SECONDARY STRUCTURE OF NASCENT DNA OF EHRLICH ASCITES TUMOR CELLS OBTAINED BY NITROCELLULOSE COLUMN CHROMATOGRAPHY

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SUMMARY: Pulse labeled DNA was isolated from EHRLICH ascites cells using different methods. Depending on the isolation procedure, the nascent DNA separated from the bulk DNA by nitrocellulose column chromatography was either entirely double stranded or contained single stranded constituents. This seems to be due to the destabilized state of the nascent DNA within a living cell causing the partial conversion of newly replicated DNA to the single stranded form when certain DNA isolation methods are applied. It is suggested that the nascent DNA separated by nitrocellulose chromatography is normally double stranded.

In the last years contradictory results have been published on the question whether the newly replicated DNA of mammalian cells has a single or a double stranded secondary structure (1-6). The investigations of Habener et al. (7) could resolve these discrepancies for the most part. These authors demonstrated that, depending on the conditions of DNA extraction, the newly synthesized DNA of HeLa cells could be isolated in either single or double stranded form. Their results suggested that a portion of the newly replicated DNA is present in the cell in a destabilized state which is characterized by a strongly increased susceptibility to denaturation during the isolation procedure. Recently, we published a method which permits the preparative separation of the nascent DNA exhibiting an increased affinity to nitrocellulose from the bulk DNA of mammalian cells (6). In order to avoid a conversion of the destabilized regions of the nascent DNA to a single stranded form, we chose our DNA isolation procedure taking into account the results of Habener et al. (7). The purified nitrocellulose binding DNA was shown to be completely double stranded. Thus, single strandedness could not be the reason for the nitrocellulose affinity of this DNA. Lately, Sato et al. (8) reported this nascent DNA frac-

tion to be single stranded as judged by CsCl centrifugation and hydroxyapatite chromatography respectively. These authors followed almost completely all conditions given by us for DNA labeling and the chromatographic separation of the nascent DNA (6), but used a different method for the preparation of the whole DNA. Especially the first steps of this method resemble the "method I" of Habener et al. (7) which was most effective in extracting the newly replicated DNA in a single stranded form. We suspected that the different secondary structures of the separated nascent DNA found by Sato et al. (8) and us (6) respectively were due to the DNA extraction procedure. In a series of experiments we tested this hypothesis and could demonstrate that the nascent DNA separated by nitrocellulose column chromatography exhibits some single stranded character when the first steps of the method used for extraction of the whole cell DNA are - in the sense of Habener et al. (7) - suitable to convert the newly formed pieces to a single stranded form.

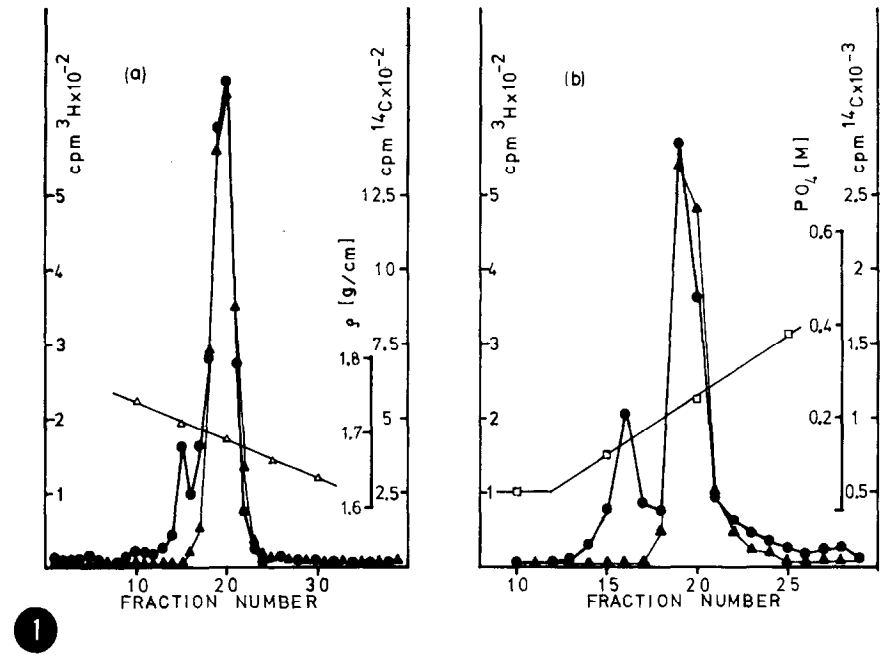
MATERIALS AND METHODS: The sources of all materials, the EHRLICH ascites cells, and the DNA labeling procedures used are described previously (6). Cells from three mice were mixed, labeled in vitro for 1 min with 100 uC/ml of ^{3}H-thymidine, washed with HANKS solution at $0^{\circ}C$, and divided into three portions. The first portion was suspended in water at a concentration of 5×10^{6} cells/ml, mixed with the same volume of 2 % SDS in 2 mM EDTA, and incubated for 3 hours at $37^{\circ}C$. After addition of an equal volume of 4 M NaCl the mixture was centrifuged for 20 min at 10 000 x g. The supernatant was extensively dialyzed against 0.05 M Tris, 0.4 M NaCl, 1 mM EDTA, pH 6.75 (TSE-buffer), and designated preparation A. The second portion was processed in the same way as the first one, but the supernatant was dialyzed against 0.14 M NaCl, 0.05 M Na-Citrate, pH 7.4 until free of SDS. Then it was mixed at room temperature with 1/3 vol. of phenol containing 10 % 1 mM EDTA and adjusted to pH 9 by adding saturated KOH solution (glass electrode). The mixture was shaken at room temperature for 40 min and then centrifuged at 12 000 x g for 2o min. The aqueous phase was carefully removed, dialyzed

against TSE-buffer until free of phenol, and designated preparation B. The remaining third of the washed cells was subjected to the DNA extraction procedure originally described in (9) and also used by us to prepare the starting material for the separation of nascent DNA by nitrocellulose column chromatography (6). But, in the present case, the extraction with ether and the precipitation of the DNA with ethanol were omitted. Instead, the aqueous phase from the second phenol extraction was dialyzed against TSE-buffer until free of phenol. The resulting solution containing over 95 % of the acid insoluble radioactivity was designated preparation C. Aliquots of all three preparations were chromatographed on 2 ml nitrocellulose columns as described earlier (6). The nascent DNA fractions eluted by TSE-buffer containing 0.5 % SDS were mixed with longtime labeled ^{14}C-DNA freed from nitrocellulose binding components as described in (6). Each of these mixtures was divided into two parts, one of which was dialyzed against 0.15 M NaCl, 0.015 M Na_3-citrate (SSC) and the other against 0.04 M K-phosphate buffer (pH 6.8) until all samples were free of SDS. The samples dialyzed against SSC were mixed with CsCl to give a density of 1.69 g/cm^3 and a total volume of 8 ml and then centrifuged for 60 h at 25°C in the 50 Ti rotor at 45 000 rpm in the Beckman L2 65B centrifuge. 0.2 ml fractions were collected from the bottom of the tubes and analyzed for radioactivity and density as already described (6). The samples dialyzed against phosphate buffer were applied to 5 ml hydroxyapatite columns. The columns were washed with 10 ml of 0.04 M phosphate buffer and then eluted with a linear gradient from 0.04 to 0.5 M K-phosphate (pH 6.8). 1.5 ml fractions were collected and analyzed for radioactivity and phosphate content as described previously (6).

RESULTS AND DISCUSSION: In the experiments described above we isolated DNA from the same ^{3}H-thymidine pulsed EHRLICH ascites cells using three different methods. The first one (A) was the method of Sato et al. (8) which we tried to follow as exactly as possible. The third one (C) was the method used in our studies on the reasons for the increased affinity of newly synthesized DNA

to nitrocellulose (6). The second method (B) was a kind of hybrid between A and C, including all steps of A and the phenol treatment of C. The separation of the nitrocellulose binding nascent DNA and the analyses by isopycnic CsCl centrifugation and hydroxyapatite chromatography respectively were done with the material from the three different preparations simultaneously and in an identical manner.

The results are shown in the figs. 1 to 3. From the nitrocellulose column loaded with preparation C, in contrast to the other two, no single stranded nascent DNA is eluted by SDS. The presence of single stranded constituents in the SDS elutable fraction originating from preparation B indicates that the phenol treatment causes no preferential loss of these constituents. Also Habener et al. (7) could not find such a loss in any case. We conclude from this that method A converts the destabilized nascent DNA to a single stranded form. Habener et al. (7) suggest that this destabilized state is maintained by factors only operative in the intact nucleus, and that disruption of the nuclear structure in the presence of the deproteinizing reagents is the essential step in the preferential conversion of newly replicated DNA to the single stranded form. On the other hand, disruption of the nuclear structure before dissociating the nucleoprotein was most effective in preventing the conversion of replicating regions to a single stranded form. Preparation C includes disruption of the nuclei and washing of the nuclear fragments before deproteinisation, while in preparation A lysis of cells and nuclei occurs simultaneously with the dissociation of nucleoprotein at slightly elevated temperatures and lowered ionic strength. The destabilizing factors are perhaps a kind of unwinding proteins which are suspected to be involved in the replication process (10). Possibly they are loosely associated with the replicating chromatin fraction within a S-phase nucleus, easily separable from the nucleoprotein when the nuclear structure is broken. The varying amounts of the total nascent DNA which are preferentially convertible to the single stranded form in different cell types (7) might be caused by varying concentrations of such pro-



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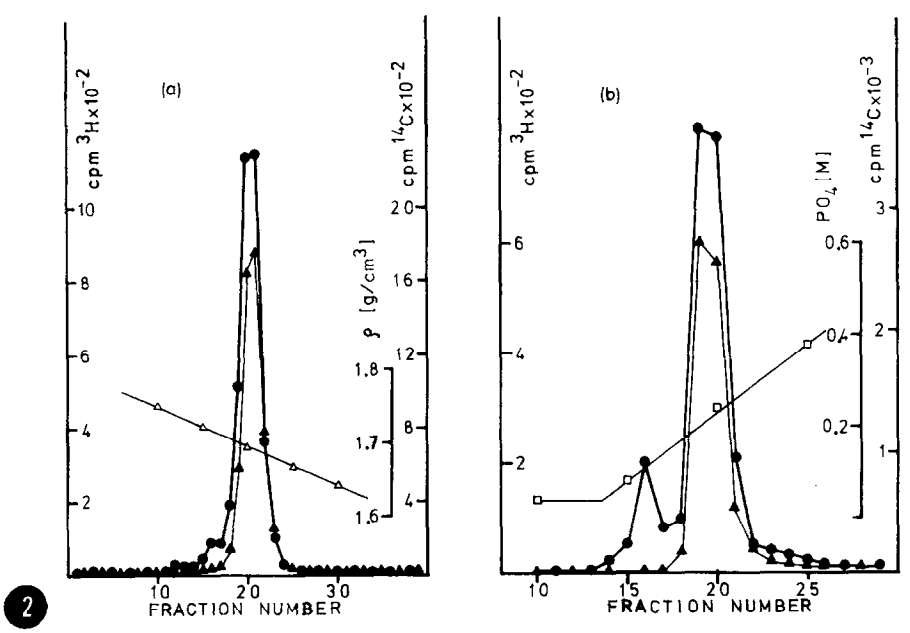
FIGS. 1 - 3: Isopycnic CsCl centrifugation (a) and hydroxyapatite chromatography (b) of the nascent DNA separated by nitrocellulose column chromatography from various DNA preparations. Each sample was mixed with ^{14}C bulk DNA as reference substance. In the case of fig. 3b additionally heat denaturated ^{14}C-DNA was added. Fig. 1: Preparation A, Fig. 2: Preparation B, Fig. 3: Preparation C. Symbols: ●—● ^{3}H pulse labeled nascent DNA separated by nitrocellulose column chromatography; ▲—▲ ^{14}C bulk DNA (in the case of fig. 3b not only native but also heat denaturated); △—△ density of the CsCl gradient; □—□ PO_4-concentration.



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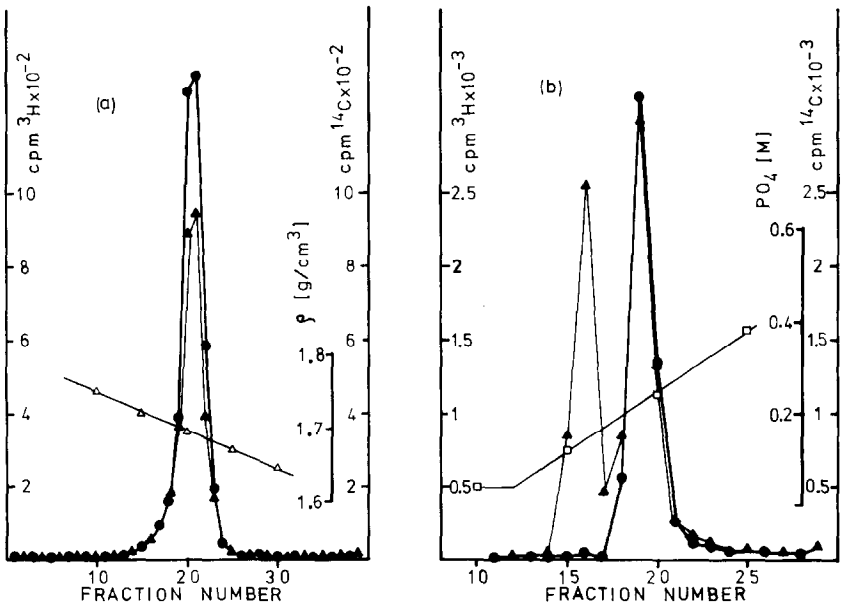

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teins in these cells. However, the nearly complete absence of constituents with the density of native DNA in the nitrocellulose binding fraction, as reported by Sato et al. (8), appears somewhat surprising. Perhaps this might be attributed to the dialysis against pure water performed by Sato et al. (8) with the nascent DNA which is reported to have a slightly increased susceptibility towards denaturation (9). It is also striking that Sato et al. (8) succeeded in eluting a DNA which behaves in neutral CsCl centrifugation as completely native with 0.01 M NaOH (pH nearly 12) from a nitrocellulose column. (Denaturation pH of EHRLICH ascites DNA is 11.5, of calf thymus DNA 11.77 (9,11).)

From the results reported here it is confirmed that newly replicated DNA has an increased affinity to nitrocellulose though lacking single stranded constituents. This is further established by the observation that nascent DNA with increased affinity to nitrocellulose can also be isolated from a DNA which has been eluted from hydroxyapatite at PO_4 concentrations higher than 0.18 M.

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