*Hin*dIII-SENSITIVE SITES PRESENT ONCE IN EVERY FOUR REPEATS OF *Eco*RI-SENSITIVE SITES IN NOVIKOFF RAT HEPATOMA DNA

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1. Introduction

Restriction endonuclease digestion followed by gel electrophoresis has been widely used to detect repeating sequences in DNA from many eukaryotes. Highly repeated fragments were found after restriction endonuclease treatment of DNA from several mammalian species, including calf, human, monkey, mouse, rat and sheep [1–4]. The α-component of African green-monkey DNA [5] was most extensively studied and the nucleotide sequence of the 172 base-pair segment of the DNA cleaved by *Hin*dIII restriction endonuclease has been determined [6]. The segment is repeated several million times in the genome.

Multiples of a unit repeat of 376 base-pairs have been found [3] after *Hin*dIII restriction-endonuclease treatment of rat-liver DNA and small repeating fragments from 60–440 base-pairs found [1] after *Eco*RI restriction-endonuclease treatment of rat-liver DNA. In the present study, DNA from Novikoff rat hepatoma cells was digested with 4 restriction endonucleases and analyzed by polyacrylamide gel electrophoresis. Interestingly, two repeating units, monomer and tetramer sizes, were found after treatment of the DNA with different restriction endonucleases. The fragments of the two repeating units come from the same GC-rich region of the DNA.

2. Materials and methods

Novikoff hepatoma ascites cells were maintained in male Holtzman rats for 6 days and DNA was extracted from the cells as in [7]. *Eco*RI, *Hin*dIII, *Hae*III and *Hin*fI restriction endonucleases were purchased from Bethesda Res. Labs (Rockville, MD).

All DNA digestions were performed in silicone-coated test tubes. DNA (usually 10 μg) in 10 μl 20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$ was incubated with various amounts of restriction enzymes for 1 h at 37°C.

Acrylamide gel electrophoresis was usually carried out at 300 V for 1 h with a vertical gel electrophoresis apparatus EC 470 (E-C Apparatus Co., St. Petersburg, FL) following the procedures in [8]. The gel was stained with 1 μg/ml ethidium bromide in water for 1 h and photographed with an ultraviolet light source using an ultraviolet-cut filter. Bands were cut out of the gel with a razor blade under ultraviolet light, the gel strips were smashed in a glass homogenizer, and the DNA fragments were extracted with water from the gel. The DNA was precipitated with ethanol, redissolved in 20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$ and redigested with different restriction endonucleases.

3. Results and discussion

Novikoff rat DNA was digested with *Eco*RI or *Hin*dIII restriction endonuclease and analyzed by 3.5% acrylamide gel electrophoresis (fig.1). Monomer to dodecamer fragments produced by partial digestion of rat hepatoma DNA with *Eco*RI are shown by arrows in fig.1A. Monomer to tetramer bands were found together with other bands after 10 μg DNA were digested with 10 units *Eco*RI for 1 h at 37°C

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(fig.1C, arrows). When 10 μg DNA were digested with up to 50 units *Eco*RI for 1 h at 37°C, the DNA cleavage pattern was the same as fig.1C. The DNA was digested with *Hin*dIII (fig.1B); a dense band was observed at the *Eco*RI tetramer position, a less dense band was found at the *Eco*RI octamer position, and a faint band was noticed at the *Eco*RI dodecamer position.

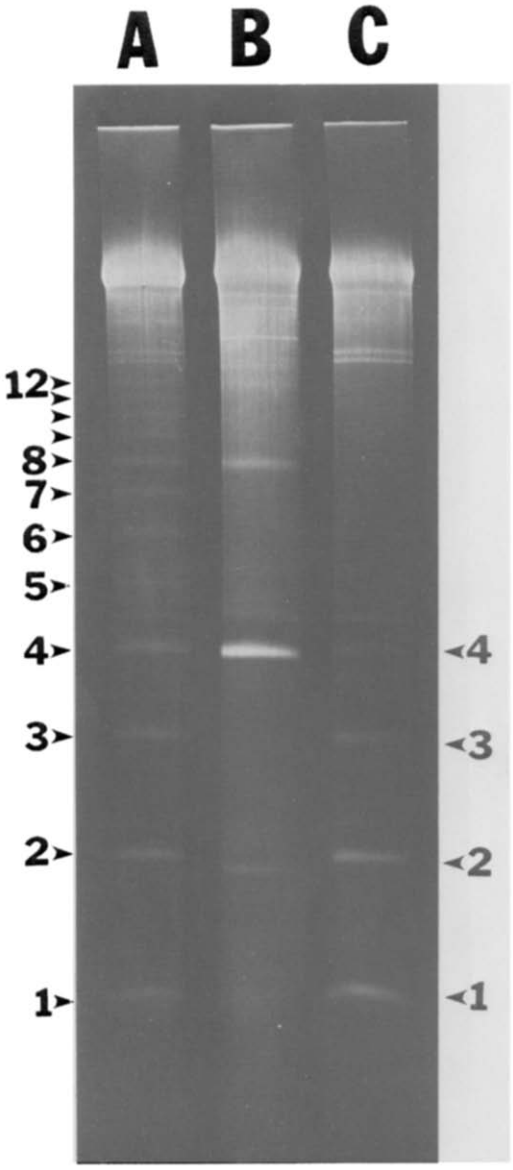

Fig.1. Novikoff rat DNA (10 μg) digested with either 5 units *Eco*RI (A), 10 units *Hin*dIII (B), or 10 units *Eco*RI (C) for 1 h at 37°C. The digests were fractionated in 3.5% acrylamide gel.

The lengths of the fragments were determined using φX174RF DNA digested with *Hin*fI as size markers. The *Eco*RI monomer was determined to be 93 ± 2 base-pairs long and *Hin*dIII fragment 1 was determined to be 372 ± 8 base-pairs long. The length of *Hin*dIII fragment 1 determined here agrees well with the length of 376 base-pairs reported in [3].

The *Hin*dIII fragment 1 (fig.2A, arrow) has the same size as *Eco*RI tetramer. It was extracted from the gel and redigested with *Eco*RI (fig.2B). The *Eco*RI monomer, dimer and trimer bands were observed in this digest, indicating that the DNA in *Hin*dIII band 1 and in *Eco*RI bands 1–3 are derived from the same region of the genome. Several bands were found which did not have corresponding bands in whole DNA digested with *Eco*RI (fig.2C). The lengths of three of these fragments in fig.2B (arrows) were 38, 55 and 131 base-pairs. The sum of the base-pair numbers in the smallest two fragments, 38 and 55, is 93, which equals the *Eco*RI monomer size. Also the sum of the base-pairs in the smallest fragment, 38, and the *Eco*RI monomer, 93, equals the base-pair number 131 in the third fragment.

A faint *Hin*dIII band which migrates a little faster than *Eco*RI band 2 (fig.1B) was also cut out and the DNA in the band was extracted redigested with *Eco*RI. No band corresponding to the *Eco*RI monomer was found in the digest.

When *Hin*dIII fragment 1 (fig.3A), which is 372 base-pairs long, was cleaved with *Hae*III, two fragments, 173 and 199 base-pairs long, were produced (fig.3B). After prolonged digestion (5 h), 87% of the *Hin*dIII fragment 1 was digested into the two smaller fragments but no other fragments were detected.

The *Eco*RI fragment 1 (fig.4A) was extracted from the gel and redigested with *Hin*dIII (fig.4B) or *Hin*fI (fig.4C). About 25% of *Eco*RI fragment 1 was cleaved by *Hin*dIII and two fragments of sizes 38 and 55 base-pairs were observed as was shown in fig.2B. *Hin*fI produced a fragment 72 base-pairs long. The complementary fragment 21 base-pairs long was hardly detectable because of its small size. About 20% of *Eco*RI fragment 1 remained intact after 0.1 μg *Eco*RI fragment 1 was digested with 10 units *Hin*fI for 5 h at 37°C. Whole Novikoff rat hepatoma DNA

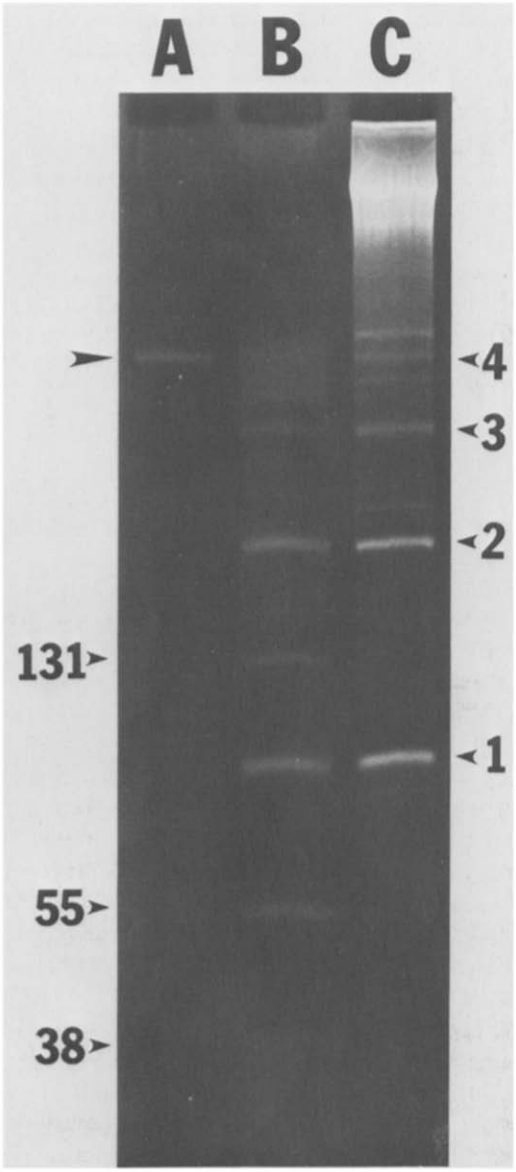

Fig.2. Purified Novikoff rat DNA *Hin*dIII fragment 1 (A) which appeared at *Eco*RI tetramer position, *Hin*dIII fragment 1 redigested with *Eco*RI (B), and whole Novikoff rat DNA digested with *Eco*RI (C). Arrows at the right show the positions of *Eco*RI monomer, dimer, trimer and tetramer. Numbers at the left show the lengths in base-pairs of the fragments in column B. Electrophoresis was performed for 1 h at 300 V in 5% acrylamide gel.

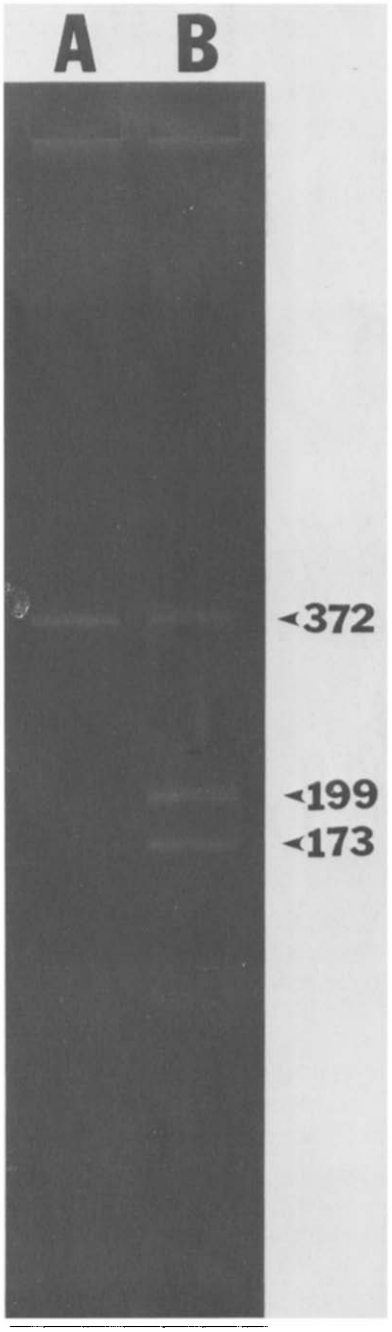

Fig.3. Intact *Hin*dIII fragment 1 (A) and *Hin*dIII fragment 1 redigested with *Hae*III (B). *Hin*dIII fragment 1 (~0.05 μg) was incubated with 1 unit *Hae*III for 30 min at 37°C. Electrophoresis was performed at 300 V for 1 h in 4% acrylamide gel. Numbers at the right show the lengths in base-pairs of the fragments.

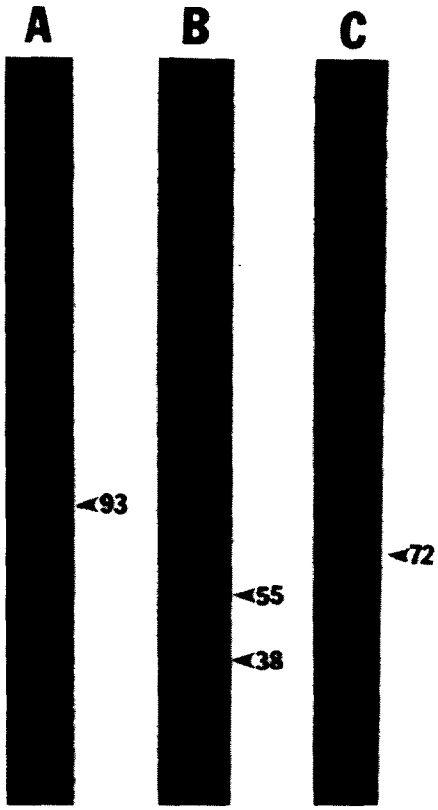

Fig.4. Novikoff rat DNA *Eco*RI fragment 1 (A), *Eco*RI fragment 1 redigested with either *Hind*III (B), or *Hin*fI (C). Fragments were analyzed in 5% acrylamide gel. Arrows point to the fragments and the numbers show the lengths in base-pairs of the fragments.

was also digested with *Hin*fI and monomer fragments 93 base-pairs long were found together with dimers and trimers in the digest (data not shown).

The cleavage maps of the highly repeated component of Novikoff rat DNA are summarized in fig.5.

The ^{32}P-labeled Novikoff rat DNA was cleaved by *Hind*III and the fragments were fractionated by gel electrophoresis. *Hind*III band 1 was cut out and radioactivity in the band was determined. It was found that 2.9% of the total DNA was in *Hind*III band 1. The repetition frequency of the fragment was calculated to be 5×10^5 times/genome, assuming that a Novikoff rat cell contains 7 pg DNA [9]. The GC content of *Hind*III fragment 1 was determined to be 68% by Whatman 3MM paper electrophoresis [10] after

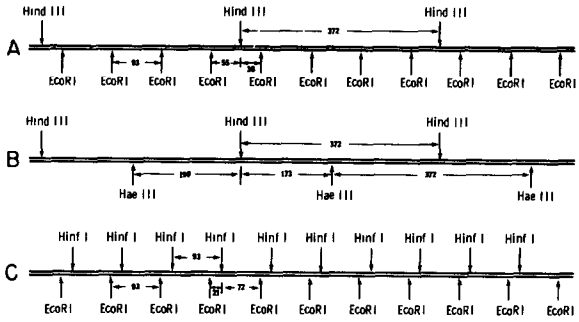

Fig.5. A cleavage map of the highly repeated component of Novikoff rat DNA by *Eco*RI and *Hind*III restriction endonucleases (A), by *Hind*III and *Hae*III restriction endonucleases (B), or by *Eco*RI and *Hin*fI restriction endonucleases (C). Numbers indicate the lengths in base-pairs of the fragments. Some cleavage sites are insensitive to the restriction endonucleases and multimers are formed.

^{32}P-labeled *Hind*III fragment 1 was digested with DNase I and venom phosphodiesterase.

The present study indicates that the *Eco*RI cleavage sites (GAATTC) and the *Hin*fI cleavage sites (GANTC) are repeated about 2×10^6 times/genome at multimers of a 93 base-pair unit, but the *Hind*III cleavage sites (AAGCTT) and the *Hae*III cleavage sites (GGCC) are repeated about 5×10^5 times at multimers of the 372 base-pair unit in the GC-rich (68%) component of Novikoff rat DNA. This might suggest that the GC-rich 93 base-pair unit DNA fragment was multiplied to about 3% of the total DNA at some stage of the evolution of Novikoff rat hepatoma cells and at a later stage of the evolution some modifications of the nucleotide sequences occurred making, some *Eco*RI or *Hin*fI sites insensitive to the enzymes and introducing *Hind*III- or *Hae*III-sensitive sites once in every 4 repeats. Another possibility is considered that the 93 base-pair unit was initially multiplied only to tetramer size and sequence changes occurred which introduced *Hind*III- and *Hae*III-sensitive sites; subsequently this 372 base-pair unit may have been multiplied to 3% of the genome.

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