# The *in vivo* demonstration of repair replication

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## *Introduction*

The excision-repair model for the reconstruction of damaged DNA *in vivo* was proposed by SETLOW & CARRIER (1964) and by BOYCE & HOWARD-FLANDERS (1964) following their demonstration of the preferential release of thymine dimers from the DNA of ultraviolet irradiated bacteria. The multistep repair process was presumed to begin with the enzymatic recognition of the damage in the DNA and the production of a single-strand break in the damaged strand near the defective region. The excision of the damage was postulated to occur by means of an exonuclease which removed the defective nucleotides, thus exposing a single-stranded region of the intact complementary strand. The repair DNA polymerase would then fill in this region with undamaged nucleotides using the complementary strand as template. Finally, a gap-closing enzyme would rejoin the repaired segment to the contiguous parental strand. This scheme has come to be known colloquially as the « cut and patch » model. An alternative sequence has been proposed, known as the « patch and cut » model, in which the excision step (or perhaps the peeling back of the damaged strand) occurs concurrently with repair replication. This model has the attractive feature that no labile single-stranded region is left exposed at any time in the course of repair. In both models the final step must be the rejoining of the repaired segment to the parental DNA.

KAPLAN, KUSHNER & GROSSMAN (1969) have purified and characterized from *M. luteus* an endonuclease that is specific for ultraviolet irradiated DNA and an exonuclease which operates upon double-stranded DNA that has been both irradiated and then treated with the endonuclease. Only a limited amount of degradation occurs and it might be supposed that in *M. luteus* the excision step normally precedes repair replication. On the other hand the recent characterization of the DNA polymerase from *E. coli* (KELLY *et al.*, 1969) strongly implicates this enzyme in a repair scheme of the « patch and cut » type. The *E. coli* DNA polymerase has been shown to

*Ann. Ist. Super. Sanità* (1969) 5, 311-317.

possess a $5' \longrightarrow 3'$ exonuclease activity that specifically facilitates the release of thymine dimers (or mismatched base sequences) from DNA as oligonucleotides. Thus, this enzyme can apparently perform both the repair replication step and the concurrent peeling away and eventual excision of the damaged strand. The presence of a high level of relatively nonspecific nuclease activity in *E. coli* extracts has thus far precluded the isolation of an activity appropriate to the recognition-incision enzyme in this system. The polynucleotide ligase is the logical candidate for the final rejoining step in repair and, in fact, an ultraviolet sentitive mutant of *E. coli* has been shown by PAULING & HAMM (1968) to be deficient in this enzyme. Thus, the excision-repair process in *E. coli* could conceivably be a three step operation involving as few as three enzymes : (1) A damage-specific endonuclease, yet to be isolated from extracts. (2) The DNA polymerase, which also performs the excision step and (3) the polynucleotide ligase to terminate the repair sequence by joining the replaced segment of nucleotides to the contiguous parental strand.

We have been interested in the measurement of repair replication in *in vivo* systems as well as in the molecular detail of the process. The first direct demonstration of repair replication of DNA in ultraviolet irradiated bacteria (PETTIJOHN & HANAWALT, 1963 ; 1964) ultilized the same essential procedure that had been used by MESELSON & STAHL (1958) for proving the normal semiconservative mode of DNA replication. The repair mode of DNA replication might be termed « nonconservative » since there is no net increase in the amount of DNA but rather a removal and replacement of parental material. Of course the repair mode is also semiconservative in the sense that the parental single-strand region opposite the damaged region is conserved. The repair replication mode has now been observed in a variety of cell types from the simplest living cells, the mycoplasmas, to complex eucaryotic organisms and even mammalian cells. Repair replication has also been demonstrated in cells after a variety of different treatments that result in structural damage to DNA. A low level of repair replication or « turnover » is even seen in the DNA of bacterial cells that are growing normally. It is very likely that repair replication of DNA has an important general significance in the maintenance of genetic stability in all types of cells and it may, in fact, account for the ubiquity of double-stranded DNA in living systems.

### *The method for demonstrating repair replication.*

The combined labeling of replicating DNA with a density label and a radioactive label is utilized to measure repair replication *in vivo* (c.f. HANAWALT *et al.*, 1969). A very short period of density-labeling results in the pro-

duction of « hybrid » DNA fragments in the course of normal semi-conservative mode of replication. (Hybrid DNA contains one strand of parental DNA and a complementary strand of newly-replicated daughter DNA). These « hybrid » DNA fragments can be separated physically from the unreplicated parental DNA in a cesium chloride equilibrium density gradient in the ultracentrifuge on the basis of their different bouyant densities. It is important to realize that in the normal procedures for isolating DNA from cells the chromosomal DNA is fragmented into many smaller pieces of molecular weights from 10 to 30 million and that in such a mixture there will be those fragments that have not replicated as well as those that have replicated during the labeling period. In repair replication it is predicted that the newly-synthesized regions of DNA will be very short relative to the size of the fragments of DNA that are being recovered from the cesium chloride gradients. In fact, these fragments may contain too little density label to appreciably shift the density from that of the normal unreplicated parental DNA. The use of a density label such as 5-bromouracil (an analogue of thymine) and a radioactive label permits the determination of the incorporation of this label into small regions of the parental DNA and this is the assay for repair replication. In the course of normal replication the incorporation of 5-bromouracil has the effect of increasing the bouyant density of the *E. coli* DNA in cesium chloride from 1.71 ml to 1.75 ml in the hybrid band. The nonconservative repair mode of replication was first observed in *E. coli* strain TAUbar following ultraviolet irradiation. It was shown that the incorporated 5-bromouracil label was in parental strands rather than in complementary daughter strands (PETTIJOHN & HANAWALT, 1964). Physical studies on the isolated DNA verified that the label was present within parental DNA and could not be separated from it by melting. Also sonic fragmentation of the DNA did not resolve 5-bromouracil containing fragments from the unlabeled parental strands. Thus, the label must have been in very short segments within the parental DNA.

A number of control experiments confirmed the interpretation that the observed phenomenon was the repair mode of replication. The thymine requiring *E. coli* strain $B_{s-1}$ that has been shown not to excise thymine dimers from its DNA was shown not to perform repair replication after UV irradiation (HANAWALT & PETTIJOHN, 1965). In strain TAUbar it was shown that reduced repair replication was seen if the cells were illuminated with visible light to facilitate photoreactivation prior to the addition of the density label (PETTIJOHN & HANAWALT, 1964). This result was as expected since photoreactivation is known to split pyrimidine dimers *in situ*, so that the excision-repair process need not function. It was shown that the DNA fragments that had incorporated 5-bromouracil by the nonconservative mode of replication were subsequently capable of replicating by the normal semi-conser-

vative mode of replication (HANAWALT, 1967). Several temperature-sensitive DNA replication-deficient bacterial mutants were shown to be able to perform the repair replication mode at the restrictive temperature (COUCH & HANAWALT, 1967). Thus, the two modes of replication clearly do not employ the identical enzyme systems. The amount of observed repair replication in these mutants after a given dose of UV was shown to be similar at the normal and the restrictive growth temperatures. Another indirect indication that the normal and repair replication systems use different polymerases was the demonstration of a strikingly different selectivity for thymine over 5-bromouracil (when a defined ratio of the base and its analogue was provided in the medium) in the repair mode as compared to the normal mode of replication (HACKETT & HANAWALT, 1966; KANNER & HANAWALT, 1968).

Possible artifacts resulting from the known pathogenicity of 5-bromouracil can be eliminated by the use of other density labels. Thus, repair replication has been shown by using $N^{15}$ and $D_2O$ as density labels (HANAWALT *et al.*, 1969) or by using $C^{13}$ and $N^{15}$ (BILLEN *et al.*, 1967; BILLEN, 1968). The quantitative result using the $C^{13}$-$N^{15}$ density labeling system (BILLEN, 1968) has correlated well with that obtained using 5-bromouracil as density label (COUCH & HANAWALT, 1967).

*Repairable damage.*

Although repair replication was first demonstrated following UV irradiation of bacterial cells it has since been shown that a variety of agents which produce structural damage in DNA can lead to the repair replication mode *in vivo*. Thus, the bifunctional alkylating agent, nitrogen mustard, which primarily attacks the 7-nitrogen position of guanine results in repair replication (HANAWALT & HAYNES, 1965). The powerful mutagen, nitrosoguanidine has also been shown to stimulate repair replication (CERDÁ-OLMEDO & HANAWALT, 1967). The attempt to detect repair replication after X-ray treatment of bacteria has led to ambiguous results and the difficulty is probably that the extensive degradation of DNA following X-ray damage obscures the repair replication mode by contributing parental label to the precursor pools. Evidence has been presented that mitomycin-C (c.f. HOWARD-FLANDERS, 1968) and methylmethanesulfonate (STRAUSS, 1968) lead to the excision-repair mode in bacteria.

It is likely that the recognized defect must be some general distortion of the DNA backbone rather than specific base damage. Regions of partial denaturation or mispairing of bases may be adequate to initiate the repair sequence.

Particularly curious was our finding (PAULING & HANAWALT, 1965) that *E. coli* strain TAUbar undergoes the repair replication mode following

a period of thymine starvation. It was not evident that thymine starvation should lead to any structural alteration in the DNA, but a clue to the mechanism of this phenomenon was seen in the fact that the effects of thymine deprivation are only manifested under conditions in which RNA synthesis was permitted (HANAWALT, 1963). It was suggested that perhaps repairable breaks are introduced into the DNA in the course of the normal process of transcription and that these breaks are normally repaired by the repair replication machinery. Under conditions of thymine starvation the breaks are still produced but not repaired and perhaps they are then enlarged so that upon subsequent readdition of thymine a more extensive repair region is observed. A mutant bacterium deficient in the polynucleotide ligase has been shown to be unusually sensitive to the process of thymineless death when thymine is withheld, as consistent with the involvement of excision-repair in this phenomenon (PAULING & HAMM, 1968).

*The generality of repair replication and details of the process.*

Most of our understanding of the excision-repair process is derived from studies on bacterial systems where mutants deficient in the various steps in repair are available. The density-labeling method of PETTIJOHN & HANAWALT (1964) has been used to provide evidence for the excision-repair mode in a number of other biological systems. SMITH & HANAWALT (1969) have shown that the simplest living cells, the mycoplasmas, perform repair replication following UV irradiation. A UV dose of 85 ergs/mm$_2$ (70 % survival of colony-forming units) induced a linear rate of repair replication that proceeded to apparent saturation after about half a generation period. The amount of repair corresponded to a 1.2 % replacement of the chromosome and this permitted a rough estimate of 150 to 600 nucleotides per repaired region. (Assuming that 85 ergs/mm$^2$ should produce 1 thymine dimer per $3.7 \times 10^6$ daltons DNA). This would seem to be a surprisingly large amount of repair but not as surprising as the estimate of 5000 nucleotides replaced per dimer in the protozoan *Tetrahymena pyriformis* (BRUNK & HANAWALT, 1967 ; 1969). In the latter case the repair label exhibited a significant shift toward greater density from that of the parental DNA strands that contained no 5-bromouracil. It was evident that only a small fraction of the parental DNA fragments contained appreciable amounts of repair label and that those that were labeled contained a sufficient amount of 5-bromouracil to detectably increase the density of those strands. It is quite conceivable that what has been reported as repair replication actually includes the contribution from at least two phenomena. A part of it is undoubtedly the result of excision-repair, but this may be generally in regions considerably shorter than our current estimates. A few of the repair regions may

be much longer than the average and these may weight the estimate to the high side and also give the result that not all of the parental strands contain appreciable amounts of label. An indeterminant amount of label may have been incorporated as a result of a recombinational mode of repair that also involves a repair replication step to complete the union of the recombining fragments of DNA. Studies on repair replication and recombination in the T4 phage system (and in mutants deficient in one or the other of these processes) may provide a model for distinguishing between the two phenomena (SOLL & HANAWALT, in preparation).

The most exciting application of the 5-bromouracil labeling method for measuring repair, was the recent demonstration by CLEAVER (1968) that normal human skin fibroblasts were capable of repair replication after UV treatment. Yet, fibroblasts from patients with the rare hereditary skin disease *Xeroderma pigmentosum* exhibited much reduced levels of repair replication. It was concluded that the failure of DNA repair must somehow be related to the fatal skin cancers that these patients generally develop upon exposure to sunlight. This is the first experimental evidence for the possible significance of the excision-repair process in the protection from or recovery from a carcinogenic transformation in human cells. Repair replication has also been shown in other mammalian cell types in culture (PAINTER & CLEAVER, 1969).

The general outline of the excision-repair mode in a variety of cell types is now apparent. The more difficult task of elucidating the details of the process remains. The density labeling approach can be used to suggest the presence of excision-repair but there are serious limitations in the quantitative determination of repair by this approach. Current studies in our laboratory are combining the density labeling approach with a chromatographic analysis of the composition of the « repaired » regions of DNA to more accurately assess the contributions of intracellular pools to the measurement of repair and to determine the mean length of the repaired regions.

This work was supported by a contract with the U.S. Atomic Anergy Commission, a grant from the Institute of General Medical Sciences of the U.S. Public Health Service, and an equipment grant from the National Science Foundation. Our studies on repair replication have been a collaborative effort with graduate students David Pettijohn, Clifford Brunk, Douglas Smith, and John Couch ; and postdoctoral associates Crellin Pauling and Lee Kanner.

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