LOCALIZATION OF HIGHLY REPEATED *Hin*dIII FRAGMENTS OF NOVIKOFF RAT DNA TO THE NUCLEOLUS

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1 Introduction

Novikoff rat ascites hepatoma cells have large nucleoli compared to normal rat cells and nucleoli from these hepatoma cells have been isolated in high yield and purity [1] The nucleolus is known to be the site of ribosomal RNA synthesis and the ribosomal RNA genes are repeated 100–500-times/diploid genome [1,2] It has been estimated that the DNA content/nucleolus is 1 3 pg and that the DNA content/cell is 8 pg for Novikoff rat cells [1] Ribosomal DNA comprised 0 008% of the total DNA [3] and ~0 05% of nucleolar DNA The remaining 99 95% of nucleolar DNA has not yet been characterized

Restriction endonuclease digestion followed by gel electrophoresis has been used to detect repeated sequences in DNA from several mammalian species [4–6] Novikoff rat hepatoma DNA was analyzed by gel electrophoresis after *Eco*RI restriction endonuclease digestion in this laboratory Small *Eco*RI fragments of Novikoff rat DNA were examined [7] by acrylamide gel electrophoresis and large *Eco*RI fragments of Novikoff rat DNA [8] by agarose gel electrophoresis The largest *Eco*RI fragment, fragment A, was reported [8] localized to the nucleolus Here, *Hin*dIII digestion patterns of total nuclear DNA, nucleolar DNA, and extranucleolar nucleoplasmic DNA have been studied by agarose and acrylamide gel electrophoresis From quantitative analysis of the amount of the smallest *Hin*dIII DNA fragment in various fractions of the nucleus, this fragment has been found to be localized to the nucleolus

2 Materials and methods

Novikoff hepatoma ascites cells were maintained in male Holtzman rats for 6 days and the cells were labeled with ^{32}P for 24 h as in [9] Nucleoli and extranucleolar nucleoplasm, i e , the nucleus minus the nucleolus, were prepared by the sucrose–Ca^{2+} procedure [1,10] After sedimentation of nucleoli by centrifugation at 1200 × *g* for 20 min from sonicated nuclei, the supernatant contained the extranucleolar nucleoplasm The extranucleolar chromatin was collected by centrifugation at 20 000 × *g* for 30 min after 2 vol ethanol were added Nucleolar contamination in the extranucleolar chromatin was minimized by monitoring the sonication process by phase microscopy to ~80% nuclear disruption

DNA was prepared generally as in [11] To eliminate small DNA fragments in the extranucleolar nucleoplasm preparation, 6 cycles of DNA spooling were repeated by adding 2 vol ethanol and dissolving the spooled DNA in SSC (0 15 M NaCl, 0 015 M sodium citrate)

*Hin*dIII restriction endonuclease was purchased from Bethesda Research Labs (Rockville, MD) and DNA digestion with *Hin*dIII and agarose or acrylamide gel electrophoresis were carried out as in [7,9] The gel was stained with 1 μg/ml of ethidium bromide in water for 1 h and photographed with an ultraviolet light source using an ultraviolet-cut filter The films were scanned by a Transidyne 2955 densitometer (Ann Arbor, MI) at 600 nm with a 0 1 mm slit width and areas under peaks were cut out and weighed Bands

of ^{32}P-labeled DNA fragments were cut out of the gel with a razor blade under ultraviolet light and the ^{32}P in the DNA in the gel was determined with a scintillation counter.

^{32}P-labeled DNA fragments, recovered from the gel as in [7], were dissolved in 10 μl 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 2 units pancreatic DNase I were added. After incubation for 1 h at 37°C, 0.001 unit venom phosphodiesterase was added to the mixture and the incubation was continued for another 1 h at 37°C. The digest was applied to Whatman 3 MM paper pre-sprayed with 5% acetic acid adjusted to pH 3.5 with ammonium hydroxide. Electrophoresis was performed at 2 kV for 1.5 h and mononucleotides were detected by autoradiography.

3. Results and discussion

Total nuclear DNA and nucleolar DNA of Novikoff rat cells were analyzed by 1% agarose gel electrophoresis before and after *Hin*dIII digestion (fig.1). Six major bands were observed after *Hin*dIII digestion of nucleolar DNA (fig.1D) and bands 1, 6 were also observed in the *Hin*dIII digest of total nuclear DNA (fig.1C). Total nuclear DNA, nucleolar DNA, and extranucleolar nucleoplasmic DNA were analyzed by 3.5% acrylamide gel electrophoresis after *Hin*dIII digestion (fig.2). Acrylamide gels resolve smaller DNA fragments better than agarose gels. Bands 1–5, shown by arrows, were observed in the *Hin*dIII digest of nucleolar DNA (fig.2B) and bands 1, 2 were also observed in the *Hin*dIII digest of total nuclear DNA (fig.2A).

The molecular weights of the fragments numbered in fig.1 were determined using the linear relationship between the logarithm of the molecular weights and the migration distances of the fragments produced by either *Hin*dIII or *Eco*RI and φX174 RF DNA fragments produced by *Hin*fI were used as size markers (see table 1). The *Hin*dIII fragment 1 of Novikoff rat DNA has the size of a tetramer of the *Eco*RI monomer

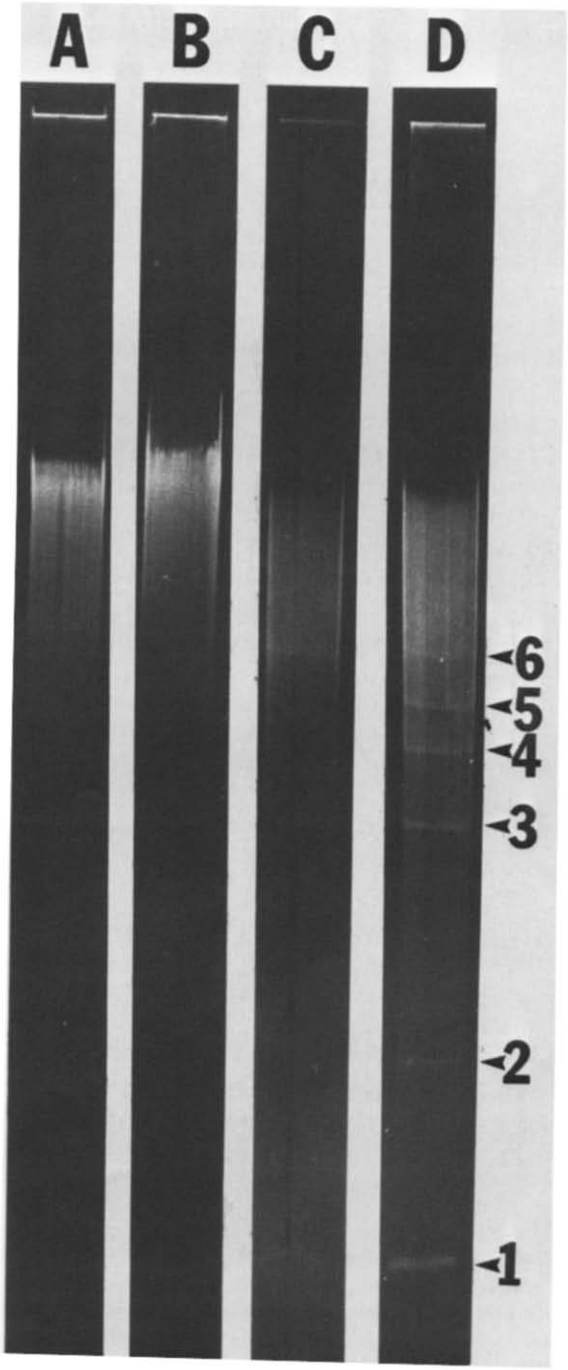

Fig.1. Agarose gel electrophoretic analysis of nuclear and nucleolar DNA before and after *Hin*dIII digestion: total nuclear DNA of Novikoff rat cells (A), nucleolar DNA of Novikoff rat cells (B), nuclear DNA digested with *Hin*dIII (C) and nucleolar DNA digested with *Hin*dIII (D). Electrophoresis was carried out at 200 V for 2 h at 19°C in a 1% agarose gel. Six major bands observed in the *Hin*dIII digest of nucleolar DNA are indicated by arrows and numbered according to their sizes.

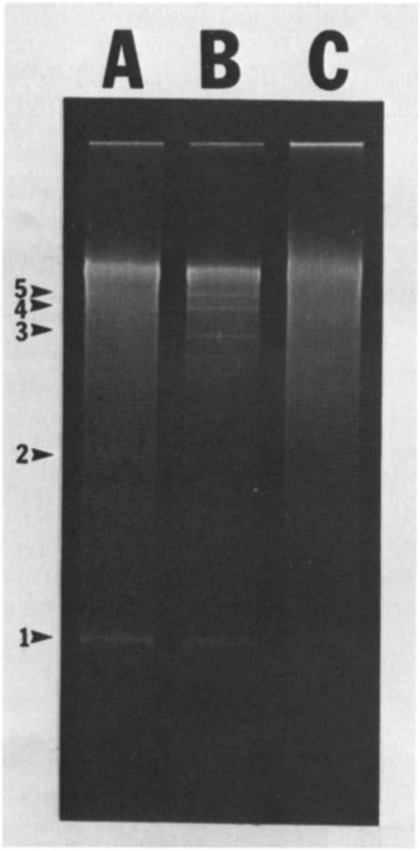

Fig 2 Acrylamide gel electrophoretic analysis of *Hin*dIII digests of 3 kinds of DNA preparations of Novikoff rat cells total nuclear DNA, 8 μg (A), nucleolar DNA, 1 3 μg (B) extranucleolar nucleoplasmic DNA, 6 7 μg (C) Electrophoresis was carried out at 300 V for 1 h at 19°C in a 3 5% acrylamide gel

fragment of Novikoff rat DNA, which has been shown to be 93 base-pairs long [7], and *Hin*dIII fragment 2 has the size of an octamer of the *Eco*RI monomer

Table 1
Sizes of highly repetitive *Hin*dIII fragments of Novikoff rat DNA

Fragment no	1	2	3	4	5	6
Mol wt (× 10^6)	0 24	0 48	1 1	1 6	1 9	2 5
Base-pair (× 10^3)	0 37	0 74	1 7	2 5	3.0	3 9

Fragments are shown in fig 1 The number of base-pairs in each fragment was calculated assuming that 1 base-pair is 640 mol wt

fragment *Hin*dIII fragment 2 has the size of a dimer of the *Hin*dIII fragment 1

All of the *Hin*dIII bands observed in nucleolar DNA must be present in total nuclear DNA Bands 3–5 were observed in nucleolar DNA but not in total nuclear DNA (fig 1,2) because these fragments are enriched in the nucleolus The background levels in fig 1,2 are high near bands 3–5 and these bands are buried under the background in the *Hin*dIII digest of total nuclear DNA *Hin*dIII fragment 6 appears to be very highly repeated (~3 × 10^4 times/genome) and band 6 can be observed above the background in the *Hin*dIII digest of total nuclear DNA (fig 1C)

Figure 2C shows a *Hin*dIII digestion pattern of extranucleolar nucleoplasmic DNA A faint band of fragment 1 was found but no other bands were observed In fig 2, 8 μg total nuclear DNA, 1 3 μg nucleolar DNA, and 6 7 μg extranucleolar nucleoplasmic DNA were loaded in each slot, which reflect the ratios of the amounts of these 3 kinds of DNA in the nucleus Figure 2 shows that fragments 1, 5 are highly localized to the nucleolus

The background level was low near band 1 and quantitative analysis was made of the DNA content of this band The average of 10 measurements of DNA in band 1 was 2 9% of the total nuclear DNA in the gel and the repetition frequency of fragment 1 was calculated to be 5 × 10^5 times/genome [7] In 4 ^{32}P experiments, band 1 was found to contain 14% of the nucleolar DNA in the gel (fig 2B), accordingly, 14/2 9 × 1 3/8 × 100 = 79% of *Hin*dIII fragment 1 DNA in the nucleus was found in the nucleolus It was also found that band 1 in fig 2C contained 0 61% of the extranucleolar nucleoplasmic DNA This was the average of 4 ^{32}P measurements Accordingly, 0 61/2 9 × 6 7/8 × 100 = 18% of *Hin*dIII fragment 1 DNA in the nucleus was found in the extranucleolar nucleoplasm In other studies, fig 2 was scanned by a densitometer and areas under peaks of *Hin*dIII fragment 1 were measured Three scanning measurements showed that 86% of *Hin*dIII fragment 1 was found in the nucleoli and 6% of *Hin*dIII fragment 1 was found in the extranucleolar nucleoplasm The results obtained by two different analyses are summarized in table 2

^{32}P-Labeled Novikoff rat DNA was digested with *Hin*dIII and fragment 1 was purified by gel electrophoresis The *Hin*dIII fragment 1 of ^{32}P-labeled Novikoff rat DNA was digested to mononucleotides with pan-

Table 2
Percent of *Hin*dIII fragment 1 DNA found in the nucleoli and extranucleolar nucleoplasm

	^{32}P measurements	Densitometer measurements
Nucleoli	79%	86%
Extranucleolar nucleoplasm	18%	6%

creatic DNase I and venom phosphodiesterase and the nucleotide composition was determined by paper electrophoresis (fig.3). *Hin*dIII fragment 1 was found to be GC-rich (68%) compared to the bulk rat DNA which contains 40–45% GC [12].

Here, 2 bands of highly repeated DNA fragments were found in the *Hin*dIII digest of total nuclear DNA of Novikoff rat cells by 1% agarose gel electrophoresis. Both of these bands were also found in nucleolar DNA and 4 other bands were also found in nucleolar DNA. Analyses of nuclear DNA, nucleolar DNA, and extranucleolar nucleoplasmic DNA digested with *Hin*dIII by 3.5% acrylamide gel electrophoresis showed that the highly repeated fragments 1–5 are localized to the nucleolus and about 80% of fragment 1 which is 68% GC-rich was localized to the nucleolus.

Two highly repeated large *Eco*RI fragments were found [8] in total nuclear DNA by agarose gel electrophoresis. These 2 fragments were also found in nucleolar DNA and another fragment of 3.6×10^6 mol. wt was also found in the nucleolus. So far, all of the highly repeated fragments found in total nuclear DNA have also been found in nucleolar DNA and nucleolar DNA contains some highly localized repeated DNA. It is well known that the nucleolus contains middle repetitive (100–500 copies/diploid genome) ribosomal DNA [1]). The findings here raise questions as to the reason for the enrichment of highly repetitive fragments in the nucleolus. Because of its high frequency of appearance in the cell and its small size, it is difficult to assign any specific functional role to *Hin*dIII fragment 1 DNA; it may be speculated that the DNA may perform some structural role in the nucleolus.

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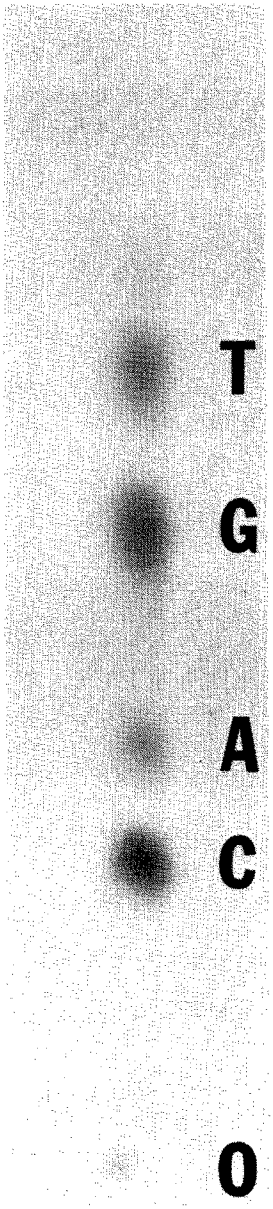

Fig.3. Nucleotide compositional analysis of *Hin*dIII fragment 1 of Novikoff rat DNA by pH 3.5 3MM paper electrophoresis. 'O' shows the origin of the electrophoresis.

References

[1] Busch, H and Smetana, K (1970) in The Nucleolus, pp 1–575, Academic Press, New York
[2] Attardi, G and Amaldi, F (1970) Ann Rev Biochem 39, 183–226
[3] Attardi, G, Huang, P C and Kabat, S (1965) Proc Natl Acad Sci USA 54, 185–192
[4] Philippsen, P, Streick, R E and Zachau, H G (1974) Eur J Biochem 45, 479–488
[5] Southern, E M (1975) J Mol Biol 94, 51–69
[6] Maio, J J, Brown, F L and Musich, P R (1977) J Mol Biol 117, 637–655
[7] Fuke, M and Busch, H (1979) FEBS Lett 99, 136–140
[8] Parker, D L, Rothblum, L I and Busch, H (1979) Cancer Res in press
[9] Fuke, M and Busch, H (1975) J Mol Biol 99, 277–281
[10] Davis, F M, Busch, R K, Yeoman, L C and Busch, H (1978) Cancer Res 38, 1906–1915
[11] Sitz, T O, Nazar, R N, Spohn, W H and Busch, H (1973) Cancer Res 33, 3312–3318
[12] Fasman, G D (1976) Handbook of Biochemistry and Molecular Biology, Nucleic Acids, vol 2, pp 1–896 CRC Press, Cleveland, OH