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In vitro Screening of Carcinogens Using DNA of the His⁻ Mutant of *Salmonella typhimurium*[1]

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Key Words. DNA strand separation · DNA replication · *Salmonella typhimurium* · Wild type (His⁺) and mutant (His⁻) strains · Carcinogens

Abstract. Carcinogens: 7,12-dimethylbenz(a)anthracene (DMBA), 2-acetylaminofluorene (AAF), 3-methylcholanthrene (MC), daunorubicin, thio-TEPA, cyclophosphamide, 1-(2-chloroethyl)2-cyclohexyl-1-nitrosourea (CCNU) and mitomycin C, which mutate *Salmonella typhimurium* tester strains in the *Salmonella*/microsome mutagenicity test, strongly stimulate the in vitro synthesis of DNA isolated from His⁻ mutants (TA-1538, TA-1537, TA-1535), and induce in vitro His⁻ DNA strand separation. Four other carcinogens, ethionine, actinomycin D, bleomycin and reserpine, which were not known as mutagens in the Ames test, also strongly stimulate His⁻ DNA synthesis and His⁻ DNA strand separation in local areas. Those substances which are neither carcinogenic nor mutagenic: *d*-lactose, fluorene, cholesterol, and saccharin, do not stimulate either His⁻ DNA (or His⁺ DNA) synthesis or DNA in vitro strand separation. Steroid hormones, which are carcinogenic only for steroid target tissues, do not react with bacterials DNA.

Introduction

In the last 10 years several short-term assays have been proposed for detection and screening of carcinogenic agents [1, 2, 12, 14, 15, 17]. The *Salmonella*/microsome test [1] which is generally used has greatly contributed to establishing the relationship between the mutagenicity and carcinogenicity of many agents [11]. Some carcinogens mutate *Salmonella typhimurium* (His⁻) tester strains into (His⁺), but 10–20% of carcinogens, among them actinomycin D, bleomycin, ehionine etc., which are carcinogenic in animals, are not mutagenic in this test [6, 11].

Very different in vitro behavior of purified cancer DNA(s) and normal DNA(s) in the absence or presence of known carcinogens led us to construct a rapid in vitro assay system (oncotest) for detection and screening of carcinogenic agents [2, 5]. In this system carcinogens induce in vitro a strong enhancement in UV absorbance (hyperchromicity) of

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cancer DNA(s) while that of normal DNA(s) is only slightly increased [5]. The UV absorbance increase, due to the local opening of the double strands of DNA by carcinogens, correlates with increase of the in vitro synthesis of cancer DNA(s) [5].

Here we present evidence that purified DNA from tester strains of His⁻ mutants (TA-1538, TA-1537, and TA-1535) of *S. typhimurium* behave in the oncotest like destabilized DNA i.e. as DNA from cancer tissues. Rapid, sensitive and inexpensive, the oncotest is a reliable method which sharply differentiates between noncarcinogenic and carcinogenic potentials.

Materials and Methods

Chemicals

Deoxyribonucleoside-5'-triphosphates (d-XTP): Miles Laboratories, USA; ^{3}H-labelled TTP (specific activity 17.5 Ci/mol), Amersham, England; pancreatic DNase, RNase, RNase T_1: Worthington Com., USA; 3-methylcholanthrene (MC), Eastman Kodak Co., USA; 2-acetylaminofluorene (AAF), Serlabo, France; 7,12-dimethylbenz(a)anthracene (DMBA), Nutritional Biochemicals Co., USA; *dl*-ethionine, Biochemicals, Co., USA; reserpine, fluorene, saccharin Krst purum, Roth, France; thio-TEPA (1-azinidyl) phosphine sulfide, Specia, France; (1-(2-chloroethyl)-3-cycloexyl)-1-nitrosourea (CCNU), bleomycin, Lab. Bellon, France; daunorubicin D, gift from Dr. *R. Maral*, Rhône-Poulenc, France; actinomycin D, Merk, USA; testosterone and estradiol, gift from Dr. *Ray*, Institut Pasteur, Paris; dl-lactose, cholesterol, gift from Dr. *M. Roumiantzeff*, Institut Mérieux, Lyon; 8-hydroxychinolin, Merck, FRG; mitomycin C, Sigma, St Louis, Mo. USA; Phenol Baker Chemicals, Deventer, Holland.

Isolation of DNA

Wild strain of *S. typhimurium* LT 2 and His⁻ mutants TA-1538, TA-1537, and TA-1535 [1] were used. Exponentially-growing cells cultured in shaken nutrient broth (Bactotryptone Difco 10 g/l, yeast extract 5 g/l and NaCl 5 g/l, pH was adjusted to 7.3) at 37 °C were used for DNA isolation. Cells collected by centrifugation (4 °C) were homogenized in Lerman's solution pH 8.0 [10] and lysed in the presence of lauryl sulfate (2% final concentration). Phenol solution containing 0.2% of 8-hydroxychinolin was added (v/v) and the mixture shaken at 4 °C for 10 min. After centrifugation at 5,000 *g* for 10 min (Sorvall SS1 centrifuge) the upper phase was saved. The interphase was retreated in the same way and centrifuged. The upper phases containing DNA were mixed and further treated by chloroform (four successive treatments and centrifugations). RNA which contaminates DNA preparations was practically eliminated by incubation with RNase [5]. After removal of RNase by three chloroform treatments, each of which was followed by centrifugation, DNA was precipitated with 2 vol of 96% alcohol, dissolved in 2SSC solution and dialyzed against the same solution for 24 h at 4 °C. Purified DNA (absorbance at 260–280 nm = 2.0) was stored at –20 °C.

Characterization of DNA

The RNA content determined by the orcinol reaction was lower than 10%. Protein content was less than 1%. The hyperchromic effect on incubation with 0.1 *N* KOH was 40–45% at 260 nm for DNA from His⁻ mutants, and 35% for DNA from His⁺ strains. The integrity of DNA was controlled by ultracentrifugation in alkaline sucrose gradient.

Conditions for DNA Synthesis

Incubation mixture for DNA in vitro synthesis contains per 0.15 ml: phosphate buffer (pH 7.65) 25 µmol; $MgCl_2$: 2 µmol; four deoxyribonucleoside-5'-triphosphates, each 5 nmol (+ ^{3}-TTP: 50,000 cpm); DNA: 0.5 µg; DNA-dependent DNA polymerase I, 60 µg. Compounds to be tested (see legend to fig. 1). Incubation 10 min at 36 °C. The reaction was stopped by the addition of trichloroacetic acid (TCA) (5% final concentration) and cooled in an ice bath. Acid-precipitable material was filtered on GF/C glass filter, washed (TCA 5%), dried, and radioactivity measured in the spectrometer Beckman (Prias). Results are expressed as cpm. The agents to be tested were dissolved in water. Those with a low water solubility were first dissolved in a minimal volume of 96% alcohol or 1% deoxycholate solution which was then diluted with water. pH was adjusted to 7.65. Dimethyl sulfoxide was avoided because of its role in DNA strand separation [5]. DNA-dependent DNA polymerase I partly

purified from *Escherichia coli* was used as it has previously been used for in vitro synthesis of DNA from normal and cancerous mammalian cells [5].

UV Absorbance (Hyperchromicity) of DNA(s)

UV absorbance at 260 nm of His⁻ mutants DNA and control DNA (20 µg in 1 ml of Tris-HCl buffer 10^{-2} *M* pH 7.65) was measured at 24 °C before and after addition of a given compound. Blank cuvette contained the equivalent amount of the same compound. Contact between DNA and compound was 1 min with gentle shaking. Results are expressed as UV absorbance increase (%).

Results

Carcinogens in the Oncotest

Under identical conditions, the oncotest evaluates the template activity of both normal and cancer DNA(s) in the presence or absence of carcinogens [5]. The latter strongly stimulate the in vitro synthesis when cancer DNA(s) are used as template, whereas syntheses with DNA(s) from healthy tissues as template is enhanced very little. The oncotest does not require the presence of microsomal enzymes for activation of carcinogens since the direct effect of carcinogens of DNA(s) is measured. Data presented in figure 1 show that DNA purified from His⁻ mutant (TA-1538) of *S. typhimurium* reacts in vitro very differently compared to DNA from wild strain. Each of four carcinogens, 7,12-DMBA, 2-AAF, ethionine and 3-MC, strongly stimulates His⁻ DNA in vitro synthesis without affecting that of DNA from wild strain of *S. typhimurium*. DNA isolated from His⁻ TA-1538 mutant cultured under the same conditions but in different batches gave reproducible results in comparison to those in figure 1 (p values between < 0.01 and < 0.001). DNA(s) from His⁻ TA-1537 and TA-1535 mutants behave for instance as DNA from TA-1538 mutant (table I). Carcinogens do not inhibit the activity of DNase which does not allow DNA synthesis (table II). It should be stressed that there is apparently no difference in template activity between human or animal cancer DNA(s) [2, 5], and His⁻ mutant DNA in the presence of DNA-dependent DNA polymerase and carcinogens. In other words, cancer DNA(s) and His⁻ mutant DNA(s) are both more susceptible to carcinogens and must therefore possess some common physicochemical properties

Table I. Effect of *dl*-ethionine on DNA in vitro synthesis using template DNA from different His⁻ mutants (*S. typhimurium*)

	^{3}H-TTP incorporated (cpm) in 10' min at 36 °C					
	TA-1538 DNA	p[a]	TA-1537 DNA	p[a]	TA-1535 DNA	p[a]
Control	1,086 ± 58	–	1,080 ± 64	–	1,298 ± 46	–
2 µg *dl*-ethionine	2,383 ± 60	< 0.001	2,256 ± 75	< 0.001	2,244 ± 100	< 0.01
4 µg *dl*-ethionine	3,832 ± 56	< 0.01	3,865 ± 82	< 0.01	3,786 ± 92	< 0.001

Incubation conditions (see text). DMBA, reserpine, actinomycin D, daunorubicin, mitomycin C, stimulate the in vitro synthesis of DNA(s) not only from TA-1538 mutant (fig. 1) but also from TA-1537 and TA-1535 strains (results not shown here).

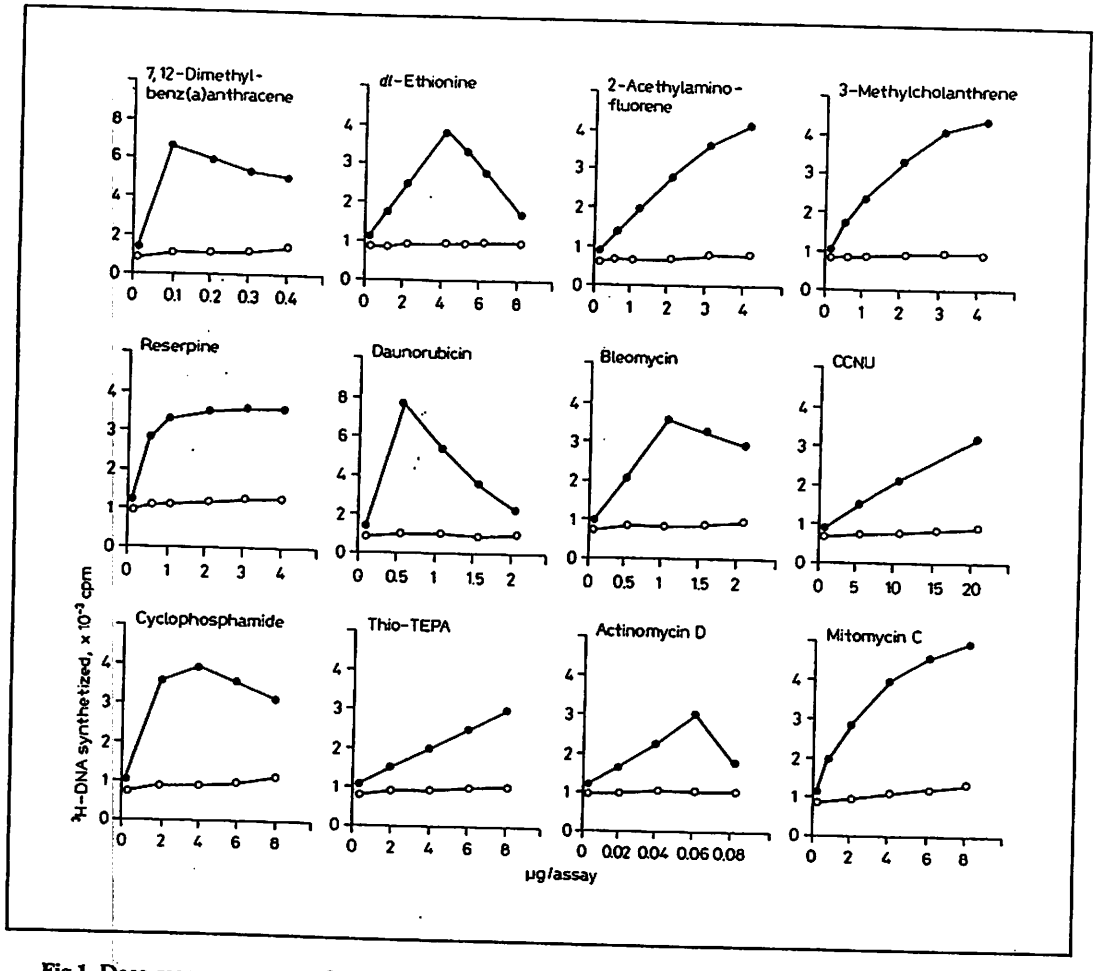
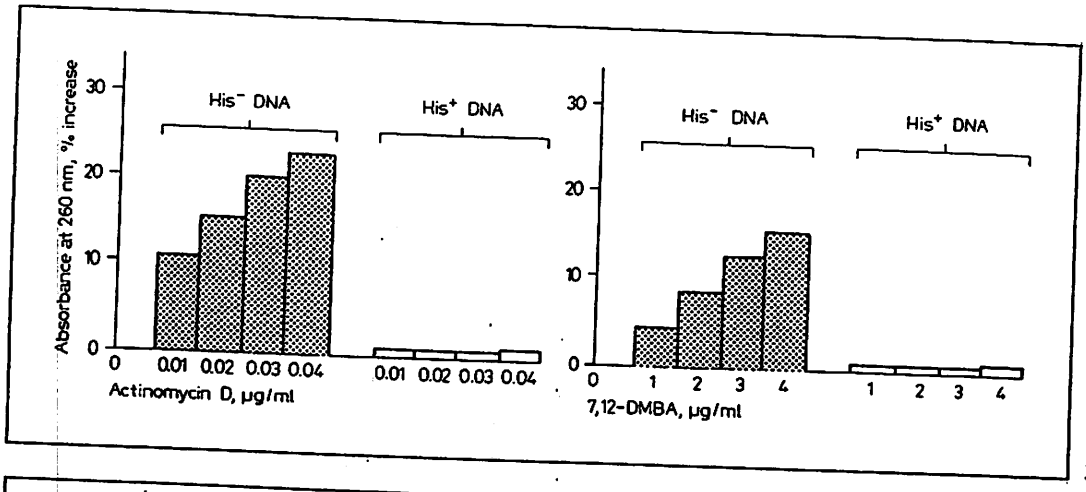
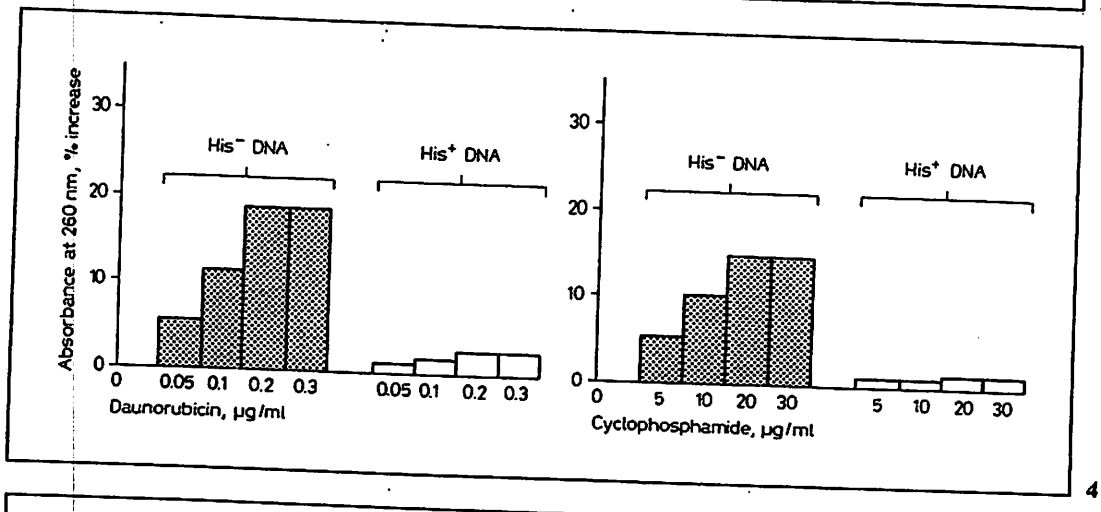


Fig 1. Dose-response curves of various chemical carcinogens and chemotherapeutic agents on His⁻ DNA (•) and His⁺ DNA (o) in vitro synthesis. Further explanation in text.

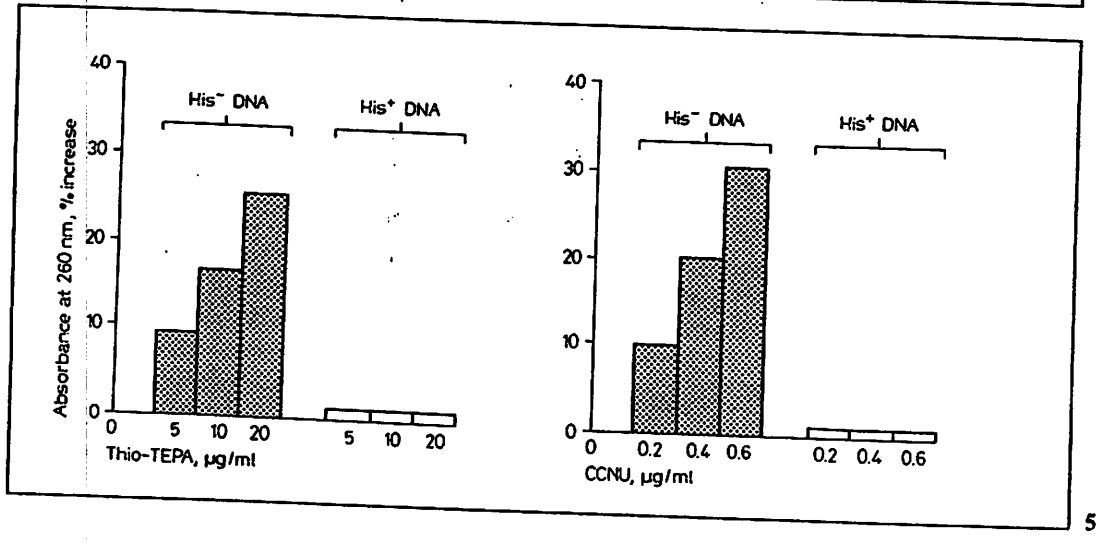
but not in His⁺ DNA. Increased template activity of His⁻ DNA is due to the separation of double-stranded regions of that DNA, as shown in figures 3, 4, and 5. In fact, 7,12-DMBA, *dl*-ethionine, daunorubicin, cyclophosphamide, actinomycin D, thio-TEPA, and CCNU bring about increased UV absorbance of His⁻ DNA (TA-1538) but not of His⁺ DNA. The increased UV absorbance of His⁻ DNA is due to local DNA strand separation, and depends on the concentrations and nature of the drugs used. Thus, actinomycin D (0.04 µg/ml) induces UV absorbance up to 23%, while cyclophosphamide (30 µg/ml) induced increase is only 15%. Additive effects of some carcinogenic agents, each consecutively added at saturating concentration to DNA solution is indicated in figure 6. The consecutive effects of daunorubicin, ethionine and DMBA results in a 35% increase of UV absorbance of His⁻ DNA while these carcinogens are practically with-


Absorbance at 260 nm, % increase
His⁻ DNA
His⁺ DNA
0 0.01 0.02 0.03 0.04
Actinomycin D, µg/ml
0.01 0.02 0.03 0.04
0 1 2 3 4
7,12-DMBA, µg/ml
1 2 3 4

3


Absorbance at 260 nm, % increase
His⁻ DNA
His⁺ DNA
0 0.05 0.1 0.2 0.3
Daunorubicin, µg/ml
0.05 0.1 0.2 0.3
0 5 10 20 30
Cyclophosphamide, µg/ml
5 10 20 30

4


Absorbance at 260 nm, % increase
His⁻ DNA
His⁺ DNA
0 5 10 20
Thio-TEPA, µg/ml
5 10 20
0 0.2 0.4 0.6
CCNU, µg/ml
0.2 0.4 0.6

5

Table III. Relationship between mutagens in *Salmonella* tester strains, carcinogens in oncotest and carcinogenesis in vivo

Compounds	*Salmonella*/microsome test	Oncotest using cancer DNA(s) or His⁻ DNA	Carcinogenesis in vivo, animals
3-Methylcholanthrene	+	+	+
7,12-Dimethylbenz(a)anthracene	+	+	+
2-Acetylaminofluorene	+	+	+
Ethionine	negative	+	+
Actinomycin D	negative	+	+
Bleomycin	negative	+	+
Acridine orange	+	+	+
Steroid hormones	negative	+ (with steroid target tissue [5])	+
Mitomycin C	+	+	+

See references [2, 5, 6, 9].

ceptible to ionic strength, and carcinogens [5], are most likely involved in DNA strand separation, i.e. unwinding in local areas.

Data presented here show that at saturating concentration each carcinogenic agent induces His⁻ DNA strand separation to a different degree indicating the existence of different reactive sites on His⁻ DNA. Additive effects of carcinogenic agents (each one used at saturation concentration) on His⁻ DNA strand separation support this view. The nature of these binding sites is not known. Although actinomycin D, bleomycin and ethionine, which are carcinogenic in animals, do not act as mutagens in the *Salmonella*/microsome test [6], each of them incubated in the presence of purified His⁻ DNA stimulates DNA synthesis and induces His⁻ DNA strand separation as efficiently as that observed with known chemical carcinogens. These observations suggest that negative results obtained with these three agents using tester strains of *S. thyphimurium* [1, 6, 9], in fact indicate a permeability barrier of bacteria. His⁻ DNA in vitro reactivity with carcinogens/mutagens or carcinogens which are not mutagens indicates that for detection of carcinogens, different bacterial tester strains [1, 7, 9] can be replaced by DNA from His⁻ mutants TA-1538, TA-1537, and TA-1535 or even from other types of mutants [*Beljanski*, unpubl. results].

The case of steroid hormones is of particular interest. They act as carcinogens with cancer DNA(s) isolated from steroid hormones target tissue (breast cancer, ovary cancer, etc.), and induce both an enhanced DNA synthesis and UV absorbance increase [5]. In contrast, DNA(s) from healthy steroid target tissues or cancer DNA(s) from nontarget tissues respond to steroid hormones weakly. Here we see that His⁻ and His⁺ *Salmonella* DNA(s) do not react in vitro to steroids or to miscellaneous substances tested.

The perfect correlation between results obtained for substances in the oncotest and results from the *Salmonella*/microsome test (table III) and from other publications, demonstrate that the increased in vitro template activity of His⁻ DNA caused by DNA un-

winding in local areas may represent in vivo a possible basic mechanism for modification of DNA template potentialities. In the case of mammalian cancer DNA we have shown that the opening of DNA double strands by a given carcinogen does not lead to chain scission [5]. The results presented here, and those previously described, suggest that under persistent in vivo action of carcinogens, DNA(s) might be destabilized, a process in which synergistic participation of cytoplasmic molecules could contribute to maintain a rather destabilized form of DNA. The results presented here, and those described elsewhere [2, 5], might help us to understand the mechanism of gene activation and to approach the problem of cancer induction.

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