# THE FIFTH BASE: A NATURAL FEATURE OF DINOFLAGELLATE DNA

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**Abstract.** The presence of 5-Hydroxymethyluracil which replaces important fractions of thymine in Dinoflagellate DNA is analyzed according to different procaryotic models of biosynthesis. The detection of HOMeU in *Amphidinium carterae* cells suggests an incorporation of this 5th base during polymerisation of DNA chains rather than a post-synthetic modification of thymines. A relationship between HOMeU and the permanent repair-like DNA synthesis observed in *A. carterae* is discussed.

## 1. Introduction

Modified bases are a common feature in DNA. It is also well-known that except in some cases, atypical bases are detected in small amounts. They can enter in the DNA by three pathways (Kornberg, 1980): (1) incorporation of a synthetic base, nucleoside or nucleotide analog; (2) incorporation of an enzymatically altered nucleotide; (3) post-synthetic modification of bases in DNA chains.

In the first case, the base analog is converted by a salvage pathway to a nucleotide 5'-tri-phosphate which can effectively compete with the natural nucleotide for base-pairing. This process has been largely used for experimental studies of DNA synthesis by incorporation of labelled or heavy analogs, in procaryotes and eucaryotes. In three species of dinoflagellates, DNA synthesis has been explored by this method (Franker *et al.*, 1974; Galleron *et al.*, 1975; Filfilan and Sigee, 1977; Galleron and Durrand, 1978, 1979).

The best examples of the second kind are certain phage DNAs which include uncommon bases (Uracil and 5-HOMeUracil in some *B. subtilis* phages, 5-HOMeCytosine in T-even phages of *E. coli* and in certain phages of *X. orizae*) even to the exclusion of thymine or cytosine. The biosynthetic pathways of HOMeC, HOMeU and U are well known and all the corresponding enzymes have been characterized (Mathews *et al.*, 1964; Reeve *et al.*, 1978). In the case of SPO1 and other closely-related phases of *B. subtilis*, the genes controlling DNA synthesis have been located in the genome. Although the pyrimidine pathway is altered in 5 steps to substitute 5-HOMeU to T, inclusion of thymine residues is tolerated up to 20% of HOMeU a proportion close to that observed in dinoflagellates. In SPO1, a phage-encoded DNA-polymerase incorporates dHOMeUTTP, dTTP, dUTP, and 5-BrUdR at identical rates (Yehle and Ganesan, 1973).

In bacterial cells as well as in animal cells, the relative pool sizes of dUTP and dTTP regulate the incorporation of uracil into DNA. The level of two different enzymes helps

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to eliminate the foreign base: when the thymidilate synthetase pathway is inhibited by methotrexate, the ratio of dUTP/dTTP in the cellular pool is raised up to 20% in spite of dUTPase which normally degrades almost completely dUTP (Nilsson *et al.*, 1980). However, under normal circumstances, it is the level of uracil-N-glycosylase, a non site-specific excision enzyme which determines the persistence of uracil in DNA. Bacterial mutants defective in this enzyme as well as methotrexate treated-cells can accumulate uracil to levels comparable with thymine levels. No other glycosylase removing pyrimidines has been reported in the literature while uracil-N-glycosylase has been found in some bacteria, phages and mammals (Kornberg, 1980).

Post-modification of the DNA occurs through the action of site-specific enzymes which protect phage and bacterial DNAs against restriction by host and phage-encoded nuclease. In phage $T_4$, for instance, α- and β-glycosyl-transferases (Josse and Kornberg, 1962) coded by the phage DNA which glycosylates the HOMeC residues are among the most important examples together with type I and type II restriction-modification methylases of *E. coli*.

Rae, in 1973, discovered that in the DNA of a marine dinoflagellate, *Crypthecodinium cohnii*, as much as 37% of thymine is replaced by HOMeU. High rates (up to 68% in *Prorocentrum micans*) have been measured since (Rae, 1976; Galleron and Durrand, 1978; Herzog and Soyer, 1982). Looking for a biological function of HOMeU by analyzing *C. cohnii* DNA, Rae and Steele (1978), found no involvement of this base in the nucleoid-like structure of the chromosomes, and no true restriction activity, i.e. no set of conditions in which nuclease activity of *C. cohnii* extracts degraded preferentially heterotypic DNA. However, he did find a definite non-randomness of the positions of HOMeU in DNA sequences. These observations led him to make the following hypothesis: the uncommon base might be the last remnant of an ancient restriction-modification system once possessed by proto-dinoflagellates.

Looking for the presence of HOMeU while exploring the cycle of DNA synthesis in *Amphidinium carterae* synchronized cultures, we made an assumption opposite to Rae's hypothesis, and tried to verify whether the large fraction (HOMeU/HOMeU + T = 62%) of HOMeU found in *A. carterae* DNA were incorporated through a phage-like mechanism.

## 2. Methods

### 2.1 Cultures of Amphidinium carterae (Hulburt) and extraction of DNA

Synchronized cultures, DNA extraction and purification, isopycnic centrifugation in CsCl gradients, *in vivo* labelling of cells by pyrimidine precursors or analogs and radioactive counting of liquid fractions have been performed as previously described (Galleron and Durrand, 1978, 1979).

### 2.2. Preparation of the acid-soluble pool of nucleotides

It was made by dissolution in 10% TCA of a cell pellet, radioactively labelled *in vivo* and

extensively washed in culture medium until no radioactivity appeared in discarded liquids. Cells were ground on ice and depigmented in methanol, ethanol and ether. Nucleotides (1-, 2- and 3-phosphates) were separated from the TCA-soluble fraction by ion-exchange chromatography: 100 µl-samples were put on top of small columns (Pasteur pipettes) loaded with Dowex 50 AG 500 WX4, Hydrogen form (Bio-Rad) in 0.1 M formic acid. Nucleosides were eluted first, then nucleotides with 0.1 M HCl. 95 % of the radioactivity was recovered in the 3 peaks.

### 2.3. Acid hydrolysis of DNA

Acid hydrolysis of DNA (extensively dialyzed against water after CsCl centrifugation) and of nucleotides extracted from the cellular pool were performed on lyophylized samples. They were dissolved in 0.5 ml 1 M HCl and boiled in sealed glass capsules. The hydrolysis was stopped at $-20\,^{\circ}C$ in ethanol and dry ice.

### 2.4. Enzymatic hydrolysis of DNA

*In vivo* labelled DNA was enzymatically hydrolyzed with DNase I and snake venom phosphodiesterase according to Ray and Hanawalt (1964). Nucleotides obtained were treated with alkaline phosphatase (Sigman, 50 µg/ml, 2 h incubation at pH 10, 37 °C). This treatment could not be applied to TCA-soluble fractions for which pH adjustment led to an excessive dilution of samples.

### 2.5. Radioactive precursors

$^{3}$H-Methyl-Thymidine ($^{3}$H-Me-TdR), ($^{3}$H-6-deoxyUridine ($^{3}$H-6-UdR), $^{3}$H-6-Uracil ($^{3}$H-6-U) and $^{14}$C-5-HydroxyMethylUracil ($^{14}$C-5-HOMeU) were purchased from CEA (Saclay, France); $^{3}$H-6-Bromo-5-deoxyUridine ($^{3}$H-Br-5-UdR) was purchased from Amersham (France). Radioactive molecules were given to cultures under conditions defined in Table I.

### 2.6. Base and nucleotide separations

They were made by thin layer chromatography (Randerath, 1971). Samples, filtered (bases) or acid-eluted, (nucleosides) from Dowex columns were evaporated to concentrations of 10 or 20 µg/100 µl of 0.1 M HCl. 5 µl spots were chromatographed in 2 dimensions on Kieselgel plates (60 F 254, Merck). The solvents were: first dimension; chloroform-methanol-$H_2O$ (4 : 2 : 1); second dimension; ethyl acetate-isopropanol-$H_2O$ (75-18-9). Spots were identified by co-migration with the corresponding commercial product visualized under UV long-wave illumination. Bases and nucleosides (T, C, U) were purchased from Sigma. HOMeU and HOMeU-deoxyUridine were purchased from Serva (Tebu-France). The HOMeU nucleotide was not commercially available.

### 2.7. Control RNA extractions

They were made according to Laulhère and Rozier (1976). Radioactive counting of solid samples was performed by elution of the spots in 10 ml Soluene (Packard) and counting in a Packard model 2425 Tricarb.

## 3. Abbreviations

$^{3}H$: tritium; $^{14}C$: radioactive carbon; C: cytosine; H, HOMeU, 5-HOMeU: 5-Hydroxymethyluracil; T: thymine; TdR: thymidine; U: uracil; UdR: deoxyuridine; Cpm: counts per minute; TCA: trichloracetic acid.

## 4. Results

### 4.1. In vivo incorporation of labelled analogs

We had previously observed that *A. carterae* cells incorporate very easily all precursors of the pyrimidine biosynthesis pathway in their DNA (Galleron and Durrand, 1978). We knew also that a permanent incorporation is superimposed to the S-phase, a

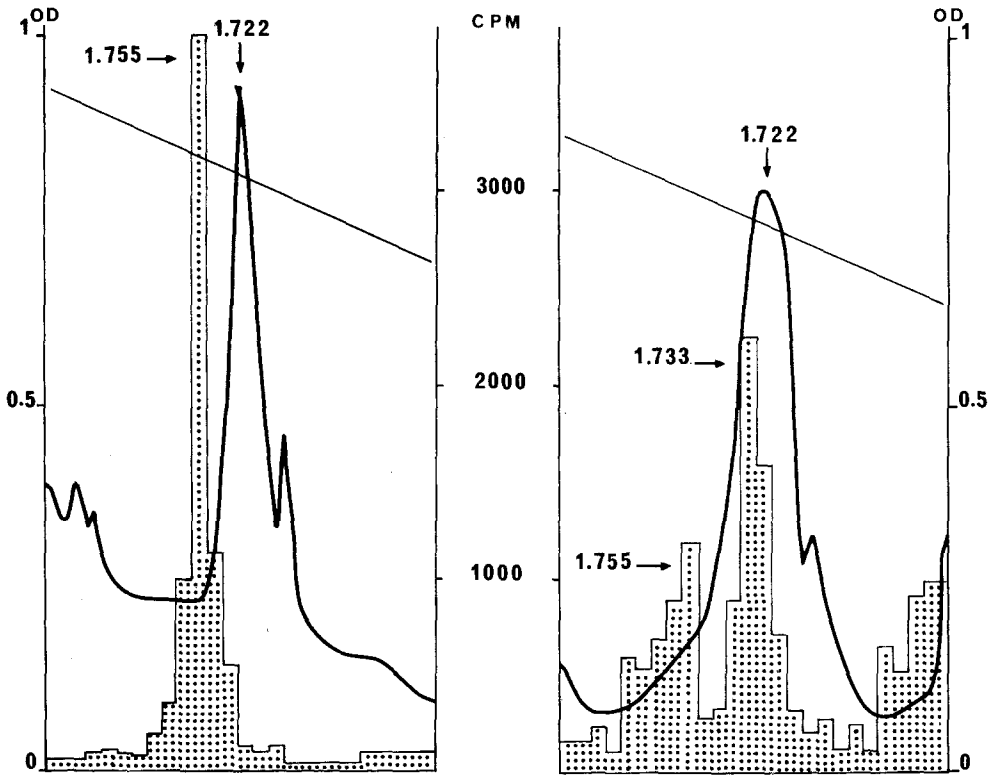



Fig. 1. CsCl centrifugation profile of *Amphidinium carterae* DNA, labelled in vivo with $^{3}H$-6-Br-5-UdR. Four synchronized 1.5-1 cultures of *A. carterae* were pooled and incubated for 4 h with $^{3}H$-6-Br-5-UdR; left: cells in S-phase; right: cells in $G_2$ phase. Nuclear DNA was isolated by centrifugation in CsCl then recentrifuged with cold *A. carterae* nuclear DNA as a density marker (d = 1.722, Galleron and Durrand, 1978). 20 drop-fractions were collected and counted for radioactivity.

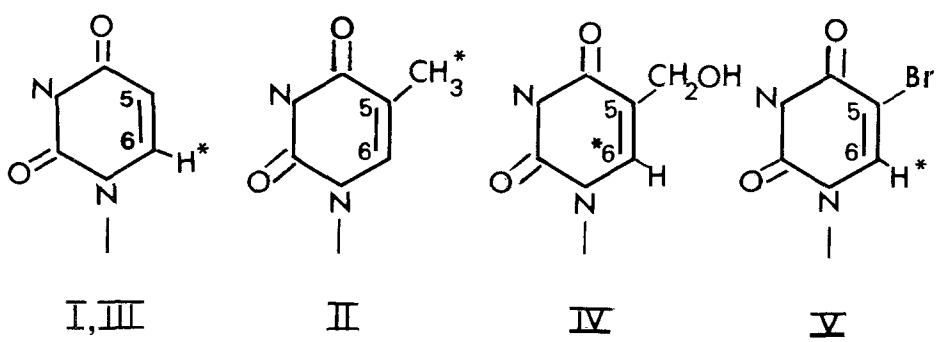
: histogram of Cpm;
: O.D. at 260 nm.

phenomenon detectable in well-synchronized cultures (Filfilan and Sigee, 1977; Galleron and Durrand, 1979). Figure 1 shows a centrifugation profile in CsCl of *A. carterae* DNA labelled *in vivo* with tritiated-bromo-deoxyuridine. The DNA extracted from cells in S-phase is heavier than the normal nuclear *A. carterae* DNA, as expected. For cells in $G_2$ phase, two DNA peaks are observed, one of which, the heavier, is probably due to a slightly imperfect synchronization of cells; the other one has a density close to, but different from the density of *A. carterae* nuclear DNA (d = 1.722, Galleron and Durrand, 1978). This slightly heavier peak corresponds to an unscheduled DNA synthesis insensitive to ethidium bromide inhibition (Galleron and Durrand, 1979) and appears to be a large scale-repair mechanism.

TABLE I

*In vivo* labelling of *Amphidinium carterae* DNA cultures with pyrimidine precursors and analogs. In all experiments, DNA was extracted, CsCl centrifuged and counted for radioactivity as cited in 'Methods'. Counts per minute (Cpm) are given after subtraction of background (less than 25 Cpm under our conditions).

| | Precursor or Thymidine analog | Spec. Act. μCi/ml | Incubation time | Chase | Spec. Act. of DNA Cpm/μg |
|---|---|---|---|---|---|
| I | $^3$H-6-Uracil (43 Ci/mM) | 2.4 | 10 min. | 1 h | $10^7$ |
| II | $^3$H-Me-Thymidine (43 Ci/mM) | 0.6 | 3h | wash | $4 \times 10^5$ |
| III | $^3$H-6-deoxyUridine (23 Ci/mM) | 0.6 | 3h | wash | $5 \times 10^4$ |
| IV | $^{14}$C-5-Hydroxy-Me-Uracil (25 Ci/mM) | 0.1<br>0.6 | 4h<br>6h | wash<br>wash | background<br>500 |
| V | $^3$H-6-Br-5-deoxy-Uridine (21 Ci/mM) | 0.625 | 4h | wash | 5000 (S-phase)<br>2000 ($G_2$-phase) |




4.2. BASE AND NUCLEOSIDE ANALYSIS IN THE CELLULAR POOL OF NUCLEOTIDES

Table I shows the efficiency with which *A. carterae* DNA is labelled by bases, nucleosides and analogs. Figure 2, Figure 3 and Table III show the labelling of bases

and nucleosides of the DNA and the labelling of the bases hydrolyzed from the pool of nucleotides (see 'Methods') in two different experiments.

Table III A displays the labelling of pyrimidines after a 2 h incubation of *A. carterae* cultures during S-phase. Thymine and HOMeU are labelled by $^{3}$H-6-UdR and also by $^{3}$H-Me-TdR with a higher rate of synthesis of HOMeU in the pool and of T in the DNA (unlike the relative contents of T and HOMeU in *A. carterae* DNA) HOMeU cannot be labelled by $^{3}$H-Me-TdR through a degradation of T and a new utilization of the free methyl group which otherwise would label DNA. Control RNA extractions have been entirely negative concerning this point.

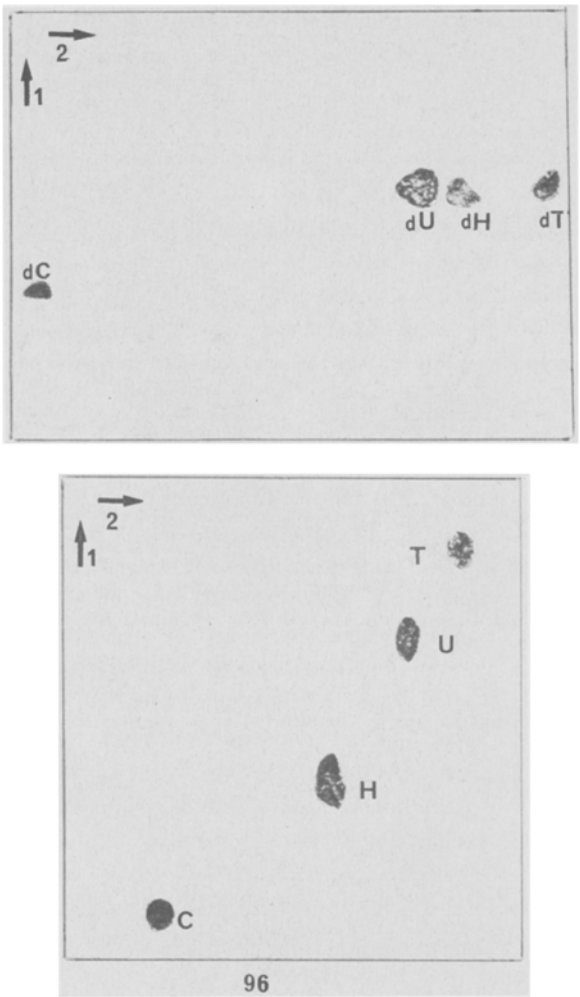



Fig. 2. Separation of pyrimidine nucleosides *(A)* and pyrimidines (B) by thin layer chromatography. Migration in two dimensions and visualization of spots have been made as described in 'Methods'. *Top*: separation of nucleosides; *bottom*: separation of bases.

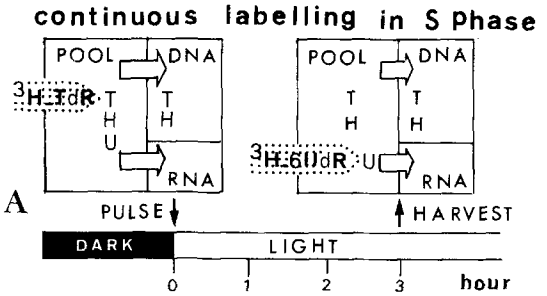



TABLE IIIA

| Labelling | Hydrolysate | | Thy | HOMeU | Cyt | Ura |
|---|---|---|---|---|---|---|
| $^3$H-Me-TdR | DNA | Nucleosides | 1000 | 100 | 0 | |
| $^3$H-Me-TdR | | Bases | 550 | 0 | 0 | |
| | Pool | Bases | 740 | 960 | 0 | 0 |
| $^3$H-6-UdR | DNA | Nucleosides | 730 | 510 | 0 | |
| $^3$H-6-UdR | | Bases | 530 | 300 | 90 | |
| | Pool | Bases | 540 | 5300 | 140 | 20 |

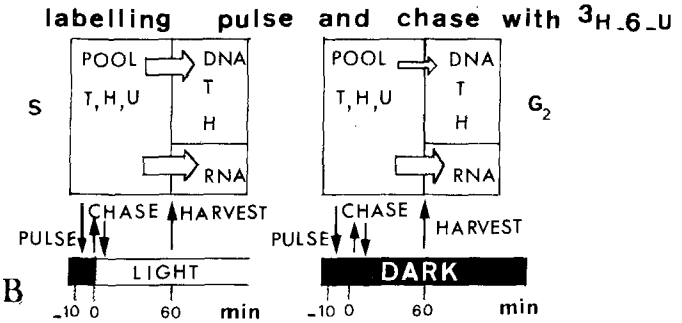



TABLE IIIB

| Cell cycle Phase | Hydrolysate | | Thy | HOMeU | Cyt | Ura |
|---|---|---|---|---|---|---|
| S | DNA | Nucleosides | 10900 | 7000 | 1600 | |
| | | Basis | 3100 | 1500 | 3000 | |
| | Pool | Bases | 100 | 24000 | 1300 | 1000 |
| $G_2$ | DNA | Nucleosides | 670 | 850 | 25 | |
| | | Bases | 950 | 1500 | 250 | |
| | Pool | Bases | 50 | 650 | 0 | 100 |

Fig. 3. and Table III. Radioactivity of pyrimidine nucleosides (Cpm) measured in hydrolysates of DNA and nucleotide pool separated by thin layer chromatography.
(A) Three 1l-cultures were labelled *in vivo* with: *left*: $^3$H-Me-TdR and *right*: $^3$H-6-UdR for 3 h under conditions described in Table I and according to the schedule drawn on the Figure above.
(B) In three 1l-cultures, 500 ml-samples were labelled with $^3$H-6-U (see Table I) and successively harvested in S- and $G_2$-phases. After 10 min-pulses, $^3$H-6-U was chased with cold uracil for 1 h. The DNA and nucleotide pool was thus extracted, hydrolyzed and analyzed as described above. Spots on chromatograms were excised and counted for radioactivity as described in 'Methods'.

As it can be seen in Table IIIB, a strong labelling of pyrimidines has been detected one hour after a 10 min-pulse of $^3$H-6-U (followed by a chase). The proportion of the labels of T and HOMeU in the pool is still opposite to the proportion measured in the DNA. HOMeU is highly labelled and accumulates in the pool. During $G_2$-phase, the reduced synthesis does not lead to a labelling of thymine.

Considered together, these observations suggest an important synthesis of HOMeU through an enzymatic pathway in the cellular pool, and a degradation of thymine residues transformed into HOMeU as is shown by the labelling of HOMeU by $^3$H-Me-TdR.

## 5. Discussion

Even in well-growing cultures of *A. carterae*, the mixture of mono-, di- and tri-phosphates that we extract from the acid-soluble fraction was not enough to allow the observation of isolated tri-phosphates on a chromatogram, after hydrolysis to bases. Nevertheless, HOMeU which is so highly labelled by exogenous thymidine, deoxyuridine, or uracil is very abundant in the cellular pool. The fact that the labelling of an exogenous HOMeU given to cultures is not observable in the DNA could be explained by the dilution of this base in the cold cellular pool.

In *A. carterae*, *C. cohnii* and *P. micans* which incorporate so efficiently pyrimidine precursors (Franker *et al.*, 1974; Galleron *et al.*, 1975; Filfilan and Sigee, 1977), there is nothing in our knowledge that could prevent algal DNA-polymerases from taking up HOMeU nucleotides as building blocks for the DNA. On the other hand, the finding by Rae (1978) of non-random positions of HOMeU in *C. cohnii* DNA, must be considered by reference to the natural DNA template. Sequencing large repetitive fractions of Dinoflagellate nuclear genome is not feasible (*A. carterae* DNA: $1.9 \times 10^{12}$ daltons per haploid cell, *C. cohnii*: $4.2 \times 10^{12}$; *P. micans*: $2.5 \times 10^{12}$, Loeblich, 1976). All we know about sequence organization is the preferential digestion of *C. cohnii* and *P. micans* total nuclear DNA by restriction enzymes which contain no T-T sequences in their total recognition site (Steele, 1980, Galleron, unpublished results). These observations are coherent with the finding by Rae (1978) of a very low frequency of 5′-HOMeU-p-T-3′ in *C. cohnii* DNA (7.9 % instead of the 29.3 % expected from GC content if HOMeU was randomly located in the genome).

In the absence of evidence for any other biological function of HOMeU in dinoflagellate DNA, we are still left with Rae's hypothesis: the persistence of a protection device against foreign endonucleases. Among the five dinoflagellate species in which HOMeU has been detected only one (*Symbiodinium microadriaticum*, Rae, 1976) is parasitic. Therefore, we cannot eliminate the interpretation of HOMeU in DNA as a vestige left in these organisms which once lived in associations either as hosts, symbionts or parasites (Sarjeant, 1974; Loeblich, 1976) and were thereby exposed to foreign endonucleases.

Further work should decide whether there is a competition between T and HOMeU regulated by their relative pool sizes, as in phage SPO1. It could be verified if, in

methotrexate treated-cells, the levels of HOMeU in DNA were raised by an inhibition of the thymidilate synthetase pathway. Southern hybridization between digested total DNA and probes made of cloned parts of the SPO1 genome coding for the enzymes of HOMeU synthesis could also bring valuable information.

Moreover, if the incorporation of pyrimidine precursors throughout the cell cycle corresponds to a repair mechanisms after preferential excision of thymines from DNA, it must work through a site-specific endonuclease still undiscovered, or through a base-specific enzyme similar to the ubiquitous uracil-N-glycolase.

Answers to these questions should give some clues to solve the mystery of the presence of HOMeU in dinoflagellate DNA, one of the strangest features of these primitive eukaryotes.

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