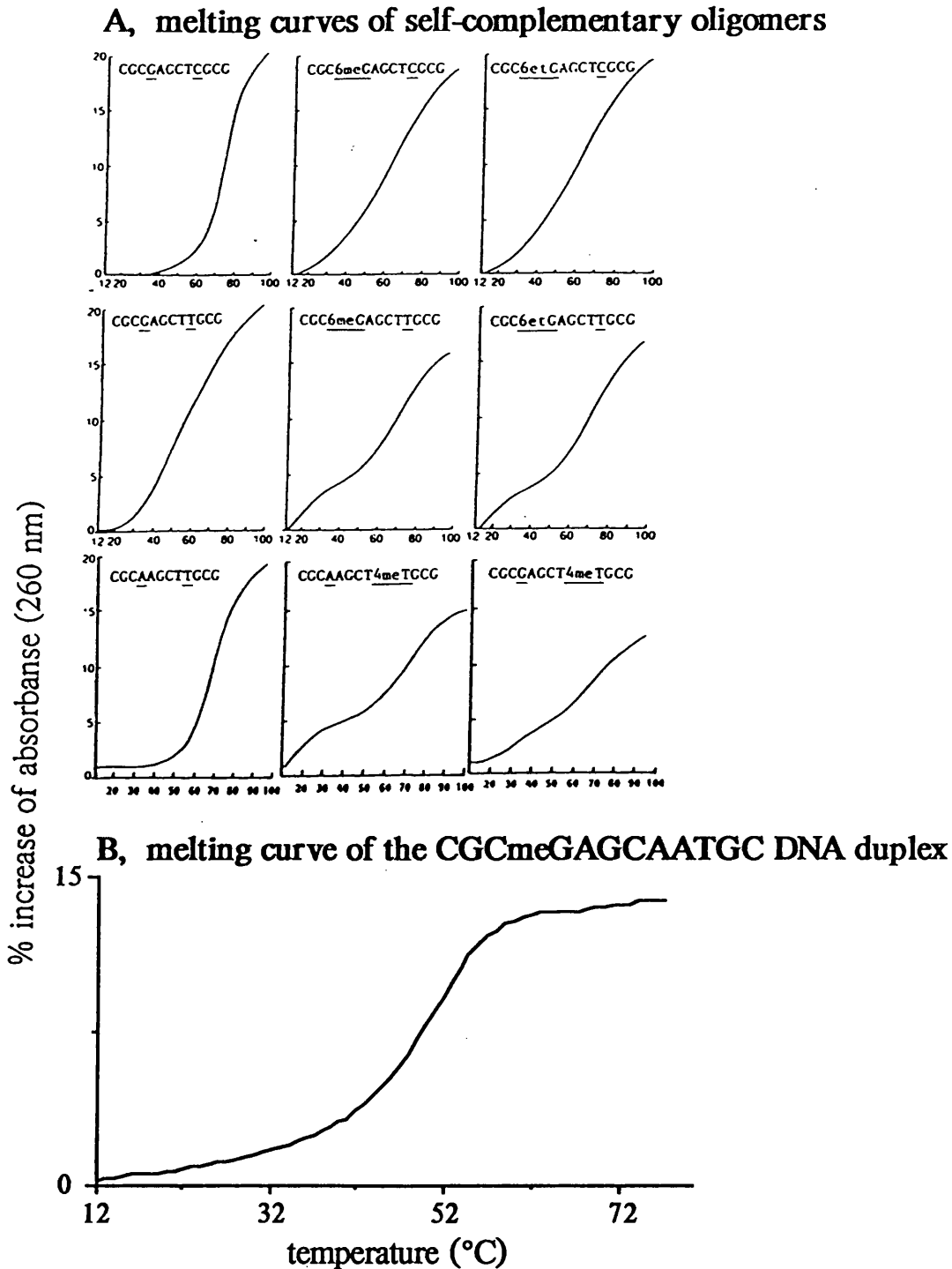
Figure 3.7 : Thermal melting curves of alkylated and parent oligonucleotides. A, self-complementary oligonucleotides. The data is from Li *et al.* (1987) and Li and Swann (1989). B, Melting curve of a non-self-complementary oligonucleotide duplex containing O^6-methylguanine, with the same sequence flanking the modified base as the self-complementary oligomers. The oligomers were in a solution 1 M NaCl, 10 mM KH_2PO_4, 1 mM EDTA pH 7. The rate of temperature increase was 1°C / min.

On the other hand, the DNA melting studies were performed at a much lower oligonucleotide concentration and the biphasic nature of the melting curves could suggest the existence of a mixture of duplex and hairpin structures. The first transition probably represents the duplex to hairpin conversion while the second one, the hairpin to coil transition (Xodo *et al.*, 1989). Therefore it is believed that oligomers in nM concentration, at medium salt concentration and moderate temperature, as used in the repair assay, would have almost exclusively the hairpin-loop structure. Further evidence arguing for the hairpin-loop structure for these self-complementary oligomers are presented in the following chapter.

CHAPTER 4

SELECTIVE REPAIR, AND CONFORMATIONAL DIFFERENCES, BETWEEN O⁶-METHYLGUANINE RESIDUES IN DIFFERENT POSITIONS IN, AND AROUND, THE 12TH CODON OF RAT H-RAS.

4.1. INTRODUCTION

In the previous chapter the effect of the nature of the alkylated adduct (i.e. alkylG or alkylT), and the size of the alkyl group (i.e. methylG or ethylG), on the repair by different alkyltransferases was presented. The purpose of the experiments described in this chapter was to study the influence of the bases surrounding an O⁶-alkylguanine residue on the efficiency of its repair by the alkyltransferase.

Most mammary tumours (Zarbl *et al.*, 1985) and skin tumours (Brown *et al.*, 1990) induced by N-methyl-N-nitrosourea have a G:C to A:T transition mutation involving the second, but not the first, G of codon 12 (normally GGA) of the H-ras protooncogene. The mutation can be detected shortly after the administration of the carcinogen and is believed to be caused by alkylation of the O⁶ of guanine (Kumar *et al.*, 1990; Miyamoto *et al.* 1990). Transfection experiments have shown that a G:C to A:T transition mutation in the first G would be equally effective in transformation of NIH 3T3 cells (Seeburg *et al.*, 1984), and it is not known why only the second G is mutated. This selectivity is also seen in eukaryotic cell lines (Dubridge *et al.*, 1987) and prokaryotes (reviewed by Gordon *et al.*, 1990). For example Richardson *et al.* (1987a, 1988) using the gene for the xanthine-guanine phosphoribosyl-transferase (gpt gene) as target for DNA alkylation by different direct-acting nitroso-alkylating agents, found a strong bias for G:C to A:T mutations to occur when the 5' flanking base is guanine and the 3' base is an adenine or thymidine. A similar bias was seen by Glickman and colleagues while studying the mutational specificities of a number of alkylating agents in the lacI gene of E. coli (Burns *et al.*, 1987; Horsfall and Glickman, 1988; Burns *et al.*, 1988a; Burns *et al.*, 1988b;

Horsfall *et al.*, 1989; Gordon *et al.*, 1988). Sequence analysis of the mutants revealed that guanines preceded 5' by a purine are ten times more likely to be mutated than those preceded by a pyrimidine residue.

There are three possible explanations for this selectivity. First, the second G in this sequence of DNA may be more readily alkylated than a guanine residue in other sequences; second, the cell may be unable to repair an O^6-alkylguanine when it is in this position; and, third, that an O^6-alkylguanine flanked by this sequence miscodes with much higher frequency than in other sequences. There has been support for all three of these possibilities.

1. Sequence selectivity in the formation of different DNA adducts by chemical carcinogens is well established. The N7-guanine adduct of aflatoxin B1, the N2-guanine adduct of benzo(a)pyrene diol epoxide, and N7-alkylguanine produced by chloroalkylating agents, are all preferentially formed within successive guanines (Boles and Hogan, 1984; Hartley *et al.*, 1986; Mattes *et al.*, 1988; Benasutti *et al.*, 1988; reviewed by Richardson *et al.*, 1990). A very elegant method was developed to determine whether O^6-methylguanine is also formed in a sequence dependent fashion (Richardson *et al.*, 1989). Oligonucleotides were synthesized containing a unique [^{3}H]-deoxyguanosine. The radiolabelled nucleotide was located at different sites within the otherwise identical base sequence of the oligomers, and after alkylation with N-methyl-N-nitrosourea, the relative amounts of [^{3}H]-O^6-methylguanine formed at each position, were quantitated. A five to six fold greater formation of O^6-methylguanine was observed when the guanine, target for alkylation, was preceded 5' by a purine than a pyrimidine. However, the sequence preference for alkylation observed in this study, was not directly reflected by the mutational spectra observed in vivo. Differences in the electrostatic potentials, and steric constrains might explain the bias for O^6-alkylguanine formation at selective sites (Topal, 1988). In addition, Buckley (1987) proposed a regio-selective mechanism for selective alkylation at the O^6 position of the second G in GG sequences. According to this mechanism, N-methyl-N-nitrosourea attacks the O^6 position of the first G in GG sequences forming an unstable intermediate

and subsequently, the alkyl group is transferred to the O^6 or N^7 of the second G. This mechanism can explain the distribution of mutations caused by direct acting alkylated agents like N-methyl-N-nitrosourea and N-methyl-N-nitroso-N'-nitroguanidine only if these agents reach the nucleus without previous decomposition to other species.

2. Topal *et al.* (1986) supported the second possibility and suggested that the uneven distribution of mutations caused by alkylating agents reflects sequence specificity in the repair process. They also proposed that the mutational specificity for the second G of the GGA 12th codon of H-ras occurs because an O^6-alkylguanine in this position cannot be repaired efficiently. This interpretation was supported to some extent by Dolan *et al.* (1988a) who observed a 2-fold slower repair of O^6-methylguanine in self-complementary oligonucleotides when the base 5' to the O^6-methylguanine was a guanine rather than a cytosine.

3. Finally, the frequency of misincorporation of thymine opposite O^6-methylguanine, during DNA replication in vitro, was found to be dependent on the flanking bases (Singer *et al.*, 1989b; Singer and Dosanjh, 1990).

None of the observations mentioned above are sufficient to explain the exclusive mutagenesis of the second guanine of the 12th codon in H-ras. In the present study Topal's hypothesis, that differences in DNA repair is a main contributor to this mutational specificity, was tested. The results support this view and suggest that the selectivity for the second G in codon 12 results from a combination of selective alkylation and defective repair with defective repair probably being the more important but neither being sufficient alone.

Short lengths of DNA (15-mers) were synthesized having the same sequence as H-ras around the 12th codon, except that the first or second guanine residue of codon 12 had been replaced by an O^6-methylguanine. Similar DNA sequences were also synthesized differing from the H-ras sequence in that one guanine was replaced by O^6-methylguanine and the neighbouring 5' or 3' base was also changed. The accurate rate constants for the repair of these DNAs by the E.coli *ada* gene O^6-alkylguanine-DNA-alkyltransferase were

measured. The *ada* alkyltransferase was chosen for two reasons. The first was practicality (the purified mammalian enzyme was not available at the time this work was done). The second was that, as has been discussed above, the mutational specificity for the 3' G in GG sequences is seen in procaryotes as well as eukaryotes and is therefore not a property peculiar to the eukaryotic system. The rate of repair differed very greatly. In particular an O^6-methylguanine in the second position of the H-ras sequence was repaired 18 times more slowly than one in the first position. Previous measurements of this type have relied on reverse-phase HPLC to separate the alkylated from the repaired oligomers, but it was found that this method could not separate the 15-mers used in these experiments. Instead the separation was done using antibodies to O^6-methyldeoxyguanosine (Souliotis *et al.*, 1989; Mironov *et al.*, 1989). This led to the unexpected finding that the avidity of the antibody to the methylguanine was influenced by the sequence in which that methylguanine was placed, and that the sequences in which the O^6-methylguanine was most accessible to the antibodies were also the sequences in which the O^6-methylguanine was most rapidly repaired. These related results show that the conformation of the alkylated base pair and the DNA around the alkylated base must be strongly dependent on the flanking sequence.

4.2. MATERIALS AND METHODS

4.2.1. Enzymes and antibodies

T4 polynucleotide kinase was obtained from Amersham International and alkaline phosphatase and phosphodiesterase (Crotalus durissus) from Boehringer Mannheim. Purified E. coli *ada* O^6-alkylguanine-DNA-alkyltransferase (19,000 Daltons) was provided by Dr D. Yarosh (Applied Genetics Inc. Freeport, N.Y).The method for raising polyclonal antibodies against O^6-methyldeoxyguanosine was previously described (Kyrtopoulos and Swann, 1980).

4.2.2. Oligodeoxynucleotides

Chemicals for oligonucleotide synthesis were obtained from Cruachem Ltd. (Glasgow,Scotland). The oligonucleotides containing O^6-methylguanine were prepared by solid phase synthesis on a Cruachem DNA synthesizer as described in chapter 2. The structures of the oligonucleotides used are shown in table 4.1. Two of the 15-mers have the sequence of part of the rat H-ras gene with the O^6-methylguanine in a position analogous to either the first or the second position of the 12th codon (i.e -T.GmeGA.G- and -T.meGGA.G) , and two have single base changes in that codon so that the bases flanking the methylguanine are changed (i.e -T.TmeGA.G, -T.meGAA.G-). The dodecamers also shown in table 4.1, were used for comparative experiments. The DNA complementary to the alkylated strands and the non-alkylated analogues of the alkylated strands were synthesized by the phosphoramidite solid phase method. The self-complementary dodecamer CGCmeGAGCTCGCG was synthesized in solution by the phosphotriester procedure (Li and Swann, 1989). The purification procedure for these oligomers was described in detail in chapter 2. The purity of each oligonucleotide was measured by enzymic digestion of the oligomer to nucleosides and chromatographic comparison of the integrated areas of the uv-absorbing peaks with those of a standard mixture of nucleosides (see chapter 2).

Table 4.1: Synthesized oligonucleotides containing O^6-methyldeoxyguanine. The underlined triplets for the pentadecamers correspond to codon 12 of rat H-ras oncogene.

Pentadecamers	abbreviation
GGCGCTmeGGAGGCGTG	-T.meGGA.G-
GGCGCTGmeGAGGCGTG	-T.GmeGA.G-
GGCGCTTmeGAGGCGTG	-T.TmeGA.G-
GGCGCTmeGAAGGCGTG	-T.meGAA.G-
GGCGCTGGAGGCGTG	-T.GGA.G-
GGCGCTTGAGGCGTG	-T.TGA.G-

dodecamers

CGCmeGAGCTCGCG

CGCmeGAGCAATGC

4.2.3. [5'-^{32}P] labelling of oligonucleotides

Fifty picomole of the O^6-methylguanine containing oligomer, 100 pmol of [gamma - ^{32}P] ATP (3000 Ci / mmol), and 10 units of T4 polynucleotide kinase were incubated at 37°C in a solution containing 70 mM tris.HCl pH 7.6, 10 mM dithiothreitol (DTT), 1 mM spermidine, 10 mM MgCl$_2$. After 30 min 10 more units of T4 polynucleotide kinase were added and after a further 30 min incubation the reaction was terminated by heating at 65 °C for 5 minutes.
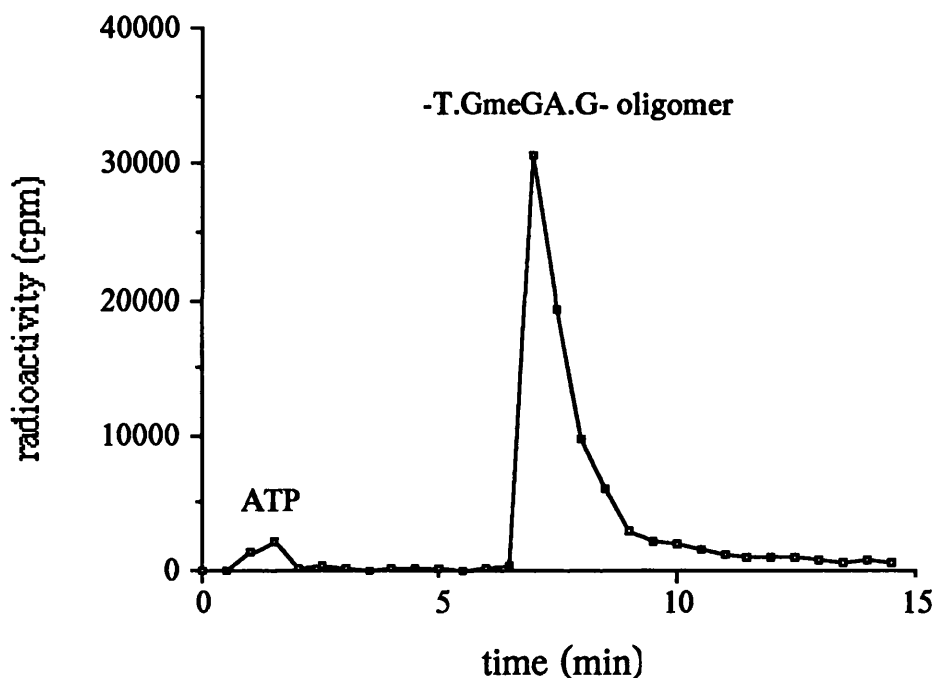
Figure 4.1 : Chromatographic analysis of the P^{32}-labelled -T.GmeGA.G- oligomer showing that almost all the radioactivity is associated with the oligomer. The column used was nova-pack C18 (phenyl), the buffers were: A, 0.33 M KH$_2$PO$_4$pH 6.3; and B, 0.33 M KH$_2$PO$_4$, 33 % CH$_3$CN pH 6.3, and the elution gradient was buffer A and a 2 % per min increase of buffer B; flow 3 ml/min

Unincorporated ATP was removed by passing the material twice through Biospin P6 columns (Biorad). HPLC of the labelled oligonucleotides showed that most of the

radioactivity was associated with the oligonucleotide peak [commonly 95-99%] (figure 4.1). Using this procedure a specific activity of 3,000 cpm per fmol of oligomer was obtained. The labelled oligonucleotide was then commonly diluted (up to 10 fold) with unlabelled oligomer. Samples were freezed dried and resuspended in a solution containing the complementary strand in 50 mM Tris HCl, pH7.6, 10 mM DTT, 1 mM EDTA . In order to ensure that the ^{32}P-labelled strand would quantitively form double stranded DNA, 20% excess of complementary strand was added. The solution containing both strands of DNA was heated to 80°C for 2 minutes and allowed to cool slowly to room temperature over a period of about 1 h to anneal.

The O^6-alkylguanine-DNA-alkyltransferase concentration was measured by its reaction with an excess of a self-complementary dodecamer containing O^6-methylguanine. The amount of dodecamer still containing O^6-methylguanine after the reaction was measured by HPLC (Graves *et al.*, 1989). The amount of the alkylated 15-mer was measured by its reaction with a known quantity of E. coli *ada* (19,000 daltons) O^6-alkylguanine-DNA-alkyltransferase.

4.2.4. DNA melting curves

These were obtained by measuring the temperature dependent changes in absorbance at 260 nm using a Unicam SP500 spectrophotometer (Pye Unicam, Cambridge, U.K), fitted with a Gilford 222 photometer and a Gilford thermoprogrammer (Gilford instruments,Oberlin OH). The oligonucleotide duplexes had an absorbance of 0.9 at 260 nm at 14 °C in 1 M NaCl, 10 mM KH$_2$PO$_4$ pH 7. The temperature was increasing by 1 °C / min. The melting temperature (Tm) values were calculated as the temperature at which the hyperchromicity was half its final value.

4.2.5. Measurement of the repair of alkylated double stranded DNA with E.coli O[6]- alkylguanine-DNA-alkyltransferase using an immunoprecipitation assay

The kinetic constants for the reaction between oligonucleotides and alkyltransferase was measured at 37 °C in a reaction mixture containing 50 mM Tris.HCl pH 7.6, 10 mM DTT, 1 mM EDTA and 200 mg/ml bovine serum albumin. At intervals after addition of the alkyltransferase to the methylated DNA, 20-50 μl of the reaction mixture were transferred to Eppendorf-like test tubes, and immediately frozen by plunging in liquid N_2 to stop the repair process. The amount of alkylated oligomer remaining in each sample was measured by immunoprecipitation using antiserum to O[6]-methyldeoxyguanosine raised in rabbits (Souliotis *et al*, 1989). The samples were transferred to an ice bucket and while still frozen, 10 x PBS (NaCl 40 g/l , KCl 1 g/l , Na_2HPO_4 5.75 g/l, KH_2PO_4 1 g/l) and antiserum (1:10) dilution were added so that the final solution was 1xPBS and 1:30 final antiserum dilution. After 30 min incubation on ice, the proteins were precipitated by adding an equal volume of saturated ammonium sulphate (pH 7). Immunoprecipitaton was left to proceed for 30 min at 0 °C. Samples were then centrifuged at 14,000 rev/min and 4 °C for 5 min, and the supernatant was carefully discarded. The precipitate was then resuspended in 100 μl of 50% saturated ammonium sulphate (pH 7) by vigorous vortexing. Following centrifugation at 14,000 rev/min and 4 °C for 5 min, the pellet was dissolved in 200 μl of 0.1 M NaOH. The solutions were then transferred to scintillation vials and the tubes were washed with water (200 μl) which was combined with the sample solutions. Scintillation fluid was then added (Ecoscint A, National diagnostics) and the radioactivity measured by scintillation counting.

If A_0 is the initial concentration of the oligomer, then the amount of unrepaired oligomer after time t is:

$$A_0\text{-}x = (\text{cpm t - cpm B / cpm 0 -cpm B}) A_0$$

Where cpm t is the radioactivity precipitated; cpm B is the background precipitation obtained after the oligomer had been fully repaired with excess enzyme; cpm 0 is the

radioactivity precipitated before the addition of the enzyme; x = amount of oligomer repaired. For a second order reaction

$$\ln [(B_0-x) A_0/ (A_0-x) B_0] = (B_0 - A_0)k\, t$$

Where k is the rate constant; and B_0 the initial concentration of the enzyme. Then a graph of $\ln [(B_0-x)A_0/(A_0-x)B_0]$ versus t is a straight line with a slope equal to $(B_0 - A_0)k$

4.2.6. Measurement of the avidity constants for the binding of O^6-methylguanine in oligonucleotides by antibodies to O^6-methyldeoxyguanosine

One hundred fmol of radiolabelled double stranded oligomer and varying amounts (over the range of 0.1-100 pmol) of the same but not labelled oligomer was incubated with antiserum as described above. The amount of antiserum added was sufficient to precipitate 40-60% of the radioactive oligomer when the non-labelled oligomer was not present into the solution. For some oligomers this precipitation was achieved using less antiserum than for others. In these cases, gamma-globulin was added in order to obtain the same total protein concentration for all the experiments. The immunoprecipitation was performed as described above except that polyethylene glycol 6000 (24% w/v in water) rather than saturated ammonium sulphate was used as the precipitating agent.

The anti-O^6-methylguanine-oligomer avidity constants were determined using the method by Steward and Petty (1980). The interaction between the antigen and antibody is a reversible reaction and from the law of mass action the Langmuir equation is easily obtained.

$$1/b = 1/K\, c\, Ab + 1/Ab \qquad (1)$$

Where b is the concentration of the bound antigen; c is the concentration of the free antigen; Ab is the total antigen binding sites and K is the avidity constant. The bound and free antigen were calculated as follows:

$$b = cpm\, 2 - cpm\, 3/ cpm\, 1 \times n \qquad (2)$$

$$c = n - b \qquad (3)$$

where: cpm 1 is the total radioactivity of the reaction mixture; cpm 2 is the radioactivity

precipitated in the presence of antiserum; cpm 3 is the radioactivity precipitated in the

absence of antiserum; n is the total concentration of oligomer.

The total antigen binding sites (Ab) were determined from equation (1) by extrapolation of

the plot of $1/b$ versus $1/c$. (When $1/c$ approaches 0 then $1/b$ approaches $1/Ab$). The

avidity constants were then obtained from the plot of $\log(b/Ab-b)$ versus $\log c$ according

to the Sip's equation:

$$\log(b/Ab-b) = \log K + \log c \qquad (4)$$

4.3. RESULTS

The purity of the synthesized oligonucleotides was determined by chromatography and nucleoside analysis . All of them gave a single peak when chromatographed by reverse phase HPLC (see figure 2.5). Enzymic hydrolysis and chromatography of the nucleosides showed that more than 98% of the A_{260} absorbing material was associated with either dC, dG, T, dA or dmeG. Neither 2,6-diaminopurine nor partially deprotected nucleosides were detected. A small amount of inosine was observed due to deamination of adenine by contaminating adenosine deaminase in the alkaline phosphatase. Comparison of the integrated areas of the nucleosides with the areas of a standard mixture of nucleosides showed that the base compositions of the oligomers were identical to those expected (see chapter 2 figure 2.5 and table 2.2).

The melting curves of the DNA duplexes were obtained and the melting temperature (Tm) values were calculated (table 4.3). In agreement with previous observations (Gaffney et al., 1984; Gaffney and Jones, 1989; Li and Swann, 1989) DNA duplexes containing O6-methylguanine had less hypochromicity and lower melting point than the parent duplexes. The Tm for the -T.GmeGA.G- and -T.meGGA.G- duplexes were 68 °C and 66 °C respectively compared to the 77 °C of the parent -T.GGA.G- duplex. (figure 4.2 A). Both -T.meGAA.G- and -T.TmeGA.G- sequences had a Tm value of 63 °C compared to the 75 °C of the parent -T.TGA.G- sequence (figure 4.2 B).

Initially attempts were made to determine the rates of repair by separating the methylated from the repaired oligomer by reverse-phase HPLC, as had been previously done with shorter oligomers (chapter 3 and Dolan et al., 1988; Graves et al., 1989). In those attempts two problems were encountered. Unlike the self-complementary 12-mers which were eluted as a single peak when chromatographed, 15-mer duplexes were eluted as a mixture of single strands and double stranded DNA (figure 4.3). Using the HPLC conditions described in chapter 3, the alkylated and parent single stranded 15-mers were eluted with almost identical retention times (figure 4.4). Thus, the conditions had to be altered in order to achieve the required separation between parent and methylated
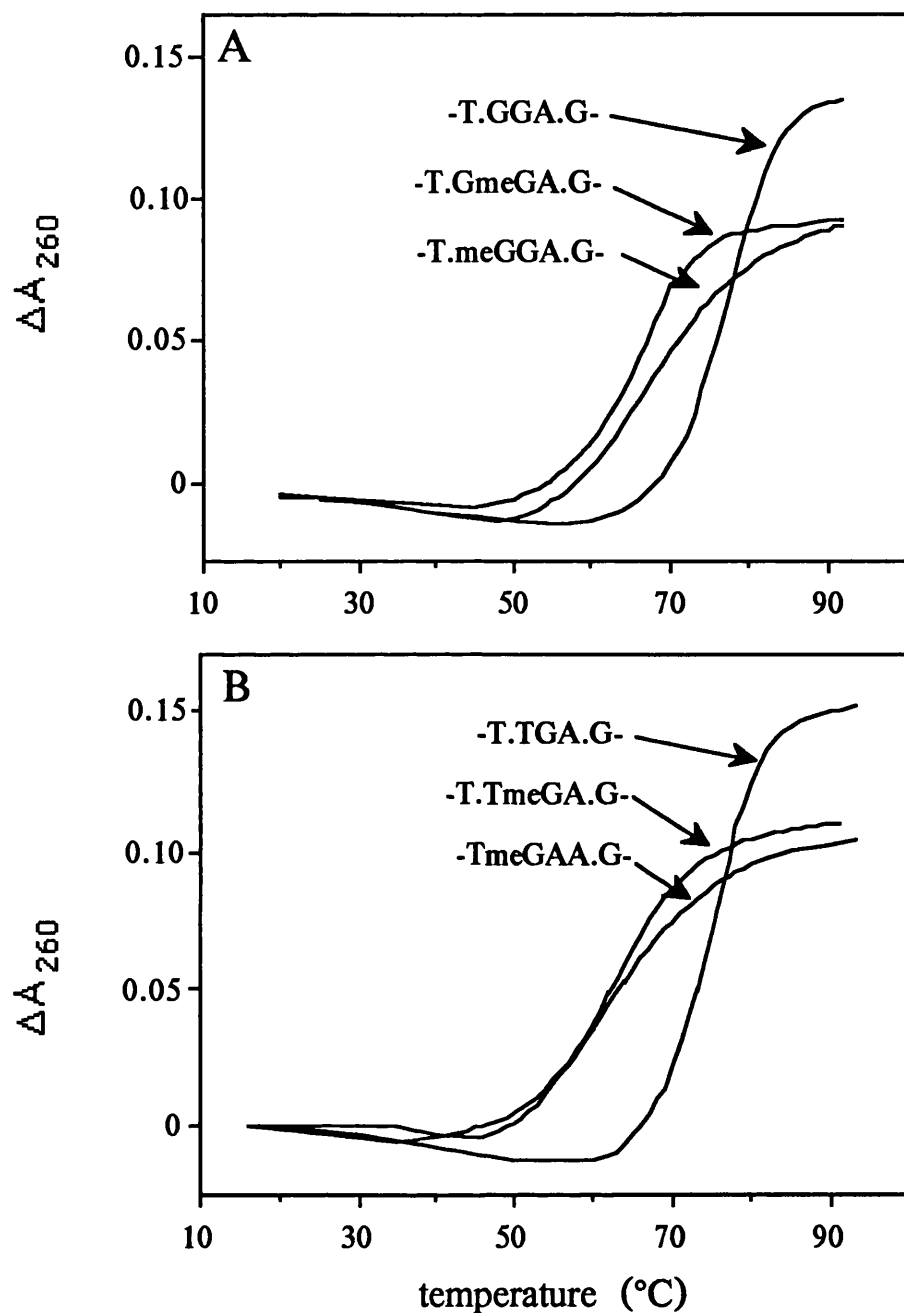
Figure 4.2: Melting curves at 260 nm for the: A, GGC.GCT.GmeGA.GGC.GTG

(-T.GmeGA.G-), GGC.GCT.meGGA.GGC.GTG (-T.meGGA.G-) and

GGC.GCT.GGA.GGC.GTG (-T.GGA.G-) duplexes and

B, GGC.GCT.TmeGA.GGC.GTG (-T.TmeGA.G-), GGC.GCT.meGAA.GGC.GTG

(-T.meGAA.G-) and GGC.GCT.TmeGA.GGC.GTG (-T.TGA.G-) duplexes. The

oligonucleotide solutions had an initial absorbance of 0.9 A260 units at 14°C.
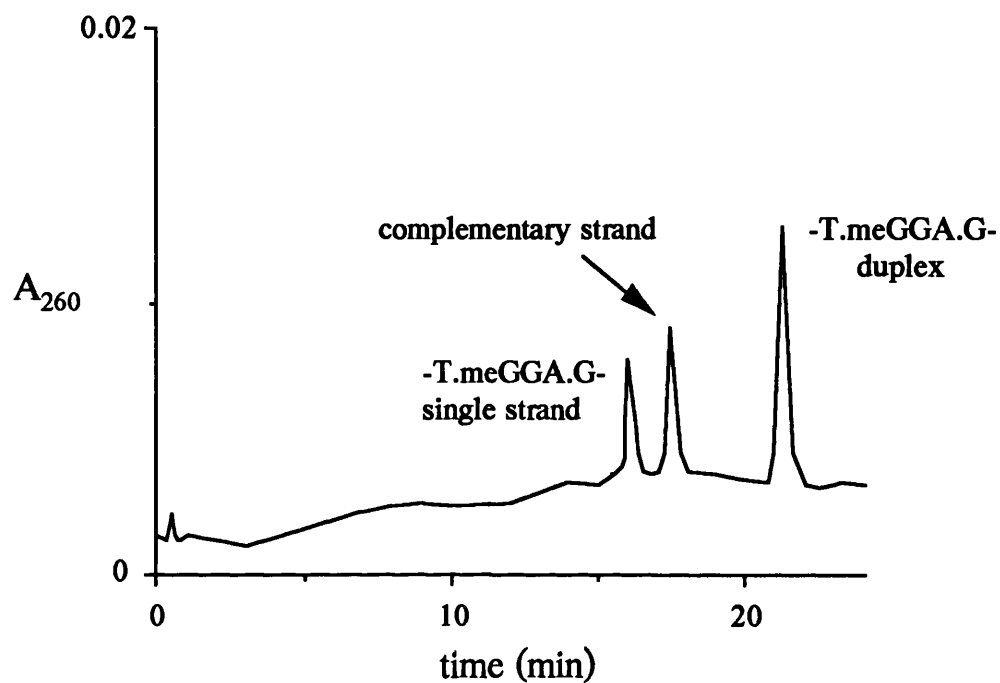
Figure 4.3 : Chromatographic analysis of the -T.GmeGA.G- duplex showing the partial

denaturation of the oligomer under the HPLC conditions. The column used was

Nova-pack C18 (phenyl), the buffers were: A, 0.33 M $KH_2 PO_4$ pH 6.3; and

B, 0.33 M $KH_2 PO_4$, 33 % CH_3 CN pH 6.3, and the elution gradient was buffer A and a

1% per min increase of buffer B; flow 3 ml/min and temperature 25 °C.
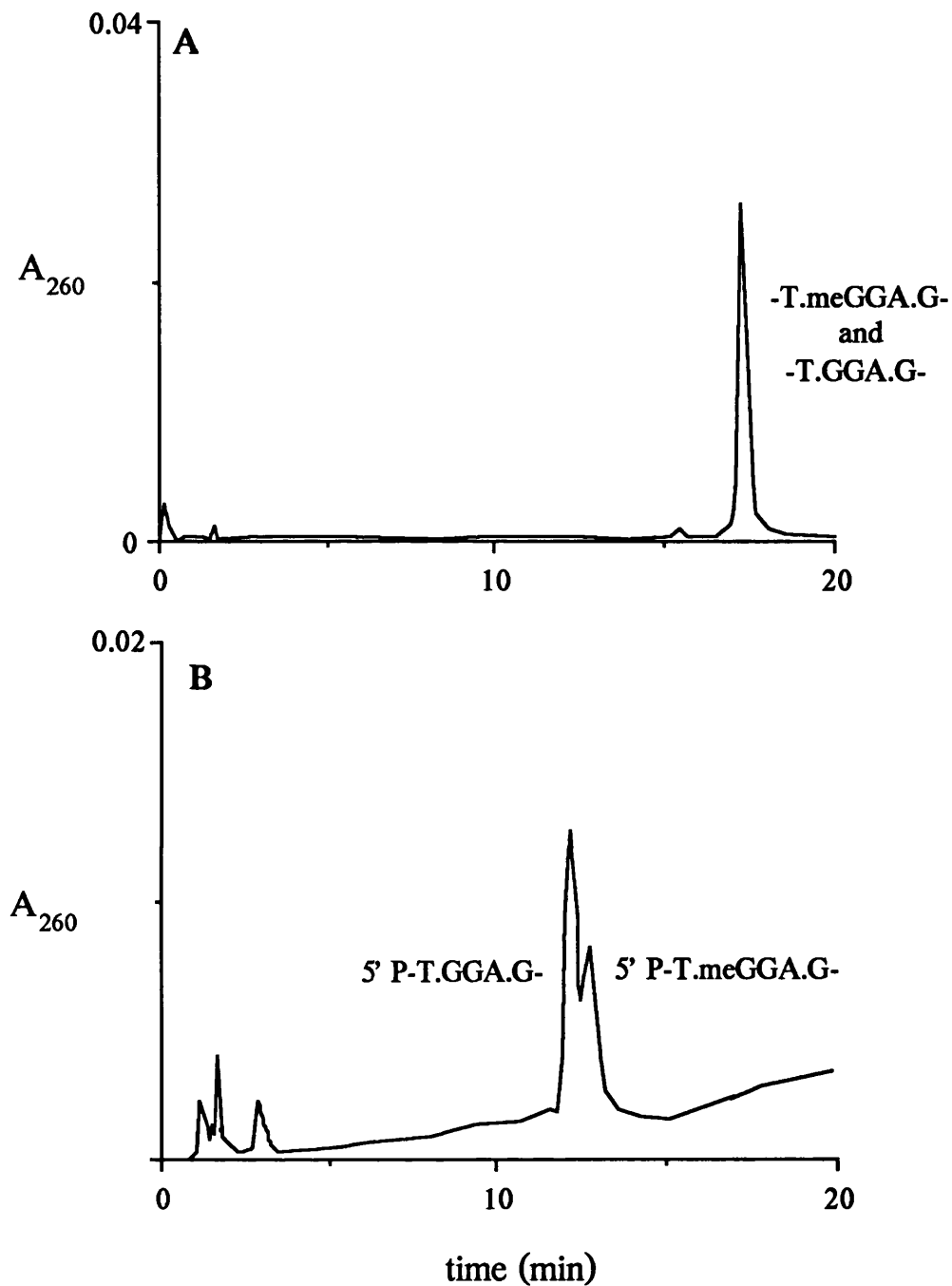
Figure 4.4 : Chromatographic analysis of a mixture of the -T.meGGA.G- and parent

-T.GGA.G- single stranded oligomers. A, 5' OH- oligomers and

B, 5' phosphorylated oligomers. The chromatographic conditions were as in figure 4.3.

oligomers, and complete denaturation of the DNA duplexes. To meet these objectives the buffers, the column, the elution gradient and the temperature were changed, but none of these changes led to satisfactory results.

However, the rate of repair can be measured by separating the DNA containing O^6-methylguanine from the repaired oligomer by immunoprecipitation with antibodies against O^6-methyldeoxyguanosine (Souliotis *et al.*, 1989; Mironov *et al.*, 1989). To validate this technique the rate of repair of the self-complementary dodecamer, CGCmeGAGCTCGCG, by the *ada* O^6-alkylguanine-DNA-alkyltransferase was measured using immunoprecipitation and the result compared to that obtained when HPLC was used to separate the alkylated from the repaired oligomer (Graves *et al.*, 1989).

The extent to which the dodecamer was precipitated depended on the amount of antiserum added, reaching a plateau in which 88-93% was precipitate when the final dilution of antiserum was 1:30. [2.5 µl antiserum / 75 µl assay volume] (figure 4.5). Complete precipitation was not achieved even when the antiserum was added undiluted to the reaction mixture. Using 1:30 final antiserum dilution less than 2% of the oligomer was precipitated after the O^6-methyl group had been removed by the *ada* gene alkyltransferase. A similar proportion of the demethylated oligomer was also precipitated when the antiserum was replaced by a gamma-globulin solution. The proportion of the methylated oligomer precipitated was independent of changes in its concentration over a range of 50-200 fmol / 75 µl assay volume, and addition of non-methylated parent oligomer over the same concentration range did not affect the precipitation of the methylated oligonucleotide (figure 4.6). These observations indicated that the antibodies were highly specific for the methylated oligomer, and that treatment with the antiserum would successfully separate the methylated from the repaired oligomer. The small proportion of the non-methylated oligomer found in the precipitate, probably resulted from a non-specific co-precipitation with the proteins, and not from a minor cross-reactivity of the antibodies. The 1/30 final antiserum dilution provided the large excess of antibodies which was required in order to obtain a constant extent of precipitation for varying amounts of the oligonucleotide. The latter was essential for the kinetic studies, because during the repair process the amount of
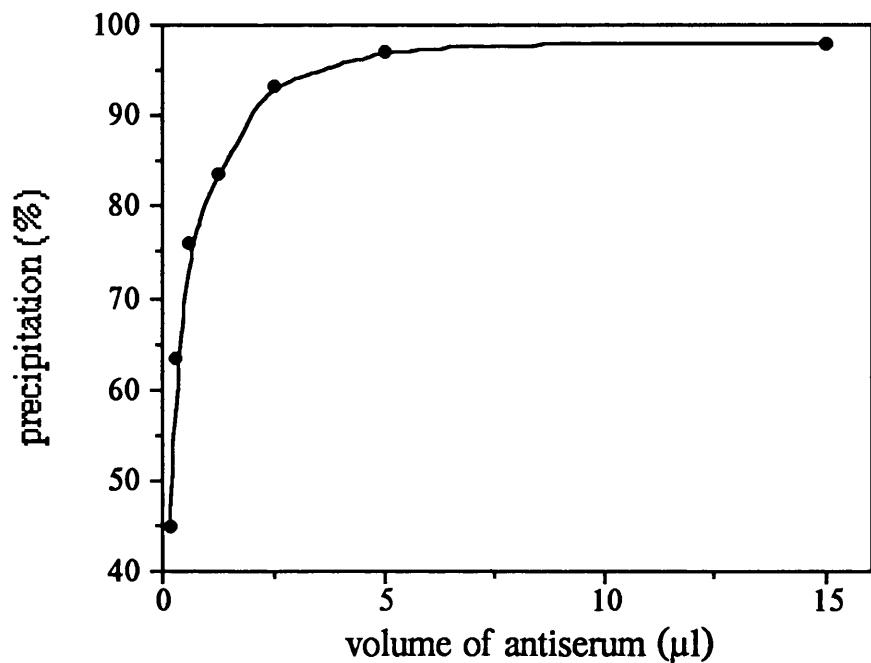
Figure 4.5 : Dependence of the precipitation of the CGCmeGAGCTCGCG, the self-complementary 12-mer, on the quantity of antiserum. 200 fmoles of oligomer in a 75 μl solution was precipitated with different antiserum dilutions as described under Materials and Methods.
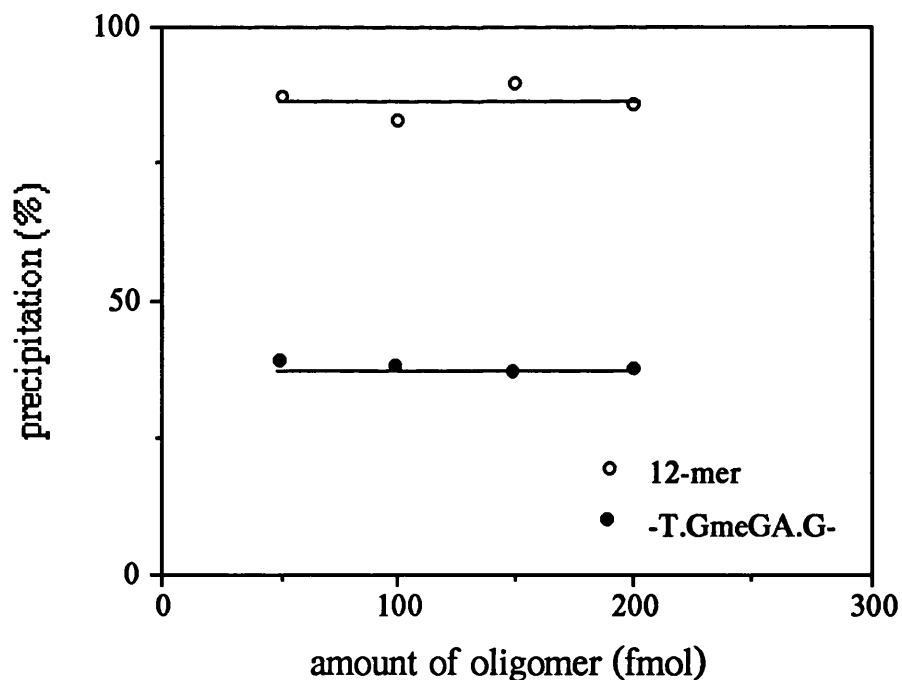
Figure 4.6 : Immunoprecipitation of different quantities of CGCmeGAGCTCGCG, the self-complementary 12-mer and the GGC.GCT.GmeGA.GGC.GTG (-T.GmeGA.G-) duplex by an 1:30 final antiserum dilution.

methylated oligomer decreases as it is converted to the non-methylated oligomer. In the previous chapter the time course for the repair reaction was obtained by quenching the samples, representing different time points, with a large excess of alkylated oligomer. This method however, cannot be applied when the repair process is to be followed by immunoprecipitation. It was attempted to stop the reaction by heat-inactivation of the enzyme, but *ada* protein is too stable to be significantly denatured after a short-lived exposure at high temperature. It was then suggested to us by Dr. Kyrtopoulos that the repair process could be followed by freezing the time-course samples with liquid nitrogen, and performing the antibody-antigen reaction at 0 °C (Souliotis *et al.,* 1990). Using this methodology a rate constant of $2.2 \times 10^7 M^{-1}sec^{-1}$ was obtained for the repair of the self-complementary 12-mer, which is virtually identical to that obtained (2.5×10^7 $M^{-1}sec^{-1}$) for the same oligomer when HPLC was used to separate the methylated from the repaired oligomer (Graves *et al.,* 1989). These results indicated that immunoprecipitation was a suitable method for following the dealkylation repair process of the the H-ras related sequences.

Only 0.6% and 0.5% of the non-methylated GGA and TGA sequences were precipitated by the antiserum (table 4.2) which was similar to the precipitation of the analogous O^6-methylguanine containing 15-mers after the methyl group had been fully removed by excess *ada* alkyltransferase. However, the antiserum did not precipitate every 15-mer containing O^6-methylguanine to the same extent. 77% of double stranded DNA formed from the -T.meGGA.G-, -T.TmeGA.G-, -T.meGAA.G- sequences was precipitated, but only about 36% of the duplex formed from the -T.GmeGA.G- sequence, in which the methylated base is in the position of the second G in the 12th codon of rat H-ras. The single stranded oligomers were more completely precipitated than the duplexes, but even in the single stranded form the GmeGA sequence was poorly precipitated (40% precipitated compared with 87% for the other sequences). Another oligomer, the dodecamer CGCAGmeGTGGTCG which contains the sequence around the 12th codon of human N-ras with the methylguanine again in the second position of the codon, was also poorly precipitated in the double stranded form (10%), but in this case the single stranded

Table 4.2 : Immunoprecipitation of the ras related and control sequences by the anti-O^6-methyldeoxyguanosine antiserum.

sequences	precipitation %		
	double strand	single strand	after total repair
-T.GmeGA.G- [1]	36	40	1.8
-T.GmeGA.G- [2]	38	43	
-T.meGGA.G-	77	86	0.8
-T.TmeGA.G-	77	87	1.3
-T.meGGA.G-	76	85	1.0
-T.GGA.G-	0.6		
-T.TGA.G-	0.5		
dodecamers			
CGCmeGAGCTCGCG[3]	89		1.4
CGCmeGAGCAATGC	61	86	0.6

The [1] and [2] indicate the two different synthesis of the GmeGA sequence. [3] Self-complementary oligomer.

DNA was precipitated well. It seemed unlikely that the poor precipitation of the -T.GmeGA.G- sequence occurred because the oligomer was impure, because when chromatographed on HPLC the oligomer was eluted as a single peak (figure 2.5 and figure 4.1) and the nucleoside analysis gave the expected base composition. However, in order to make certain that the poor precipitation of the GmeGA sequence was a property of that sequence rather than an artifact, the oligomer was resynthesized. The product of the second synthesis, gave very similar results to those obtained from the first synthesis (table 4.2).

For all DNA duplexes, including the -T.GmeGA.G- sequence, the proportion precipitated was independent of the duplex concentration, over the range used in the experiments, meaning that the rate constant for the repair of that sequence could be as accurately determined as the rates for the sequences that were more completely precipitated (figure 4.6).

The progress of the repair differed significantly between the sequences examined (figure 4.7). In order to quantitate the observed differences, the removal of the methyl group by the alkyltransferase was analysed as a second order reaction (Dolan et al., 1988; Graves et al., 1989) and graphs like these in figures 4.8 - 4.11 were obtained. Subsequently the rate constants were calculated as described in "Materials and Methods". The constants shown in table 4.3 are the mean of at least two independent determinations. The rate of the repair of O^6-methylguanine was 18 times slower when present at the second position (-T.GmeGA.G- sequence) than at the first position (-T.meGGA.G- sequence) of codon 12 of the ras-related oligomers. Substitution of the guanine preceding the O^6-methylguanine in the -T.GmeGA.G- sequence by a thymine (-T.TmeGA.G- sequence) increased the rate of the reaction by 25 times. Substitution of the second guanine of the -T.meGGA.G- sequence with an adenine to give the -T.meGAA.G- sequence reduced the rate of the repair by 2.6 times.

The rate constants determined for these ras-related pentadecamers were 2 orders of magnitude less than that observed for the self-complementary dodecamer CGCmeGAGCTCGCG. To discover whether the rapid repair of the self-complementary
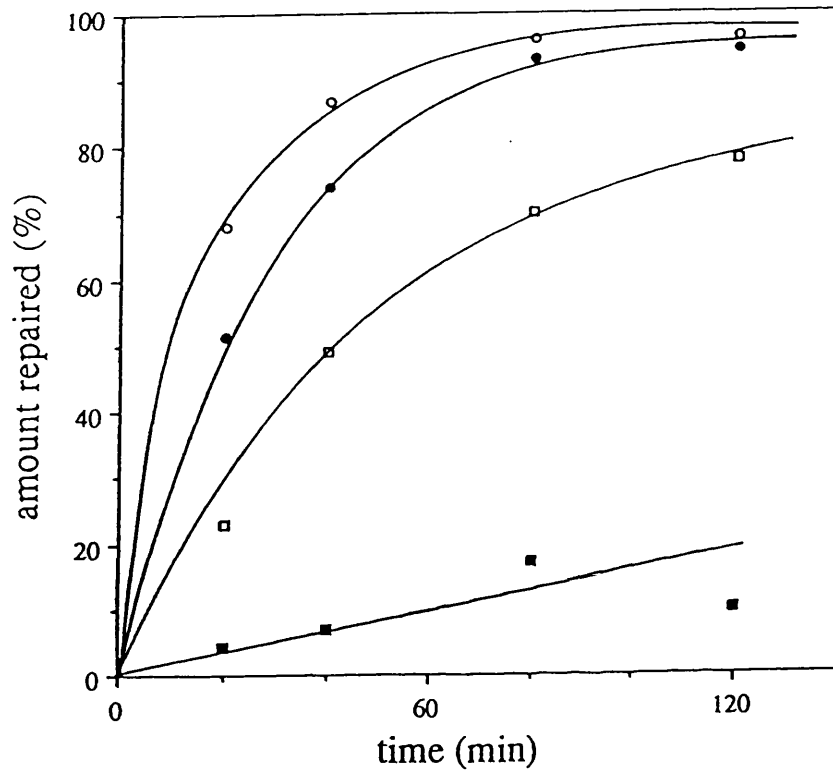
Figure 4.7 : Differential repair for the ras related sequences by the E. coli *ada*

alkyltransferase. 400 fmol of *ada* protein was incubated with 60 fmol of each oligomer in a

volume of 40 μl as described under Materials and Methods.   o   -T. TmeGA.G- duplex;

●   -T.meGGA.G-;   ▫   -T.meGAA.G- and   ▪   -T.GmeGA.G-

duplexes.
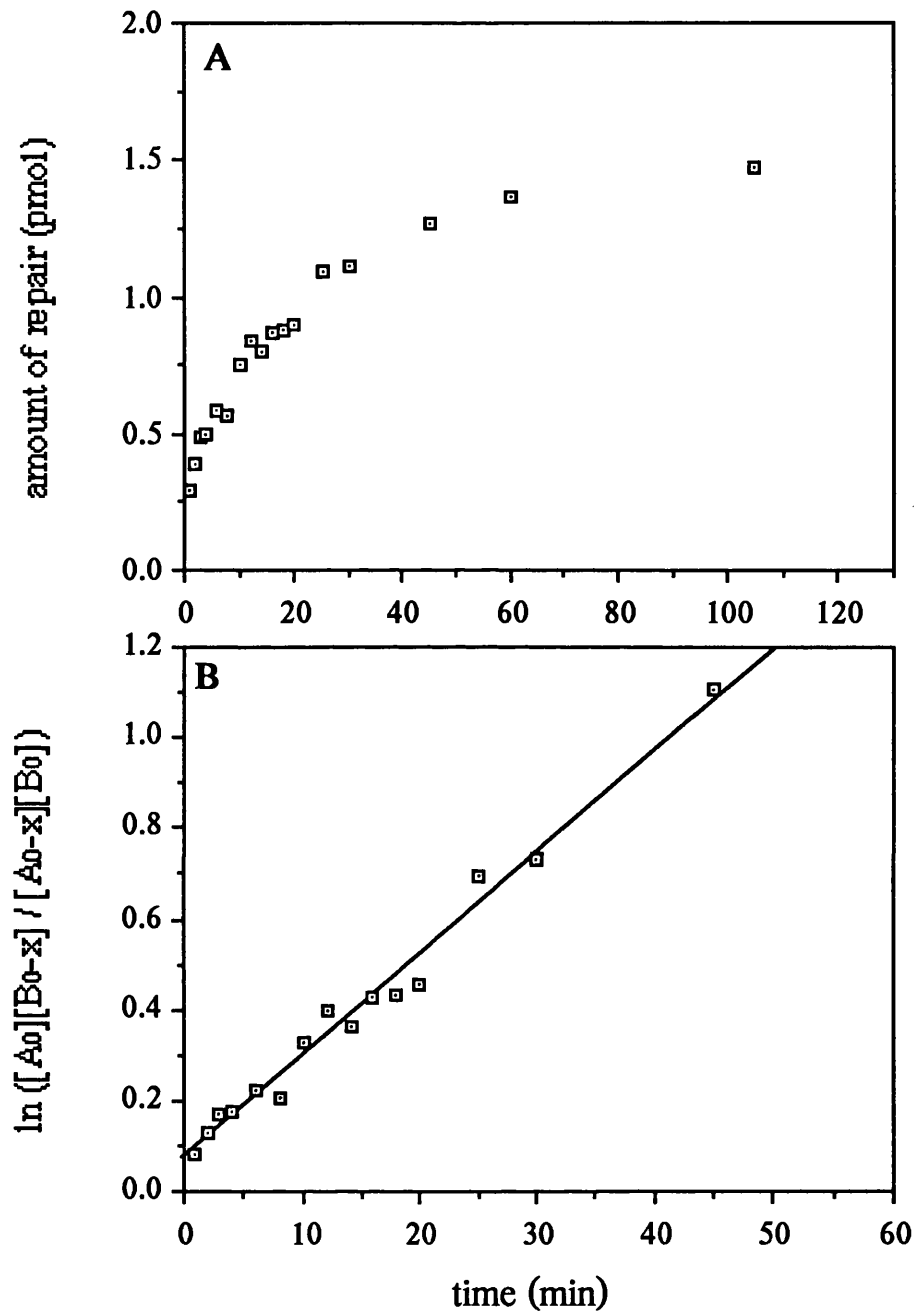
Figure 4.8 : Rate of repair of O^6 -methyldeoxyguanine in the -T.meGGA.G- duplex (2.5 pmol) by the ada alkyltransferase (1.5 pmol). A, the rate of formation of the parent oligomer by repair of the alkylated oligomer. B, graph of ln ($[B_0-x][A_0]/[B_0][A_0-x]$) as a function of time. A_0 and B_0 are the initial concentrations of oligomer and enzyme respectively, x is the amount reacted after time t. The slope of the line is equal to $k(A_0-B_0)$ where k is the rate constant.
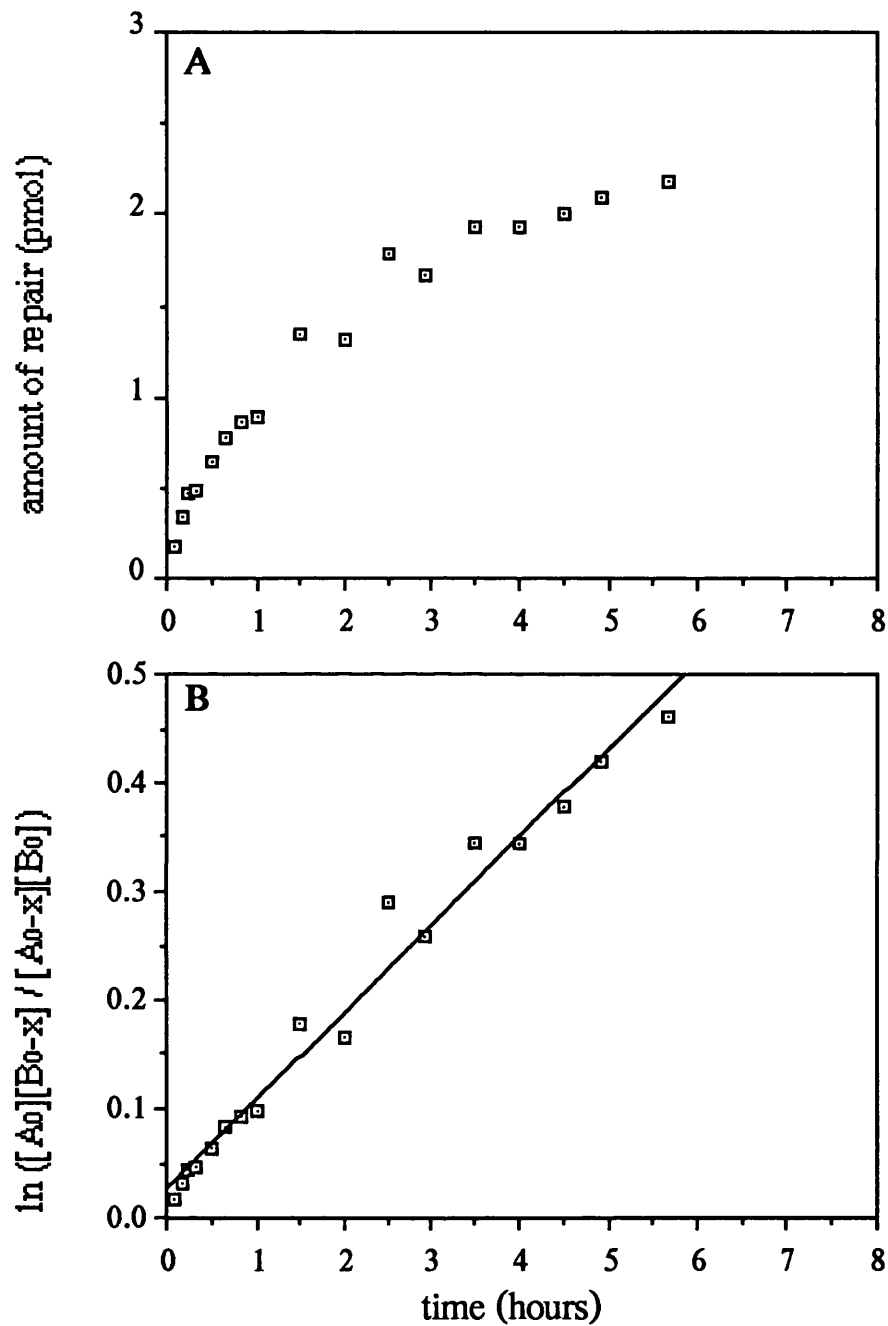
Figure 4.9 : Rate of repair of O^6 -methyldeoxyguanine in the -T.GmeGA.G- duplex (4.2 pmol) by the ada alkyltransferase (3 pmol). A, the rate of formation of the parent oligomer by repair of the alkylated oligomer. B, graph of ln ([Bo-x][Ao]/[Bo][Ao-x] as a function of time.
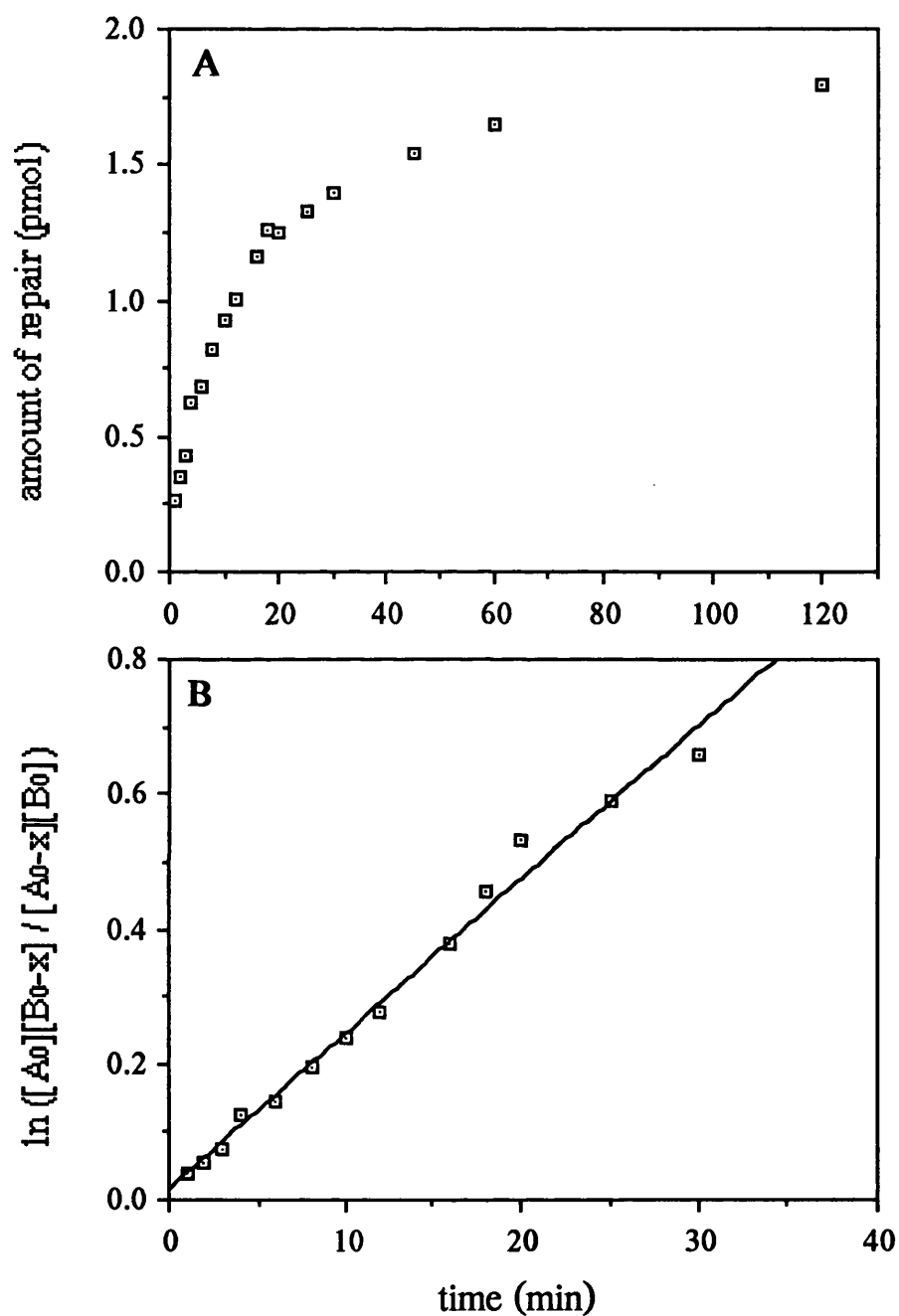
Figure 4.10 : Rate of repair of O^6 -methyldeoxyguanine in the -T.TmeGA.G- duplex (2.5 pmol) by the ada alkyltransferase (1.8 pmol). A, the rate of formation of the parent oligomer by repair of the alkylated oligomer. B, graph of ln ($[B_0-x][A_0]/[B_0][A_0-x]$) as a function of time.
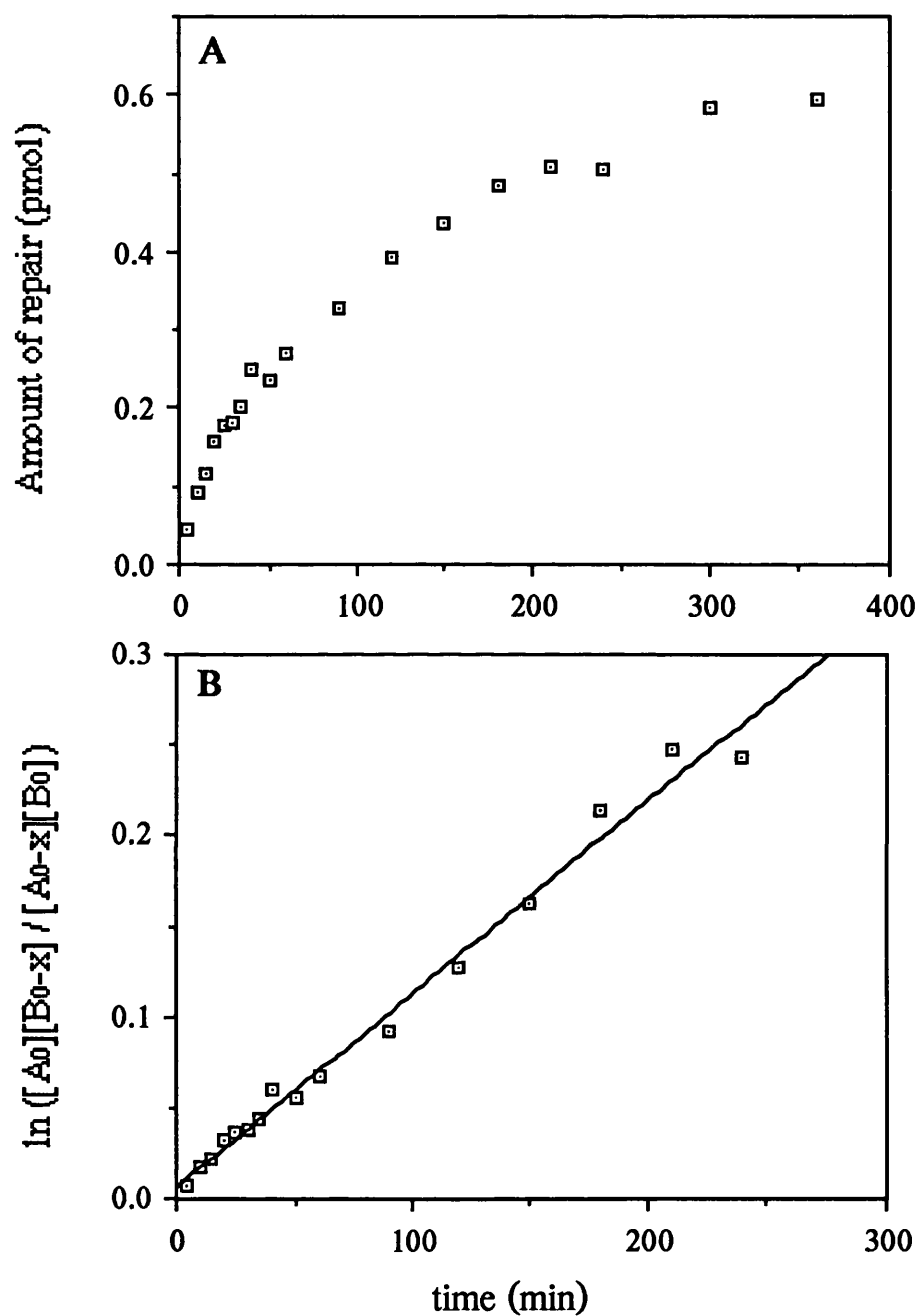
Figure 4.11 : Rate of repair of O^6 -methyldeoxyguanine in the -T.meGAA.G- duplex

(0.82 pmol) by the ada alkyltransferase (0.7 pmol). A, the rate of formation of the parent

oligomer by repair of the alkylated oligomer. B, graph of ln ([Bo-x][Ao]/[Bo][Ao-x]) as a

function of time.

oligomer was a consequence of its sequence or a consequence of it being self-complementary, a dodecamer CGCmeGAGTAATGC was synthesised and annealed to its complementary strand. This was repaired an order of magnitude slower than the self-complementary oligomer, even though the base composition around the O^6-methylguanine was the same for both oligonucleotides.

DNA-anti-DNA interactions are commonly studied using either saturated ammonium sulphate or polyethylene glycol (PEG) to precipitate the antibody-antigen complex. PEG has the advantage of precipitating low avidity as well as high avidity DNA-anti-DNA complexes, whereas the ammonium sulphate precipitation is selective for high avidity interactions, possibly because it dissociates the low avidity DNA-anti DNA complexes (Smeenk and Aarden, 1980 Smeenk *et al.*, 1980). Figure 4.12 shows the precipitation of the -T.GmeGA.G- and -T.TmeGA.G- duplexes at different antiserum dilutions using either ammonium sulphate or PEG. While, both gave similar precipitation of the -T.TmeGA.G- duplex, there was a 2-fold difference in the amount of the -T.GmeGA.G- duplex precipitated. PEG gave always the higher precipitation. At an 1:30 final antiserum dilution, 60% and 38% of the -T.GmeGA.G- duplex was precipitated using the PEG and ammonium sulphate respectively. Smeenk and Aarden (1980) implemented the PEG precipitation in an immuno-assay for high molecular DNA. They observed that as well as the DNA-antibody complexes, PEG also precipitates the very weak complexes formed between DNA and the low density lipoproteins of the antiserum. However, this was not observed in the immunoprecipitation of the 15-mers, since the parent oligomer was precipitated to a similar extent using either PEG or ammonium sulphate.

PEG was thus, preferred for the determination of the avidity constants. Different amounts of antiserum were used to give 40-60% precipitation for different oligomers. The most concentrated antiserum used, was for the precipitation of the -T.GmeGA.G- oligomer (1:42 dilution). The most diluted was for the precipitation of the self-complementary CGCmeGAGCTCGCG and the single stranded

Table 4.3 : Relationship between the rate of repair and recognition by the anti-O^6-methyldeoxy-guanosine antibodies. All the sequences except the CGCmeGACGTCGCG which is self-complementary, were present with an excess (20%) of the complementary strand so that a double helix whould be formed. However, the avidity of antibodies to O^6-methyldeoxy-guanosine was also measured for the CGCmeGAGCAATGC 12-mer in the absence of the complementary strand (single st.). The ΔTm are also included[a].

Sequences	Rate of repair	avidity constant	ΔTm
GGCGCTGmeGAGGCGTG	1.1×10^4 M^{-1}s^{-1}	0.24×10^7 M^{-1}	9 °C
GGCGCTmeGAAGGCGTG	7.7×10^4 M^{-1}s^{-1}	N.D	12 °C[b]
GGCGCTmeGGAGGCGTG	2.0×10^5 M^{-1}s^{-1}	0.86×10^7 M^{-1}	11 °C
GGCGCTTmeGAGGCGTG	2.8×10^5 M^{-1}s^{-1}	1.85×10^7 M^{-1}	12 °C
CGCmeGAGCAATGC	2.0×10^6 M^{-1}s^{-1}	5.0×10^7 M^{-1}	
CGCmeGAGCAATGC (single st.)		1.5×10^8 M^{-1}	
CGCmeGAGCTCGCG	2.2×10^7 M^{-1}s^{-1}	2.0×10^8 M^{-1}	>20 °C[c]
dmeG (free nucleoside)	--	1.0×10^9 M^{-1}	--

[a] The ΔTm was calculated by subtracting the Tm value of the parent duplexes from each observed value. [b] Value calculated by subtracting the Tm value of the -TGA- duplex which has the same flanking bases. [c] value calculated from Li and Swann (1989).

N.D: No data available
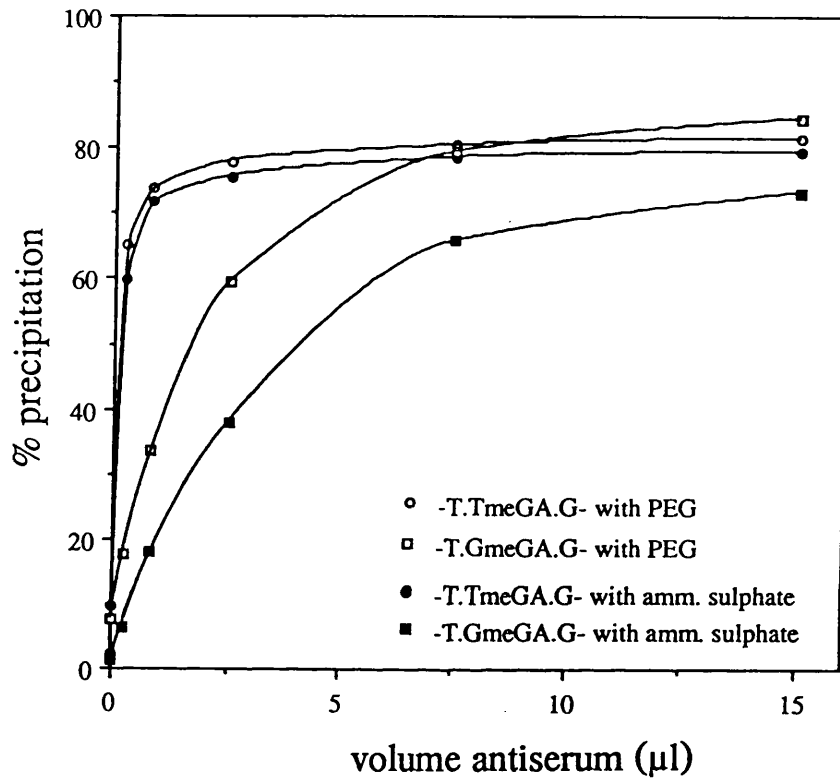
Figure 4.12 : Comparison of the amount of the -T.TmeGA.G- and -T.GmeGA.G-
duplexes precipitated using either saturated $(NH_4)_2SO_4$ or PEG as precipitating agents.
All the samples had the same total protein concentration of 30 mg/ml.
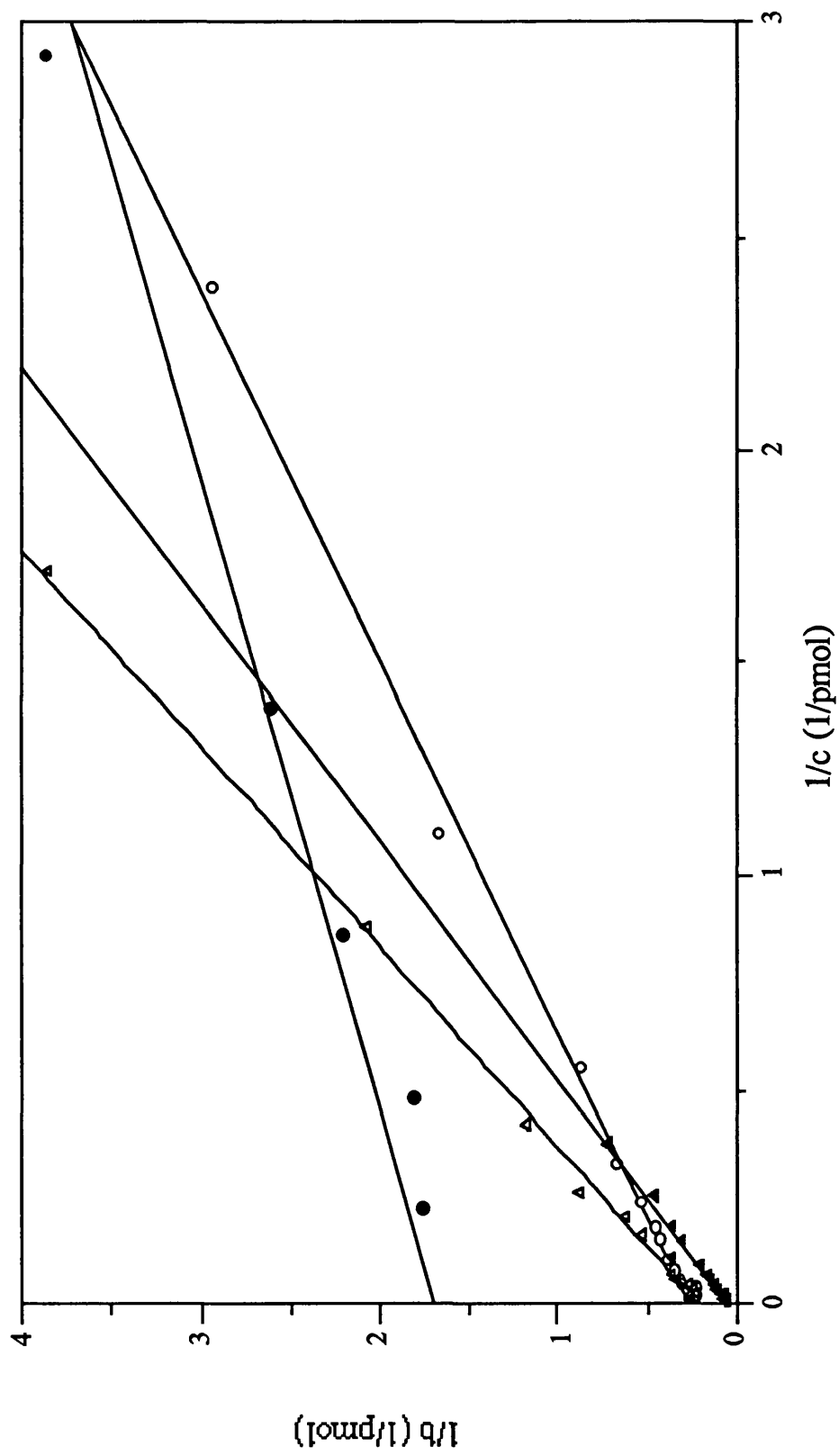
Figure 4.13 : Binding curves for the interaction of the antibody against O ⁶methyldeoxyguanosine with the ▲ -T.GmeGA.G-, ▲ -T.meGGA.G-, o -T.TmeGA.G-, and ● the CGCmeGAGCTCGCC oligomers. For the curve of the last oligomer data points that lay beyond the borders of this graph were also taken into account.
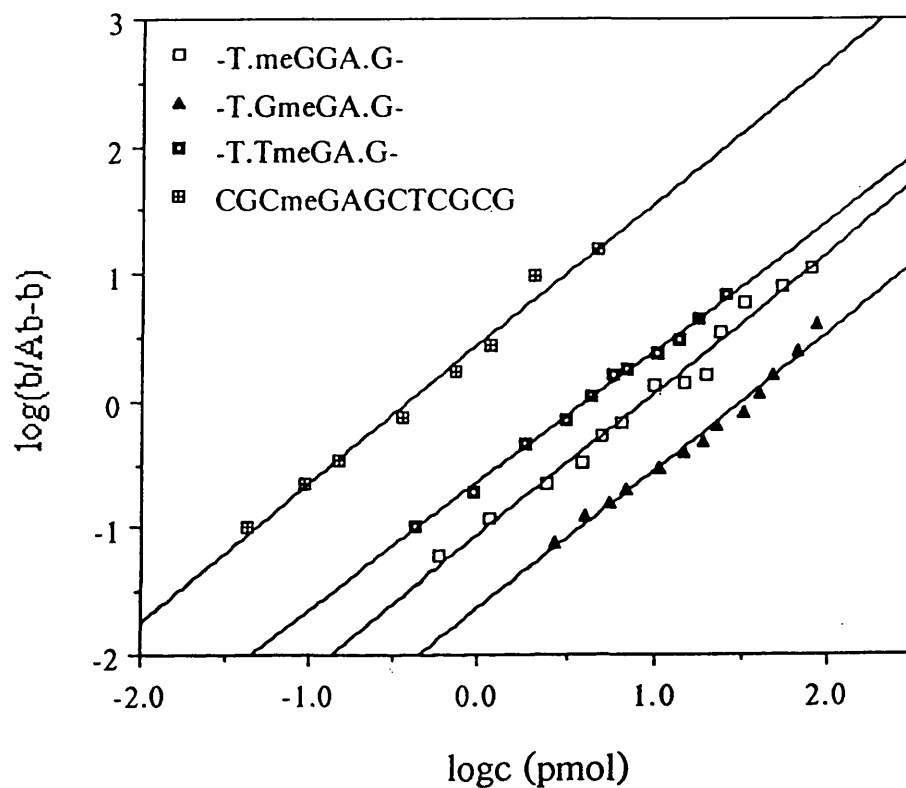
Figure 4.14 : Sips' plots for the DNA-anti-DNA interactions of the -T.TmeGA.G- , -
-T.meGGA.G-, -T.GmeGA.G- duplexes and of the CGCmeGAGCTCGCG self-
complementary oligomer . The avidity constants of table 4.3 were obtained from the y-
intercept of these graphs as described under Materials and Methods
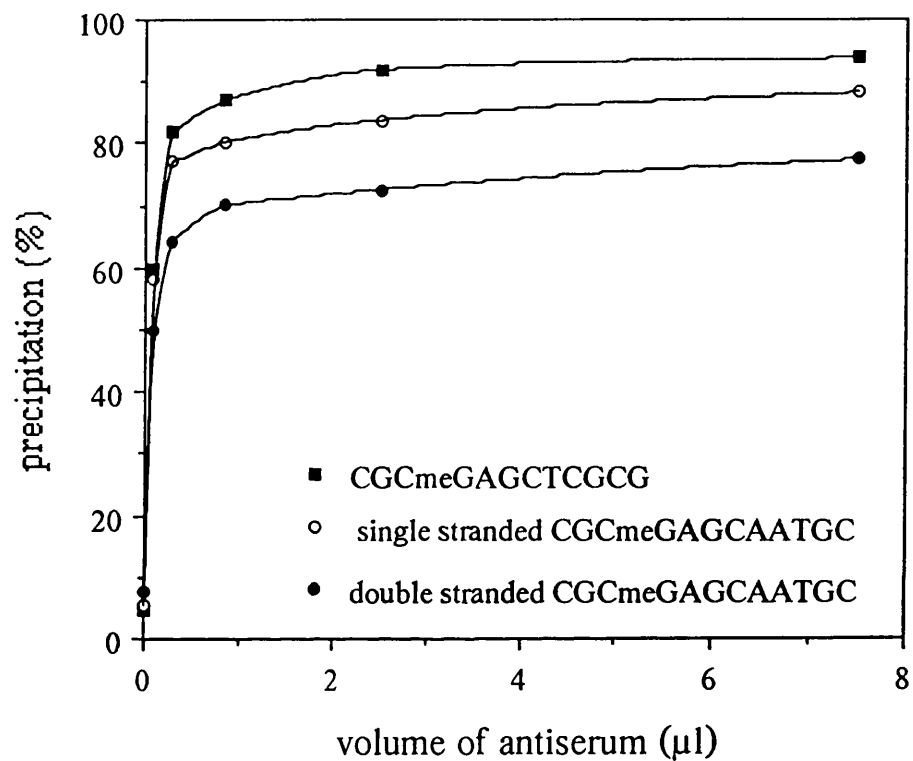
Figure 4.15 : Comparison of the interaction with the antibodies of

CGCmeGAGCTCGCG, the self-complementary 12-mer, and the single or double stranded

CGCmeGAGCAATGC. The precipitating agent was PEG.

CGCmeGAGCAATGC oligomers (1:1200 dilution). The total antigen binding sites were

calculated (figure 4.13) and the Sip's plots were obtained as described in "Materials and

Methods" (figure 4.14) . From the intercepts of these plots the avidity constants (table 4.3)

were calculated. [log(b/Ab-b) = 0 , log c = - log K]. The ranking order for the avidity

constants parallel with the order of the rates of repair. In particular the -T.GmeGA.G-

sequence was repaired 25 times slower, and had an avidity constant 8 times lower than the

-T.TmeGA.G- sequence. The interaction of the self-complementary

CGCmeGAGCTCGCG oligonucleotide with the antibodies resembles most closely the

interaction of the single stranded CGCmeGAGCAATGC oligomer and not this of the

double stranded oligomer (figure 4.15). The avidity constants for both the

self-complementary and single stranded oligonucleotides were an order of magnitude

higher than that of the -T.TmegA.G- sequence (table 4.3) and had values close to that of

the free nucleoside ($1 \times 10^9 M^{-1}$), as it was previously determined using the same

antiserum (Salih and Swann, 1982).

4.4. DISCUSSION

In this study the effect of the flanking bases on the repair of O^6-methylguanine by E.coli *ada* alkyltransferase was examined. Synthetic 15-mers were used as substrates and the repair reaction was followed by immunoprecipitation instead of the HPLC-based method described in the previous chapter. The reason for this change in the methodology was that reverse-phase HPLC could not separate the methylated from the parent 15-mers. Very recently it was observed that anion exchange chromatography can separate the two forms of DNA, even for oligomers more than 15 bases long. However, the HPLC method is labour-intensive and time-consuming method and should be avoided whenever antibodies against the DNA adduct of interest (i.e O^6-methylG, O^6-ethylG,O^4-methylT, O^4-ethylT) are available and can be used to follow the repair processes.

The antibodies used in this study were raised against an O^6-methyldeoxyguanosine-BSA conjugate (Kyrtopoulos and Swann, 1980) and exhibit a high affinity in their interaction with the free nucleoside ($K= 1 \times 10^9 M^{-1}$) (Salih and Swann, 1982). It was found however, that they also react with O^6-methylguanine residues within short oligomers, albeit with reduced efficiency (table 4.3). Very recently, Nehls and Rajewsky (1990) reached similar conclusions for the affinity of anti-O^6-ethyldeoxyguanosine antibodies in their reaction with high molecular weight DNA containing O^6-ethylguanine.

Single stranded oligomers interacted with the antibodies better than the respective DNA duplexes. The differences however, between the precipitation of the two forms of DNA varied significantly between different oligomers. The greater difference was observed for the CGCAGmeGTGGTCG oligomer and the smallest for the -T.GmeGA.G- oligomer (table 4.2). The -T.GmeGA.G- sequence in particular, was the only one among the oligomers used in the present study, which had the O^6-methylguanine residue surrounded by two purines. Probably the stacking interactions between the adjacent purines, render the O^6-methylguanine residue unaccessible to the antibodies in either the single or double

stranded DNA.

The oligonucleotides used in this study could not be quantitively precipitated even when concentrated antiserum was added in the reaction mixture (figures 4.5, 4.12, 4.15). In two other studies similar incomplete precipitation was observed and was attributed to minor conformations of the substrate oligonucleotide with reduced alkyl group accessibility (Souliotis *et al.*, 1989; Mironov *et al.*, 1989). An alternative explanation could be that DNA-anti-DNA complexes are dissociated in a small extent during the precipitation of the proteins. However, incomplete precipitation of the methylated oligomer was not a major drawback of the immunoprecipitation method since the proportion precipitated was constant for small changes in the oligomer concentrations, even for the most poorly precipitated -T.GmeGA.G- sequence (figure 4.6).

The rate of the repair of O^6-methylguanine was found to differ as much as 25- fold depending on the flanking base sequence (table 4.3). Surprisingly, the DNA duplexes -T.TmeGA.G- and -T.meGAA.G-, which have the same bases flanking the alkylated base were repaired with rates differing by 3.6 times. A possible explanation for this observation could be a next-to-neighbour effect as suggested by Topal *et al.* (1986). The -T.GmeGA.G- sequence was the most poorly recognized by the alkyltransferase and was repaired 18 times slower than the -T.meGGA.G- sequence. The effect of sequence on the rate of repair seems to be a reflection of the conformation and accessibility of the alkylated base because the association of antibodies to O^6-methyldeoxyguanosine with the DNA duplexes was affected by the base sequence flanking the alkylated base. Those duplexes which were repaired most slowly were also those where the methylguanine was least accessible to the antibody (table 4.3).

Differences were also observed in the thermal denaturation profiles of these oligomers. All DNA duplexes containing O^6-methylguanine were significantly less stable than the parent oligomers as expected from previous studies (Gaffney and Jones, 1989; Li and Swann, 1989). Unlike the self-complementary oligomers (chapter 3 figure 7) denaturation occurs within a very narrow range of temperature and the melting curves were monophasic, indicating a single helix to coil transition. The least, and most stable

oligomers were also these with the slowest and fastest rates of repair respectively (table 4.3). However, the properties of the other two oligomers (T.meGGA.G- and -T.meGAA.G- were not compatible with an inverse correlation between stability, as reflected in the melting point (Tm), and rate of repair. A Tm dependence upon the flanking base sequence was also reported in two recent studies by Pauly *et al.* (1988) and Voigt and Topal (1990). In another study, the enthalpy for the dissociation of oligomeric duplexes containing O^6-methylguanine was measured, and their circular dichroism spectra were obtained (Bishop and Moschel, 1991). The oligomers had the sequence of the rat H-ras gene around codon 12, and it was found that the duplex with the modified base at the second G of the GGA codon 12 was more stable, and its dichroism spectrum resembled more the normal non-alkylated oligomer, than the oligomer with O^6-methylguanine at the first position of the same codon. Voigt and Topal (1990) observed that sequences, very similar to those used in the present study, have different electrophoretic mobilities on a non-denaturing polyacrylamide gel. These observations indicate that O^6-methylguanine could have a global effect in the structure of DNA which is greatly dependent upon the flanking base sequence.

The most poorly repaired methylguanine, also interacted most weakly with the antibody and was surrounded by the same sequence as the second G of the 12th codon of H-ras gene. Therefore, the results presented in this chapter argue strongly for a contribution of repair specificity in the observed non-random distribution of mutations observed in both eukaryotes and prokaryotes.

However, two previous observation have seemed inconsistent with the existence of repair specificity in vitro. When Mitra *et al.* (1989) put the rat H-ras oncogene containing an O^6-methylguanine placed in either the first or second position of codon 12, into a shuttle vector, and studied the mutations caused upon transfection of Rat 4 (TK-) cells, they found that the mutation frequency , although very low ($\approx$1%), was independent of the position of O^6-methylguanine (Mitra *et al.*, 1989) . However in this system a small number of plasmids enter each cell and the alkyltransferase molecules would always be in a

vast excess over the number of alkylated bases. This is not comparable to the situation where mammary tumours are induced by administration of NMU (Sukumar *et al.*, 1983), where more O^6-methylguanine residues are produced than the available alkyltransferase molecules. Under such conditions, the difference in rates of the repair we observed, could have a disproportionate and greater effect in the mutation frequency because the enzyme molecules would be exhausted on the repair of the most favoured sites while the least repairable sites would remain untouched, and thus, a higher frequency of mutations at these positions is to be expected.

In another study a similar mutational spectrum was obtained in a plasmid DNA grown in unadapted ie low *ada* alkyltransferase content, adapted i.e high alkyltransferase content, or *ada* deficient E. coli cells, and exposed to N-methyl-N-nitroso-N'-nitroguanidine (Richardson *et al.*, 1987b). However, an unusual aspect of this study was that the mutations observed were almost exclusively at the antisense strand and such strand specificity was not observed when the chromosomal E.coli lacI gene was used as target for alkylation (review by Gordon *et al.*, 1990). Nevertheless, there are some indications that preferential repair had taken place and similar evidence can be seen in experiments with mer+ and mer- mammalian cells (Sikpi *et al.*, 1990). Positions 128 in Richardson *et al.* (1987) and 129 in Sikpi *et al.* (1990) appear to be stronger mutagenic hotspots in the repair deficient cells, while others appear to be stronger mutagenic hotspots in the repair proficient cells (positions 402 and 123 in the two studies respectively). Similar differences would be expected if non-random repair process follows a non-random alkylation process. There is significant evidence that N-nitroso compounds form O^6-alkylguanine more frequently in some sites in DNA than others but the distribution of mutations does not reflect the alkylation pattern (Richardson *et al.*,1989). This is probably because some alkylation sites are significantly intensified as sites of frequent mutagenesis by an inefficient repair, while others are diminished by a very effective repair.

The rate constants for the repair of O^6-methylguanine, obtained in the present study, are 1-2 orders of magnitude slower than the rates determined previously (Dolan *et*

al., 1988b; Graves *et al.*, 1989), and show a much greater effect of the sequence than was found by Dolan *et al.* (1988a). The reason for this difference in results seems to be that previous authors have all used shorter and self-complementary oligomers. Comparison of the rate of the repair of a non-self complementary and a self-complementary 12-mer with the same bases flanking the O^6-methylguanine showed that the self-complementary oligonucleotide was repaired with unusual speed. The reason is probably that in repair assays low concentration of oligomer, in low salt concentration and at 37 °C, is used. Under these conditions self-complementary oligonucleotides exist mainly in a hairpin-loop conformation (see chapter 3). The avidity constants between the antibodies against O^6 -methyldeoxyguanosine and the self-complementary oligomer we used is very high and close to that between the antibody and the free nucleoside suggesting that, as one would have suspected, the O^6-methylguanine present in an oligomer with a hairpin-loop structure would probably spend more time frayed out of the helix than stacked into it and hence could be more accessible and removed faster by the alkyltransferase.

In summary it seems likely that the high specificity for activating mutations in the H-ras gene results as a combination of selectivity of alkylation and inefficient repair with the latter amplifying to a great extent the effect of the first.

CHAPTER 5

O^4-ALKYLTHYMINE PRODUCES SITES OF DNA HYPERFLEXIBILITY

5.1. INTRODUCTION

Most investigators have focused their research with O^6-alkylguanine or

O^4-alkylthymine to the study of their promutagenic properties. However, both adducts

have reasonably long biological half-lifes in animals treated with alkylated agents (Den

Engelse *et al.*, 1986; Den Engelse *et al.*, 1987), and they were also found in human tissues

of healthy individuals (Wild, 1990). The persistence of these lesions in DNA might cause

changes in physiological cellular functions by means not associated with their mutagenicity,

probably by altering the efficiency with which certain proteins interact with DNA.

O^6-methylguanine residues in a sequence containing the SV40 origin of replication affects

significantly the binding of the SV40 large T antigen (Bignami and Lane, 1990). The same

DNA adduct has been shown to influence DNA-protein interactions for several restriction

enzymes (Voigt and Topal, 1990). In addition, the presence of O^6-methylguanine in DNA

alters the efficiency of (cytosine-5)-methyltransferase for DNA alkylation and the

magnitude of this effect depends upon the position of O^6-methylguanine relative to the

cytosine residue, target for methylation by this enzyme (Tan and Li, 1990).

It is not known if changes as such in cellular functions can be related to

oncogenesis by alkylating agents, but the possibility should not be excluded.

Carcinogenesis is a complex process which has been historically divided into two stages,

initiation and promotion. Nitrosamine-induced cancer is now believed to be initiated by

point mutations, most likely caused be miscoding of O^6-alkylguanine or O^4-alkylthymine

during replication (Zarbl *et al.*, 1985; Kumar *et al.*, 1990). The nitroso compounds

however, are complete carcinogens and can bring about all the multistage processes of

carcinogenesis, presumably including the subtle changes in gene expression associated

with promoters such as the phorbol esters. The mechanism by which nitroso compounds carry out these changes might be associated with conformational changes in DNA following alkylation. The results of the previous chapter showing that the conformation of an O^6-alkylguanine residue is dependent upon the DNA sequence in which it resides, is relevant to this discussion because it shows that conformation has to be considered in the context of specific sequences.

One of the distortions of DNA that might be caused by O^6-alkylguanine or O^4-alkylthymine is DNA curvature. Natural DNA becomes curved when certain sequences are repeated in phase with the periodicity (10.5 base pairs) of B-DNA (recent reviews by Crothers et al., 1990; Hagerman, 1990). Most of the DNA loci of biological significance now considered to be curved, among them promoters (Struhl, 1985; Ryder et al., 1986; Plaskon and Wartell, 1987; Kawamoto et al., 1989) and origins of replication (Dean et al., 1987; Zahn and Blattner, 1987; Williams et al., 1988; Eckdahl and Anderson, 1990) , share the same motif of dA:dT tracts half a helical turn long separated by an integral number of helical turns (Wu and Crothers, 1984). There are a number of reports suggesting that a correlation exists between between curvature and DNA recognition by proteins, and degree of DNA curvature and biological function (Deb et al., 1986; Zahn and Blattner, 1987; Bracco et al., 1989; Collis et al., 1989). Zahn and Blattner (1987) found that binding of the lambda initiator of replication protein O is associated with the intrinsic curvature of certain DNA sequences within the lambda origin of replication. Elimination of the central dA:dT tract which resides within one of these sequences abolishes curvature and binding of the O protein. Bracco et al. (1989) reported that placing curved DNA 40 to 80 base-pairs upstream of the start site of the gal promoter in E. coli can restore promoter activity, despite deletion of the -35 site and deletion of an upstream binding-site for the transcriptional activator cAMP-CRP (catabolite activator protein). Minimal changes in the location of the curvature strongly affected the level of expression. However, the question whether curvature per se is of functional importance, or whether the dA:dT tract is important with curvature being fortuitous and insignificant, is yet to be answered. The postulate (Husain et al., 1988) that some DNA repair enzymes recognize loci of DNA

curvature rather than the DNA sequence itself is particularly attractive because a damaged base has to be recognised irrespective of the surrounding DNA sequence.

For these reasons the extent of DNA bending and other conformational changes produced by the presence of O^6-alkylguanine and O^4-alkylthymine was measured. This was assessed by non-denaturing gel electrophoresis. Curved DNA migrates significantly slower than normal DNA during electrophoresis on a non-denaturing polyacrylamide gel. This property, first observed in a study of kinetoplast DNA (Marini *et al.*, 1982; Marini *et al.*, 1984), led to the discovery of DNA curvature and has been used in almost every subsequent investigation of DNA curvature. The melting point (Tm) of the alkylated DNA was also determined and provided a measure of the destabilizing effect of the altered base-pair.

Within the oligomer used was a sequence 5' GGT.CAA.GAG 3' corresponding to codons 60, 61 and 62 of the mouse K- ras gene. Ras genes are activated by mutation of codons 12, 13, or 61 in a large number of animal tumours induced by chemical carcinogens (reviewed by Balmain and Brown, 1988). This particular sequence was chosen because mouse lung tumours induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), an important carcinogen in tobacco-induced cancer, had the K-ras oncogene activated via point mutations in codon 61 (normally CAA) as well as in codon 12 (Belinsky *et al.*, 1989; Belinsky *et al.*, 1990). The T:A to C:G mutations occurred in the second position of this codon and could be explained by miscoding following the formation of O^4-alkylthymine.

While this work was in progress Voigt and Topal (1990) reported that O^6-methyl-guanine produces a small distortion of DNA. However as will be shown below, O^4-methyl-thymine and O^4-ethylthymine produce very much greater conformational distortion of the DNA helix and this conformational change was primarily a great increase in the flexibility of the DNA and was produced by both O^4-alkylT:A and O^4-alkylT:G base pairs. In general this flexibility did not have a preferred direction. Only in the case of O^4-alkylT:A at pH 8.3, had this flexibility an anisotropic component, which apparently was lost when the pH was reduced to 6.5, so that only in this limited case was the DNA actually bent.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals and Enzymes

Acrylamide: methylene-bis-acrylamide (19:1 w/w) was from Sigma and all other general chemicals from Aldrich or BDH. [gamma -^{32}P]ATP (specific radioactivity 3000 Ci/mmol) was from the NEN Division of Du Pont; T4 DNA ligase and polynucleotide kinase were from New England Biolabs; alkaline phosphatase and snake (Crotalus durrissus) venom phosphodiesterase from Sigma.

5.2.2. Oligonucleotide synthesis

The oligonucleotide sequences are shown in figure 5.1. The modified bases were O^4-methyl- and O^4-ethylthymine at either positions 1 or 2 and O^6-methylguanine at position 3. The oligomers containing O^6-methylguanine were prepared as before (Smith et al., 1990). Those containing O^4-methyl- and O^4-ethylthymine were prepared by a new technique in which a chemically reactive pyrimidine derivative is incorporated into the oligonucleotide, and then converted into O^4-alkylthymine after synthesis (see chapter 2). This technique has the advantage that the product of a single oligonucleotide synthesis can be subdivided and a number of 4-substituted pyrimidines made from it. Deprotection and purification was carried out as described in chapter 2. The presence of the correct modified base and the completeness of the deprotection was confirmed by base analysis (see chapter 2).

5.2.3. ^{32}P- labelling and formation of ligation ladders

The oligomers containing the modified base, i.e the top strand in figure 5.1, was 5'-phosphorylated using 5 μl 10 mM ATP and 10 units T4 kinase in 40 μl, 50 mM tris-Cl, 10 mM $MgCl_2$, 7 mM dithiothreitol (DTT). After 30 min. incubation at 37 °C the kinase was inactivated by heating at 65 °C for 10 min. The complementary DNA strand, i.e the bottom strand in figure 5.1, was [5'-^{32}P]- labelled with 50 μCi [gamma - ^{32}P]- ATP under

A) 21-mer

123
GGACC.TCC.CTC.TTG.ACC.TGC.T
G.AGG.GAG.AAC.TGG.ACG.ACCTG

↓

21bp
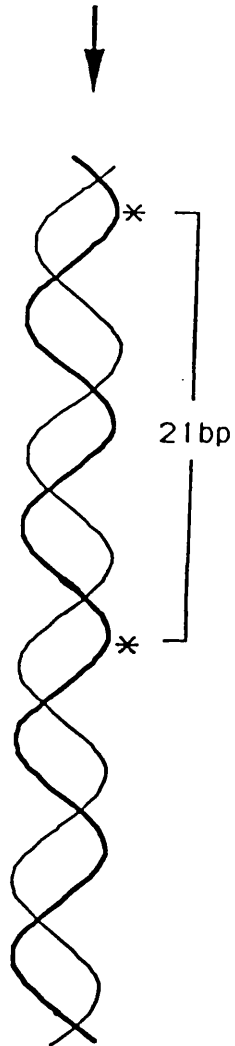
B) 16-mer

123
GGACC.CTC.TTG.ACGC.T
G.GAG.AAC.TGCG.ACCTG

↓

16bp
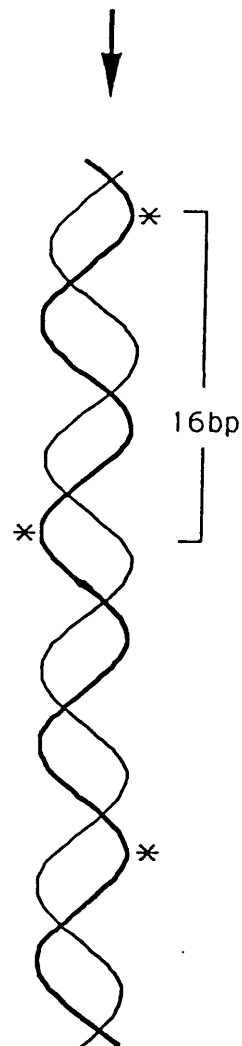
Figure 5.1 : Sequences used for the formation of ligated polymers containing base pair

substitutions, A, in phase and B, out of phase with the helical repeat. The numbers

above each duplex refer to the positions where base-pair substitutions were introduced.

The polymer DNA duplexes consisting of 21-mers have the base-pair substitutions on the

same side of the helix, whereas the polymers consisting of 16-mers have the base-pair

substitutions on opposite sides of the helix. (*) indicates the position of the substitution.

the conditions described above except that after the initial 30 min. incubation, 5 μl 10 mM ATP was added and the incubation continued for 30 min. to ensure complete 5'-phosphorylation. Forty pmols of the top strand in figure 5.1 and 20 pmols of the ^{32}P labelled complementary strand were annealed in 67 μl, 66 mM tris·Cl pH 7.6, 6.6mM MgCl$_2$, 1 mM ATP by heating to 80 °C and slow cooling to room temperature.

Subsequently 7.5 μl of 100 mM DTT and T4 DNA ligase (0.1 Weiss units) were added and the ligation reaction carried out at room temperature. At 5, 10, and 15 minutes 25 μl were transferred into a single tube containing 25 μl 2% SDS and 1 μl 500 mM EDTA . Mixing aliquots of different incubation times was found to give more even size distribution of oligomers. Samples were extracted with 100μl phenol:chloroform (1:1) and DNA was precipitated by addition of 300 μl ethanol. After 40 min at -70 °C, samples were centrifuged at 14,000 rev / min and 4 °C for 30 min. The supernatant was discarded, the precipitate washed with 75 % cold ethanol (1 ml), and after a second centrifugation, the DNA pellet was resuspended in 30μl tris·EDTA pH 8 containing 5% glycerol, bromophenol blue and xylene cyanol.

5.2.4. Gel electrophoresis

The ligation products were electrophoresed on a non-denaturing 4% polyacrylamide gel at room temperature until the bromophenol blue had migrated 25 cm. The buffer for electrophoresis at pH 8.3 was 89 mM tris·borate, 10 mM EDTA, and that for electrophoresis at pH 6.5 was 89 mM tris·phosphate, 10 mM EDTA. The voltage applied at pH 8.3 was 10 V/cm, but the gel formed in the pH 6.5 buffer had a much lower resistance and this voltage drop would have produced significant heating of the gel. Any heating of the gel is undesirable in these experiments since it has been shown that DNA duplexes undergo temperature dependent conformational changes even in temperatures below the Tm (Dieckmann, 1987). To avoid heating, a voltage drop of 5 V/cm was applied which gave the same power input (4-5 watts) as at pH 8.3. Following electrophoresis the gels were dried and autoradiographed. The autoradiographs were scanned by a

Joyce-Loebl scanning densitometer (Chromoscan 3, Joyce-Loebl, Marquisway, Tyne and Wear, England). The relative mobilities were calculated by dividing the distance migrated by each alkylated oligomer by the distance of migration of the same length control oligomer. The K-factor (Dieckmann, 1987) is the ratio of apparent length relative to actual chain length and was obtained using as molecular weight markers the $(dN_{16})_x$ polymers. A graph of the logarithm of the molecular weight vs the mobility of the control $(dN_{16})_x$ oligomers gave the linear equation from which the apparent lengths of the other oligomers were calculated. Relative lengths were then plotted vs the number of repeats of each polymer.

5.2.5. DNA melting curves

Oligonucleotides 21 bases long were synthesized having sequence complementary to that of the bottom strand of figure 5.1 so that blunt ended 21-mer duplexes could be formed. For the Tm determination, 1 nmol of each complementary strand was annealed in a volume of 0.6 ml and in a solution containing 1 mM tris·Cl pH 8.3, 0.1 mM EDTA, 1 mM $MgCl_2$, 25 mM NaCl. The temperature dependent changes in absorbance at 260 nm was followed using a CARY 3 spectrophotometer connected to a Cary temperature controller. (Varian Techtron Pty ltd, Australia). The rate of temperature increase was 0.5 °C/min. The Tm was determined from the graph of the first derivative of the absorbance vs temperature, as the temperature at which the first derivative aquires the maximum value.

5.3. RESULTS

5.3.1. Electrophoretic mobility of oligomers containing O^4-alkylthymine

Oligonucleotides containing O^4-methylthymine, O^4-ethylthymine and O^6-methylguanine were synthesized, 16 or 21 bases in length, and annealed with the complementary strands forming 12 and 17 base-pair duplexes with 4 residues single strand overhangs (figure 5.1). In the duplexes O^4-alkylthymine was paired with either adenine or guanine, and O^6-methylguanine was paired with cytosine. To see whether the neighbouring bases have an effect on the structural change in DNA caused by O^4-alkylthymine the modified base was placed in two of the positions normally occupied by thymidine. O^6-methylguanine replaced the guanine in the G:C following the T:A base-pairs which in other oligomers were replaced with O^4-alkylthymine. Analogous sequences containing the normal T:A base-pair, and sequences containing T:G and C:A mismatches were also included for comparison.

Assuming that there are 10.5 base-pairs per helical turn of B-DNA, ligation of the 21-mers containing one modified base would give a series of oligomers $(dN_{21})_x$ with the lesion always on the same side of the DNA helix [in phase] (figure 5.1). Any asymmetric distortion of the DNA produced by these modified bases would be additive, producing a plane curved structure. A curved DNA molecule has less electrophoretic mobility than normal DNA. However, bending is not the only cause of abnormal migration (Hagerman, 1990). Changes in mobility not connected with bending were evaluated by electrophoresis of ligated oligomers with 16 base-pair sequence repeats $(dN_{16})_x$. These would have the alkylated bases positioned every one and a half helical turns, and the asymmetric effect of one alkylated base would be counteracted by the next alkylated base because it would lie on the other side of the helix (figure 5.1). Electrophoresis was carried out at the normal alkaline pH 8.3 and also at pH 6.5 .

A typical autoradiograph of the gel after electrophoresis of ligated 16-mers at pH 8.3 is shown in figure 5. 2. The autoradiograph was scanned by a densitometer
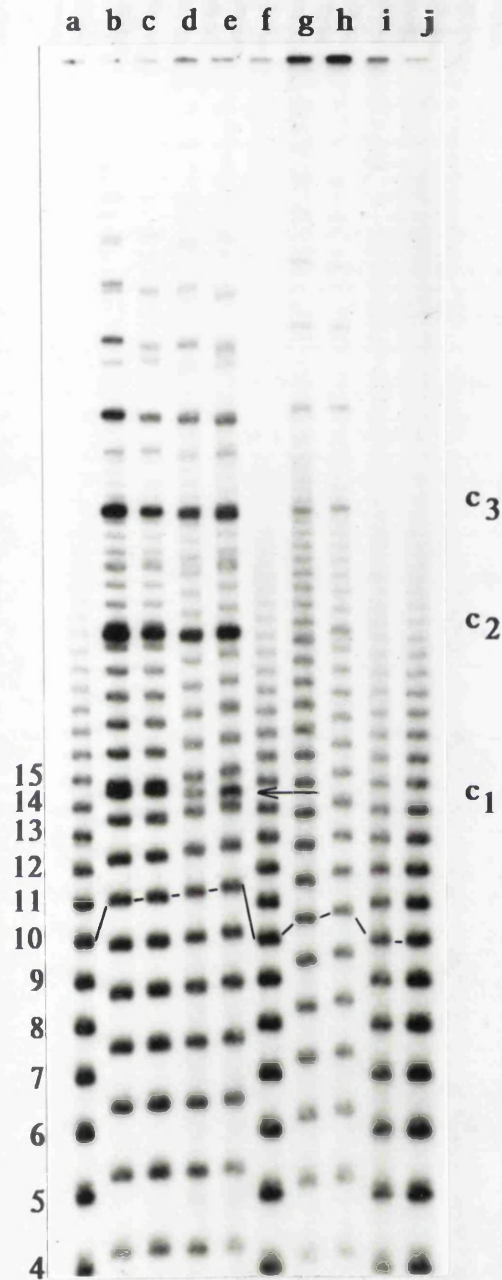
Figure 5.2 : Nondenaturing gel electrophoresis of ligated 16-mers oligomers on a 4% polyacrylamide gel at pH 8.3. Oligomers were ligated and electrophoresed as described under Materials and Methods. The number of 16 base-pair monomers in each band are indicated along the left side of the autoradiograph. The cyclic products are indicated along the right side of the autoradiograph. Bands corresponding to multimers of the same length (160 base-pairs) are indicated. Control ligation ladder lanes a , f and j. Oligomers containing at position 2, meT:A lane b; etT:A lane c; meT:G lane g; etT:G lane h; T:G lane i. Oligomers containing at position 1, meT:A lane d; and etT:A lane e .
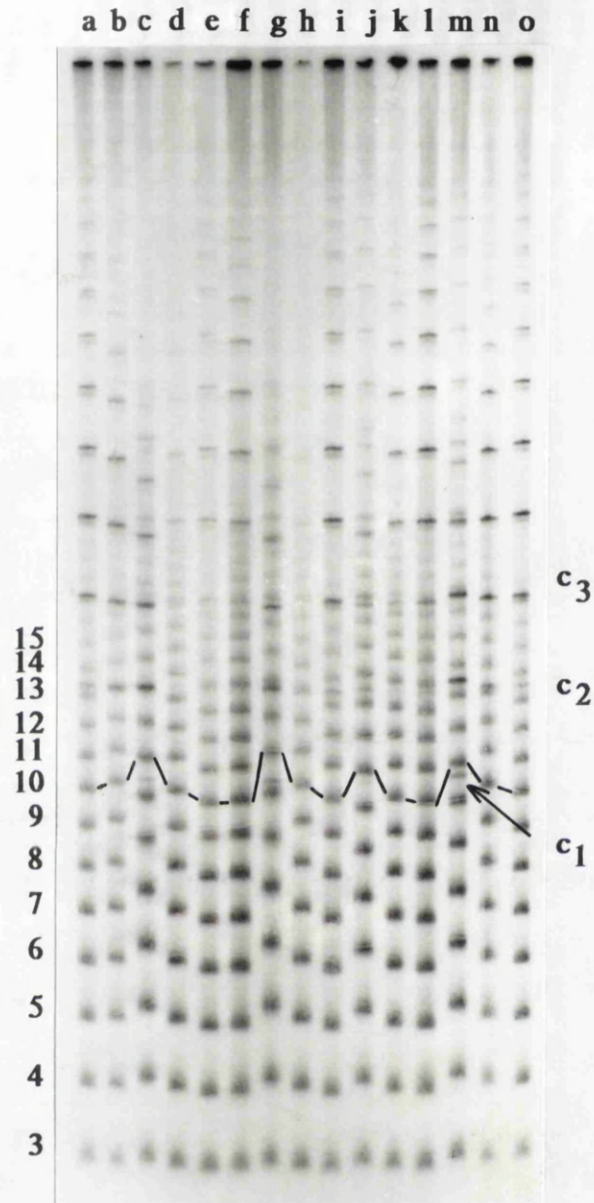
Figure 5.3 : Nondenaturing gel electrophoresis of ligated 21-mers on a 4% polyacrylamide gel at pH 8.3. The number of 21 base-pair monomers ligated in each band are indicated along the left side of the autoradiograph. The cyclic products are indicated along the right side of the autoradiograph. Bands corresponding to multimers of the same length (210 base-pairs) are indicated. Control ligation ladder lanes a , i and o. Oligomers containing at position 2, C:A lane b; meT:A lane c; meT:G lane d; T:G lane e; etT:A lane g; etT:G lane h. Oligomers containing at position 1, meT:A lane j; meT:G lane k; T:G lane l; etT:A lane m;   etT:G lane n. Oligomers containing at position 3 meG:C; lane f.

A, Multimers of 16 base-pair repeats containing meT:A at position 2

distance of migration
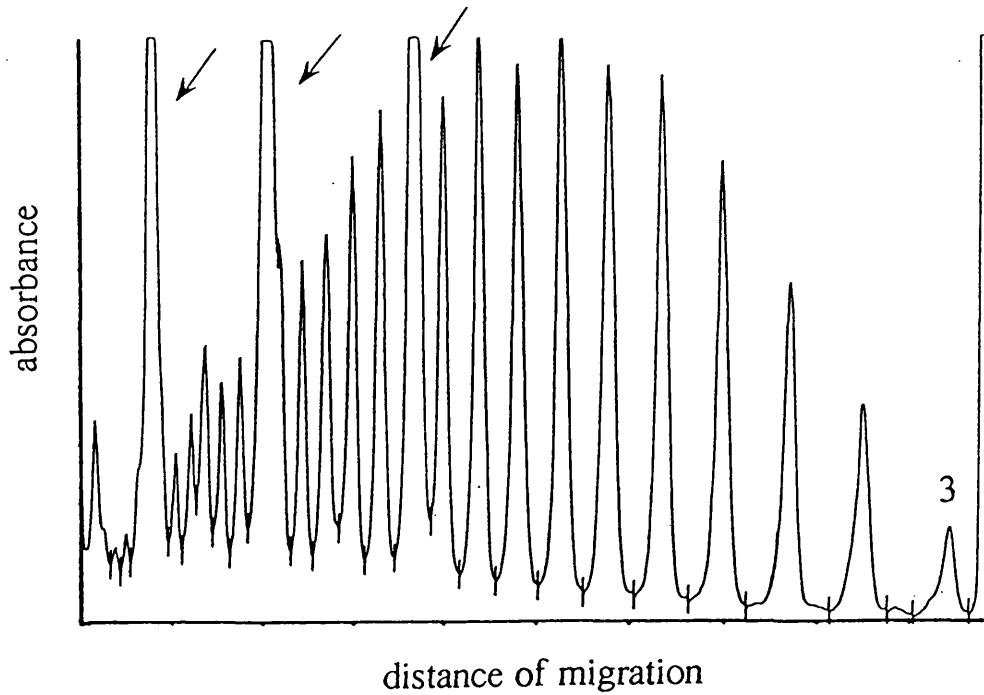
B, Multimers of 21 base-pair repeats containing meT:A at position 2

distance of migration
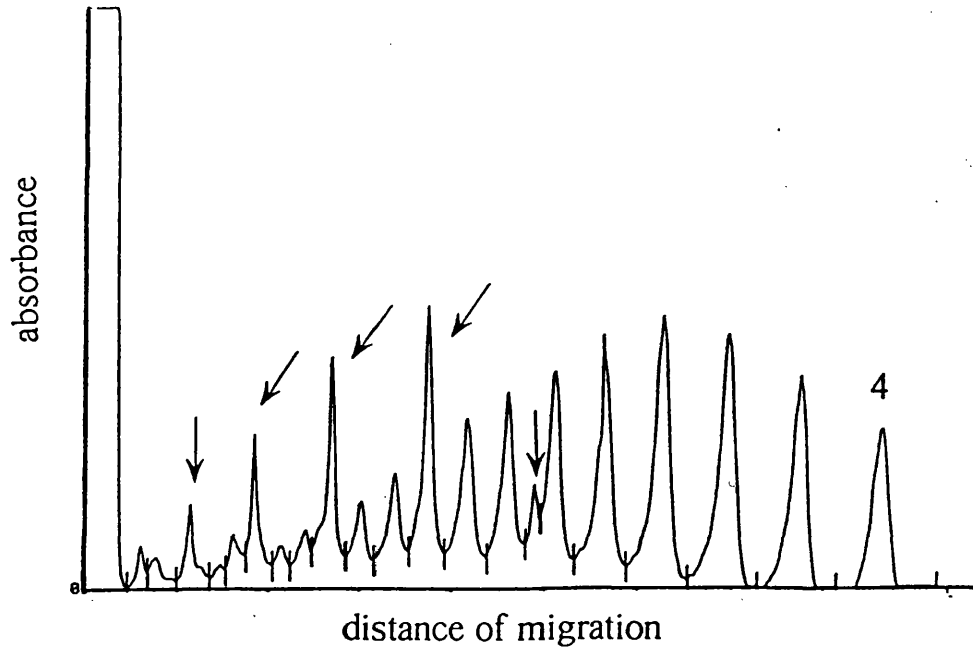
Figure 5.4 : Example of traces after scanning of the autoradiographs using a densitometer.

200 mm of the autoradiographs were scanned. A, is the scanning trace of lane b of figure

5.2; B, is the scanning trace of lane c of figure 5.3. The circles are indicated by arrows.
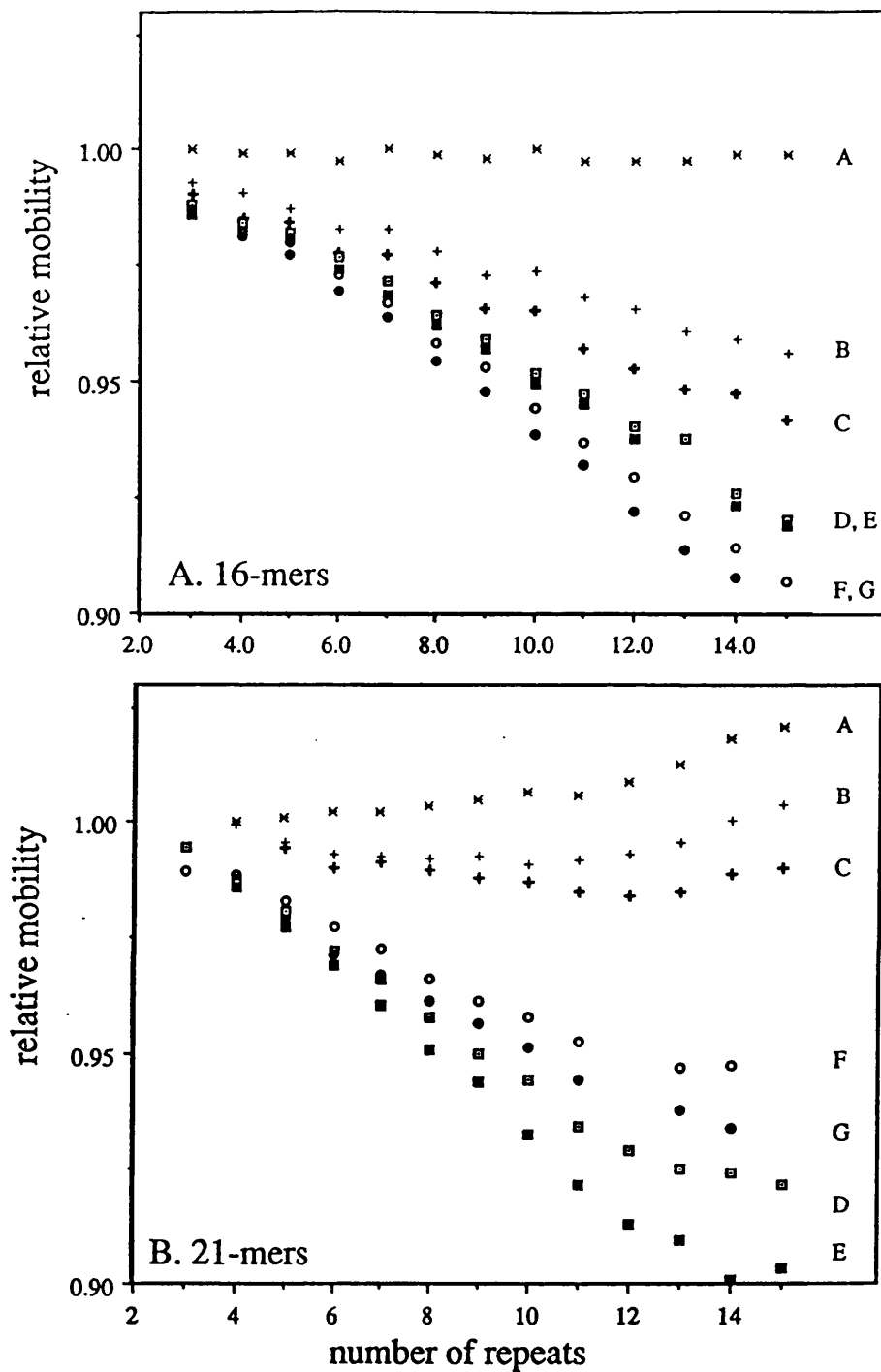
Figure 5.5 : Changes in relative electrophoretic mobilities of A, multimers with 16 base-pair repeats and B, 21 base-pair repeats as a function of the number of repeats. The relative mobilities were calculated from the polyacrylamide gels shown in figure 5.2 and 5.3 as described under Materials and Methods. The figure shows that the presence of an alkylT reduces the mobility of the DNA and that alkylT:A pairs have a greater effect than alkylT:G pairs. The values for polymers with base pair substitutions at position 2 are: □ (D) meT:A; ■ (E) etT:A; + (B) meT:G; + (C) etT:G; ✕ (A) T:G. For substitutions at position 1 are: o (F) meT:A; ● (G) etT:A.

(figure 5.4 A) and relative mobilities were obtained as described in "Materials and Methods" and plotted versus the number of 16 base-pair repeats in the polymer (figure 5.5 A). The DNA containing a T:G mismatch at every 16th base-pair migrated at the same rate as the control sequence. All oligomers containing O^4-alkylthymine migrated more slowly than the corresponding control sequences. In summary : 1. sequences containing alkylT:A migrated considerably more slowly than the corresponding sequences containing alkylT:G ; 2. sequences containing ethylT paired either to A or to G migrated more slowly than the corresponding sequences containing methylT; 3. the effect of alkylT was greater when it was in position 1 than in position 2. The rank order of *reduced* mobility for the $(dN_{16})_x$ oligomers is shown in figure 5.6 A.

These $(dN_{16})_x$ ligation ladders showed two families of bands (figure 5.2 and 5.4 A). The first comprised the linear DNA molecules while the other consisted of circular molecules. The rate at which T4 DNA ligase converts linear DNA fragments into circles reflects the flexibility of the DNA molecule (Shore *et al.*, 1981). There was a correlation between the formation of circles and the degree of retardation of the linear molecules containing O^4-alkylthymine. The most slowly migrating sequences, i.e. those containing alkylT:A, produced circles of smaller diameter and a greater proportion of circles than the alkylT:G containing sequences (figure 5.2). The control $(dN_{16})_x$ ligation mixture and the sequences containing T:G mismatches, which had normal mobility, did not produce short length circles.

Non-denaturing electrophoresis of the ligated 21-mers gave the autoradiograph shown in figure 5.3 and scanning traces like this in figure 5.4 B. In summary the results were: 1. the sequences containing alkylT:A base pairs migrated most slowly; 2. sequences containing ethylT base paired to either A or G migrated more slowly than the analogous sequences containing methylT (figure 5.5 B); 3. The most slowly moving linear sequences formed the smallest circles; 4. in contrast to the considerable retardation caused by the presence of alkylT, the presence of meG:C caused only a small reduction in mobility which was even less than that produced by the C:A mismatch.

Figure 5.6 : Rank order of reduced mobility for A, ligated 16-mers consisting of 11

monomeric repeats and B, ligated 21-mers with the same number of repeats; pH 8.3.

The rank order of reduced mobility for the $(dN_{21})_x$ polymers is shown in figure 5.6 B. The exact comparison between the migration of the 16-mers and 21-mers is discussed more fully below but in general terms there was a parallel between the electrophoretic behaviour of the ligated 21 base-pair oligomers $(dN_{21})_x$ (figure 5.3) and that of the ligated 16 base-pair oligomers $(dN_{16})_x$ (figure 5.2).

One respect in which the results with these $(dN_{21})_x$ oligomers did not parallel those from the $(dN_{16})_x$ oligomers, was in the effect of the position of the alkylT:A base pair on the mobility. An alkylthymine in position 2 in the $(dN_{21})_x$ oligomers had a greater effect than an alkylthymine in position 1 (figure 5.6 B). This was the opposite of what was observed for the ligated 16-mers (figure 5.6 A).

While carrying out this work two unexpected observations were made. The first was that the ligation product of the control 21 base-pair oligomer contained relatively small circles (figure 5.3). As is discussed below, these small size circles would not be expected to form in normal DNA (Shore and Baldwin, 1983), and they were not seen in the ligation ladder of the control 16 base-pair sequence (figure 5.2). The second was that although relative electrophoretic mobilities were reduced as polymer size increased, this relationship was not linear for multimers with high number of repeats (the curves of figure 5.5 B bent upwards). This contrasts with the linearity observed for the $(dN_{16})_x$ polymers which was maintained even for very long oligomers (figure 5.5 A). It was suspected that the reason for these findings was that the sequence used as control has itself anomalous electrophoretic mobility. The 16-mer sequence had 3 fewer bases at the 5' end and 2 fewer bases at the 3' end than the 21-mer but the relative base composition was almost identical to that of the 21-mer sequence. Thus, the 21-mer and the 16-mer should have very similar relative electrophoretic mobilities. However, electrophoresis in parallel of the control $(dN_{21})_x$ and $(dN_{16})_x$ ligation mixtures showed that the $(dN_{21})_x$ oligomers migrated more slowly than expected (figure 5.9). This is indicative of a sequence dependent curvature and can explain both the above mentioned observations. Interestingly, when the adenine in position 1 or 2 was replaced by a guanine, forming a T:G mismatch, the resultant $(dN_{21})_x$

ligation ladder migrated faster than the control 21-mer ligation ladder (figure 5.6 B).

5.3.2. pH effect

In addition to the normal pH 8.3 gels, electrophoresis was also carried out at pH 6.5 because the structure of alkylT:A pairs in solution is known at acidic pH but is not as yet known at alkaline pH (Kalnik *et al.*, 1988a,b). At pH 6.5 the rank order of *reduced* mobility for the $(dN_{16})_x$ and the $(dN_{21})_x$ polymers is shown in figure 5.7.

All the oligomers containing base pair substitutions, whether in the $(dN_{21})_x$ or the $(dN_{16})_x$ series, had less relative retardation at pH 6.5 than at pH 8.3. However the pH effect was not of the same magnitude for all the sequences. There was an extraordinary difference between relative mobilities of $(dN_{21})_x$ multimers containing alkylT:A or C:A mismatches at the two pH's (Figure 5.8 B), but there was only a moderate change in relative mobilities of $(dN_{21})_x$ multimers containing meG:C, T:G, or alkylT:G. The relative mobilities of all the $(dN_{16})_x$ oligomers were changed only slightly by the change in the pH and the extent was similar for all the sequences (figure 5.8 A).

Because of the differences in electrophoretic behaviour of the 16-mer and 21-mer multimers used as controls one can not distinguish a phase dependent effect on mobility over and above the phase independent effect from the data as shown in figure 5.5 A and B. However, the studies at the two different pHs allowed the phase dependent effect to be distinguished from the phase independent component of retardation of the $(dN_{21})_x$ multimers.

The $(dN_{21})_x$ and the $(dN_{16})_x$ ligation ladders were run in parallel at both pH conditions and the K-factor (Diekmann, 1987) of the oligomers was obtained (see Materials and Methods) using as molecular weight markers the $(dN_{16})_x$ multimers. Higher K-factors for the $(dN_{21})_x$ than these of the $(dN_{16})_x$ multimers is an indication of an anisotropic flexibility or bending of DNA. In order to avoid the possibility that the differences observed in retardation at the two pH conditions was an artefact caused by the difference in the voltage drop previously used for the acidic and basic gels (see Material and Methods)
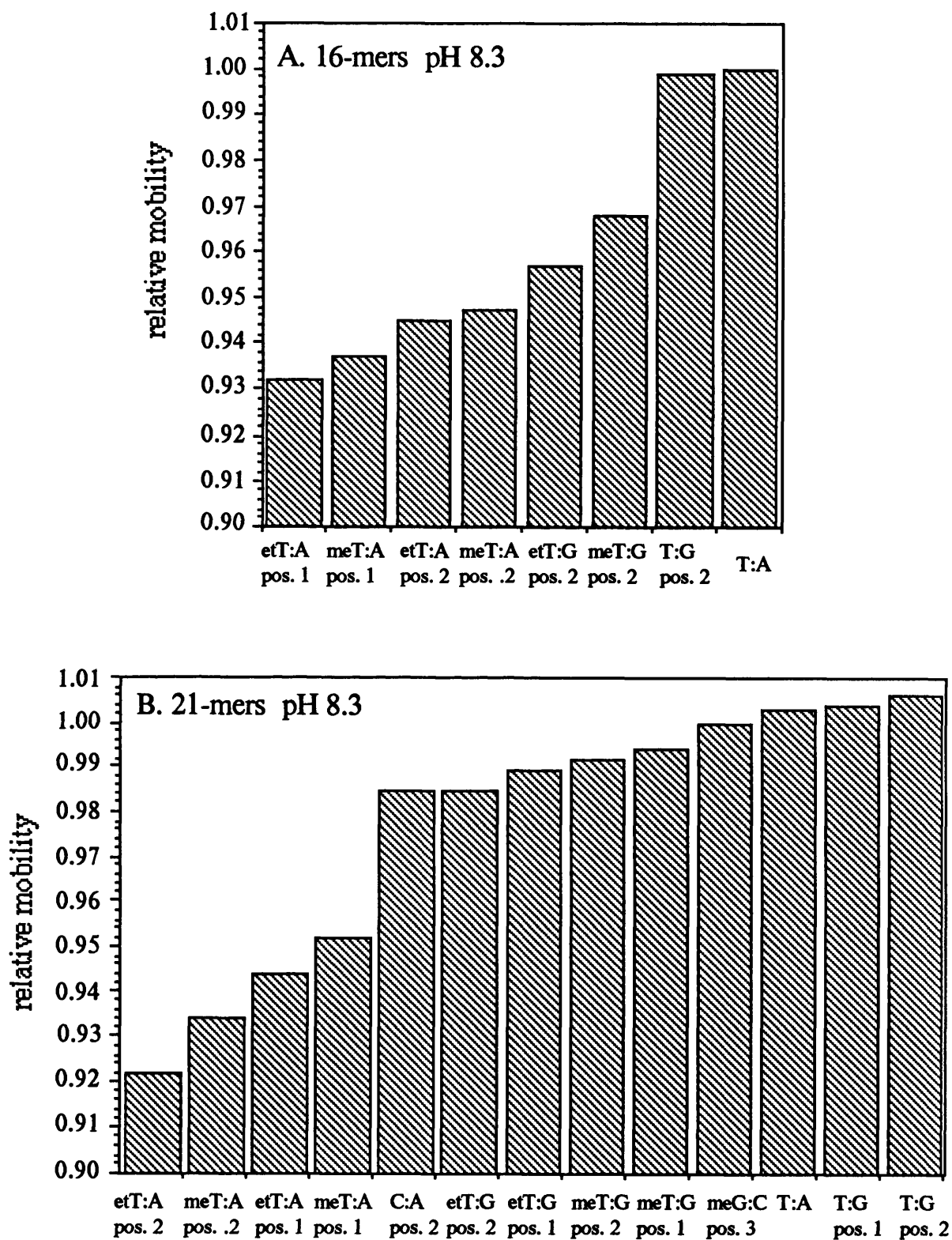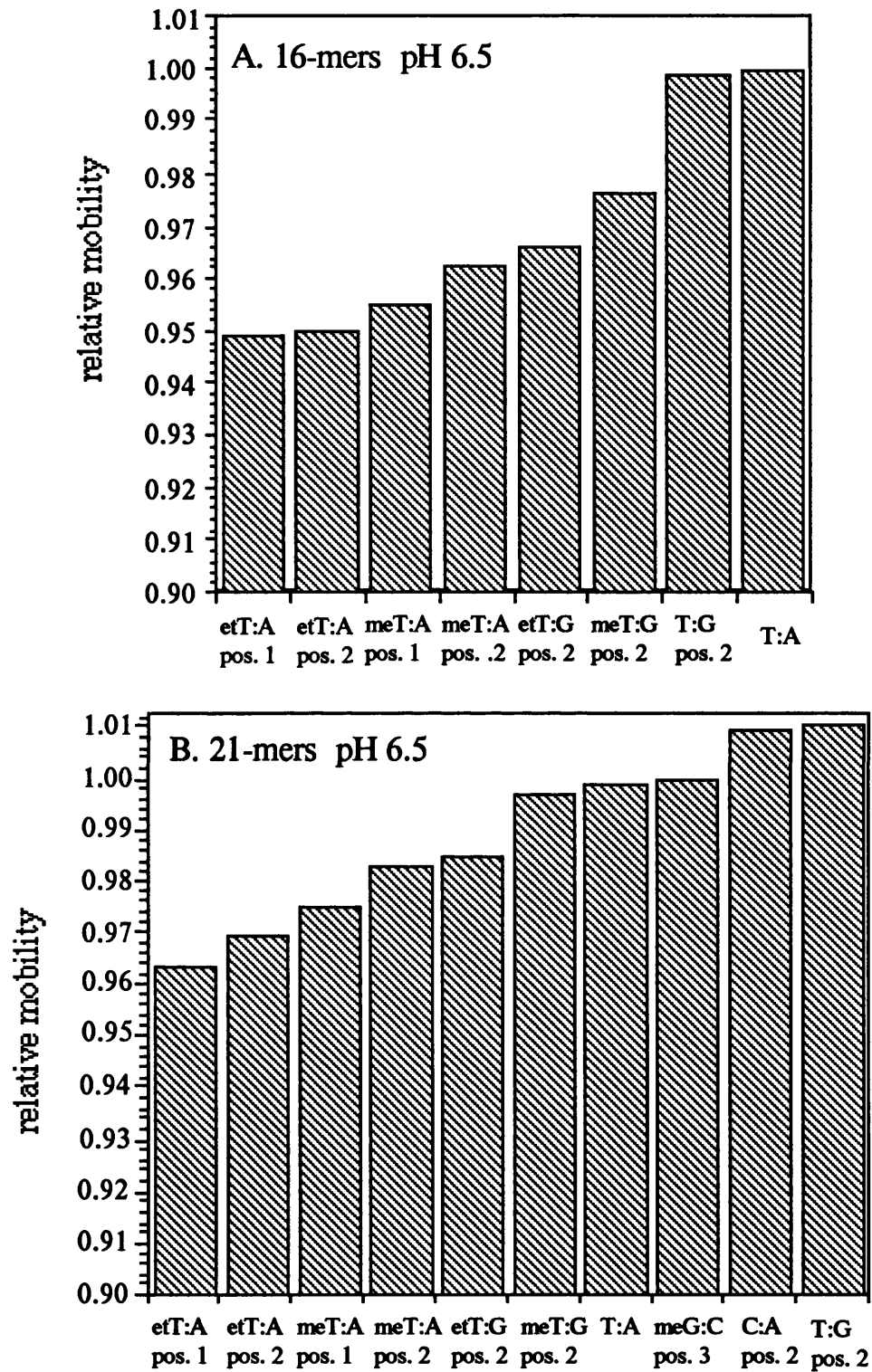
Figure 5.7 : Rank order of reduced mobility for A, ligated 16-mers consisting of 11 monomeric repeats and B, ligated 21-mers with the same number of repeats; pH 6.5.
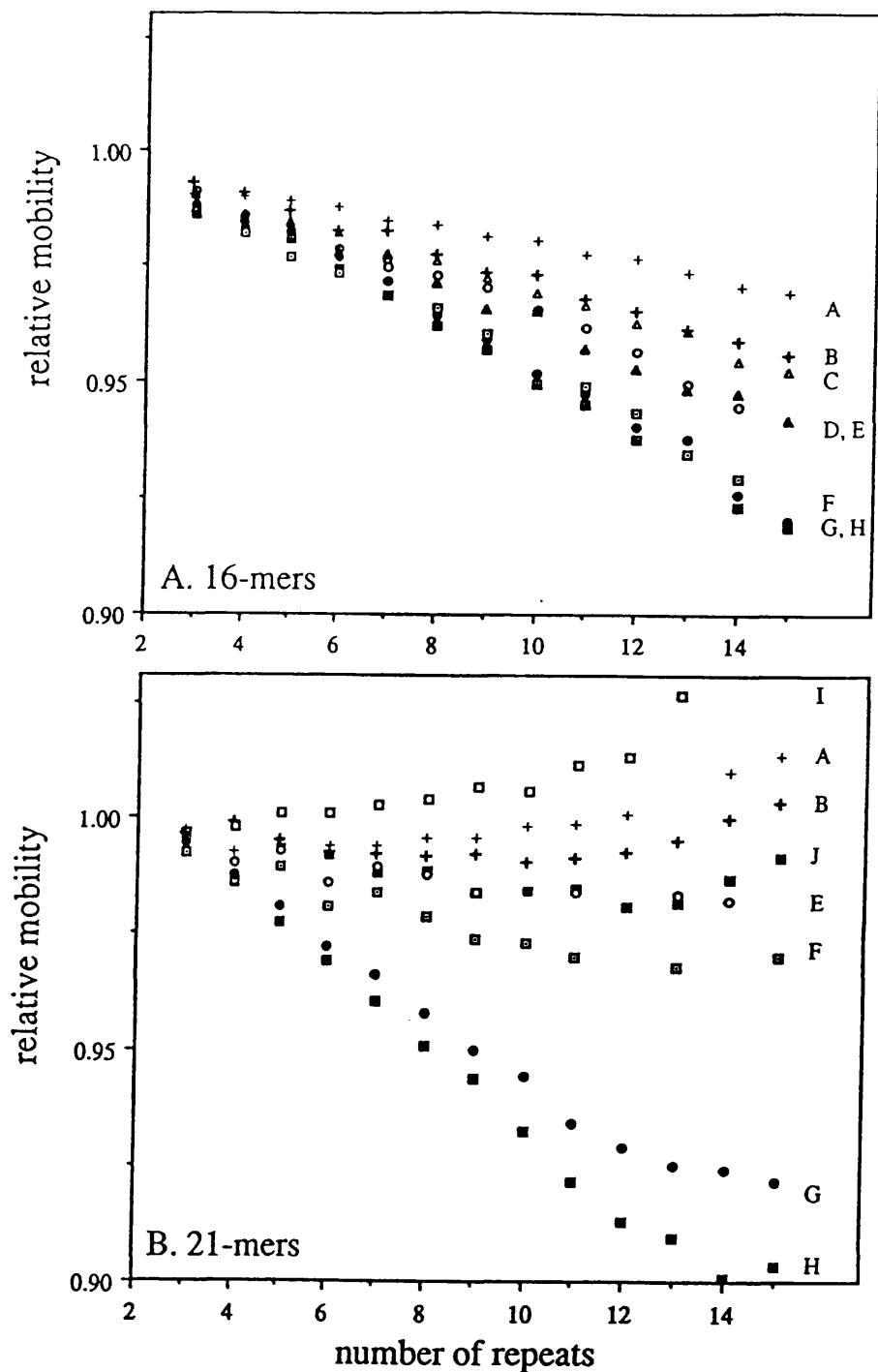
Figure 5.8 : The effect of pH on the relative mobility of A, 16 base-pair and B, 21 base-pair multimers containing O⁴-alkylthymine and 21 base-pair multimers containing C:A mismatches. The figure shows results from sequences in which the alkylthymine was in position 2. Very similar results were obtained with sequences where the substitution was in position 1. The closed or bold symbols correspond to the values at pH 8.3 and the open symbols to the values at pH 6.5. ● ○ (G, E) meT:A; ▣ ▢ (H, F) etT:A; ▪ ▢ (J, I) C:A; + + (B, A) meT:G; ▲ △ (D, C) etT:G.
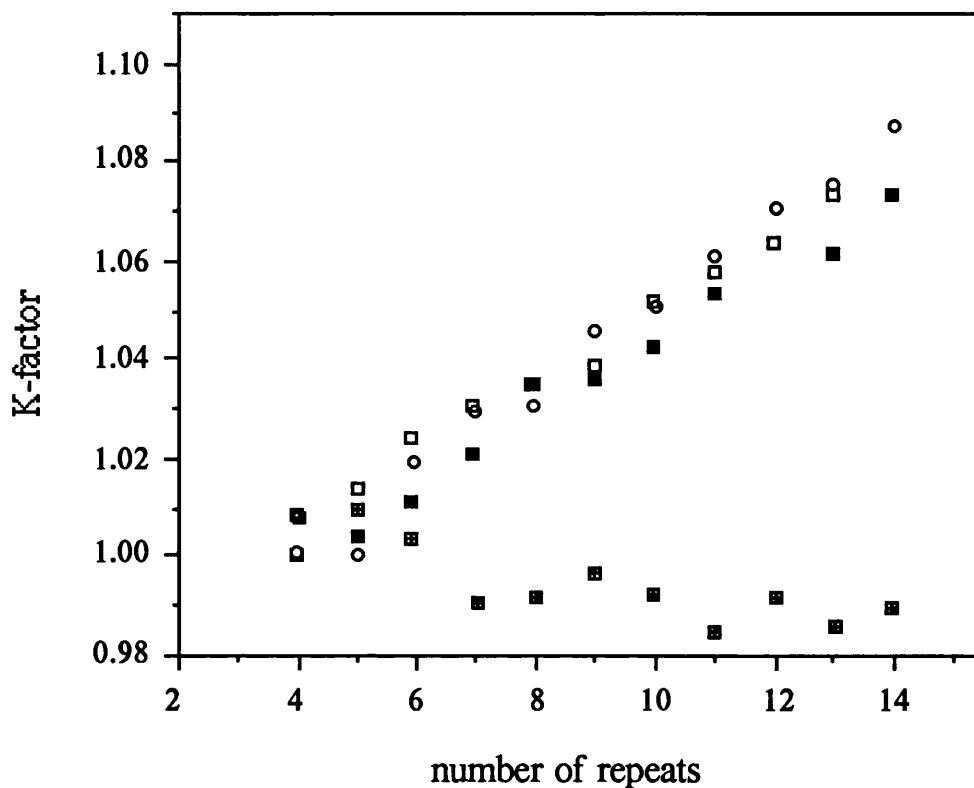
Figure 5.9 : The effect of pH and voltage gradient on the K-factor of the control 21

base-pair multimers as function of the number of repeats. The K-factor determination was

based on the migration of the control 16 pase-pair multimers (see Materials and Methods).

The figure shows that at both pH 6.5 and 8.3 the DNA migrated more slowly than

expected, and that this slow migration was not affected by pH or the voltage gradient in

the gel.   o   Electrophoresis at 10 V / cm and pH 8.3 ; Electrophoresis at 5 V / cm

□   at pH 8.3 and   ■   at pH 6.5. ▩   Multimers of a 21-mer which had the exact

sequence of K-ras gene around codon 61.
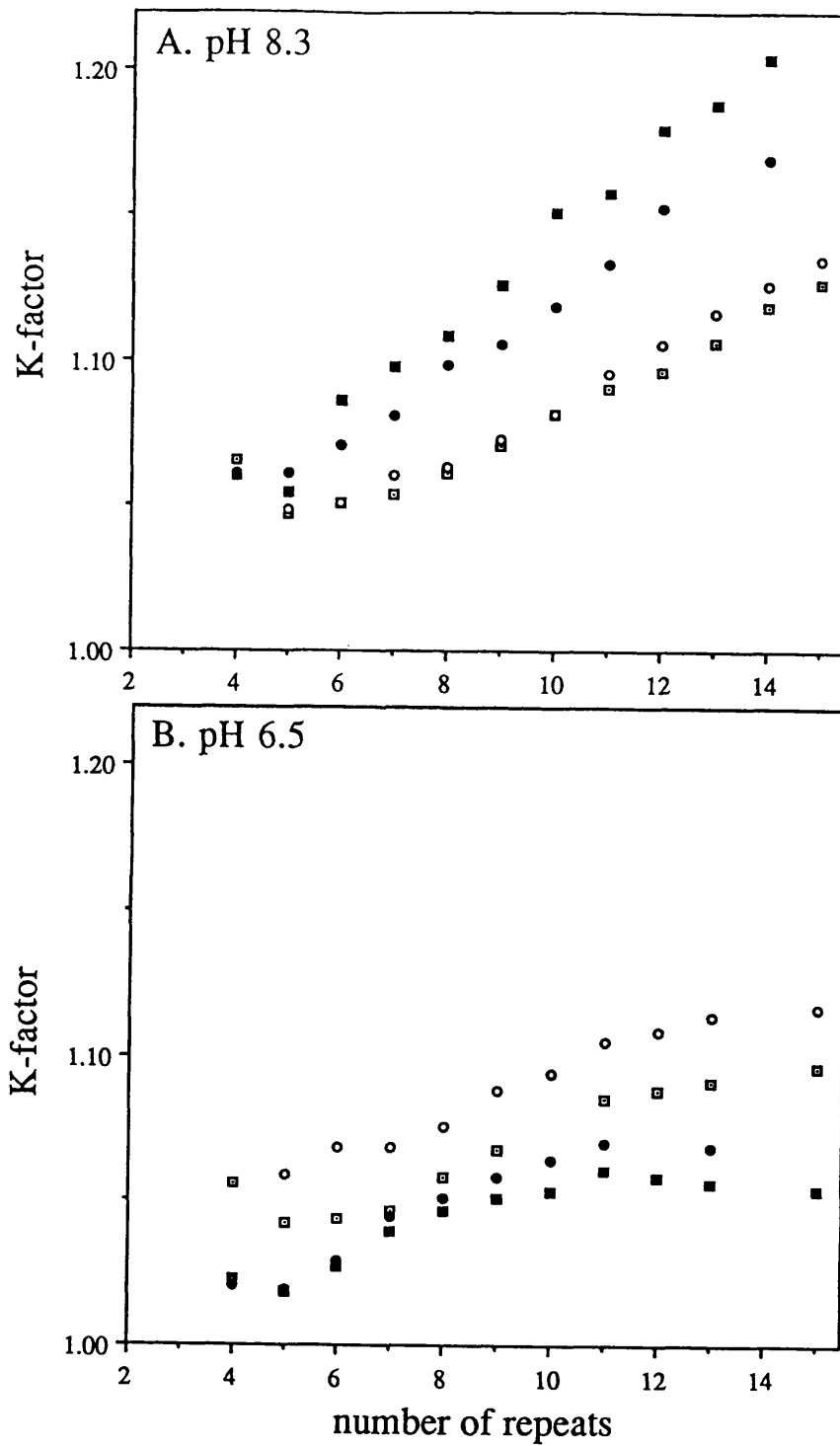
Figure 5.9 : Evidence for a degree of anisotropic flexibility, or bending, of sequences containing methylT:A base pairs at pH 8.3 (A) but not at pH 6.5 (B). The K- factors were calculated as in Materials and Methods using the 16 base-pair multimers as control. □ 16-mers with meT:A at position 2; ○ meT:A at position 1. ■ 21-mers with meT:A at position 2; ● meT:A at position 1.
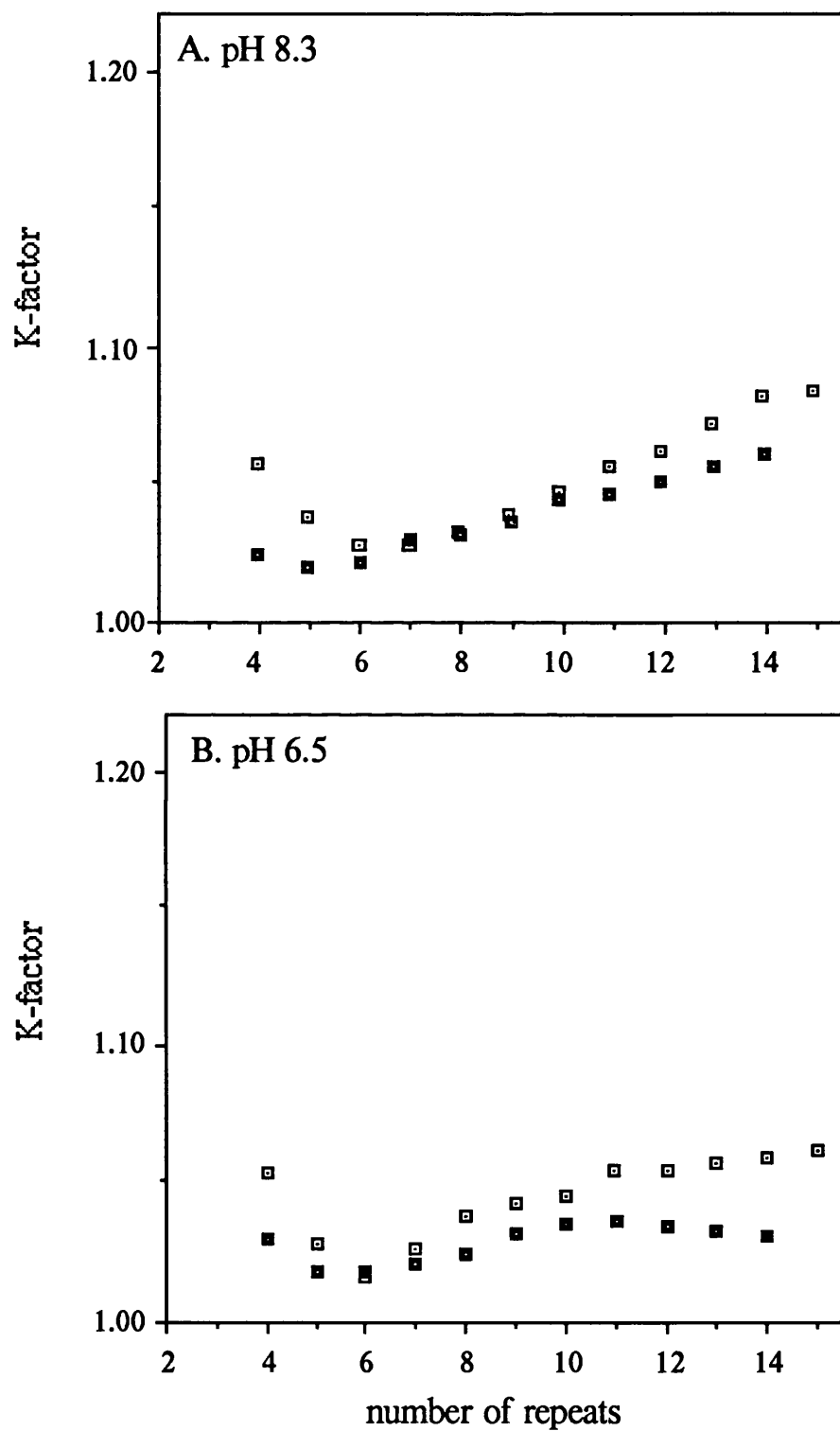
Figure 5.11 : K-factor vs number of repeats for multimers containing methylT:G

base-pairs at pH 8.3 (A) and at pH 6.5 (B). □   16-mers with meT:G at position 2;

■   21-mers with meT:G at position 2.

the same voltage drop of 5 V/cm was used for both pHs.

The K-factor increase as a function of the number of repeats for the $(dN_{21})_x$ control sequences, was similar at both pHs (figure 5.9). However, the $(dN_{21})_x$ polymers containing alkylT:A base-pairs had significantly higher K-factors than the respective $(dN_{16})_x$ polymers, at pH 8.3 but not at pH 6.5 (figure 5.10 A and B). The K-factor difference between 16 and 21 base-pair multimers was greater when the alkylT:A base-pair was in position 2 than when it was in position 1. Both $(dN_{21})_x$ and $(dN_{16})_x$ multimers containing alklylT:G base-pairs had similar K-factors at both pHs (figure 5.11 A and B).

5.3.3. Stability of 21-mer duplexes

All base-pair substitutions caused a decrease in the melting point (Tm) (figures 5.12 A and B). The smallest decrease in Tm was caused by the T:G base-pair at position 2 while the greatest decrease was caused by an meG:C base-pair at position 3 (table 5.1). In all cases examined, the duplex containing the alkylT:G base-pair was more stable than the duplex containing the alkylT:A base-pair. All base-pair substitutions were more destabilizing when present at position 1 than at position 2. The size of the alkyl group (methyl or ethyl) did not have any significant effect on the Tm values.
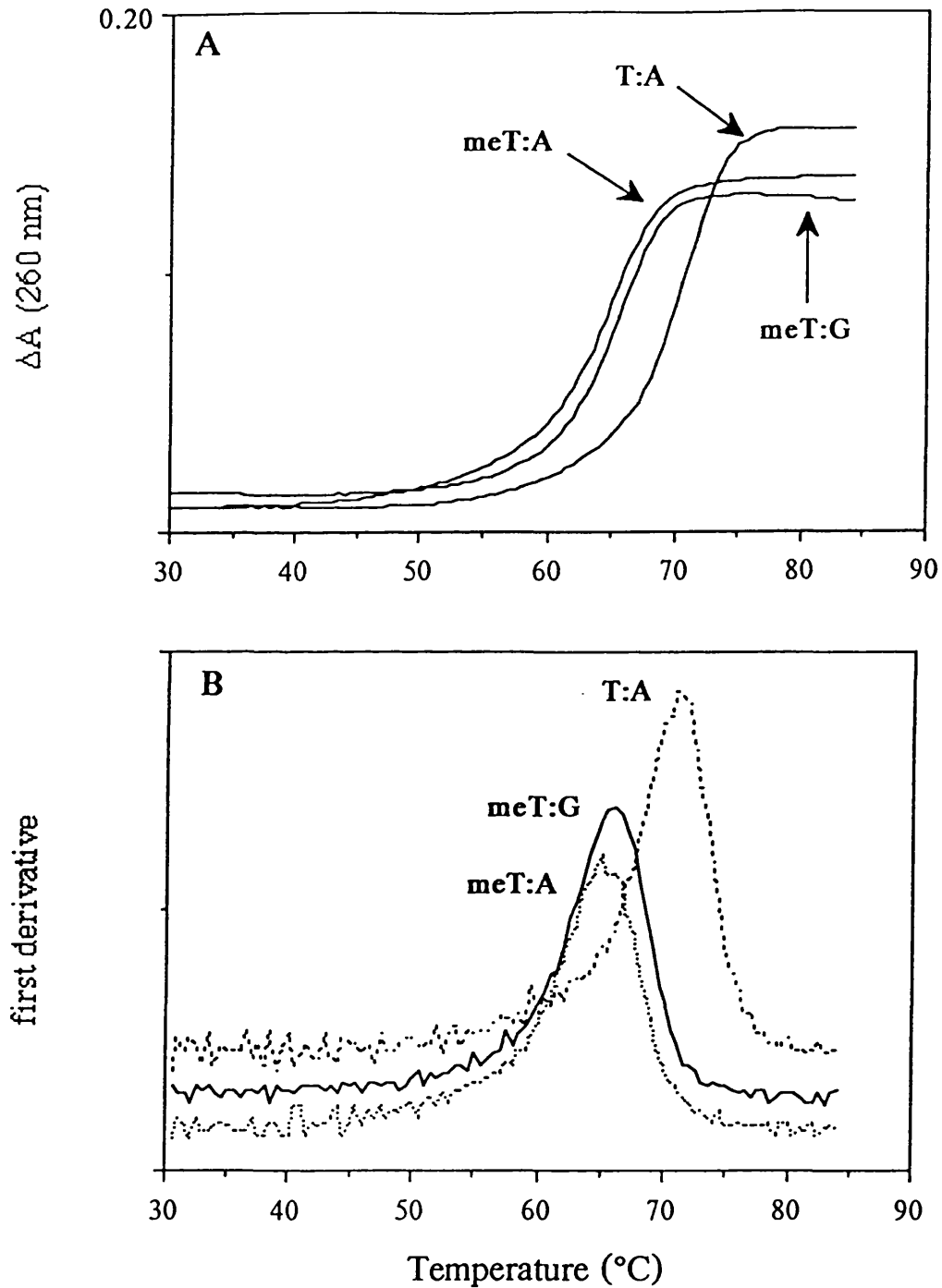
Figure 5.12 : Thermal denaturation of 21-mer duplexes containing O^4- methylthymine at position 2. A, melting curves at 260 nm. B, graphs of the first derivative of absorbance vs temperature. The Tm was determined as the temperature in which the first derivative of absorbance had the maximum value.

Table 5.1 : Tm values for 21-mer duplexes with base pair substitutions at position 1 2 and 3

base pair	position 1		position 2		position 3	
	Tm (°C)[a]	δTm(°C)[b]	Tm (°C)	δTm (°C)	Tm (°C)	δTm (°C)
control	70.9	--	--	--	--	--
C:A	ND	ND	64.2	6.7		
T:G	ND	ND	67.7	3.2		
meG:C					63.1	7.8
meT:A	63.6	7.3	64.9	6.0		
etT:A	63.3	7.6	64.9	6.0		
meT:G	64.8	6.1	65.7	5.2		
etT:G	64.8	6.1	65.6	5.3		

[a] Tm values were calculated as described under Materials and Methods. [b] δTm values were calculated by subtracting the Tm value of the control oligomer from each observed value. ND: no data available.

5.4. DISCUSSION

The control sequence used in the present study was unexpectedly found to be curved (figure 5.9). The degree of gel retardation [e.g the $(dN_{21})_{15}$ multimer had a K-factor of 1.1], is considerable for a low percentage acrylamide gel (Ulanovsky et al., 1986) and suggests a significant curvature. Substitution of one of the A's in the TTG/CAA by a G gave $(dN_{21})_x$ oligomers which migrated faster than the respective control multimers. Trifonof and Sussman (1980) suggested a model for DNA curvature based on the difference between the wedge (roll and tilt) angle of the dinucleotide ApA and that of any other dinucleotide. Thus, when this curvature was first observed it seemed to suggest that it was centred in the ApA dinucleotide, and that the K-ras gene might also have curvature centred on the 61st codon. However, the sequence used is only partially similar to that of the K-ras and subsequently it was found that the sequence 5' AC.ACA.GCA.GGT.CAA.GAG.GAG.T 3' which corresponds exactly to codons 58 to 63 of the mouse K-ras gene has no electrophoretic abnormality (figure 5.9). Therefore, the cause of the observed curvature is not clear. Unfortunately the theoretical understanding of DNA curvature is not yet complete. Several DNA bending models suggest a contribution of the roll and tilt angles of every dinucleotide step in the overall curvature (Ulyanov and Zhurkin, 1984; Calladine and Drew, 1986; Calladine et al., 1988). In these models, presence of a dA:dT tract is not always required as a prior condition for predicting curvature, and recently sequences without any ApA dinucleotide step were experimentally found to be curved (Brukner et al, 1991). However, any single model cannot explain all the available relative mobility data (Tan and Harvey, 1987).

While much evidence exists for the direction (Zinkel and Crothers, 1987; Koo and Crothers, 1988) and the magnitude (Koo et al., 1990) of DNA curvature induced by oligo dA:dT tracts, little is known about the distortions of the DNA helix caused by modified bases. Electrophoretic mobility studies have been performed for 2-acetylaminofluorene-guanine adducts (Schwartz et al., 1989), thymine-dimers (Husain, et al., 1988) and O^6-methylguanine (Voigt and Topal, 1990). The presence of all these

modified bases retarded the migration of the oligomers through the gel. The 2-acetylaminofluorene-guanine-induced retardation was not phase dependent (Schwartz *et al.*, 1989), but the thymine-dimer (Husain *et al.*, 1988) and O^6-methylguanine (Voigt and Topal, 1990) induced retardation was much greater when the lesions were repeated in phase with the 10.5 base-pair helical turn of the DNA. Therefore, it was suggested that 2-acetylaminofluorene produces isotropic flexibility of DNA while thymine dimers and O^6-methylguanine induce largely asymmetric (anisotropic) flexibility (see below for definition).

O^4-alkylthymine produced a considerable reduction of the electrophoretic mobilities of oligomers either when it is repeated in phase or out of phase with the helical repeat. This effect was much greater when the modified base was paired to adenine than when paired to guanine, and the magnitude of the effect depended on the size of the alkyl group. O^4-ethylthylmine always caused greater retardation than O^4-methylthymine.

One possible reason for this retardation is "static" curvature which is defined as a fixed curvature of the helical axis, superimposed on a background of normal thermal motions of DNA. One characteristic of curved DNA is that gel retardation is observed only if the sequence responsible for the bending is repeated in phase with the helical turn [≈10.5 base-pairs] (Koo *et al.*, 1986). There are a number of other reasons for gel retardation which do not have this phasing dependence and in which the degree of retardation is proportional to the number of lesions but independent of their alignment in relationship to the helical repeat. These include cruciforms, localized single-strandedness or bulges in the DNA sequence, chemical or physical interaction with the gel matrix, and isotropic flexibility. Isotropic in this context implies an increased flexibility in which the time average distribution of the DNA fluctuations is symmetric with respect to the linear helical axis. In contrast, anisotropic flexibility is defined as the flexibility which is symmetrical around the helical axis. Anisotropic flexibility like the static curvature results in a phase-dependent gel retardation. Although the distinction between static curvature and anisotropic flexibility is a subtle one, the term "anisotropic flexibility" is used in this discussion referring to modified DNA, while the term "curvature" is used to describe the

intrinsically curved natural DNA.

Oligomers used in this study lack any self-complementarity that could induce hairpin or cruciform formation. Furthermore, any physical or chemical interaction of the modified thymidine with the gel matrix is unlikely to be the cause of the retardation because in that case one would expect the same effect when the alkylated thymine is paired either with guanine or adenine. Voigt and Topal (1990) suggested that the gel retardation they observed, was due in part to a localized bubble formation during electrophoresis, which lead to a frictional delay in the mobility of O^6-methylguanine containing oligomers. This does not seem to be the case for the O^4-alkylthymine containing oligomers because, although no extensive study of the distribution of small circles was performed, there seemed to be a correlation between retardation and the formation of small circles.

The rate of ligation of DNA molecules into circular forms depends on the ring closure probability, commonly called the j-factor, which is a function of three probability densities [figure 5.13] (Levene and Crothers, 1986).

1. The probability of the two ends of the DNA to lie within the same infinitesimal small volume ($R \longrightarrow 0$).

2. The probability of the tangents to the helical axis at the two ends of the molecule to be nearly parallel ($\pi \longrightarrow 0$).

3. The probability of the torsion angle Ω between the chain termini to lie within a small increment about the helix twist angle ($\Delta\Omega$) between adjacent base-pairs. This is equivalent to specifying that the phosphate backbone be nearly continuous at the point where the two ends are to be joined.

The first two parameters give the measure of the dependence of the j-factor on the flexibility of the DNA molecule. The more flexible a DNA molecule, the higher the probability of the two ends coming together and being ligated to form a circle (Zahn and Blattner, 1985; Ulanovsky et al., 1986). For normal (non-bent) DNA, these parameters obtain significant high values only when the number of base-pairs exceeds a critical value (persistence length). Shore et al. (1981) and Shore and Baldwin (1983) showed that DNA fragments above 500 base-pairs can be efficiently self-ligated and form circles, but cyclization
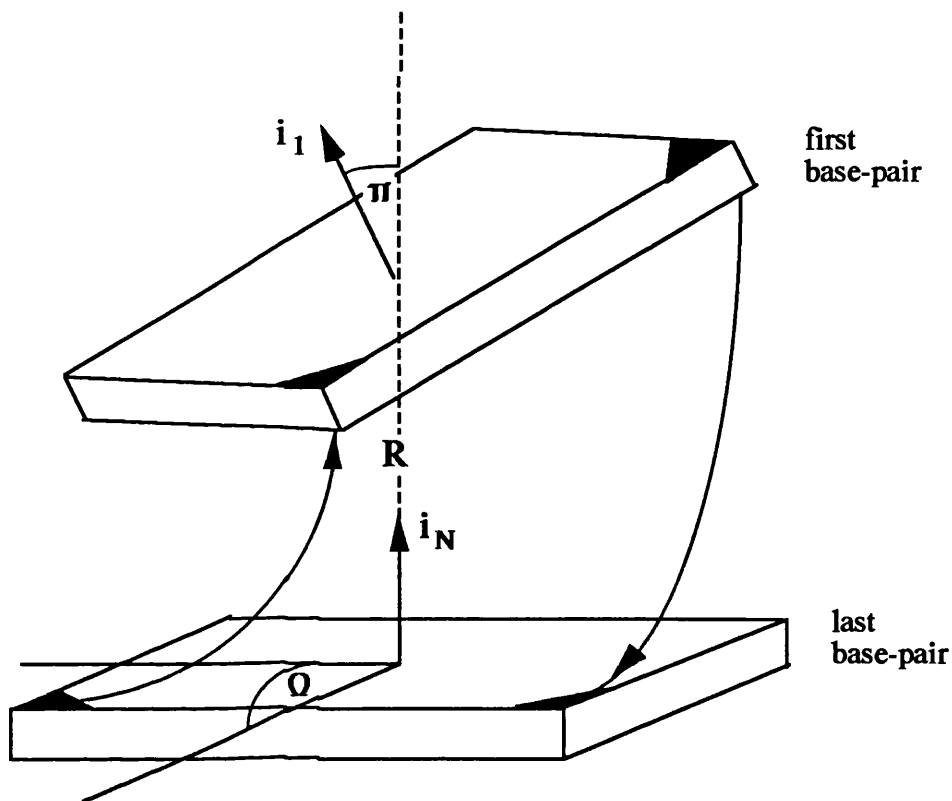
Figure 5.13 : Parameters that affect the rate of ligation of DNA molecules into circular forms. In the scheme the planes of the first and last base-pair of a linear DNA molecule are shown. Shaded corners locate attachments of bonds to sugar C1' atoms. R is the distance between the first and last base-pair; π is the angle between the

vectors i_1 and i_N. These vectors are vertical to the respective base-pair planes. Ω is the tortion angle between the base-pairs. Curved arrows indicate the 5'-3' direction of each backbone when the circle is formed.
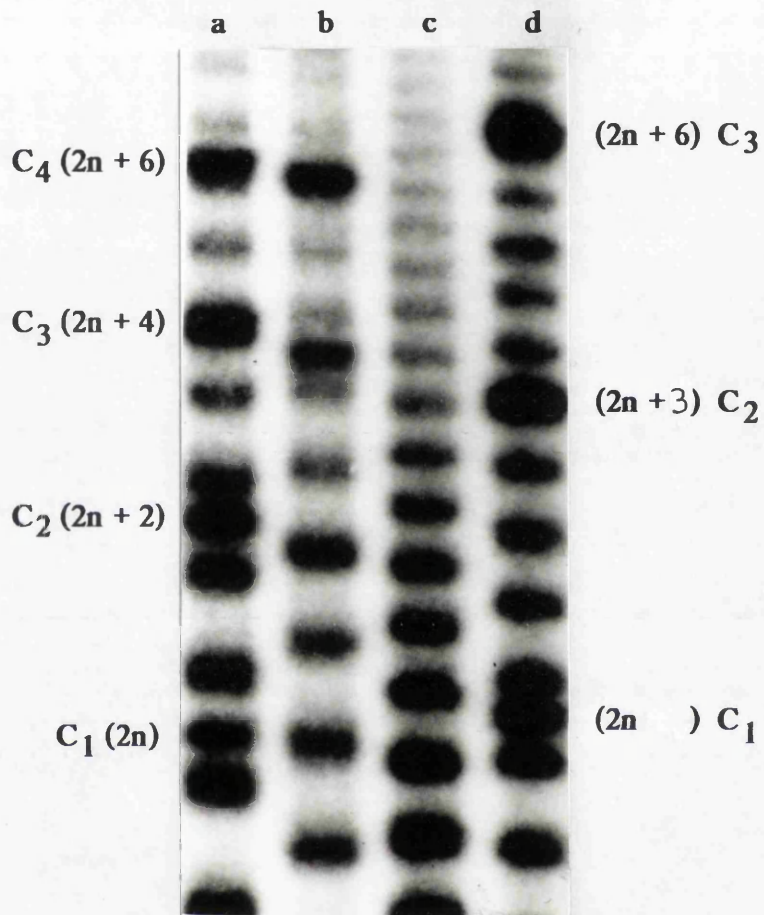
Figure 5.14 : Comparison of the circles formed in the (dN21)x and (dN16)x ligation
mixtures. Lane a, (dN21)x polymers containing O^4-methylT:A at position 2; lane d, (dN16)x
polymers containing O^4-methylT:A at position 2; lanes b and c, (dN21)x and (dN16)x control
ligation ladders respectively. The circilar products of the (dN21)x and (dN16)x ligation
mixtures are indicated along the left and right side of the autoradiograph respectively. In
parenthesis are the number of helical turns for the respective circles.

becomes progressively more difficult as DNA length decreases, reaching a zero probability

for circle formation at about 150-240 base-pairs. Ligation of oligomers containing

O^4-alkylthymine however, formed circles of smaller diameter than the control sequences

(figure 5.2 and 5.3), and this observation could only lead to the conclusion that

O^4-alkylhtymine produces sites of DNA hyperflexibility. As figure 5.2 shows

O^4-alkylT:G containing oligomers which were less retarded than the alkylT:A containing

multimers, produced also a smaller proportion of circles. This is in agreement with the

predicted properties of DNA molecules with induced flexibility. Localized

single-strandedness or bulges in these polymers could reduce the mean circle distribution

only if these regions were also regions of increased flexibility.

The contribution of the continuity of the phosphate backbone to the probability of

circle formation can also be seen by comparing the distribution of circles in the $(dN_{21})_x$

and $(dN_{16})_x$ ligation mixtures (figure 5.14). The 5' and 3' end of all the multimers with 21

base-pair sequence repeats are separated by an integral number of DNA turns, and only

small torsional fluctuations are required to bring them into register for ring closure. Thus,

all the multimers have the same probability (maximum), in terms of the continuity of the

phosphate backbone, to form a circle. In contrast, the ends of the $(dN_{16})_x$ multimers are

separated by integral (even number of monomeric repeats) or half integral helical turns

(odd number of monomeric repeats). It can be seen from the spacing between the circles in

the $(dN_{16})_x$ ligation ladders that only circles of multimers with even number of repeats are

formed (figure 5.14). Polymers with odd number of repeats were not formed because these

molecules must undergo torsional deformations of the order of half a helical turn to achieve

torsion angle compliance.

The flexibility we observed, could be either isotropic, i.e bending in all directions

is equally possible, or anisotropic, i.e bending in some directions is thermodynamically

favoured. Both $(dN_{21})_x$ and $(dN_{16})_x$ containing O^4-alkylthymine were significantly

retarded relative to the control sequences (figure 5.5 A, B). Thus, it was initially assumed

that the flexibility caused by this DNA adduct is centrosymmetric with respect to the helical

axis. Two observations however, were not compatible with the isotropic model for DNA flexibility. The first was that alkylT:G containing $(dN_{21})_x$ multimers had significantly lower relative mobility than the respective $(dN_{16})_x$ multimers, in contrast to the alkylT:A containing polymers which had similar mobilities for both the $(dN_{21})_x$ and $(dN_{16})_x$ ligation ladders. The second was that the ranking order of reduced mobility of the $(dN_{21})_x$ polymers containing alkylT:A, was the inverse of that of the $(dN_{16})_x$ polymers. When it was subsequently found that the control $(dN_{21})_x$ polymers have anomalous electrophoretic mobility, it was suspected that the relative mobilities as shown in figure 5.5 B give an underestimation of the effect of the O^4-alkylthymine residues on the mobility of the $(dN_{21})_x$ polymers. To test the issue of phasing the $(dN_{16})_x$ and $(dN_{21})_x$ ladders were run in parallel and the control $(dN_{16})_x$ ligation ladder was used as a reference. The increase in the K-factor of the oligomers containing the alkylT:A base-pair was far greater when the modified base was repeated every 21 base-pairs, i.e. in phase with the helical repeat, rather than every 16 base-pairs, i.e. with the alkylated base alternately on one side of the helix then on the other (figure 5.10 A). The phasing factor was greater at position 2 than at position 1 of the sequence. Evidence that the phase dependence of the retardation was caused by the alkylT:A base-pair, and not by inherent DNA curvature came from the studies at pH 6.5. The K-factor of the control $(dN_{21})_x$ sequence was virtually unaffected by the decrease of pH (figure 5.9), but the K-factor of the alkylT:A containing $(dN_{21})_x$ multimers was reduced (figure 5.10 B), so that at pH 6.5 the retardation of the $(dN_{21})_x$ multimers, in which the alkylT:A pair was in phase, was similar to the retardation of the $(dN_{16})_x$ oligomers in which the alkylT:A pair was out of phase.

These observations lead to the conclusion that there is a significant difference in the structure of the oligomer containing alkylT:A pairs at pH 8.3 and pH 6.5. At pH 8.3 the conformational change and flexibility produced by an alkylT:A pair is asymmetric (anisotropic). A greater anisotropic factor for the alkylT:A at position 2 could explain the differences observed in the ranking order of reduced mobilities of the $(dN_{16})_x$ and $(dN_{21})_x$

polymers. At pH 6.5 similar retardations, and ranking orders were observed suggesting that the conformational change and flexibility produced by the alkylT:A base-pair is isotropic (Koo *et al.*, 1986). The anomalous migration caused by the alkylT:G base-pair at pH 8.3 did not have a phasing characteristic, and no significant difference was observed in the decrease of retardation of the alkylT:G containing $(dN_{21})_x$ and $(dN_{16})_x$ oligomers when the pH was reduced to 6.5 (figure 5.11 A and B). In this case, an isotropic rather than an anisotropic flexibility model would be plausible.

A pH effect, similar to the one observed for the oligomers containing alkylT:A base-pair, was observed for the oligomers containing C:A base-pair which suggests that this pH effect could be a reflection of similarities in the structures for these mismatches. NMR studies have shown that methylT:A base-pairs are more stable at acidic than neutral pH, and that at the lower pH there is a strong similarity between the structure of the alkylT:A and C:A base-pairs (Kalnik *et al.*, 1988a,b; Swann, 1990). These NMR studies and X-ray crystallography of DNA duplexes containing C:A mismatches (Hunter *et al.*, 1986) suggest that the C:A and methylT:A pairs have one hydrogen bond between the NH_2 of adenine and N3 of the pyrimidine, and possibly a second hydrogen bond between N1 of adenine and the O^2 of the pyrimidine, with the hydrogen on N1 resulting from protonation of the adenine. Thus the protonation of adenine at pH 6.5 (but not at pH 8.3), with the formation of a second hydrogen bond may be the explanation for the difference in the structure and flexibility of DNA at pH 6.5 and pH 8.3.

The effect of the position of the alkylT containing base-pair on the mobility of the $(dN_{16})_x$ oligomers at pH 8.3, and of both the $(dN_{16})_x$ and $(dN_{21})_x$ oligomers at pH 6.5, could be explained by the difference in stacking interactions. The alkylT at position 2 is in the sequence 5' -T.alkylT.G- 3', and in position 1 it is in the sequence 5'-C.alkylT.T- 3' (figure 5.1). The theoretical expectation that the pyrimidine-purine combination of nearest neighbours at position 2 should be more effective at stabilizing the O^4-alkylthymine in an intrahelical stacked conformation than the pyrimidine-pyrimidine of position 1 (Saenger, 1984), is supported by the melting point (Tm) measurements of 21-mer duplexes (table 5.1). These results show that the sequences with the alkylT in position 2 have higher

melting point than those with the alkylT at position 1.

Another interesting aspect of this study is the difference in migration anomaly caused by O^4-alkylT:A and O^4-alkylT:G base-pairs. The anomalous migration caused by the O^4-alkylT:A base-pair was greater than that caused by the O^4-alkylT:G base-pair. In addition, the retardation caused by O^4-alkylT:A was dependent upon phasing of the alkylated base-pairs with the helical turn, while that caused by O^4-alkylT:G base-pair was not. A difference would have been predicted, because previous NMR studies suggest that the O^4-alkylT:G retains the Watson-Crick alignment, while the O^4-alkylT:A base-pair adopts a wobble conformation (Kalnik *et al.*, 1988a,b). Furthermore, this difference relates with the relative decrease in the melting point (Tm) of the two base-pairs (table 5.1). Although previous studies on a self complementary dodecamer in our laboratory suggested that O^4-alkylT:A is more stable than the O^4-alkylT:G base-pair (Li *et al.*, 1987), measurements on non-self-complementary 21-mers (table 5.1), shows that the opposite is in fact the case. While as mentioned above, a relationship seems to exist between gel retardation and stability of DNA duplexes containing O^4-alkylthymine, this association was not observed in comparison with the O^6-methylG:C and C:A containing oligomers. Both mismatches caused a significant decrease in the melting temperature but only a moderate gel retardation relative to these caused by the O^4-alkylT:A or O^4-alkylT:G base-pairs. Probably parameters other than stability of the DNA helix contribute in the structural alterations which are detected by non-denaturing gel electrophoresis.

One striking aspect of these results is the discovery that O^4-alkylthymine causes a much greater effect on the flexibility of DNA than O^6-methylguanine (figure 5.6 B, and Voigt and Topal, 1990). These differences may be relevant to the differences in the recognition of these modified bases by repair enzymes, and even suggest a different role for them in the process of carcinogenesis. N-nitroso compounds can produce all these changes associated with promotion as well as with initiation of carcinogenesis. If alkylation of DNA does produce epigenetic changes then one would predict that alkylT in DNA would have a greater role than alkylG because of the greater change in DNA

conformation which it produces.

CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVES

Barbacid's discovery that rat mammary carcinogenesis by N-methyl-N-nitrosourea is associated with a single G:C to A: transition mutation in the H-ras oncogene, was probably the most exciting event in nitrosamine carcinogenesis of the last decade. The mutation was always observed in the second position of the 12th codon of this gene (normally GGA), and was attributed to alkylation of the guanine to form O^6-methylguanine following by miscoding during replication. However, the bias for mutations in this position is totally unexpected in its apparent magnitude. Point mutations in other positions can also lead to amino acids substitutions that confer transforming properties to the mutated protein. In particular, substitution of Gly12 by any other amino acid except proline results in oncogenic activation of the p21 protein. Most interestingly a similar specificity for mutation in the middle G of GGA sequences was also observed in bacteria, which indicates that the reason for the preference can be elucidated by study of the bacterial system.

Three possible explanations for this selectivity immediately present themselves: 1) the alkylating agents selectively alkylate the second guanine; 2) an O^6-methylguanine at the second position cannot be repaired; 3) an O^6-methylguanine at the second position miscodes with much greater frequency than it would have in another sequence. While all three possibilities are not mutually exclusive, the results presented in this thesis suggest that the selectivity for mutations greatly depends on the fact that an O^6-methylguanine in this position is poorly repaired. Short oligonucleotides were synthesized resembling the sequence of H-ras gene around codon 12. Measurements of the rate of their repair showed that an O^6-methylguanine in the second position of codon 12 is repaired 18 times slower than one in the first position. The method used for the assessment of the rate constants was based on immunoprecipitation and it was coincidentally found that the most poorly repaired sequence was also poorly recognized by the antibody against O^6-methyldeoxyguanosine. Measurements of the avidity constants between antibody and each sequence confirmed that

a correlation exists between the rate of repair and the accessibility of O^6-methylguanine by the antibody. It is suggested that the sequence flanking an O^6-methylguanine residue influences the orientation of this adduct, with effect on its repa rability by the alkyltransferases. This is the first time antibodies were used to detect structural differences in DNA, and one can assume that their application for similar purposes can be extended and for other DNA adducts. Topal (1988) found that unrepaired O^6-methylguanine residues in the ampicillinase gene of a bacteriophage f1/ pBR 322 plasmid chimera are within sequences that exhibit a significant (75%) homology with the sequence of rat H-ras around codon 12. Thus, H-ras activation in mammary cells appears to be the unfortunate coincidence of a DNA position which when alkylated cannot be repaired effectively and the resulting mutation is pathologic in its biological endpoint.

Another aspect of the effect of an alkylated base on DNA conformation has been the discovery that O^4-alkylthymine residues in DNA produce localized flexibility in the relatively rigid DNA helical rod. The question that immediately arises is does this flexibility have any biological significance? DNA-protein interactions often involve or induce specific structural features of DNA. Some proteins intercalate into the DNA duplex by melting out the complementary strands; some others bind specifically to bent DNA, while others induce DNA curvature. Thus, an interesting possibility is that the alkylthymine, providing it is not in a position where the 4-O oxygen is normally involved in a hydrogen-bonded contact between the protein and DNA, might, through the increased flexibility of the DNA, increases the affinity between DNA and protein by maximising the crucial contacts between them. If this were so, and the protein in question was a transcription factor, one could envisage that increased flexibility could lead to up-regulation of genes. Up-regulation of genes is often associated with the promotion of carcinogenesis. Some N-nitroso compounds, as complete carcinogens, can possibly promote as well as initiate carcinogenesis, but no theory has been suggested yet as to the mechanism of such a promotion.

The effect of the presence of O^4-alkylthymine residues in DNA in the protein-DNA

interactions is currently being examined in our laboratory by Ms H.B. Tan. The products of two nuclear oncogenes, fos and jun, form a heterodimer, bind to a specific sequence which is found upstream of a number of genes (e.g. collagenase and stromelysin) and so, regulate their expression. Preliminary results indicate that an O^4-methylthymine immediately 5' of the fos-jun binding sequence increase significantly its affinity for DNA binding.

Previous studies showed only similarities in the effects of O^4-alkylthymine and O^6-methylguanine in the structure and stability of DNA containing these adducts. However, significant differences are reported in this thesis.

1) Polymers containing O^6-alkylguanine or O^4-alkylthymine residues migrate slower than the parent polymers in a non-denaturing polyacrylamide gel. The extent of gel retardation, as shown in chapter 6 and in comparison with the data by Voigt and Topal (1990), is significantly higher for the O^4-alkylthymine containing oligomers. This result probably indicates that an O^4-alkylthymine residue renders the DNA helix more flexible than an O^6-alkylguanine residue does.

2) Polymers containing O^4-alkylT:A base-pairs migrate more slowly than polymers containing O^4-alkylT:G base-pairs which is the opposite of what was observed for oligomers containing O^6-alkylguanine residues.

3) Thermal denaturation studies showed that oligomers containing O^4-alkylT:A base-pairs are more stable than oligomers containing O^4-alkylT:G base-pairs, in contrast with the current consensus, and the available data for O^6-alkylguanine containing oligomers.

It is believed that these differences should be taken into consideration when examining the role of these DNA adducts in mutagenesis and carcinogenesis by alkylating agents

When the comparative studies on the efficiency of repair by the *ada*, *ogt*, and human alkyltransferases were initially carried out, the prime interest was to see if the newly discovered E. coli *ogt* protein resembles the human enzyme in its biochemical properties

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more than the *ada* protein does. The consensus opinion was that the human alkyltransferase, unlike the *ada* protein, cannot repair O^4-alkylthymine and therefore it was important to know whether the *ogt* protein could repair it. It could and it was quite remarkably active against O^6-ethylguanine. The relative rates of repair of O^6-methylguanine, O^6-ethylguanine and O^4-methylthymine were different to those with the *ada* alkyltransferase. In particular, the repair by the *ogt* protein of O^6-ethylguanine was about 200 times faster and that of O^4-methylthymine 80 times faster, than with the *ada* alkyltransferase. These results suggest that when E. coli cells are exposed to methylating agents in concentrations insufficient to trigger the *adaptive* response, the O^6-methylguanine and O^4-methylthymine produced are mainly repaired by the *ogt* and not by the *ada* protein. In addition, the high efficiency of the repair of O^6-ethylguanine by the *ogt* protein, in association with the inefficiency of longer chain alkylating agents to act as *adaptive* response inducers, could suggest that the repair by the *ogt* protein constitutes a major protection mechanism of the cell against longer alkyl DNA adducts.

The human alkyltransferase, when cloned, was also tested for its activity in the repair of the same synthetic oligomers. The relative rates of repair of O^6-methylguanine and O^6-ethylguanine showed that the human enzyme, like the *ogt* protein, is less sterically hindered by the size of the alkyl group than the *ada* protein. Unexpectedly, the human alkyltransferase was able to remove the alkyl group from the O^4-methylthymine containing oligomer. The rate however, of its repair was very slow and put in doubt that this process has any biological significance in vivo. It was recently suggested that an other repair mechanism might exist specific for the repair of O^4-alkylthymine, and this possibility should be further exploited in the future.

The structure of the alkyltransferases is still unknown and the reason for the observed differences in the repair efficiencies can only be speculative. All known alkyltransferases contain a conserved pentapeptide, pro-cys-his-arg-val, which contains the alkyl-acceptor cysteine residue of the *ada* alkyltransferase and possibly of the other proteins. The *ada* and ogt proteins however, have another conserved peptide, leu-arg-thre-

ileu-pro-cys-gly, which also contains a cysteine residue. This sequence is not present in the human or the dat1 bacillus subtilis alkyltransferases and one might suggest that it plays a particular role in the repair of O^4-alkylthymine. Thus, it would be of interest to compare the efficiencies of the dat1 and human alkyltransferases in the repair of O^4-alkylthymine. However, X-ray crystallographic studies are needed to unveil the structure of the active centre of these enzymes and the amino acids that participate in its formation. In these studies, use of substrate analogues might help to elucidate the repair mechanism. As such one might suggest oligonucleotides containing O^6-thiomethylguanine, due to its apparent similarity in structure to O^6-methylguanine and the inability of the alkyltransferases to repair this adduct.

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