A Novel Origin of Some Deoxyribonucleic Acid Thymine and Its Nonrandom Distribution*

Pasquale Grippo, Elio Parisi, Clementina Carestia, and Eduardo Scarano†

ABSTRACT: [*methyl*-$^2H_3$]Thymine is found in DNA extracted from developing sea urchin embryos cultured in the presence of L-[*methyl*-$^2H_3$]methionine. The [*methyl*-$^2H_3$]thymine is only a very small fraction of the total DNA thymine and for this reason is called the minor thymine. In embryos cultured up to the gastrula stage, the minor thymine is found only in the monopyrimidine isostichs, namely, between two purines. In embryos cultured up to the prism stage, the minor thymine is found both in the monopyrimidine and in the oligopyrimidine isostichs, although its concentration is higher in the monopyrimidine isostichs. Hence, the points shown by the data are: (1) some [*methyl*-$^2H_3$]thymine is found in DNA of developing sea urchin embryos when [*methyl*-$^2H_3$]methionine is the precursor; (2) transfer of the intact CD_3 unit of methionine is required to explain this finding; (3) the distribution of this [*methyl*-$^2H_3$]thymine along the DNA chain is nonrandom. It is suggested that the DNA minor thymine arises at the polymer level by enzymatic deamination of DNA 5-methylcytosine. Base transitions G·C to A·T, following the formation of the minor thymine, and a round of DNA duplication might be primary chemical events of cell differentiation.

We have previously reported on the possible existence in DNA of developing sea urchin embryos of a small fraction of the thymine (minor thymine)[1] (Scarano, 1969; Scarano *et al.*, 1967), whose methyl group would arise from the methyl group of methionine by direct transfer at the polymer level. The existence of this thymine was suggested by the finding of a nonrandom distribution, in the thymines from DNA pyrimidine isostichs,[2] of label arising from the methyl group of methionine (Scarano, 1969; Scarano *et al.*, 1966, 1967; Grippo *et al.*, 1968a,b). The occurrence of an enzymatic deamination of DNA 5-methylcytosine to thymine at the polymer level would account for this finding. However, other interpretations are compatible with the previously published experimental data, for instance, nonrandom excision and repair of a small fraction of DNA nucleotides, heterogeneity in DNA and/or in cell population.

The mere finding of label arising from methyl-labeled methionine in the methyl group of DNA thymine does not demonstrate the transfer of the intact methyl group of methionine. In fact, in developing sea urchin embryos (Scarano *et al.*, 1965) the methyl group of methionine, by oxidation and the reactions of the one-carbon-unit metabolism, enters also into the purine ring of adenine and guanine and into the methyl group of thymine.

Direct evidence for the possible formation of the minor thymine in DNA by transfer of the intact methyl group of methionine was investigated by using L-methionine labeled with three deuterium atoms in the methyl group.

In this paper are reported experiments performed with developing sea urchin embryos cultured in the presence of L-[*methyl*-$^2H_3$]methionine. The results indicate the formation of a small fraction of DNA thymine (the minor thymine) labeled with all three deuterium atoms in the methyl group and distributed nonrandomly among the pyrimidine isostichs. This confirms the previous finding of the nonrandom distribution of the minor thymine in DNA (Scarano *et al.*, 1967). The results strongly suggest that the minor thymine component arises at the polymer level. In addition, the data suggest that the synthesis of this thymine is related to the stages of embryonic development.

Materials and Methods

L-[*methyl*-$^2H_3$]Methionine (99% content in deuterium) was synthesized according to Rachele *et al.* (1955), by New England Nuclear Corp. (Boston, Mass.). All other chemicals were reagent grade of the highest purity available.

Russell's viper venom was obtained from the Hafkine Institute (Parel, Bombay), sea urchins (*Paracentrotus lividus* and *Sphaerechinus granularis*) were obtained from the Zoological Station of Naples.

The following chromatographic solvents were used: (a) 95% ethanol–1 M ammonium acetate saturated with sodium tetraborate (70:30, v/v) (Plesner, 1955); (b) isopropyl alcohol–H_2O–concentrated NH_4 (85:15:1.3, v/v) (Hershey *et al.*, 1953); (c) 1-butanol–H_2O (86:14, v/v) (Markham and Smith, 1949).

Sea urchin embryos (5000/ml) were cultured with continuous gentle stirring in sea water at 18°. In all experiments penicillin G (5 mg/l.) and streptomycin sulfate (10 mg/l.) were added.

DNA from sea urchin embryos was extracted essentially as previously described (Augusti-Tocco *et al.*, 1968). Pyrimi-

* From the International Institute of Genetics and Biophysics, Naples, Italy and the Cathedral of Molecular Biology, University of Palermo, Palermo, Italy. *Received February 2, 1970.*

† Inquiries and requests for reprints should be addressed to Professor E. Scarano, International Institute of Genetics and Biophysics, 80125, Naples, Italy.

[1] This thymine is called minor thymine fraction or simply minor thymine.

[2] The uninterrupted sequences of pyrimidine nucleotides have been called "pyrimidine isostichs" (Shapiro and Chargaff, 1964).

TABLE I: Relative Intensity of Peak of Mass 129 of Samples of Commercially Available Thymine and of Thymine from DNA of *Paracentrotus lividus* Embryos.[a]

Expt	$\frac{m/e\ 129}{m/e\ 126} \times 100$
a	0.040 ± 0.005
b	0.038 ± 0.005
c	0.043 ± 0.003
d	0.056 ± 0.009
e	0.050 ± 0.008
f	0.045 ± 0.009

[a] Thymine samples, obtained from Calbiochem were used for analysis a, b, and c. Sample d is thymine from DNA of *P. lividus* embryos at prism stage. Samples e and f were thymines from monopyrimidine and from oligopyrimidine clusters, respectively, of a DNA obtained from *P. lividus* embryos at the pluteus stage. In this table and in the following ones, the data are reported as the average ± the standard deviation. The average was calculated from at least 6 mass spectra.

dine isostichs were prepared according to Burton and Petersen (1960) and separated on a column of DEAE-cellulose using a linear gradient of LiCl at pH 5 (Spencer and Chargaff, 1963). Each isostich fraction was lyophilized and the salt was removed by extraction with a mixture of ethyl ether–isopropyl alcohol (2:1, v/v).

The following experiment was performed to check whether 5-methylcytosine might be hydrolyzed to thymine during the preparation of the pyrimidine isostichs: [*methyl*-^{14}C]-5-methyldeoxycytidine (300,000 dpm) was treated with 2% diphenylamine in formic acid in the presence of 4 μmoles of deoxythymidine. After 18 hr at 30°, diphenylamine was removed by extraction with diethyl ether. Deoxythymidine and the labeled 5-methyldeoxycytidine were separated on a Dowex 50-X8 column. Deoxythymidine recovery was 95%. No radioactivity was found in deoxythymidine.

The two monopyrimidines (pTp and pCp) were separated by high-voltage electrophoresis. Each pyrimidine isostich was hydrolyzed to the nucleoside level with alkaline phosphatase and snake venom; the deoxyribonucleosides were chromatographed in solvents a and b. The deoxythymidine spot was eluted from the chromatogram, concentrated, and hydrolyzed to thymine by 70% perchloric acid. After hydrolysis the bases were chromatographed in solvents c and b. The thymine samples were lyophilized and analyzed by mass spectrometry.

High-voltage electrophoresis was performed according to Smith (1955) on washed Whatman No. 3MM paper.

Snake venom and alkaline phosphatase hydrolysis were performed as previously described (Grippo *et al.*, 1968b).

Deoxyribonucleosides were hydrolyzed to bases with 70% perchloric acid according to Bendich (1955).

The mass spectra measurements were made with a Consolidated Electrodynamics Co. double-focusing mass spectrometer. Samples of 6–100 μg were introduced as dry powder in the ionization chamber of the instrument. The spectra were recorded at ionization voltage of 15 and 25 eV and at 120°. The data are reported as intensity of the 127, 128, and 129 mass peaks relative to the peak of mass 126, the most intense peak of thymine. For each value the standard deviation is also reported.

Results

Samples of commercially available thymine of high purity and samples of DNA thymine from developing sea urchin embryos were analyzed by mass spectrometry. The results are listed in Table I. The peak of mass 129 of these thymine samples arises from the natural isotopic composition of the elements C, N, O, and H. For instance, a thymine of mass 129.050477 has the formula $^{12}C_4{}^{13}C^{16}O^{18}O^{14}N_2{}^1H_6$, the mass of thymine of isotopic composition $^{12}C_5{}^{14}N_2{}^{16}O_2{}^1H_3{}^2H_3$ is 129.061759. The resolving power of the mass measurements in our experiments does not permit us to distinguish the above thymine isomers, which are detected as one peak.

In Table II are reported the relative intensities of peak of mass 129 of thymine from DNA monopyrimidines and from DNA oligopyrimidines of developing sea urchin embryos cultured in the presence of L-[*methyl*-$^2H_3$]methionine. By comparing the data of Table I and of Table II it is clear that the DNA thymines of embryos grown in the presence of the labeled methionine contain an excess of the peak 129, with the exception of the thymine of the oligopyrimidines of experiments a, b, and c.

The excess of the 129 peak of the thymine samples listed in Table II should have the isotopic composition $^{12}C_4{}^{14}N_2{}^{16}O_2{}^1H_3{}^{12}C^2H_3$ because it is detected in samples of highly purified thymine and because it appears only when L-[*methyl*-$^2H_3$]methionine is present in the culture medium.

In each experiment of Table II the 129 peak of the monopyrimidine thymine is higher than that of the oligopyrimidine thymine. The Student test applied to all the values of the monopyrimidines and to all the values of the oligopyrimidines, listed in Table II, indicates that the two sets of values are different with a probability of 95%. The standard deviations for the Student test are calculated considering as if each value of Table II would be the result of one measurement. Though it might appear not orthodox to apply the Student test to a sample of experiments not done under identical conditions, the outcome of the test is highly significant. Analysis of the data of Table II by the pairing method (Mandel, 1964) reveals that the two sets of data are different with a probability of 99.5%. Pairing of the data of each experiment of Table II is justified by the fact that the monopyrimidine thymine and the oligopyrimidine thymine were obtained from the DNA of a single culture of developing embryos.

Moreover, the data of Table II show that no excess of the 129 peak is found in the oligopyrimidines when *Paracentrotus lividus* and *Sphaerechinus granularis* embryos are cultured soon after fertilization for 20 and 26 hr, respectively. However, an excess of the 129 peak occurs also in the oligopyrimidines in embryos cultured for more than 42 hr.

The pairing method of the Student test applied to the values of the monopyrimidine thymines and to the values of the oligopyrimidine thymines of experiments a, b, and c of Table II, tells that with a probability between 90 and

TABLE II: Relative Intensity of Peak of Mass 129 of DNA Thymine from Sea Urchin Embryos Grown in the Presence of L-[*methyl*-$^2H_3$]Methionine.[a]

Expt	Sea Urchin Species	Culturing Time (hr)	$\frac{m/e\ 129}{m/e\ 126} \times 100$ Thymine from DNA Monopyrimidines	$\frac{m/e\ 129}{m/e\ 126} \times 100$ Thymine from DNA Oligopyrimidines
a	*Paracentrotus lividus*	20	0.08 ± 0.012	0.05 ± 0.008
b	*Paracentrotus lividus*	20	0.06 ± 0.006	0.04 ± 0.007
c	*Sphaerechinus granularis*	26	0.10 ± 0.020	0.05 ± 0.020
d	*Paracentrotus lividus*	42	0.15 ± 0.025	0.09 ± 0.010
e	*Paracentrotus lividus*	62	0.12 ± 0.013	0.10 ± 0.010
f	*Sphaerechinus granularis*	65	0.11 ± 0.025	0.07 ± 0.014

[a] Experiments a and b: *P. lividus* embryos cultured for 20 hr until late blastula stage. L-[*methyl*-$^2H_3$]Methionine (5.0 mg/l.) was added 15 hr after fertilization when embryos were at the early blastula stage. Experiment c: *S. granularis* embryos cultured until gastrula stage in the presence of 10^{-3} M deoxythymidine. L-[*methyl*-$^2H_3$]methionine (8.0 mg/l.) was added 2 hr after fertilization. Two further additions of 5 mg/l. were made at intervals of 12 hr. Experiment d: *P. lividus* embryos cultured until the prism stage, in the presence of L-[*methyl*-$^2H_3$]methionine. Some embryos were still at the late gastrula stage. The labeled methionine (3.3 mg/l) was added 4 times at intervals of 10 hr. Experiment e: *P. lividus* embryos cultured until the prism stage. Labeled methionine (5.0 mg/l.) was added 3 times at intervals of 20 hr. The first addition of labeled methionine was done in expt d and e 2 hr after fertilization. Experiment f: *S. granularis* embryos cultured until the prism stage in the presence of 10^{-3} M deoxythymidine. Some embryos were still at the late gastrula stage. L-[*methyl*-$^2H_3$]Methionine (7.3 mg/l.) was added 2 hr after fertilization. Seven additions of 6.0 mg/l. were made at intervals of 9 hr.

95% the two sets of values are different. The same test applied to the values of the monopyrimidine thymines and to the values of the oligopyrimidine thymines of experiments d, e, and f of Table II, tells also that the two sets of data are different with the same probability. Identical conclusions are obtained on the monopyrimidines of experiments a, b, and c *vs.* the monopyrimidines of experiments e, f, and d, and for the oligopyrimidines of the two sets of experiments. Thus, the data of Table II can be arranged in four groups, namely: (1) thymines of the monopyrimidines of experiments a, b, and c; (2) thymines of the oligopyrimidines of experiments a, b, and c; (3) thymines of the monopyrimidines of experiments d, e, and f; (4) thymines of the oligopyrimidines of experiments d, e, and f. The chances that the data of Table II would have been arranged in four groups by accident are so slight and the connection with developmental stages so clear that there is little doubt of the significancy of the data.

In developing sea urchin embryos grown in the presence of methyl-labeled methionine, the methyl group of thymine is labeled also through the one-carbon pool, to which the methyl group of methionine contributes by oxidation and subsequent transfer to the folic acid coenzymes of the one-carbon-transfer metabolism (Scarano *et al.*, 1967). Thus, in the experiments in which the embryos were grown in the presence of [*methyl*-$^2H_3$]methionine one expects to find an excess of the isotopic peaks of mass 127 and 128 in the DNA thymines. However, the thymines of mass 127 and 128 should be randomly distributed in the DNA pyrimidine isostichs because the 127 and the 128 thymines are incorporated into DNA by the DNA polymerase from the dTTP pool.

The data of Table III show in three experiments (a, b, and c) that the thymines of mass 127 and 128 are randomly distributed in the DNA monopyrimidines and in the DNA oligopyrimidines, while the 129 thymine is nonrandom. In Table III are also reported the data of a sample of thymine of *P. lividus* embryos cultured in the absence of [*methyl*-$^2H_3$]-methionine.

The nonrandom distribution on DNA of the 129 thymine peak and the random distribution of the 127 and 128 thymine peaks demonstrate that the minor thymine cannot arise by oxidation of the methyl group of methionine through the one-carbon unit metabolism and addition of two or one deuterium atoms to the natural isotopic isomers 127 and 128, respectively.

In one experiment, Table IV, in which embryos cultured in the presence of [*methyl*-$^2H_3$]methionine did not develop normally and did stop development at the early gastrula stage, the 129 peak was found randomly distributed in the DNA monopyrimidines and in the DNA oligopyrimidines just as in every instance for the 127 and 128 peaks.

Discussion

Two main findings are reported in this paper. First, an excess of the peak of mass 129 occurs in the DNA thymines from embryos cultured in the presence of [*methyl*-$^2H_3$]-methionine in comparison with that of the DNA thymines from embryos cultured without the labeled methionine. This demonstrates the existence in the DNA of developing sea urchin embryos of a trace amount of thymine, the minor thymine, of isotopic composition $^{12}C_4{}^{14}N_2{}^{16}O_2{}^1H_3{}^{12}C^2H_3$, which appears to arise by the transfer of the intact methyl

TABLE III: Relative Intensity of Peaks of Mass 127, 128, and 129 of DNA Thymine from Sea Urchin Embryos Cultured with (a, b, and c) and without (d) L-[*methyl*-$^2H_3$]Methionine.[a]

Expt	Thymine from	$\frac{m/e\ 127}{m/e\ 126} \times 100$	$\frac{m/e\ 128}{m/e\ 126} \times 100$	$\frac{m/e\ 129}{m/e\ 126} \times 100$
a	DNA monopyrimidines	6.0 ± 0.2	0.61 ± 0.02	0.08 ± 0.012
	DNA oligopyrimidines	6.1 ± 0.4	0.60 ± 0.05	0.05 ± 0.008
b	DNA monopyrimidines	7.5 ± 0.3	0.69 ± 0.05	0.10 ± 0.020
	DNA oligopyrimidines	7.6 ± 0.6	0.70 ± 0.07	0.05 ± 0.020
c	DNA monopyrimidines	7.9 ± 0.5	0.74 ± 0.09	0.11 ± 0.025
	DNA oligopyrimidines	7.6 ± 0.2	0.76 ± 0.06	0.07 ± 0.014
d	DNA	5.8 ± 0.4	0.54 ± 0.05	0.050 ± 0.008

[a] a, b, and c are mass spectra analyses of DNA thymines from embryos cultured in the presence of L-[*methyl*-$^3H_2$]methionine as described in experiments a, c, and f of Table II, respectively. d is a mass spectrum analysis of a DNA thymine from *Paracentrotus lividus* embryos at the pluteus stage.

group of methionine. Second, the minor thymine has a nonrandom distribution in DNA, and consequently appears to be synthesized at the polymer level.

These findings can be accounted for by three types of explanations. The first type of explanations is all trivial and can be discussed under two headings, namely: chemical deamination during the procedure for the preparation of the thymine samples; presence of an impurity in the thymine samples analyzed by mass spectrometry. That chemical deamination of some DNA 5-methylcytosines to thymines might occur at the level of the pyrimidine isostich preparation is excluded by the experiment described under method. At the level of the hydrolysis step to bases, no chemical deamination can occur because the hydrolysis is performed on deoxythymidine after it has been separated from 5-methyldeoxycytidine. The presence of an impurity is excluded by the purification procedures used at the levels of DNA and of pyrimidine isostich preparation, and finally by the electrophoretic and chromatographic purifications. For instance, even a trace amount of ribothymidine derivatives from tRNA would have been separated by the chromatography with the borate solvent (Plesner, 1955).

The second type of explanations is formation of ^{12}C,-$^2H_3$-labeled thymine in the acid-soluble fraction as dTTP and subsequent incorporation in DNA by the polymerase step or by a mechanism of excision and repair of DNA segments. These explanations appear highly unlikely because, in addition to the existence in the acid-soluble fraction of a [^{12}C,$^2H_3$]dTTP, they require the simultaneous occurrence of two conditions to account for the nonrandom distribution of the minor thymine, namely, an increase in the ratio of [^{12}C,$^2H_3$]dTTP over dTTP of natural isotopic composition at the time of synthesis or repair of DNA richer in thymine in the monopyrimidine sequences. An analogous explanation was discussed to account for the origin of the minor thymine from the acid-soluble fraction in our earlier experiments (Scarano *et al.*, 1967). It appears to be exceedingly unlikely that these two very specific explanations are correct because, in the two types of experiments, two different *ad hoc* mechanisms for the appearance of the label in dTTP would have to occur concomitantly with the change in composition of the DNA being synthesized.

The results presented here and in our previous paper indicate the existence of two types of thymine in DNA and also the nonrandom distribution of the minor thymine. The nonrandom distribution of the minor thymine was emphasized in our first paper on the subject (Scarano *et al.*, 1966). Explanation of our data must account for the nonrandom distribution of the minor thymine.

Thus, several lines of evidence indicate that the origin of the minor thymine is not from the acid-soluble fraction through the DNA polymerase step or through a repair mechanism.

TABLE IV: Relative Intensity of Peaks of Mass 127, 128, and 129 of DNA Thymine from Abnormally Developing Sea Urchin Embryos Grown in the Presence of L-[*methyl*-$^2H_3$]-Methionine.[a]

Thymine from	$\frac{m/e\ 127}{m/e\ 126} \times 100$	$\frac{m/e\ 128}{m/e\ 126} \times 100$	$\frac{m/e\ 129}{m/e\ 126} \times 100$
DNA monopyrimidines	8.6 ± 0.20	0.86 ± 0.05	0.08 ± 0.01
DNA oligopyrimidines	8.9 ± 0.20	0.84 ± 0.04	0.08 ± 0.01

[a] *P. lividus* embryos were cultured in the presence of L-[*methyl*-$^2H_3$]methionine (5.0 mg/l.). Starting 2 hr after fertilization, four additions of labeled methionine at intervals of 11 hr were made. At the end of the experiment, the embryos were abnormal gastrulae, while normal embryos would have reached the pluteus stage.

The third type of explanations includes all the possible mechanisms of formation of the minor thymine at the polymer level. The nonrandom distribution of the thymine peak of mass 129, and the random distribution of the thymine peaks of mass 127 and 128 strongly indicate that the minor thymine is synthesized at the polymer level. We have already discussed the enzymatic deamination of specific 5-methylcytosines to thymines in DNA as a possible mechanism for the formation of the minor thymine (Scarano *et al.*, 1967; Scarano, 1969).

The present data on the higher concentration of the DNA minor thymine in the monopyrimidine isostichs confirm our previous report obtained with a completely different experimental approach (Scarano *et al.*, 1967). However, in our previous work, because of the intrinsic limitations of the experimental approach used, it was possible to demonstrate only a nonrandom distribution on DNA of the minor thymine and no information could be inferred on the occurrence of the minor thymine in the oligopyrimidines. However, the data of Table II of the present paper suggest that the minor thymine is found also in the DNA oligopyrimidines of embryos cultured until they reach the prism stage while in embryos cultured until the early gastrula stage no minor thymine is found in the oligopyrimidines. This indicates that the synthesis of the minor thymine is related to stages of embryonic development and would support the hypothesis by Scarano (Scarano, 1969; Scarano and Augusti-Tocco, 1967; Scarano *et al.*, 1967) of sequential enzymatic modifications of DNA as the basic mechanism of cell differentiation.

Because of technical difficulties we have not yet been able to perform experiments in which the nuclear DNA is analyzed after separation from the mitochondrial DNA. However, no significant mitochondrial DNA synthesis occurs until the late blastula stage, and from the gastrula stage to the prysm stage the mitochondrial DNA synthesis is a very small fraction of the nuclear DNA synthesis (Gustafson and Lenicque, 1952; Pikò *et al.*, 1967). Thus, it appears plausible to conclude that the minor thymine is synthesized on the nuclear DNA.

Another point worth discussing is the finding of a random distribution of the minor thymine in the DNA from embryos that did not develop beyond gastrulation. This indicates a correlation of an abnormal biochemical event at the DNA level, namely, the genotype level with abnormal development.

Recently Sneider and Potter (1969) reported that in amethopterin-blocked cultures rescued by deoxythymidine, a significant amount of ^{14}C label arising from the methyl group of methionine is found in DNA thymine. They suggest that this thymine arises at the polymer level, but no studies were made on the distribution of the labeled thymine in DNA. In sea urchin embryos a fraction of the label in the methyl group of methionine, even in aminopterin-blocked and deoxyadenosine- and deoxythymidine-rescued embryos, is found in the methyl group of DNA thymine (Scarano *et al.*, 1967). This phenomenon masks the formation of the minor thymine when investigated with methionine labeled in the methyl group. Only by double-labeling experiments, with deoxythymidine and methionine was it possible to obtain indirect evidence of the minor thymine by its nonrandom distribution on DNA.

To conclude, the findings reported in this paper indicate that in the developing sea urchin embryo a small fraction of the DNA thymine is synthesized at the polymer level. The existence of the DNA minor thymine might imply that a mechanism of base changes in DNA would underlie the stepwise transitions by which cell differentiation and embryonic development are realized.

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