SPECIAL COMMUNICATION

THE INTERACTION OF METABOLIC SYSTEMS WITH DAMAGED DNA¹⁾

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The International Genetics Symposia in 1956 was one of the first international meetings held in Japan after the war and it was a great success. The scientific sessions were impressive and we Americans were absolutely fascinated by your country. The opening address was given by George Beadle and many of you will remember the closing lecture given by J. B. S. Haldane, a huge man, both physically and intellectually, ruddy faced, English, and in Punjabi dress.

After two weeks in your country I knew that I wanted to return for a longer period. As a result of having been trained in Beadle's *Neurospora* group at the California Institute of Technology it had been my good fortune to meet Francis Ryan, one of the first of our geneticists to come to Japan as a visiting Professor in the 1950's. Professor Ryan played a major role in bringing the new methodologies of microbial genetics to Japan and in inspiring a generation of geneticists. I went to him for advice as to where I might work in Japan. After some thought, Francis suggested the Department of Genetics at Osaka University under Professor Hideo Kikkawa as the best environment for me. This seemed a good choice, especially since Kikkawa's pioneering work with kynurenine and the pathway of tryptophan metabolism in insects (Kikkawa 1941; 1953) was an important part of the intellectual path that led to the *Neurospora* work and the precise formulation of the gene-enzyme relationship.

My desire to work in your country was strengthened by a fruitful association with Dr. Teiichi Minagawa who came to work with me at Syracuse before going on to Cold Spring Harbor. When I did arrive in Japan in 1958, the group in Osaka was particularly cordial and helpful and I was "taken in hand" by Yasuyuki Takagi who helped with many practical details both at home and in the laboratory. Like many geneticists in the late 1950's I had decided that it was important to work on DNA and after some attempts to study the transmutation effect of isotope decay in *Neurospora* (Strauss 1958) I decided to concentrate on mutation in bacteria. It seemed to me that there were interesting things to be learned about chemical mutagens and as the result of the

1) Delivered at the 51st Annual Meeting of the Society, in memory of the late Dr. Shunzo Okubo, Professor of Genetics, Faculty of Medicine, Osaka University, who died on October 9th, 1978, during the 50th Annual Meeting.

work of Auerbach (1957) and Westergaard (1957) I picked on the alkylating agents as mutagens. At Osaka I had just started on experiments to test the role of protein synthesis on the production of mutations in *Escherichia coli* when Dr. Takagi suggested that a student in Professor Kikkawa's department was interested in switching from work with *Drosophila* to bacterial genetics, and that it might be profitable for both of us if he were to help me, and so Okubo and I started to collaborate (Strauss and Okubo 1960).

Specificity of induced mutation

This was in late 1958 and it is interesting to consider for a moment our view of induced mutation at about that time. To begin with, there was not much similarity in the approach of those investigators studying the mutagenic effects of radiation and those working on chemically-induced mutation. Radiation was the first agent clearly shown to induce mutation and the field was dominated by biophysical questions, particularly by the question of the validity of target theory and the role of the indirect effect of radiation (Lea 1947). A series of intriguing observations by Witkin (1956) and by Doudney and Haas (1959) showed that protein synthesis might play an important role in the establishment of a certain class of mutations induced by ultraviolet light, but it was not obvious which metabolic events might be involved.

The situation with chemical mutagens was clarified by the analysis of the nature of mutations induced by simple chemicals in both plant (Mundry and Gierer 1958) and bacterial viruses (Freese 1959a). Freese was able to classify many mutations as transitions, produced and reversed by base analogs such as bromouracil and resulting at the molecular level from substitutions of purine for purine and pyrimidine for pyrimidine in DNA (Freese 1959a). A second class of mutation, resistant to reversion by base analogs (Table 1) was first classified as transversions, the substitution of a purine for a pyrimidine or a pyrimidine for a purine (Freese 1959b). Transversions do in fact occur, as shown by the amino acid sequence analysis of mutant proteins, but the mutations produced by proflavin and not reversed by bromouracil (Table 1) were recog-

Table 1. Reversion induction of spontaneously reverting mutants

RII Mutants induced by	No. of mutants tested	Mutants found (%)		Approximate spontaneous background of non-reversion inducible mutants
		Inducible to revert by AP and/or BD	Non-inducible to revert by neither AP nor BD	
2-Aminopurine	98	98	2	2
5-Bromouracil	64	95	5	2
Hydroxylamine	36	94	6	4
Nitrous Acid	47	87	13	15
pH 5, 45°C	115	77	23	15
Ethyl ethanesulfonate	47	70	30	10
Proflavin	55	2	98	—
Spontaneous	110	14	86	—

from Freese, 1963.

nized by the group at Cambridge as being addition and/or deletion mutants (Brenner *et al.* 1961) and these "frame shifts" served as the basis for the genetic analysis of the genetic code as triplet in nature (Crick *et al.* 1961).

This brilliant deduction, confirmed by biochemical analysis, validated the molecular analysis of the mode of mutagen action which was extended by Freese and others to the alkylating agents and to organisms other than viruses (see Freese and Freese, 1966 for a typical review). There was continuing interest in the problem of mutation as a metabolic process, but the Freese analysis was so elegant and appeared to account for so many facts on the basis of a simple theoretical framework that it was soon included in even the elementary textbooks of biology. Although investigators such as Charlotte Auerbach might insist (1967) that: "Mutagenesis . . . must be thought of as a biological process in which a chemical change in DNA is only a first step, although an indispensable one", interest centered on the chemical alterations that might be produced in DNA bases and on the pairing changes that might occur as a result of the purely chemical nature of these alterations.

Significance of radiation sensitive mutants

The genetic mutants which led to a change in these views were even then being isolated. If mutation is the result of chemical or physical change produced in DNA by external agents, then the genetic constitution of the treated organism should make little difference in the response (assuming equal reactivity). The isolation of mutants of bacteria particularly sensitive to the lethal effect of ultraviolet radiation (Hill 1958) (Fig. 1) was the first in a chain of investigations which convincingly demonstrated that

Fig. 1. Ultraviolet sensitivity and host cell reactivation deficiency of bacterial mutants. Left: Ultraviolet survival of wild-type *Bacillus subtilis* and two UV-sensitive mutants. Right: Survival of UV-irradiated *B. subtilis* phage SP02 as assayed in the host cell recovery deficient mutant (lower curve), the wild-type and a host cell recovery proficient (*hcr*⁺), UV-sensitive bacterial host (upper curves). Data from Okubo and Romig (1966).

Fig. 2. Excision repair synthesis in Raji and XPA-3 lymphoblastoid lines. Exponentially growing cells were preincubated with 10 mM hydroxyurea and 1 μ M FdU for 30 min at 37°C. Open symbols, Raji; closed symbols, xeroderma.

A. Repair induced by UV irradiation. The medium was removed and the cells suspended in warm PBS containing hydroxyurea and FdU and UV irradiated. After irradiation warm medium and [3 H]dT (μ Ci/ml; 13 Ci/mmol) was added. The cells were incubated for 60 min and the amount of repair synthesis determined.

B. Repair as a function of time induced in Raji cells by treatment with chemical carcinogens. Cells preincubated with hydroxyurea and FdU were treated with carcinogen for 15 min at 37°C. After washing and resuspension in medium containing hydroxyurea and FdU, the cells were divided into equal aliquots. At hourly intervals, [3 H]dT (10 μ Ci/ml, 13 Ci/mM) was added and repair was allowed to proceed for 1 h. The value for the 1st h was obtained from a separate culture of cells to which [3 H]dT and carcinogen were added. Cumulative values were obtained by addition. Circles, control, no addition; squares, AAAF, 10 μ g/ml; triangles, MNNG, 5 μ g/ml; stars, MMS, 200 μ g/ml.

C. As above but with xeroderma cells.

Note: Raji is a cell line derived from a Burkitt's lymphoma. XPA-3 is a lympho-blastoid line derived by J. Robbins from a xeroderma pigmentosum complementation group C patient. From Altamirano-Dimas *et al.* (1979).

the metabolic capabilities of an organism determine its response to chemical and physical agents. The lesion produced by a mutagenic agent can actually be removed by a process now called excision repair (Setlow and Carrier 1964; Boyce and Howard-Flanders 1964), or the response of the organism can be altered so that it becomes "mutagen stable" and, although exquisitely sensitive to the killing effect of a particular treatment, no longer responds by producing mutations (Miura and Tomizawa 1968; Witkin 1969; Kondo *et al.* 1970). Analogs of some of the bacterial mutants were discovered in human cells, starting with the recognition that xeroderma pigmentosum cells were unable to initiate UV-induced excision repair (Cleaver 1968) (Fig. 2). The analysis of mutator and antimutator mutants of bacteriophage T4 (Drake 1973) indicated that the replicative DNA polymerase might control mutation rate by editing or failing to edit out mutations as a result of the action of the polymerase associated 3'-5'

EXCISION REPAIR PATHWAYS

Fig. 3. Excision repair pathways.

nuclease activity (Muzyczka *et al.* 1972). These observations all vindicated the view of Auerbach (1967) and of Kondo *et al.* (1970) that the response of the organism was critical and the analysis of the response of the organism to mutagenic alteration has turned out to be a complex and interesting problem of biochemistry (Drake and Baltz 1976).

The most studied of the interactions between organism and altered DNA is the system(s) of excision repair (Fig. 3). Excision repair in mammalian cells is best understood by supposing that at least two separate pathways are involved, nucleotide excision and base excision (or apurinic/apyrimidinic, AP) repair each with its own system of enzymes (Duncan *et al.* 1976). The steps of nucleotide excision are well known: an initial incision adjacent to the site of a lesion is followed by a strand displacement which as a result of exonuclease action results in removal of a stretch of nucleotides including the damage, accompanied by resynthesis of an undamaged replacement using a parental, undamaged strand as a template. A final ligation completes the process.

The recognition of this overall process by Setlow and Carrier (1964) and by Boyce and Howard-Flanders (1964) prompted numerous investigations with other organisms in an attempt to work out the details of the process. It seemed to Shunzo Okubo that analysis of mutants of organisms in which the DNA could be analyzed by bacterial

transformation would be desirable and he therefore set out to isolate an excision repair defective *Bacillus subtilis* mutant (Okubo and Romig 1965, 1966). (It should be remembered that the techniques for transformation of *E. coli* were not discovered until much later.) Okubo's previous studies on the viruses of *Bacillus subtilis* were extremely helpful for this purpose since he had demonstrated that transfection by the large phage, SP01, required some recombination process (Okubo *et al.* 1964), whereas transfection by the DNA of the smaller phage SP02 required only single molecules (Okubo and Romig 1966). This made it possible to determine not only whether host cell reactivation was deficient in mutants, but also whether recombinational processes were involved.²⁾ The availability of the *B. subtilis* phage, SP01, was also of great help when Dr. Searashi came to my laboratory and isolated a strain of *Bacillus subtilis* deficient in repair capability for methyl methanesulfonate-induced damage (Searashi and Strauss 1965) and, as later shown by Dr. Cozzarelli's group (Gass *et al.* 1971), also deficient in DNA polymerase I activity.

The Enzymology of excision repair processes

The observation that a crude extract of *Micrococcus lysodeikticus* (now called *M. luteus*) would recognize DNA damage induced by ultraviolet light or by methyl methanesulfonate (Strauss 1962) was used as the starting point for a series of investigations on the enzymology of nucleotide excision in which Shunzo Okubo played a central role along with Drs. Sekiguchi, Takagi, and Nakayama (Nakayama *et al.* 1967; Okubo *et al.* 1967; Shimada *et al.* 1967; Takagi *et al.* 1968; Nakayama *et al.* 1971; Okubo *et al.* 1971). As part of the program of fractionation and purification of the *M. luteus* extract, Okubo and Nakayama (1968) developed a methodology for the study of bacterial transformation in *M. luteus* which was then used to investigate the *in vivo* role of the endonuclease. This role is complex and the detailed enzymology of nucleotide excision as it occurs *in vivo* in free living organisms is still unknown, notwithstanding the elegant enzymological investigations on the subject (cf. Riazuddin and Grossman 1977; Minton *et al.* 1975). It may be that the UV-endonuclease induced on infection of *E. coli* with T4 bacteriophage and extensively purified by Dr. Sekiguchi and his group (Sekiguchi *et al.* 1970; Yasuda and Sekiguchi 1976) is the clearest example of an enzyme with an *in vivo* function since this enzyme has specificity for pyrimidine dimers, processes a variety of UV-irradiated DNA's in cells so that they can be repaired in otherwise excision defective organisms (Tanaka *et al.* 1977) and is affected by mutations which alter the UV sensitivity of the infecting virus.

The situation with *Escherichia coli* itself and in mammalian cells is more complicated. There are several enzymes which recognize UV-irradiated DNA. Some recognize photoproducts other than dimers, others recognize partially denatured regions and there are also enzymes with real specificity for pyrimidine dimers (Waldstein *et al.* 1979). In general, the isolated endonucleases are small protein molecules with no cofactor

2) Separate from these studies but of great interest was the cooperative endeavor using the suppressor sensitive mutants (Okubo and Yanagida 1968) in which Okubo and Yanagida in Japan and Fujita and Ohlsson-Wilhelm working with Peter Geiduschek analyzed the genetics of bacteriophage SP01 (Okubo *et al.* 1972).

requirement. In *Escherichia coli* three genes have been reported as involved in the incision of dimers and in permeabilized cell systems ATP is required for *uvr*⁺-dependent incision. A complementation assay system has been used to show that ATP and at least two gene products contribute to an excision complex of high (170,000) molecular weight (Seeberg 1978). A similar large enzyme complex obtained from calf thymus incises UV-induced dimers (Waldstein *et al.* 1979). Since the smaller endonucleases are also dependent on the *uvr* gene products (Braun *et al.* 1976), the simplest way of reconciling the data currently published is to suppose that an *in vivo* complex can be broken up in some way to yield smaller endonucleases.

This view has the advantage of accounting for the peculiarities of nucleotide excision. It is known that single genetic defects in bacteria can affect not only the ability to repair UV damage but also damage induced by nitroquinoline oxide, mitomycin C and psoralen plus light. The xeroderma pigmentosum genetic alteration leads to deficiency in the removal of not only pyrimidine dimers but also adducts added by polyaromatic hydrocarbons such as benzpyrene diolepoxide (BPDE) and acetoxy acetylaminofluorene (see the review by Hanawalt *et al.* 1979). The configuration of DNA after reaction with this variety of mutagens is certainly different enough to require a remarkable single enzyme and it is easier to suppose that the xeroderma alteration results in disruption of an enzyme complex. Furthermore, the sequence of reactions that make up nucleotide excision appear to occur in a coordinated fashion. For example, DNA with single strand breaks occurs relatively rarely (Karran *et al.* 1977) making it unlikely that nucleotide excision proceeds via a series of sequential intermediates with significant half lives.

Nakayama, Okubo and Takagi (1971) recognized that an endonuclease activity found in *M. luteus* and active against DNA treated with methyl methanesulfonate differed from the UV-specific enzyme. This implies that the pathway of repair for methylating agent induced damage differs from the UV repair pathways. Such a hypothesis accounts for the finding that the UV-sensitive mutant of *B. subtilis* (and similar *uvr* mutants of *E. coli*) is not sensitive to methyl methanesulfonate. However, since the mutant isolated as sensitive to methyl methanesulfonate by Dr. Searashi (Searashi and Strauss 1965) is also sensitive to UV it is possible that a later stage in a sequential repair process is affected. The finding that the *alk* *E. coli* mutant, sensitive to methyl methanesulfonate but not to UV, is deficient in repair involving polymerase I (the *pol* A product) (Yamamoto and Sekiguchi 1979) supports this hypothesis. However, because the xeroderma strains do repair MMS-induced damage (Fig. 2), I prefer to believe that these excision repair reactions are not part of the nucleotide excision repair complex in mammalian cells.

Recent investigations, particularly by Lindahl (1976), have made the differences between nucleotide and base excision more understandable. A variety of glycosylases with specificities for different altered nucleotides have been isolated. For example, enzymes have been detected with specificity for 3-methyladenine, hypoxanthine, uracil and even for 7-methylguanine with imidazole rings opened by treatment with alkali (Lindahl 1979). Uracil glycosylase has been found by Sekiguchi and his collaborators in human placenta (Sekiguchi *et al.* 1976) and a mutant of *B. subtilis* defective in the

Fig. 4. *In vitro* repair of alkali-labile sites in phage T7 DNA. The figure gives the results of alkaline-sucrose gradient centrifugation of:

- a) Untreated T7 DNA ······
- b) MMS-treated DNA (12.5 mM MMS for 10 min plus 1 h at 70°C in pH 7.4 after removal of the MMS to give 10 alkali-labile sites per strand) plus nuclease plus polymerase plus ligase ●—●
- c) Complete system minus polymerase ○ ○
- d) Complete system minus polymerase and ligase □ □
- e) Complete system minus ligase ■ ■

From Bose *et al.* (1978)

glycosylase has been reported by Makino and Munakata (1977). The uracil glycosylase plays a key role in protecting normal DNA from the introduction of uracil residues.

Glycosylase action results in apurinic or apyrimidinic (AP) sites. AP DNA sites can arise in cells both as a result of the chemical depurination of alkylated (and normal) bases and via glycosylase action and are substrates for AP endonucleases which are widely distributed in cells. *In vitro* reconstruction of a repair system using AP DNA, AP endonuclease, exonuclease, polymerase and ligase is possible and has been reported by Verly *et al.* (1974) using prokaryotic enzymes. We have prepared such a system using T7 DNA and with the enzymes AP endonuclease, exonuclease and DNA polymerase α prepared from human lymphoma cells (Bose *et al.* 1978). The *in vitro* reaction has many of the properties observed for methyl methanesulfonate-induced repair *in vivo* including a repair "patch" of only a few nucleotides (Fig. 4). It is convenient to refer to this sequence of reactions as the AP repair pathway, or the base excision pathway. The AP pathway may be distinguished from nucleotide excision in cells by the smaller size of the repair patch (Regan and Setlow 1964) and by the relative prevalence of DNA with single strand breaks produced as an intermediate in AP repair (Karran *et al.* 1977).

Table 2. Alkylation products in the presence and absence of chloramphenicol

MNNG treatment	7-N-methylguanine		O ⁶ -methylguanine		mg DNA ^a recovered	Mutation ^b		Mutations ^c genome
	dpm/mg DNA	adducts/genome	dpm/mg DNA	adducts/genome		mutants scored	proportion mutant	
40 min 1.25 µg/ml	4731	268	43	5	7.05	2/2516	.0008	0.08
40 min 1.25 µg/ml and chloramphenicol	6606	373	705	79	7.40	23/1550	.015	1.5
40 min 2.5 µg/ml	15425	876	133	12.1	6.27	10/983	.010	1.3
40 min 2.5 µg/ml and chloramphenicol	20654	1173	2549	233	5.07	79/648	.122	15

^amg DNA determined from the absorbancy of guanine in column elute.

^bmutation to *ara*, *aceAB*, *arg*, and *met* determined by replica plating.

^ccalculated by multiplying the average number of mutations per locus by 4000 (possible number of genes which could mutate).

Data of R. Sklar.

There is still much to be learned about the way in which cells rid their DNA of potentially mutagenic alterations. One of the most interesting observations concerns the adaptive response of bacterial cells to treatment with methyl nitronitrosoguanidine (MNNG). This substance produces O⁶-methylguanine adducts which result in mutation by mispairing. Cairns and his group have demonstrated that MNNG treatment of *E. coli* induces an enzyme(s) which removes the adduct (Samson and Cairns 1977; Schendel and Robins 1978). The reaction is not catalyzed by a glycosylase and methanol is liberated from one of the first products (Karran *et al.* 1979). A similar enzyme system operates in mammalian cells (Pegg 1978) but it is not clear whether it is inducible. The biological results of the induction in bacteria may account for some of the observed high mutagenicity of MNNG at DNA growing points (Cerdeira-Olmeda *et al.* 1968). Protein synthesis is required for induction and chloramphenicol can therefore prevent enzyme induction. Cells incubated with chloramphenicol along with MNNG have a higher mutation frequency and higher residual O⁶-methylguanine in their DNA (Table 2). The induction process occurs in *E. coli* within 10 minutes as shown by the effect of chloramphenicol addition at different times after the start of MNNG treatment (Fig. 5). Mutagenic adducts more than 10 minutes away from the growing point will be removed by the inducible process before DNA synthesis reaches their locale and therefore an enrichment of mutations near the growing point will be observed. The fact that chloramphenicol has very different effects on mutation induced by either UV or by ethyl sulfate, as well as the observation that the frequency of ethyl sulfate-induced mutation was increased by incubating cells with the amino acid analog, trypta-

Fig. 5. Effect of time of chloramphenicol addition on the yield of MNNG-induced mutation. Exponential cells were treated with 2.5 $\mu\text{g/ml}$ MNNG and chloramphenicol (50 $\mu\text{g/ml}$) was added at the time indicated after treatment, up to 40 min. Treated cultures were washed, resuspended in growth medium, grown to stationary phase and plated on rifampin-containing plates. Data of R. Sklar.

zan, was reported by Okubo and myself in 1960 (Strauss and Okubo 1960). It seems likely that we were seeing the mutagenic effect of inhibiting an inducible repair system but one cannot be sure.

Bypass mechanisms

The systems of excision repair are extensive and complex but in some ways are not relevant to the problem of mutation. UV-excision defective bacterial mutants are hypermutable after UV-irradiation and this implies that the excision system, which removes UV lesions, reduces the amount of mutation (Hill 1965). Preventing the replication of animal cells until chemical or radiation induced lesions have been removed from the DNA reduces the amount of mutation finally obtained (Maher *et al.* 1977). The excision repair mechanisms are therefore "error free". This conclusion implies that it is the presence of lesions in cells during DNA synthesis which results in mutation.

Although UV irradiation or treatment of cells with many mutagenic chemicals can inhibit DNA synthesis, both bacterial and eukaryotic cells can replicate their DNA even though inhibitory lesions are present. This paradox can be demonstrated by calculation, for example, of the number of non-excisable pyrimidine dimers required to produce a lethal hit in either bacteria (Howard-Flanders and Boyce 1966) or xeroderma cells (Cleaver 1970), and also by the direct demonstration of chemically induced lesions in replicated DNA. *Uvr* mutants of bacteria can replicate even when carrying 60 pyrimidine dimers per cell and a series of experiments from different laboratories (e.g., Dipple and Roberts 1977; Strauss *et al.* 1979) can be summarized by saying that repli-

Fig. 6. Model of recombination repair *via* strand displacement.

— parental DNA
 ---- and . . . , daughter DNA
 ● block to replication

Note: a description of post replication repair by gap filling would start with the structure on the upper left and fill in the gap by new (. . .) synthesis.

cation of polyaromatic hydrocarbon adducts in human cells can occur when there is up to about one lesion per replicating unit.

At least two mechanisms account for the ability of the replicating system to bypass damage in bacteria. One of these mechanisms is probably "error free", the other is an inducible mechanism associated with the production of mutation. The constitutive bypass mechanism in bacteria occurs when DNA synthesis reaches a lesion and hesitates on one strand while continuing along the other parental template. Further downstream synthesis then reinitiates on the damaged strand. As a result of a strand displacement reaction, a portion of the parental strand of correct polarity is inserted into the "gap" in the newly synthesized strand, thereby bypassing the lesion (Fig. 6). The newly produced gap in the parental strand is filled by the nick translation which has displaced the parental strand. Ligation of the ends results in a complete new molecule still containing lesions (Ganesan 1974; Rupp *et al.* 1971).

The inducible reaction is identical with the system of Weigle reactivation for phage and involves the *rec* and *lex* gene products (Witkin 1976). After induction, DNA synthesis bypasses lesions without recombination. An ingenious explanation of how this could occur has been furnished by Radman and his group (Villani *et al.* 1978; Caillet-Fauquet *et al.* 1977). The replicative bacterial DNA polymerase contains a 3'-5' nuclease which serves an "editing" function by removing bases which do not form correct pairs. Mutations which change the ratio of polymerase to editing nuclease activity in bacteriophage T4 can be recognized as being either mutator or antimutator genes (see above). A lesion which makes normal base pairing impossible should block DNA synthesis since as soon as an incorrect base is inserted opposite the damaged parental

nucleotide, the editing nuclease will recognize that a normal base pair has not been formed and will remove the newly inserted base. A continual turnover of nucleotides without chain elongation will result (Villani *et al.* 1978). An "error prone" bypass could occur if as a result of an inducible process, the editing nuclease on the replicating polymerase were suppressed so that the insertion of a mismatched base at the distorted site of a pyrimidine dimer (for example) would not be recognized and removed. Even though an error correcting nuclease were removed, one would still expect most mistakes to occur at the site of the lesion and it is found the most UV-induced mutations in *E. coli* occur at or next to sites of pyrimidine dimers (Coulondre *et al.* 1978). Mutagen stability and high lethality would result from genetic inactivation of the inducible mechanism (as in *recA* or *lex* mutants) since error prone bypass would not occur.

Regardless of whether this hypothesis is correct in all details, the situation in bacteria is clearer than in eukaryotic cells. One of the evidences that *rec* and *lex* gene products are involved in bacterial mutation is the mutagen stability for ultraviolet-induced mutation of *rec* and *lex* strains (Witkin 1976). On the other hand, *recA E. coli* strains do yield mutations after treatment with alkylating agents such as MNNG and ethyl methanesulfonate (Kondo *et al.* 1970) indicating that base mispairing mechanisms play a role in mutation induction in this organism.³⁾ The isolation of strains of yeast which are mutagen stable not only for UV but also for mutation induced by alkylating agents (Prakash 1976) means that for these eukaryotes almost all induced mutation must be mediated by some metabolic process. (Spontaneous mutation may result from the operation of a different mechanism since mutagen stable strains may have high spontaneous mutation frequency).

Although mammalian cells can bypass in both UV- and chemically-induced lesions, extensive recombinational repair of the type observed in bacteria does not occur (Lehmann and Kirk-Bell 1978). When cells damaged with UV or with chemical mutagens are given short pulses of radioactive thymidine, DNA is synthesized in smaller pieces. On incubation these smaller sized pieces reach the size attained in untreated controls. This enlargement of smaller pieces has been used to define the process of post replication repair (Lehmann 1972). One explanation is that synthesis "hesitates" at the site of the block but continues unabated along the sister strand. At some point downstream from the lesion, synthesis again takes up on the damaged strand leaving a stretch of single stranded DNA. Postreplication repair, as postulated, involves filling in the gap and is operationally defined as the elongation of daughter strands in alkaline sucrose gradients (see Fig. 6). Since recombinational mechanisms are excluded, some unspecified mechanism involving bypass of the lesion must account for the eventual filling in of the gap.

The process of DNA synthesis in eukaryotes is complicated by the occurrence of separate replicating units (replicons) which appear to be called into active synthesis in

3) R. Sklar in my laboratory has found that the *rec A* mutation (but not *lex*) does eliminate the high frequency of closely linked mutation seen in MNNG mutagenesis but this is a minor effect compared to single site mutation.

Fig. 7. Model of replication repair by branch migration (after Higgins *et al.* 1976).

clusters (Hand 1978). The size of these units is variable being of the order of 45 m μ in rodent cells and 15 m μ in human cells (Kapp and Painter 1978). The replication of DNA in untreated cells generally proceeds bidirectionally and it is possible (but not demonstrated) that replicons terminate at defined positions to await the arrival of DNA synthesized by adjacent replicating units. When replicons are small, it is difficult to distinguish the joining of adjacent replicons from the elongation observed in post replication repair. There is therefore some debate as to the actual existence of the gapped regions in all damaged cells. The older view that there are gaps in the DNA is supported by the autoradiographic experiments of Doniger (1978) who showed stretches of newly synthesized DNA much longer than the distance between dimers and by the observation by Menighini (1976) of single-strand endonuclease sensitive sites in DNA synthesized by UV-irradiated cells. However, there is no evidence that the single stranded regions are opposite dimers as required by the hypothesis (Menighini and Hanawalt 1976).

An alternative hypothesis was suggested by Higgins (Higgins *et al.* 1976) and by Fujiwara (Fujiwara and Tatsumi 1976) based on the finding that bifilarly substituted DNA was observed immediately on incubating damaged cells in medium containing bromodeoxyuridine. A process involving strand displacement and branch migration followed by use of the daughter DNA strand made off the undamaged parental strand as a template could, after reversal of the branch migration, lead to bypass of lesions (Fig. 7). The bypass reaction might then be error free and a consequence of the normal replication mechanism. However, Dr. K. Tatsumi in my laboratory showed that most or all of the bifilar DNA observed was formed from DNA growing points as a result of an *in vitro* process on extraction of the DNA (Tatsumi and Strauss 1978) so that *in*

in vivo demonstration of the branch migration mechanism has not been accomplished. Using the measurement of the bifilarly substituted DNA as an indication of the amount of DNA at growing points, Tatsumi (Tatsumi and Strauss 1979) has shown that growing points from newly initiated replication units accumulate when cells are allowed to synthesize DNA in the presence of caffeine. A similar accumulation of growing point material occurs after treatment with chemical mutagen (Strauss *et al.* 1979) suggesting that the critical events in DNA synthesis on damaged templates occur in the growing point regions.

Yet another view is taken by Cleaver who supposes that there are no definite termination sites for replicating units but that lesions, such as the pyrimidine dimer, serve as such termination sites (Park and Cleaver 1979). Cleaver's view has the advantage of accounting for the "magic number" of lesions (about one per replicon) which can be tolerated by a cell but it, like all the other hypotheses does not provide a molecular model of the events involved as is available for prokaryotic bypass. It should be clear that none of the mechanisms proposed excludes the others and, as in bacteria, there may be more than one bypass mechanism with only particular modes of bypass being error prone.

The discovery of inducible repair mechanisms in bacteria has provided a rationale for looking for similar mechanisms in eukaryotic cells. At present the evidence is suggestive but not conclusive. The most interesting observations involve the study of the host cell reactivation mechanisms of animal viruses. Just as with bacterial viruses, adeno- and herpes viruses can be reactivated by systems present in the host cell (Day 1978). The recent observation that the survival of ultraviolet-irradiated SV40 virus can be enhanced by infecting cells which have been pretreated with small doses of a chemical carcinogen or UV is particularly suggestive of an inducible mechanism (Sarasin and Hanawalt 1978).

The Development of an in vitro system

The problem remains: how do polymerases get around lesions and are errors generated in the process? The introduction of a probability factor, (P), by Park and Cleaver (1979) to indicate the chance of DNA synthesis being blocked by a lesion with the probability a function of time, merely indicates the general reticence of all hypotheses in providing mechanistic details. It has therefore seemed necessary to develop an *in vitro* system to approach the problem of bypass.

We would like to develop a system in which bypass occurs and to learn what factors are necessary for its operation and whether the system automatically generates errors. A major start on this problem has already been made by those investigators who are studying the fidelity of different polymerases and the frequency with which polymerases make errors. Many of these experiments have been carried out by Loeb and his collaborators (Loeb *et al.* 1974) who have concluded from measurements in which the incorporation of "wrong" bases is determined using synthetic templates that the frequency of error is influenced by the nature of the polymerase and by the ionic environment, for example the substitution of Mn^{++} for Mg^{++} . These experiments could be criticized on the basis of the special nature of synthetic templates and Loeb

Fig. 8. Use of ϕ X174 DNA to locate the site of lesions. Single circles, ϕ X174; double circles, replicative form; solid circles, sites of lesions; dashed line, newly synthesized DNA; PAGE, polyacrylamide gel electrophoresis.

has now developed a system using ϕ X174 DNA which takes such objections into account (Weymouth and Loeb 1978; Gopinathan *et al.* 1979). DNA is extracted from an amber mutant of ϕ X174 and this DNA is then used as a template for *in vitro* DNA synthesis using a priming fragment with a 3' OH terminus 83 nucleotides from the amber mutation. The resulting DNA is then completed by hybridization with a restriction enzyme fragment mixture and analyzed for amber reversions in a spheroplast transfection assay. More reversions are observed when synthesis is catalyzed by AMV reverse transcriptase polymerase activity than by DNA polymerase I (Gopinathan *et al.* 1979).

The ability to use a completely sequenced DNA (Sanger *et al.* 1977) is a major dvantage and both Radmans group (Caillet-Fauquet *et al.* 1977) and group led by S. Weiss at the University of Chicago (Hsu *et al.* 1977) have used ϕ X174 DNA to show that a single pyrimidine dimer or a single benzpyrene residue can be an absolute block to DNA synthesis catalyzed by *E. coli* DNA polymerase. We are using ϕ X174 DNA to determine the nucleotide sites at which reaction is blocked (Moore and Strauss 1979). We have adapted a variant of Loeb's procedure (Fig. 8). ϕ X174 DNA is prepared and reacted with mutagen. Restriction fragments are prepared by Hae II digestion of RF, separated by gel electrophoresis, and single fragments are then hybridized with the reacted single stranded DNA. This template plus primer is then used for *in vitro* DNA synthesis. Following reaction with all four deoxynucleoside triphosphates including α - P^{32} TTP, the mixture is treated with the original restriction enzyme (HaeII), which separates newly synthesized DNA from primer, and then denatured and electrophoresed. Four reaction mixtures with different dideoxynucleotide chain terminators (Sanger *et al.* 1977) are run as sequence standards. The gels after electrophoresis are covered with X ray film

and after exposure the film is developed to give a series of bands corresponding to the positions at which DNA synthesis has been arrested.

The total amount of synthesis obtained with pol I as the catalyst is proportional to the number of mutagen groups added to the DNA. Furthermore, as the number of groups is increased, those restriction fragments furthest from the primer are no longer synthesized, with the fragments most distal being lost first (Moore *et al.*, in preparation). In order to study effects at the nucleotide level we have reacted DNA with mutagen so that in general there is at least one adduct within about 50 nucleotides from the primer; e.g., about 250 acetoxy acetylaminofluorene adducts per virus molecule.

One of the most obvious and reassuring of results is that the pattern of bands obtained with UV-treated templates differs from the pattern observed with either benzpyrene diolepoxide or with acetoxy acetylaminofluorene (AAAF) and that the pattern observed with benzpyrene diolepoxide is very similar but not identical with that given by AAAF-reacted templates (Fig. 9). Closer analysis of the electrophoresis pattern of the products of the reaction mixtures using *E. coli* polymerase I as the enzyme shows that: a) bands are observed near the site of G on the template for AAAF and near the site of possible pyrimidine dimers for UV and b) although multiple bands may be obtained, the major band occurs as though synthesis terminated one base prior to the reacted site on the template. This observation may be related to the finding with synthetic mRNA translation systems that the inhibitory effect of combination with AAAF "was limited mainly to the base immediately adjacent to the modified G residue" (Weinstein and Grunberger 1974).

We have attempted to learn more about the factors which determine where synthesis will terminate (Moore *et al.*, in preparation⁴). First, we have shown that termination is not due to degradation of the template since electrophoresis on agarose of reacted templates shows the great majority to be of a size larger than single stranded- and smaller than double stranded relaxed circles. Next we tested the behavior of different polymerases. For this purpose, AAAF-treated templates were used as substrates for reaction catalyzed by either DNA polymerase I or III of *E. coli*, a T4 DNA polymerase, AMV reverse transcriptase and a DNA polymerase α isolated from the human lymphoma line, Daudi. The prokaryotic polymerases have associated 3'-5' nuclease activity, our preparation of DNA polymerase α is devoid of measurable nuclease activity, and the reverse transcriptase is reported as without exonuclease activity (Samuel *et al.* 1979).⁵

4) The original work reported in this paper was done at Chicago by Peter Moore, Kallol Bose, Kouichi Tatsumi, Robert Sklar, Karen Kato, N. Patrick Higgins, and Samuel Rabkin. The work was supported by grants from the National Institutes of Health (GM 07816, CA 14599, CP 85669) and the Department of Energy (EY 765-02-2040). We are grateful to the Chemical Carcinogenesis Program, NCI for supplying radioactive benzpyrene diolepoxide and acetoxy-acetyl aminofluorene.

5) DNA polymerase I is a commercial preparation. We thank Dr. N. Cozzarelli for samples of DNA polymerase III and for T4 DNA polymerase. AMV reverse transcriptase was obtained from NCI. Some preparations of this enzyme are reported as having associated endonuclease activity (Samuel *et al.* 1979). DNA polymerase α was prepared from a Daudi, human Burkitt's lymphoma line by K. Bose.

Fig. 9. Polyacrylamide gel analysis of the products synthesized by pol I on DNA templates reacted with AAFA or anti-BPDE or irradiated with UV. About 265 AAFA adducts or 140 anti-BPDE adducts were added per ϕ XDNA molecule. The DNA for the UV template was irradiated with 1000 J/m² of UV at a DNA concentration of 100 μ g/ml. The primer used was HaeII fragment 5. Lanes A, C, G, T are sequence standards. Numbering is from the center of the HaeII recognition site. The reaction conditions are as previously published (Moore and Strauss 1979).

Fig. 10. Polyacrylamide gel analysis of the products synthesized on an AAAF-treated DNA template by pol I, AMV reverse transcriptase, pol III, DNA polymerase α and T4 DNA polymerase⁴. The DNA template was primed with HaeII restriction fragment 5 and contained 146 AAF residues per ϕ X174 molecule. Data of P. Moore.

All of the polymerase reaction mixtures give a pattern of bands indicating that synthesis is blocked, at least temporarily at or near the sites of adducts (Fig. 10). Analysis of the patterns reveals significant differences between the reaction mixtures. The three prokaryotic polymerase catalyzed reactions stop one base short of the AAAF adduct which is assumed to be with G (Miller and Miller 1969). The bands for the AMV reverse transcriptase catalyzed reaction mixture are displaced and stop at the level of the reacted template nucleotide. The question as to which base(s) is inserted opposite the lesion is being investigated by Dr. P. Moore. The DNA polymerase α

reaction is more complex in that at some positions it behaves like pol I whereas at others it behaves like AMV reverse transcriptase. At the five positions we can analyze where the enzyme behaves like pol I, there is either a 3' CG 5' or a 3' GG 5' template sequence. In four positions where the behavior is reverse transcriptase-like, the template sequence is 3' TG 5'. It appears as though there may be a difference in enzyme behavior depending on whether an AT or a GC base pair precedes the lesion. As yet we have investigated only this one preparation of DNA polymerase α , we do not know whether other enzymes, including enzymes from non-tumor sources will behave this way. The differences in site of inhibition may depend on the differences in stacking interaction between AAF and neighboring bases (Weinstein and Grunberger 1974) and it is clearly important to study the behavior of the enzymes with a set of different carcinogens since we observe these differences only with AAF-reacted templates. All the enzymes behave in a similar fashion with UV- and with benzyrenedioxepoxide-reacted templates with inhibition occurring at a site one base previous to either a pyrimidine dimer or to a G on the template strand.

Certain preparations of *E. coli* DNA polymerase I do show light bands at the level of reacted nucleotide. At least two factors other than the enzyme preparation are involved. The ionic environment plays a critical role since substitution of Mn^{++} for Mg^{++} leads to an alteration such that synthesis is blocked at the site of the AAF lesions as with AMV reverse transcriptase (Fig. 11). We observe this phenomenon with *E. coli* DNA polymerase I but not with the preparation of T4 DNA polymerase we have used. Mn^{++} changes the polymerase to nuclease ratio of the enzymes but polymerase activity is inhibited more than nuclease (Table 3). We also find that the nucleotide concentration in the reaction mixture can affect the results, since at high nucleotide concentrations DNA polymerase I appears to insert bases at the level of the reacted nucleotide (Fig. 11).

The nature of the enzyme, the type of lesion, the base sequence adjacent to the lesions, the nucleotide concentration and the ionic environment therefore all play a role in determining the exact site of inhibition of *in vitro* DNA synthesis. We cannot exclude a role for the 3'-5' nuclease but it does not seem to be necessary since all enzymes respond identically to some lesions regardless of the presence or absence of nuclease. Even the effect of Mn^{++} in altering the site of inhibition is not easily ascribed to changes in editing nuclease since Mn^{++} substitution for Mg^{++} inhibits polymerase more than nuclease activity (Table 3). Our results do not require the hypothesis that it is the 3'-5' nuclease which regulates DNA synthesis. However, our methodology is relatively insensitive in detecting "readthrough". It does seem that AAF adducts are absolute blocks to DNA synthesis by polymerase I and by polymerase α but we have not made similar determinations with the other enzymes and could not in any case detect readthrough of a few percent.

The observation of the effect of Mn^{++} and of the difference in site of inhibition of AMV reverse transcriptase and polymerase I bears a resemblance to the results of the Loeb group on misincorporation (Sirover *et al.* 1979; Gopinathan *et al.* 1979) but we do not understand what the meaning of this resemblance between two rather different phenomena might be. The Loeb group has measured the misincorporation of

Fig. 11. The effect of nucleotide concentration and choice of divalent cation on the sites of termination by pol I on AAAF-treated DNA. Reaction mixtures contained 50 mM Tris HCl, pH 8.0, 5 mM dithiothreitol, 0.06 p moles AAAF-treated template, 0.2 units pol I and the following concentrations of divalent cation and the four deoxynucleotide triphosphates (dNTPs): 1) MgCl_2 8 mM, dNTPs 20 μM ; 2) MgCl_2 8 mM, dNTPs 50 μM ; 3) MgCl_2 8 mM; dNTPs 100 μM ; 4) MnCl_2 1.0 mM; dNTPs 100 μM . Data of P. Moore and S. Rabkin.

Table 3. Effect of divalent cation on DNA polymerase and 3'-5' exonuclease activity

Enzyme	Mg ²⁺	Mn ²⁺	Polymerase Activity		Exonuclease Activity	
			cpm	%	cpm released	%
<i>Pol 1</i>						
(Large fragment)	8 mM	—	3096	100	3393	100
	—	0.2 mM	257	8.3	2624	77.3
	—	0.5 mM	2370	76.6	2901	85.5
	—	0.75 mM	880	28.4	2344	69.1
	—	1.0 mM	771	24.9	1531	45.1
<i>T4</i>	5 mM	—	3211	100	3254	100
	5 mM	0.2 mM	3438	107.1	2019	62.0
	5 mM	0.5 mM	2982	92.9	1460	44.9
	5 mM	0.75 mM	969	30.1	1335	41.0
	5 mM	1.0 mM	666	20.7	724	22.2

Data of K. Bose

nucleotides and our methodology visualizes the sites of inhibition after mutagen treatment of DNA. Our results do indicate how complicated an understanding of the mutational process will be. If the end result of an alteration in the DNA depends not only on the nature of that alteration but also on the particular position in the DNA in which it occurs and on the structure of the enzymes which process it, then the view of Auerbach (1967) and of Kondo *et al.* (1970) is fully vindicated and the metabolic systems of the organism must play a crucial role in determining the nature of any mutation. Findings that "the position of the base pairs can play an important role in not only determining the rates of mutations but also in the types of base changes" (Prakash and Sherman 1973) may soon be understandable on the enzymatic level. Of course we need to develop better methods to measure low levels of "readthrough" and to determine the relation between our observations and those of Loeb. However, it does seem likely that a variety of systems now being developed will permit the analysis of the bypass of lesions and the resulting biological events.

It is now twenty-one years since I came to work in the Department of Genetics at Osaka University and first met Shunzo Okubo. These have certainly been exciting years for our science. New discoveries come so fast that one thinks of rapid change as the normal state. The tools for ever more sophisticated analysis of biological phenomena seem to be continually generated. It was Shunzo Okubo's good fortune to have contributed significantly to an exciting field at a time of rapid growth. I consider myself fortunate to have known and worked with him and I am particularly pleased that a portion of his work was done in my country. I like to think that his stay in Chicago and later in California contributed to his skill, to his career, and most of all to the pleasure he took in his work. I hope that the kind of association we had, in which we learned from one another, will continue with your country and mine during what promises to be an equally exciting time to come.

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