DEVELOPMENT OF IMMUNOCHEMICAL METHODS TO DETECT POINT MUTATIONS IN THE FORM OF MISMATCHED BASE PAIRS IN DNA

by

PAUL C. TURNER

A thesis submitted to the
Faculty of Science and Engineering
at the university of Edinburgh
for the degree of Doctor of Philosophy.

Department of Chemistry
West Mains Road
University of Edinburgh
Edinburgh
EH9 3JJ

December 1995

ACKNOWLEDGEMENTS

I would like to thank Prof. Tom Brown for the supervision he gave me throughout the course of the work. He was always enthusiastic and continually helped direct the project in times of difficulty, I am extremely grateful for that. The projects funding came from SRC, Shell UK Ltd., Kent. I would like to thank my industrial supervisors, Dr. W.P. Watson and Dr. J.P. Aston, for establishing the project and for their help and advice. A considerable period of time was made available for me to work in the immunochemistry group at SRC. I thank the staff (Dr. J.P. Aston, Eric Hitchings, Lathan Ball and Erica Ackerman) for their support, advice and for keeping me in my place. Additionally, I would like to acknowledge the support and advice of Dr, A.S. Wright of SRC who provided considerable background to the project.

I thank John Gryzbowski for the use of the DNP monomer, used to facilitate assay development. The production of the oligonucleotides was carried out using the automated DNA synthesis machines in the Oswel DNA Service. I thank the staff for their patience to the continual interruptions to their working day. I thank the chemistry department for the use of facilities. Finally, I thank the many colleagues, friends and family who have helped me get this far.

ABSTRACT

Advances in molecular biology techniques have permitted the development of radical new approaches to analyse point mutations i.e. the substitution of one base pair for another, at the molecular level. However, such approaches focus on only a small, pre-defined region of the genome and, because methods are dependent on prior phenotypic selection of mutant cells, they do not actually detect mutation. The occurrence of a point mutation at a given locus, i.e. base pair, in the genome is a very rare event and this presents a formidable obstacle to the development of a general method to detect point mutations.

A generic method is needed to enrich these mutations. Direct enrichment is not feasible due to the fact that the base pair constituting the mutation is perfectly normal, at least from a chemical standpoint. It is the precise location of this base pair within the DNA duplex that is abnormal. Nevertheless, the 'normal' chemical structures of mutant base pairs can be specifically transformed into manifestly aberrant structures by denaturing the mutated duplex and reannealing with normal, i.e. non-mutated, complementary strands to produce a DNA base pair mismatch at the mutated loci. Immunoanalytical methods, based on monoclonal antibodies may prove effective in detecting and enriching mismatches. This has been investigated.

A series of 15mer single stranded oligonucleotides of defined sequence linked chemically by means of a tetraethylene glycol linker to a complementary 15mer strand were synthesised in such a way that the intramolecular duplex contains a specific central mismatched base pair. Since the two strands were chemically linked, a highly stable duplex, indicated by UV melting studies, was formed which should persist *in vivo*. These synthetic duplexes were used to evoke an immune response by direct immunisation, or as methylated BSA complexes. In a related approach a series of 75mer duplexes have been constructed such that they contain four evenly spaced mismatched base pairs. Each sequence was extended by the addition of a 5mer phosphorothioate oligonucleotide at the 3' end to protect the DNA from nuclease degradation. In a third approach, a C100mer phosphorothioate sequence was produced. Antibodies recognising cytosine in C100mer may cross react with mismatches containing this base (A:C; C:C; T:C).

Hybridoma cell lines obtained after fusions from 15mer duplex immunised mice were screened for antibody production using the ELISAs initially developed. These experiments failed to produce antibodies recognising mismatched base pairs. There was concern that cell lines producing the desired antibodies may not have been identified during the early stages of the project while developing screening assays. More recently, an ELISA format in which biotin labelled oligonucleotide duplexes were immobilised on streptavidin coated plates was used as a solid phase. For immunisations with the larger duplexes the improved screening methods were used to monitor antibody production. Although anti-mismatch antibodies were detected using AC75 and GT75 no monoclonal cell lines were isolated. The C100mer produced antibodies that bound strongly to single stranded DNA containing cytosine, and cross reacted with duplexes containing AC and CC mismatches. Monoclonal cell lines were isolated from these experiments with retained antibody specificity. These are cryopreserved at Sittingbourne Research Centre.

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Abbreviations

A	adenine
ABTS	2,2-azinobis-(3-ethylbenzthiazoline) sulfonic acid
A-DNA	a specific double helical form of DNA
ASO	Allele Specific Oligonucleotides
B-DNA	a specific double helical form of DNA
BSA	bovine serum albumin
C	cytosine
C100PS	cytosine 100mer phosphorothioate oligonucleotide
CCM	Chemical Cleavage of Mismatch
CDI	carbodiimide
cDNA	complementary DNA
CDR	complementarity determining regions
dA	deoxyadenine
dC	deoxycytosine
dG	deoxyguanine
DGGE	Denaturing gradient gel electrophoresis
dGm^5dC	dG-5methylcytosine dinucleotide
DMSO	dimethylsulphoxide
DMTrCl	dimethoxytrityl chloride
DNA	deoxyribonucleic acid
DNase	DNA nuclease enzyme
DNP	2,4-dinitrophenyl
dsDNA	double stranded DNA
DSS	disuccinimyl suberate
E. coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
G	guanine

HAT	hypoxanthine, aminopterin and thymine
HPLC	high perfomance liquid chromatography
HT	hypoxanthine and thymine
IAC	immunoaffinity chromatography
MAM	Mismatch amplification mutation
MBS	3-maleimidobenzoyl-N-hydroxy succinimide ester
MeBSA	methylated ester of BSA
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PASA	PCR amplification of specific alleles
PBS	phosphate buffered saline
PBS/Tw	PBS + 0.05% Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PTE	PBS/Tw + EDTA
RFLP	Restriction fragment length polymorphism
RNA	ribonucleic acid
RNA-DNA	a hybrid duplex of RNA and DNA
RNase A	Ribonuclease A
RPMI	Roxwell Park Memorial Institute-1640 tissue culture medium.
SLE	Systemic Lupus Erythematosus
Sp2	Sp2/1-Ag14 plasmacytoma cell
SSCP	Single strand conformation polymorphism
ssDNA	single stranded DNA
T	thymine
TBS	tris buffered saline
TBS/Tw	TBS + 0.05% Tween 20
TEA	triethylamine
THF	tetrahydrofuran
Tween 20	polyoxyethylenesorbitane monolaurate

U	uracil
UV	ultra violet
UvrABC	a DNA repair enzyme
V-region	variable region
VSP	very short patch
w/v	weight / volume
Z-DNA	a double helical form of DNA
5BrdC	5-bromodeoxycytosine
5BrdU	5-bromodeoxyuridine
5MedC	5-methyldeoxycytosine

CHAPTER 1 : MUTATIONS IN DNA

1.1 INTRODUCTION

In the Western world advances in medical care, public health measures and living conditions have reduced disease associated with infection and malnutrition (Emery and Rimoin, 1983). Genetic disorders as a group now represents a significant fraction of chronic disease and mortality. Genetic disorders account for 7% of still births and neonatal deaths, and approximately 8.5% of childhood deaths (U.S. Congress, 1986).

Approximately 3000 different genetic diseases have been identified. Some genetic disease will manifest themselves shortly after birth, others will show indirect effects later in life, e.g. increased susceptibility to some forms of heart disease, diabetes, or cancer. In the case of cancer there is now overwhelming evidence of the multi-stage nature of the disease. There may be a combination of an inherited genetic disorder and subsequent mutations through external exposures to certain mutagenic agents. Much work has been conducted to identify the cause, the type and the location of all forms genetic mutation.

There are a considerable number of types of mutation that can occur. Alterations in DNA function can be a consequence of the change to an entire gene or a single base pair. The latter can be as devastating to health as the former, however its detection is considerably more complex. Methods to detect base pair mutations often rely on the artificial formation of base pair mismatches. Identification of duplexes containing mismatched base pairs indicate the presence of a point mutation. This project aimed to develop immunoanalytical methods to detect mismatched base pairs, so as to improve the ability to detect mutations that occur at the level of the base pair. These methods should offer the possibility of highly sensitive techniques for identifying changes in the human genome and thereby estimating the genetic risk associated with such mutations.

1.2 NUCLEIC ACIDS

Nucleic acids are macromolecules composed of several monomeric units called nucleotides. The sequence of the nucleotides in the polymeric structure determines the function in the storage and transfer of genetic information. Nucleic acids can be divided into two main classes, the deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). However, only the structure of DNA will be discussed.

The monomeric units of DNA are deoxyribonucleotides. These consist of a phosphate, a sugar, and one of four nitrogenous bases (adenine, (A), cytosine (C), guanine (G), or thymine (T)). The sugar, β-D-2'-deoxyribose, is joined via a β-glycosyl linkage to one of the four bases to give a nucleoside. Successive nucleosides are joined via the 3'- and 5'- hydroxy groups of the sugar by phosphodiester bonds to give oligodeoxyribonucleotides. Thus the backbone consists of alternating phosphate and sugar groups, with nitrogenous bases as side chains to this backbone, see figure 1.

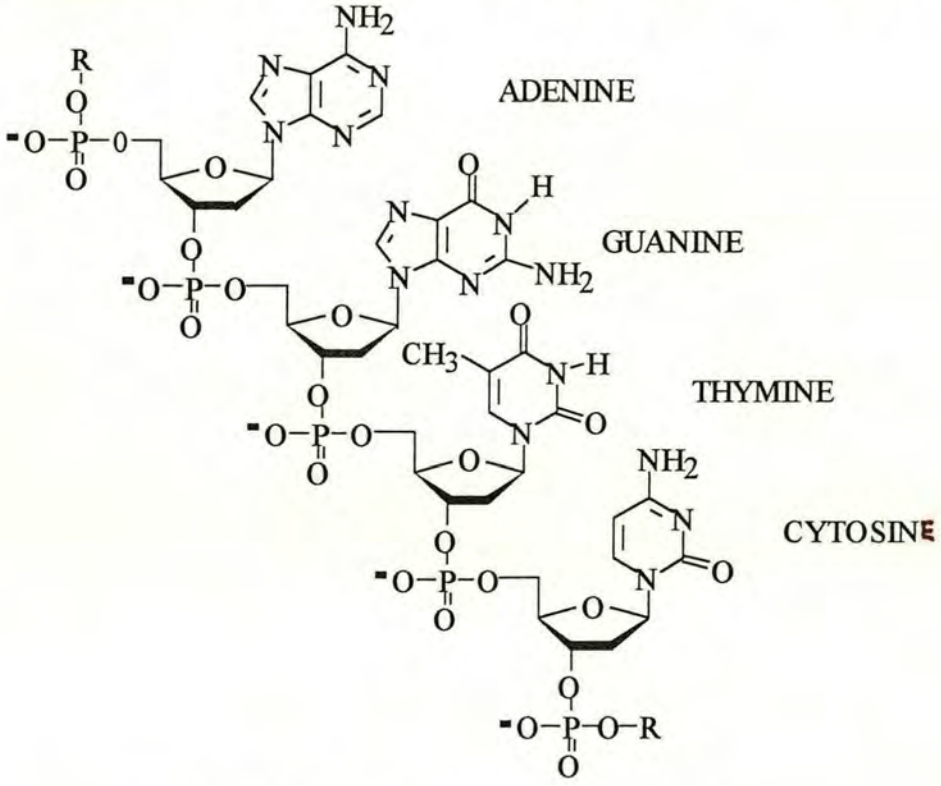


FIGURE 1. MONOMERIC UNITS OF DNA

Native DNA from most organisms consists of two oligonucleotide chains in a right handed double helical arrangement, known as B-DNA. The double helix is stabilised by hydrogen bond formation between the heterocyclic bases, see figure 2. Adenine (A) pairs with thymine (T), and cytosine (C) pairs with guanine (G). The helix is further stabilised by base stacking within the helix.

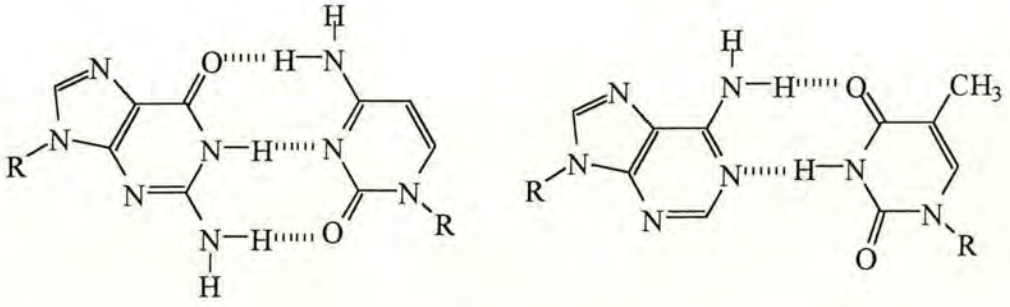


FIGURE 2. WATSON CRICK BASE PAIRS

The interaction of DNA with its environment, especially proteins are central to its function. The original description of DNA based on the classical Watson and Crick model (Watson and Crick, 1953), gave the impression of rigidity. In fact DNA is structurally dynamic and the original B-form DNA structure proposed by Watson and Crick can adopt several conformations depending on environmental conditions such as the nature and the concentration of the counterions and relative humidity and, in synthetic polynucleotides with the nature of the sequence. DNA double helixes exist in a right handed form in A-DNA and B-DNA. B-DNA can be further subdivided into 8 forms with closely related structures. There also exist a left handed variety known as Z-DNA. This can be subdivided into four closely related groups. The various structures of DNA have been reviewed (Saenger, 1984).

The nucleotide sequence in DNA codes for the regulation of gene function or for a protein product of transcription or translation.

The nucleotide sequence is arranged in triplets of nucleotides known as codons. A series of codons that code for a protein or regulate cell function are called genes. Chromosomes are made up of thousands of genes, dispersed amongst a large amount of non-coding DNA, in a complex arrangement with proteins. The entire genome is made up of many chromosomes in eukaryotes. The human genome contains 46 chromosomes, comprising of approximately 3 billion nucleotide base pairs.

1.3 TYPES OF MUTATIONS IN DNA

1.3.1 Definitions of Mutation

Mutations are changes in the composition of the genetic material, DNA. The types of mutation are to some extent a function of the cell line and the gene locus in which they occur. Mutations are influenced by numerous chemical and physical agents. They are generally divided according to size into gene mutations and chromosome mutations.

Depending on the nature and location of the mutation, and on the function of the genes in which they occur, mutations may in theory be beneficial, neutral or harmful to the individual. It is generally thought that newly arising mutations in regions of DNA that directly determine the structure and regulation of proteins are more likely to be detrimental rather than beneficial. Therefore, efficient detection of any mutation is essential to avoid progression towards disease.

1.3.1.1 Gene Mutations

Gene mutations occur within or across a single gene. They result from either the substitution of one nucleotide for another, or the rearrangement, deletion or insertion, of several nucleotides within the gene. They can also be a consequence of duplication or deletion of an entire gene. For example, sickle cell anaemia is caused by the substitution of a single base pair. This point mutation results in one

codon within the globin gene being altered such that the amino acid valine replaces glutamic acid (Ingram, 1957). In comparison, α-thallassemia occurs where the gene coding for the α-globin is missing (Weatherall and Clegg, 1982).

In somatic cells, dominant oncogenes are generated by mutations. Oncogenes are genes whose protein products may be involved in processes leading to transformation of a normal cell to a malignant state. They are part of a normal cellular gene that has been activated by mutation. There are various types of oncogenes involved in cell transformation. Most oncogenes are similar to genes that act along growth control pathways.

There are four basic groups of oncogene protein products. Those that code for growth factors e.g. *sis* oncogene; those that produce mutant cell surface or intracellular receptors e.g. *ros*, *neu*, *erb*A; those that code for mutant intracellular signalling proteins, e.g. mutant G proteins formed by *ras* oncogenes, protein kinases formed by *src* oncogenes; and those that effect transcription rates within the nucleus e.g. *jun*, *myc* and p53 oncogenes (Darnel, 1990).

The mutation can be in the form of a point mutation e.g. for *ras* oncogenes, or as gene rearrangements e.g. amplification of c-myc genes (Bishop, 1987; Varmus, 1984). The literature on the activation of oncogenes is vast and beyond the scope of this text. The fact that activation of oncogenes by mutation is implicated in the multistage process leading to cancer has had many reviews (Reddy *et al.*, 1982; Tubin *et al.*, 1982; Yuasa *et al.*, 1983; Bos *et al.*, 1985, 1987; Barbacid, 1987; Forrester *et al.*, 1987; Brookes, 1989; Grand and Owen, 1991; Ohgaki, 1991).

1.3.1.2 Chromosome Abnormalities

Major structural changes affecting more than one gene are described as chromosomal abnormalities. These involve loss, addition, or displacement of all or part of a chromosome. Occasionally in the production of germ cells an entire extra chromosome may be included. In Down Syndrome the resulting offspring has 3 copies of chromosome 21. Chromosome

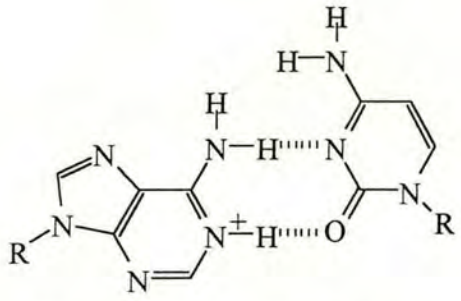
translocations also occur at a high frequency in some tumours, suggesting they play a role in the development of these tumours, e.g. Philidelphia chromosome translocations associated with chronic mylegenous leukaemia (Rowley, 1973).

1.3.2 Mutational Causation

There are several known causative agents for mutation. Gene mutations occur spontaneously, and are induced by the interaction of chemical and physical agents with DNA. Evidence that ionising radiation causes mutation was presented by Muller (1927). Methods to detect mammalian mutations were discussed in the 1930's (Snell, 1935). The investigation of mutation induction by chemical agents was initiated by Cattanach (1966). One of the major aims of the investigation of chemical mediated mutations is to evaluated associated risk of chemicals, natural and synthetic, within the environment and work place (Ehling, 1991). According to Chemical Abstracts there were 10 million registered chemicals in 1984, most of these had not been tested for toxicity (U.S. Congress, 1986).

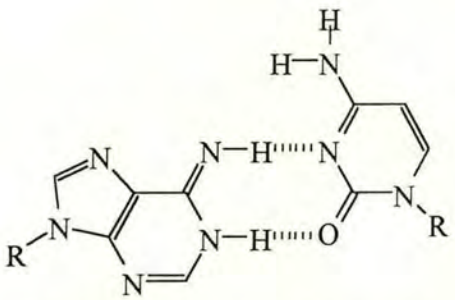
1.3.2.1 Spontaneous Mutations

Mistakes in the normal duplication of DNA can give rise to spontaneous mutations, but they are rare events (Singer and Kusmierek, 1982). Misincorporation may occur as a result of spontaneous tautomerisation leading to mispairing of iminocytosine with adenine, iminoadenine with cytosine, enolguanine with thymine, or enolthymine with guanine. In addition guanine can mispair with adenine in several forms (Kennard, 1987). Mispairs produced by rare tautomeric forms of the bases (Topal and Fresco, 1976a,b) are sterically equivalent to Watson-Crick base pairs, see figure 3 and 4, and are thought not to significantly destabilise the double helix (Kennard and Hunter, 1991).



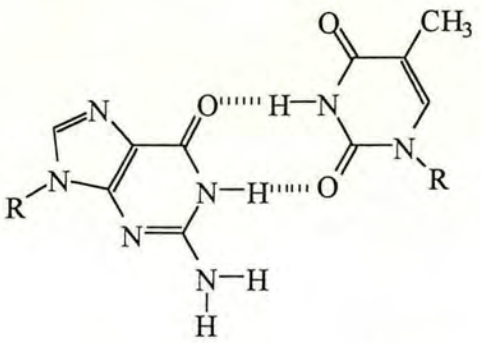
ADENINE CYTOSINE

A : C Wobble Base Pair (Protonated)



ADENINE CYTOSINE

A : C Wobble Base Pair (Imino Tautomer)



GUANINE THYMINE

G : T Wobble Base Pair

FIGURE 3. A:C and G:T WOBBLE BASE PAIRS

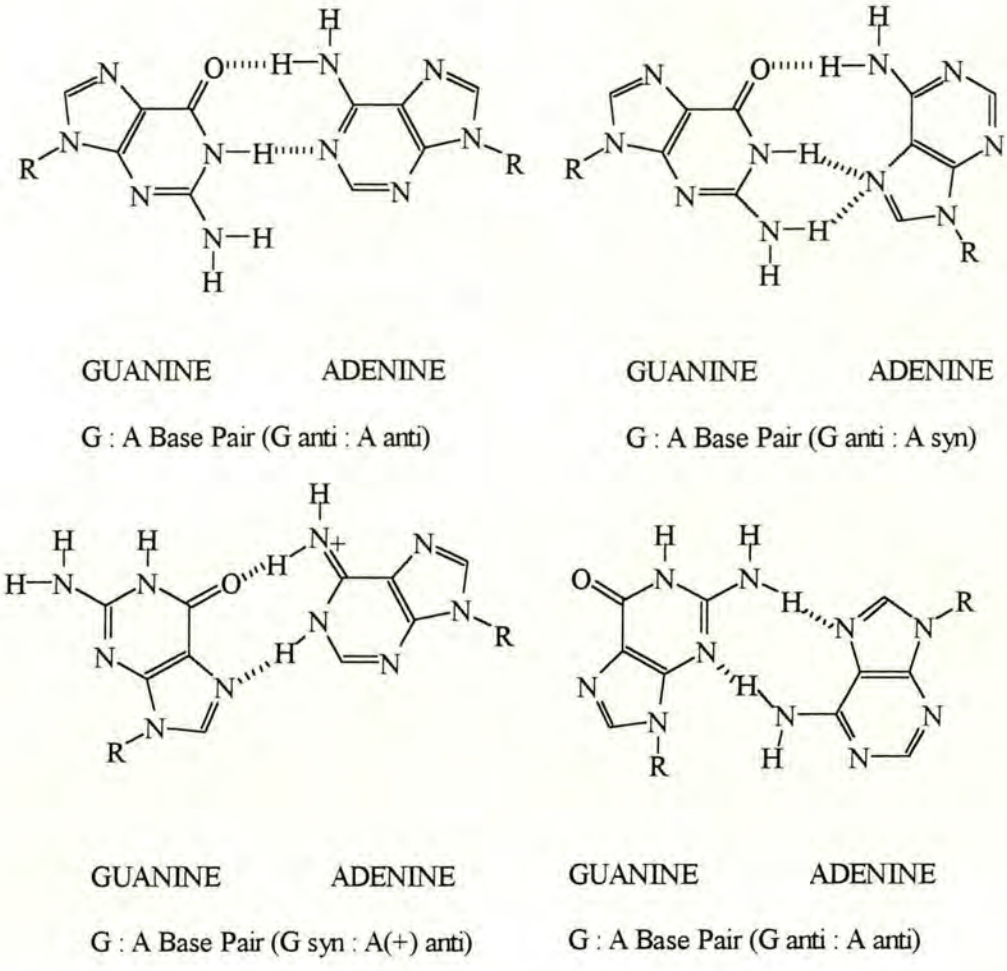


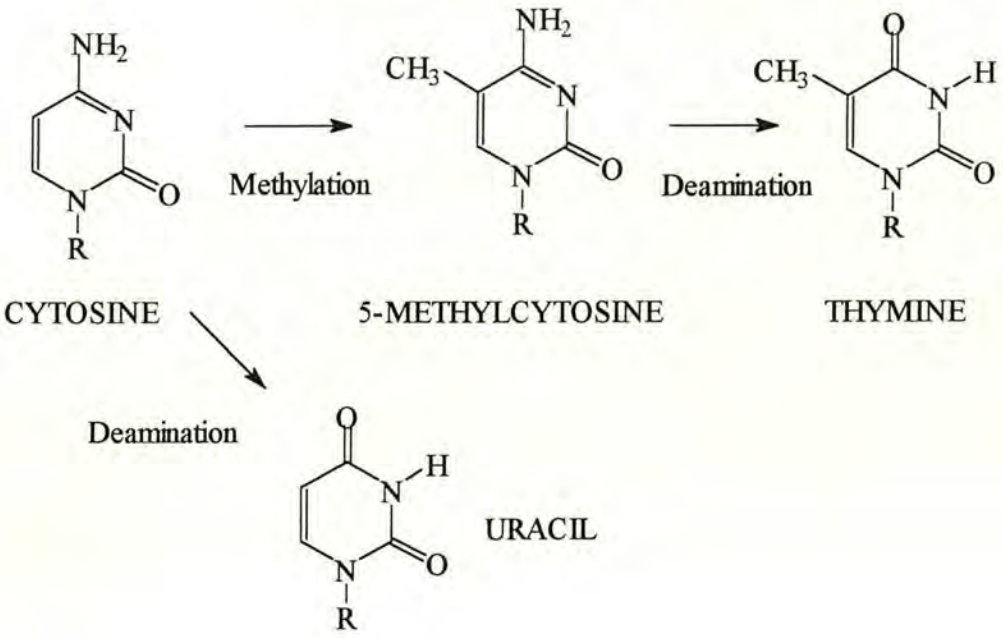
FIGURE 4. G : A BASE PAIRS

1.3.2.1.1 Methylation and Spontaneous Mutation

Cytosines in CpG dinucleotides are methylated to 5-methylcytosine (5MedC) in many animal systems, and these are known to be premutagenic lesions in human DNA (Jones *et al.*, 1992). 5MedC was first shown to be mutagenic in bacteria (Coulondre *et al.*, 1978). The mutagenic potential is attributed to the deamination process. Cytosine will deaminate to uracil, not normally found in DNA, and will therefore be corrected by repair enzymes. However, 5MedC deaminates to thymine, a normal component of DNA, and thus does not represent an unusual site for the action of repair enzymes, see figure 5. If DNA replication at the site of the resultant T:G mismatch occurs, before mismatch repair has taken

place, a C:G to T:A transition mutation may occur for one daughter strand, i.e. a point mutation arises as a result of 5MedC deamination.

FIGURE 5. DEAMINATION OF CYTOSINE AND 5-METHYLCYTOSINE



The rate of 5MedC deamination has been found to be more rapid than for cytosine in dsDNA (Erlich *et al.*, 1986), this rate of 5MedC deamination further increasing in ssDNA. During DNA replication, regions of dsDNA are dissociated to ssDNA, therefore 5MedC deamination during replication may represent a major mutational occurrence. There is 200kda specific G:T mismatch repair enzyme that repairs approximately 90% of G:T mispairs to the original G:C matched pair (Brown and Jiricny, 1987). However, this repair system is not completely error free.

It has been calculated (Sved and Bird, 1990) that methylation at CpG increases the possibility of transitions by a factor of 12. Further, it has been suggested that methylation at CpG in the human germline may account for 30-40% of all point mutations and restriction fragment length polymorphisms (Cooper and Youssoufian,

1988; Barker *et al.*, 1984). In colon cancer 63% of point mutations in p53 gene occur at CpG sequences. The mutation being consistent with deamination of 5-methylcytosine, leading to C:G to T:A transitions at the CpG sites. These observation suggest that mutations in the p53 gene associated with tumour progression in colon cancer are induced predominantly by an endogenous process (Vogelstein, 1990). The role of DNA methylation and mutation has recently been reviewed (Holliday and Grigg, 1993). It should be noted that there is no evidence that CpG dinucleotides, independent of methylation, are inherently unstable.

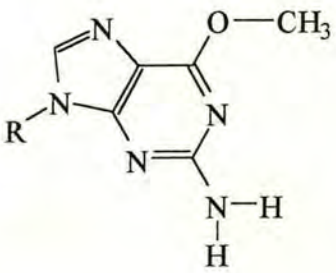
1.3.2.2 Non-Spontaneous Mutations

For many mutations exogenous agents are the predominant causative factors. In lung cancer transversion mutations that are not associated with 5MedC deamination predominate (Jones *et al.*, 1991; Sommer, 1990; Chika *et al.*, 1990). These mutations are more likely to be associated with exogenous factors such as those found in tobacco smoke (Chika *et al.*, 1990). The following have been identified as mutagenic agents :

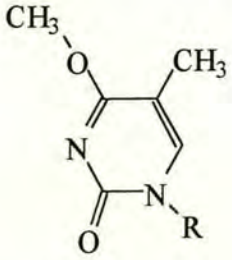
(***A***) Alkylating Agents
(***B***) Intercalating Agents
(***C***) Radiation

1.3.2.2.1 Alkylating Agents

Alkylating agents are either electrophilic compounds, or compounds that are metabolised to electrophilic species, which can bind to nucleophilic centres. In DNA these are predominantly the nitrogen and oxygen atoms within the bases. Most of the nitrogen and oxygen atoms in DNA present potential targets for the attack of alkylating agents (Singer and Kusmierek, 1982; Pegg, 1983; Horsfall *et al.*, 1990). The alkylation sites thought to be predominantly responsible for mutation development are O(6)-alkylguanine and O(4)-alkylthymine, see figure 6. These give rise to G:C to A:T, see figure 7, (Saffhill *et al.*, 1985) and A:T to G:C transitions respectively (Singer *et al.*, 1986; Preston *et al.*, 1986; 1987).

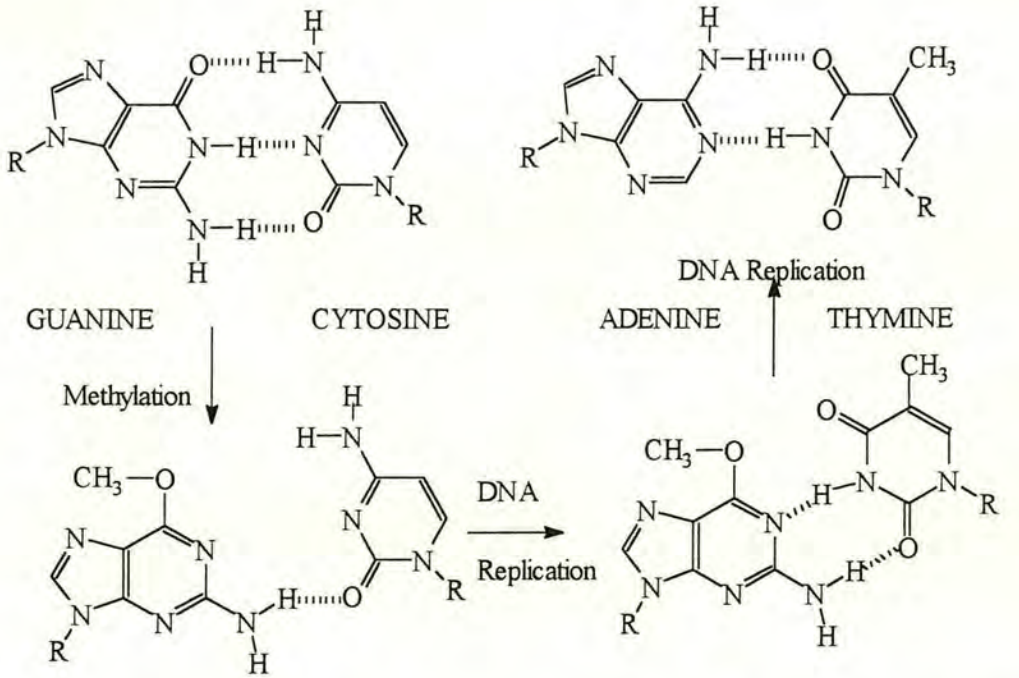


O(6)-METHYLGUANINE



O(4)-METHYLTHYMINE

FIGURE 6. PRE-MUTAGENIC ALKYLATION PRODUCTS OF GUANINE AND THYMINE



O(6)-METHYLGUANINE CYTOSINE O(6)-METHYLGUANINE THYMINE

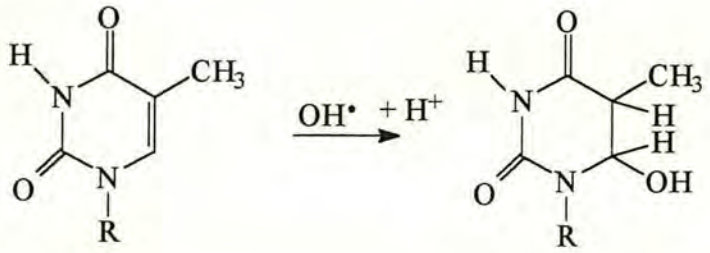
FIGURE 7. G : C TO A : T POINT MUTATION

1.3.2.2.2 Intercalating Agents

Acridine dyes, e.g. ethidium bromide, are flat molecules that are able to insert themselves between the stacked bases in DNA double helixes. The binding causes some unwinding of the DNA duplex, which interferes with subsequent DNA transcription. The anthracenes and benzopyrenes are oxidised and react at the 2-amino group of guanine before intercalating.

1.3.2.2.3 Radiation

Two forms of radiation, ionising and ultra violet (UV), interact with DNA and can lead to mutation. Single strand breaks in the sugar-phosphate backbone are a major lesion induced by ionising radiation, double stranded breaks occur more rarely. In addition, breakage of the glycosidic bond may occur, leading to a depyrimidated or depurinated site. Ionising radiation also produces short lived hydroxyl free radicals, after interaction with water. These can cause indirect damage by interaction with the bases of DNA, see figure 8 and 9.



THYMINE HYDROLYSIS PRODUCT

FIGURE 8. HYDROXY RADICAL ATTACK OF THYMINE

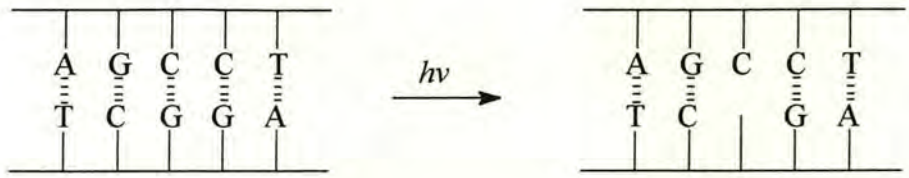
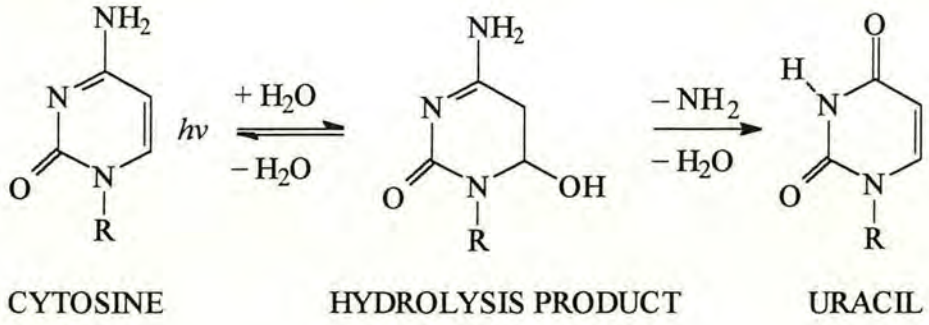


FIGURE 9. DEPURINATION OF DNA

UV radiation is not sufficiently energetic to directly ionise DNA, but can produce a short-lived excited, and chemically reactive state. UV radiation can cause alteration of bases due to the addition of a water molecule across the 5, 6 double bond of an excited pyrimidine base, see figure 10. This can spontaneously dehydrate forming the original base, or in the case of cytosine may deaminate and dehydrate forming uracil.



CYTOSINE HYDROLYSIS PRODUCT URACIL

FIGURE 10. HYDROLYSIS AND DEAMINATION OF CYTOSINE

An excited pyrimidine may also interact chemically with a neighbouring pyrimidine to form a cyclobutane linked dimer, see figure 11. An excited base may also interact and form a covalent link with a protein.

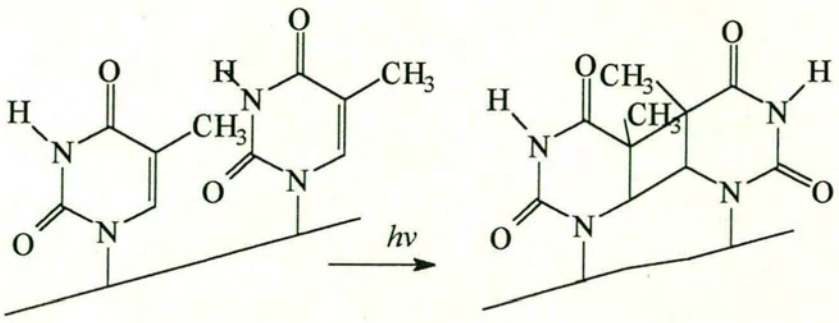


FIGURE 11. FORMATION OF THYMINE DIMERS

1.4 MUTATION RESEARCH

1.4.1 Introduction

Much of our knowledge about how substances interact with DNA, and how they may cause mutations is derived from studies conducted on experimental animals. The results from animal studies are then extrapolated to estimate the associated risk to humans and associated mutation rates. For many years the validity of data obtained in this way has been open to question. Many compounds undergo transformation within the host animal prior to exerting their effect. Metabolic pathways for chemical transfomations vary from species to species, thus a precise interpretation of all results is difficult.

Other methods to investigate mutation occurrence have included looking for gross changes in chromosome structure or number, or looking for the occurrence of mutant proteins. The presence of a mutant protein is indicative of alteration to the gene or genes that codes for the synthesis of that protein.

With increasing developments in molecular biology, and in particular the extensive use of the Polymerase Chain Reaction (PCR), the exact molecular changes associated with mutation are now being investigated. This is extremely important for very localised mutations, e.g. single base pair substitutions, and insertions and deletions involving only a few nucleotides. Gross mutations in DNA such as amplifications, gene rearrangements, and losses of genes, are successfully detected by Southern blot hybridisation. In general, detection of single nucleotide substitution require PCR based methods (Caskey, 1987).

Techniques for the physical and chemical characterisation of DNA damage and potential premutational lesions are continually being developed and improved. This area of research encompasses detection of DNA adducts and the detection of mutation. Many point mutations arise as a consequence of replication occurring before repair processes have taken place, or where incorrect repair of

adducted DNA has occurred. It is important to understand the consequences of chemical-DNA interactions and the mechanisms by which DNA damage are fixed as mutations.

1.4.2 Detection of DNA-Adducts

Formation of carcinogen-DNA adducts is recognised as a necessary step in the development of most chemically induced cancers (Yuspa and Poirier, 1988). Although it is difficult to predict absolutely the relationship between adduct/mutation occurrence, methods to detect, identify and quantify DNA adducts have arisen. These serve to estimate the cancer risk in humans from environmental or endogenous exposures.

Physical methods for the detection of carcinogen-DNA adducts include gas chromatography/mass spectrometry (Shuker *et al.*, 1992), fluorescence spectroscopy (Weston *et al.*, 1990), electrochemical conductance (Floyd *et al.*, 1986) and atomic absorbance spectrometry (Reed *et al.*, 1988). Several of these methods have recently been reviewed (Weston, 1993).

Other methods to detect carcinogen DNA adducts in humans include ^{32}-P post-labelling assay (Reddy and Randerath, 1987) and enzyme immunoassay (Poirier, 1990). The combination of HPLC with ^{32}-P postlabelling of DNA adducts is an emerging technique (Gorelick, 1993, and references therein). Antisera against DNA adducts and carcinogen modified DNA (Poirier, 1981,1984; Muller and Rajewsky, 1981) are used to quantify and localise chemical induced DNA damage (Leng, 1985; Strickland and Boyle, 1984; Phillips, 1990; Santella, 1988) and to monitor human exposure (Weston *et al.*, 1989; Poirier and Weston, 1991).

A major obstacle has been the requirement to find very low levels of adduct, in a vast majority of non-adducted material, where only limited quantities of DNA are available. Enrichment techniques have thus been needed prior to adduct quantification. Immunoaffinity chromatography (IAC) columns have been developed for this

purpose. One such method involves immobilisation of a monoclonal antibody against O(6)-methyldeoxyguanosine (Cooper *et al.*, 1992) for the analysis of DNA from human sera. IAC/HPLC post labelling has been applied to the detection of both O-6-methyldeoxy guanosine and benz(a)pyrene adducts in human samples (Sheilds *et al.*, 1993).

The above combination of techniques provides an improvement upon immunoassay methods used in isolation as usually less DNA will be required. The method is dependent upon the specificity of the antibodies produced. However, the lack of specificity of the antibody for an antigen can be utilised for some applications, e.g. where a class of adducts such as methyl, ethyl, and propyl groups are under investigation. The application of mixed antibody (IAC) columns may also be utilised in some instances (Cooper *et al.*, 1992).

1.4.3 Detection of Mutation

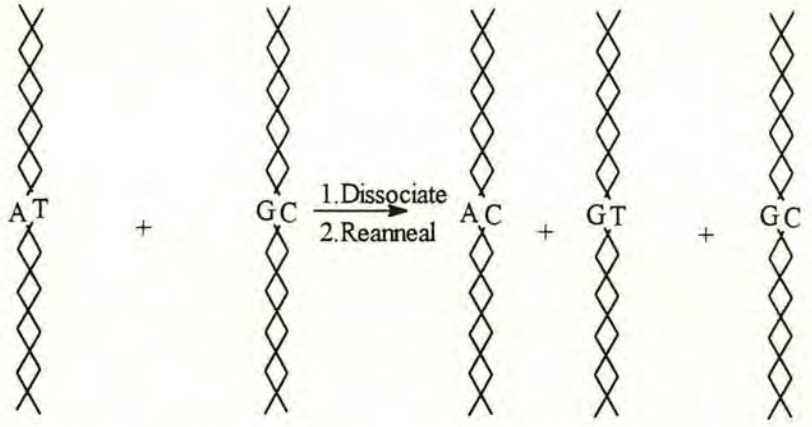
There are two main reasons for the identification of human mutations. Firstly, unknown mutations are screened for in a gene suspected to be the locus of a given disease. The second area is the application of mutation detection for genetic diagnosis. These are usually used in the detection of inherited diseases in which a few known mutations account for all cases of the disease.

The first disease causing mutation in a human gene was identified in one of the globin genes (Flavell *et al.*, 1978). Since then many more mutations involved in human disease have been discovered. As the number of known mutations increases, so techniques to screen and diagnose mutation improve. There are several chemical based methods, enzyme specific methods, and gel electrophoretic methods. Increasingly many of the methods involve DNA amplification, using PCR techniques, to either improve sensitivity, or as an integral part of the technique. Only techniques involved in the detection of point mutations will be discussed. These are often detected as a mismatched base pair artificially created at the site of a point mutation.

1.4.3.1 Point Mutations and Mismatches

The detection of point mutations has to a large extent been dependent upon the production of heteroduplexes. These are formed by combining single stranded DNA from the two sources, mutant and wild-type DNA. The mutant DNA is usually the DNA being tested (target DNA). The wild-type is usually a probe of DNA or RNA that is complementary to the non-mutated form of the target DNA, see figure 12. Mutant DNA being detected by the differing properties of the heteroduplex containing a mismatched base pair, compared to a fully complementary duplex containing no mismatched base pairs. The immunochemical basis of the project will rely on the differing properties of heteroduplex compared to homoduplex DNA.

FIGURE 12. ARTIFICIAL PRODUCTION OF MISMATCHES



In an excess of normal DNA duplexes the mutant strand can be dissociated into single strands. When allowed to reanneal the two mutant strands will hybridise with normal strands to form mismatch containing duplexes.

Any locus can exist as one of 4 possible base pair combinations, and for any given base pair there are 3 possible point mutations. Therefore, there are 12 possible point mutations.

Mutations of base pairs from Purine:Pyrimidine to Pyrimidine:Purine are known as transversion mutations, e.g. A:T to C:G, those which convert Purine:Pyrimidine to Purine:pyrimidine as transitions, e.g. A:T to G:C.

When producing artificial heteroduplexes, *in vitro*, transition mutations give rise to two duplexes each containing a different purine:pyrimidine mismatched base pair, see Table 1 (1, 4, 9 and 12). Transversion mutations will give rise to one duplex with a purine:purine mismatch and one duplex with a pyrimidine:pyrimidine mismatched base pair, see Table 1 (2, 3, 5, 6, 7, 8, 10 and 11).

Table 1. Types of Point Mutation

Mutation No.	Mutation	Mismatches	Type
1	C:G to T:A	C:A and T:G	Transition
2	C:G to A:T	C:T and A:G	Transversion
3	C:G to G:C	C:C and G:G	Transversion
4	T:A to C:G	T:G and C:A	Transition
5	T:A to A:T	T:T and A:A	Transversion
6	T:A to G:C	T:C and G:A	Transversion
7	A:T to C:G	A:G and C:T	Transversion
8	A:T to T:A	A:A and T:T	Transversion
9	A:T to G:C	A:C and G:T	Transition
10	G:C to C:G	G:G and C:C	Transversion
11	G:C to T:A	G:A and T:C	Transversion
12	G:C to A:T	G:T and A:C	Transition

The following mispairings can exist : A:A, C:C, G:G, T:T, A:C, A:G, C:T, and G:T. Methods to detect all of these mismatches would allow detection of all possible mutations in any given ssDNA from a DNA duplex. However, if both strands of DNA from a duplex are used to form heteroduplexes then only four mismatch base pair detection systems are required. To detect all possible types of point mutation combinations of detection systems are required to be investigated.

Mutation types 1, 4, 9, and 12 (see table 1) would be identified by detecting heteroduplexes containing A:C or G:T mismatches. It would not be necessary to look for both mismatches. Similarly mutations 2, 6, 7, and 11 would be identified by detecting heteroduplexes containing A:G or C:T. Mutations 3 and 10, and mutations 5 and 8 would be identified by detecting heteroduplexes containing C:C or G:G, and T:T or A:A, respectively. Therefore, to identify all types of mutation you would need to be able to detect one type of mismatch from each of the four pairs of mismatch containing heteroduplexes.

For any base pair a random process of mutation would give rise to 1/3 transition mutations and 2/3 transversion mutations. However, transition mutations predominate in DNA, representing 59% of all point mutations (Li *et al.*, 1984). Generally, transition mutations occur due to modification of one base in base pair, e.g. 5MedC deamination converts 5MedC to thymine and thus generates a G:T mispair. Where this mispair is not identified and corrected a G:C to A:T point mutation in one daughter strand during replication will occur. In general, errors during replication will favour mismatches that form wobble base pairs. These mismatches are more likely to be purine:pyrimidine mispairs and so produce transition mutations.

The only disadvantage of this approach is that analysis of both strands is required. The analysis of the products of mismatch analysis usually uses labelled DNA probes, and thus analysis of both strands increases the cost and where radiolabels are used the amount of hazardous waste. The four mismatch detection method does however reduce the number of methods required to detect mismatches.

1.4.3.2 Effect of Base Sequences

The detection of a mismatch may be further complicated by the nature of the duplex base sequence in which it is positioned. This may take two forms:

(***A***) In G:C rich regions duplex formation is a result of base pairs with three hydrogen bonds, compared to only two in A:T rich regions. G:C rich regions are therefore energetically more stable. In a G:C rich region recognition of given mismatch by a mismatch recognition process (proteins or chemicals) may not be equivalent to the same mismatch in an A:T rich sequence. This difference in mismatched base pair exposure to the environment may give rise to significant variation for a mismatch specific antibodies affinity for a mismatched base pair.

(***B***) The immediate neighbouring base pairing may greatly influence the recognition of a given mismatch, independently from the general DNA sequence. For all mismatches there will be any one of four base pairs (A:T, T:A, C:G, G:C) at a position 3' and 5' immediate to the mismatch. Thus, there are 16 possible neighbouring conformations for each mismatch, giving a possible 128 conformations. For mismatches with the same base (A:A, C:C, G:G, T:T) some of the configurations are equivalent. For mismatches with different bases all 16 configurations are different

3'-A-A-A-5' is the same as 3'-T-A-T-5'
5'-T-A-T-3' 5'-A-A-A-3'

whereas,

3'-A-A-A-5' is not the same as 3'-T-A-T-5'
5'-T-C-T-3' 5'-A-C-A-3'

There are 10 different sequence configurations for the four mismatches formed with the same base pairing. This gives a total of 104 possible arrangements. For some of the methods to identify mismatches, e.g. denaturing methods, sequence configurations may not be an important factor. However where specific recognition is

important (e.g. mismatch repair enzymes) these factors may become increasingly important. For the current study where monoclonal antibody production against mismatched base pairs is being investigated, recognition could be affected by neighbouring base pairs. This could prove a significant obstacle in this approach.

1.4.4 Current Methods of Detecting Point Mutations.

Mutations can be found by directly sequencing the amplified products of the PCR (Gyllensten and Erlich, 1988; Murray, 1989). However, this method is fairly laborious, and also PCR is not error free and thus could create mutations.

It is possible that hot spots for mutations, *in vivo*, may also be predominant sites for PCR error? DNA sequencing is commonly used to identify or confirm the exact nature and position of a mutation, previously detected by a screening method. Methods for the detection of mutation have recently been reviewed (Cotton, 1993, 1992; Prosser, 1993). Only a brief description of those methods currently being used will be discussed. The importance of sensitivity of these methods will be emphasised.

1.4.4.1 Single Strand Conformation Polymorphism (SSCP)

Single strands of DNA will form unique tertiary structures depending on their nucleotide sequence. This allows single strands of DNA differing by one or more nucleotide to be separated by electrophoresis in a non-denaturing polyacrylamide gel. This method has been described (Orita *et al.*, 1989a) as Single Strand Conformation Polymorphism (SSCP). By combining SSCP with PCR a simple and sensitive method for the detection of point mutations was obtained (Orita *et al.*, 1989b; Hayashi, 1991). If mRNA is converted into cDNA by reverse transcriptase it can also be analysed by PCR-SSCP (Murakami *et al.*, 1991a,b) for the detection of single or multiple nucleotide mutations.

The advantage of this method is that it can detect a single base substitution at unknown positions within target DNA fragments. The efficiency of detection of a single base substitution, in fragments less than 300 base pairs, is greater than 90% (Sekiya, 1993). DNA fragments for analysis from cancer specimens are often obtained where the tumour cells are in the minority. By physically separating normal strands from mutant strands, this method allows subsequent PCR amplification of even the faintest bands. This allows full characterisation of minute amounts of mutant DNA by sequencing.

PCR-SSCP has been used to detect mutations in DNA associated with human cancers, e.g. ras gene mutations in lung carcinomas (Suzuki *et al.*, 1990); and hereditary diseases in humans, e.g. gene mutation leading to cystic fibrosis (Dean *et al.*, 1990).

1.4.4.2 Denaturing Gradient Gel Electrophoresis (DGGE)

A modification of standard electrophoretic gel procedures, allows DNA to be separated not only on the basis of size, but also on the basis of nucleotide sequence (Fischer and Lerman, 1983). The dissociation of dsDNA occurs on heating, and in the presence of denaturing chemicals, e.g. formamide or urea. Polyacrylamide gels for electrophoresis can be prepared such that they contain denaturing chemicals. In, addition they can be produced such that a gradient of denaturing chemicals exists. The concentration of denaturing chemicals increases as the DNA migrates through the gel by electrophoresis. DNA is separated according to size and it also moves into an increasing concentration of denaturing chemicals.

Duplex dissociation to single strands occurs as they reach their distinctive critical concentration of denaturing chemicals. Single stranded DNA has greater flexibility than duplex DNA and can become stuck in the pores of the gel. Thus as single stranded domains are formed by the denaturing conditions the DNA effectively cease to migrate.

The concentration of denaturing chemicals that will cause duplex dissociation is sequence dependent. When DNA heteroduplexes are formed between mutant and wild type sequences, any point mutation will produce a mismatch site. As with thermal melting, chemical denaturing is greatly facilitated by the presence of a mismatch. Thus, mismatch heteroduplexes will melt and migration will cease at a lower denaturant concentration than fully matched sequences. The method usually requires radiolabelled probes to provide the necessary sensitivity.

Originally DGGE only detected about 50% of all mutations, and this is attributed to differing melting domains. If the mismatch occurs in a high melting domain, a lower melting domain may have already caused dissociation and arrested migration before the mismatch can influence melting. PCR facilitates the attachment of a high melting point 40-base pair GC rich sequence (GC clamp) (Sheffield *et al.*, 1989, 1992; Abrams *et al.*, 1990). Thus all mutations should fall into low melting domain regions, and nearly all point mutations should be detected.

1.4.4.3 Allele Specific Oligonucleotides (ASO)

This method relies on hybridising chemically synthesised oligonucleotides to genomic DNA pre-immobilised to a solid support. These ASOs hybridise to perfectly paired sequences, but poorly where target DNA will form a mismatched base pair (Connor *et al.*, 1983). DNA hybridisation can be detected by labelling the oligonucleotide probe.

1.4.4.4 Restriction Fragment Length Polymorphism (RFLP)

Certain nucleotide sequences in DNA are specifically recognised and cleaved by bacterial restriction enzymes. There are a variety restriction enzymes all recognising different restriction sites. Genomic DNA is isolated from white blood cells and treated with restriction enzymes. The number of fragments produced is determined by the occurrence of a particular enzyme restriction site. Fragments can be processed by two methods.

(***A***) Fragments can be cloned, or amplified by PCR, to produce large quantities of DNA fragments. These can then be cut into smaller fragments, with a further set of restriction enzymes, and separated by gel electrophoresis. The presence of extra or reduced bands is indicative of mutation.

(***B***) Fragments from an entire human genome are separated by gel electrophoresis. Double stranded DNA is then dissociated into single strands, within the gel. The DNA is then transferred to a plastic membrane (Southern blotting), and complementary DNA probes are then annealed.

Cloning, and to a lesser extent PCR, may introduce new mutations, but does not rely on the availability of DNA probes. The method using probes is however more rapid. The major shortfall of the method is its reliance on mutation recognition only at restriction sites, and not the entire gene sequence.

1.4.4.5 PCR Amplification of Specific Allele (PASA)

For PCR amplification to occur *Taq* polymerase requires the primer sequence and target sequence to form perfect base pairing. If two alleles of a gene differ by only one nucleotide then the allele that is perfectly complementary with the primer sequence will amplify in preference to the allele forming a mismatched base pair. The closer the proximity of a mismatched base pair, formed between the primer and target sequence, to the 3' end of the primer the less likely the PCR can occur. This technique has been successfully applied to screen for hereditary disorders, e.g. screening humans for abnormal factor VIII gene, and mutations in phenylalanine hydroxylase gene (Somner *et al.,* 1992).

1.4.4.6 RNase A

This method relies on the ability of the enzyme ribonuclease A (RNase A) to cleave double stranded RNA:DNA heteroduplexes at the site of a mismatched pair (Myers *et al.,* 1985). DNA is cut into

fragments with restriction enzymes, then heat denatured into single strands. These are then reannealled with radiolabelled single strands of wild-type RNA to form a heteroduplex. Treatment of the heteroduplex with RNase A will then cause strand breakage at mismatched base pairs. RNase A products are then separated by gel electrophoresis according to size. Where cleavage occurs the loss of one band on the gel and the occurrence of two new bands of lower molecular weight are seen.

However, RNase A is an inefficient cutter of all mismatched base pairs in heteroduplexes, only cutting pyrimidine:pyrimidine mismatches well. There are 12 possible mismatched base pairs, only 4 of which are pyrimidine:pyrimidine, see Table 1. If both single strands from the mutant duplex are used for the analysis then 2/3rds of the mismatch types can be identified, see 1.4.2.1.

1.4.4.7 Chemical Based Methods

Chemicals have been used to study nucleic acids in a variety of ways. These include studies on single stranded DNA (Kochetkov and Budvoskii, 1972), on RNA structure (Metz and Brown, 1969a, 1969b), and on Z-DNA (Johnson and Rich, 1985). DNA sequencing (Maxam and Gilbert, 1977, 1980) was the first chemical method to be able to identify point mutations in DNA. Maxam-Gilbert sequence analysis uses base selective partial chemical cleavage to determine the DNA sequence.

A second method known as the Sanger dideoxy-sequence analysis relies upon the use of 2',3'-dideoxynucleoside 5'-triphosphates as base specific chain terminators of the synthesis of a complementary DNA copy by a DNA polymerase (Sanger, 1977, 1981, 1988). Both of these methods rely on gel electrophoresis to interpret the chemical reactions.

PCR amplification coupled to sequencing by the Sanger method has been carried out (Ruano and Kidd, 1991). Both methods require many manipulations, and are fairly time consuming. Although some laboratories still use sequencing methods to find

mutations, more often they are used as a reference method to determine the exact nature of a mutation, after a more rapid screening method has identified a gene sequence containing a mutation.

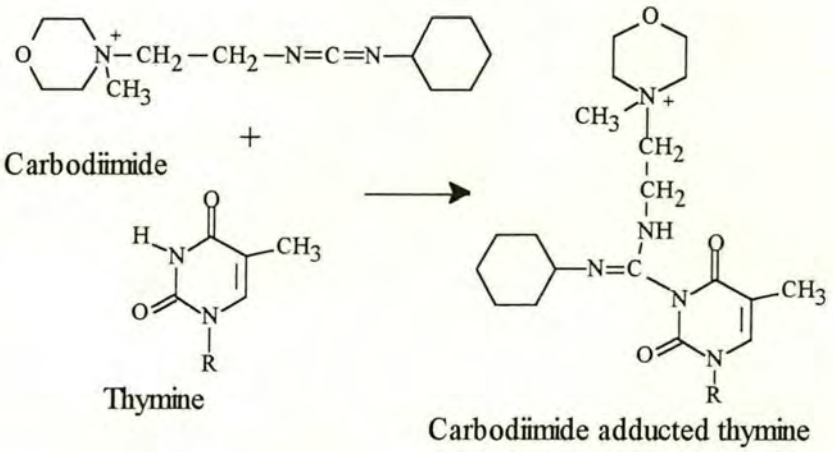
Some chemicals that react with single stranded DNA or variants of normal DNA structure, such as Z-DNA, can react with mismatched base pairs. Mismatched base pairs can be formed artificially, at sites of point mutation, by annealing mutant and wild type DNA. Two chemical methods for the detection of point mutation, based on mismatch recognition, have emerged. Both rely on specific modification at the site of a mismatched base pair in an otherwise normal DNA duplex.

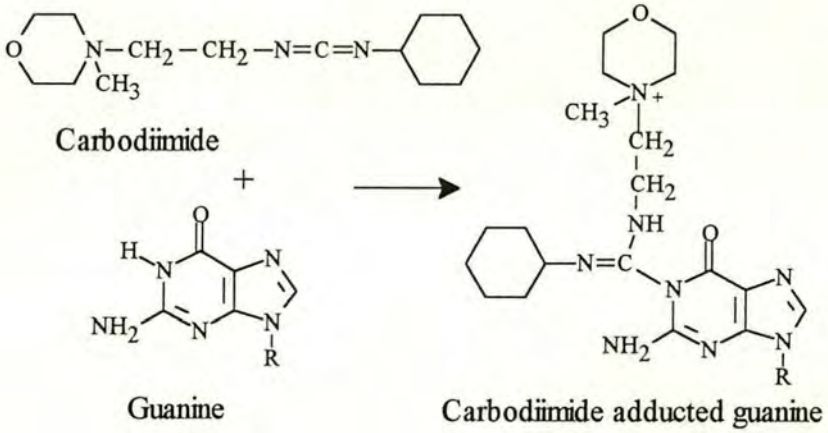
These methods have been successfully used in the detection of previously unknown mutations. In addition they not only detect mutation, but also locate the mutation (to within approximately 10 nucleotides). This is advantageous when compared to some of the simpler methods such as (DGGE) (Myers *et al.*, 1985), and (SSCP) (Orita *et al.*, 1989a). Both methods have the potential to detect all mutations in relatively long stretches (up to 2kb) of DNA.

1.4.4.7.1 Carbodiimide (CDI)

It was shown (Metz and Brown, 1969a) that modification of RNA and DNA occurs with 1-cyclohexyl-3-[2-(morpholinyl) ethyl]-carbodiimidemetho-p-toluene sulphonate (carbodiimide or CDI). CDI will react with unpaired thymine and uracil (at N3) and guanine (at N1), see figure 13. The difference in electrophoretic mobility caused by the modification is then used to separate mutant and wildtype strand (Novack *et al.*, 1986). The method thus having similarities with DGGE and SSCP, in that mutation is detected but location is unknown.

FIGURE 13 REACTIONS OF CARBODIIMIDE





An improvement to this method was the use of the *Escherichia coli* (*E. coli*) repair enzyme UvrABC exonuclease. The enzyme cleaves both sides of the modified region of DNA (Thomas *et al.*, 1986). However, this enzyme has only recently become readily available. Immunochemical methods for the detection of CDI modified DNA were investigated. Specific antisera have been produced (Wani *et al.*, 1989; Ganguly *et al.*, 1989), and the identification of a previously unknown mutation (Ganguly *et al.*, 1991) has been carried out. The use of PCR (Ganguly and Prockop, 1990) has been incorporated into this method. The modification to the heteroduplex prevents the PCR reaction from proceeding, thus indicating the presence of a point mutation.

1.4.4.7.2 Chemical Cleavage of Mismatch (CCM)

Several chemicals have been tested for their ability to modify mismatched base pairs, such that subsequent treatment with piperidine would cause cleavage of the nucleic acid backbone. Those tested include hydrazine, potassium permanganate, formic acid, sodium hydroxide, diethylpyrocarbonate, methylene blue, hydroxylamine, and osmium tetroxide (Cotton *et al.*, 1988).

Hydroxylamine, which modifies mismatched cytosines, and osmium tetroxide, which modifies mismatched thymines, proved to be most successful. Combining the two methods provides a technique capable of detecting all possible mutations. However, T/G mismatches are often poorly recognised (Forrest *et al.*, 1991; Cotton *et al.*, 1993). This is related to the stability of the mismatch. This method has also been applied to mutations in RNA, where mismatches in RNA:DNA heteroduplexes, formed by annealing with a DNA probe, are recognised (Cotton and Wright, 1989; Dahl *et al.*, 1989). There are two main advantages of the CDI and CCM methods :

(***A***) They can detect all eight possible mismatches and therefore all point mutations. Unlike other cleavage methods, e.g. RNase A method that fail to recognise approximately 30%.

(***B***) They disclose the region of the DNA duplex containing the mutation. This is not possible using methods such as DGGE and SSCP, where only the presence of a mutation is identified.

CDI and CCM thus serve as less labour intensive methods to provide the information obtainable by DNA sequencing. They are however more labour intensive than some other methods. Both methods rely on PCR to amplify DNA sequences for heteroduplex formation. The major disadvantage of the CCM method is its reliance on very toxic chemicals, e.g. osmium tetroxide. The use of alternatives to osmium tetroxide have been investigated, e.g. replacement with potassium permanganate (Gogos *et al.*, 1990). However, to date this replacement has not become routinely used.

1.4.5 Sensitivity of Mutation Detection

The ability to detect the majority, preferably 100%, of mutations is not the only important development of research into mutation detection. In heritable genetic disorders 50 or 100% of the target DNA will contain the mutation. However, in the somatic mutations of tumour tissue, sensitivity, i.e. the ability to measure the proportion of mutant material to normal DNA, is important. During biopsy normal as well as tumour tissue will be excised, often mutated cells will represent a considerable minority of the total cell population excised. Therefore, techniques are needed to identify a very small percentage of the total DNA which has undergone mutation.

Most of the above detection methods will detect mutations if mutant DNA represent 5-20% of the total DNA extracted. DGGE for example will detect mutations where 10-15% of the DNA being analysed contains the mutation (Heimdal *et al.*, 1993), for CCM (osmium tetroxide) 2-5%, and CCM (hydroxylamine) 1-2% (Prosser, 1993) of the DNA needs to contain the mutation. A few technique have been developed to address this problem. They are usually used for the detection of previously identified mutations, rather than discovering previously unidentified mutations.

1.4.5.1 Mismatch Repair Enzymes

The method relies on the high specificity of Mut Y, an E. *coli* mismatch repair protein (Lu and Chang, 1988; Au *et al.*, 1988; Tsai-Wu *et al.*, 1991), to recognise mismatches. Mut Y specifically recognises and nicks G:A mismatches only (there is a weak activity for A:C mismatches). The method is analogous to the chemical cleavage methods in that a mismatched base pair artificially produced at a site in DNA containing a point mutation will lead to cleavage of the heteroduplex, producing two strands of DNA. Wild-type DNA will not be recognised by Mut Y, and therefore will not be cleaved.

This method has been used to detect mutations in 1% of starting material (Lu and Hsu, 1992). Other mismatch enzymes will be needed to screen for all types of mutations. If more types of mismatches were to be detected utilising repair enzymes, they may become very useful tools in mutation detection.

1.4.5.2 Mismatch Amplification Mutation (MAM) Assay

The MAM assay is a modification of the PCR Amplification of Specific Alleles (PASA) assay, previously described (1.4.3.5). The PASA assay relies on the inability of PCR to amplify sequences containing mismatches at or near the -3' end. Wild type target DNA and wild type primer will form perfect Watson-Crick base pairings, and thus will undergo the PCR reaction. Production of a mismatch occurs between mutant target DNA and wild type primer DNA, therefore amplification is greatly reduced. The technique permits preferential amplification of one allele relative to another.

The technique is rapid and simple in comparison to methods such as RFLP and oligonucleotide hybridisation, and can be used to detect mutations in nanogram quantities of DNA. However, it lacks the sensitivity of detection of other methods. RFLP in combination with PCR has been reported to be able to detect a point mutation in genomic DNA samples occurring at a frequency of 1 in 10,000 (Kahn *et al.*, 1991; Kumar and Barbacid, 1988), as compared to about 1 in 40 for PASA (Sarker *et al.*, 1990). The low sensitivity is due to an estimated 15-50% amplification per cycle of most mismatch containing sequences under a variety of PCR conditions (Kwok *et al.*, 1990).

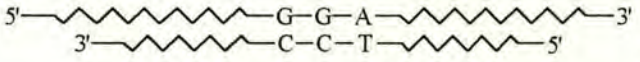
This method has recently been modified to allow the detection of mutations with comparable sensitivity to the RFLP-PCR method (Cha *et al.*, 1992). The modifications are based on the ability to design specific primers for known mutations, where the primers are designed to be complementary to the known mutation product, and has a second mutation adjacent to the mutation being detected.

Hybridisation between mutant target DNA and mutant primer, plus an additional mutation, yield a heteroduplex with one mismatch. Under suitably modified PCR conditions amplification of single mismatch heteroduplex sequences can be carried out. However, hybridisation between wild type target DNA and the above primer yield a heteroduplex with two mismatches. The presence of a double mismatch reduces PCR efficiency to such an extent that amplification will be effectively non-existent, see figure 14, (the extension of the assay method from PASA is shown). PCR conditions have been optimised and this method has been used to detect the G to A transition in the 12the codon in the rat c-Ha-*ras* gene (Cha *et al.*, 1992). They report the ability to detect mutations where present as low as one in 10^5 to background wild type DNA.

FIGURE 14. BASIS OF MAM ASSAY

Basis of PCR Allele Specific Amplification (PASA) Assay

Wild Type allele hybridised with Wild Type probe: A and C



No Mismatch
Good amplification

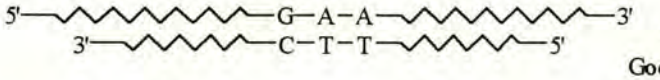
Mutant allele hybridised with Wild Type probe: B and C

One Mismatch
Poor amplification

Wild Type allele hybridised with Mutant probe: A and D

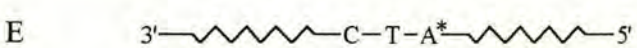
One Mismatch
Poor amplification

Mutant allele hybridised with Mutant probe: B and D



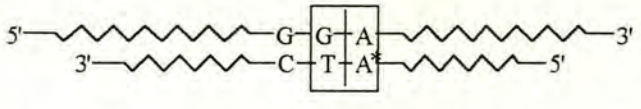
No Mismatch
Good Amplification

Basis of MAM Assay



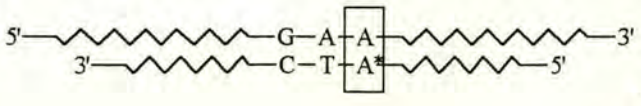
Mutant probe
plus mutation*

Wild Type allele hybridised with Mutant probe plus mutation *: A and E



Two Mismatches
No Amplification

Mutant allele hybridised with Mutant probe plus mutation: B and E



One Mismatch
Good amplification
(Modified PCR)

1.5 MISMATCHED BASE PAIRS AS IMMUNOGENS

The exact nature of the immune response to B-DNA is uncertain. It forms part of the immune response in SLE, though the induction of antibodies by B-DNA that will bind to native B-DNA has not been reported (see chapter 3). It is hoped that modification of duplexes such that they contain a mismatched base pair will suitably alter the structure of B-DNA so that immune recognition at this site may be possible. Most of the methods to detect point mutations in DNA duplexes rely on the *in vitro* production of mismatched base pairs. The properties of the subsequent heteroduplexes formed allowing them to be distinguished from perfectly paired sequences.

1.5.1 Mismatch Structure

Protein DNA interactions are involved in many of the fundamental processes that occur within cells. These include packaging, replication, recombination, restriction and transcription. The requirement for sequence specific recognition of DNA binding protein to DNA varies greatly. Many proteins, e.g., histones and DNA polymerases, have little sequence specificity. Others proteins, e.g. repressors and restriction enzymes have very high sequence specificity, possibly binding to a single site of 10-20 base pairs in a background of 10^6 to 10^9 base pairs, .

It is now known that many mismatches that can be formed in DNA do not cause gross perturbations in the double helix. Proteins that recognise and repair these must therefore identify the DNA at the level of the individual base pair (Freemont *et al.*, 1991). The neighbouring base pairs may also have a role in mismatch repair enzyme recognition. To discover the molecular mechanisms underlying mismatch repair a great deal of work has focused on the elucidation of the structure of a variety of synthetic DNA duplexes containing mismatched base pairs.

Polyacrylamide gel electrophoresis (PAGE) has been used to determine the longer range effects of mismatched base pairs on DNA sequences. A combination of three other techniques have been used to study the structure of mismatches in DNA, these are :

(*A*) Ultraviolet Melting Studies.
(*B*) X-Ray Crystallography.
(*C*) Nuclear Magnetic Resonance (NMR) Spectroscopy.

The literature on this subject is vast, key references only have been used in this discussion. Mismatches in DNA duplexes has recently been reviewed (Brown *et al.*, 1993, and references therein).

1.5.1.1 Polyacrylamide Gel Electrophoresis (PAGE)

Although techniques such as NMR spectroscopy can give us very detailed information on the spatial arrangement of the atoms, these are not useful for looking at long range structural changes, e.g., duplex bending. Gel electrophoresis can provide such information on gross structural alterations in the duplex, which affect duplex migration within the gel. It has been found (Bhattacharya and Lilley, 1989) that all mismatches are associated with minimal or no global structural changes (in a 458 base pair fragment studied).

1.5.1.2 Ultraviolet Melting Studies

The relationship between thermal melting and mismatch repair efficiency has been investigated (Werntges *et al.*, 1986). All 16 possible base pair arrangements were investigated in an octadecameric oligonucleotide duplex. The melting temperatures of all mismatched base pairs were lower than for fully matched duplexes. However, the melting temperatures did not correlate to the repair efficiency of the mispair. There was however a correlation between the type of mispair formed and the repair efficiency. Those mispairs that can form wobble base pairs e.g., T:G, G:G, C:A are best repaired, those forming weak base pairing or permanent loops e.g., T:T and C:C are generally poorly repaired.

It was shown (Kramer *et al.*, 1984) that mismatches that are most often formed show the highest repair efficiency and vice versa. Base pair mismatches most likely to occur during DNA replication are those that will form the best, all be it non Watson-Crick, base pairing i.e. wobble base pairs. Mismatches forming loops, i.e.., Pyrimidine:Pyrimidine pairing are not likely to be stable enough to be incorporated into newly synthesised strands. Thus evolution has developed a system to repair the most frequently occurring errors.

1.5.1.3 X-Ray Crystallography and NMR Spectroscopy

Crystals have been grown for various forms of synthetic oligonucleotides including those forming B-DNA, A-DNA and Z-DNA, RNA-DNA hybrids, and RNA (reviewed by Kennard and Hunter, 1989). The first mismatched base pair to be characterised by X-ray crystallography was the purine:pyrimidine G:T mismatch (Hunter *et al.*, 1987). This base pair adopts a wobble configuration, viable non Watson-Crick hydrogen bonds between the base pairs. The thymine base projects into the major groove and the guanine base in the minor groove. The mismatched base is accommodated into the normal double helix by small adjustments in the configuration of the sugar phosphate backbone.

The geometry of the G:T mismatch is essentially identical in all structures so far studied (B-, A-, and Z- form DNA). The A:C mismatch in DNA has a similar structure to the G:T mismatch. It can theoretically exist in two forms (Hunter *et al.*, 1986). The adenine base may be either protonated or exist in a rare tautomeric form.

X-ray crystallography cannot provide molecular details at the level of individual hydrogen atoms. However, ultraviolet melting studies and NMR spectroscopy indicate that DNA duplexes containing A:C base pairs are unusually stable at low pH, i.e.., under conditions favouring protonation at N-1 of adenine (Brown *et al.*, 1990). It is possible to postulate base pairs with rare tautomers (Topal and Fresco, 1976a,b) previously discussed in section 1.2.3.1. However, it is not thought that these are likely to occur, and no direct evidence has been provided.

The G:A mismatch is unusual in that it can adopt many different forms and is repaired less efficiently than G:T and A:C mismatches. Four possible configurations have been proposed after characterisation studies using X-ray crystallography and NMR (Prive *et al.*, 1987; Gao and Patel, 1988; Brown *et al.*, 1986; Leonard *et al.*, 1990; Ebel *et al.*, 1992; Lane *et al.*, 1992).

1.5.1.4 Conclusions

The small degree of distortion associated with mismatches implies that recognition of gross structural perturbations will be difficult, and therefore direct recognition of the mismatched bases must occur by repair enzymes. X-ray crystallography indicates that duplexes with mismatches are not distorted. However, not all mismatches will allow crystal formation.

It should be noted that the methods for analysing the structure of base pair mismatches may influence the results obtained. It has been argued that this may be particularly true when the method of analysis is X-ray crystallography. It has been suggested that the duplex conformation in solution, by interpretation of NMR spectroscopy data, may not be identical with the static picture provided by X-ray diffraction in the crystal state (Roongta *et al.*, 1990). Structural variations observed in the X-ray crystallographic studies maybe a result of crystal packing forces. X-ray crystallographers argue that the crystals formed by DNA homo- and heteroduplexes are very different from those obtained by small molecules, they are in fact highly hydrated (up to 50%), and can to some extent be regarded as in solution. As many duplexes, some containing mismatches do not form crystals they argue against the forced nature of DNA crystal formation.

The combined evidence of X-ray crystallography, NMR spectroscopy, ultraviolet melting and gel electrophoresis suggest that mispairs can be incorporated into the double helix with only slight distortion of base stacking within the helix. Although the thermodynamic stability varies with the bases involved, the

stereochemical nature of the mispair is likely to determine the recognition of mismatched bases by repair enzymes.

Those forming wobble base pairs (T:G, C:A, G:G) will produce more rigid, if slightly distorted, structures, and are therefore more likely to provide recognition sites for the repair enzymes. They are consequently more likely to form recognition sites for the immune system when synthetic duplexes containing these mismatch sites are used as immunogens. Mispairs where no hydrogen bonding (C:C, T:T, C:T) occur present no real recognition site to repair enzymes as they are not bulky and can easily be accommodated within the helix. However, they are thought to rotate in and out of the helix reasonably freely. Therefore antibodies to cytosine or thymine in the single stranded state may also recognise these bases in mispairs. Immunisations with single stranded DNA of either cytosine or thymine may produce antibodies that recognise mismatched base pairs containing their respective bases, i.e.. C:C, C:T, and T:T mispairs.

1.5.2 Mismatches in DNA and Mismatch Repair

1.5.2.1 Introduction

The recognition of errors or mutations in DNA is essential to maintain the normal biological processes of all organisms. For example a system exists to remove DNA damage by O(6)-alkylation of guanine (O(6)-dG). An enzyme O(6)-alkyl guanine DNA transferase removes the alkyl group of O(6)-dG, by means of an internal cysteine residue (Pegg *et al.*, 1983) thus preventing this pre-mutagenic lesion developing.

Another enzyme system of great interest is the nucleotide excision repair in *E. coli*, known as the UvrABC complex, recently reviewed (Houten, 1990). This enzyme system recognises and repairs a large variety of different modifications to the Watson-Crick double helix, based on the overall structural perturbation caused by modification, rather than the specific agent causing the disruption. It

was thought that such an enzyme system may recognise the structural disruption due to the presence of mismatched base pairs. However, UvrABC does not recognise mismatches unless they contained G or T residues that were first chemically modified with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide, a reagent specific for unpaired G and T residues (Thomas *et al.*, 1988). Thus UvrABC cannot be described as part of mismatch recognition systems.

DNA replication is not error free, and a proofreading mechanism exists (Brutlag and Kornberg, 1972) which dramatically increases the fidelity of the base-pairing mechanism. However, this mechanism on occasions may fail and a normal, but incorrect nucleotide is introduced. This incorrect base forms a mismatched base pair site, and will lead to a point mutation on subsequent DNA replication. Mismatch repair systems exists that recognise and correct base pairing errors within the Watson-Crick helix.

The efficient correction of mismatches that arise in DNA during replication is dependant on two main factors:-

(***A***) Mismatch recognition - guides the repair enzymes to the error;
(***B***) Strand discrimination - recognises the incorrect base.

1.5.2.2 Mismatch Repair Systems

A mismatch repair system was proposed in the 1960s (Holliday, 1964) in eukaryotic cells, as a mechanism for gene conversion in fungi. However, most of our knowledge is based on repair processes first suggested in the bacteria *E. coli* (Nevers and Spatz, 1975). Bacteria possess multiple mismatch repair systems in two classes (Claverys and Lacks, 1986; Modrich, 1987; Radman, 1988; Radman and Wagner, 1986):

(***A***) A long patch repair system that processes a variety of mispairs in a strand specific manner involving excision-repair tracts of a kilobase or more.

(***B***) A short patch repair system that corrects a particular type of mismatch e.g. G:A in a strand specific reaction that involves a few nucleotides in the excision-repair tracts.

In *E. coli* the long patch repair system (Wagner and Meselson, 1976) and one short patch pathway (Au *et al.*,1988; Radicella *et al.*, 1988) function to eliminate DNA biosynthetic errors. A different short patch system is involved in the correction of G:T mispairs resulting from the spontaneous deamination of 5-methylcytosine in m^5C:G base pairs (Lieb, 1985; Lieb *et al.*, 1986; Jones *et al.*, 1987; Zell and Fritz, 1987).

1.5.2.2.1 Methyl-directed (long excision tract) Mismatch Correction

This pathway in *E. coli* functions in the correction of base pair mismatches and small insertions and deletions generated through errors in DNA replication (Wagner and Meselson, 1976). The DNA strand to be repaired is identified by virtue of the methylation state of adenine, a post replicative process that occurs at d(GATC) sequences (Pukkila *et al.*, 1983; Lu *et al.*, 1983; Wagner *et al.*, 1984; Kramer *et al.*, 1984; Dohet *et al.*, 1986; Raposa and Fox, 1987).

There are five components involved in mismatch repair, Mut S, Mut L, Mut H, Mut U (also known as uvrD) and a single strand DNA binding protein. Mut S recognises all eight mismatches although not equally (Modrich, 1989; Radman and Wagner, 1988). After recognising and binding large regions of DNA, up to several kilobases, are excised and resynthesised during the course of repair (Wagner and Meselson, 1976; Su *et al.*, 1988). The system corrects a variety of mismatched base pairs, but not all with equal efficiency (Kramer *et al.*, 1984; Dohet *et al.*, 1985; Jones *et al.*, 1987c; Su *et al.*, 1988; Lahue *et al.*, 1989).

1.5.2.2.2 Mismatch Specific (short excision tract) Repair Systems

Two systems exist that are independent of d(GATC) methylation. They have restricted mismatch correction specificity.

(*A*) Very Short Patch (VSP) Mismatch Repair

The VSP repair system was first identified by Lieb (1983, 1985). This repair system recognises and corrects G:T mismatches that arise in resting DNA through spontaneous deamination of 5-methylcytosine in m^5C:G base pairs (Zell and Fritz, 1987). The recognition process is sequence specific. The repair usually involves the excision of less than 10 base pairs, and uses Mut L and Mut S gene products (Jones *et al.*, 1987b; Lieb, 1987; Fritz and Zell, 1987).

(*B*) Mut Y Dependant G:A to G:C Correction

The Mut Y repair system functions independently of Mut H, Mut L, Mut S, and Mut U gene products. The Mut Y protein is involved in a repair pathway that corrects G:A mismatches to G:C Watson-Crick base pairs (Radicella *et al.*, 1988). The nicking activity of Mut Y is specific for G:A containing (weakly for A:C containing) DNA fragments, and has no activity on DNA containing other mispairs or homoduplex DNA. Mut Y removes the mispaired adenines of G:A (or A:C) mismatches, its endonuclease activity then cleaves at the first phosphodiester bond 3' to the apurinic/apyrimidic sites.

The high specificity of Mut Y has recently allowed it to be used in a highly specific manner to detect mismatches in DNA artificially produced (Lu and Hsu, 1992). Thus, one of the biological methods to ensure DNA fidelity can be used in vitro to detect sites of point mutations.

1.5.2.3 Mismatch Correction in Eukaryotes

Mismatch repair systems in eukaryotes have received considerable attention in the last decade. Base pair mismatches and small insertions and deletions have been shown to be repaired by a mismatch repair system in the fungi *Saccharomyces cerevisiae* (Bishop and Kolodner, 1986). The efficiency of correction is dependent upon the nature of the mismatch pair (Bishop *et al.*, 1987, 1989). The order of mismatch repair efficiency was found to be:

G:G > A:C, G:A, G:T > T:C > T:T, C:C, A:A.

These results were confirmed (Kramer *et al.*, 1989) except that C:C mispairs were not found to repair.

1.5.2.3.1 Human Mismatch Repair Systems

Transfection experiments in mammalian cells (Brown and Jiricny, 1987, 1988) have demonstrated that all mispair types are repaired. However they are repaired with different efficiencies and specificity's. Mismatches are divided into two classes, heterogeneous (involve different bases) and homogeneous (involve the same bases), and the type of repair reflects the class.

For heterogeneous repair, repair to G:C predominates in all cases, with G:T showing both highest efficiency of repair and that repair preferring G:C correction. The picture is more complicated for homogeneous repair. The type of repair is highly dependent upon flanking sequences. The mismatch repair efficiencies for homogeneous base pairs was found to be:

G:G >> C:C > A:A > T:T.

The overall efficiencies for both classes was :

G:T, G:G > A:C > C:T > C:C > A:A >> A:G, T:T.

This general eukaryotic mismatch repair pathway (Holmes *et al.*, 1990; Thomas *et al.*, 1991) is believed to be analogous to the Mut H, Mut L, Mut S pathway (Lu *et al.*, 1983).

A specific pathway may exist that recognises G:T and corrects to G:C in the absence of strand discrimination. G:T mismatches arising from deamination of 5-methylcytosine in resting i.e.. non-replicating and non-recombining, DNA. They are repaired by a system that specifically removes the thymine and replaces cytosine (Wiebauer and Jiricny, 1989). This pathway not only prevents mutation it also maintains methylation patterns in DNA by restoring CpG sites.

A 200kd protein extracted from HeLa cells, first thought to be a specific G:T binding protein (Jiricny *et al.*, 1988), has been recently purified and further characterised (Hughes and Jiricny, 1992). The protein binds independently of flanking sequences and has a high affinity for G:T mismatches. It has also been found to bind, albeit with lower affinity, to A:C, G:U, G:G, and G:A mismatches. This protein is now regarded as a more general mismatch recognition protein, similar in function to Mut S in *E. coli*.

Eukaryotes posses another mismatch binding protein. A 100kda polypeptide has been found in extracts of a Burkitts lymphoma cell line (Stephenson and Karran, 1989). This binds specifically to heteroduplexes containing A:C, C:T, an T:T mismatches.

1.6 CONCLUSIONS

Mismatched base pairs in DNA are a result of errors in DNA replication, spontaneous deamination of 5-methylcytosine to thymine, and occur during recombination processes. To avoid mismatch occurrence leading to point mutation, highly specialised repair systems exist. These function to maintain DNA fidelity by the recognition of mismatch sites and the ability to strand discriminate.

Although most of the work has been carried out in *E. coli* there appears to be some correlation between their mismatch systems and those in mammalian systems. However, there is still a large amount to be discovered about mismatch repair.

Mismatch repair processes are of great interest for their potential in screening for point mutations. Of particular note is the recent work in the development of a screening method for point mutation by using purified Mut Y to detect artificially produced mismatches (Lu and Hsu, 1992). By using several specific, or one general mismatch recognition protein(s) (with relatively high affinity) these proteins could be used to screen for all point mutation.

Additionally, mismatch repair processes provide information on the types of mispairs that are best recognised. Where immunochemical methods to detect mismatches in DNA are to be investigated, the ability of mispairs to be recognised by the repair system may reflect the type of mispairs to be used as antigens in the production of anti-mismatched base pair antibodies.

The proposed experimental work aimed to produce antibodies that are capable of specific recognition and binding to a variety of mismatched base pairs in DNA. This necessitates mismatched base pairs being structurally distinct from Watson-Crick base pairs. The physical evidence, provided by PAGE, UV melting, X-ray crystallography, and NMR, in addition to the methods of detecting point mutations supply considerable indication that duplexes containing a mismatched base pair are significantly dissimilar from fully complementary strands. This evidence is strongly supported by

the discoveries of both prokaryotic and eukaryotic systems capable of recognising mismatched base pairs. This evidence provides considerable confidence at the outset of the project. The production of specific antibodies to mismatched base pairs would provide a substantial tool in mutation research.

CHAPTER 2 : SYNTHESIS OF IMMUNOGENIC DNA SEQUENCES

2.1 INTRODUCTION

The object of the study was to produce antibodies that recognise mismatched base pairs in DNA duplexes. Synthetic oligonucleotides were used throughout the study. Production of synthetic oligonucleotides is now routinely carried out using automated DNA synthesisers and is briefly described below. There were several major concerns with the use of duplex oligonucleotides for immunisations.

Firstly, the duplexes produced must remain thermally stable throughout the immunisation regime. Therefore, duplexes must only dissociate into single strands at temperatures above physiological temperature. The thermal stability of short DNA duplexes of less than 30 base pairs is greatly reduced by the inclusion of a mismatched base pair. The thermal stability of a duplex formed by the association of single strands is also dependent upon the concentration of the strands. This is a crucial factor during immunisation as relatively small amount of duplex are used, diffusion and therefore dilution of DNA will occur during the immunisation regime.

The thermal stability of an intramolecular duplex, formed by the folding of a self complementary single strand, is independent of the concentration of single strand present. It was hoped that the production of a suitable linker would allow the production of oligonucleotide duplexes containing a mismatched base pair that would not dissociate at physiological temperatures. A central non-complementary region within an otherwise self complementary single strand could be used. However, during immunisation this would be likely to induce an immune response to single stranded DNA. The presence of anti-single stranded DNA antibodies, in addition to the desired anti-mismatched base pair antibodies, may interfere with the screening processes.

Secondly, DNA is unstable in serum due to the presence of nucleases (DNase enzymes). The oxidation step in the production of synthetic oligodeoxyribonucleotides can be altered such that a phosphorothioate (a sulphur analogue of phosphate), instead of phosphate group is produced. Phosphorothioate oligodeoxyribonucleotides are resistant to the effects of DNase enzyme degradation. They can be used parallel studies with standard oligonucleotides where DNA degradation is a concern.

Thirdly, molecules with molecular weights <10,000 are generally considered to be too small to be immunogenic. They are usually covalently attached to larger carrier protein (e.g., Bovine Serum Albumin (BSA), Chicken Gamma Globulin (CγG), Keyhole Limpet Haemocyanin (KLH) prior to immunisations. A short double stranded oligonucleotide containing 20 or 30 bases is still relatively small to be used as an immunogen. It may therefore be necessary to covalently attach the oligonucleotide to a protein carrier. Due to the recent increase in non-radioactive DNA probe technology, there are several specific methods available to bind DNA and oligonucleotides to proteins.

Where synthetic oligonucleotides are being produced this can be achieved by the use of modified nucleotide phosphoramidites, containing a specific linker function for conjugation (Urdea *et al.*, 1988; Jablonski *et al.*, 1986; Zuckermann *et al.*, 1987). These can be incorporated as part of automated DNA synthesis such that the conjugation to the protein will occur at a defined site in the oligonucleotide. Secondly there are methods where the synthetic oligonucleotide is produced normally and then modified to allow conjugation to proteins (Renz and Kurz, 1984; Borel *et al.*, 1984; Murakami *et al.*, 1989; Chu and Orgel, 1988; Pollard-Knight *et al.*, 1990). However, the earliest methods to enhance immunogenicity of DNA relied on simple complex formation between positively charged methylated BSA (MeBSA) and negatively charged DNA (Sueko and Cheng, 1962).

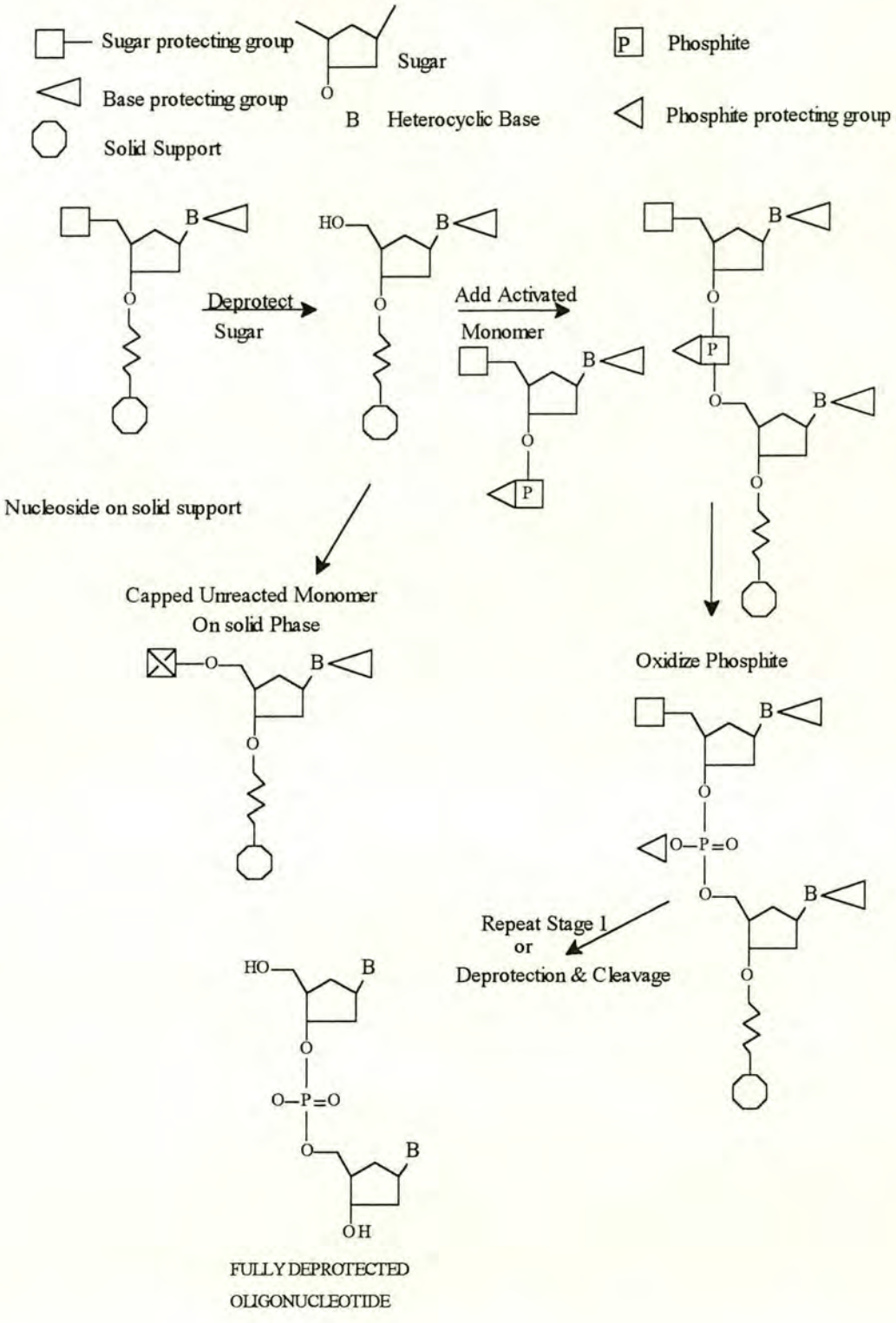
2.2 OLIGODEOXYRIBONUCLEIC ACID SYNTHESIS

Production of synthetic oligonucleotides is now routinely carried out using the solid phase phosphoramidite technique. Over the past 10 years this process has been automated, allowing the rapid production of synthetic oligodeoxyribonucleotides sequences of up to about 100 bases. Modern machine-aided oligonucleotide synthesis has recently been reviewed (Brown and Brown, 1991) so only an overview will be presented here.

The process uses the addition of successive, protected, cyanoethyl phosphoramidites to a nucleoside attached at the 3'-terminus to a solid phase. The incoming phosphoramidite binds to the deprotected 5'-terminus of the solid phase nucleoside. An excess of reagent is used to drive the reaction to completion, any unreacted 5' solid phase is then capped, rendering it inert to further monomer additions. The phosphite group is then oxidised to produce a phosphatetriester group. The dimer is then ready for subsequent phosphoramidite additions.

On the completion of monomer additions, to produce the desired oligonucleotide sequence, the protecting groups can be removed and the sequence cleaved from the solid support, see figure 15. The major advantage of this process is that a large number of reactions can be carried out without the need for elaborate purification procedures between each reaction. Excess reagents are simply removed by repeated solvent wash steps, and the process is rapid.

FIGURE 15 OVERVIEW OF AUTOMATED DNA SYNTHESIS



2.3 RESULTS AND DISCUSSION

2.3.1 Production of an Oligonucleotide Linker Molecule

2.3.1.1 Synthesis of Linker Molecule, Compound [3]

Two molecules (tetraethylene glycol and 1,12-dodecanediol) were investigated as candidate molecules to be used as the DNA interstrand linker. The interstrand linker was to be incorporated at a specific site into an oligonucleotide during automated DNA synthesis. Automated DNA synthesis occurs *via* successive addition of phosphoramidites, therefore the candidate linker molecules need to be converted into phosphoramidites. Both tetraethylene glycol and 1,12-dodecanediol can be readily converted by reaction with dimethoxytrityl chloride followed by 2-cyanoethyl N,N-disopropylchlorophosphoramidite (see experimental) to produce phosphoramidites.

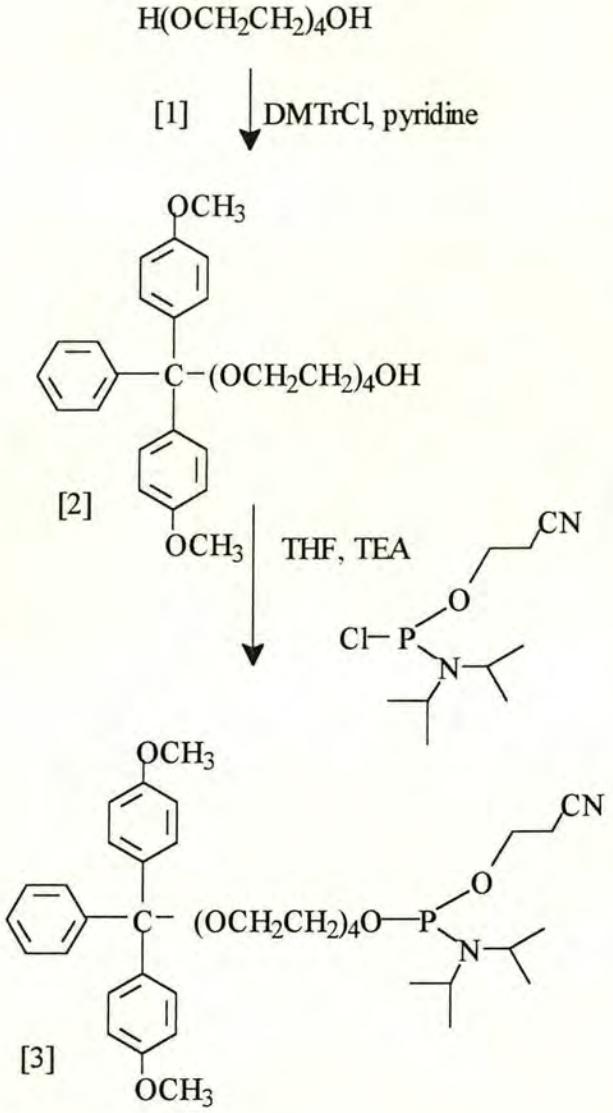
The purification of the products for the conversion 1,2-dodecanediol to a phosphoramidite proved more difficult when compared to that for tetraethylene glycol. Therefore, tetraethylene glycol was chosen as the linker molecule. Fig 16 shows the synthesis of the tetraethylene glycol monomer, for solid phase oligonucleotide synthesis, compound [3].

2.3.1.2 Coupling Efficiency during Oligonucleotide Synthesis

The coupling efficiency of the tetraethylene glycol phosphoramidite during solid phase synthesis, as measured by the amount of trityl cation release (measured at 494nm), was 80%. This coupling efficiency was low when compared to that observed for the normal (A, C, G and T) DNA phosphoramidite synthesis monomers (coupling efficiency >98%).

The time allowed for phosphoramidite coupling to the 5' end of the oligonucleotide chain is known as the wait step. For normal DNA phosphoramidite synthesis monomers this wait step was 30 seconds. The coupling efficiency for the tetraethylene glycol

FIGURE 16. SYNTHESIS OF TETRAETHYLENE GLYCO MONOMER FOR DNA SYNTHESIS



DMTrCl - Dimethoxytrityl chloride
THF - Tetrahydrofuran
TEA - Triethylamine

monomer was improved to greater than 94% when an extended wait step of 3 minutes was carried out for its addition. The chemistry for solid phase synthesis has been optimised for the use of normal phosphoramidite bases. The use of extended wait procedures is not uncommon practise where unusual phosphoramidite additions are being carried out.

During automated DNA synthesis it was essential that each incoming monomer couples to the immobilised oligonucleotide with high efficiency. If this does not occur the overall yield will be very low. Ideally 100% coupling is desirable, in practise >98% was acceptable. For one addition of an unusual monomer a reduced coupling efficiency can be tolerated. All of the normal bases added prior to or after compound [3] coupled at >98%. Compound [3] was stored at -20°C under nitrogen prior to use, and was stable for at least 3 months.

Three other non-nucleotide monomer phosphoramidites were used in this project. The coupling efficiency of the three monomers in solid phase synthesis was high. Amino Link 2 monomer (see figure 17) coupled at >98%, the Biotin monomer (see figure 18) coupled at >97%, and the Dinitrophenyl monomer (see figure 19) coupled at >95%. The use of these three monomers will be discussed later.

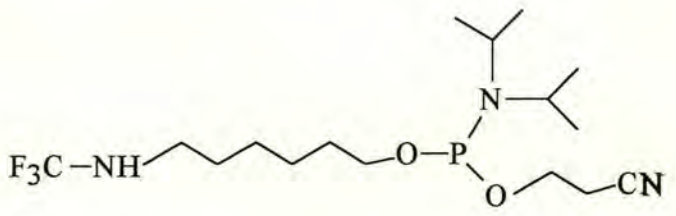


FIGURE 17. AMINO LINK 2 PHOSPHORAMIDITE

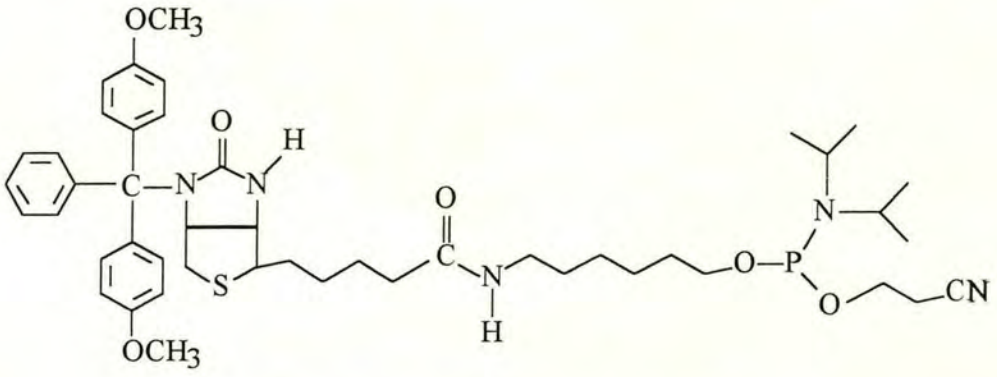


FIGURE 18 BIOTIN PHOSPHORAMIDITE

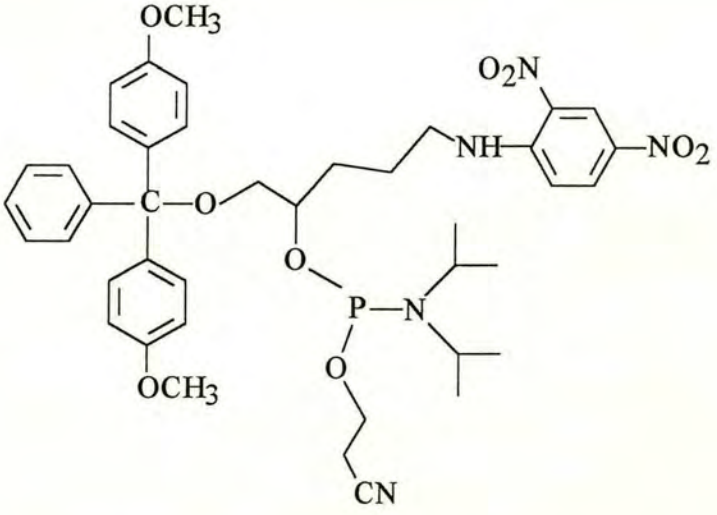
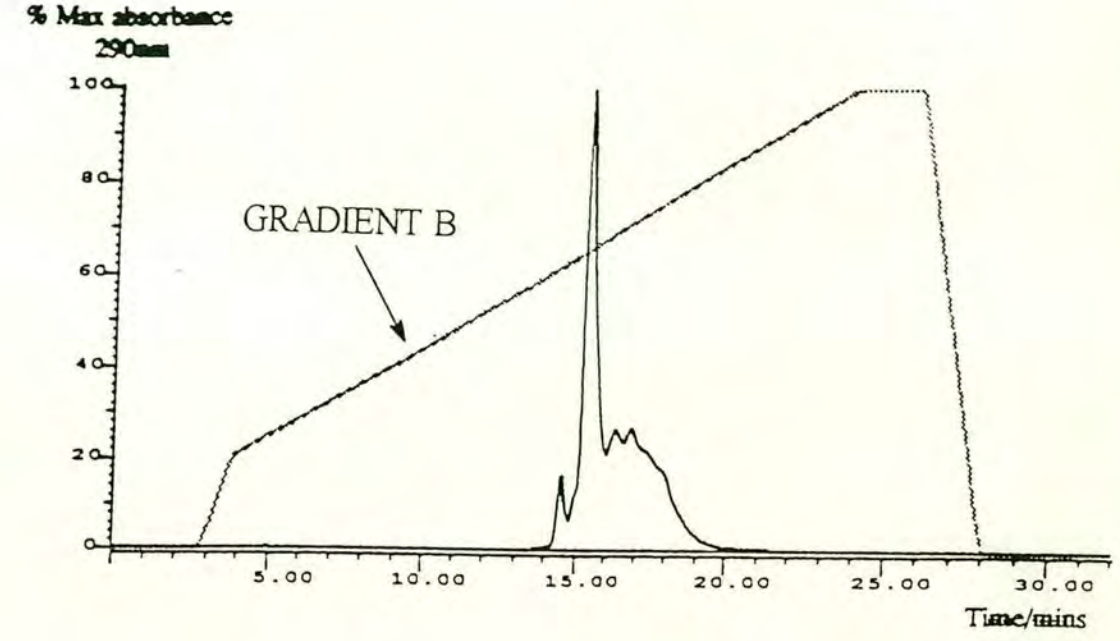


FIGURE 19. DINITROPHENYL MONOMER

2.3.2 Purification of DNA Sequences

All of the phosphoramidite monomers used contain a 5'-4,4-dimethoxytrityl group. After addition of each nucleotide this group remains attached to the oligonucleotide. The group was removed, under acidic conditions, to allow the next nucleotide to react. This group is very hydrophobic and thus DNA sequences with this group attached are retained by reverse phase HPLC columns relative to DNA sequences without this group. Oligonucleotide purification by reverse phase HPLC was thus facilitated by synthesising sequences and leaving the 5' terminal trityl group attached. All failure sequences will have had this group removed prior to the terminal nucleotide addition. Therefore, only the full length oligonucleotide, the desired product, will contain a terminal 5' 4,4-dimethoxytrityl group.

Apart from the cytosine 100mer phosphorothioate (C100PS) all sequences were purified by both reverse phase HPLC and gel filtration. C100PS is to be used as an immunogen to raise anti-cytosine antibodies that may also interact with mismatches containing cytosine. All C100PS failure sequences will contain only cytosine bases and thus HPLC purification was unnecessary. Figure 20 shows a typical HPLC purification of an oligonucleotide.



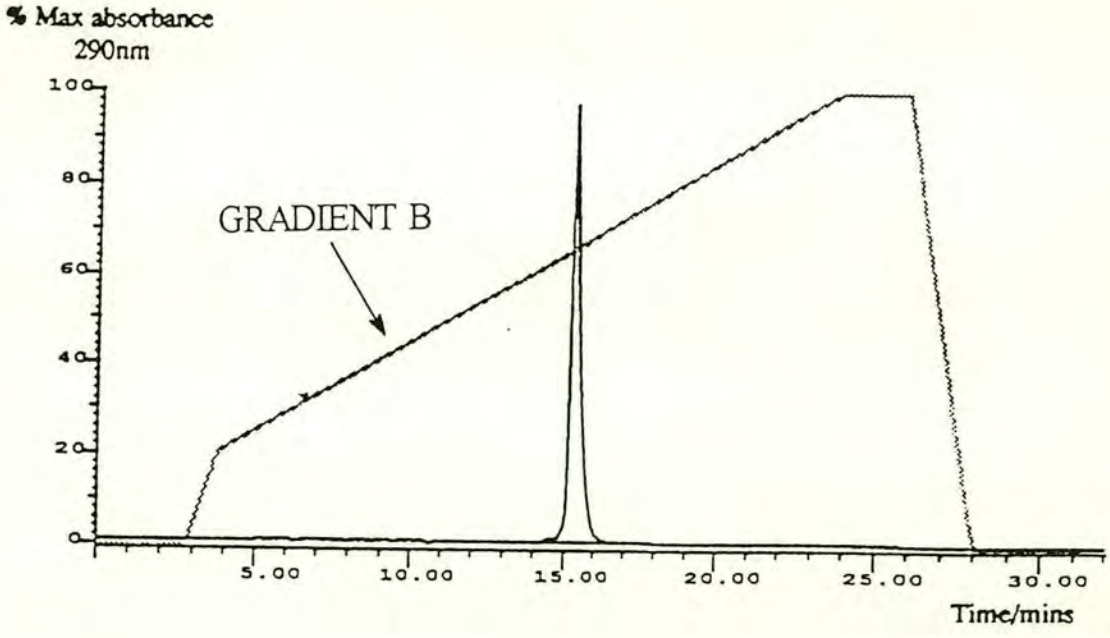


FIGURE 20. HPLC PURIFICATION AND ANALTICAL HPLC OF COLLECTED PEAK FOR A12XT12

2.3.3 Oligonucleotide Sequences Produced.

Oligonucleotides were synthesised for three main purposes :

[*A*] To establish the thermal stability of covalently linked duplexes.

[*B*] The production of sequences for immunisation.

[*C*] The production of sequences to establish screening procedures.

2.3.3.1 12mer Duplexes for Thermal Stability Studies

A series of short oligonucleotides were synthesised to investigate the effect on thermal stability of duplexes linked *via* the tetraethylene glycol monomer, compared to duplexes formed by the association of independent complementary strands. Sequences were produced such that they were fully complementary, or contained a central base pair mismatch, see Table 2. An additional sequence was produced incorporating two consecutive additions of the tetraethylene glycol monomer. This was to confirm that the linker was of an optimum length for duplex formation.

Table 2, Oligonucleotides for Thermal Stability Study

NAME	SEQUENCE
A12	$A_5\underline{\mathbf{A}}A_6$
T12	$T_6\underline{\mathbf{T}}T_5$
T6AT5	$T_6\underline{\mathbf{A}}T_5$
AT12	$A_5\underline{\mathbf{A}}A_6$-X-$T_6\underline{\mathbf{T}}T_5$
AA12	$A_5\underline{\mathbf{A}}A_6$-X-$T_6\underline{\mathbf{A}}T_5$
AT2X	A_{12}-X-X-T_{12}

Where X is the tetraethylene glycol linker, $-(CH_2CH_2O)_4O-$.

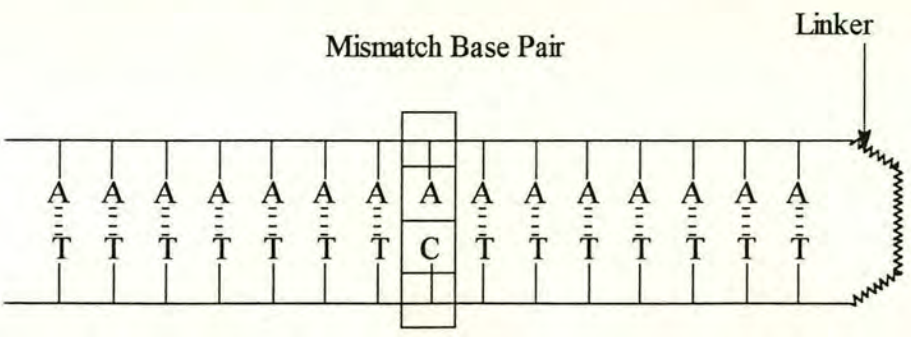
2.3.3.2 15mer DNA Duplexes

15mer DNA duplexes were synthesised to carry out immunisation experiments with, see table 3. The first five DNA sequences AT15-CC15 are 15mer duplexes, stabilised with an inter strand linker, and the duplexes contain a central matched base pair AT or mismatched base pair AA, AC, GG or CC, see figure 21. DNA sequences AA15P-CC15P were similar except the sequences were "complete phosphorothioate oligonucleotides".

Table 3, 15mer Duplexes for Immunisation

NAME	SEQUENCE
AT15	A_7**A**A_7-X-T_7**T**T_7
AA15	A_7**A**A_7-X-T_7**A**T_7
AC15	A_7**A**A_7-X-T_7**C**T_7
GG15	A_7**G**A_7-X-T_7**G**T_7
CC15	A_7**C**A_7-X-T_7**C**T_7
AA15P	A_7**A**A_7-X-T_7**A**T_7
AC15P	A_7**A**A_7-X-T_7**C**T_7
GG15P	A_7**G**A_7-X-T_7**G**T_7
CC15P	A_7**C**A_7-X-T_7**C**T_7

FIGURE 21. 15mer DUPLEX FOR IMMUNIZATIONS

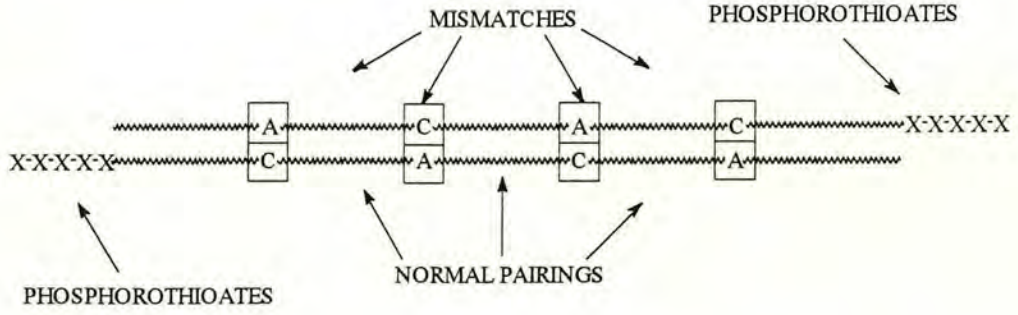


2.3.3.3 75mer DNA Duplexes

Two 80mer single stranded DNA sequences were synthesised such that on hybridisation a 75mer duplex containing normal nucleotides would form. The nucleotides in the duplex formed were predominantly A and T, but contained 4 evenly spaced **AC** mismatched base pairs. The 3'-termini of each oligonucleotide overhangs the duplex by five nucleotides and all of these were thymine phosphorothioates, see figure 22.

Two 80mer single strands were synthesised as above incorporating 4 evenly spaced **GT** mismatched base pairs. For this duplex the five overhanging 3'-thymine phosphorothioate nucleotides were replaced with adenine phosphorothioates. The duplexes, named AC75 and GT75 respectively, were used to carry out immunisation experiments.

FIGURE 22. 75mer DUPLEXES FOR IMMUNIZATIONS



Additionally 80mer single strands were synthesised as above, except that on hybridisation the sites for the mismatched base pairs were replaced with **GC** paired nucleotides. This was to be used as control DNA during screening experiments for anti-mismatched base pair antibodies. It was thought that the presence of single stranded regions, that contained one of the bases present in the mismatch of interest should be avoided. When screening for specific mismatch antibodies they may give rise to false positives. Therefore, two types of duplex containing GC matched pairs were produced. To screen

against antibodies for AC mismatched base pairs the 3'-terminal phosphorothioate nucleotides were T's, and for GT mismatched base pairs they were A's, named GC75T and GC75A respectively.

2.3.3.4 C100PS Single Stranded Oligonucleotide

A complete phosphorothioate oligonucleotide containing 100 cytosines (C100PS) was synthesised to carry out immunisation experiments. This single stranded sequence will not directly be able to elicit an immune response such that mismatched base pairs are recognised. However, it may produce antibodies to single strands containing cytosine. It was hoped that mismatched base pairs containing cytosine may be sufficiently displaced from the duplex that some recognition by anti-polycytosine antibodies may occur.

2.3.4 Additional Oligonucleotide Sequences Produced

A series of additional oligonucleotides were synthesised as the project developed. These were generally produced in response to the development of screening techniques. These modified oligonucleotides will be briefly described here. Their role in assay development will be elaborated upon in the relevant sections of chapter three.

2.3.4.1 Single Strands and Biotin Labelled Oligonucleotides

A variety of single stranded oligonucleotide sequences were synthesised, see Table 4, to develop one of the immunoassays to screen for antibodies specific for mismatched base pairs. The assay was based on the binding of a biotin end labelled single stranded oligonucleotide to streptavidin, pre-immobilised on a solid support. A fully complementary oligonucleotide strand (second strand), or one that would form a single central mismatched base pair, would then be allowed to hybridise to the immobilised strand to form a duplex.

Table 4, Biotin-Oligonucleotides for Immobilisation

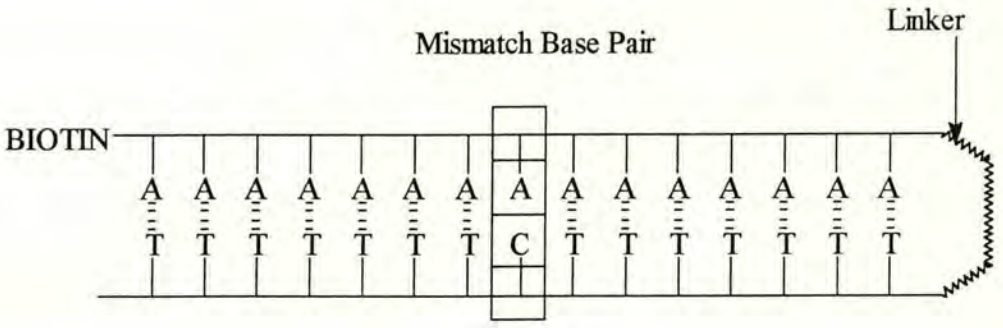
NAME	SEQUENCE
TTT	$T_{10}\underline{\mathbf{T}}T_{10}$
TCT	$T_{10}\underline{\mathbf{C}}T_{10}$
TAT	$T_{10}\underline{\mathbf{A}}T_{10}$
AAAB	$A_{10}\underline{\mathbf{A}}A_{10}$-Biotin
TTT-DNP	$T_{10}\underline{\mathbf{T}}T_{10}$-Dinitrophenyl
TCT-DNP	$T_{10}\underline{\mathbf{C}}T_{10}$-Dinitrophenyl
TAT-DNP	$T_{10}\underline{\mathbf{A}}T_{10}$-Dinitrophenyl
AC15B	Biotin-$A_7\underline{\mathbf{A}}A_7$-X-$T_7\underline{\mathbf{C}}T_7$
C20B	C_{20}-Biotin

To ensure equivalent duplex formation occurred, for fully matched and mismatched duplexes, and was maintained through the time course and conditions of the immunoassay, a method to quantitate duplex formation was required. It was decided that labelling the second strand with 2,4-dinitrophenyl (DNP) would allow us to compare duplex formation for fully matched and mismatched sequences. The amount of DNP labelled solid phase duplex formed could be measured using an anti-DNP antibody.

A series of DNP end labelled second strands were synthesised such that duplexes would either be fully matched or contain a single base pair mismatch. The DNP monomer is a non-nucleoside phosphoramidite produced by a colleague in Edinburgh (Grzybowski *et al.*, 1993). The DNP labelled oligonucleotide was synthesised such that on hybridisation to the immobilised biotin labelled oligonucleotide the biotin and DNP groups will be at opposite ends of the duplex. Therefore, the detection of the DNP group will not be sterically hindered by its proximity to the solid phase. Additionally, the hybridisation of the second strand to the support may also be hindered, by the presence of the DNP group, if the DNP group was positioned at the site adjacent to the solid phase.

As an alternative method to immobilise duplex oligonucleotides to a solid phase a sequence was produced as a 15mer copy of AC15 (used for immunisation experiments) with the addition of a 5'-terminal biotin group, see figure 23. A cytosine 20mer was also synthesised end labelled with biotin. This was attached to solid phases as above to screen C100P immunisations.

FIGURE 23. BIOTIN LABELLED 15mer DUPLEX



2.3.4.2 Extended Biotin-Linker Labelled Oligonucleotides

A series of single stranded and duplex (*via* tetraethylene glycol linker) oligonucleotides were synthesised, see Table 5. They were end labelled to biotin directly (as previously described), or linked *via* an additional tetraethylene glycol monomer. The additional linker serves to extend the distance between the biotin group and oligonucleotide. An additional series of oligonucleotides were synthesised, incorporating a dinitrophenyl group as well as the biotin group. This provides a readily detectable label to establish oligonucleotide duplex binding to immobilised streptavidin, as previously described. The DNP group was incorporated into the oligonucleotide such that it forms an integral part of the interstrand linker, thus the DNP group and the biotin group are at opposite ends of the duplex.

Table 5, Biotin-Linker Oligonucleotides

NAME	SEQUENCE
AT10B	A_{10}-DNP-X-T_{10}-Biotin
AT10BL	A_{10}-DNP-X-T_{10}-X-Biotin
C10B	DNP-C_{10}-Biotin
C10BL	DNP-C_{10}-X-Biotin
C11B	DNP-C_{11}-Biotin
AC30BL	A_{10}**A**A_{10}**A**A_{10}-X-T_{10}**C**T_{10}**C**T_{10}-X-Biotin
GT30BL	A_{10}**G**A_{10}**G**A_{10}-X-T_{10}**T**T_{10}**T**T_{10}-X-Biotin
C20BL	C20-X-Biotin

Where DNP is Dinitrophenyl.

2.3.5 UV Melting Curve Determinations

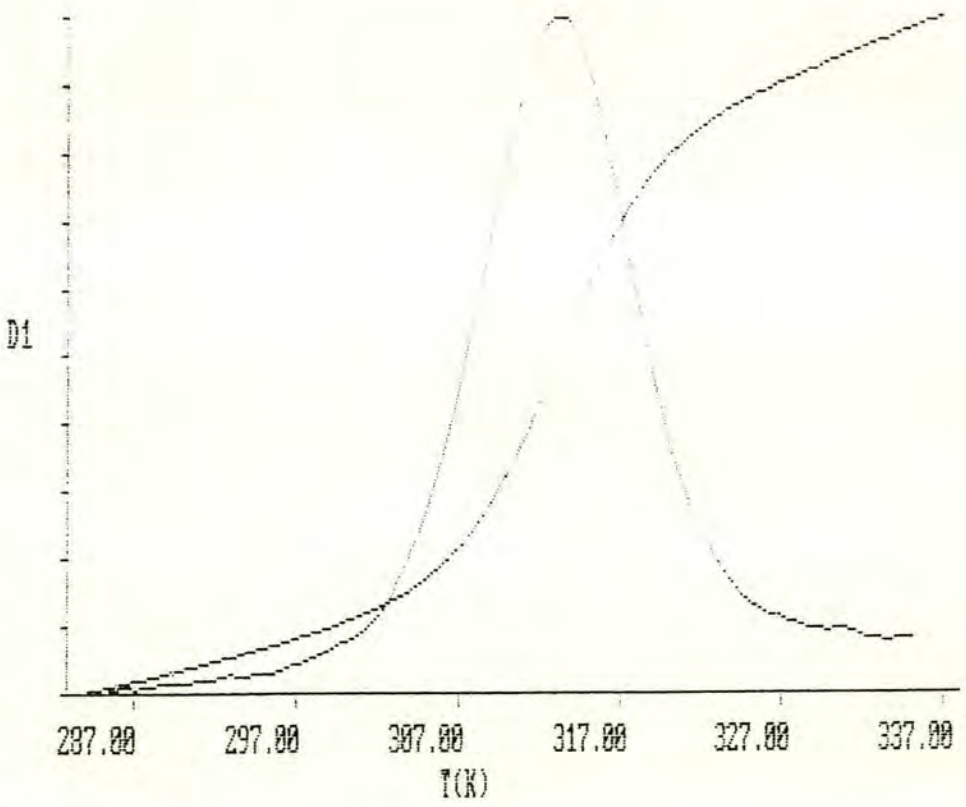
The thermal dissociation of the strands in a double helix is called melting because it occurs abruptly at a specific temperature. The melting temperature (Tm) is defined as the temperature at which half of the helical structure is lost. The melting of DNA was monitored by measuring the increased absorbance, hyperchromism, caused by the unstacking of the base pairs as the duplex dissociates. The absorbance was monitored using a UV light source at 264nm. The Tm values presented are the mean value, of triplicates, obtained by UV melting experiments. Tm values in all cases are +/- 0.5°C or less.

2.3.5.1 12mer Duplexes

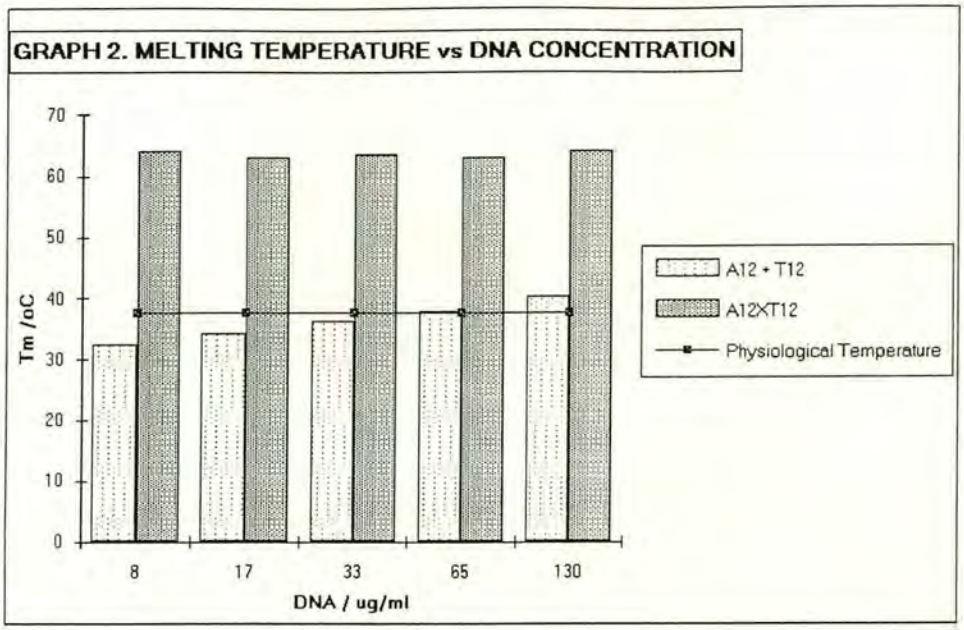
Concentration dependent UV melting curve determinations were carried out to compare the thermal stability of 12mer duplexes. The duplexes were formed by hybridisation of separate single stranded oligonucleotides and a self complementary sequence covalently linked, *via* a central tetraethylene glycol, at one end. The thermal stability of fully matched duplexes and those containing a central base pair mismatch were studied.

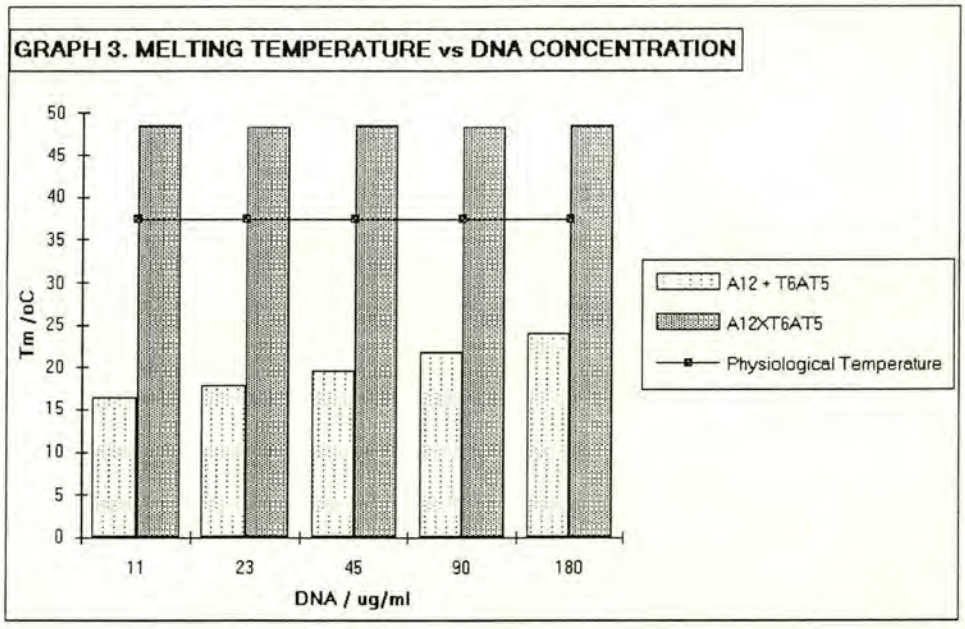
Graph 1 shows the results of typical UV melting curve, and derivative of the curve for a 12mer duplex produced by combining A12 and T12. The characteristic DNA melting curves were obtained for all DNA concentrations tested. The temperature at the maximum point of the first derivative of the melting curve was the melting temperature (Tm).

GRAPH 1 UV MELTING CURVE OF A12 + T12



The Tm's of fully matched, graph 2, and mismatch containing, graph 3, duplexes (formed by separate single stranded oligonucleotides and a self complementary sequence (described above) were measured at several DNA concentrations.





For all of the DNA concentrations tested the Tm of sequences was increased if the strands were covalently linked with tetraethylene glycol, see Graph 2 and 3. In the absence of the linker the Tm of the sequences (fully matched or containing a central mismatched base pair) decreases by about 2°C as the concentration of the DNA is halved. The tetraethylene glycol linker greatly increased the melting

temperature of the duplex with and without the mismatch. It would be possible to envisage the tetraethylene glycol linked single strands forming a larger duplex *via* hybridisation of two separate self complementary strands. However, for both matched and single mismatched base pair containing sequences the melting temperature was found to be independent of DNA concentration. Thermal melting of duplexes formed by independent self complementary DNA strands is concentration dependent. Therefore, as expected the linker was creating a duplex formed by a single strand forming a hairpin loop.

It was essential that the duplex produced was not only formed *via* a hairpin loop, but also that the hairpin loop allowed all base pairing to occur. If the linker was of insufficient length then some of the nucleotides would be involved in forming the hairpin loop and thus exist as short single strand regions, either side of the linker molecule. This would slightly reduce the thermal stability, but more importantly would provide an extra, unwanted, form of DNA. These short single stranded regions could significantly influence the immune response during immunisation experiments. This situation would be unacceptable for our purposes.

Concentration dependant UV melting studies on the 12mer duplex AT2X (incorporating two tetraethylene glycol linkers) were undertaken to investigate the length of the linker. The Tm of AT2X was equivalent to that of AT12 (the same duplex with only one linker) at all concentrations tested (8, 17, 33, 65, 130 μg/ml). If the Tm for AT2X was higher than AT12 this would indicate that AT2X was forming a more stable duplex. Therefore, this study clearly indicates that the linker molecule was of optimum length for the stabilisation of oligonucleotide duplexes.

Duplexes produced by the hybridisation of short independent oligonucleotides, such that the duplex contained a mismatched base pair, will be unsuitable for immunisation. Synthetic DNA duplexes will be used for immunisation experiments at a concentration < 1000μg/ml. Diffusion of the duplexes during the immunisation regime will further decrease the concentration of the duplexes. The

Tm of 1000μg/ml of a 12mer, containing a central base pair mismatch, could very roughly be estimated by extrapolation (based on the observation on page 64) of the results presented in graph 3. The Tm at 1000μg/ml would be about 28.7°C by this process. The DNA will thus remain fully dissociated during the immunisation as its Tm will be well below physiological temperature. The concentration of this duplex with a Tm at physiological temperature would be about 20mg/ml.

The incorporation of the linker has not only increased the Tm to above physiological temperature it also means that the sequence will remain as a duplex even at high dilution. Although the Tm of stabilised duplexes for perfectly matched 12 mer duplexes is very high, the Tm of mismatch containing sequences is only about 10°C above physiological temperature. The Tm is the temperature at which the half of the duplexes will be dissociated. It is important that majority of the DNA is in the form of a duplex. Melting of sequences occurs over a 15-20°C range, about 10°C either side of the Tm. The use of slightly longer sequences should increase the duplex stability, such that a Tm > 50°C will be obtained.

It was decided to synthesise a series of 15mer duplexes of a similar nature to the tetraethylene glycol stabilised 12mer duplexes, described above, to be used in immunisations experiments.

2.3.5.2 15mer Duplexes

UV melting studies were carried out on 15mer duplexes containing a variety of mismatched base pairs, stabilised by the tetraethylene glycol linker, see Table 6. Concentration dependent studies were not carried out on the 15mer duplexes as the thermal stability of hairpin loop duplexes has been previously demonstrated to be independent of the DNA concentration.

Table 6, UV Melting of 15mer Duplexes.

SEQUENCE NAME	Tm / ^{0}C
AT15	>75
AA15	51.0
AC15	50.0
GG15	53.0
CC15	44.0
AA15P	18.5
AC15P	27.0
GG15P	17.5
CC15P	20.0
AC15B	50.0

All the normal oligonucleotide duplexes containing a mismatched base pair have a melting temperature well above physiological temperature. These sequences should remain as duplexes during immunisation experiments.

The Tm's for complete phosphorothioate 15mer duplexes, are all below physiological temperature. This will cause the sequences to dissociate into single stranded oligonucleotides after immunisations. These sequences will not present a mismatched base pair within a duplex to the immune system and so were unsuitable for immunisation experiments. Phosphorothioates contain a sulphur atom in the phosphate backbone of DNA instead of one of the oxygen atoms. Sulphur atoms are bulkier than oxygen atoms. The decrease in Tm may reflect constriction of duplex formation caused by all the phosphate linkages being replaced with bulkier phosphorothioates. Further studies could investigate the use of phosphorothioate linkages dispersed amongst normal phosphate linkages within a duplex, such that thermal and enzymic stability are combined.

2.3.5.3 75mer DNA Duplexes

The melting temperatures for the 75mer duplexes was high, see Table 7. These longer sequences were not chemically stabilised by an inter strand linker. Although AC75 and GC75 contained four instead of one mismatched base pair, the greater number (71) of Watson-Crick base pairs affords reasonable duplex stability to these sequences. The thermal stability of DNA duplexes of this length were independent of concentration and they will therefore remain hybridised even at high dilution. The five phosphorothioate bases should reduce nuclease degradation of these DNA duplexes during immunisation experiments. The Tm of the two fully complementary strands, GC75T and GC75A are equivalent and slightly higher than mismatched sequences, as expected..

Table 7. UV Melting of 75mer Duplexes

NAME	Tm / ^{o}C
AC75	54.0
GT75	56.5
CC75	52.2
GC75T	64.8
GC75A	64.5

2.3.5.4 Biotin Labelled Duplexes

AT10B and AT10LB are tetraethylene glycol stabilised 10mer duplexes. They were synthesised such that biotin was directly attached or attached *via* a 13 atom spacer arm at the free 5'-terminal of the duplex. They were produced to investigate possible steric hindrance, for biotin/streptavidin interaction caused by the close proximity of the bulky duplex DNA to the biotin molecule.

The tetraethylene glycol linker for these duplexes was extended by the additional adjacent incorporation of the DNP monomer. The incorporation of this monomer simply creates a larger hairpin loop, and the thermal stability of the duplex is still high, see Table 8, as seen for AT2X. These duplexes should remain fully associated over the time course of the assay.

Table 8. UV Melting of Biotin Linker Duplexes

NAME	Tm / ^{o}C
AT10B	60.5
AT10BL	60.5
AC30	65.1
GT30	68.4

The Tm's of the two 30mer duplexes was higher than physiological temperature, thus, during the immunoassay for screening anti-DNA antibody production the duplex will remain associated. They also each provide two mismatched base pair binding sites for antibody recognition, compared to AC15B which contained only one.

2.4 OLIGONUCLEOTIDE PROTEIN COMPLEXES

The 15mer duplexes containing mismatched base pairs were to be used for immunisation experiments. These duplexes have a relatively low molecular weight, <10,000, compared to the type of proteins that are commonly used as immunogens, molecular weight usually >50,000. Because of this, and the inherently low immunogenicity it was decided to investigate the conjugation of the duplexes to large proteins. Compounds of low molecular weight to be used to evoke an immune response (haptens) are routinely bound to large "carrier proteins" to aid their immunogenicity.

DNA sequences are degraded by serum nucleases if injected into animals. The tetraethylene glycol linked terminus may afford some protection as it presents an unusual structure to the nuclease enzymes. If the non-linked terminus of the duplex is attached to a carrier protein then it will be shielded from direct attack of serum nucleases. The proximity of the protein to the whole oligonucleotide duplex should also afford some nuclease protection to the sequence by sterically hindering the approach of serum nuclease enzyme. Thus conjugation may aid immunisation by increasing the size of the immunogen and the serum half life of the oligonucleotides.

Antibody production from immunisation / fusion experiments is generally detected by means of an enzyme linked immunosorbent assay (ELISA). This requires adsorption of antigenic material to plastic microtitre plates. In general, proteins bind well to these solid phases, whereas binding of duplex DNA is limited. Therefore, the production of oligonucleotide/protein conjugates could also be used to enhance the binding of the duplexes to the microtitre plates for screening of antibody production.

Heterobifunctional linkers were used to conjugate oligonucleotides to proteins. These methods involved the synthesis 5'- amino linked oligonucleotides, Fig. 17. The reactions included the use of disuccinimidyl suberate (DSS) and 3-maleimidobenzoyl-N-hydroxy succinimide ester (MBS). DSS links amino modified oligonucleotides to primary amine sites, e.g. lysine residues, in proteins. MBS links through a primary amine at one end and thiol at the other. To obtain a stable thiol modified oligonucleotide, amino modified oligonucleotides were reacted with N-succinimidyl-3-(2 pyridyldithio) propionate. The proteins free primary amines are functionalised using MBS. The stable thiol modified oligonucleotide can then be reduced, with dithiothreitol, to give a sulphydryl group just prior to reaction with an MBS modified protein.

A variety of purification methods were investigated to try to separate the products of the conjugation reactions. HPLC methods were unsuccessful, and gel filtration was attempted. Using sephacryl S-400 no separation of protein and oligonucleotides was observed.

Sephadex G50 separated the oligonucleotide from protein, however, we were never confident that the protein/oligonucleotide peak obtained was specific conjugation products or simply a non-covalent protein/oligonucleotide complex. To dissociate possible protein/oligonucleotide complexes, increased salt concentration in the gel filtration elution buffers were used. However, the increase in salt concentration affected the separation of the reaction products. Thus we were unable to confirm specific conjugation had occurred.

Nucleic acid immunisations are often carried out as complexes formed by mixing with MeBSA. It was decided to abandon the specific conjugation procedures and use nucleic acid/MeBSA complexes. These complexes should also provide protection from serum nuclease enzymes during immunisation experiments. For larger oligonucleotides, 75mer duplexes and C100PS single strand, the greater molecular weight should obviate the need for a carrier protein. The addition of phosphorothioate nucleotides for all of the larger oligonucleotides should provide nuclease resistance.

2.5 EXPERIMENTAL

General Reagents

All solvents were of analytical grade. All reagents, specified laboratory grade, were supplied by FSA Laboratories Supplies unless otherwise stated. Tetraethylene glycol and dimethoxytrityl chloride were supplied by Aldrich Chemicals Co. Ltd., 2-cyanoethyl N,N-diisopropylchloro phosphoramidite was supplied by Fluka. Methyl ester of Bovine Serum Albumin (MeBSA), Tris (Tris [hydroxymethyl) aminomethane) base and Tris HCl were supplied by Sigma Chemicals Company. Acetonitrile (HPLC grade) was supplied by ABI, ethylenediaminetetraacetic Acid (EDTA) disodium salt, BDH Ltd., toluene p-sulphonic acid (AR) by May and Baker Ltd., and chloroform-d by Goss Scientific Instruments Ltd.

Anhydrous Reagents

Anhydrous pyridine was obtained by distillation from potassium hydroxide. Anhydrous tetrahydrofuran was obtained by distillation from sodium-benzophenone. Anhydrous dichloromethane was obtained by distillation from calcium hydride. Anhydrous triethylamine was obtained by distillation from calcium hydride.

NMR

^{1}H-NMR spectra were obtained using a Brucker WP-200 spectrometer (200.13 MHz). ^{13}C-NMR spectra were obtained using a Brucker WP-200 spectrometer (50.32 MHz). ^{31}P-NMR spectra were obtained using an FX-90Q spectrometer (81 MHz).

Mass Spectra

Mass spectra were obtained using a Kratos MS5OTC spectrometer.

Chromatography

Flash column chromatography was carried out using silica gel 60 (220-440 mesh ASTM), (Fluka). Thin layer chromatography (TLC) was carried out on aluminium sheets, silica 60 F_{256}, 0.2mm (Merck). Six solvent systems were used for TLC (each contained 1% triethylamine).

Solvent Systems for TLC

SOLVENT	MOBILE PHASE	Ratio (v/v)
A	Dichloromethane : Ethyl acetate	1:1
B	Dichloromethane : Ethyl acetate	9:1
C	Dichloromethane : Methanol	95:1
D	Ethyl acetate	
E	Ethyl acetate : Diethyl ether	1:1
F	Ethyl acetate : Toluene	7:3

Synthesis of Compound [2]. 11-(4,4'-Dimethoxytrityloxy)-3,6,9-trioxa-undecan-1-ol.

Dimethoxytrityl chloride (1.0eq., 9.72g, 28.7mmol), dissolved in anhydrous pyridine (40 mls), was added dropwise to tetraethylene glycol (5.0eq., 27.90g, 144mmol) which had previously been dried by co-evaporation with anhydrous pyridine (2x50mls). The solution was allowed to stir at room temperature for two hours. Dichloromethane (175 mls) was added to quench the reaction and the organic phase extracted with 5% aqueous sodium bicarbonate (2x100 mls). The organic phase was dried over sodium sulphate, filtered, then evaporated in vacuo. The residue was then dissolved in minimum solvent B and purified, by flash column chromatography on a silica gel column, to give an oil (8.713g, 17.5mmol, 61%). R_F: 0.18 (solvent A); 0.15 (solvent B); 0.31 (solvent C). FAB ms $(M+H)^+$ m/z 496. NMR data δ_H ($CDCl_3$) 2.82-2.95 (1H, brs, OH), 3.21-3.26 (2H, t, $CH_2\underline{CH_2}OC(Ar)_3$, J=5.2Hz), 3.55-3.71 (14H, m, [$(C\underline{H_2}C\underline{H_2}O)_3$ and $(OC\underline{H_2}CH_2O(Ar)_3)$]), 3.71 (6H, s, OCH_3), 6.79-6.85 (5H, m, aryl), 7.18-7.49 (8H, m, Ar-0-); δ_c($CDCL_3$) 54.94, 61.46, 62.90, 70.15, 70.50, 72.30, 76.25, 76.89, 77.52, 85.70, 112.80, 126.42, 127.51, 127.96, 129.83, 136.07, 144.85, 158.13.

Synthesis of Compound [3] (Tetraethylene glycol monomer). 2-Cyanoethyl-[11-(4,4'-dimethoxytrityloxy)-3,6,9-trioxa-undecan-1-yl] N,N-diisopropylphosphoramidite.

To a stirring solution of [2] (1.0eq., 1.592g, 3.2mmol, which had previously been dried by coevaporation with anhydrous tetrahydrofuran (10 mls)) in 20 mls of anhydrous tetrahydrofuran was added diisopropylethylamine (4.0eq., 1.18g, 1.59 mls, 9.1mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (1.5eq.,1.14g, 1.07 mls, 4.8mmol). The reaction was quenched after 30 minutes with ethyl acetate (50 mls). The organic phase was extracted with 10% aqueous sodium bicarbonate (2x80 mls) followed by saturated aqueous sodium chloride (50 mls). The organic phase was dried over sodium sulphate, filtered and evaporated in vacuo. The residue was then dissolved in solvent D and purified by flash column chromatography. The appropriate fractions were then precipitated from excess hexane at -78°C. The product was obtained as an oil (1.20g, 1.72mmol, 54%). R_F: 0.68 (solvent D); 0.64 (solvent E); 0.53 (solvent F). ^{31}P-NMR data ($CDCl_3$) : 148.751ppm. FAB ms $(M+H)^+$ m/z 697. NMR data: δ_H ($CDCl_3$) 1.14-1.19 (m, 14, $N[C\underline{H}(C\underline{H}_3)_2]_2$), 2.56-2.63 (2H, t, $OC\underline{H}_2CH_2CN$, J=6.5Hz), 3.18-3.24 (2H, t, $OCH_2C\underline{H}_2CN$, J=5.2 Hz), 3.62-3.70 (16H, m, $(C\underline{H}_2C\underline{H}_2O)4$), 3.77 (6H, s, $OC\underline{H}_3$), 6.79-7.84 (13H, m, aryl); δ_C ($CDCl_3$) 20.07, 20.20, 24.41, 24.49, 42.76, 43.01, 55.04, 58.17, 58.54, 62.24, 62.97, 70.57, 70.99, 71.13, 76.29, 76.93, 77.56, 85.76, 112.87, 117.62, 126.49, 127.59, 128.04, 129.90, 136.16, 144.93, 158.21.

Oligonucleotide Synthesis

Synthesis of oligonucleotide sequences was carried out by the solid phase phosphoramidite method, using Applied Biosystems ABI 380B DNA synthesisers. Cyanoethyl phosphoramidite monomers (Adenine (A), Cytosine (C), Guanine(G), Thymine (T)) for DNA synthesis were supplied by ABI, and were used as 0.1M solutions in DNA synthesis grade acetonitrile (Applied Biosystems). The tetraethylene glycol monomer, compound [3], was a non-nucleoside cyanoethyl phosphoramidite, and was used as a 0.2M solution in

anhydrous dichloromethane. The biotin monomer was a non-nucleoside cyanoethyl phosphoramidite (Cambridge Research Biochemicals), the Aminolink-2 phosphoramidite was supplied by ABI, both were used as normal phosphoramidite monomers. The dinitrophenyl (DNP) monomer (Grzybowski *et al.*, 1993) a non-nucleoside cyanoethyl phosphoramidite (2-cyanoethyl [1-(4,4'-dimethoxytrityloxy)-4-oxa-6-(2,4-dinitrophenylamino) hept-2-yl] N,N-diisopropylamino phosphoramidite). It was used as normal DNA synthesis monomers, and was kindly donated by Mr. John Grzybowski.

Normal cyanoethyl phosphoramidite monomers for DNA synthesis are coupled for 30 seconds during which they react with the terminal end of the oligonucleotide. The tetraethylene glycol and DNP monomers required an extended wait step of 3 minutes during its addition to the oligonucleotide. All sequences were deprotected in 33% ammonia at 55°C for 5hours), they then underwent gel filtration on NAP-10 columns pre-equilibrated with distilled water. All sequences (except aminolink-2) were synthesised leaving the 5'-dimethoxytrityl group attached to aid purification by reverse phase HPLC (see below). All aqueous solutions and buffers were prepared using distilled water and filtered through 0.45μm Sartolon (nylon 66) filters (Sartorius GmbH).

Reversed Phase HPLC

Column - Brownlee Aquapore Reverse-Phase Octyl (C8)

Dimensions	: 25cm x 10mm
Loop volume	: 1ml
Buffer A	: 0.1M ammonium acetate pH 7.0
Buffer B	: 0.1M ammonium acetate pH 7.0 + 50% acetonitrile
Flow rate	: 3ml/min.

Gradient:

Time/minutes	% Buffer A	% Buffer B
0:00	100	0
2:00	100	0
3:00	80	20
23:00	20	80
24:00	0	100
27:00	0	100
28:00	100	0
30:00	100	0

Detection: UV 264nm 1.0μM scale synthesis analytical
UV 290nm 1.0μM scale synthesis preparative.

Gel Filtration using NAP-10 Columns

NAP-10 columns (Pharmacia) are prepacked disposable columns containing Sephadex G25 Medium of DNA Grade. They are used for the removal of low molecular weight (<1000) compounds from nucleic acids and oligonucleotides (>10 nucleotides) in aqueous solution. Removal of low molecular weight compounds was carried out by equilibrating the column with 3x5ml of distilled water (or the desired buffer), under gravity. The nucleic acid/oligonucleotide sample was then loaded in 1ml of aqueous solution. 1.5ml of eluting solution (distilled water or buffer) was added and the eluant collected. If the column is pre-equilibrated with, and the loaded sample eluted with, a buffer solution other than the buffer solution originally containing the oligonucleotide then an effective buffer exchange occurs. Thus nucleic acids can be purified from low molecular weight contaminants, and transferred to an alternative buffer solution if required, rapidly.

Detritylation of Trityl-On Purified Sequences

After HPLC purification, solutions containing 'Trityl-On' oligonucleotide sequences were evaporated to dryness, and detritylated with 3% acetic acid (10mls). After 30 minutes they were evaporated to dryness and dissolved in appropriate buffer. Low molecular weight contaminations were removed by gel filtration using NAP-10 columns, as described above.

Oligonucleotide Sequences Produced.

Sequences for 12 mer DNA duplexes

Name	Sequence
AAA1	5'- AAA AAA AAA AAA -3'
TTT1	5'- TTT TTT TTT TTT -3'
TAT1	5'- TTT TTA TTT TTT -3'
AT12	5'- TTT TTT TTT TTT XAA AAA AAA AAA A -3'
AA12	5'- TTT TTA TTT TTT XAA AAA AAA AAA A -3'
AT2X	5'- TTT TTT TTT TTT XXA AAA AAA AAA AA-3'

15 mer DNA duplexes

Name	Sequence
AT15	5'- TTT TTT T**T**T TTT TTT XAA AAA AA**A** AAA AAA A -3'
AA15	5'- TTT TTT T**A**T TTT TTT XAA AAA AA**A** AAA AAA A -3'
AC15U	5'- TTT TTT T**C**T TTT TTT XAA AAA AA**A** AAA AAA A -3'
GG15	5'- TTT TTT T**G**T TTT TTT XAA AAA AA**G** AAA AAA A -3'

- CC15 5'- TTT TTT T**C**T TTT TTT XAA AAA AA**C** AAA AAA A -3'

- AA15P 5'- $T_sT_sT_s$ $T_sT_sT_s$ T_s**A**$_sT_s$ $T_sT_sT_s$ $T_sT_sT_s$ $X_sA_sA_s$ $A_sA_sA_s$ A_sA_s**A**$_s$ $A_sA_sA_s$ $A_sA_sA_s$ A -3'

- AC15P 5'- $T_sT_sT_s$ $T_sT_sT_s$ T_s**C**$_sT_s$ $T_sT_sT_s$ $T_sT_sT_s$ $X_sA_sA_s$ $A_sA_sA_s$ A_sA_s**A**$_s$ $A_sA_sA_s$ $A_sA_sA_s$ A -3'

- GG15P 5'- $T_sT_sT_s$ $T_sT_sT_s$ T_s**G**$_sT_s$ $T_sT_sT_s$ $T_sT_sT_s$ $X_sA_sA_s$ $A_sA_sA_s$ A_sA_s**G**$_s$ $A_sA_sA_s$ $A_sA_sA_s$ A -3'

- CC15P 5'- $T_sT_sT_s$ $T_sT_sT_s$ T_s**C**$_sT_s$ $T_sT_sT_s$ $T_sT_sT_s$ $X_sA_sA_s$ $A_sA_sA_s$ A_sA_s**C**$_s$ $A_sA_sA_s$ $A_sA_sA_s$ A -3'

- AC15B 5'- BTT TTT TT**C** TTT TTT TXA AAA AAA **A**AA AAA AA -3'

X = $(CH_2CH_2O)_4O$;
N = A, C, G, T, or X;
N_s = Phosphorothioate link.

75 mer Duplexes

Two 80mer single stranded DNA sequences were synthesised, shown below, such that on hybridisation a 75mer duplex, containing normal DNA bases, would form containing 4 evenly spaced AC mismatched base pairs. The 3'-termini of each end overhung the duplex by 5 bases, all of these were phosphorothioate bases. Two 80mers were synthesised as above incorporating 4 evenly spaced GT or CC mismatched base pairs. Sequences forming GC matched base pairs at the sites of the above mismatches were also synthesised.

Oligonucleotide 1

5'- TTA TTT ATT ATA TTT $\mathbf{X_1}$AA TAT AAA TTA TTA $\mathbf{X_2}$AT TAA TAA ATT ATA $\mathbf{X_3}$TA TAA ATT TAA TAA $\mathbf{X_4}$AT AAT TAA TAA TTT $P_SP_SP_S$ P_SP_S -3'.

Oligonucleotide 1

5'- AAA TTA TTA ATT AT$\mathbf{X_1}$ TTA TTA AAT TTA TA$\mathbf{X_2}$ TAT AAT TTA TTA AT$\mathbf{X_3}$ TAA TAA TTT ATA TT$\mathbf{X_4}$ AAA TAT AAT AAA TAA $P_SP_SP_S$ P_SP_S -3'.

For duplex forming :

AC75 mismatch	$\mathbf{X_1}$ = A, $\mathbf{X_2}$ = C, $\mathbf{X_3}$ = A, $\mathbf{X_4}$ = C and P_S = T
GT75 mismatch	$\mathbf{X_1}$ = G, $\mathbf{X_2}$ = T, $\mathbf{X_3}$ = G, $\mathbf{X_4}$ = T and P_S = A
CC75 mismatch	$\mathbf{X_1}$ = C, $\mathbf{X_2}$ = C, $\mathbf{X_3}$ = C, $\mathbf{X_4}$ = C and P_S = T
GC75A match	$\mathbf{X_1}$ = G, $\mathbf{X_2}$ = C, $\mathbf{X_3}$ = G, $\mathbf{X_4}$ = C and P_S = A
GC75T match	$\mathbf{X_1}$ = G, $\mathbf{X_2}$ = C, $\mathbf{X_3}$ = G, $\mathbf{X_4}$ = C and P_S = T

Single Strands to form Immobilised Duplex

- TTT 5'- TTT TTT TTT T**T**T TTT TTT TTT -3'
- TCT 5'- TTT TTT TTT T**C**T TTT TTT TTT -3'
- TAT 5'- TTT TTT TTT T**A**T TTT TTT TTT -3'
- AAAB 5'- BAA AAA AAA AA**A** AAA AAA AAA A-3'
- TTTDNP 5'- DTT TTT TTT TT**T** TTT TTT TTT T -3'
- TCTDNP 5'- DTT TTT TTT TT**C** TTT TTT TTT T -3'
- TATDNP 5'- DTT TTT TTT TT**A** TTT TTT TTT T -3'

DNP = 2,4-Dinitrophenyl

C100PS was synthesised with all bases having phosphorothioate linkages, the sequence was only purified by gel filtration.

Biotinylated Sequences

- C20B 5'- BCC CCC CCC CCC CCC CCC CCC -3'
- AT10B 5'- BTT TTT TTT TTX DAA AAA AAA AA -3'
- AT10BL 5'- BLT TTT TTT TTT XDA AAA AAA AAA -3'
- C10B 5'- BCC CCC CCC CCD -3'
- C10BL 5'- BLC CCC CCC CCC D -3'
- C11B 5'- BCC CCC CCC CCC D -3'
- AC30BL 5'- BLT TTT TTT TTT **C**TT TTT TTT TT**C** TTT TTT TTT TXA AAA AAA AAA **A**AA AAA AAA AAA **A**AA AAA AAA AAA A-3'
- GT30BL 5'- BLT TTT TTT TTT **T**TT TTT TTT TT**T** TTT TTT TTT TXA AAA AAA AAA **G**AA AAA AAA AA**G** AAA AAA AAA A -3'
- C20BL 5'- BXC CCC CCC CCC CCC CCC CCC C -3'

B = Biotin; X and L = $(CH_2CH_2O)_4O$; D = 2,4-Dinitrophenyl.

DNA Monomer Coupling Efficiency

Coupling efficiencies of phosphoramidites were measured by comparison of the absorbance, at 494nm, of the dimethoxytrityl cations produced in the detritylation steps of successive synthesis cycles. The coupling efficiency was calculated as follows:

Coupling efficiency = [A_{494}(cycle n)/ A_{494}(cycle n-1)] x 100. The absorbance values were measured as dilutions of the detritylation product fractions in 125ml of p-Toluene 4-sulphonic acid (0.1M) in acetonitrile. Triplicate values for each trityl colour were measured.

UV Melting Curve Determination

DNA UV melting curves were obtained using the Lambda 15 UV/VIS Spectrophotometer (Perkin-Elmer), the Digital Temperature Controller for UV Spectrophotometer (Perkin-Elmer) and PECSS/peccs2 and PECSS/tconv3 programmes for UV spectroscopy (Perkin-Elmer) , all programmes run on an IBM Personal Systems/2, Model 50 computer.

Fully complementary or partially complementary strands, containing one or more base pair mismatches, were monitored for their melting curves between 5°C - 75°C. Where single stranded sequences were combined equimolar concentrations of each were used. Each sequence under investigation was measured in triplicate. All sequences were dissolved in sodium dihydrogen phosphate buffer (10mM), EDTA (1.0mM) and sodium chloride (0.20M), pH 7.2 prior to melting curve determinations.

CHAPTER 3 PRODUCTION OF ANTI-MISMATCHED BASE PAIR ANTIBODIES

3.1 ANTIBODIES TO NUCLEIC ACIDS

3.1.1 Introduction

The immunogenicity of nucleic acids has received considerable attention. In general, mammalian DNA, most notably dsDNA in the B-conformation, is poorly immunogenic, even when complexed to a carrier protein and administered in adjuvant (Plescia and Braun, 1967). However, other forms of nucleic acids, from a variety of sources, have been found to be immunogenic. These include ssDNA, Z-DNA, DNA-RNA duplexes, ssRNA, dsRNA, triple helix DNA, and mono, di and tri-nucleotides.

Production of antibodies by immunisation with nucleic acids has not produced an immune response directed to dsDNA. However, a major area of research has focused on the autoimmune disease systemic lupus erythematosus (SLE). The hallmark of this disease are the presence of autoantibodies to the B-form of dsDNA (Tan *et al.*, 1966; Schwartz and Stollar, 1985; Stollar, 1986; Braun *et al.*, 1986). The production of these types of antibodies provided evidence of a possible immunogenic role for nucleic acids. The development of methods to monitor anti-DNA antibodies occurring in autoimmune diseases aids the development of methods to detect the production of anti-nucleic acid antibodies after immunisations. Autoantibodies for nucleic acids have also served as a source of information on protein-nucleic acid interactions (Anderson *et al.*, 1988).

Antibodies that recognise nucleic acids are also of interest as probes of different nucleic acid structures. Antibodies have been produced that recognise and study Z-form DNA duplexes (Nordheim *et al.*, 1981; Lee *et al.*, 1984a), triple helical structures (Lee *et al.*, 1987), and various ribopolynucleotides (Rudkin and Stollar, 1977).

3.1.2 Historical

The earliest reported production of antibodies to nucleic acids, by direct immunisation, came in the 1940's. A globulin was detected in equine sera, pre-inoculated with pneumococci, possessing an activity for nucleic acids (Lackman *et al.*, 1941). Similar globulins were not found in normal sera. The reaction was inhibited by purine nucleotides, nucleosides and bases. Pyrimidines showed weak inhibition, and pentoses and phosphates showed no inhibition. Related methyl purines such as caffeine and theophyllin also gave rise to inhibition. In addition the reaction was sensitive to ionic strength and dependant upon the type of ions present.

There have been other reports on antibodies raised by nucleic acid immunisations (Blix *et al.*, 1954; Phillips *et al.*, 1958). At about the same time antibodies were detected in the sera of patients with SLE, that reacted with DNA (Deicher *et al.*, 1959).

Another source of anti-DNA antibodies was discovered using ruptured T4 bacteriophage (Levine *et al.*, 1958). Antibody production appeared to be directed towards the DNA. Further work on a polyclonal antisera obtained from immunisation with ruptured T4 bacteriophage was undertaken. Antisera was found to react with thermally denatured DNA from T4 bacteriophage, but showed no reactivity with native T4 DNA or non T4 DNA (Levine *et al.*, 1960). The T4 bacteriophage DNA is known to contain modified bases. These are repeating units of glucosylated 5-hydroxymethylcytosine. The reactivity of the antisera was suspected to be directed towards these modified bases. Murakami *et al.*, (1961) showed that antibodies raised by ruptured T4 bacteriophage DNA was directed towards glucosylated 5-hydroxymethylcytosine, rather than DNA that was unmodified.

During the early 1960 evidence was presented that RNA may be immunogenic. Barbu and Panigel (1960) produced antibodies that reacted with RNA. The reactivity was directed to the modified bases which are present in RNA.

The validity of the results of all of the assay systems used to detect anti-DNA antibodies described above are open to criticism. The purity of the nucleic acid preparations and characterisation of the DNA in the antigen-antibody reactions are inconclusive. Murakami *et al.*, (1961) suggested that DNA contaminated with 0.5% w/w amino acids obtained from DNA hydrolysates was sufficiently pure, and assumed that the antibodies were specifically recognising the DNA. Any contaminating material present may interfere with correct characterisation of antibody specificity. The antibody may recognise DNA, contaminant, or DNA-contaminant as a non covalent complex. Additionally, non-covalent interactions of any contaminant (small molecules or proteins) may alter the conformation adopted by the complex (Pohl *et al.*, 1973; Thammen *et al.*, 1981; Sinha and Patterson, 1983).

Chemically pure DNA was found not to induce antibody formation (Plescia *et al.*, 1964). Antibody production was possible where DNA-protein complexes were used, however, the antibodies produced were directed against thermally denatured DNA. Characterisation of such antibodies is difficult because denatured DNA will contain single stranded regions, but may also fold into secondary structures where self complimentary regions allow some internal base pair formation. The induction of anti-dsDNA antibodies has proved more difficult.

Over the last 20 years many laboratories have been investigating the ability of the immune system to produce antibodies to modified bases, nucleosides, nucleotides, oligonucleotides and DNA. The aim of the research is the quantitation and localisation of DNA adducts using antisera elicited against DNA adducts and carcinogen modified DNA. Highly specific polyclonal and monoclonal antibodies have been raised to a large number of adducted species including various alkylation products, and metabolites of benzo(a)pyrene (Poirier *et al.*, 1980; Santella *et al.*, 1984) and aflatoxin-B1 (Hsieh *et al.*, 1988).

Antibodies have been generated with two different types of immunogens. Modified DNA can be electrostatically complexed to methylated bovine serum albumin, or monoadduct derivatives can be covalently coupled to carrier proteins. However, a detailed review of immunodetection of adducted or modified bases in DNA is beyond the scope of this text. There have been several recent reviews on the subject (Poirier, 1981; Poirier, 1984; Strickland and Boyle, 1984; Santella, 1988; Poirier 1991) and references there in.

3.1.3 Antibodies to Nucleosides and Nucleotides.

Immunisations using immunogenic forms of DNA, e.g. T4-bacteriophage DNA (Levine *et al.*, 1960) did not produce DNA specific antibodies. In the production of anti-nucleic acid specific antibodies, nucleosides and nucleotides (nucleic acid building blocks) have been used as immunogens. BSA-purine (adenine and guanine) conjugates have been used to immunise rabbits (Butler *et al.*, 1962). Antibodies released into the serum of these immunised animals were found to react with denatured but not native DNA. BSA ribonucleoside and ribonucleotide conjugates have been shown to be immunogenic (Erlanger and Beiser, 1964). Antibodies binding to adenine, guanine and cytosine recognised bases in RNA and denatured DNA. Antibodies binding to uridine recognised RNA but not DNA. BSA-deoxyadenylate conjugate produced antibodies to deoxyadenylate (Humayun and Jacob, 1973). They found that the sugar and phosphate backbone were important immunogenic components as well as the heterocyclic bases.

Antibodies have been raised to dinucleoside phosphates (Wallace *et al.*, 1971) and trinucleoside phosphates (Bonavida *et al.*, 1972) conjugated to BSA. These antibodies recognised the immunogen and also the individual nucleosides. The nucleoside coupled to the protein carrier and not the terminal nucleoside was found to form the immunodominant portion of the antigenic determinant.

In the autoimmune disease SLE antibodies have been found in the serum that recognise dsDNA (Tan *et al.*, 1966). This has raised the question of whether dsDNA antibodies are inducible or only formed after some kind of somatic mutation. The specificity of inducible antibody formation for a variety of nucleic acid structures has thus received considerable attention.

3.1.4 Antibodies to DNA from SLE patients.

Systemic lupus erythematosus (SLE) is characterised by an exuberant immune response. This is manifested by the presence of autoantibodies with a wide spectrum of specificity's, and by their presence in high concentration. There are at least 11 separately distinct antigens in SLE ranging from DNA to various nucleic acid binding proteins (Tan, 1989). As previously stated the hallmark of SLE are antibodies to dsDNA, although there is a greater frequency of autoantibodies to ssDNA (Stollar *et al.*, 1962).

Antibodies to DNA were the focus of research in the 1960s and 1970s. The subject has been reviewed (Tan, 1982), and more recently (Stollar, 1986). In general antibodies defined as reactive with ssDNA recognise polymers of purine and pyrimidine bases which are available for reaction with antibody in the single stranded form but not accessible in the dsDNA form. Antibodies reactive with dsDNA recognise mainly the deoxyribose phosphate backbone and, therefore, are reactive with both dsDNA and ssDNA. In certain cases antibodies recognise helical structures on dsDNA and only react with ssDNA where secondary structures were possible. Secondary structure can occur as a consequence of folding caused by base pairing of self complementary nucleotide sequences.

The use of antisera from SLE patients to evaluate anti-DNA responses was limited by the multitude of the immunogenic response. However, antibodies to DNA arise spontaneously in different strains of murine lupus (Theofilopolous and Dixon, 1985). This is an autoimmune disease of inbred mice, that develops in a similar manner to human SLE. New Zealand Black (NZB) and the F-1 hybrids

produced by the mating of NZB and New Zealand White were the first described models of spontaneous lupus-like autoimmune disease (Heylen and Howie, 1963). Two other strains were subsequently described in the literature (Andrews *et al.*, 1978). A lupus like form of human SLE in a non-human primate (rhesus macaque) has recently been proposed (Anderson and Klein, 1992).

Hybridoma technology allowing the production of specific monoclonal antibodies has provided information on the precise characterisation of autoantibodies to DNA. Hybridomas have been produced by fusing the spleens of autoimmune diseased mice to create a variety of cell lines secreting anti-DNA monoclonal antibodies (Pollard, *et al.*, 1986; Pisetsky and Caster, 1982; Andrezejewski *et al.*, 1981). Monoclonal autoantibodies to DNA are being used both as probes for nucleic acid structure and also to try and elucidate the nature of the immune response in the production of anti-dsDNA antibodies that cannot be mimicked by immunisation regimes.

Surprisingly, murine lupus models respond poorly to exogenous immunisations with mammalian dsDNA (Stollar, 1981; Madaio, 1984). The ability of lupus individuals to generate high affinity responses to self endogenous antigen, despite poor responses to immunisations with self antigens or foreign antigen thus remains an enigma. To elucidate possible mechanisms of SLE antibody production, comparisons with two anti-DNA producing systems have been investigated. Firstly, the production of antibodies to DNA by bacterial immunisations, and secondly the drug induced lupus like symptoms seen in several mammalian systems.

3.1.5 Immunisation with Bacterial DNA

Bacterial DNA has been shown to be immunogenic in mice (Gilkeson *et al.*, 1989a,b; Messina *et al.*, 1992). A sex difference for *E. coli* DNA induced antibodies in Balb/c immunised mice has been reported (Palmer *et al.*, 1992), with the greater response to all test antigens occurring in sera of female mice. The induction of anti-

DNA antibodies in normal mice with *E. coli* DNA is attributed to the presence of unusual nucleotide sequences that are rare in mammalian DNA (Gilkeson *et al.*, 1989a,b; Pisetsky *et al.*, 1990). Monoclonal antibodies produced from Balb\c mice immunised with *E. coli* DNA bind predominantly to denatured DNA containing AATGTG sequences (Messina *et al.*, 1992), rather than denatured DNA in general. Antibody production has also been shown to occur after immunisation with viral DNA (Schattner and Ragar-Zisman, 1990; Flaegstad, 1988), and from parasites (Jones, 1977; Fischer *et al.*, 1981, Ternynck *et al.*, 1990).

The sera of non-SLE patients infected with *E. coli* have been found to contain anti-DNA antibodies (Robertson and Pisetsky, 1992). They reacted to both ssDNA from *E. coli* and denatured DNA from a variety of eukaryotic sources. They also reacted to synthetic oligonucleotides poly dC and poly dT. Although *E. coli* induced antibodies to DNA showed high cross reactivity to DNA, as seen in SLE, no anti-mammalian dsDNA reactivity has been found for *E. coli* induced antibodies.

Monoclonal antibodies produced after Balb/c mice were immunised with *E. coli* DNA were found to bind ssDNA, and to non-nucleic acid antigens, e.g., β-galactosidase, cardiolipin, and various nuclear antigens (Pyun *et al.*, 1993). This resembles lupus anti-DNA antibodies in the pattern of cross reactivity. However, no binding of these induced anti-DNA antibodies was observed with mammalian dsDNA, the hallmark of SLE.

Genetic analysis of variable regions of anti-DNA antibodies from *E. coli* immunised and autoimmune mice have been carried out (Foster *et al.*, 1992; Gilkeson *et al.*, 1993). Similarities in the amino acid sequences in the complementarity determining regions (CDRs), (part of the variable (V) region which forms the antigen binding site), have been observed. Many spontaneous anti-DNA antibodies have multiple arginine residues in the V region, particularly in part of the CDR, not seen for antibodies from bacterial DNA immunised animal (Gilkeson *et al.*, 1993). These differences may account for the inability of induced anti-DNA antibodies to bind mammalian dsDNA.

This may point to important mechanisms underlying the production of antibodies to DNA in SLE patients. They suggest that as well as DNA presentation, to initiate the immune system to respond, immunoregulatory abnormalities may exist in SLE development. Normally immunoregulation removes autoantibody producing cell precursors from the immune system. In SLE immunoregulation abnormalities prevent the elimination of autoimmune cell precursors thus an autoimmune response occurs.

3.1.6 Z-DNA and Drug Induced Lupus

The right handed B-form of DNA is the usual double helical structure as proposed by Watson and Crick. The Z-form is a left handed helix which occurs in nucleic acid with alternating G-C purine-pyrimidine sequences under certain conditions of solvent composition or helical stress. Z-DNA was first discovered using atomic resolution X-ray crystallographic analysis of the hexanucleoside pentaphosphate (CpGpCpGpCpG), (Wang *et al.*, 1979). In this conformation the DNA forms a double helix with Watson-Crick base pairs and anti-parallel sugar phosphate chains, the sugar phosphate back-bone following a zig-zag course, hence Z-DNA.

Z-DNA can be induced from solutions of B-DNA by altering the ionic strength (Pohl *et al.*, 1973; Thammen *et al.*, 1981) provided that the DNA has alternating purine-pyrimidine sequences. Brominated dC (5BrdC) in poly (dG-dC) : poly (dG-dC) sequences remains in the Z-form independent of ionic strength (Lafer *et al.*, 1981). The methylated polymer, poly (dG-5MedC) : (dG-5MedC), (methylation at position 5 of cytosine) was converted to Z-DNA at relatively low concentrations of $MgCl_2$ (Behe and Felsenfield, 1981).

While it is the base sequence or primary structure of nucleic acids that contains the genetic information, the helix conformation is also important for site specific interactions. Protein-nucleic acid interactions are critical for regulation of gene expression, replication and packaging of genetic material. It is also possible that a small

region of Z-DNA within a DNA molecule that is mostly in the B-DNA form could act as a signal for protein binding. There is evidence to suggest a role for Z-DNA in recombination (Kmiec and Hollomen, 1986). Unusual structures such as cruciforms (Lilley, 1980; Panayotatos and Wells, 1981), and bent helixes (Marini *et al.*, 1982; Zahn and Blattner, 1987) might constitute protein recognition sites. Therefore, there is great interest in the factors influencing nucleic acid conformation and recognition of such by proteins.

The structural features of Z-DNA as compared to B-DNA were detected by the use of immunochemical methods, Z-DNA was found to be highly immunogenic in experimental animals (Lafer, *et al.*, 1981a,b; Zarling *et al.*, 1984, Gunnia *et al.*, 1991). Antibodies have been produced from immunisations with Z-DNA that are highly specific for Z-DNA, showing no cross reactivity with B-DNA, RNA, DNA-RNA hybrids and several synthetic polynucleotides (Lafer *et al.*, 1981b).

It has been reported that drugs such as hydralazine bind to DNA duplexes (Sinha and Patterson, 1983), and can facilitate the transition of poly (dGm^{5}dC) from the B-form DNA to Z-form DNA (Thomas and Messner, 1986). The Z-DNA helix differs from B-DNA in several structural features that render it highly immunogenic. The purine bases of Z-DNA are rotated into a *syn* rather than the *anti*-conformation found in B-DNA. (Stollar, 1986). To allow the pyridine bases to hydrogen bond their deoxyribose groups are rotated. With this alternating different geometry of adjacent bases, there are associated changes in the backbone geometry, the base stacking, and the orientation of the base pair axis to the fibre axis. With the alternating pyrimidine-purine sequence, the repeating unit of Z-DNA is a dinucleotide, rather than a mononucleotide seen for B-DNA.

From an immunological point of view the deoxyribose-phosphate backbone in Z-DNA follows a zig-zag left handed course compared to the smoother helix of right handed B-DNA. The interphosphate distances differ from those of B-DNA, and the helix is slimmer with more base pairs per helical turn. In addition, the major

groove in B-DNA is replaced by a convex surface on which the purine N7 and C8 and the pyrimidine C5 positions are exposed and are accessible to recognition by proteins (Stollar, 1986). Therefore, the structure of Z-DNA is quite immunological distinct from B-DNA.

It is believed that the production of autoantibodies to dsDNA in drug related lupus might involve the induction and stabilisation of Z-DNA (immunologically distinct from B-DNA) by drugs such as hydralazine (Thomas and Messner, 1986; Thomas *et al.*, 1993).

3.1.7 Conclusions

There is great interest in the study of antibodies that bind to nucleic acids. Antibodies which bind DNA occur spontaneously in the autoimmune disease Systemic Lupus Erythematosus and have been implicated in the pathogenesis of the disease (Schwartz and Stollar, 1985). These antibodies have a large range of specificity's. There is also great interest in studying general DNA binding protein interactions. Hybridoma technology allows the production of large amounts of anti-DNA antibody (a DNA binding protein) to be produced. Many of the types of interactions between antibodies and DNA are likely to be similar to those between DNA binding proteins and DNA.

The production of antibodies by classical hybridoma technology relies on the immunogenicity of DNA to evoke a response in immunised animals. Although the B-conformation of DNA is generally considered to be non-immunogenic, other forms of DNA have proved useful in the production of anti-DNA antibodies. In addition, several strains of mice have developed a form of SLE and spontaneously produce anti-DNA duplex antibodies. The fusion of splenocytes, from lupus mice, with a myeloma cell line has allowed monoclonal antibodies to duplex DNA to be produced (Stollar, 1986). Also the synthetic duplex poly d(TC). poly d(GA) has been found to be weakly immunogenic, and monoclonals have been produced (Lee *et al.*, 1985).

3.2 PRODUCTION OF ANTIBODIES : GENERAL

3.2.1 The Immune System

The vertebrate immune system is a network of molecules and cells capable of distinguishing self from non-self. It functions to protect against the invasion of foreign micro-organisms and their toxins. Part of the vertebrates immune system is an adaptive response (Hilderman and Reddy, 1973; Marchalonis, 1976). This works by continually scanning its environment and initiating the destruction of any foreign material found. This adaptive system learns from experience and remembers its encounters. It is thus specific, adaptive and possesses memory.

There are two types of leukocytes involved in the adaptive immune response, the B-lymphocyte and T-lymphocyte. Soluble proteins known as antibodies are products of antigenically activated B-lymphocytes (plasma cells). The antibodies are highly specific recognition elements of the "humoral" immune response. Each antibody has a specific affinity for the foreign material that stimulated its production. T-lymphocytes also aid the humoral response by helping stimulate the changes that transform resting B-lymphocytes to antibody producing plasma cells.

Immunisation of an animal causes the production of a wide range of antibodies capable of binding to the immunogen, the antibodies are released into the serum of the immunised animal. On antigenic challenge the majority of the B-lymphocytes remain unstimulated. Only a small number possessing the specific receptors are stimulated, the receptors being cell surface immunoglobulins on the B-lymphocytes (Ada, 1970; Raff, 1971). Antigenic stimulation initiates B-lymphocyte activation to antibody producing cell. Thus several related but not identical highly specific immunoglobulins all recognising the same antigen are released into the animals circulation.

3.2.2 Monoclonal Antibodies

For many purposes the use of polyclonal antisera is perfectly acceptable, and good accuracy and precision of assays utilising these sera will be obtained. For certain applications polyclonal antisera may be unsuitable, e.g. they may not be able to distinguish very closely related molecules. In these instances affinity purification of a polyclonal antisera may be used to increase the specificity of the remaining antibody content of the antisera. However, it is often more desirable to use a monoclonal antibody.

A monoclonal antibody is defined as the antigen specific homogeneous immunoglobulin product of an immortalised plasma cell clone. Monoclonal antibodies are therefore the selected product secreted from a hybrid cell line produced by the fusion of a myeloma cell line with an antibody producing plasma cell (Kohler and Milstein, 1975, 1976). Cell fusion technology now allows the immortalization of specific antibody producing cells, followed by their growth in tissue culture systems to generate potentially unlimited quantities of monoclonal antibody. Monoclonal antibody secreting hybridomas will probably be needed where specific recognition of a mismatched base pair in a DNA duplex, compared to fully matched DNA, is required.

Each fusion experiment combines spleen cells with a plasmacytoma cell line. For the first two weeks post fusion the supernatants from cell culture wells are frequently changed. This serves two purposes. Firstly, it ensures a constantly high concentration of nutrients around the cells. This is particularly important in terms of hypoxanthine (H) and thymine (T) concentration. These are essential for hybrid cell growth in the presence of aminopterin (A) put in the medium to kill unfused plasmacytoma cells and hybrid cell formed by fusions between two plasmacytoma cell lines. The selective medium is normal growth medium containing H, T and A, usually called 'HAT'

Secondly, it ensures the removal of dead material from the wells. Fusion wells will contain other cellular products in addition to

the desired splenocyte-plasmacytoma hybrid cell line. Each fusion will also give rise to plasmacytoma-plasmacytoma hybrids, splenocyte-splenocyte hybrids, and unfused plasmacytomas and unfused splenocytes. All of the cell types apart from splenocyte-plasmacytoma hybrids (the desired cell type) will die in the selective 'HAT' culture conditions used. The dead cells do not remain strongly attached to the plastic culture wells and are removed over successive changes of medium.

Production of hybridoma cell lines by cell fusion is essentially a random process and occurs at low frequency (about 1 in 10^5). Immunisation greatly increases the percentage of active spleen cells producing the desired antibody. Thus, immunisation greatly increases the probability that the relatively rare cell fusion events occur between myelomas and the desired antibody producing spleen cells. Additionally, fusions are carried out with large numbers of spleen cells (about $1\text{-}2\text{x}10^8$). The stimulatory effect of immunisation will vary with immunogenicity of the test antigen. As previously stated dsDNA is generally regarded as poorly immunogenic. There are two main disadvantages when trying to produce monoclonal antibodies to potentially poorly immunogenic material.

(A) Low affinity antibodies may be produced, these will be harder to detect at low concentration immediately post fusion.

(B) Immunisation with poorly immunogenic material will not be as effective in stimulating the immune system, compared immunisation with highly immunogenic material. The ratio of the desired antibody producing splenocytes compared to irrelevant splenocytes will be reduced. If this occurs than a smaller proportion of the splenocytes that undergo fusion will produce the desired hybridoma cell line.

These problems may be increased for antibody production where the antigenic site only represents a small proportion of the injected immunogen. Thus, where the production of hybridomas secreting antibodies against the antigen of interest occurs at very low

frequency, extremely effective and rapid screening will be required to avoid missing the few positives that occur.

3.2.3 Detecting Monoclonal Antibody Production

As previously stated (section 3.2.2) cell fusions yield a mixture of cellular products. The growth medium used allows only the growth of myeloma cell lines. However, after every immunisation/fusion experiment irrelevant hybridomas will be produced. Fusion products are grown in multiwell containers (usually hundreds of wells for each fusion). Each well may contain cells produced by separate fusion events. Each separate fusion event may produce colonies of cells producing the desired antibody product, an irrelevant antibody product, or no antibody at all.

The first few weeks post-fusion are a critical period, during which identification of hybridomas secreting the desired antibody must occur. The desired cell lines must then be isolated from other cell lines, producing monoclonal cell lines. If the identification and cloning procedures are not rapidly undertaken then a cell line producing the desired antibody may become overgrown by an irrelevant cell line. Hybridoma cell lines that do not produce antibody usually grow more rapidly than antibody producing hybridomas. Therefore, it is vital to have a rapid and reliable screening assay. The essential features of a screening assay for monitoring hybridoma production of antibody are :

(A) It must be sensitive and capable of detecting very small amounts of antibody.

(B) It must be accurate, false positives as well as false negatives must not occur.

(C) It must be rapid to perform and capable of handling large numbers (hundreds) of samples.

(D) It must be reproducible and reliable.

Antibody secreting hybridomas are usually detected by methods that measure the antibody content of the spent media. There are several methods of detecting antibody. the two most commonly used methods are radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA).

RIA uses radiolabelled antibody or antigen as the means of detecting specific antibody antigen interactions. It many formats it is desirable as it is often regarded as a positive screen for antigen. This point will be discussed later in this chapter. Although RIAs are generally very sensitive assays there are some disadvantages in their use. There are hazards associated with the handling of radioisotopes. When screening for antibody production the antigen must be radiolabelled. For DNA radioactivity is most easily incorporated as ^{32}P labelled ATP and polynucleotide kinase. This incorporates ^{32}P phosphate at the 3'-OH of deoxyribose.

Large numbers of screening experiments are required for each fusion carried out. Separation steps are major contributors to reproducibility problems in immunoassay. RIAs are generally solution phase assays, and require specific separation methods e.g. antibody precipitation, immobilised secondary antibodies (often on insoluble beads) or exclusion using dextran coated charcoal (charcoal cannot be used to separate antibody from macromolecules). Efficient separation steps using radiolabelled materials becomes less amenable to rapid throughput when large numbers of samples are being assayed.

The use of microtitre format solid phase immunoassay provides easy and highly reproducible separation of bound and non-bound antibody. Alternative labels such as enzymes have been developed to detect antibody antigen interactions on solid phases. Due to the high reproducibility, sensitivity and ability to handle the large numbers of samples ELISA is the most commonly used method to monitor antibody production from fusion experiments.

3.2.4 ELISA Techniques

There are two types of ELISA for measuring antibody production of hybridoma cell lines. They are the non-competitive ELISA and competitive ELISA.

In non-competitive ELISA the presence of antibody that binds to some component of the test compound is detected. A solid phase, usually a 96 well microtitre plate, is coated with a fixed level of test compound (immunogen or hapten-carrier protein conjugate) by passive adsorption. Antibody secreted into the growth medium by hybridoma cells is added to the microtitre wells, antigen specific antibody will bind to the coating material. The growth medium is removed by washing, leaving antigen specific antibody bound to immobilised coating material. An enzyme labelled species specific secondary antibody is added. This will bind to immobilised primary antibody in the well. Excess second antibody is then washed away and the amount of enzyme remaining bound is measured. This is usually carried out by adding a colourless enzyme substrate that produces a coloured product that can be measured spectrophotometrically. The amount of colour produced is directly proportional to the amount of antigen specific antibody that binds to the solid phase coating material. This reflects the amount and/or the affinity of the antigen specific antibody in the growth medium tested.

In competitive ELISA information may be obtained about the specificity of antibody binding. This is particularly important where antibodies against small molecules are being produced. These will either be compounds that needed to be coupled to a carrier protein, or where antibodies are required that only recognise a specific part of a large molecule, i.e. a mismatched base pair in a DNA duplex. In competitive ELISA, a limiting amount of antibody is incubated with antigen. The antibody/antigen solution is added to antigen pre-coated wells. Antibody will compete for binding to antigen in solution and antigen immobilised to the solid phase. Maximum antibody binding to the solid phase will occur for test samples where no antigen is added. As the amount of antigen in solution increases the availability of antibody that binds to the solid phase will

decrease, described as inhibition. The amount of immobilised antibody is detected as for non-competitive ELISAs. Inhibition of binding to the solid phase is thus a measure of specific antigen binding, and for specific antigens their concentration.

The results from competitive ELISA provide greater certainty regarding the specificity of the antibodies. In the development of antibodies to small molecules binding of antibody to the carrier protein or to the link between the hapten and the carrier protein may occur giving rise to false positive results. Additionally, for all antigens non-specific binding of antibodies to uncoated portions of the solid phase may occur. Detergents and irrelevant proteins are often added to reduce non-specific binding.

3.3 ANTI-DNA ANTIBODY ASSAYS

3.3.1 Introduction

A variety of immunoanalytical methods exist for the measurement of anti-nucleic acid antibodies. These include precipitation from solution, precipitation in gels, complement fixation, immunofluoresence, passive haemaglutination, and radioimmunoassay, and are reviewed by Stollar (1973). Although successful, most of these methods have been developed for the screening of either serum from patients with SLE or bleeds from immunised animals.

These methods are useful when analysing relatively few samples (tens of samples), however, this is not the case when screening hybridomas where up to 1000 samples may be screened. The advances in hybridoma technology coupled with a desire to produce monoclonal antibodies to nucleic acids (from autoimmune and nucleic acid immunised animals) has led to the development of anti-DNA antibody screening using ELISA techniques (Klotz, 1982; Silvestris *et al.*, 1987).

3.3.2 ELISA Techniques

A variety of anti-nucleic acid ELISAs have been described, though to date there is not a generally accepted method. There are two main areas of variability in ELISA techniques in the literature :

(1) Methods of immobilisation of nucleic acid to the solid phase.

(2) Buffer requirements, especially the inclusion of EDTA, during the assays.

Several ELISA methods have been established to test for anti-nucleic acid antibodies in serum from SLE patients, others are used with purified monoclonal antibodies from studies on murine lupus. Differing assay requirements to some extent reflect the differing requirements of the test systems.

3.3.2.1 Immobilisation Techniques

Methods for the direct attachment of DNA to polystyrene have not been standardised. Interactions with polystyrene are predominantly hydrophobic (Kochwa *et al.*, 1965) so that dsDNA molecules present a particular problem because the most hydrophobic portions of the macromolecule are held within the duplex. Dissociation of duplexes to produce ssDNA increases the possibility of interaction with the polystyrene, and has been used for direct fixation (Pesce, 1974).

The nature of the diluent used for immobilisation of single stranded DNA has also been investigated. The addition of $MgCl_2$ (0.1M) to phosphate buffered saline was found to dramatically enhance the adsorption of DNA onto the wells (Negata, *et al.*, 1985), a similar effect was seen with Ca^{2+} ions but not with Na^{+} ions.

The ionic strength of the diluent also affects the adsorption of ssDNA to polystyrene. It was found that 1.5M NaCl or 0.5M ammonium sulphate gave maximum adsorption of a 642 base pair sequence (Inouye and Hondo, 1990). Other studies have combined

highly alkaline buffers (pH 11-12) with high ionic strength (1.2-1.3M NaCl) to increase adsorption (Lacy *et al.*, 1989; Loon *et al.*, 1992). The influence of buffer conditions on adsorption is also believed to be dependant upon the size of the DNA fragments and on the type of microtitre plates used, the latter occurs commonly in all ELISA techniques. In addition there appears to be an optimal concentration for DNA adsorption, and above this optimal concentration less immobilisation of DNA occurs (Guesdon,1992).

For dsDNA, indirect methods have been used that involve electrostatic interactions of the negatively charged phosphate backbone in DNA and positively charged proteins, such as protamine sulphate (Klotz *et al.*, 1979), poly-L-lysine (Fish and Ziff, 1981) and methylated BSA (Rubin *et al.*, 1983). A major concern with these methods is the possibility of increased non-specific binding of antibody to these charged proteins. For protamine sulphate precoated wells it has been recently suggested (Rupin *et al.*, 1993) that complete saturation of the precoat with dsDNA removed false positives, that had previously been reported using this method.

There is also concern that specific structural features of the duplex may be altered by interaction with positively charged proteins. Immunisations with nucleic acids are generally carried out as complexes with MeBSA. Thus MeBSA enhanced coating of dsDNA would present obvious cross reactivity problems during antibody screening assays. It has also been suggested that binding of B-DNA to MeBSA coated plates could induce certain regions of it to form Z-DNA (Lafer *et al.*, 1981). Therefore, in experiments to produce anti-B-DNA antibodies by immunisations with B-DNA/MeBSA complexes, antibodies to Z-DNA may be produced. These may be incorrectly described as anti-B-DNA antibodies if MeBSA enhanced B-DNA coating methods were used. It has also been suggested that anti-Z-DNA antibodies may be able to induce susceptible B-DNA sequences to the Z-DNA conformation (Lafer *et al.*, 1981), this may be thought as analogous to the ionic strength induction of Z-DNA previously reported (Pohl *et al.*, 1973; Thammen *et al.*, 1981).

More recently pre-treatment of polystyrene microplate wells with UV light has been shown to enhance the binding of both ssDNA, dsDNA and synthetic oligonucleotides (Zouali and Stollar, 1986). The mechanism whereby UV irradiation increases the binding capacity of polystyrene wells is unknown. Hydrocarbon polymers with saturated backbone chains are susceptible to oxidation involving singlet oxygen (Rabek *et al.*, 1985).

UV treatment of polystyrene in the presence of air has been associated with ring opening oxidation and formation of chemically reactive muconic dialdehyde structures (Rabek *et al.*, 1985). This may occur through formation of a dioxetane intermediate. During photoxidation of polystyrene, polyene structures in the main chain can also be generated. In this case O_2 oxidation may generate reactive peroxides on the polymer.

Over the last few years more specific methods of immobilisation of nucleic acid have been introduced. The modification of ssDNA with biotin has allowed attachment to microtitre plates that have been precoated with either anti-biotin antibodies (Coutlee *et al.*, 1989a,b), or streptavidin (Emlen *et al.*, 1990). Immobilisation of ssDNA has also been reported by direct covalent linkage to modified microtitre plates (Rasmussen *et al.*, 1991). Covalink NH plates (Nunc) have a secondary amine on a spacer arm, this allows reaction *via* carbodiimide to the 5'-terminal phosphate group of DNA.

3.3.2.2 Assay Conditions

In addition to immobilisation techniques the type of antibody/DNA interactions are reported to be dependant upon the buffer system used in the ELISA. The most commonly used buffers used are phosphate buffered saline (PBS) and Tris buffered saline (TBS). Two anti-DNA monoclonal antibodies, from autoimmune mice, showed higher titres when assayed in TBS compared to PBS (Pisetsky and Semper, 1984). Similar results were obtained for sera from some human SLE patients. Other sera showed higher titres with PBS compared to TBS. They suggest that antigenic sites on

DNA may be variably affected by the conditions of the assay, altering quantitative and qualitative assessment.

To validate the production of antibodies by immunisation with Z-DNA high ionic strength buffers were used to induce the transition from B-DNA to Z-DNA. Phosphate buffer containing up to 4M NaCl was used to induce the Z-DNA transformation in susceptible duplexes, during the assay (Moller *et al.*, 1982). It should be noted that the use of such high ionic strength buffers during an assay is unusual. Immunoassays are usually carried out in physiological buffer using ~ 0.13 - 0.14M NaCl. The harsh salt conditions to transform B-DNA to Z-DNA (Moller *et al.*, 1982) would normally reduce antibody antigen interaction.

Nucleic acids are degraded in serum by the action of nuclease enzymes. These enzymes can also be found in actively growing cells *in vitro*. This can be due to the addition of animal serum proteins to supplement the growth medium, or possibly the release of nuclease enzymes from dying cells within the cell culture. Clearly, when screening for anti-DNA activity in such medium the elimination or inactivation of such enzymes is highly desirable.

There are conflicting suggestion as to the use of nuclease inhibitors during anti-DNA ELISAs. To a certain extent the nature of the assay system may influence the requirement for nuclease inhibitors. Where immobilisation involves the interaction of random multiple sites of the nucleic acid to a solid phase, and where binding of antibody to the bound DNA only will be measured, the use of nuclease inhibitors may not be essential. In these case the proximity of the DNA to the solid phase will reduce the accessibility of the DNA to nuclease enzymes. Where purified monoclonal anti-DNA antibodies are being used the use of nuclease inhibitors may also be unnecessary.

However, the immobilisation of DNA to microtitre plates can be specifically carried out at one terminus of the DNA molecule, e.g. covalent linkage through the terminal phosphate group. This allows the DNA sequence greater freedom of movement and interaction

with components within the solution around it. Additionally, competitive ELISA use DNA (as inhibitor) free in solution. In both of these situations the DNA will be more susceptible to nuclease degradation, and thus attempts to reduce DNA degradation may need to be investigated.

The most commonly used nuclease inhibitor is EDTA. EDTA binds divalent cations which are essential for nuclease enzyme activity. Although EDTA has been used by several workers (Lacy and Voss Jr., 1989; Moller *et al.*, 1982), it may interfere with anti-DNA antibody binding to DNA. Attempts have been made to develop standard incubation conditions, while inhibiting serum enzymes that break down nucleic acids (Brosalina *et al.*, 1988). The addition of nuclease inhibitors, EDTA, urea, and diethylpyrocarbonate to the serum not only inhibited the action of the nucleases, but also led to a sharp decrease in the binding of anti-DNA antibodies to oligonucleotides. They suggested the addition of an excess of an irrelevant oligonucleotide as an inhibitor. More recently the effect of EDTA on anti-DNA binding has been confirmed. The presence of 10mM EDTA has been reported (Pyun *et al.*, 1993) to lead to a 50% decrease in binding of monoclonal antibodies to bacterial DNA.

During the production of anti-DNA antibody secreting hybridomas it was reported that DNA may be introduced into the growth medium due to the loss from cells in culture (Ehrenstein *et al.*, 1993). Anti-DNA antibodies secreted into the cell growth medium may then not be available for interaction with DNA when screening is carried out. Cell lines producing antibody may then be incorrectly discarded after screening for anti-DNA activity. This is particularly important where low quantities of high affinity anti-DNA antibodies have been released into the growth medium, i.e. at early screening stages post fusion or post cloning. They suggest that supernatants harvested from cell culture should be always be pre-treated with DNase 1 (a nuclease enzyme) in the presence of 1mM $MgCl_2$ and 0.02mM $CaCl_2$, for 1hr at 37°C. The reaction should then be stopped by the addition of 15mM EDTA. This process may of course affect subsequent DNA binding for some anti-DNA

antibodies. In the production of anti-DNA antibodies the presence of nucleic acids in the growth medium has not been reported in other studies. However, this report adds to the uncertainty when developing screening methods.

3.3.3 Conclusions

Development of screening methods to detect the release of antibodies, into the growth medium, that specifically recognise and bind to mismatched base pairs is not a trivial task. This is in sharp contrast to screening methods for classical haptens. If classical haptens are immunogenic screening is relatively straightforward and useful monoclonal antibodies will usually be obtained within 6-12 months. For anti-mismatch base pair antibodies the diversity of immobilisation methods and assay buffer requirements generates considerable confusion as to the choice for assay conditions. For our purposes we are using B-form oligonucleotide duplexes (generally considered to be poorly immunogenic or non-immunogenic) containing a mismatched base (of unknown immunogenicity). We are therefore attempting to devise suitable screening methods for antibody production (based on the varied methods in the literature) to detect the possible production of antibodies from material of unknown, but probably weak, immunogenicity.

The choice of immunogen as well as screening strategy is clearly of great importance in the outcome of this work. For these reasons a variety of potentially immunogenic materials were produced. There are clearly many variables involved in developing an effective screening method for detecting any anti-DNA antibody production. A considerable amount of time was therefore spent in devising, developing and testing of ELISA screening methods.

3.4 SCREENING ASSAYS FOR ANTI-MISMATCHED BASE PAIR ANTIBODIES

Apart from the unknown immunogenicity, and the immobilisation and buffer conditions for assaying, detection of antibodies to mismatched duplexes presents particular problems in the initial screening. In terms of immunisation, a mismatched base pair to some extent can be regarded as a hapten attached to a larger carrier molecule, analogous to a small molecule (hapten) attached to a carrier protein. If the mismatch is immunogenic, antibodies will be produced which can be detected by screening using an ELISA.

A variety of different antibodies will be produced that will recognise different components of the immunogenic material. Some of the antibodies will recognise the hapten (of interest) and some will recognise the carrier protein. When the antisera are screened in an ELISA they will bind both the hapten and the carrier protein if the same protein is used for the immunisation and the coating material. To avoid detection of antibodies to the carrier protein, the hapten is attached to a different protein, where possible, using a different means of attachment. Therefore detection of any antibodies should indicate specific recognition of the hapten.

However, for a mismatched base pair the 'hapten' is an integral component of the duplex. Therefore, this cannot be isolated and placed in another non-B-DNA duplex for screening. In this case, a positive screen using a mismatch containing duplex only implies anti-duplex antibodies are present. The mismatch could be screened for in a different duplex, i.e. immunise with poly d(AT) and screen with poly d(GC) containing the same mismatch. This may be sufficiently different to aid specific mismatch recognition. However, the antibody recognition of the mismatch structure may be affected by local base sequence. Thus recognition of an AC mismatch by an antibody may be different in different sequences.

If every mismatched base pair were to present a different site for recognition in every different sequence there would be limited use of anti-mismatched antibodies. It is more likely that certain

sequences will present the same mismatches slightly differently. Thus anti-mismatch base pair antibodies may have a range of affinities for the same mismatched base pair if different DNA sequences.

This may still create problems during screening. Antibody screening for classical haptens usually avoids non-specific binding associated with the carrier protein. Antibody recognition should only occur for hapten/antigen specific antibodies. For DNA duplexes containing a mismatched base pair, overall sequence differences that do not effect recognition of the mismatch may mean the sequences are very similar. Antibodies produced that recognise irrelevant (fully matched) regions of the DNA duplex would probably cross react with the irrelevant regions of the alternative, but similar, duplex used for screening. This may be compared to immunising with an antigen bound to rat albumin and screening for antibody using human albumin. Although they are different there will also be considerable identity/similarity between the protein carriers. Binding of antibody to irrelevant (non-antigen) sites is extremely likely.

The successful production of monoclonal antibodies usually relies on the ability to carry out cloning experiments as early as possible. Therefore, the results from the first screen are crucial to avoid loss of desired hybridomas by overgrowth of non-secretors or irrelevant secretors. For classical haptens detection of antibody binding in a non-competitive assay suggests antigen specific antibody is present. Competitive ELISA then confirms the specificity. As previously discussed, a mismatched base pair cannot be isolated and thus inhibition studies need to be carried out with matched and mismatch containing duplexes. Clearly with anti-mismatch antibody detection, the amount of information obtained by the initial, non-competitive, screen will be less than those involving classical haptens and carrier proteins.

In addition, for a classical hapten, high binding in the first screen (assuming no non-specific binding) may imply good recognition of the hapten, and these cell supernatants will be chosen for competitive studies. The difference in immunogenicity of

matched compared to mismatched base pairs in a B-DNA duplex is unknown, but matched base pairs are more abundant in the duplex for screening. Therefore, there will be uncertainty selecting cell supernatants for competitive ELISA prior to cloning experiments.

3.5 RESULTS AND DISCUSSION

3.5.1 Immunisations and Serum Analysis

Mice were immunised with 15mer duplexes either as a free oligonucleotide or complexed with MeBSA. 75mer duplexes and C100PS were immunised as free DNA. All immunisations were carried out with the oligonucleotide (or complex with MeBSA) in adjuvant. This not only helps evoke an immune response, it also acts as a depot for the prolonged release of the test immunogens. Three mice were immunised per immunogen, individual mice are referred to by number e.g. AC15-1, AC15-2 etc. Sera from mice bleeds were obtained to assess anti-DNA antibody production, prior to fusion experiments.

3.5.2 15mer Oligonucleotide Duplexes

One of the main challenges in the immunochemical aspects of the work has been the establishment of reliable screening methodology. It was decided that ELISA was the most appropriate assay format, for reasons discussed previously. The first concern was to physically attach an oligonucleotide duplex to a 96 well microtitre plate and still present a mismatched base pair to any anti-DNA antibodies. Initially pre-coating methods using poly-L-lysine and MeBSA were investigated to enhance oligonucleotide binding to the solid phase. Poor end point colour development was observed using poly-L-lysine precoat and non-specific binding was occurring for plates precoated with MeBSA. The MeBSA method was also unsuitable for all serum taken from mice immunised with oligonucleotide/MeBSA complexes, for obvious reasons.

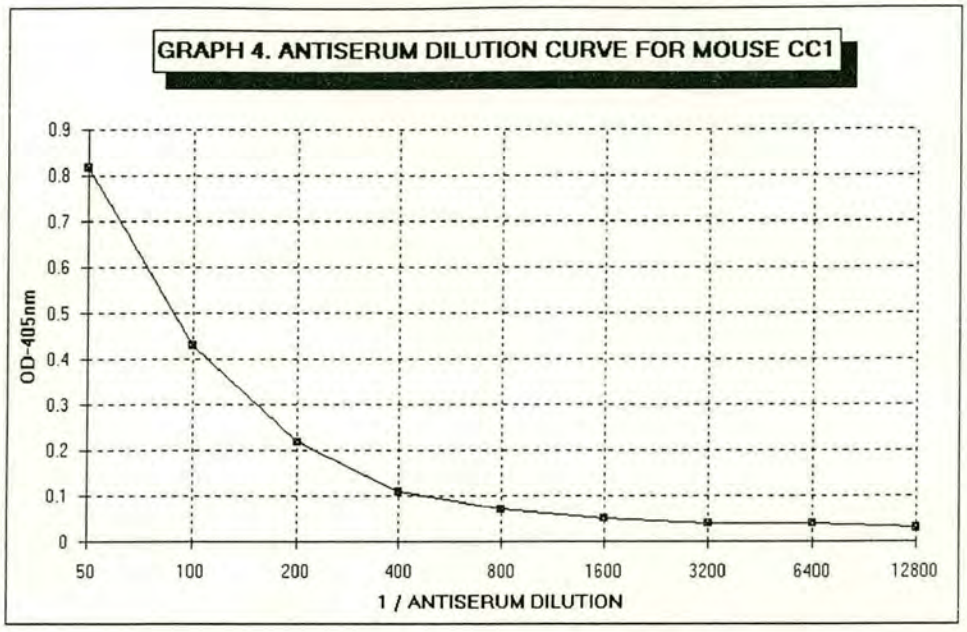
Results from plates coated by simple incubation with oligonucleotide, with no precoat, indicated limited or no binding of antibody occurred, this probably reflects the weak binding of duplex DNA to microtitre plates. Although single stranded DNA binds well to the polystyrene plates used, duplex DNA will bind much less efficiently. The reduced binding for duplex DNA was probably a consequence of the purine and pyrimidine groups being unavailable

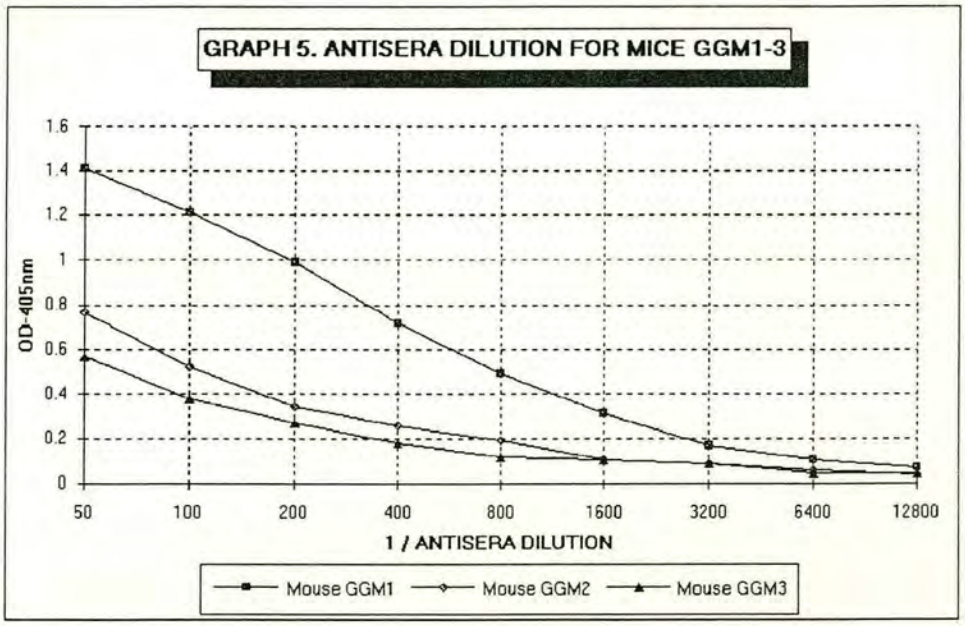
for interaction with the polystyrene as they are hidden by base pair formation. Duplex oligonucleotide binding was enhanced by UV irradiation of microtitre plates prior to oligonucleotide incubation. Increased binding of antibody to DNA coated plates that were pre-treated by UV-irradiation was seen. Antibody was not found to non-specifically bind to UV-irradiated plates.

3.5.2.1 Non-competitive Assay of Serum

All of the serum collected from mice after immunisations with 15mer duplexes (AC15, AA15, CC15, GG15) gave some binding to DNA coated plates in non-competitive ELISA, using non specific coating methods 1A-D (see experimental). Antibodies were produced from immunisations using both free DNA and DNA complexed to MeBSA.

Only results for microtitre plates coated with oligonucleotide by means of UV-irradiation are reported here, see Graph 4 and 5. All values plotted were means of triplicates (standard deviation <5% for these and subsequent graphs). Each mouse yields only a small amount of blood for serum analysis, typically 5-40μl. A large number of assays were carried out using several of the coating methods before binding of antibody to DNA was observed in non-competitive ELISAs. For a number of the mice all of the serum obtained was used in non-competitive assays and thus competitive assays could not be carried out. Competitive assay were carried out to test sera from three mice.


GRAPH 4. ANTISERUM DILUTION CURVE FOR MOUSE CC1
OD-405nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
50
100
200
400
800
1600
3200
6400
12800
1 / ANTISERUM DILUTION


GRAPH 5. ANTISERA DILUTION FOR MICE GGM1-3
OD-405nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
50
100
200
400
800
1600
3200
6400
12800
1 / ANTISERA DILUTION
Mouse GGM1
Mouse GGM2
Mouse GGM3

3.5.2.2 Competitive Assay of Serum

A limiting dilution of serum obtained from mice CC15-1, GG15M-1 and GG15M-3, from the results of non-competitive ELISA, were used for competitive ELISA. The concentration of DNA added was 5μg/ml, see table 11.

Table 11, Competitive ELISA using Mice Sera

Test Serum	Serum Dilution	Inhibitor	% Inhibition
CC15-1	1:50	CC15	42
GG15M-1	1:200	GG15	30
GG15M-3	1:50	GG15	10

The results from competitive ELISA confirm that antibody was being produced that bound to both immobilised DNA duplexes and that this binding was inhibited in the presence of competing DNA. Although duplexes were chosen that contained the relevant mismatches, it should be noted, as previously discussed (section 3.4), the detection of antibody binding to a DNA duplex containing a mismatched base pair does not provide absolute identification of anti-mismatched base pair antibodies. This situation does not present itself for classical haptens, where inhibition in competitive assay does provide antibody binding identification.

Where competitive studies with DNA duplexes containing a mismatched base pair show no inhibition there is clearly no recognition of the mismatch. Where positive inhibition with a mismatch containing duplex occurs a further competitive ELISA using duplex DNA without a mismatched base pair could be carried out. If competitive assays gave no inhibition for duplexes without a mismatch, but inhibition occurred for a mismatch containing duplex, this would indicate the presence of anti-mismatched base pair antibodies. However, when immunogenic material is administered to animals a wide range of antibodies are released into the serum. There will be variations in both specificity as well as affinity of

antibodies in the sera. Thus, where an anti-DNA antibody response occurs, competitive assay of serum indicating inhibition with both mismatch containing and non-mismatch containing duplexes is highly probable. Therefore, until monoclonal antibodies are produced an absolutely conclusive screen for anti-mismatched base pair antibodies will not be possible. This problem is fundamental to this project and would occur whatever screening strategy was employed.

These assays indicate that the 15mer oligonucleotide duplex sequences being used as immunogen were evoking an immune response. Fusion experiments were conducted using the spleens of mice where competitive ELISA of sera showed inhibition.

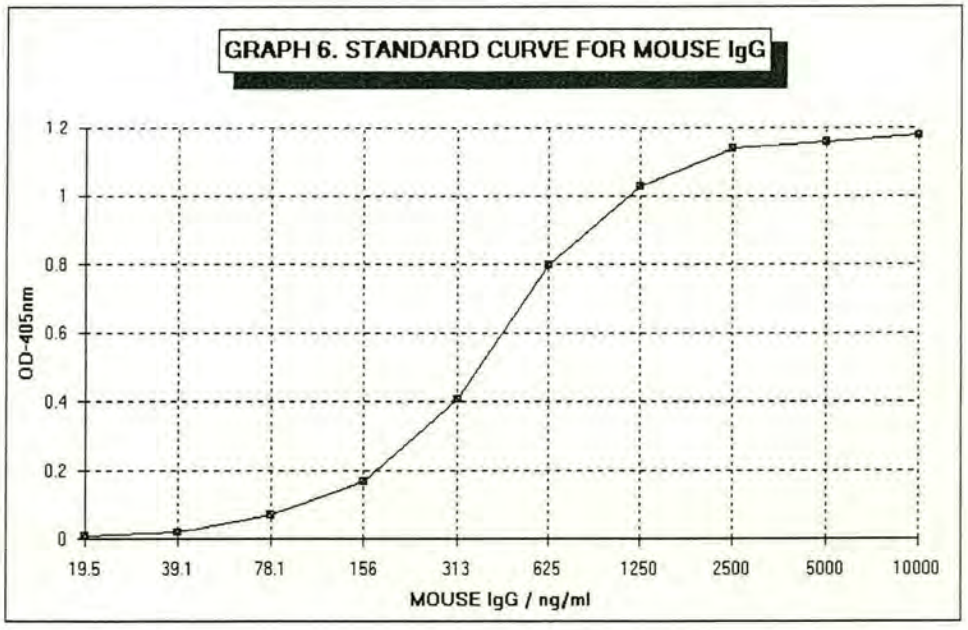
3.5.3 Fusion Experiments

After fusion experiments the cells were observed under a microscope for the first two weeks post fusion. The first fusion carried out (mouse spleen GG15M-1) failed to produce any actively growing hybrid cells. They were discarded after 10 days. At this time fusions were routinely working when carried out by more experienced staff on other projects. Therefore, there was no concern regarding any reagents being used. Due to the failure of the first fusion it was decided prudent to practice the fusion procedure.

Spleens from non-immunised mouse could be used, but if successful would produce no useful material. Several of the sera obtained from mice immunised with 15mer duplex were only screened using the non-competitive ELISA, see section 3.5.2.1. All sera (CC15-1, GG15M-1 and GG15M-3) tested in competitive ELISA gave inhibition. It was thought reasonable to assume some of the other supernatants would have shown inhibition. Five fusion experiments were carried out prior to fusions on CC15-1 and GG15M-1. All five test fusions and subsequent fusion experiments produced live hybrid cells immediately post-fusion. In addition, plasmacytoma and splenocytes were observed to die over the first five days.

When hybrid colonies were sufficiently large, just visible without the aid of a microscope (~ 0.5-1.0mm diameter), samples of the growth medium were removed so that antibody secreted into it could be measured. The growth medium of actively growing cells were first screened for the presence of mouse immunoglobulin G (IgG), IgG ELISA. Positive IgG producers were then screened for the presence of anti-DNA antibodies.

The IgG screen in the format used serves to establish the wells producing IgG so that supernatant from these wells could be selected for further investigation. Typically, this assay reduced the number of test wells for anti-DNA assays by about 20-40% of the total number of wells produced by fusion experiments. The levels of antibody in established hybridoma culture supernatants are typically 10-75 µg/ml. Fusion wells may contain less than this, but the assay was able to detect 0.1µg/ml of mouse IgG, see Graph 6, and thus all IgG producers should be detected.



Usually greater than 50% of the wells contained colonies that were sufficiently large to be screened at about the same time. Using the ELISA format it was more convenient to screen all fusion wells at

the same time. Where supernatants from fusion wells gave no binding in the IgG ELISA were kept if the well contained very small colonies. This would be re-screened when the colonies increased in size.

3.5.3.1 15mer Oligonucleotide Duplexes

Fusion experiments produced hybrid cell lines that were :

(a) actively growing in cell culture medium,
(b) secreting mouse IgG ;
(c) secreting IgG that recognises some structural component of DNA.

Table 12. 15mer Fusion Assay Results

MOUSE/IMMUNOGEN	IgG ASSAY	DNA ASSAY
AC15-1	Positive	Positive
AC15M-1	Positive	Positive
AC15M-2	Positive	Positive
AA15-1	Positive	Positive
CC15-1	Positive	Positive
GG15M-2	Positive	Positive
GG15M-3	Positive	Positive

The DNA assay was a non-competitive ELISA using the appropriate mismatch containing duplex as coating material. Competitive ELISA failed to identify any colonies producing anti-mismatch base pair specific antibodies. For the five test fusions no competitive assays were carried out using the sera. It was unknown if inhibition was likely to occur. For GG15M-3 the inhibition seen when screening the sera was small, only 10%. This low inhibition may not be significant. Alternatively it may reflect a limited immune response. This means relatively few spleen cells would be producing the relevant antibodies. Fusion experiments rely on immunisation boosting the numbers of relevant splenocytes.

Significantly, when spleens were obtained from mice immunised with DNA/MeBSA complexes (e.g. GG15M-3) all the spleens were approximately double the size of the spleens after immunisations with non-complexed DNA. The MeBSA may be forming the immunodominant regions of the immunogen. The use of MeBSA to complex DNA may be useful to produce a polyclonal antisera containing anti-DNA antibodies, but may be less useful for producing monoclonal antibodies.

As competitive assays using the supernatants from hybridomas obtained by fusion experiments were being conducted for mice immunised with 15mer duplexes it became apparent that modifications in the ELISA and fusion procedure needed to be addressed. The UV-irradiation procedure coats the DNA in a random orientation onto the surface of the test wells. This may obscure or alter a mismatch base pair recognition site. Alternative assay procedures need to investigated.

Secondly, the cells growth medium contains a high concentration (10%) of foetal calf serum (FCS). When supernatants were removed from growing cells the FCS will be a potential source of nuclease enzymes. No positive competitive inhibition studies were obtained from supernatants where fusions were carried out using spleens from mice immunised with 15mer duplexes. The presence of FCS may have significantly influenced competitive ELISA results.

3.5.4 75mer Oligonucleotide Duplexes and C100P

At the same time as alternative coating methods 2(A-C) involving the use of biotin/streptavidin binding were being investigated, alternative immunogens were produced. These were AC75, GC75 and C100P, see chapter 2. Immunisations were carried out using these immunogens without the formation of MeBSA complexes.

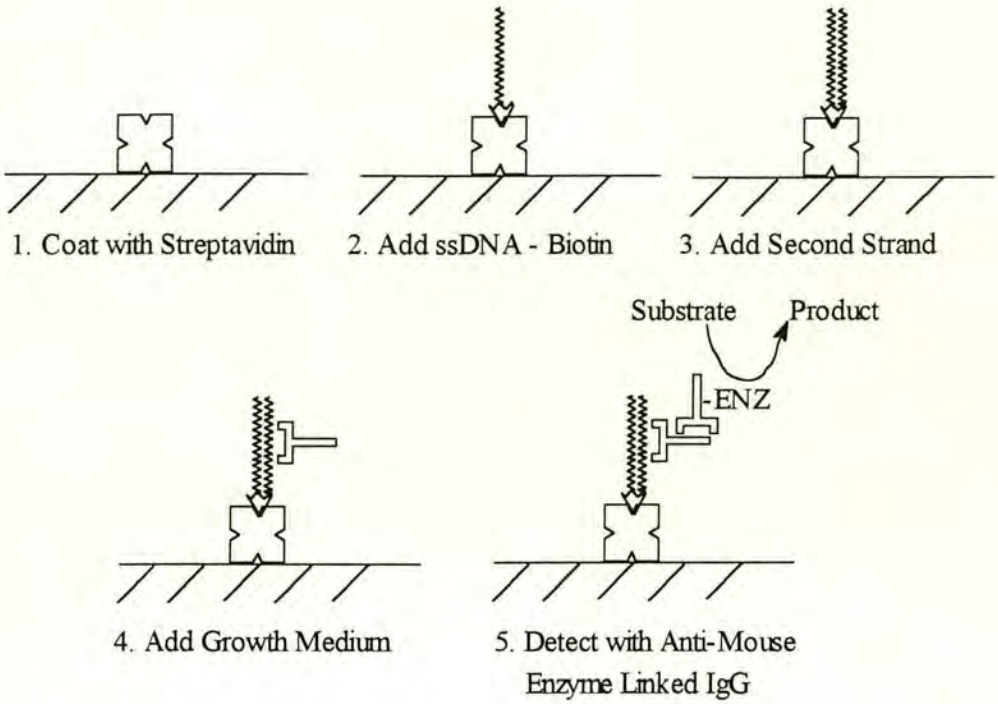
3.5.5 Assay Development using Coating Methods 2(A-C)

Alternative coating procedures included the use of streptavidin and biotinylated oligonucleotide, coating methods 2(A-C). The streptavidin/biotin binding system was chosen due to the strong and selective affinity of the complex $K_D = 10^{-15}$. The complex is stable at extremes of pH, and various buffer conditions, making the reaction essentially irreversible. Although avidin could have been used, streptavidin was preferred. Streptavidin lacks the carbohydrate residues associated with avidin. Although these residues would aid binding of avidin to microtitre plates, they may also increase non-specific binding of other assay components during the assay. The isoelectric point of streptavidin is in the neutral range. This is the pH at which most ELISAs are carried out so this will reduce non-specific interactions during the assay.

3.5.5.1 Confirmation of Duplex Formation by Coating Method 2(A).

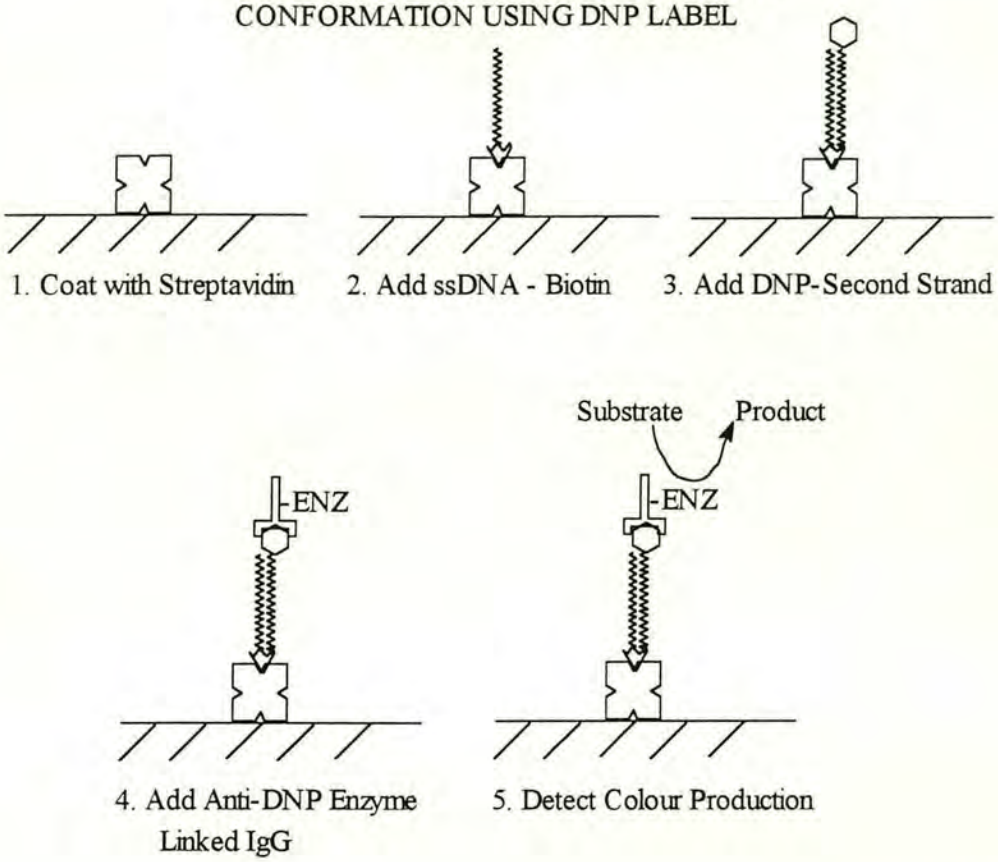
Coating method 2(A) allows a specifically 5'- biotinylated single strand oligonucleotide to bind to plates precoated with streptavidin (see figure 24). A second strand, either fully complementary or containing a single base pair mismatch, was introduced.

FIGURE 24. BIOTIN/STREPTAVIDIN ELISA

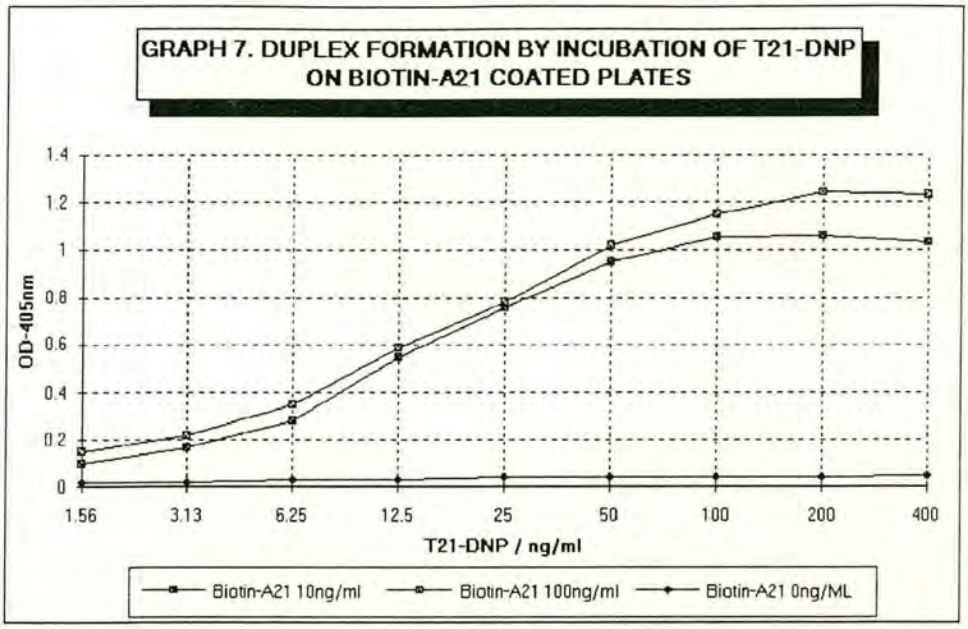


To ensure the binding of the second strand was occurring an additional series of 5'- specifically labelled second strand were produced. These second strands would also form either fully complementary duplexes or would contain a central base pair mismatch. The label used was Dinitrophenyl (DNP), and detection of DNP was *via* an enzyme labelled anti-DNP antibody, and colour development. The use of DNP labelled oligonucleotides would provide information on first strand binding to streptavidin coated plates and second strand forming a duplex, (see figure 25).

FIGURE 25. BIOTIN/STREPTAVIDIN ELISA CONFORMATION USING DNP LABEL



For fully complementary strands a dose response curve was obtained, see Graph 7. As the concentration of DNP labelled oligonucleotide (T21-DNP) was increased, the amount of DNP detected also increased. In the absence of Biotin-A21 no binding of T21-DNP was observed. This indicates that duplex formation was responsible for the availability of DNP detection. The binding of T21-DNP appears to reach saturation at greater than 50ng/ml T21-DNP.



These experiments were repeated using DNP labelled oligonucleotides similar to T21-DNP but the central base was replaced with A or C (TAT-DNP and TCT-DNP respectively). When incubated on Biotin-A21 coated plates they would create an AA or AC base pair mismatch. Unfortunately, weaker dose response curves were obtained where duplexes were formed using these sequences. This means that the amount of duplex oligonucleotide formed was less where a mismatch was formed on introducing the second strand. Although there will still be duplex oligonucleotide present, in this format there will also be a proportion of biotinylated single stranded oligonucleotide in the assay.

The T_m of two non-covalently attached single stranded oligonucleotides forming a duplex is also dependent upon the concentration of the duplex. The concentration of oligonucleotide used to coat wells in ELISA experiments is very low and the amount of oligonucleotide that actually binds is even less. The T_m of two non-covalently attached single stranded oligonucleotides forming a duplex with a mismatched base pair is considerably lower than that for a fully matched sequence. The anti-DNA assay also has an extra incubation and wash step compared with the anti-DNP detection

assay. Once the oligonucleotide duplex is formed in coating the plates and excess unbound oligonucleotide is washed away, the concentration of oligonucleotide will be so small that any dissociated strands will never re-associate.

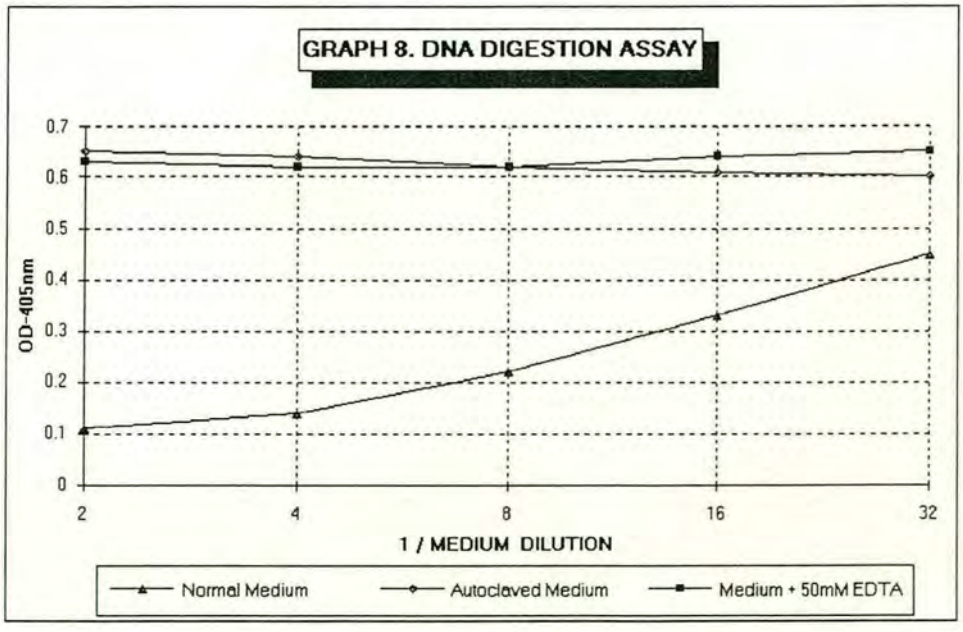
Additionally, it cannot be assumed that biotin-A21, or any other biotin labelled oligonucleotide immobilised, *via* the biotin, at one end to a solid phase, presents all bases for hybridisation to another complementary oligonucleotide. The first, or even second, base might be sterically hindered by its proximity to the streptavidin. This will cause the incoming second strand to slightly overhang the biotin immobilised strand. Thus a mixture of fully matched DNA duplex, a mismatch base pair site and single stranded DNA may be presented for antibody recognition even where duplex formation has occurred. The uncertainty surrounding the completeness of duplex oligonucleotide and the presence of single stranded oligonucleotide means that this assay format would not be suitable for our requirements.

The use of streptavidin/biotin labelled oligonucleotides was modified to produce a more effective coating procedure. A DNA duplex was synthesised to be of a similar nature to the 15mer duplexes used as immunogen in previous experiments, stabilised by an inter strand linker, but with an additional 5'-terminal biotin attached. The T_m of the biotin-AC15mer duplex (AC15B) was 50°C in UV melting studies. This was the same as the unbiotinylated AC15 duplex. Duplex melting is a unimolecular process and, therefore, independent of oligonucleotide concentration, as previously described. Hence, when AC15B binds to streptavidin coated plates it will remain as a duplex. This coating method was used for anti-DNA experiments with subsequent fusions.

3.5.5.2 Detection of Nuclease Enzymes by DNA Digestion Assay

3.5.5.2.1 Nuclease Degradation

In an assay system in which oligonucleotides are coated with random orientation 'effectively flat' (methods 1(A-D)) onto the surface of microtitre plates, the oligonucleotides are probably not available for interaction with nuclease enzymes. However, in competitive assays and where the oligonucleotides are coated by the biotin/streptavidin method, oligonucleotides will probably be more accessible to nuclease enzymes. A DNA digestion assay was carried out to investigate the extent of oligonucleotide degradation. Plates coated with single stranded DNA (cytosine 20mers end labelled with biotin, (C20B) were incubated with cell culture medium, autoclaved cell culture medium, and cell culture medium containing EDTA (a nuclease inhibitor). DNA remaining undigested was subsequently detected using a commercially available anti-DNA antibody, see Graph 8.

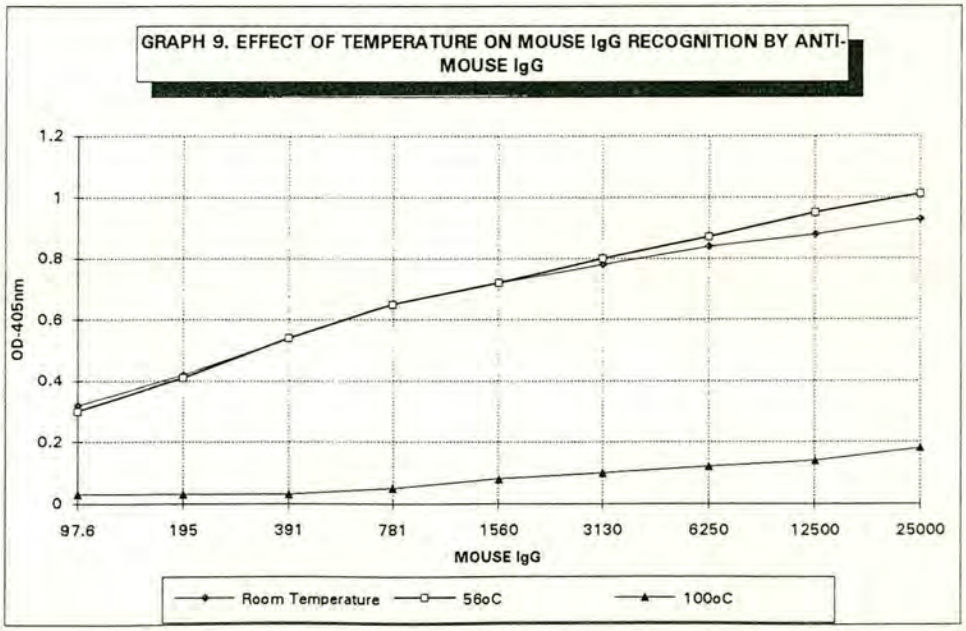


The results of the DNA digestion assay clearly shows that DNA digestion occurs with concentrated medium, but that the amount of digestion is reduced by :

- decreasing the concentration of the medium, i.e. diluting the enzyme concentration;
- heat inactivation by autoclaving;
- addition of EDTA as a nuclease inhibitor.

The effect of nuclease enzymes could be reduced by any of the above measures. However, supernatants from cell lines may only contain small amounts of antibody. Dilution of the supernatants may reduce the concentration of antibody such binding during ELISA was no longer detected. Anti-DNA antibody producing cell lines may then be missed, especially if low affinity antibodies are being produced. B-DNA is known to be poorly immunogenic. The enhancement of immunogenicity by the presence of a mismatch is unknown. The amount of dilution before loss of signal occurs will vary greatly between cell lines/antibody produced.

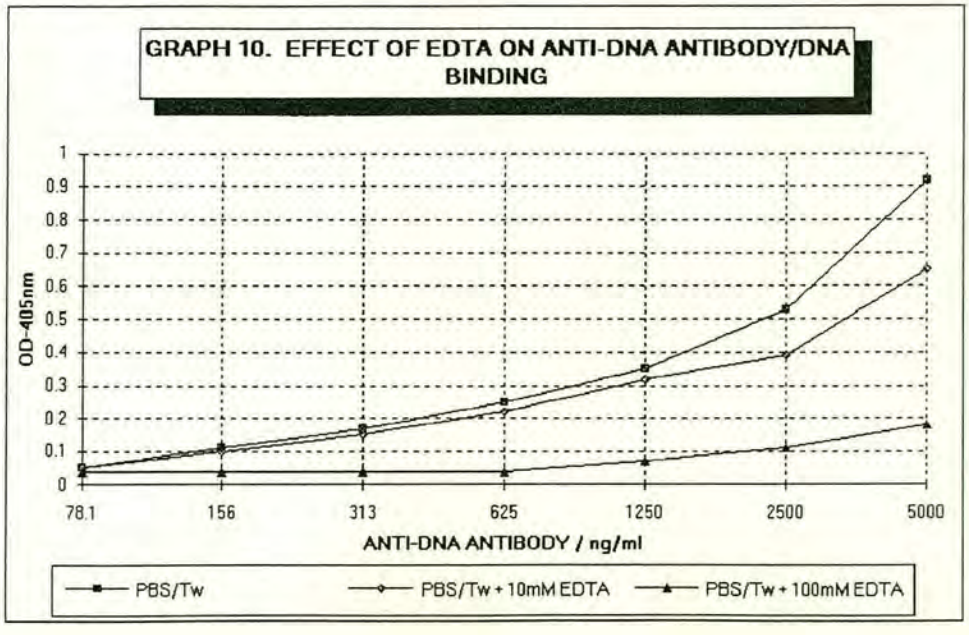
Heat inactivation of supernatants by autoclaving would mean loss of antibody function, so this is unsuitable. We have shown, see Graph 9, that a commercially available murine IgG can be heated to 56°C, for 30 minutes, and still be recognised as a murine monoclonal, using an anti-murine IgG assay.



EDTA is used as a nuclease inhibitor in most molecular biological work and was adopted for use. Unfortunately the first set of fusion assay results using EDTA in all wash and buffer solutions gave low end point colour production. The use of EDTA was further investigated to establish its suitability in anti-DNA ELISAs.

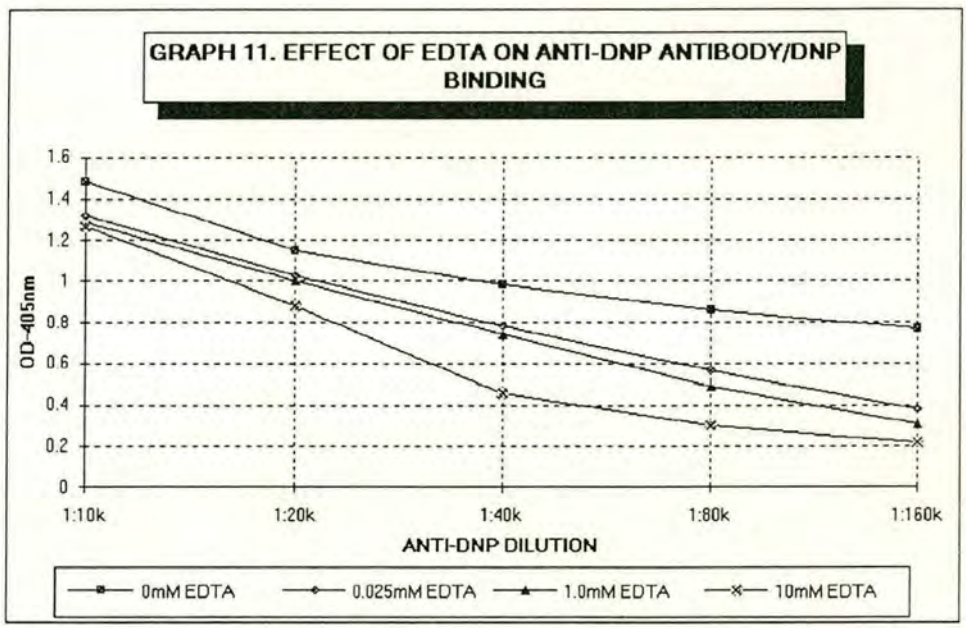
3.5.5.2.2 The Effect of EDTA using an Anti-DNA Antibody.

Biotin end labelled single strand (C20B) was attached to streptavidin coated plates. Anti-DNA antibodies at various concentrations were then allowed to bind with the immobilised oligonucleotide. All of the antibody incubation steps and wash steps were carried out using a buffer containing 0, 10 and 100mM EDTA. Graph 10 shows the results of including EDTA in an anti-DNA antibody ELISA, the immobilised DNA being detected was the single stranded oligonucleotide (C20B). The antibody used was commercially available, not an in house product of previous work. It is clear that 100mM EDTA completely abolishes detection of antibody binding. In the absence of EDTA antibody binding was detected at anti-DNA antibody concentrations between 1.0 and 5.0µg/ml. In the presence of 10mM EDTA the amount of binding was reduced by about 50%.



3.5.5.2.3 Effect of EDTA on Anti-DNP Antibody/DNP Binding

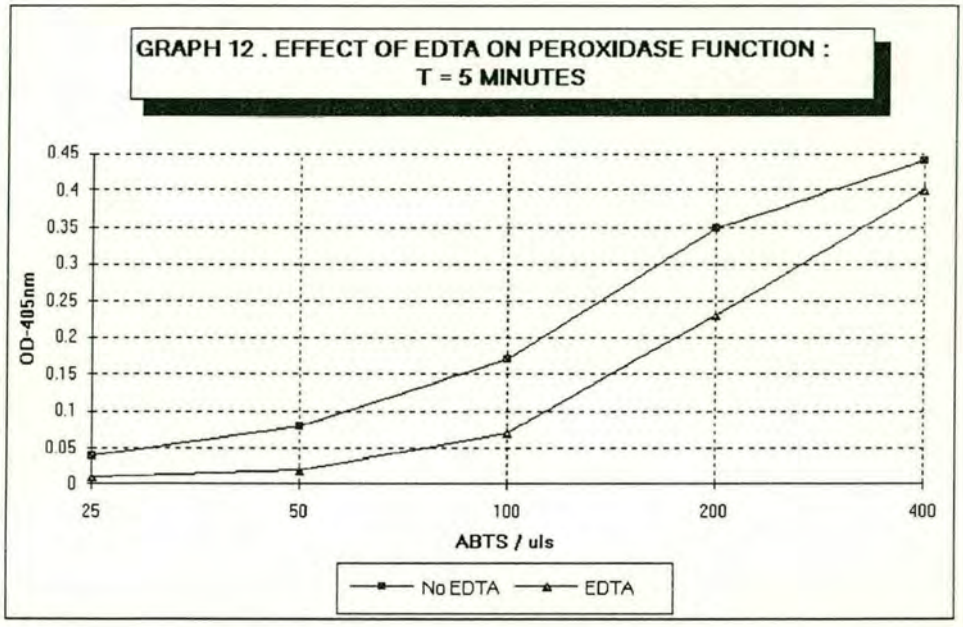
The ELISA was investigated to establish where EDTA was affecting the assay, the anti-DNA antibody/DNA interaction or some other component of the assay. The assay was carried out using a duplex oligonucleotide (AT10BL) labelled at one end with biotin and the other with DNP. Graph 11, shows the result of EDTA added to the buffers and wash solutions using an oligonucleotide duplex, labelled at one end with biotin to allow binding to streptavidin coated plates, and at the other end with DNP to allow detection *via* an anti-DNP antibody.

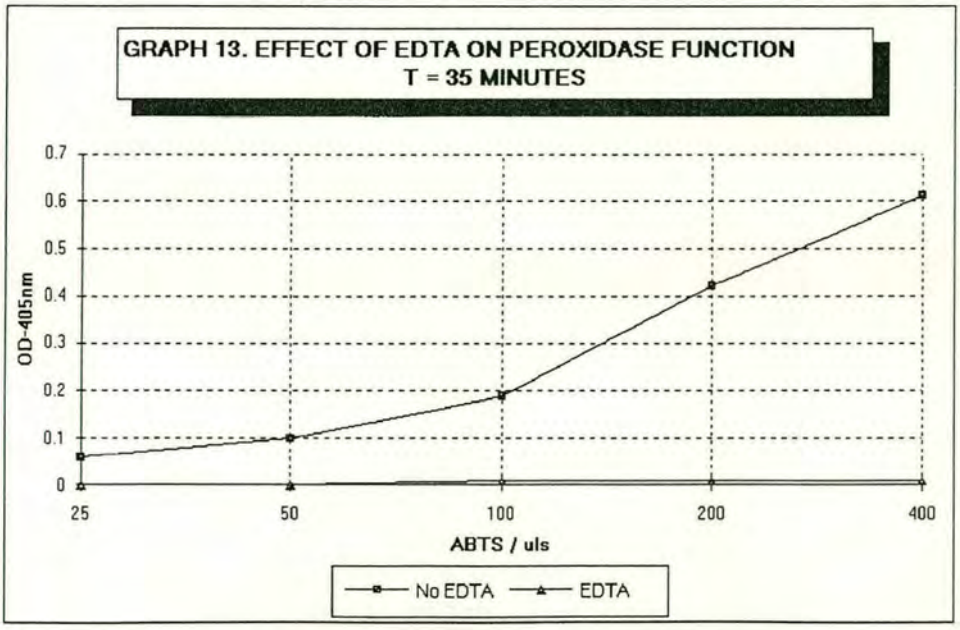


In the absence of EDTA a dose response was observed as anti-DNP antibody was diluted. As the amount of EDTA added was increased there was a corresponding decrease in signal at all anti-DNP antibody dilutions investigated. It can clearly be seen that the presence of as little as 25 x 10^{-3}mM EDTA causes a deleterious effect in the assay. It was uncertain as to the nature of the effect. Although a similar effect was reported for other anti-DNA ELISAs (Brosalina *et al.*, 1988; Pyun *et al.*, 1993), it was not reported for anti-DNP assays. It was decided to look at the effect of EDTA on peroxidase function specifically.

3.5.5.2.4 Effect of EDTA on the Enzyme Activity of Antibody-Peroxidase Conjugate with ABTS

The peroxidase conjugated second antibody used to detect anti mouse IgG was incubated with various concentrations of ABTS (the peroxidase enzymes substrate used in our ELISAs) with and without the addition of 1mM EDTA. Graph 12 and 13, shows the effect of EDTA on peroxidase function after 5 minutes and 35 minutes.





After 5 minutes there was an increase in colour development with increasing ABTS substrate with or without EDTA. Although EDTA does appear to have a slight inhibitory effect on peroxidase function at all ABTS concentrations. In the absence of EDTA the ABTS continued to be converted to a colour product at all ABTS concentrations, graph 13. However, in the presence of EDTA the green product did not increase over the time course of the assay (35 minutes). The amount of coloured product did not simply remain low (similar to amounts seen at 5 minutes), rather it actually decreased to virtually zero at all concentrations of ABTS used. EDTA must alter the enzymic activity of horseradish peroxidase, or it interacts with and alters the coloured product of the reaction.

As colour production does occur initially the enzyme must be at least partially functional. EDTA may form a colourless complex with the coloured product of the reaction? Alternatively it may allow the enzyme to further interact with the ABTS product forming a secondary, colourless product? Whatever the cause, EDTA clearly cannot be used at this stage of the assay. It was decided that the buffers and wash steps used after supernatant incubation would have to be carried out in the absence of EDTA. It is believed the supernatant will be most likely source of nuclease related problems. Additionally, once anti-DNA antibody binding to DNA has occurred the antibody will probably afford sufficient protection to the DNA of any further nuclease interaction with DNA.

3.5.5.3 Immobilisation of AC15B

When using the commercially available anti-DNA antibody to test the effects of EDTA, we obtained weaker signals if AC15B was used, and stronger signals if the single strand C20B was used to coat plates. It was uncertain if this reflected the fact that the duplex was weakly antigenic for the anti-DNA antibody, compared to C20B, or if the duplex was not binding to streptavidin coated plates efficiently. Biotin is internalised by streptavidin, positioned 9.0 angstroms below the surface of the protein. Single stranded DNA may be sufficiently flexible to allow binding of end labelled biotin to streptavidin. By comparison duplex DNA is far more rigid and binding between biotin

and streptavidin may be sterically hindered giving reduced interaction at the binding site, and hence reduced coating of duplex DNA. Although the commercially available biotin phosphoramidite monomer contained a linker arm, it was decided that extension of this linker may enhance DNA duplex coating.

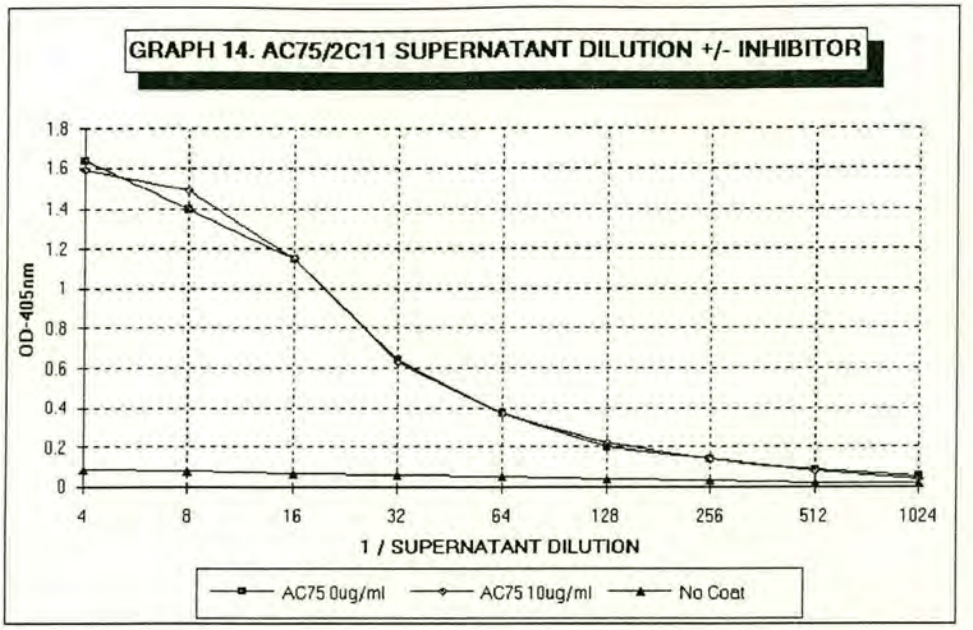
3.5.5.3.1 75mer DNA Duplex and C100PS

Before synthesis of this modified biotin labelled oligonucleotide could be carried out, two fusion experiments had been carried out using the spleens from AC75 immunised mice and one fusion from a C100P immunised mouse. The growth medium of hybridomas formed were awaiting screening. Cytosine 100mer (C100PS) was synthesised as a single strand to raise antibodies that may interact with cytosine containing mismatches. Therefore, it may be possible to immobilise antibodies to cytosine containing mismatches, such as AC or CC, by coating with single stranded cytosine (C20B). Fusion wells from AC75 and C100PS immunisations were subsequently tested for anti-DNA activity using plates coated with AC15B duplex and C20B single stranded DNA.

After visual analysis the IgG ELISA and non-competitive anti-DNA ELISA all three fusions produced hybrids that were actively growing in cell culture medium, were secreting mouse IgG, and were secreting IgG that recognised some structural component of DNA.

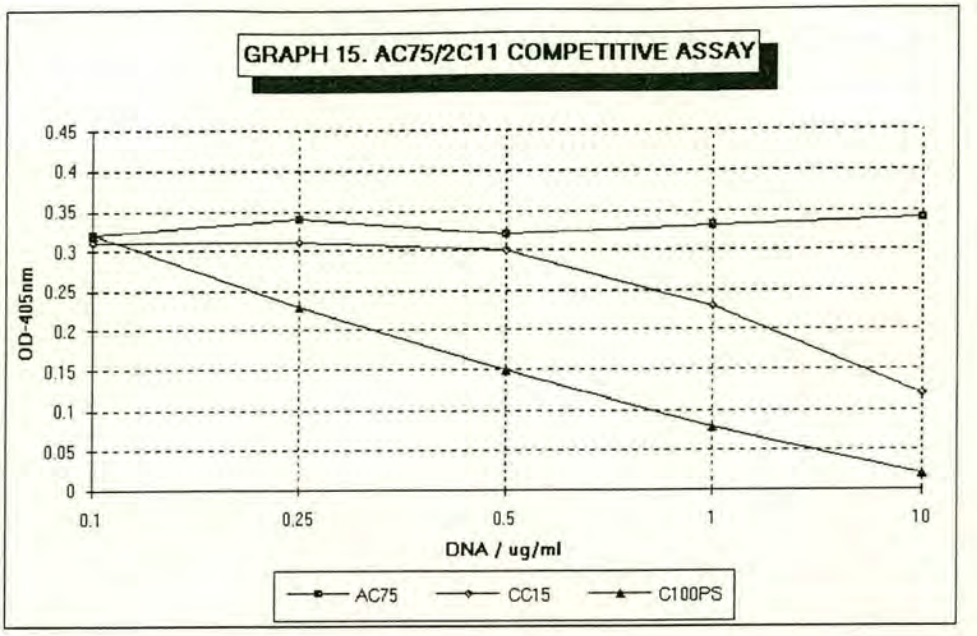
3.5.5.3.1.1 AC75mer Duplexes

Antibody production was screened for using C20B and AC15B as coating material. Increased amounts of antibody binding were observed when C20B was used compared to AC15B duplex as coating material. For several supernatants tested antibody binding was detected using C20B, but was negligible using AC15B. The supernatant from only one well gave inhibition in competitive studies. The results of a series of competitive assays obtained using the growth medium of fusion well 2C11 (well location coded AC75/2C11) using C20B as coating material are shown in Graphs 14 and 15.



Graph 14 shows the result of a competitive assay for supernatant AC75/2C11 at a range of supernatant dilutions. In the absence of competing DNA (AC75) a decrease in antibody binding was observed as the supernatant was diluted. In addition in the absence of C20B as coating material no non-specific binding was detected. Antibodies are thus recognising and binding to some component of the DNA coat (single stranded cytosine). However, no inhibition of antibody binding was detected when incubation of supernatant with AC75 (10μg/ml) was carried out.

Graph 15 shows the result of a competitive assay at a fixed, and limiting, supernatant dilution for AC75/2C11 using several concentrations of AC75, CC15 and C100PS as inhibitor. As expected antibody binding was not inhibited when AC75 was used as competitor at 0.1-10μg/ml. Inhibition of antibody binding was detected when using either C100P single strand and CC15mer duplex as competitor. The inhibition with C100PS was greater than that for CC15. This may reflect the greater number of cytosine bases in C100PS. CC15 and C20B contain no phosphorothioate bases, therefore, the response appears to be directed towards normal DNA. The antibodies do not appear to be recognising AT matched pairs or AC mismatched pairs. Therefore, it appears that the antibodies are recognising the CC mismatched base pair.



Cloning experiments were carried out in attempts to produce a monoclonal cell line from this fusion well. Cloning experiments produced many wells in which single colonies were observed. However, none of them produced antibodies that showed inhibition to any test antigens AC75, CC15 or C100PS, at 10μg/ml. Problems associated with cloning experiments will be discussed later in this chapter and chapter 4.

3.5.5.3.1.2 C100PS Single Strand

One of the fusion wells (3C6) from mice immunised with C100PS single stranded oligonucleotide produced antibodies that bound to the coating material (single stranded C20B) and gave positive inhibition with AC75 in competitive ELISA. Cells from well 3C6 were used for cloning experiments in an attempt to produce a monoclonal cell line. Cloning experiments produced 27 wells containing single colonies, 10 of these produced antibody that bound to the coating material and, therefore, competitive assays were carried out, see Table 13.

Table 13, C100P/3C6 First Clone Test Results

WELL	IgG ASSAY	DNA ASSAY	% INHIBITION
D10	Positive	Positive	0
E1	Positive	Positive	46.7
G7	Positive	Positive	60.0
G9	Positive	Positive	60.3
G11	Positive	Positive	49.7
G12	Positive	Positive	47.4
H1	Positive	Positive	57.1
H2	Positive	Positive	34.4
H3	Positive	Positive	61.8
H6	Positive	Positive	49.3

Inhibitor was AC75 10μg/ml

Nine of the wells produced antibody that gave positive inhibition with AC75 in competitive ELISA. Although there was variation in the amount of inhibition produced by the supernatant of each well, this does not necessarily mean that different monoclonal cell lines have been produced. The variation in percentage inhibition may reflect differences in cell numbers and/or antibody titres when the cell growth medium was sampled.

These results clearly demonstrate that an antibody produced recognises structural features of single stranded DNA, and a duplex composed of AT base pairs and AC mismatched base pairs. Inhibition studies were then conducted using GC75 as inhibitor. GC75 was an identical duplex to AC75 except that the four AC mismatched base pair sites in AC75 were replaced with GC matched base pairs. Similar inhibition results were obtained using this non-mismatched duplex in competitive ELISA as for those using mismatch containing duplexes. Therefore, these monoclonal antibodies do not specifically recognise mismatched base pairs in dsDNA.

3.5.6 Enhanced Biotin - Duplex Immobilisation

The previous results were obtained using ssDNA as coating material, while testing for mismatches in DNA duplexes. Although anti-DNA antibody production was detected, the use of ssDNA as coating material was not desirable and some cell lines producing anti-DNA mismatched base pairs antibodies may be missed.

Oligonucleotide sequences similar to the biotinylated 15mer duplex, but containing an extra interstrand linker between the biotin and the oligonucleotide were synthesised. The inclusion of the extra interstrand linker should reduce possible steric hindrance and allow DNA duplexes to be bound to plates efficiently. Sequences of this nature were synthesised containing AC and GT mismatches. All subsequent fusions were screened using this assay. Sequences containing a dinitrophenyl group were also synthesised to allow optimisation of conditions for the binding of this modified oligonucleotide to streptavidin coated plates.

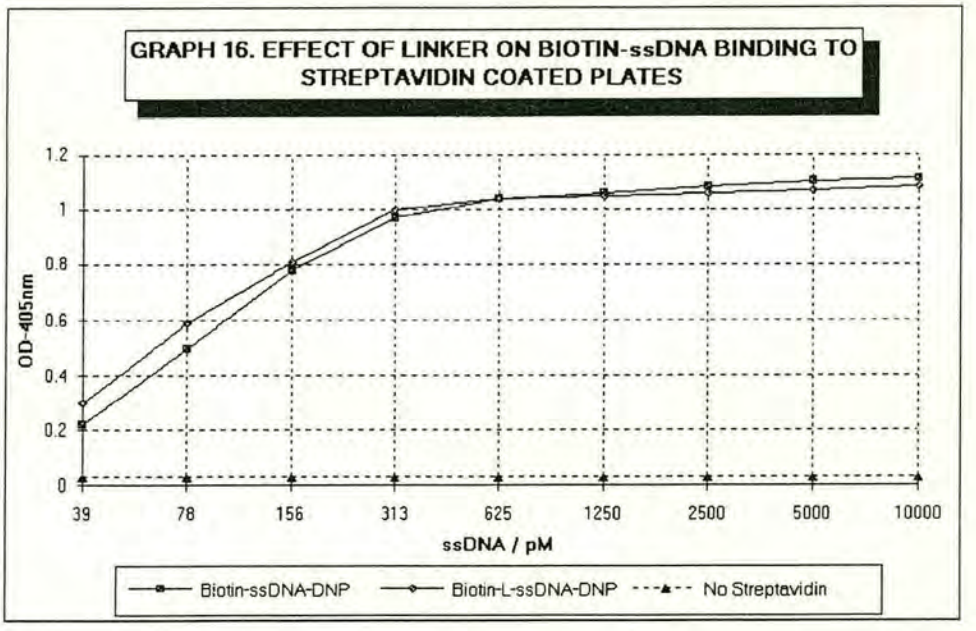
3.5.6.1 Optimisation of Assay Conditions

To investigate the possibility that steric hindrance of the binding of the biotinylated oligonucleotides to streptavidin coated onto microtitre plates was occurring, a series of modified oligonucleotides were synthesised, see Table 14. Oligonucleotides were synthesised such that single strands and duplexes would present DNP at one end, and biotin directly linked (as previously carried out) or biotin linked to the oligonucleotide *via* an extended spacer arm (to reduce steric hindrance) at the other.

Table 14, Oligonucleotides for Assay 3 Optimisation

NAME	SEQUENCE
C10B	DNP-C_{10}-Biotin
C10BL	DNP-C_{10}-X-Biotin
C11B	DNP-C_{11}-Biotin
AT10B	A_{10}-DNP-X-T_{10}-Biotin
AT10BL	A_{10}-DNP-X-T_{10}-X-Biotin

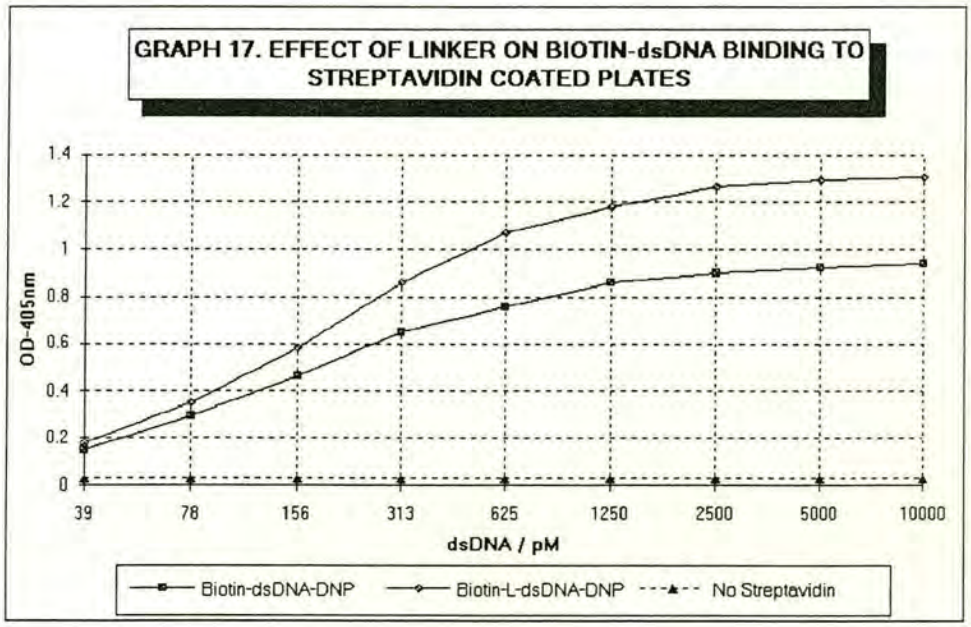
Oligonucleotides C10B and C10BL were synthesised to investigate whether the incorporation of a linker between biotin and the oligonucleotide increased the amount of oligonucleotide binding to streptavidin coated plates. Graph 16 shows a comparison of the binding of these two strands, at a variety of oligonucleotide concentrations (100-0.39 $x10^{-10}$M). Binding of oligonucleotides to the solid phase was detected using a peroxidase conjugated anti-DNP antibody, described previously (sections 3.5.5.2.3). As the amount of biotin-ssDNA-DNP added to coated wells was increased the amount detected increased. Binding reached a maximum at above 300pM of DNA. This probably reflects saturation of streptavidin binding sites.



The binding of the oligonucleotide with an extended linker resulted in almost identical binding. The attachment of biotin *via* a linker to single strands was clearly unnecessary, as biotin directly attached to the oligonucleotide did not appear to cause steric hindrance. In the absence of streptavidin coating neither type of ssDNA was found to bind, at all concentrations of ssDNA.

The presence of the linker in C10BL displaces the DNP group from the solid phase to a greater extent compared to the DNP in C10B. To confirm that these observations reflected the effect of biotin linker on streptavidin binding and not proximity of the DNP group to a solid phase, these experiments were repeated using C11B. The binding of C11B, C10B and C10BL to streptavidin coated plates at all oligonucleotide concentrations tested were equivalent, results not shown.

The above experiment was repeated using oligonucleotide duplexes, AT10B and AT10BL, instead of ssDNA. Graph 17 shows the result of the dilution of these duplexes on streptavidin coated plates. There was clearly a difference in the amount of binding between the two duplexes. The duplex with biotin attached *via* a linker (AT10BL) binds to a greater extent at all concentrations under the assay conditions. It should be noted that the duplex with biotin directly attached does bind to streptavidin coated plates.



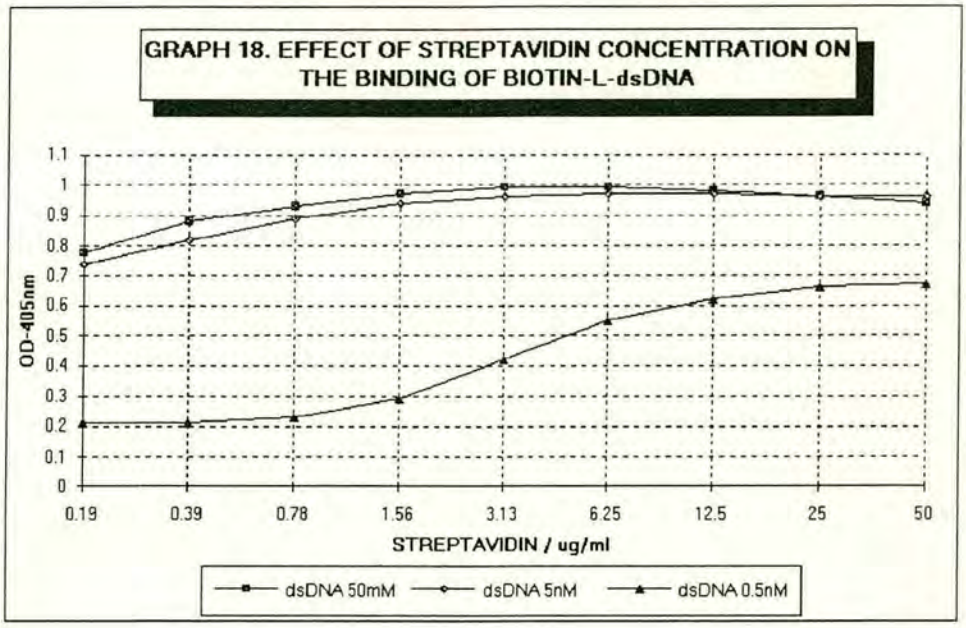
For both duplexes the amount of bound material becomes saturated. The maximum binding of AT10BL was at a concentration of 5 x10^{-9}M. This represents an approximate 10 fold reduction in

concentration of oligonucleotide compared to that used in previous experiments. The use of DNP labelled oligonucleotides allowed us to confirm that the duplexes were bound to the solid phase. Control experiments using AT10BL showed that in the absence of streptavidin no binding of duplex was detected, see graph 17.

The peroxidase conjugated anti-DNP labelled antibody was also shown to give no binding to either streptavidin coated plates or non-DNP labelled oligonucleotides attached (*via* biotin) to streptavidin coated plates. In addition, incubations with non-biotinylated oligonucleotides labelled with DNP using the previous assay conditions produced no colour end point. This indicates that the assay specifically involves biotin labelled oligonucleotides binding to streptavidin coated plates. As DNP labelled oligonucleotides could be used to monitor the binding of oligonucleotide duplexes it was decided to use these to optimise assay conditions.

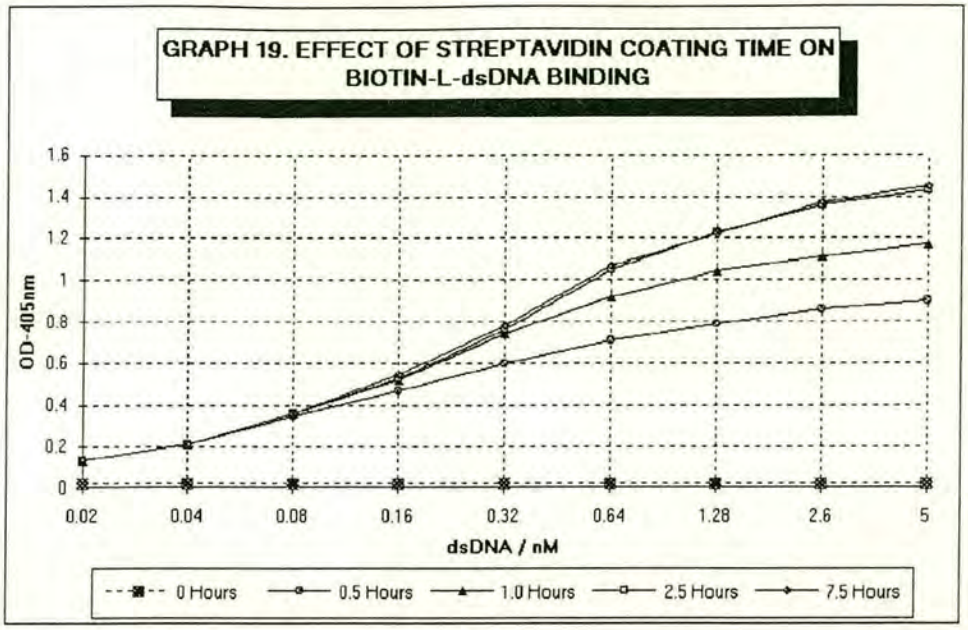
3.5.6.1.1 Effect of Streptavidin Concentration on Duplex Binding.

Streptavidin was used to coat microtitre plates at a variety of concentrations (50-0.2μg/ml). Each streptavidin coated plate was then incubated with AT10BL (0.5, 5.0, and 50 $x10^{-9}$M). Binding was detected in the usual way *via* the presence of DNP groups. Graph 18 shows the effect of various coating concentrations for streptavidin at three oligonucleotide concentrations. There appears to be no difference in oligonucleotide binding for AT10BL between 5 and 50 $x10^{-9}$M, for all of the streptavidin concentrations. Maximum binding occurs for streptavidin coated at 1μg/ml and AT10BL at 5 $x10^{-9}$M. At the lower AT10BL concentration (0.5 $x10^{-9}$M) there was a clear dose response dependant upon the concentration of streptavidin used. However, the maximum binding at 0.5 $x10^{-9}$M AT10BL was less than that for 5 $x10^{-9}$M AT10BL.



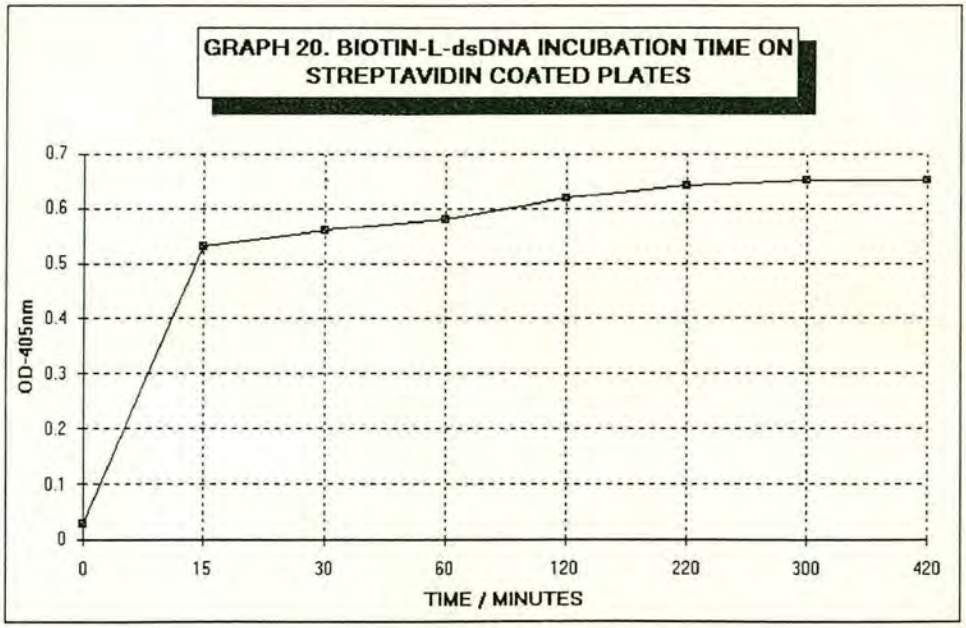
3.5.6.1.2 Effect of Streptavidin Coating Time.

Streptavidin (1μg/ml) was used to coat microtitre plates for 0, 0.5, 1.0, 2.5, 5.0, 7.5, 15, and 24 hours. Duplex oligonucleotide AT10BL at various concentrations (5.0-0.02 $x10^{-9}$M) was allowed to bind. Graph 19 shows the result of duplex binding at various streptavidin concentrations. In the absence of streptavidin, no binding for any AT10BL concentrations tested was observed. Maximum binding occurred at 2.5 hours with AT10BL at a concentration of 5.0 $x10^{-9}$M. Incubation of streptavidin for 7.5, 15 or 24 hours gave no increase in duplex binding.



3.5.6.1.3 Effect of Incubation Time on AT10BL Binding.

Plates were coated with streptavidin (1μg/ml) for 2.5 hours, and then incubated with AT10BL (5.0×10^{-9}M) for a time scale of 0-420 minutes. Graph 20 shows the effect of AT10BL binding versus incubation time. Binding of AT10BL was very rapid (about 80% bound in the first 15 minutes). The binding eventually reached a maximum after 120 minutes.



The coating of the biotin-linker-oligonucleotide duplex to streptavidin coated plates was now optimised as outlined below. Plates were coated with streptavidin (1μg/ml) for 2.5 hours, and then incubated with appropriate oligonucleotides (5.0×10^{-9}M) for 2 hours. For screening the antibodies produced by AC75 immunisations AC30BL was used to coat microtitre plates, for GT75 immunisations GT30BL was used, and for C100P immunisations AC30BL and C20BL were used. C20BL was used as antibodies that may bind to this may also bind to CC75 duplexes (containing CC mismatched base pairs). The duplex CC30BL was not available at this time.

3.5.7 Use of Serum Free Medium

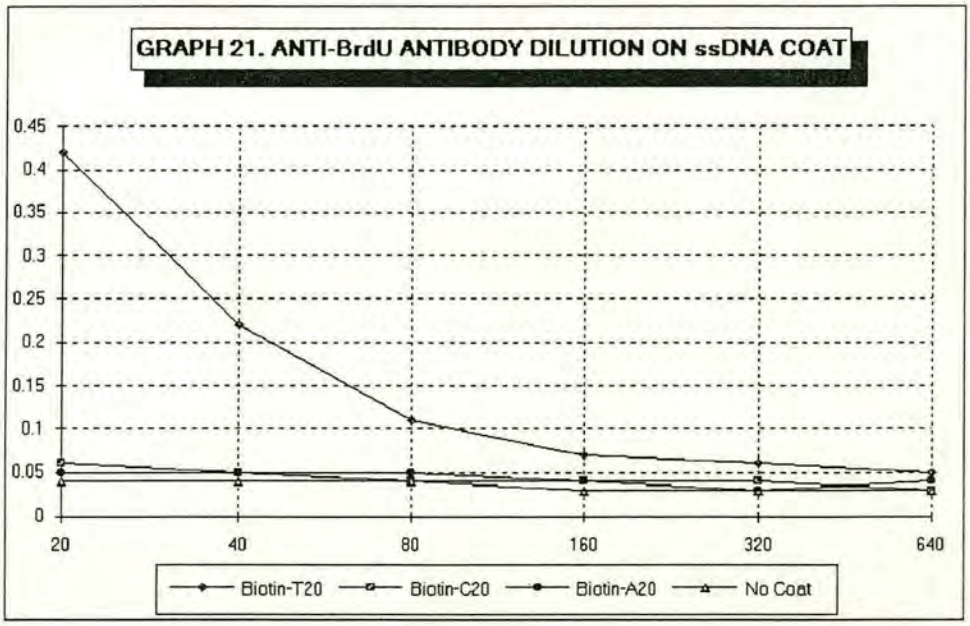
As previously discussed there was concern as to the possible inhibition of anti-DNA antibody/oligonucleotide binding in the presence of EDTA. Dr. P Aston and E. Ackerman (SRC) were testing various serum free media for the production and growth of hybridoma cell lines. Serum free medium would not only reduce the cost, they also simplify subsequent antibody purification. For our purposes the use of serum free medium may greatly decrease nuclease contamination in test supernatants.

Fusions from immunised mice were subsequently carried out using a 3:1 mix of serum free:complete medium. Thus fusions experiments were conducted in a medium containing 2.5% (instead of 10%) foetal calf serum (FCS). The medium was changed as normal except, after 5 days the FCS was reduced to 1%, and then 0.5% after 10 days, screening usually being carried out after 14 days. For all of the fusion experiments carried out using reduced FCS there was no significant change in the number wells produced containing actively growing hybridomas (typically >90%) compared to fusions using normal serum levels. When using low FCS concentrations the degree of attachment of cells to the culture plates was slightly reduced, but not sufficiently to cause cell loss when changing or testing supernatants. Therefore, it was acceptable to use reduced serum levels when carrying out fusion experiments.

3.5.8 Studies with Anti-Bromodeoxyuridine Antibodies

The structure of 5-bromodeoxyuridine (BdU) is similar to that of thymine. Antibodies raised against BdU are commercially available. It was thought that if these antibodies bound to single stranded oligonucleotides containing thymine then they may also bind to thymine containing mismatches, in a manner analogous to the proposed binding of anti-C100PS antibodies to cytosine containing mismatches.

Oligonucleotides T20B, C20B, and A20B were attached to streptavidin coated plates. Graph 21 shows the results of the binding of various concentrations of anti-BdU antibodies to coated plates. There was clearly a dose response, although weak, for the binding of these antibodies to single stranded oligonucleotides containing thymine (T20B). No binding of anti-BdU antibodies occurred using immobilised A20B or C20B, or in the absence of oligonucleotides.



The binding of anti-BdU antibodies to plates coated with duplexes containing a GT mismatch (GT30BL) or AC mismatch (AC30BL) was investigated. These antibodies showed no binding to either duplex at all antibody concentrations tested. Further studies using these antibodies were not carried out.

3.5.9 Screening Using Improved DNA Immobilisation

Procedures for coating duplex oligonucleotides containing a mismatched base pair were optimised. Reduction of potential nuclease enzyme interference was addressed. There was greater confidence that the screening assay was presenting a mismatched base pair such that it was available for recognition by any anti-mismatch specific antibodies that may be produced by immunisation and fusion experiments.

3.5.9.1 GT75 Fusions

Two fusion experiments were carried out using splenocytes from GT75 immunised mice. Supernatants were tested by non-competitive ELISA, 27 wells were positive. Of these 15 gave positive inhibition studies when GT75 (10μg/ml) was used as a competitive inhibitor, see Table 15.

Table 15, Competitive Inhibition Studies on GT75 Fusion.

WELL LOCATION	% INHIBITION
1D10	53.2
2E6	84.6
2F3	71.1
2F5	55.7
3D3	23.4
3G6	12.1
4D7	52.6
4E6	7.5
5C9	26.3
5D2	49.4
5D7	70.0
5D9	43.6
5F2	58.5
6E9	56.1
6G9	73.0

Inhibition studies were then carried out comparing GC75 inhibition with the GT75 inhibition, shown above. All of the test wells indicated no difference in inhibition with the exception of test supernatants taken from 5C9 and 5D7, see Table 16.

Table 16, Competitive inhibition studies GT75/ 5C9 and 5D7

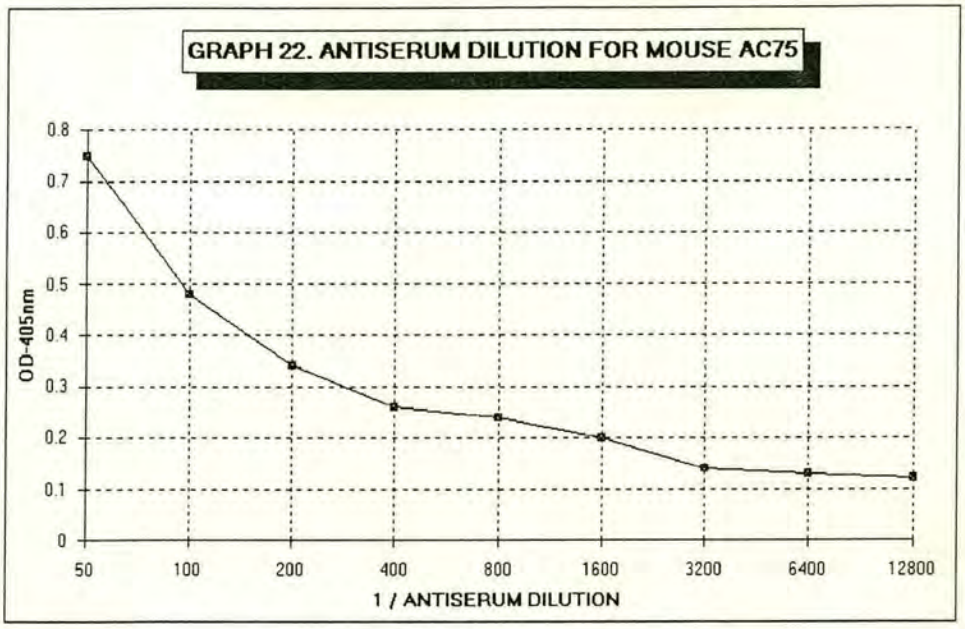
WELL LOCATION	% INHIBITION GT75	% INHIBITION GC75
5C9	26.3	0.0
5D7	54.3	41.7

5C9 showed some inhibition (26.3%) in the presence of a duplex containing a mismatch, GT75, and no inhibition with a similar sequence without a mismatch, GC75, suggesting specific recognition of the mismatch had occurred. The absorbance measured in the ELISA was low, indicating low affinity antibodies or a low concentration of antibody was present in the test supernatant. The colonies were relatively slow growing compared to other hybridoma colonies. This may reduce the amount of antibody released into the growth medium. The inhibition for 5D7 was greater, but the difference in inhibition was slight. It was decided to carry out cloning experiments for both of these cell lines.

For cells from fusion well 5C9 no colonies were obtained from fusion experiments, despite the addition of hybridoma growth medium supplement to the cells. For 5D7 cloning experiments produced 21 wells containing single colonies. However, none of these monoclonals gave inhibition in competitive studies. One mixed clone well, containing several colonies, did show inhibition in competitive studies, but the inhibition was equivalent for both GT75 and GC75 as inhibitor. No other wells were found to give positive inhibition.

3.5.9.2 AC75 Fusion

A non-competitive ELISA was carried out using dilutions of the serum from the pre-fusion bleed of an AC75 immunised mouse, graph 22.



Antibody was found to be present that bound to the coating material. A fusion was carried out producing 43 wells indicating binding in non-competitive assays. Seven of these gave positive inhibition in a competitive assay, see Table 17.

Table 17, Competitive Assay from AC75 Fusion

WELL LOCATION	% INHIBITION
1C8	46.1
1C10	62.4
1G3	42.3
2D2	10.7
3F9	23.0
4D6	47.2
5G2	24.1

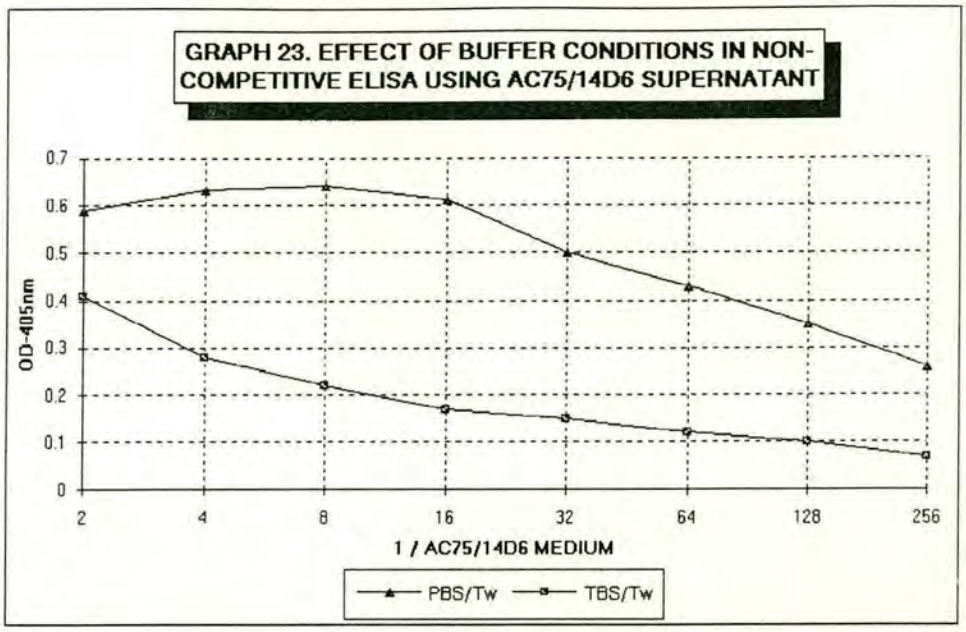
Inhibitor was AC75, 10μg/ml.

Similar percentage inhibitions were found when using GC75 (10μg/ml) as inhibitor. It was possible that some of the wells contained a mixed population of hybridoma cell types e.g. one colony producing anti-mismatch antibodies and another colony producing anti-DNA antibodies. Competitive studies may then not reflect the true inhibition of one colony but rather inhibition of a mixed antibody population.

It was decided to carry out cloning experiments on wells whose test supernatants gave high inhibitions. Cloning experiments were carried out on 1C8, 1C10, 1G3 and 4D6. Many wells were produced containing single colonies, however, they all gave inhibition to duplexes with or without mismatched base pairs in competitive assays. One of the 4D6 single colony wells was further cloned to produce the monoclonal cell line AC75/4D6/4.12. This cell line was subsequently used to compare oligonucleotide binding in different assay buffers (phosphate buffer versus tris buffer), see below.

3.5.9.3 Effect of Buffer Conditions

It was reported (Pisetsky and Semper, 1984) that buffer conditions can influence the binding of anti-DNA antibodies to DNA in ELISA experiments. We had produced a monoclonal antibody that bound to all of the duplex oligonucleotides tested. Non-competitive and competitive assays were carried out using PBS/Tw or TBS/Tw in the anti-DNA antibody binding step only. Graph 23 shows the result of a non-competitive ELISA for AC75/4D6/4.12.



Although both produce dose dependent dilution curves, the shapes of the curves were clearly very different. The binding of the antibody to coated plates in PBS/Tw was greater at all dilutions of antibody. Competitive studies were then carried out, see Table 18, at three antibody dilutions. Antibody dilutions were selected from the result of non-competitive studies.

Table 18. Competitive Assay on AC75/4D6/4.12 in Two Buffers

BUFFER	PBS/Tw pH 7.2	TBS/Tw pH 7.2
% Inhibition AC75	45	<5
% Inhibition AC75	39	<5
% Inhibition AC75	44	<5

Inhibitor AC75 10μg/ml

The buffer used was greatly influencing the amount of inhibition in competitive studies. There was virtually no inhibition of binding in the presence of TBS compared to PBS. Pisetsky and Semper (1984) found that the binding of antibodies to DNA can vary greatly depending on the buffer. Some antibodies were found to have relatively increased binding to DNA in TBS, others in PBS.

Pisetsky and Semper (1984) gave no explanation to this variability in binding of anti-DNA antibodies. It is worth noting that in non-competitive ELISA anti-DNA antibody binding to DNA coated plates was reduced for TBS/Tw compared to PBS/Tw. Subsequently high inhibitions were observed in competitive ELISAs using PBS/Tw as buffer, and virtually no inhibition when using TBS/Tw as buffer.

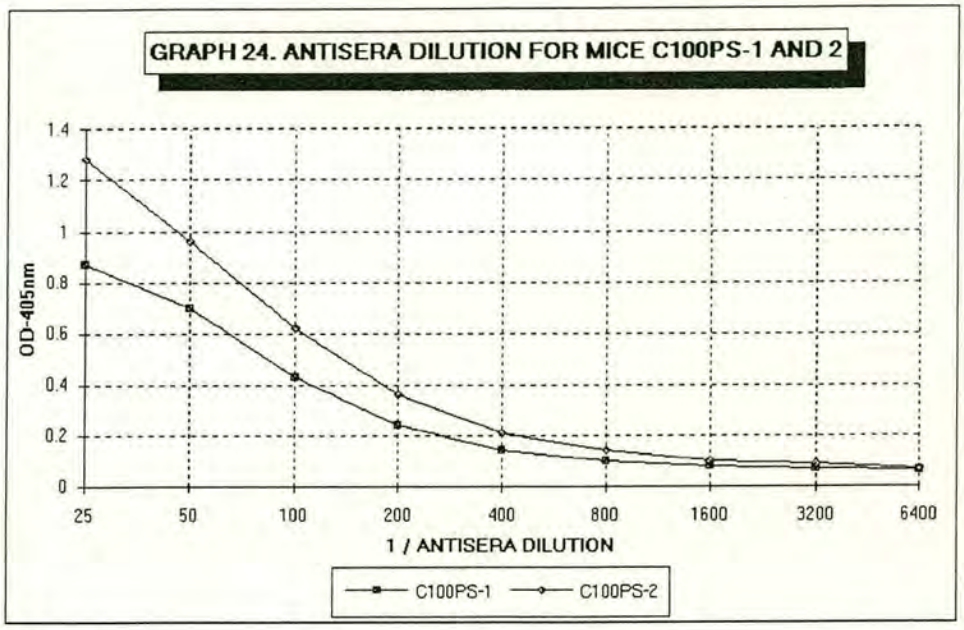
Tris may alter conformation adopted by the antibody binding site and/or the DNA. Conformational changes in the antibody binding site could give rise to reduced antibody binding to DNA, indicated in graph 23. However, as a limiting amount of antibody was used in the competitive ELISA some inhibition (albeit different to that seen for PBS/Tw) would be expected to occur.

Alternatively, the conformation of the DNA duplex may be altered in the presence of TBS/Tw. It could be argued that the effect of TBS/Tw on altering the conformation of the immobilised DNA and inhibitor DNA may be different. The immobilised DNA was a relatively short duplex. It was a single strand modified with a central tetraethylene glycol linker allowing it to form a stable self complimentary duplex. In addition it was immobilised (*via* biotin streptavidin interactions) at one end to a solid phase. By comparison the DNA used as inhibitor was a relatively long duplex. It had none of the potential restrictions suggested for the immobilised strand for conformational alteration. In addition it contains twice as many mismatched base pairs, and these may aid conformational change.

Anti-DNA antibodies in TBS/Tw may thus be able to recognise relatively unaltered immobilised DNA (non-competitive ELISA). However, they may be unable to recognise conformationally altered inhibitor DNA (competitive ELISA), producing no inhibition. There are of course other buffers that may be used in antibody screening experiments. Clearly it would be impossible to test all of them while screening fusion and cloning experiments for anti-mismatch antibodies. However, it should be noted that useful antibodies may be lost by not examining a variety of buffer conditions.

3.5.9.4 C100PS Fusions

Non-competitive ELISAs were carried out using sera from pre-fusion bleeds for two C100PS immunised mice, see graph 24.



The serum of both mice contained antibodies that bound to the coating material, the titre for mouse 2 was slightly higher. Fusions were then carried out. 26 fusion wells indicated antibody binding in non-competitive ELISA. Five of these gave positive inhibition against C100PS, three of these (wells 1-5C7 and 2-3G7, 2-3G10) gave positive inhibition in competitive assay against duplexes, see Table 19.

Table 19. Competitive ELISA of C100P/ 1-5C7, 2-3G7 and 2-3G10.

Well	Inhibition (%) C100P	Inhibition (%) AC75	% Inhibition (%) CC75	Inhibition (%) GC75
1-5C7	>80	40.2	39.5	19.8
2-3G7	>80	10.0	79.4	<5
2-3G10	>80	<5	11.2	<5

Inhibitor at 10μg/ml

As expected, positive wells show very high inhibition when incubated with C100PS. Fusion well 1-5C7 appears to contain antibodies that recognise duplexes containing AC and CC mismatches, to a slightly greater extent than duplexes containing GC base pairs in place of the mismatches. This may represent antibodies that recognise mismatched base pairs in a duplex, but also cross react with non-mismatched sequence. It should also be noted that a fusion well may contain a mixed cell population and thus inhibition with GC75 may represent binding by antibodies other than those recognising the mismatch containing duplexes.

The binding of antibody from 2-3G7 appear to be directed towards duplexes containing a CC mismatch, compared with AC mismatched or GC non-mismatched duplexes. 2-3G10 showed only slight inhibition with CC75, but virtually no inhibition with either GC matched or AC mismatch containing duplexes. Cells from these wells were used for cloning experiments.

Using our protocol perfect cloning experiments should produce approximately 36 wells that contain single colonies. For single colony cell lines producing the desired antibody further cloning experiment will be carried out to ensure monoclonality. However, in practice single colony wells from the first cloning experiment probably are monoclonal. Cloning experiments over the course of this study have generally been reasonably efficient, producing greater than 25 single colony wells in most cases. However, when screening cloned cell lines many of them have not given results similar to those seen for fusion wells from which the cloned cells were obtained. Where this occurs, predominantly anti-DNA binding activity seems to be lost.

This could suggest that some of the fusion results generated false positives, and there could be a fundamental problem with the screening strategy. However, the fusions discussed in sections (3.5) did produce clone wells producing antibodies with similar properties to the fusion wells from which they were obtained.

It is known that genetic instability of hybridomas occurs immediately post fusion. This can lead to a loss in antibody production and/or secretion. In addition, the loss of desired antibody secreting cells may occur because the cells secreting antibody of interest represent the minority cell type in the fusion wells. If the anti-DNA antibody secreting cells were in the minority then cloning experiments would rarely isolate single colony cell lines with the desired antibody production. Increasing the number of cloning experiments from one fusion well should increase the probability of isolating the desired clone. To this end two cloning experiments were conducted in subsequent cloning experiments, to improve the chances of obtaining the desired monoclonals.

Ideally the entire fusion well may be cloned to ensure the desired clone was isolated. This approach would generally be disregarded as unpractical, due to the huge numbers of cells present (thousands) when fusions are screened. Keeping thousands of monoclonal cell lines in cell culture would be expensive and time consuming. More importantly, depending on the relative colony growth rate, the ability to accurately screen these numbers of cell lines becomes a problem. This will be discussed further in section 4.

More than 50 single colony wells were obtained for each fusion well in cloning experiments. Despite this no anti-mismatched base pair antibodies were identified, by competitive ELISA, for 2-3G7, 2-3G10. Three wells containing single colonies were obtained for 2-3G10 that produced anti C100PS antibodies, these were used to further investigate the effect of EDTA on antibody/DNA binding, see later. Anti-dsDNA duplex antibodies were obtained for the monoclonal cell lines derived from C100PS/1-5C7.

3.5.9.4.1 Monoclonals from C100PS/1-5C7

As previously discussed for antibodies from AC75/4D6/4.12 cell line the type of buffer used in the anti-DNA antibody binding reaction influenced the results obtained. Non-competitive assays on monoclonals from C100PS/1-5C7 were carried out using both PBS/Tw and TBS/Tw. For all C100PS/1-5C7 monoclonals tested

higher antibody titres were observed if the anti-DNA antibody incubation was carried out in PBS/Tw, than in TBS/Tw. In many cases the amount of binding detected using TBS/Tw as buffer were so low that the hybridomas producing the test supernatants would have been discarded. It was also found that using PBS/Tw at half strength increased the amount of antibody detected in non-competitive assays. Antibody/antigen interaction are usually directly proportional to salt concentration, so this was not unexpected.

Cloning experiments from C100PS/1-5C7 produced 51 wells containing single colonies, 40 of these produced antibodies giving positive inhibition of greater than 70% with C100PS (10μg/ml). Inhibition studies with duplex oligonucleotides were then carried out. Table 20 shows the results from competitive studies carried out on appropriate dilutions of antibody containing supernatants in 0.5xPBS/Tw.

Table 20. Competitive Inhibition Studies on C100P/1-5C7

WELL LOCATION	% INHIBITION AC75	% INHIBITION CC75	% INHIBITION GC75
1.1	**67**	**89**	**65**
1.2	65	89	54
1.4	49	75	30
1.6	58	82	38
5.1	62	89	7
5.2	65	91	28
5.4	59	88	<5
5.9	50	86	<5
5.10	38	75	<5
5.11	38	72	<5
5.12	57	87	<5
5.19	62	75	<5
5.23	52	81	<5

Inhibitor at 10μg/ml

These results represent inhibition studies at fixed antibody dilution at one relatively high concentration of inhibitor. It can be seen that CC75 gives the best inhibition and thus was recognised in preference to non-mismatched sequences, and some of those containing AC mismatched base pairs. The stability of the duplexes was in part dependent upon the salt concentration of the buffer, therefore, the use of a reduced salt buffer may increase the exposure of cytosine containing mismatches, at AC and CC mismatch base pair sites, to antibodies in our experiments.

This was the first set of experiments demonstrating the presence of monoclonal antibodies with some reactivity to a mismatched base pair in a DNA duplex. Further experiments should be carried out to test the specificity of these antibodies, and on the sensitivity of assays that can be produced using these antibodies. It should be noted that these were not mismatch specific antibodies, and were not analogous to mismatch repair proteins. Instead they represent antibodies that recognise single stranded oligonucleotides containing cytosine, with sufficient cross reactivity to detect 10μg/ml of duplexes containing CC and to a lesser extent AC mismatches. A great deal of work would be required to establish all of the cross reactivities of these antibodies. Details of this will be discussed later.

3.6 EXPERIMENTAL

Production of monoclonal antibodies

All of the procedures leading to the production of monoclonal antibodies, including immunisations, splenocyte preparation, general cell culture, fusion and cloning experiments were based on procedures routinely used by the immunochemistry group at Shell Research Centre (SRC). They were adapted from the techniques of Kohler and Milstein (1975 and 1976).

Immunisations

Immunisations were carried out by Dr J.P.Aston (SRC), on female BALB/c mice. Three mice were used for each immunogen. Primary injections and secondary boost injections were subcutaneous into the nape of the neck. Pre-fusion boosts were given by intraperitoneal and intravenous injection on days 3 and 2 prior to fusion experiments.

15mer Duplexes

Primary injections were carried out with a mismatched DNA duplex (100μg) in isolation from a carrier protein or complexed with the methyl ester of BSA (MeBSA). The immunogen was injected as an emulsion in Freunds Complete Adjuvant (Sigma Chemical Company).

Secondary injections were carried out after three weeks. The same procedure as above was used except Freunds Incomplete Adjuvant (Sigma Chemical Company) was used to form the emulsion. Pre-fusion boosts were carried out at least 1 month after secondary injections. Pre-fusion boosts contained DNA (100μg) in aq. NaCl (0.15M).

75mer Duplexes and C100PS Single Strand

Primary injections were carried out with duplex or single stranded DNA (100μg) in isolation from a carrier protein. The immunogen was injected as an emulsion in Hunters Titermax Adjuvant. Secondary injections were carried out as above using Freunds Incomplete Adjuvant. Pre-fusion boosts were injected with DNA (100μg) in aq. NaCl (0.15M).

Bleeds were taken from immunised mice one week after secondary injections. Serum from bleeds was obtained by centrifugation. Serum was tested for the presence of anti-DNA immunoglobulins by Enzyme Linked Immunosorbent Assay (ELISA), described later.

Hybridoma production

Cell culture reagents

Roxwell Park Memorial Institute cell culture medium - 1640 (RPMI-1640 or RPMI) was supplied by Gibco Europe Ltd. Complete medium is RPMI containing L-glutamine (4mM), sodium pyruvate (1mM), penicillin (100U/ml), streptomycin (100U/ml), Myoclone plus foetal calf serum (10%) supplied by Gibco Europe Ltd. HT medium is complete medium containing hypoxanthine (100mM), and thymidine (16mM) supplied by BDH Ltd. HAT medium is complete medium containing hypoxanthine (100mM), thymidine (16mM), aminopterin (0.4mM) supplied by BDH Ltd. Serum free/protein free medium was supplied by Gibco Europe Ltd. Complete serum free medium is made as complete RPMI, omitting the FCS. Polyethylene glycol (PEG) and dimethylsulphoxide (DMSO) both hybridoma grade were obtained from Sigma Chemicals Company. Hybri-plus media supplement was obtained from Sera-Lab.

Splenocyte Preparation

Splenocytes, for fusion experiments, were obtained from spleens of mice, two days after the second pre-fusion boost injections. Spleens were removed from terminally anaesthetised mice and placed in RPMI (20ml), at 4°C. All subsequent operations were carried out in a cell culture laboratory in laminar flow hoods using aseptic techniques. All cell culture fluids were pre-warmed to 37°C before being used.

Spleens were trimmed of connective tissue, then transferred to a sterile Petri dish. A superficial longitudinal cut was made along the length of the spleen with a scalpel. Splenocytes were gently dispersed from the organ using two 10ml syringes fitted with 26-gauge needles, each containing RPMI (10ml). One syringe was used to anchor the spleen, the other to puncture it and expel fluid in successive small areas, using the fluid from first one and then the other syringe. The cell suspension, typically 0.6-1.2 x 10^8 cells, was then pelleted by centrifugation at 1000 rpm for five minutes.

Fusion of Splenocytes with Plasmacytoma Cell Line

Sp2/1-Ag14 (Sp2) a non-immunoglobulin producing plasmacytoma cell line was used as the fusion partner in fusion experiments. For each fusion three 200ml culture flasks were grown to near confluency with the Sp2 cell line. The Sp2 cells adhere to the plastic surface of culture flasks. They were carefully removed by gentle scraping, with a rubber tipped rod, from the flasks and pelleted by centrifugation at 1000 rpm for five minutes.

Sp2 cells and splenocytes were resuspended separately in RPMI (10ml) and were measured for cell viability by trypan blue exclusion. Trypan blue cannot pass through the membranes of live cells, but readily passes into dead cells. The total cell yield per spleen was usually 0.6-1.2 x 10^8 with greater than 90% of the cells able to exclude trypan blue. Sp2 and splenocyte cell suspensions were combined to give 10^7 Sp2 cells and 10^8 splenocytes. The

mixed cell suspension was then divided into six equal volumes and each pelleted at 1000 rpm for five minutes, each pellet serving as a separate fusion experiment.

For each fusion the supernatant was removed from the mixed pellet and PEG (300µl) added dropwise over 30 seconds with continuous agitation of the pellet. RPMI (6ml) was then added dropwise over 60 seconds with gentle shaking to resuspend the cell mixture. The cell suspension was then pelleted by centrifugation at 1000 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in complete medium (6ml). The cell suspension was then dispensed in aliquots (60 x 100µl) to each of the central wells of a 96 well cell culture plate. HAT medium (100µl) was added to each of the wells. The cell culture plates were then placed in a humid incubator at 37°C with a filtered gas supply of 5% CO_2 in air.

Post-fusion cell culture

Cell culture supernatant (100µl) was removed from each fusion well and replaced with HAT medium (100µl) on day 1, 3, 5 and 7 post fusion. This was continued on day 9, 11 and 13 using HT medium, after which complete medium was used. Usually by day 10 post fusion post fusion the cells were of sufficient density to initiate screening for anti-DNA antibodies. Positive anti-DNA antibody producing cell lines then underwent cloning experiments in an attempt to produce monoclonal cell line by limiting dilution. Occasionally Hybri-plus media supplement (10%v/v) was added if poor growth was observed. All 96 well culture plates were monitored for up to four weeks post fusion to allow slow growing hybridomas to reach sufficient density to initiate screening.

Cloning by Limiting Dilution

Cloning by limiting dilution aims to produce monoclonal hybridoma cells from actively growing hybridomas from fusion experiments. This was achieved by diluting cell suspensions into 96 well cell culture plates such that several rows should contain a single cell per well. A feeder layer of cells, providing cell-cell contact and

growth factors was required to help maintain cell viability, this is important for extremely low density cell dispersions. Thymocyte cell suspensions were used, and obtained as follows.

A 4-6 week old female BALB/c mouse was killed by overdose anaesthesia. Using sterile dissecting instruments an incision was made along the mid line of the thorax to the neck. The thoracic cavity was opened and two thymus lobes, lying anterior to the heart, were removed and transferred to a sterile container containing RPMI (10mls).

All subsequent operations were carried out in a cell culture laboratory in laminar flow hoods using aseptic techniques. Thymus lobes were placed in a sterile petri dish containing RPMI (10mls), at 37°C. Thymocytes were dispersed by gently teasing the thymus with forceps. The cell suspension was then pelleted by centrifugation at 1000 rpm for five minutes and the pellet was resuspended in complete medium (20mls).

Thymocyte suspension (4mls) was added to approximately 200 hybridomas. Thymocyte/hybridoma cell suspension (2.4mls) was added to the first 2 rows of a 96 well cell culture plate (100µl per well), leaving 1.6mls unused. Thymocyte suspension (2.4mls) was then added to the remaining thymocyte/hybridoma cell suspension (1.6mls), gently mixed, then dispensed as above into rows 3 and 4, again leaving 1.6mls unused. Thymocyte suspension (1.6mls) was then added to the remaining thymocyte/hybridoma cell suspension (1.6mls), gently mixed, then dispensed as above into rows 5 and 6, leaving 0.8mls unused. Finally the remaining thymocyte/hybridoma suspension (0.8mls) was added to the thymocyte suspension (1.6mls), and dispensed into rows 7 and 8.

Complete medium (100µl) was added to each of the 96 wells, and the cell culture plates were then placed in a humid incubator at 37°C with a filtered gas supply of 5% CO_2 in air. Theoretically each set of two rows will contain on average 5, 2, 1, and 0.5 hybridoma cells per well, respectively.

After 10-14 days the plates were examined microscopically for colony formation and those producing actively growing colonies were screened for the presence of anti-DNA antibodies. To ensure monoclonality, cloning by limiting dilution was repeated twice for any positive wells from the first round of cloning.

Freezing Cells

Approximately 10^6 cells were pelleted by centrifugation at 1000 rpm for 5 minutes. The pellet was resuspended in foetal calf serum containing 10% DMSO (0.5mls) at 4°C. The cell suspension was transferred to a cryotube, placed into a freezing container containing propan-2-ol, and stored overnight at -70°C. Cryotubes were then transferred to liquid nitrogen for long term storage.

Thawing Cells

Cells were rapidly thawed from cryotubes, in a 55°C water bath, with gently shaking to resuspend the cells. The cell suspension was diluted with RPMI (10mls), prewarmed to 37°C, then pelleted by centrifugation at 1000 rpm for 5 minutes. The pellet was resuspended in complete medium (2mls), prewarmed to 37°C, and dispensed (1ml) into two 2ml wells of a cell culture plate. These were then placed into a humid incubator at 37°C, with a filtered gas supply of 5% CO_2 in air.

Enzyme Linked Immunosorbent Assay (ELISA)

Reagents

2,2-Azinobis (3-ethylbenzthiazoline) sulfonic acid (ABTS), hydrogen peroxide (30%), polyoxyethylene sorbitan monolaurate (Tween 20 or Tw), sodium carbonate, and streptavidin were obtained from Sigma Chemical Company. Citric acid, disodium hydrogen orthophosphate, sodium chloride and sodium hydrogen carbonate were obtained from BDH Chemical Company. Phosphate buffered saline (PBS) tablets were obtained from Oxoid Ltd. Reduced Mg^{2+} and Ca^{2+} PBS was obtained from ICN Flow.

Coating buffer (pH 9.6) contains sodium bicarbonate (1.6g), sodium hydrogen carbonate (2.9g) in 1 litre of distilled water. PBS (pH 7.2) contains 10 PBS tablets in 1 litre of distilled water. PBS/Tw contains 10 PBS tablets, and Tween 20 (0.5g) in 1 litre of distilled water. Reduced strength PBS/Tw (0.5x PBS/Tw) contains 5 PBS tablets, and Tween 20 (0.5g) in 1 litre of distilled water. TBS/Tw contained 0.01M Tris, 137mM NaCl and 0.05% (w/v) Tween 20, pH 7.2. Peroxidase buffer contains disodium hydrogen orthophosphate (4.41g), citric acid (4.58g), 30% hydrogen peroxide (180µl) in 500ml distilled water. ABTS concentrate contains ABTS (0.75g) in 50ml distilled water. Peroxidase buffer and ABTS concentrate are filtered (0.2µ filter) and stored at 4°C. ABTS ($0.6mgml^{-1}$) in peroxidase buffer was prepared on the day of use for all ELISA experiments. Colour was allowed to develop for 30 minutes prior to measuring.

Mouse IgG standards were obtained from Sigma. Rabbit anti-DNA antibodies were obtained from Boerhringer Mannheim. Peroxidase conjugated swine anti-rabbit antibodies, peroxidase conjugated rabbit anti-mouse antibodies, rabbit anti-mouse antibodies and mouse anti-bromodeoxyuridine antibody (anti-BrdUrd, Bu 20a) were obtained from Dako Ltd. All assays were carried out using 96 well microtitre plates (Nunc Maxisorb). End point colour development was measured using a 96 well microplate reader (Molecular Device VMax), the data was analysed and stored using a NEC Powermate SX/20/VI computer.

ELISA Plate Coating Conditions

1. Non-specific adsorption of DNA to microtitre wells.

(A) Untreated plates : 96 well microtitre plates were coated (100µl/well) with 15mer DNA duplexes (AA15, AC15, GG15 or CC15 ; 1µg/ml) in Tris buffered saline (TBS, Tris 10mM, sodium chloride 150mM, pH 7.2). The DNA was allowed to passively bind overnight at room temperature. Unbound material was then removed by washing with PBS (4 x 200µl). Non-bound sites on the plates were then blocked (200µl/well) with gelatin (0.1%), in distilled water

for one hour. Plates were then washed as above using PBS/Tw and then sealed and stored at 4°C until required.

(B) Poly-L-Lysine treated plates (adapted from Fisher and Ziff, 1981): Plates were precoated (100µl/well) with poly-L-lysine (50µg/ml) in distilled water for two hours then washed with TBS/Tw (4 x 200µl). 15mer DNA duplexes as described above were then allowed to bind as 1.(A) for two hours. Unbound material was removed by washing with PBS (4 x 200µl). Non-bound sites on the plates were blocked (200µl/well) with gelatin (0.1%), in distilled water, or (200µl/well) with poly-L-glutamate (50µg/ml), in distilled water, for one hour. Plates were then washed and sealed as above.

(C) MeBSA treated plates (adapted from Rubin *et al.*, 1983): Plates were precoated (100µl/well) with MeBSA (0.1%), in distilled water, for two hours then washed with TBS/Tw (4 x 200µl). 15mer DNA duplexes as described above were then allowed to bind as 1.(A) for two hours. Plates were then washed, blocked and stored as described in 1 (A).

(D) UV-Irradiated plates (adapted from Zouali and Stollar,1986): Plates were UV-irradiated at 254nm for 18 hours. DNA binding, blocking and storage was carried out as 1(C).

2. Specific attachment of DNA to microtitre plates (adapted from Emlen *et al.*, 1990)**.**

(A) Duplex coat formation *via* complementary single strands : Plates were coated (100µl/well) with streptavidin (1µg/ml) in coating buffer (pH 9.6). Unbound material was removed by washing with PBS (4 x 200µl). Non-bound sites on the plates were blocked (200µl/well) with gelatin (0.1% (w/v)), in distilled water for one hour. Plates were washed as above using PBS/Tw prior to coating (100µl/well) with biotin end labelled single stranded DNA sequence AAAB (200ng/ml), in PBS/Tw containing 0.2mM EDTA (PTE). Plates were washed with PTE (4 x 200ml). A duplex was formed by adding a single stranded DNA sequence (100µl/well), in PTE, either fully complementary TTT (400ng/ml), or such that a single base pair

mismatch would form TCT or TAT (400ng/ml), for two hours. Plates were washed with PTE (4 x 200µl), and stored as previously described (see also detection of duplex formation).

(B) Coating with thermally stabilised 15mer duplex :
Plates were coated in the same way as 2 (A) except a biotinylated duplex AC15B (400ng/ml) was used instead of biotin labelled single stranded oligonucleotides. AC15B was a 15mer duplex containing a central AC mismatch, stabilised at one end with a tetraethylene glycol linker, and biotinylated at the opposite end (cf. AC15 used as immunogen or coating material above). Plates were blocked and stored as described in 2 (A).

(C) Coating with single stranded DNA :
Plates were coated as 2(A) with a biotin labelled cytosine 20mer C20B (400ng/ml), blocked and stored as described in 2(A).

Detection of Duplex Formation in 2 (A).

Detection of duplex formation was evaluated by using DNP end labelled single stranded DNA sequences. These would be either fully complementary TTTDNP (400ng/ml), or such that a single base pair mismatch would form TCTDNP or TATDNP (400ng/ml), when incubated on plates precoated with AAAB (as in 2 (A)). Plates were then washed with PTE (4 x 200µl). Formation of the duplex was measured by detection of the DNP group. A peroxidase enzyme conjugated anti-DNP antibody (100µl/well) was added (1:15,000 dilution), in PBS/Tw. Plates were washed with PBS/Tw (4 x 200µl), then a colourless peroxidase substrate (100µl/well) of ABTS (0.6mg/ml) in peroxidase buffer was added. In the presence of peroxidase enzymes this produces a coloured product which can be measured spectrophotometrically at 405nm.

Anti-DNA antibody screens of mice sera

Serum from mice were screened for the presence of anti-DNA antibody using non-competitive and competitive ELISA techniques using the coating procedures described in 1(A-D). Initially non

competitive ELISAs were carried out. The serum was diluted in PBS/Tw (1:50), then double diluted to give 9 or 10 dilutions. These were incubated (100µl/well) on microtitre plates for two hours. Plates were washed as above using PBS/Tw. A peroxidase conjugated rabbit anti-mouse antibody (100µl/well) diluted in PBS/Tw (1:1000), was added for one hour. Plates were washed as above using PBS/Tw. Colour development was produced and monitored as previously described.

Non-competitive ELISAs of serum produce dilution-response curves. Where good dilution-response curves were obtained for mice serum, competitive ELISAs were carried out. A limiting dilution of serum was incubated with a range of concentrations of DNA (100-0.1µg/ml) in PBS/Tw. These were then incubated on coated plates (100µl/well), and developed as in the non-competitive ELISA described above.

3.6.1 Screening of fusions for anti-DNA antibody production

Mouse IgG ELISA

Plates were coated (100µl/well) with a rabbit anti-mouse antibody (1:1000) in coating buffer, for two hours. Plates were washed with PBS/Tw as previously described. Supernatants from fusion wells (100µl/well) were transferred to these plates, and incubated for one hour. Plates were washed as above. Peroxidase conjugated rabbit anti-mouse antibody (100µl/well) diluted in PBS/Tw (1:1000), was added for one hour. Plates were washed and ABTS was added. Colour development was monitored as previously described.

Assays using coating procedures 1(A-D).

Non-competitive ELISA.

Supernatants from fusion wells (100µl/well) were transferred to coated plates, and incubated for two hours. Plates were washed as above, and then a peroxidase conjugated rabbit anti-mouse antibody

(100µl/well) diluted in PBS/Tw (1:1000), was added for one hour. Plates were then washed as above using PBS/Tw. Colour development was monitored as previously described.

Competitive ELISA.

Supernatants from fusion wells (100µl) were incubated with a range of concentrations of DNA (100-0.1µg/ml in 100µl) in PBS/Tw and with PBS/Tw (100µl) only as a control. These assay solutions were transferred to coated plates (100µl/well), and incubated for two hours. Plates were washed as above. Assay procedure and colour development was carried out as previously described.

Assays using coating procedures 2(A-C)

Non-competitive ELISA.

Supernatants from fusion wells (50µl/well) were transferred to coated plates containing PBS/Tw with EDTA (1.0 or 0.2mM), and incubated for two hours. Plates were washed with PBS/Tw with EDTA (1.0mM) as above. Assay procedure and colour development was carried out as previously described.

Competitive ELISA.

Supernatants from fusion wells (50µl) were incubated with a range of concentrations of DNA (100-0.1µg/ml in 100µl) in PBS/Tw with EDTA (1.0 or 0.2mM). These assay solutions were transferred to coated plates (100µl/well), and incubated for two hours. Plates were washed with PBS/Tw with EDTA (1.0mM) as above. Assay procedure and colour development was carried out as previously described.

DNA Digestion assay

Tissue culture medium, autoclaved tissue culture medium, and tissue culture medium with EDTA (50mM) were diluted in PBS/Tw. The medium was incubated (100µl/well) on plates coated with single

stranded cytosine 20mer end labelled with biotin (C20B) by procedure 2(C). After one hour wells were washed with PBS/Tw as above, and incubated (100µl/well) with a commercially available rabbit anti-DNA antibody (0.1mg/ml) in PBS/Tw for two hours. Plates were washed in PBS/Tw and a peroxidase conjugated swine anti-rabbit antibody was added (100µl/well) in PBS/Tw (1:500) for one hour. Plates were washed and colour development was carried out as previously described.

Investigating Anti-Bromodeoxyuridine Antibodies

Plates were coated (100µl/well) with streptavidin (1µg/ml) in coating buffer (pH 9.6), for 2.5 hours. Unbound material was removed by washing with PBS (4 x 200µl). Non-bound sites on the plates were blocked with gelatin as previously described. Plates were then coated (100µl/well) with either biotin-linker end labelled single stranded DNA sequence T20B, C20B, and A20B or biotin-linker end labelled duplexes AC30B, and GT30B ($5x10^{-9}$M), in PBS/Tw, for 2 hours. 30 wells were coated for each sequence. A further 30 streptavidin coated wells were incubated with PBS/Tw only to serve as a series of controls. Plates were washed with PBS/Tw (4 x 200µl).

Anti-BrdUrd was diluted 1:20 in PBS/Tw and then double diluted to give 9 dilutions (1:20 - 1:5120), in PBS/Tw. PBS/Tw was also used as a control blank for the antibody. Anti-BrdUrd dilutions (100µl/well) were then incubated in oligonucleotide coated or blank wells, in triplicate, for 1 hour. Plates were washed and developed with a peroxidase conjugated rabbit anti-mouse antibody as previously described.

Testing Biotin-Linker Coating Procedure

Standard procedure

Streptavidin and oligonucleotide concentration and incubation times were varied to establish coating conditions. All streptavidin coated plates were blocked with gelatin as previously described.

Detection of DNP labelled oligonucleotides was carried out using peroxidase enzyme conjugated anti-DNP antibody (100µl/well) diluted 1:15,000 in PBS/Tw. Colour development was previously described.

Single Stranded Oligonucleotides

Plates were coated with streptavidin (1µg/ml), or with coating buffer only as a control. Single stranded oligonucleotides C10B, C10BL, and C11B (100 $x10^{-9}$M) were double diluted in PBS/Tw to give 9 dilutions (100-0.39 $x10^{-9}$M), PBS/Tw only serving as a blank. These oligonucleotides were 5'-biotin and 3'-DNP labelled. Oligonucleotide dilutions (100µl/well) were incubated on streptavidin coated or blank wells, for 2 hours. Further assay development was carried out as previously described.

Double Stranded Oligonucleotides

The above assay was repeated except duplex forming oligonucleotides AT10B and AT10BL were used in place of single stranded oligonucleotides. All coating and assay conditions were identical. N.B. These duplexes were 5'-biotin labelled, with a central DNP group as part of the tetraethylene glycol linker such that on duplex formation the biotin and DNP groups are at opposing ends of the duplex. The Biotin binding at the solid phase (streptavidin).

Optimisation of Biotin-Linker-Oligonucleotide Immobilisation

Streptavidin Concentration

Streptavidin (50µg/ml) in coating buffer was diluted with coating buffer to give 9 dilutions (50-0.20µg/ml). Plates were coated with various streptavidin concentrations (100µl/well), coating buffer only serving as a blank, for 2 hours. AT10BL (50, 5 and 0.5 $x10^{-9}$M) in PBS/Tw were added (100µl/well) for each coating concentration, in triplicate, for 2 hours. Further assay development was carried out as previously described.

Streptavidin Coating Time

Plates were coated (100µl/well) with streptavidin (1µg/ml) in coating buffer for 24, 15, 7.5, 5, 2.5, 1.0 and 0.5 hours, coating buffer only was used as 0 hour blank. AT10BL (5.0×10^{-9}M) in PBS/Tw was double diluted, to give 9 dilutions ($5.0\text{-}0.02 \times 10^{-9}$M), in PBS/Tw. AT10BL concentrations (100µl/well) were then added to each of the above coated wells, in triplicate, for 2 hours. Plates were washed and developed as previous described.

Oligonucleotide Coating Time

Plates were coated (100µl/well) with streptavidin (1µg/ml) in Coating buffer for 2.5 hours. AT10BL (5×10^{-9}M) in PBS/Tw was added (100µl/well) to coated plates for 420, 300, 220, 120, 60, 30, and 15 minutes, PBS/Tw only serving as 0 minute blank. Plates were washed and then developed as previously described.

Optimised Coating Conditions

Subsequently all anti-DNA antibody production was screened using the optimised oligonucleotide immobilisation procedure. N.B. The oligonucleotides used for screening were not DNP labelled. The assay procedure was as outlined below:-

Plates were coated (100µl/well) with streptavidin (1µg/ml) in coating buffer, for 2.5 hours. Plates were washed and blocked with gelatin (0.1% (w/v)) and were ready for immediate use, or could be stored sealed, at 4°C, for several months. Oligonucleotides A20BL, C20BL, T20BL, AC30BL, and GT30BL (5×10^{-9}M) in PBS/Tw were added to streptavidin coated plates (100µl/well), for 2 hours. Plates were washed and used for screening anti-DNA antibodies in serum or cell culture supernatants.

Antibody containing supernatants from fusion and cloning experiments were always screened using plates freshly coated with oligonucleotide. Supernatants were always diluted 1:1 with PBS/ Tw

for non-competitive assays. Any positives underwent competitive assays as previously described.

Effect of ETDA on Assay using C20B-Oligonucleotide

Buffers and wash solutions were adjusted with EDTA such that they contained 0, 10 and 100mM EDTA. Oligonucleotide C20B (400ng/ml) in PBS/Tw with or without EDTA were added (100µl/well) to streptavidin coated plates and allowed to bind for 2 hours. Plates were washed and a commercially available anti-DNA antibody, (5µg/ml) was double diluted in PBS/Tw with or without EDTA to give 7 dilutions (5-0.6µg/ml). These were added (100µl/well) for 1 hour. Plates were then washed and developed as previously described.

Effect of ETDA on Assay using DNP-Oligonucleotide

PBS/Tw containing EDTA (10, 1.0, 0.1, 0.025, 0.0025mM) or PBS/Tw only was used in all buffer and wash solutions. AT10BL ($5x10^{-9}$M) in PBS/Tw +/- EDTA was added (100µl/well) to streptavidin coated plates for 2 hours. Plates were washed and then developed using PBS/Tw +/- EDTA at all stages except in the peroxidase substrate buffer.

Effect of EDTA on Peroxidase Function

Peroxidase conjugated rabbit anti-mouse antibody (1:1000) was added (10µl) to peroxidase buffer (1ml) with or without EDTA (1mM) containing either 300, 200, 100, 50 or 25µl ABTS (0.6mg/ml) in peroxidase buffer. Colour development was measured in duplicate after 5 and 30 minutes, using 100µl/well in a microtitre plate.

Immobilised Oligonucleotide Serum Incubation

Oligonucleotide C20B (400ng/ml) in PBS/Tw was added (100µl/well) to streptavidin coated plates for 2 hours. Myoclone plus FCS untreated, or heat inactivated in an autoclave, was double diluted in PBS/Tw to give (Neat, 1:2, 1:4, 1:8, 1:16, 1:32). Serum

dilutions were added to coated wells (100µl/well) and allowed to incubate for 1 hour. Plates were washed and a commercially available anti-DNA antibody (1µg/ml) in PBS/Tw was added (100µl/well) for 1 hour. Plates were then washed and developed as previously described. Control wells were set up containing no serum with or without anti-DNA antibody, and no oligonucleotide with normal or treated serum.

CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

The analysis of the interaction of DNA with genotoxic agents is fundamental to monitoring human carcinogen exposure and assessment of the associated risk. Much work has been carried out on the methodology to detect DNA adducts. However, many genotoxic agents will interact with DNA which then may undergo repair or replication. These processes will remove the adducted base but may lead to a point mutation at the site of interaction. The use of the PCR coupled to DNA sequencing has provided the most general method to detect abnormal DNA sequences. Although PCR is relatively simple the overall process is still labour intensive.

Alternative methods of detection have focused on converting the point mutation into a base pair mismatch, by denaturation and annealing of the mutated strand in an excess of non-mutated complementary DNA. Detection of mismatched sites are then carried by techniques such as single stranded conformation polymorphism, oligonucleotide hybridisation, denaturing gradient gel electrophoresis, RNase A cleavage of RNA:DNA duplexes and others discussed in chapter one.

More recently mismatch repair enzymes (such as Mut Y and thymidine DNA glycosylase) have been used to detect point mutations in the form of base pair mismatches in duplexes (Hsu *et al.*, 1994). The enzymes being used in a similar way to RNase A. However, these methods are limited in the sensitivity of the assay systems. At best they only detect mutations if there are mutations in greater than 2% of the DNA being tested. Where the mutation is occurring at very low frequency enrichment methods are required.

Mismatch repair proteins may serve as a potential means to enrich mismatch base pair containing duplexes. The assay conditions would need to be modified such that recognition occurred with no cleavage. The major draw back at the moment is these enzymes are not readily available in a highly purified form.

Monoclonal antibodies raised against mismatched base pairs may serve as a highly specific method for immuno-enrichment of DNA sequences containing point mutations. This type of analysis would provide a means of determining mutation at any locus at the molecular level with the sensitivity and precision required to assess low level cancer risk. The study has focused on the production of monoclonal antibodies to specific base pair mismatches in synthetic DNA duplexes. Two distinct areas of research were produced. Firstly, the production of DNA duplexes containing mismatched base pairs for immunisation experiments and antibody screening was described in chapter 2. Secondly, immunisation and fusion experiments to produce antibody, and subsequent identification of antibody binding properties was described in chapter 3.

A considerable amount of data is available concerning the structure of mismatched base pairs in DNA duplexes. Mismatched base pairs have been shown to be structurally and thermodynamically different from normal Watson-Crick base pairs. These differences have been detected by physical, chemical and biological techniques. Of particular interest is the finding that mismatched base pair recognition enzymes exist in both prokaryotes and eukaryotes. This means protein recognition of mismatched base pairs in DNA duplexes occurs.

Although the immune system can be stimulated to produce antibodies to certain types of nucleic acid, e.g. Z-DNA, RNA-DNA hybrids, mammalian B-DNA has not been shown to be immunogenic in test animals. The immunogenic nucleic acids all possess non-B-DNA structures/conformations. A mismatched base pair provides an altered structure to B-DNA and thus provides a possible recognition site for immune recognition. However, for most occurrences of anti-nucleic acid antibodies a large proportion, or the entire immunogen, is in the altered conformation, not just one base pair.

The current study has aimed to develop a novel method to detect rarely occurring point mutations in DNA, with high sensitivity. The point mutation will be detected by the artificial production of a mismatched base pair at the site of mutation, and subsequent

detection of the mismatched base pair. A variety of DNA duplexes were constructed containing mismatched base pairs, for immune recognition.

The use of automated DNA synthesisers allowed us to produce large amounts of specific DNA sequences, rapidly and such that purification was easily achieved. A major concern was the thermal stability of relatively short duplexes, especially as the presence of a mismatch will destabilise the duplex. In order to ensure thermal stability, single stranded sequences were chemically linked at one terminus *via* a tetraethylene glycol linker to a complementary strand such that the duplex contained a central base pair mismatch. The chemical linkage was carried out as part of the automated synthesis. Duplexes of this nature were found to be thermally stable, and additionally the thermal stability was independent of concentration, thus they would not dissociate even at high dilution.

Attempts to increase the duplexes stability to nuclease degradation was carried out. Duplexes were prepared as described above except the phosphate groups were all converted into phosphorothioate groups, which affords stability to nuclease enzymes. However, these duplexes were found to be thermally unstable and, therefore, unsuitable for immunisation experiments. It may be worth investigating the use of mixed phosphate and phosphorothioate containing duplexes for immunisation. The thermally stable duplexes were used to immunise mice alone or as complexes with methylated BSA. Methylated BSA may also reduce nuclease degradation by sterically hindering the approach of the enzyme to the DNA.

A considerable amount of time was then devoted to developing a screening method capable of detecting the production of anti-mismatched antibodies. There is a great deal of uncertainty as to the possible immunogenic nature of mismatched base pairs, and devising a suitable assay was a considerable task. When using the initial screening methods we were uncertain if the assay was capable of detecting mismatched based pairs in DNA duplexes. Where anti-DNA antibodies and specifically anti-mismatched base pair

antibodies were not detected we were uncertain if the assay had failed or that we were simply not achieving the desired immune response. It is possible that antibody screening methods used during the first 6-12 months of the immunochemical portion of the study may have not detected potentially useful antibodies. The difficulty and uncertainty in developing a screening assay was fuelled by the varied and contradictory requirements for anti-DNA antibody assays presented in the literature.

In initial screening procedures oligonucleotides were immobilised in a random orientation to the surface of microtitre plates. This random orientation may not have allowed the antibody to interact with the base pair mismatch. Limited information was obtained from these experiments and no anti-mismatched base pair antibodies were detected.

As improved assay procedures were being developed alternative sources of immunogen were investigated. Larger duplex, 75mers, were constructed such that they contained four evenly spaced single base pair mismatches. Additionally these duplexes had five phosphorothioate containing base pairs at the 3' terminus to improve stability against possible nuclease degradation. In addition, a complete C100mer single stranded phosphorothioate (C100PS) was constructed. Antibodies that recognise cytosine bases should be produced and these may cross react with cytosine containing mismatches (A:C; C:C; T:C).

Subsequent methods to screen for antibody production concentrated on improved immobilisation techniques. Methods to specifically immobilise duplexes by end labelling with biotin followed by incubation with streptavidin coated plates were investigated. It was found that biotin labelled duplexes stabilised by a tetraethylene glycol linker, as previously discussed, were required. In addition the biotin had to be attached to the duplex *via* a second linker or steric hindrance, caused by the proximity of the fairly rigid duplex to the biotin, reduced the subsequent binding of biotin to streptavidin coated plates.

Using this coating procedure we investigated antibody production in a variety of assay conditions. Variations in buffers e.g. PBS/Tw vs TBS/Tw, produced considerable differences in antibody binding during both non-competitive and competitive assays. For the monoclonal antibody tested (AC75/14D6/4.12) TBS/Tw gave less antibody binding in non-competitive assays (compared to PBS/Tw) and no inhibition in competitive assays. Pisetsky and Semper (1984) noted some monoclonal anti-DNA antibodies had altered antigen binding, dependant upon buffer used.

Alterations in binding may reflect slight structural changes in duplex conformation caused by the buffer. Alterations in binding were only investigated using mismatch containing duplexes. The presence of a mismatched pair may facilitate duplex conformational change. However, the duplexes investigated by Pisetsky and Semper (1984) were fully matched, thus a mismatch site is not prerequisite for this phenomena. This finding was of great concern as the variety of possible buffers is huge. Clearly it would be impossible to screen the hundreds of test wells produced in fusion and cloning experiments to determine the ideal or effective assay conditions for different anti-DNA antibody populations.

To reduce possible nuclease enzyme degradation of DNA, EDTA in wash solutions and buffers was investigated. EDTA was found to greatly interfere with the peroxidase/ABTS reaction and to reduce anti-DNA antibody/DNA interactions. Thus the use of this compound to preserve the DNA in the test system was then detrimental to the detection of the DNA in the test system. Serum free/protein free cell culture medium was used for fusion and cloning to reduce nuclease contamination. The use of reduced FCS in these experiments did not effect the numbers of hybridomas produced or the ability to produce wells containing single colonies in cloning experiments.

For several of the supernatants obtained from fusion wells (containing mixed cell populations) inhibition studies indicated antibodies were present that had some specificity to mismatched base pairs in duplexes. However, we were unable to generate monoclonal

cell lines with similar specificity. The cloning procedure used routinely obtained greater than 25 single colony wells per cloning experiment. The hybridomas produced may be genetically unstable, losing the ability to secrete antibody and/or produce antibody.

In addition, hybridoma formation is rare event. The production of monoclonal antibodies would not be possible if it were not for the ability to increase the relative amount of the desired splenocytes, for fusion, by immunisation. If this were not possible it would be extremely unlikely that the desired clones could be isolated. Where the mismatched based pair provides only a weak immunogenic stimulus to the immune system, splenocytes producing the desired antibody will be in the minority, compared to good immunogens. Where this occurs the desired hybridoma production will be an extremely rare event. Hybridomas secreting the desired antibody have a greater probability of being over grown at the post fusion stage, and thus less likely to be isolated in cloning experiments.

The cell growth medium for clone and fusion wells are not identical, and this may also explain some of the differences observed. Clone wells contain a large amount of dead material from the feeder cells and unlike fusion wells this material will not be removed. The growth medium is not changed between carrying out the cloning procedure and the screening for antibody production. The degraded and partially degraded DNA within the supernatants may interact with antibody released into the supernatant prior to screening, as suggested by Ehrenstein *et al.*, (1993).

Where C100PS was used as an immunogen we obtained hybridomas secreting antibody into the growth medium that showed high titres against single stranded cytosine containing oligonucleotides. Monoclonal antibody producing cell lines were produced that recognised duplexes containing C:C and A:C mismatches but not to fully matched duplexes. It should be noted that these are not mismatch specific antibodies as they bound to single stranded oligonucleotide. However, they did not bind to the duplex containing fully matched base pairs.

Alternative antibody production technology

There are potentially two other methods to generate antibodies, both of which may overcome drawbacks of the traditional immunisation and hybridoma technology methods. Firstly, splenocytes can be isolated from non-immunised mice and antigenic material can then be introduced *in vitro*. At the moment this method is not used routinely, although it has certain advantages over the classical *in vivo* approaches. The main advantage is that the environment in which immunisation occurs is under greater control. Problems associated with *in vivo* metabolism (including nuclease enzyme degradation) are greatly reduced. Also weaker immunogens have been shown to produce immune responses with this technique, important when using DNA. In addition it requires less antigen and the antigen does not need to be coupled to a carrier protein.

Secondly, over the last few years there has been an increasing amount of work focusing on recombinant phage display antibody production first described at the start of the decade (McCafferty *et al.*, 1990; Clarkson *et al.*, 1991). This technology serves to by-pass the hybridoma techniques by genetically engineering monoclonal antibodies (reviewed by Winter and Milstein, 1991). Briefly, the technology allows the mRNA coding for the antibody to be isolated and then converted to cDNA. This cDNA sequence can then be incorporated into a phage, such that antibodies may be produced in unlimited quantities. More importantly it is possible to manipulate the binding site of the antibodies produced, by the introduction of mutations at the antigen binding regions in the cDNA. This could allow the manipulation of anti-DNA antibodies such that anti-mismatched base pair antibodies are produced.

Both of these techniques potentially provide alternative methods to produce anti-mismatched base pair antibodies. The later probably requiring some initial work with traditional hybridoma techniques in order to have mRNA, from anti-DNA antibody secreting hybridomas, to manipulate.

Conclusions

In conclusion, a great deal of work has led to the development of improved assay methods aimed at detecting the production of anti-mismatched base pair antibodies after immunisation experiments. This study did not produce any monoclonal cell lines producing specific anti-mismatched base pair antibodies. However, cell growth medium after fusions contained antibodies that showed inhibition to mismatch containing duplexes in competitive assays. Cloning experiments failed to isolate these monoclonal cell lines producing antibodies. Further work may need to focus on the cloning procedure. However, part of the difficulty could be sheer numbers of experiments that need to be carried out. It was reported, see main text, that 20,000 fusion wells were screened in the development of a monoclonal to the immunogen Z-DNA, this work produced one useful monoclonal cell line.

It was discussed in the introduction to chapter 3 that ELISA was not the only screening method available. However, for the number of samples we were handling it was the preferred method. Throughout the course of the screening experiments improvements in assay methodology were developed. Each step appearing to give us an improved assay. When screening for antibodies produced by a poorly immunogenic material it would be expected that isolating a desired monoclonal would be an extremely rare event, similar to the production of anti-Z-DNA antibodies. The coating methods developed could be used for non-competitive and competitive ELISA, and thus should be used to screen a large number of fusion experiments.

I feel that much has been learned regarding the types of immunogen that should be used to produce anti-mismatched antibodies. The larger duplexes containing multiple mismatched base pairs and end labelled with phosphorothioate bases have proved useful. These may be rendered more stable to nuclease attack during immunisations by the inclusion of phosphorothioate groups within the duplex. The use of poly-cytosine 100mer as a complete phosphorothioate produced antibodies that were able to recognise

duplexes containing CC and AC mismatches. These antibodies could be used to detect or enrich DNA duplexes containing mismatched base pairs single stranded DNA was not present. To date poly-cytosine 100mer is the only sequence used that produce anti-mismatched base pair antibodies, poly T, A and G have yet to be studied. A number of suggestions for continued work on this project are presented.

4.1 FUTURE WORK

The production of monoclonal cell lines that produce antibodies with specific recognition of a base pair mismatch in DNA compared to native DNA has proved elusive. However, pre-clonal stages of the work provided implications that the production of antibodies to a mismatched base pair was possible. Outlines of possible future work aimed at producing anti-mismatched base pair antibodies are discussed below.

The use of hybridoma cell lines already produced.

- Continued use of the established ELISA methodology to screen the production of hybridoma cell lines of current interest.

- Extensive screening of the large numbers of cell lines produced by fusion experiments prior to the improved ELISA.

- Production of a solution phase immunoassay, possibly utilising radiolabelled DNA.

The synthesis of a more immunogenic source of DNA.

- The original 15mer duplexes were synthesised as normal phosphate oligonucleotides. One major concern is the degradation of these duplexes during the immunisation regime (see also 4.1.4). By replacing an oxygen atom in DNAs phosphate group with a sulphur atom (producing a phosphorothioate) the serum half life will be increased. Creating complete phosphorothioate sequences proved unsuccessful due to thermal instability. However, this could be overcome by the selective addition of phosphorothioate groups at some but not all positions within the duplex.

- The use of DNA-RNA hybrids as a source of immunogenic material. The native B-DNA conformation is poorly immunogenic whereas RNA-DNA hybrids are more immunogenic. Mismatched base pairs could be incorporated into this hybrid in a manner

similar to the synthetic DNA duplexes. The enhancement in the immunogenicity of the entire duplex may increase the immunogenicity of a mismatched base pair within the duplex. This approach could be coupled to selective phosphorothioate inclusion.

- Experiments to specifically conjugate oligonucleotides to proteins proved unsuccessful. The use of streptavidin/biotin immobilisation of oligonucleotides could be adapted such that streptavidin/biotin oligonucleotides could be used as an immunogen. This would greatly increase the molecular weight of the immunogen and possible provide some hindrance to nuclease enzymes.

- The use of poly-thymine, poly-guanine, and poly-adenine 100mers as complete phosphorothioates as immunogens.

Cloning procedures

- Investigation of the DNA content of clone wells may be important. If DNA levels produced by dying feeder cells was high cloning procedures in the absence of feeder cells may need to be investigated. Cloning could be carried out using cell culture medium enhanced with growth factors.

Alternatives antibody production methodologies

- Use of phage display technology to modify anti-DNA antibody binding such that anti-mismatched base pair antibodies are produced.

- Use of *in vitro* antibody production to reduce the effect of nuclease degradation of antibody.

CHAPTER 5 : REFERENCES

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