Analysis of Extrachromosomal DNA from Normal and Tumor Cells

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Two independent methods (pulse-electrophoresis of intact DNA isolated from agarose-encapsulated cells and differential extraction of intact DNA from Celite-immobilized nucleoproteins) yielded evidence of the presence of free (extrachromosomal) DNA in cells of various origin, both malignant and normal, cultured and isolated from animal tissues. Free DNA from all cell types studied form three discrete electrophoretic bands of 400, 250-300, and 50 thousand base pairs. According to the data on ^{3}H-thymidine incorporation, free and chromosomal DNA differ in their metabolic properties. By means of the polymerase chain reaction using c-myc and L-myc primers it is established that free DNAs of HT1080 cells contain structural genes. Application of the method of conformational polymorphism of single-strand fragments revealed their identity to the corresponding genes localized in the chromosomes.

Key Words: *extrachromosomal DNA; DNA pulse electrophoresis; DNA chromatography; DNA metabolism*

Extrachromosomal DNA plays an important role in the reorganization and functioning of the genome [10,12,14]. Amplified oncogenes in the form of extrachromosomal double microchromosomes are often found in human tumors and probably greatly contribute to the disease progression [12]. It is generally accepted that amplification is an exclusively rare event which can be detected only retrospectivelly, by selection and expansion of the corresponding cell clone(s) [14]. Besides the well-known amplified genes and circular DNAs, there seem to exist certain other, less investigated forms of extrachromosomal DNA [4,5].

Recently the possibility arose of a biochemical study of extrachromosomal elements under conditions minimizing the probability of intrachromosomal DNA fragmentation. Thus, the invention of the technique of pulse electrophoresis of DNA obtained from cells pre-encapsulated in agarose with subsequent proteinase K treatment made it possible to analyze giant molecules [13] and, in certain cases, to detect the presence of free (chromosome-unbound) forms of a size of 50-650 thousand base pairs (tbp) [2,8,15].

On the other hand, several years ago we elaborated a method of DNA differential extraction from immobilized nucleoproteins which also minimizes DNA fragmentation at the stage of deproteinization [6,7]. Using this method we succeeded in demonstrating the existence of a heterogeneous population of free molecules. As the latter were found in cell populations not having undergone any pre-selection, and thus could not be assigned to known types of amplified sequences, the further investigation of their nature was of obvious interest. Two alternative

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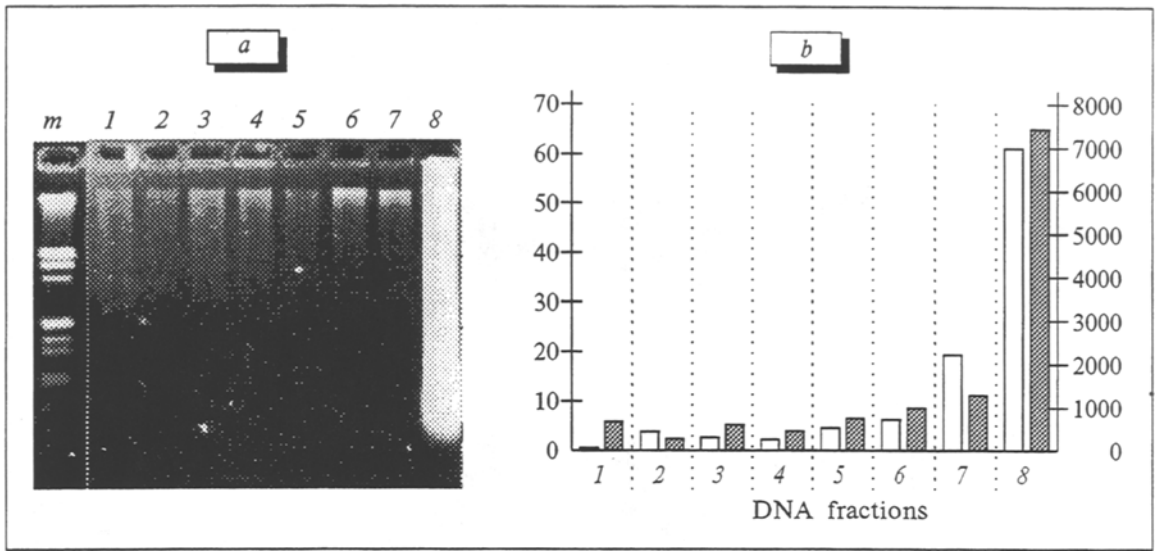

Fig. 1. Free JHF DNAs obtained by chromatography: electrophoretic (*a*) and quantitative (*b*) distribution. *a*) electrophoresis in 1% agarose gel of DNA fractions extracted from immobilized JHF lysates. Figures at top designate DNA fractions (fractions *1–7* represent free DNA, fraction *8* is chromosomal DNA tightly bound to the nuclear matrix); *m*: markers (DNA of λ phage restricted with HindIII and EcoRI). Diffuse spot in the low-molecular region of band *8*: cellular RNAs. Staining with ethidium bromide (0.5 μg/ml). *b*) quantitative determination of DNA (percentage of total amount, white bars) using spectrofluorometry of DNA complexes with Hoechst 33258 (ordinate, left axis). Specific radioactivity of JHF DNA fractions estimated after 1-day labeling of cells with ^{3}H-thymidine in a concentration of 0.25 μCi/ml (cpm/μg; shaded bars; ordinate, right axis).

viewpoints regarding the origin of these molecules can be postulated: a) the appearance of free molecules as a result of artificial fragmentation of the chromosomal DNA, and b) the occurrence of free DNA molecules *in vivo* as normal cellular components with unknown functions. In the present work we attempted, first, to isolate free DNAs from cells of various origin (normal and tumor cells, cultured and obtained from animal tissues) using two independent methods (pulse electrophoresis of DNA obtained from agarose-encapsulated cells and differential extraction of DNA from immobilized nucleoproteins), and, second, to compare the metabolic properties of free molecules and chromosomal DNA. The putative metabolic differences could serve as direct confirmation of a physiologically normal presence of free DNAs in the cell, thus ruling out their artificial origin.

MATERIALS AND METHODS

Experiments were carried out on cultured cell lines (HeLa; human lung fibroblastic fibrosarcoma HT1080; mouse neuroblastoma N1E115; SV40-transformed Jungarian hamster fibroblasts (JHF), line 4/21; human skin primary fibroblasts) and animal tissues (brain, liver, thymus, and spleen of mouse and rat; heart muscle of rabbit and dog). Free DNA was isolated by either of two methods described below.

Method 1: cells were washed in L buffered solution (0.1 M EDTA, pH 8; 0.01 M Tris-HCl, pH 7.6; 0.02 M NaCl), embedded in blocks of 0.5% low-melting agarose, and incubated in L buffer supplemented with 1 mg/ml proteinase K and 1% sarkosyl for 48 hours at 50°C [11]. DNA-containing blocks were placed on vertical 1% aga-

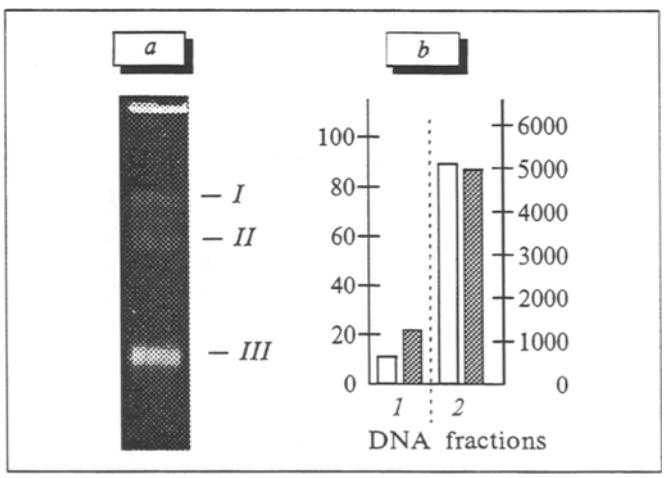

Fig. 2. Free JHF DNA obtained by RPGE method: electrophoretic (*a*) and quantitative (*b*) distribution. *a*) pulse electrophoresis in vertical 1% agarose gel of native DNA isolated from agarose-encapsulated cells. At the start (in agarose blocks): chromosomal DNA; bands in the gel: free DNAs (fractions *I*, *II*, and *III* of approximate size 400, 250–300, and 50 tbp, respectively). *b*) quantitative estimation (percentage of the total amount) of chromosomal DNA and summated free DNA (ordinate, left axis, white bars). Specific DNA radioactivity, cpm/μg (ordinate, right axis, shaded bars). Conditions of JHF labeling – see legend to Fig. 1.

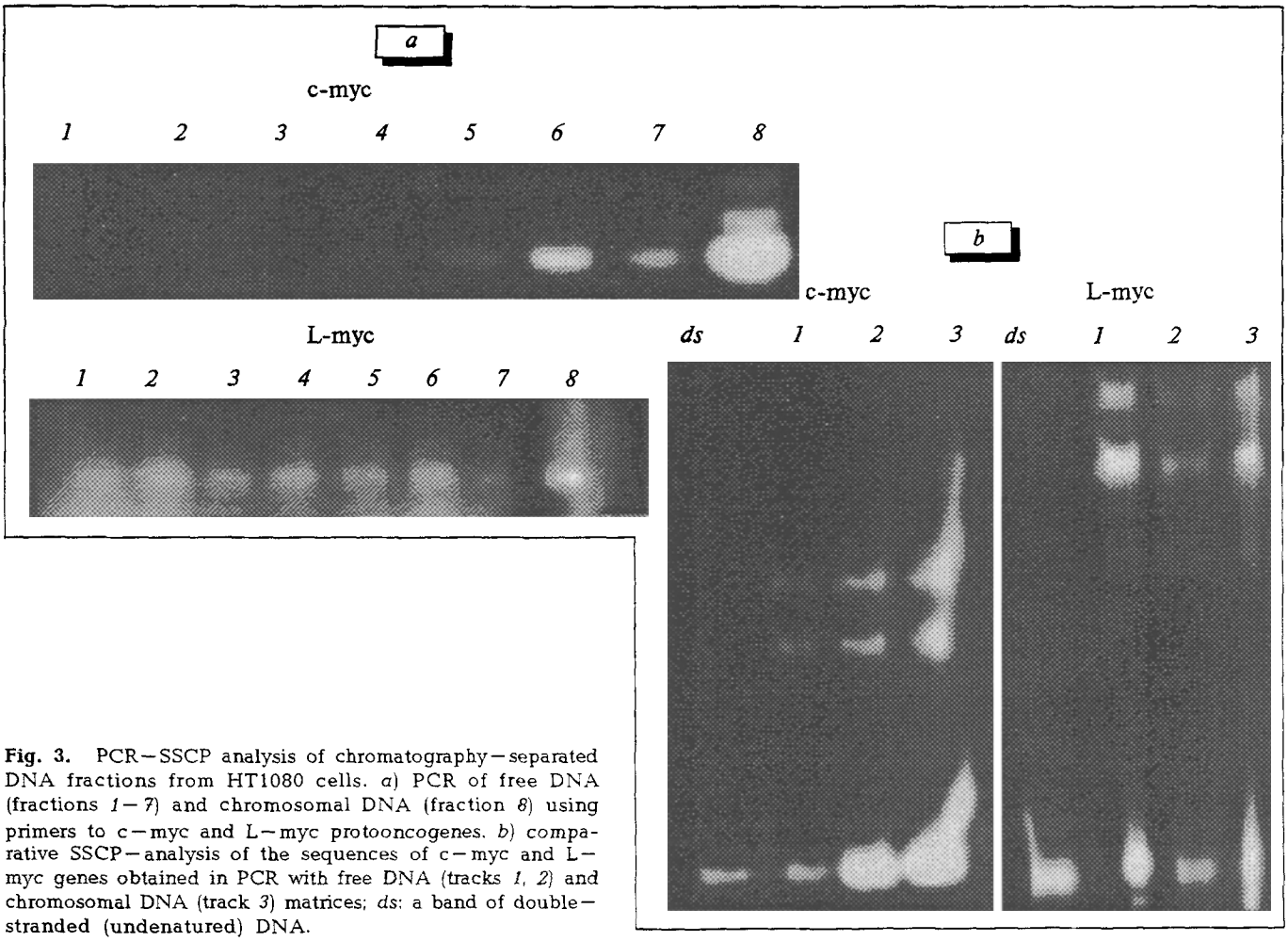

Fig. 3. PCR–SSCP analysis of chromatography–separated DNA fractions from HT1080 cells. *a*) PCR of free DNA (fractions *1–7*) and chromosomal DNA (fraction *8*) using primers to c–myc and L–myc protooncogenes. *b*) comparative SSCP–analysis of the sequences of c–myc and L–myc genes obtained in PCR with free DNA (tracks *1, 2*) and chromosomal DNA (track *3*) matrices; *ds*: a band of double–stranded (undenatured) DNA.

rose gel and subjected to reverse-pulse gel electrophoresis (RPGE) [3] on Hoefer PC 750 apparatus under the following conditions: 200 V, 3 hours, pulse forward 0.3 sec, pulse backward 0.1 sec (ramp factor 6.6). DNA multimers of λ phage (Bio-Rad) served as markers. Gels were stained with ethidium bromide (0.5 μg/ml).

Method 2: cells were lysed with Triton X-100; the lysed proteins were immobilized by mixing with Celite 545 that served as an adsorbent, and the suspension was loaded on a chromatography column. Stepwise DNA extraction was performed by increasing the concentration of dissociating agents [1,6].

DNA fractions were precipitated with isopropanol and deproteinized by treatment with proteinase K and sarkosyl. Analysis of the specimens thus obtained included electrophoresis in 1% agarose gel, estimation of specific radioactivity, and the polymerase chain reaction (PCR). PCR products were examined by the method of single-strand conformation polymorphism (SSCP) [9]. PCR was conducted using primers to exon 3 of human c-myc protooncogene (5'-ACGCAGCGCCTCCCTCCACT sense primer; 5'-GCTCGTTCCTCCTCTGGCGC antisense primer; fragment length equal to 183 bp) and human L-myc protooncogene (5'-AGTTCACT CACAGGCCACAT sense primer; 5'-TGCATAT CAGGAAGCTTGAG antisense primer; fragment length equal to 267 bp).

RESULTS

The results of JHF DNA differential extraction followed by the electrophoresis of fractions in 1% agarose gel are presented in Fig. 1, *a*. In accordance with the earlier obtained data [1], the bulk of cellular DNA (nearly 70%) is very tightly bound to the nuclear matrix; it is extracted under the most stringent conditions (4 M LiCl, 8 M urea, 95°C), and, accordingly, is eluted in the last fraction [fraction 8; DNA (8)]. In addition, a set of minor fractions is detected [fractions 1-7; DNAs (1-7)], that are relatively weakly bound to the protein, as can be concluded from the conditions required for their extraction (1-3 M NaCl, 2-4 M

LiCl, and 4-8 M urea, 2°C). As can be seen, their size exceeds 20 tbp. An analogous distribution was observed in all cell types tested.

DNA fractions obtained by the above-mentioned method from JHF that were preincubated for one day with ^{3}H-thymidine (0.25 μCi/ml), were quantitatively evaluated by spectrofluorometry of samples stained with Hoechst 33258, after which radioactive label incorporation was recorded on the scintillation counter. The specific radioactivity of the DNA fractions shows considerable differences. The corresponding index of the main fraction (DNA 8; operationally named below as chromosomal DNA) exceeds that of free DNA several times (Fig. 1, *b*). This regularity discovered by us during an analysis of other cell types (HT1080, HeLa, N1E115 cell lines, human skin primary fibroblasts, etc.) apparently indicates autonomy of the free DNAs.

For a check of this hypothesis, another method of free DNA isolation was applied (reverse-pulse gel electrophoresis of DNA from agarose-encapsulated cells). This approach makes it possible to practically exclude both mechanical and enzymatic fragmentation of the chromosomal DNA. Despite these mild conditions, in all objects studied by us free DNAs with remarkably regular properties were found (Fig. 2, *a*). The major portion of chromosomal DNA (nearly 85-90%) remains at the start (i.e., in agarose blocks), while the remaining 10-15% of DNA forms three discrete bands of approx. 400, 250-300, and 50 tbp (bands *I*, *II*, and *III*, respectively). The regular distribution pattern of DNA of all cell types provides indirect evidence against attributing the phenomenon to artificial degradation of the chromosomal DNA. If this is true, one should expect a heterogeneous population of products varying for different tissues depending on the level of activity of an individual nuclease. Comparison of the specific radioactivity of the RPGE-obtained chromosomal and free DNA fractions from JHF showed the same regularity observed by us earlier,i.e., this index was significantly higher for the chromosomal DNA (Fig. 2, *b*).

Comparison of the two approaches used, however, reveals differences in the size of free DNA isolated by different methods, namely, nearly 25 tbp after chromatography and much more (50-400 tbp) after RPGE. Perhaps the first approach results in partial DNA degradation during the chromatographic procedures.

Thus, the results of two independent methods point to two principal facts: a) the existence in cells of various origin (normal and malignant, cultured and derived from animal tissue) of free, chromosome-unbound, DNAs of large size, and b) metabolic autonomy of free DNAs. It was of interest to elucidate their informational content, and in particular to determine whether they contain structural genes and, if so, whether these genes are identical in the chromosomal and free DNA. In the following experiments we attempted to answer these questions.

Results of an analysis of HT1080 DNA chromatographic fractions using PCR are presented in Fig. 3, *a*. The use of human instead of hamster cells as in previous experiments is connected with the lack of a hamster gene bank. With the aid of corresponding primers, the sequences of c-myc and L-myc protooncogenes were detected in the free molecules (Fig. 3, *a*). The SSCP method [9] was used to check for possible point nucleotide substitutions in the "free" structural genes as compared to their chromosomal homologs. This method makes it possible in most cases to detect such mutations in PCR products. Results of a comparison of c-myc and L-myc protooncogene fragments synthesized by means of PCR using matrices from free and chromosomal DNA are reflected in Fig. 3, *b*. Proceeding from the analogous electrophoretic mobility of denatured DNA strands, one can conclude that the "free" sequences under study do not differ from the corresponding "chromosomal" sequences.

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