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Cloning and Characterization of a Highly Reiterated 5.8-Kilobase Pair Nucleolar *Eco*RI DNA Fragment Found in Novikoff Hepatoma Ascites Cells†

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ABSTRACT: The DNA of Novikoff hepatoma ascites cells was found to contain a 3.6-megadalton *Eco*RI restriction fragment, referred to as *Eco*RI fragment A (Parker et al., 1979). C_0t analyses demonstrated an enrichment of fragment A sequences in Novikoff hepatoma genome relative to normal rat liver DNA. This fragment was cloned in λ gtWES to determine its molecular structure and sequence organization. The DNA from a positive clone was labeled by nick translation and hybridized to a Southern blot of *Eco*RI digested Novikoff DNA. Distinct hybrids formed with the region corresponding to fragment A. The greater degree of hybridization to the nucleolar fraction suggested a nucleolar enrichment of fragment A. Fragment A has a *Pst*I site approximately 300 base pairs from one terminus which was used to generate mono-5′-^{32}P-labeled fragments. The larger *Pst*I subfragment, 5500 base pairs, labeled at a single terminus, was used to evolve a restriction enzyme map. The 300 base pair fragment was partially sequenced, revealing the presence of a repetitive sequence "island", $TT(GTCT)_8(GAAT)_5G$-. C_0t analysis, utilizing the purified clone as a probe, confirmed the enrichment of fragment A sequences in the tumor relative to the normal rat liver control.

When DNA prepared from the nucleoli of Novikoff hepatoma ascites cells was digested with any of a number of restriction endonucleases and subjected to agarose gel electrophoresis, various dense ethidium bromide staining bands were found which ranged in size from approximately 1000 base pairs to 7500 base pairs (Parker et al., 1979; Fuke & Busch, 1979). When similar digestions were carried out with whole nuclear DNA prepared from Novikoff hepatoma cells, a distinct subset of these bands was not seen. When the DNA of normal rat liver nuclei or nucleoli was digested similarly, this subset of bands was not found (Parker et al., 1979; unpublished observations).

Digestion of Novikoff nucleolar DNA with the restriction endonuclease *Eco*RI generated three ethidium bromide staining bands (in the molecular weight range greater than 1000 base pairs) (Parker et al., 1979). These fragments, referred to as fragments A, B, and C, contained 5800, 2100, and 1400 base pairs, respectively. Fragment A was not found in similar digests of either normal rat liver nuclear or nucleolar DNA whereas fragments B and C were seen. Comparative *Eco*RI digestions of Novikoff hepatoma nucleolar and nuclear DNA suggested that fragment A was localized predominately in the nucleolus. Nucleoli prepared by several methods (Busch & Smetana, 1970) all contained relatively similar amounts of fragment A. Previous hybridization studies, utilizing a radiolabeled fragment A probe, demonstrated that fragment A was amplified in the Novikoff hepatoma cells in comparison to normal rat liver cells. Fragment A comprised approximately 5% of nucleolar DNA and did not cross-hybridize with rRNA or any known rDNA fragments. Its reiteration number, approximately 6000, as well as its large size suggested that it was not part of the rDNA repeats which number 250–500 (Parker et al., 1979).

In the present study, we report on the successful cloning of the *Eco*RI fragment A in λ gtWES. Using this clone as a hybridization probe we were able to confirm a nucleolar

† From the Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030. *Received April 29, 1980.* These studies were supported by Cancer Center Grant CA-10893, P1, awarded by the National Cancer Institute, Department of Health, Education and Welfare, National Institute of General Medical Sciences Grant BRSG-79-P8, and the Bristol-Myers Fund.

‡ Predoctoral trainee of Houston Pharmacological Center, Grant GMO-7405-03.

0006-2960/81/0420-0762$01.00/0

localization of the fragment and have determined a more accurate estimation of the amplification factor of the fragment in the Novikoff hepatoma. Southern images using the cloned probe have been used to identify related but not identical species found in normal rat liver. In addition, a restriction enzyme map of the fragment is reported, along with partial sequence data.

Materials and Methods

Preparation of DNA, Restriction Endonuclease Digestions, and Agarose Gel Electrophoresis. All DNA samples were isolated and prepared as described previously (Parker et al., 1979). Restriction endonuclease digestions were performed as recommended by the supplier. Agarose gel electrophoresis was carried out in a buffer containing 40 mM Tris, pH 7.8, 20 mM sodium acetate, and 2 mM EDTA for 16 h at 35 V.

Preparation of λ gtWES Arms, Ligation of DNA Fragments, and Transfection of E. coli. The λ gtWES cloning kit was generously provided by Dr. P. Leder of NIH. A stock of λ gtWES λ B was first prepared as described by Tiemeier et al. (1977). Phage λ arms were prepared by digestion of λ gtWES λ B DNA with *Eco*RI, followed by sucrose density gradient centrifugation. *Eco*RI digested Novikoff nucleolar DNA was ligated together with the λ arms and used to transfect *E. coli* LE392 cells as described by Tiemeier et al. (1977).

Screening of Clones. Recombinant clones were screened by in situ hybridization by the method of Kramer et al. (1976). The fragment A probe used for screening was gel isolated (Parker et al., 1979) from *Eco*RI digestions of total Novikoff nucleolar DNA. The probes were nick-translated by the method of Mackey et al. (1977). Minilysates of positive clones were grown, and DNA from each clone was isolated (Blattner et al., 1972). These DNAs were screened by restriction enzyme digestion and Southern blot hybridization (Wahl et al., 1979).

Sequencing of a Fragment A Subfragment and Placement of Restriction Endonuclease Cleavage Sites within Fragment A. In a typical protocol, clone λ A_3 DNA was first digested with *Eco*RI and the resulting 5′ termini were ^{32}P labeled (Maxam & Gilbert, 1977). The labeled fragment A was isolated by agarose gel electrophoresis. Fragments labeled at a single terminus were obtained by redigestion of the fragment with *Pst*I enzyme, which cleaves approximately 300 base pairs from one terminus. DNA sequencing was performed by the Maxam–Gilbert technique using the *G, A + G, C and C + T* reactions (1980).

Partial restriction endonuclease digestions of the large mono-5′-labeled fragment were used to determine the correct placement of the various subfragments and thus place the restriction sites within the cloned fragment A (Smith & Birnstiel, 1976).

C_0t Analysis of Cloned Fragment A. Cloned fragment A was gel isolated from *Eco*RI digested total λ A_3 clone DNA. The fragment was nick translated and hybridized with various DNA samples as drivers. Control hybridizations were performed using *E. coli* DNA as a "mock" driver. All driver DNAs were sheared to a uniform size of 200–400 base pairs in a French pressure cell. The probe concentration was kept below 3 ng/mL and the hybridizations were completed in 2–3 h. The hybridization reactions were performed in 20-μL aliquots, overlayed with 30 μL of paraffin oil in 1.5-mL Eppendorf tubes. The hybridizations were done at 62 °C in 0.18 M NaCl, 10 mM Tris, pH 7.4, and 2 mM EDTA and stopped by immersion in dry ice–acetone. The extent of hybridization was assayed by S1 nuclease digestion (Samal & Bekhor, 1979), and radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

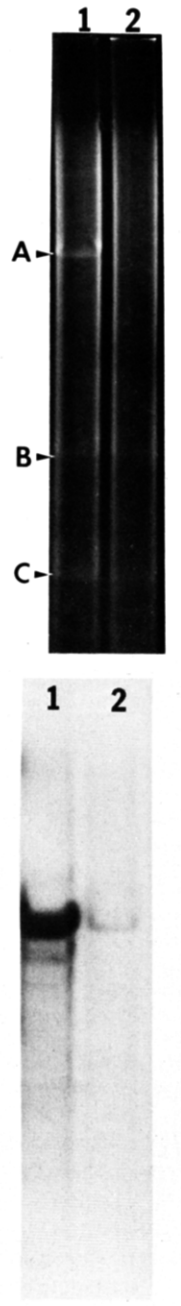

FIGURE 1: (A) *Eco*RI digestion pattern of (1) Novikoff nucleolar DNA and (2) Novikoff nuclear DNA: 3 μg of each sample was electrophoresed in a 1% agarose gel for 16 h at 40 V. The sizes for fragments A, B, and C are 5.8, 2.1, and 1.4 kilobase pairs, respectively. (B) Southern blot hybridization of gel shown in Figure 1A to ^{32}P-nick-translated clone λ A_3. Each channel represents 3 μg of either Novikoff nucleolar (1) or Novikoff nuclear DNA (2).

Results

The initial step used to characterize the structure of fragment A was to clone it in λ gtWES by using *Eco*RI digested Novikoff nucleolar DNA (Figure 1a). A positive clone, designated λ A_3, was selected for by in situ hybridization to a probe enriched in fragment A. The clone was radiolabeled and hybridized to a Southern blot (Southern, 1975b; Wahl et al., 1979) of Figure 1A (Figure 1B). A very strong signal was observed in the region corresponding to fragment A in the Novikoff nucleolar track (lane 1) when compared to the Novikoff nuclear track (lane 2), suggesting a nucleolar en-

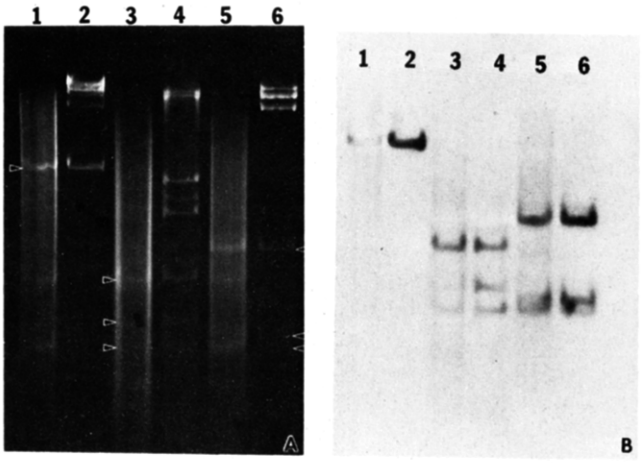

FIGURE 2: (A) Comparative restriction enzyme digestion of clone λ A_3 (lanes 2, 4, and 6) and Novikoff nucleolar DNA (lanes 1, 3, and 5). Enzymes are as follows: (1 and 2) *Eco*RI, (3 and 4) *Hin*dIII, (5 and 6) *Pst*I. Pointers indicate fragment A sequences. (B) Southern blot of gel shown in (A) hybridized to ^{32}P-nick-translated fragment A isolated from clone λ A_3.

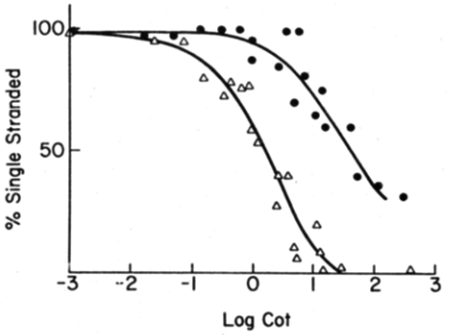

FIGURE 3: C_0t curve analysis of a nick translated fragment A probe from the λ A_3 clone hybridized with an excess of Novikoff hepatoma nuclear DNA (Δ) or normal rat liver nuclear DNA (●) as drivers. A consistent snapback of 35% was observed, independent of DNA concentration; 35% was subtracted from all points.

richment or localization. The presence of the fragment A insert in clone λ A_3 was further confirmed by comparative restriction endonuclease digestion and Southern blot hybridization with Novikoff nucleolar DNA (Figure 2).

The reassociation curves shown in Figure 3 confirm the amplification of fragment A sequences in the Novikoff hepatoma cell relative to normal rat liver cells using a cloned fragment A probe. The $C_0t_{1/2}$ values obtained for the reassociation of the fragment A probe with Novikoff hepatoma nuclear and normal rat liver nuclear DNA, 1.1 and 45, respectively, indicated an approximately 40-fold enrichment of fragment A sequences in the tumor relative to normal rat liver nuclei. The fragment A probe did not hybridize to completion when normal rat liver nuclear DNA was used as driver.

Two hybridization experiments were conducted to determine the nature of the fragment A sequence in the normal rat liver cells and its relationship, if any, to fragment A from Novikoff hepatoma. Figure 4 is a Southern blot hybridization comparing *Eco*RI digested Novikoff nuclear and normal rat liver nuclear DNAs after hybridization with nick-translated λ A_3. In the center lane, containing Novikoff hepatoma nuclear DNA (Figure 4, lane 2), strong hybridization was detected in the area corresponding to fragment A (arrow) and to a lesser extent with two smaller fragments (pointers). The sizes of these two fragments were 4.1 and 4.5 kbp. In the lanes containing normal rat liver DNA (Figure 4, lanes 1 and 3) hybridization was detected only to the two smaller bands.

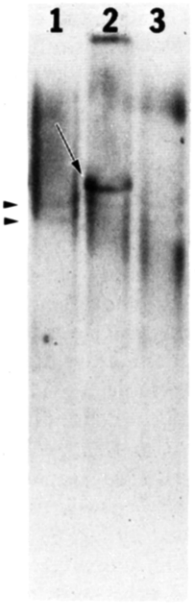

FIGURE 4: Southern blot hybridization of nick-translated fragment A (clone A_3) to 2 μg of *Eco*RI digested Novikoff hepatoma nuclear DNA (lane 2) and either 4 or 2 μg of *Eco*RI digested normal rat liver nuclear DNA (lanes 1 and 3, respectively).

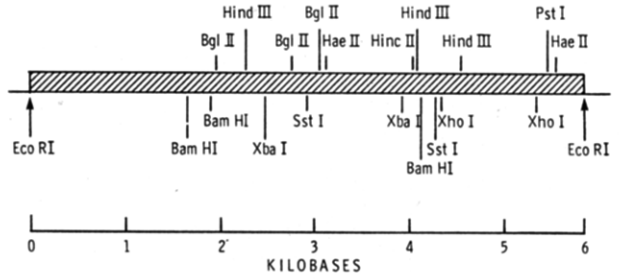

FIGURE 5: Composite restriction map of fragment A.

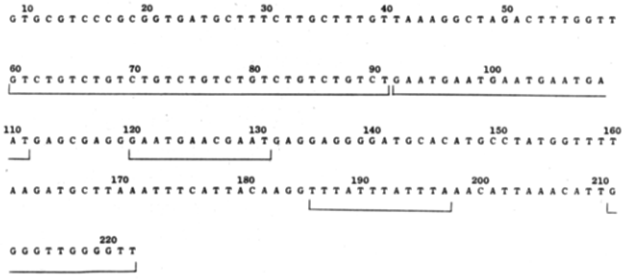

FIGURE 6: Partial sequence of small *Pst*I subfragment of fragment A: base pairs 9–212. Repetitive regions are underlined.

A detailed map of this fragment A insert was evolved (Figure 5) by partial restriction endonuclease digestion of mono-5′-^{32}P-labeled fragment A. Fragment sizes were confirmed by total restriction enzyme digestion of the isolated fragment A insert. Previous hybridization studies using fragment A as probe (Parker et al., 1979) had suggested the presence of snapback regions comprising up to 35% of the fragment, but the restriction enzyme map did not reveal lower order repeating units. Therefore, direct sequence analysis was initiated utilizing the small (300 base pair) *Pst*I subfragment of fragment A.

A partial sequence of the *Pst*I subfragment is shown in Figure 6. Repetitive "islands" are present within the portion that was sequenced. One repeat, extending from base pair no. 60 to no. 111 consists of eight sets of GTCT followed by five sets of GAAT. Smaller repeats can be seen in base pairs

120–130 and 186–197. Another large repetitive island has been noted approximately 100 base pairs further into the structure. It is not known if these repetitive areas are a generalized phenomenon throughout the fragment A sequence.

Discussion

The presence of a repetitive class of DNA in the nuclei of most eukaryotes has led to much speculation in recent years as to their possible function (Appels & Peacock, 1978; John & Miklos, 1979; Skinner, 1977). A number of repetitive genes, for example, those genes coding for rRNA and in some cases the histone genes, exhibit "dosage repetition" (Long & Dawid, 1980). These repeated genes have presumably arisen due to a large cellular demand for their products at some particular time. Similarly, a cell population placed in a sufficiently demanding environment will be selected against according to its ability to survice in that environment. This phenomenon has been shown in some cases to result in the selection of cell variants containing a specific amplified gene (Schimke et al., 1978; Miller et al., 1979).

Another class of repetitive DNA, often referred to as "satellite" or "highly repetitive" DNA, is characterized by a repetition number of between 1000 and 100000 copies per cell and small, often tandem, repeating units (John & Miklos, 1979). In certain instances, these highly repetitive elements have been found associated with centromeric and heterochromatic staining regions of chromosomes (Pardue & Gall, 1970; Musich & Skinner, 1972; Kurnit et al., 1973). Transcription products have also been detected (Harel et al., 1968; Cohen et al., 1973; Varley et al., 1980), but it appears unlikely that these sequences code for proteins. Studies by Scheller et al. (1978) suggested that certain highly repetitive DNA sequences are transcribed into RNAs that are sequestered within the nucleus.

It is unclear to which, if either, of these classifications fragment A belongs. The $C_0t_{1/2}$ value obtained for the fragment in Novikoff hepatoma nuclei, 1.1, suggests that the sequences are repeated between 1000 and 10000 times per cell. The fact that fragment A sequences are enriched in Novikoff hepatoma relative to the control liver suggests a selection-related amplification, perhaps associated with the use of (dimethylamino)azobenzene, a chemical carcinogen, for the original tumor induction (Novikoff, 1957). However, no co-migrating species of fragment A, as defined by *Eco*RI cuts, were detected in the normal rat liver. This might indicate that the two smaller species detected are parent or familial genes (Long & Dawid, 1980).

Because of its size, 5800 base pairs, the fragment A repeat appears to be more complex than highly repetitive DNAs that have been previously investigated. Preliminary evidence (data not shown) suggests that the 5800 base pair fragment A is actually a subfragment of a 7300 base pair repeat structure that is arranged in a tandem array. The scattered arrangement of restriction sites within this fragment argues against the prresence of any consistent lower order repeat unit as found in rat satellite I (Fuke & Busch, 1979; Pech et al., 1979). The tetranucleotide repeats reported here suggest that there may be "islands" of lower order repeats which were not observed after restriction enzyme digestion. The $G(A)_nTG$ sequence, where $n = 1$–5, which comprises almost 50% of mouse satellite (Southern, 1975a; Biro et al., 1975; Horz & Zachau, 1977) was present in the region of fragment A which was sequenced.

Currently, we are attempting to determine the genomic structure of the fragment A gene and whether this gene is transcribed. Also of interest is the relationship of fragment A to the fragment A related sequences found in the normal rat liver cells. Such studies may provide answers pertaining to the relationship of fragment A to the increased growth rate of the Novikoff hepatoma.

Acknowledgments

We wish to thank R. K. Busch for the injection of rats with tumor cells and Dr. J. Reiners for his many helpful suggestions and comments.

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