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Genetics

Short DNA sequences from the cytoplasm of mouse tumor cells induce immortalization of human lymphocytes *in vitro*

(cytoplasts/extrachromosomal DNA/DNA–protein complexes)

HINRICH ABKEN*†, RALF HEGGER, CHRISTOPH BÜTZLER, AND KLAUS WILLECKE

Institut für Genetik, Abteilung Molekulargenetik, Universität Bonn, Römerstrasse 164, D-5300 Bonn 1, Germany

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ABSTRACT **Cytoplasts of mouse L929 and Ehrlich ascites tumor cells harbor DNA sequences that induce unlimited proliferation ("immortalization") of human lymphocytes after transfection *in vitro*. By equilibrium centrifugation of cytoplasmic lysates in a neutral CsCl gradient, the immortalizing activity was recovered together with extramitochondrial fractions at high salt densities (1.85–1.87 g/cm³). Unexpectedly, these fractions contain linear DNA molecules of 50–500 bp in length. In contrast, cytoplasts of primary, senescent cells (mouse embryo fibroblasts, human lymphocytes) do not harbor DNA in the corresponding fractions. Cytoplasmic DNA isolated from high-density fractions of mouse tumor cells was cloned in subset libraries, and of 45 DNA sequences we identified 2 clones—one from L929 cytoplasts (203 bp) and another one from the cytoplasm of Ehrlich ascites cells (372 bp)—that induce unlimited proliferation of human lymphocytes *in vitro*. Immortalized lymphoid cells harbor 1–5 copies of transfected DNA integrated into chromosomal DNA, whereas about 100 copies were found as episomal DNA in the cytoplasmic fraction. No immortalization could be induced by transfection of nuclear DNA randomly fragmented to 200–500 bp. Although the cloned DNA sedimented at 1.70 g/cm³, after transient transfection into lymphocytes, these DNA sequences form salt-stable complexes that sediment in fractions at the same high density (1.82–1.88 g/cm³) from which they were originally cloned. The high-density banding of these cytoplasmic DNA sequences may be due to association with RNA and/or with (metallo-) proteins *in vivo*. Since both cloned DNA sequences with immortalizing activity have stop codons for protein translation in all possible reading frames, immortalization may be induced by insertional inactivation or functional suppression of genes that are needed to be expressed during cellular senescence or programmed cell death.**

Normal lymphocytes from peripheral blood are quiescent cells that exhibit a limited life span in culture, enter crisis, and undergo senescence resulting in cell death. However, resting lymphocytes can be activated to clonal proliferation *in vitro* by repeated stimulation with mitogens or thymus-independent antigens or by a pathway that requires T-cell recognition of antigen and accessory cells. Thus, by continuous and appropriate stimulation, long-term cultures of factor-dependent B cells can be established *in vitro*—e.g., by addition of certain cytokines in combination with immobilized antibodies (1, 2). On the other hand, infection with transforming viruses leads to establishment of autonomously proliferating B- and T-cell lines—e.g., after infection with Epstein–Barr virus or herpesvirus saimiri (3) or with human T-cell leukemia virus type I, respectively. Many, but not all, tumor cell lines exhibit autonomous and unlimited growth in culture. Cellular senescence may be a mechanism to prevent tumor formation *in vivo* (4), whereas escape from senescence appears to be frequently a predisposition for neoplastic transformation. However, immortality *in vitro* is not sufficient for tumor induction *in vivo*.

Previously, we reported (5) that human lymphocytes can be induced to escape senescence *in vitro* by fusion with cytoplasts from established tumor cell lines ("immortalization"). The immortalized cells proliferate continuously *in vitro* by secretion of four cytokines that cooperate in their activity of autocrine growth promotion (6). However, the immortalized cells do not induce tumors or lymphoproliferative syndromes after injection into immunodeficient mice. Human B-cell clones were established that secrete immunoglobulins (IgM, IgG, and IgA) with monoclonally restricted heavy and light chain classes. The active agent responsible for immortalization by fusion with L929 cytoplasts was shown to be extrachromosomal, cytoplasmic DNA (7). For immortalization of human lymphocytes, DNA from L929 cytoplasts was 100-fold more efficient than nuclear DNA. Cytoplasmic DNA with immortalizing activity seems to persist predominantly in tumor cell lines exhibiting unlimited growth potential (e.g., L929, Ehrlich ascites cells, Ag8.653, HS-Sultan) (8), whereas primary senescent cells (e.g., mouse embryo fibroblasts, human peripheral blood lymphocytes) do not harbor immortalizing activity in the cytoplasm. Since the activity to induce lymphocyte proliferation could be detected in a mitochondria-depleted fraction of cytoplasts, we concluded that the immortalizing DNA is located extramitochondrially in the cytoplasm of L929 cells (7).

Here we report the isolation of immortalizing DNA of L929 cytoplasts by equilibrium centrifugation in a CsCl density gradient. Unexpectedly, the immortalizing activity sediments with fractions at high densities that harbor short, linear DNA molecules. From these cytoplasmic fractions of mouse L929 and Ehrlich ascites cells, we cloned two DNA sequences (203 bp and 372 bp)‡ that are capable of inducing unlimited proliferation of human lymphocytes after transfection *in vitro*. We speculate that immortalization of mitogenically stimulated cells by transfer of these DNA sequences may be due to insertional inactivation or suppression of genes required to enter senescence.

MATERIALS AND METHODS

Cells and Cell Lines. Mouse L929 (ATCC CCL 1) and Ehrlich ascites cells (ATCC CCL 77) were cultured in Dulbecco's modified Eagle's medium (DME medium). Cytoplasts were induced by incubation with cytochalasin B as described (5). Mononuclear cells were isolated from fresh

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Abbreviation: DIG, digoxigenin.

*To whom reprint requests should be addressed.
†Present address: Medizinische Klinik I, Forschergruppe "Maligne Lymphome", LFI, Universität Köln, Josef-Stelzmann-Strasse 9, D-5000 Cologne 41, Germany.

‡The sequences reported in this paper have been deposited in the GenBank database (accession nos. L 12036 for the LC108 DNA sequence and L 12037 for the EFC38 DNA sequence).

samples of human peripheral blood and cultured in Iscove's modified Dulbecco's medium (IMD medium) supplemented with 1 unit of insulin and 10 μg of transferrin per ml, 1 mM pyruvate, 1 mM oxaloacetic acid, and 10% (vol/vol) fetal calf serum as described (7). Only cultures that contained <1% spontaneous blast cells after 24 hr of culture were used for further experiments. Lymphocytes were transfected with DNA by using DOTAP (*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride) reagent (Boehringer Mannheim) as described (9). Briefly, 70 μl of DOTAP (1 mg/ml) was added to 180 μl of 20 mM Hepes/150 mM NaCl and mixed with 250 μl of a solution containing DNA in 20 mM Hepes/150 mM NaCl. After incubation for 10 min at room temperature, 10^7 cells in 2 ml IMD medium were added and incubated for 12 hr at 37°C. Cells were washed, resuspended in fresh medium, and cultured in 96-well plates (10^6 cells in 100 μl of medium per well). For the next 3 weeks, 50 μl of medium was changed every 4 days. The culture was subdivided for the first time 5–6 weeks after transfection, when continuously proliferating colonies were observed.

DNA Analyses. High molecular weight DNA and plasmid DNA were prepared, transferred by blotting techniques onto nylon membranes, and hybridized according to standard techniques (10). DNA was radioactively labeled by the random-primed labeling procedure (11) or by reaction with polynucleotide kinase. Alternatively, DNA was labeled with digoxigenin(DIG)-11-ddUTP by using the terminal transferase reaction. DIG-labeled DNA was detected by the chemiluminescence procedure according to the supplier's instructions (Boehringer Mannheim). DNA sequencing was performed by the chain-termination method (12).

For isolation of cytoplast DNA, 5×10^9–10^{12} cytoplasts were resuspended in 500 μl of 50 mM Tris·HCl/10 mM EDTA/1.5 mM $MgCl_2$, pH 7.5, and lysed by repeated freezing and thawing. Cellular debris was sedimented at $1000 \times g$ for 5 min, and the supernatant (300 μl) was centrifuged to equilibrium in a neutral CsCl gradient (1.70 g/cm^3) at 180,000 × *g* and 10°C. Fractions were dialyzed into 10 mM Tris·HCl/1 mM EDTA, pH 7.5, and subsequently incubated with 100 μg of RNase A per ml for 1 hr at 37°C and with 0.1% SDS/100 μg of proteinase K per ml for 12 hr at 60°C. Finally, DNA was extracted with phenol and chloroform/isoamyl alcohol.

Polymerase chain reactions (PCRs) (13) were performed with primer oligonucleotides A (5′-GATCCAATCAGCTCAGCCACCCCCA-3′) and B (5′-AAAACCAGGGCCTCCCACATG-3′) for amplification of EFC38 DNA. Primers C (5′-GATCTTGAGTTTCCTCGTTGTAGGT-3′) and D (5′-GATCCAAAGCCCTCTGCTGGCCTCC-3′) were used for amplification of LC108 DNA. Thirty cycles (30 s at 60°C; 90 s at 72°C; 60 s at 95°C) were performed.

RESULTS

Extramitochondrial DNA Exhibiting Immortalizing Activity Sediments with Fractions at High Salt Density. Cytoplasts of mouse L929 and Ehrlich ascites tumor cells harbor DNA that induced unlimited proliferation of human peripheral blood lymphocytes after transfection *in vitro* (7) (Table 1). For isolation of the cytoplasmic DNA, 10^9 L929 cells were grown in the presence of [^{3}H]thymidine for 50 hr, the lysate of cytoplasts was centrifuged to equilibrium in a neutral CsCl gradient, and DNA of each fraction was precipitated onto glass fiber filters. The profile of [^{3}H]thymidine incorporation revealed three different fractions (A, B, and C) containing DNA (Fig. 1*a*). Hybridization analyses indicated that fraction B harbored mitochondrial DNA [with pAM-1 DNA (14) as probe] (Fig. 1*b*) and fraction C contained chromosomal DNA [with a repetitive DNA sequence of the B2 family (pMR142) (15) as probe] (Fig. 1*c*). No hybridization was obtained with DNA in fraction A. On the other hand, with fraction A DNA as probe, hybridization was recorded predominantly with DNA from fractions at 1.85–1.87 g/cm^3 (Fig. 1*d*). Thus, fraction A harbors predominantly nonmitochondrial, cytoplasmic DNA of L929 cells. Preincubation of the cytoplasmic lysate with RNase A or with proteinase K did not alter the property of the cytoplasmic DNA to sediment in fractions of high salt densities during centrifugation to equilibrium. In Ehrlich ascites tumor cells, nonmitochondrial DNA was detected in fractions at 1.85–1.87 g/cm^3 too (data not shown). However, DNA from fraction A of Ehrlich ascites cells did not hybridize to DNA of the corresponding fractions of L929 cytoplasts, implying that both extrachromomal fractions predominantly harbor different DNA sequences. By equilibrium centrifugation from cytoplasmic lysates of mouse embryo fibroblasts or human blood lymphocytes, no DNA could be detected in fractions at high salt densities. This suggests that primary senescent cells that do not harbor immortalizing activity to human lymphocytes do not contain cytoplasmic DNA in high density fractions, whereas cytoplasts from L929 and Ehrlich ascites tumor cells exhibit immortalizing activities and harbor extramitochondrial DNA in these fractions.

Table 1. Frequency of immortalization of human lymphocytes from peripheral blood by DNA transfection

Cell fraction from which DNA was isolated/cloned DNA	DNA transfected, μg	Immortalized colonies, no.
L929 cells		
Nuclei	10	0
Cytoplasts	3	52
Fraction A	≈0.0001	20
Fraction B	1	0
Fraction C	10	0
Cloned DNA		
pLC108 (circular)	0.2	43
pLC108 (linear)	0.2	19
LC108 (PCR)	0.01	35
pAT153	0.2	0
Ehrlich ascites cells		
Nuclei	10	0
Cytoplasts	3	65
Fraction A	≈0.001	10
Fraction B	1.0	0
Fraction C	10	0
Cloned DNA		
pEFC38 (circular)	0.2	51
pEFC38 (linear)	0.2	32
EFC38 (PCR)	0.02	34
pUC19	0.2	0

In each experiment, 10^8 lymphocytes were transfected with the amount of DNA indicated. DNA of fractions A, B, and C was isolated from the corresponding fractions of a CsCl density gradient (see Fig. 1). Circular and linearized plasmid DNA were transfected. LC108 DNA (203 bp) and EFC38 DNA (372 bp) were amplified by PCR techniques ("PCR"). pAT153 and pUC19 were used as cloning vectors for LC108 and EFC38 DNA, respectively. Nuclear DNA isolated from nuclei and from fractions C of a CsCl density gradient exhibited an average size of about 15 kb; mitochondrial DNA from fractions B, of 16 kb; and extramitochondrial DNA in fractions A, of about 0.4 kb. No immortalized lymphocytes were obtained after transfection with the other pLC and pEFC DNA sequences cloned from the same fractions as LC108 and EFC38 DNA, respectively.

Short, Cytoplasmic DNA Sequences Immortalize Human Lymphocytes After Transfection. To identify DNA with immortalizing activity, human lymphocytes from peripheral blood were transfected (*i*) with DNA from fraction A of L929 cytoplasts, (*ii*) with mitochondrial DNA isolated from fractions B, and (*iii*) with chromosomal DNA from fractions C. About 8 weeks after transfection with DNA from fraction A, lymphoid cell colonies were obtained that showed unlimited proliferation potential (Table 1). At this time, lymphocytes

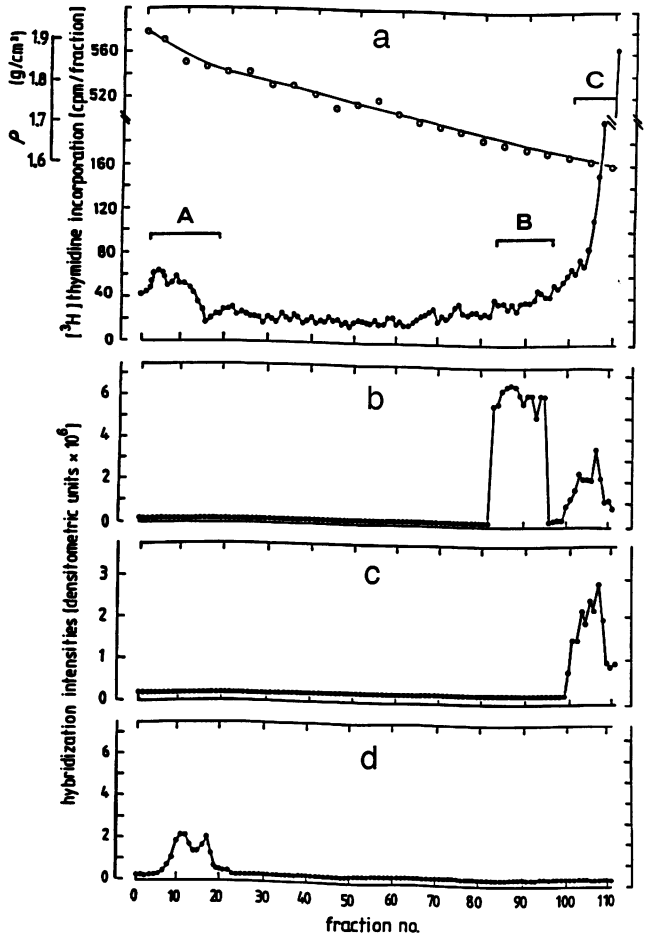

FIG. 1. Equilibrium centrifugation of a cytoplasmic lysate from L929 cytoplasts in a CsCl density gradient. L929 cells were cultured in the presence of [^{3}H]thymidine. Cytoplasts were induced by incubation with cytochalasin B and isolated, and the lysate was centrifuged to equilibrium in a CsCl density gradient. DNA of each fraction was precipitated with trichloroacetic acid onto glass fiber filters. (*a*) Radioactivity incorporated into DNA was determined by liquid scintillation spectrometry, and CsCl density was recorded by refractometry. (*b–d*) DNA of each fraction was hybridized (*b*) to cloned mitochondrial DNA (pAM-1) (14), (*c*) to a mouse repetitive DNA sequence (pMR142) (15), and (*d*) to DNA isolated from fractions A of the gradient. Hybridization intensities were monitored by densitometric scanning of the autoradiographs.

transfected with DNA from fractions B or C had entered crisis and died without establishment of continuously growing colonies. Accordingly, human lymphocytes were immortalized by DNA of fraction A isolated from the cytoplasm of Ehrlich ascites cells but were not by DNA from fractions B and C (Table 1). Incubation of fraction A with 50 μg of DNase I per ml for 2 hr at 37°C destroyed the immortalizing activity, whereas incubation with 100 μg of RNase A or proteinase K per ml had no effect.

After radioactive labeling (using T4 polynucleotide kinase) and analysis by gel electrophoresis, DNA from fraction A of L929 and Ehrlich ascites cells was found to contain DNA molecules of 50–500 bp in length (Fig. 2). The signal was destroyed by incubation with DNase I and with nuclease S1 plus exonuclease III but not with nuclease S1 alone or with RNase A, RNase T1, RNase H, or proteinase K. Therefore, and because the cytoplasmic DNA could be radioactively labeled by polynucleotide kinase and by the terminal transferase reaction, we conclude that fraction A predominantly contains linear DNA molecules. However, it cannot be

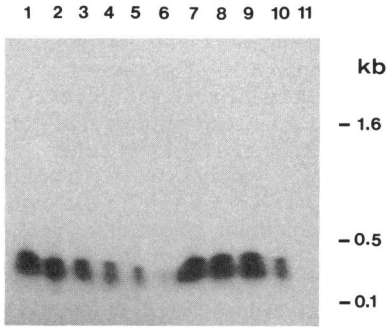

FIG. 2. Gel electrophoresis of cytoplasmic DNA from Ehrlich ascites cells after treatment with different nucleases. Cytoplasmic DNA from Ehrlich ascites cells was labeled with ^{32}P by using the polynucleotide kinase reaction (lane 1) and was digested with DNase I (0.09 units) for 5 min (lane 2), 10 min (lane 3), 15 min (lane 4), 20 min (lane 5), and 30 min (lane 6) at 37°C. Furthermore, DNA was incubated with RNase A (100 μg/ml) and RNase T_1 (1000 units/ml) (lane 7), RNase H (1 unit/μl) (lane 8), nuclease S1 (100 units/μl) (lane 9), exonuclease III (175 units/μl) (lane 10), and with nuclease S1 and exonuclease III (lane 11), each for 1 hr at 37°C. DNA was electrophoresed through a 4% neutral polyacrylamide gel.

excluded that, in addition, circular DNA molecules may be present.

For molecular cloning, DNA from fraction A (isolated from a lysate of 5×10^9 L929 cytoplasts) was ligated into the *Bam*HI site of pAT153 DNA. We obtained recombinant clones (designated pLC) with DNA inserts of 43–260 bp. Some of these clones harbor identical inserts, whereas the other sequences have no homology to each other. In summary, we cloned 20 different LC DNA sequences from fraction A of the cytoplasm of L929 cells. Similarly, after cloning of cytoplasmic DNA of Ehrlich ascites cells (3×10^{12} cells) into the *Bam*HI site of pUC19 DNA, >300 recombinant clones were obtained (designated pEFC). However, DNA sequence analyses of >100 clones revealed 25 different EFC insert sequences (59–438 bp). DNA sequences cloned in independent experiments were found to be completely identical to 1 of the 25 different EFC DNA sequences. Therefore, it is very unlikely that the short DNA sequences cloned represent random fragments of chromosomal DNA generated during cloning procedures. The cloned pLC and pEFC insert DNA sequences hybridize to DNA of the cytoplasmic fractions A of L929 and Ehrlich ascites tumor cells, respectively (data not shown).

To identify the immortalizing DNA sequence, DNA samples of the 20 different pLC and of the 25 pEFC clones were transfected separately and in pools of 4 clones into human lymphocytes. As summarized in Table 1, transfection of pLC108 DNA (203-bp insert) and of pEFC38 DNA (372-bp insert) induced lymphocytes to proliferate continuously *in vitro*. Fig. 3 depicts the nucleotide sequences of these two cloned DNA inserts. A search in the EMBL nucleotide data bank [release no. 35 (March 9, 1993)] did not reveal significant homology to any known DNA sequence. Immortalization was obtained after transfection of circular and of linearized pLC108 or pEFC38 plasmid DNA as well as of insert DNA amplified by PCR techniques (Table 1). Immortalized lymphocytes cloned by dilution to single cells harbor 1–5 copies of transfected LC108 DNA integrated into high molecular weight DNA as shown by Southern blot hybridization (Fig. 4*a*). We noticed some cross-hybridizations, however, with endogenous sequences of the human genome. By centrifugation of a cytoplasmic lysate from immortalized cells in a CsCl gradient and dot-blot hybridization of each fraction with LC108 DNA, we found that the immortalized cells carry about 100 episomal copies of transfected LC108 DNA in addition to 1–5 copies integrated into chromosomal DNA.

LC108

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GATCTTGAGT TTCCTCGTTG TAGGTCTTCC CTGGCTCTGC TCACGCTCTC ACTGACTTCT  60
CTCAGCTCAG TCACAGTGTC TATTTCTTTC CACTTAAAGA TGTGCATTTT TATTTGATGC 120
GTGCAGGTGT TTTGCCTGCA TGGATGGCTG TGCACCATGT ATGGGACCTG GTGCTCTTGG 180
AGGCCAGCAG AGGGCTTTGG ATC
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EFC38

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GATCCAATCA GCTCAGCCAC CCCCAGCTCT CCTGTATGTA TGGCTCCAAT GCTGTTCATC  60
CCTCAGCATA AATCAATCAT TTGGTTTAGA TTCCTCCCTT TGACTTATTG CTACTATTAG 120
TATCAGTGAC TCTTCAGCCG ATTCTTTTCA GACATTGGAA CCCCAGCCTC AGATCACAGT 180
TGTAGAACAA ATATTAAAAG AGTAAATTAT TATATCATTG AACATTCAAA AGTGCTTTGC 240
AGTCATTGAC ACATAATAAT AATGAAGCCT AAACAGTAAC ATGAAAATGT GGAATTGTAT 300
TAATGTAAAA TCAAGGCCTG GGGCATAGCT CATTGGTGGA TGTTTGCCTA TCATGTGGGA 360
GGCCCTGGTT TT
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FIG. 3. DNA sequence of LC108 DNA cloned from L929 cytoplasts and of EFC38 DNA cloned from the cytoplasm of Ehrlich ascites cells.

Transfection with DNA of the cloning vectors (pAT153; pUC19) did not induce lymphocyte proliferation. None of the further 43 pLC and pEFC DNA sequences, transfected separately or in combination, exhibited immortalizing activities. Furthermore, no immortalization was obtained after transfection of fragmented nuclear DNA (20 μg) of 200–500 bp from L929 or Ehrlich ascites cells obtained by partial digestion with *Sau*3A, *Alu* I, and *Hpa* II. Thus, immortalization does not seem to be due to transfer of short DNA fragments of random sequences.

To verify that the cloned pEFC38 DNA (372 bp) is a complete DNA sequence from the cytoplasm of Ehrlich ascites cells, DNA from fraction A was tailed with dATP and amplified by PCR techniques using oligo(dT) nucleotide primers. Sequence analyses of three independent clones hybridizing to EFC38 DNA confirmed in all cases the entire nucleotide sequence of the pEFC38 insert (372 bp).

After Transient Transfection, Cloned Cytoplasmic DNA Sequences Form Complexes that Band at High Salt Densities. We analyzed whether LC108 and EFC38 DNA are capable after transient transfection to form DNA complexes that band at high-density fractions. Cloned LC108 (203 bp) and EFC38 (372 bp) insert DNA were amplified by PCR techniques, labeled with DIG-ddUTP by reaction with terminal transferase, and subjected to equilibrium centrifugation in a CsCl density gradient. These DNA sequences were recovered from fractions at 1.70 g/cm^3 as expected for naked DNA (Fig. 5). After transient transfection into human lymphocytes (0.5 μg of DNA per 10^7 cells) and centrifugation of the cytoplasmic lysates in a CsCl density gradient, DIG-labeled DNA was detected in fractions at 1.82–1.88 g/cm^3 in addition to the naked DNA in fractions at 1.70 g/cm^3 (Fig. 5). Dot-blot hybridizations confirmed that these fractions harbor the transfected DNA sequences. After transient transfection of the 322-bp *Pvu* II fragment of pUC19 DNA, on the other hand, the pUC19 DNA was reisolated in cytoplasmic fractions at 1.70 g/cm^3 but could not be detected in fractions at high densities (data not shown). We conclude that after transient transfection LC108 and EFC38 DNA specifically form salt-stable complexes that can be recovered from fractions at the same high density from which the DNA sequences were originally cloned.

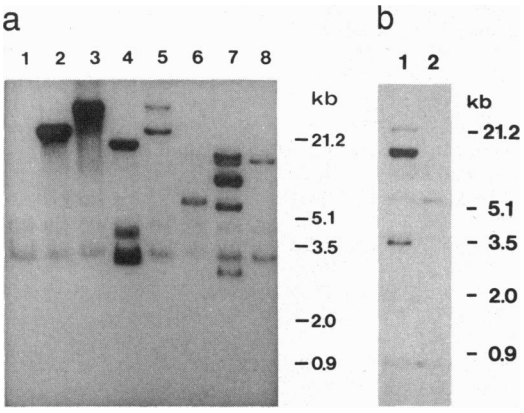

FIG. 4. Southern blot hybridization of human lymphoid cell lines immortalized by transfection with pLC108 DNA (*a*) and of mouse tumor cells (*b*). High molecular weight DNA (7 μg) was cleaved with *Hin*dIII and electrophoretically separated, and the Southern blot was hybridized to LC108 DNA (203 bp). (*a*) DNA of peripheral blood lymphocytes (lane 1) and of human lymphoid cell lines immortalized by transfection with pLC108 DNA and cloned by dilution to single cells (lanes 2–8). (*b*) DNA of L929 cells (lane 1) and Ehrlich ascites cells (lane 2). The results show that different transfectants harbor different amounts of transfected LC108 DNA integrated in the genome at different positions.

DISCUSSION

We have previously reported (7, 8) that human lymphocytes can be immortalized by transfection with DNA that is located extramitochondrially in the cytoplasm of L929 and Ehrlich ascites cells. The immortalizing activity is now shown (*i*) to band with cytoplasmic DNA fractions at high salt densities after equilibrium centrifugation in a neutral CsCl gradient and (*ii*), unexpectedly, to be represented by two short DNA sequences [LC108 (203 bp) and EFC38 (372 bp)] cloned from these fractions. Induction of unlimited proliferation of human

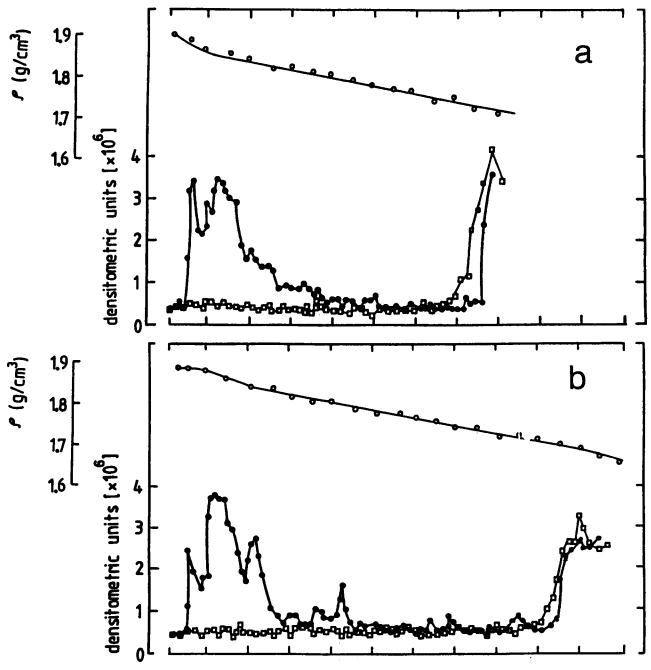

FIG. 5. Reisolation of LC108 and EFC38 DNA after transient transfection into human lymphocytes. LC108 DNA (203 bp) (*a*) and EFC38 DNA (372 bp) (*b*) were labeled with DIG-ddUTP and transfected into human lymphocytes. Twelve hours after transfection, a cytoplasmic lysate of cells transfected (●) was centrifuged to equilibrium in a CsCl density gradient. As controls, DIG-labeled DNA (□) was centrifuged as well. DNA from each fraction was dotted onto nylon membrane and detected by anti-DIG antibodies by the chemiluminescence technique. Signal intensities were monitored by densitometric scanning of the autoradiograph. ○, Density (g/cm^3) of the fraction; ●, DIG-labeled DNA transfected into lymphocytes; □, DIG-labeled DNA.

lymphocytes *in vitro* is a specific activity of each of these two cloned DNA sequences: neither transfection of a further 43 short DNA sequences cloned from the same cytoplasmic fraction nor transfection of chromosomal DNA fragments of the same average length (200–500 bp) lead to lymphocyte immortalization. Cloning experiments using DNA tailing reactions and PCR amplification indicate that the entire EFC38 DNA sequence cloned is likely to exist as linear DNA molecule in the cytoplasm of Ehrlich ascites cells and does not represent a random fragment of chromosomal DNA generated during cloning procedures.

Our analyses show cytoplasmic DNA in fractions with a density of about 1.86 g/cm^3 after equilibrium centrifugation in a neutral CsCl density gradient. Banding of DNA in fractions of high density is unusual, since mammalian DNA purifies at 1.699–1.700 g/cm^3 in the case of nuclear DNA (16) or at 1.686–1.712 g/cm^3 in the case of satellite DNA (17). Cloned LC108 and EFC38 DNA sediment in fractions at 1.70 g/cm^3 (see Fig. 5) as expected for naked, double-stranded DNA. On the other hand, our analyses demonstrate that these DNA sequences persist in cytoplasmic fractions that band at high salt density. Thus, we speculate that these DNA sequences may not persist as naked double-stranded DNA molecules in the cytoplasm of mouse tumor cells but may be partially single-stranded and/or may be associated with RNA or metallo-proteins or both molecules. These assumptions are supported by detection of the protein label [^{35}S]methionine and the RNA label [^{3}H]UTP in high-density cytoplasmic fractions (unpublished results). However, pretreatment of cytoplasmic DNA with protease K or with RNase A did not alter the property of cytoplasmic DNA to sediment with fractions of high densities. The signals for formation of high density complexes are likely to be encoded by the appropriate DNA sequences because after transient transfection the LC108 and EFC38 DNAs sediment in fractions at the same density from which the DNA sequences were originally cloned. In contrast, other short DNA molecules (e.g., 322-bp fragment of pUC19 DNA) do not form salt-stable complexes after transfection. Furthermore, preliminary experiments show *in vitro* interaction of cloned LC108 and EFC38 DNA sequences with a crude lysate of cytoplasmic proteins *in vitro* in the presence of Zn^{2+} and Na^+, respectively (ref. 18, unpublished results). The molecular structure of the cytoplasmic DNA–protein complexes, however, remains to be elucidated, especially how DNA binding proteins contribute to the immortalizing activity of LC108 and EFC38 DNA.

The notion of DNA–polypeptide complexes in cytoplasmic fractions at high salt density furthermore assumes very tight interactions between DNA and polypeptides because the complex is stable to high salt concentrations (6.9 M CsCl in fractions at 1.86 g/cm^3) and to detergents (0.1% SDS) and is resistant to degradation by proteinase K, RNase A, and RNase H. Tight DNA–polypeptide interactions were reported for chromosomal DNA sequences suggested to be part of nuclear matrix structures of Ehrlich ascites cells, of human placenta, and of other sources (19). The salt-stable DNA–protein interaction of nuclear DNA–polypeptide complexes was recently shown to be due to phosphotriester bonds between hydroxyamino acid residues in peptides and internucleotide phosphates of the DNA (20). Covalent bonds between DNA and polypeptides would explain the unusual stability of the cytoplasmic complexes, in particular, the resistance to detergents, high salt concentrations, or alkali (unpublished results). Furthermore, the shift in density after transfection of cloned DNA sequences to fractions at high salt densities implicates *de novo* formation of tight DNA complexes *in vivo*.

One possible mechanism to induce immortalization of proliferating cells may be integration of DNA fragments into gene sequences that are needed for induction of cellular senescence. This possibility seems to be unlikely, however, since lymphocyte proliferation could neither be induced by transfection of short and random fragments of nuclear DNA nor by any of 43 short DNA sequences cloned in addition from the same cytoplasmic fraction. Immortalization by a protein gene product encoded by the immortalizing DNA seems to be unlikely, too, since both DNA sequences LC108 and EFC38 have stop codons for protein translation in all possible reading frames. Preliminary experiments, however, indicate that these DNA sequences exhibit promoter as well as silencer activities when assayed in gene constructs with the chloramphenicol acetyltransferase reporter gene (B. Reifenrath and H.A., unpublished results). Possibly, immortalization of mitogenically stimulated lymphocytes by transfection of LC108 and EFC38 DNA may be initiated by functional suppression of genes that are needed to be expressed during cellular senescence and/or programmed cell death.

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