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Membrane-Associated DNA in the Cytoplasm of Diploid Human Lymphocytes

(electron microscopy/gradient centrifugation/DNA characterization/IgG)

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ABSTRACT **A species of DNA that is apparently associated with the plasma membrane of diploid human lymphocytes that are continuously growing is described. This DNA differs from nuclear or mitochondrial DNA by virtue of its location in the cell, time of synthesis in the cell cycle, and various physical properties. At present, the role of this DNA is unknown.**

Gene expression in prokaryotic and eukaryotic cells can be altered by events at cell surfaces. Some examples of this phenomenon are the effects of colicins on bacteria, contact inhibition in mammalian cells, and induction of an immune response. However, the mechanism by which a cell responds to an event at its surface is unknown. In the immune system, the problem can be clearly defined since a single molecular species, the immunogen, induces cellular proliferation in a limited number of reactive cells. Furthermore, recent studies suggest that the union of immunogen with membrane-associated immunoglobulin (M-IgG) is the event that initiates cellular proliferation and differentiation (1, 2).

Conceptually, the union of immunogen with M-IgG could alter gene expression by several mechanisms. Among these are: (*a*) liberation of a small intermediary molecule, such as cyclic AMP, that might act on either transcription or translation, (*b*) initiation of the translation of membrane-associated RNA, which could ultimately result in the synthesis of a regulatory polypeptide, or (*c*) initiation of either the replication or transcription of membrane-associated DNA, leading to gene amplification or cell regulation. The feasibility of the first two possibilities is illustrated by the role of cyclic AMP in the regulation of hormone synthesis and the association of RNA with cytoplasmic membranes in other systems (3, 4). The present investigation was designed to determine whether there is an association between the plasma membrane and DNA that might support the third mechanism. Continuously growing, diploid human lymphocytes (WIL_2) known to have M-IgG were studied (5). A species of cytoplasmic DNA that is apparently associated with the plasma membrane is described. This DNA differs from the cytoplasmic DNA studied by others by its location in the cell and characteristics that distinguish it from nuclear and mitochondrial DNA (6–13). These results have recently been presented in preliminary form (14).

Abbreviations: EthBr, ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthidium bromide); M-IgG, membrane-associated immunoglobulin.

METHODS AND RESULTS

Nature of cytoplasmic DNA

To determine whether nonmitochondrial, cytoplasmic DNA exists in human lymphocytes and to investigate its subcellular localization, cellular DNA was uniformly labeled with [2-^{14}C]thymidine, and cells were lysed with the nonionic detergent NP-40 (15). This detergent lyses the plasma membrane, but not the nuclear membrane, as shown by phase-contrast microscopy; nuclei can be quantitatively removed from lysates by low-speed centrifugation. The lysate obtained after removal of nuclei was separated into several fractions. To ensure removal of any contaminating high molecular weight DNA, material obtained from the individual fractions was selectively extracted with 1% sodium dodecyl sulfate followed by precipitation with 1 M NaCl (16). Linear and supercoiled DNA molecules were then separated in ethidium bromide–cesium chloride (EthBr–CsCl) equilibrium density gradients (17).

Virtually all cytoplasmic DNA was found in a fraction sedimenting at 13,000 × *g* and, of this, less than 10% was supercoiled (Fig. 1). Cytoplasmic DNA represented about 0.5% of the total cellular DNA that incorporated isotope during two cell generations. A small amount of DNA was also found in the fraction sedimenting at 100,000 × *g* (Fig. 1), but its unusually high density in EthBr–CsCl suggested its association with more dense molecules such as RNA. The nature of this high-density DNA is being investigated; it will not be considered in this report. DNA was not found in the supernatant fluid from the 100,000 × *g* fraction (Fig. 1).

DNA from peak fractions (Fig. 1, *a–d*) of the EthBr–CsCl gradients was further characterized by velocity sedimentation in CsCl (Fig. 2). Two species of DNA, which sedimented at about 35 S and 16 S, were recovered (Fig. 2*a–c*). The 35S DNA had the expected sedimentation characteristics of mitochondrial DNA, whereas the 16S DNA presumably represented a unique species. A 16S component was not found in the DNA that remained after selective extraction of nuclei (Fig. 2*d*).

Association of DNA with cytoplasmic membranes

Since the bulk of cytoplasmic DNA sedimented at 13,000 × *g*, its association with larger cytoplasmic structures was suggested. To study the nature of the complex, we labeled cellular DNA uniformly with [2-^{14}C]thymidine and lysed the cells with NP-40. Aliquots of cytoplasm were either not treated further, digested with phospholipase C, or treated with sodium deoxycholate and sedimented in linear sucrose gradients. All

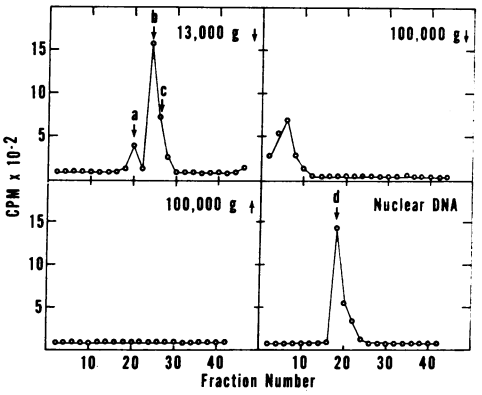

FIG. 1. Equilibrium centrifugation of DNA from cell fractions in EthBr–CsCl density gradients. DNA from 5.0 × 10^8 cells was labeled with ^{14}C for two cell generations as in Table 2. Cells were collected by centrifugation for 2 min at 800 × *g*, washed three times in cold (4°C) Earle's salts, suspended in 2.0 ml of RSB buffer (1.0 × 10^{-2} M NaCl–1.5 × 10^{-3} M $MgCl_2$–1.0 × 10^{-2} M Tris·HCl, pH 7.4) and lysed by the addition of 0.2 ml of 5% NP-40. Nuclei were sedimented from the lysate by centrifugation at 1000 × *g* for 15 min at 4°C. The supernatant fluid was carefully decanted and material sedimenting at 13,000 × *g* (13,000 G ↓) was prepared by centrifugation at 4°C for 15 min. Material sedimenting at 100,000 × *g* (100,000 G ↓) was prepared from the 13,000 × *g* supernatant by centrifugation for 60 min at 4°C. The final supernatant fluid was decanted (100,000 G ↑). Nuclei and the 13,000 G ↓ and 100,000 G ↓ fractions were suspended in 1.5 ml of STE buffer (1.0 × 10^{-1} M NaCl–1.0 × 10^{-3} M EDTA–5.0 × 10^{-2} M Tris·HCl, pH 7.4) and made 1% in sodium dodecyl sulfate. Each cell fraction was selectively extracted (16). Supernatant fluids were centrifuged in 3.0 ml of CsCl (ρ = 1.571) containing 100 μg/ml of EthBr for 48 hr at 31,000 rpm in the SW50L rotor. Fractions were collected and the radioactivity in a 0.02-ml sample of each fraction was measured after precipitation with 5% Cl_3CCOOH. *a*, *b*, *c*, and *d* (*arrows*) indicate fractions that were studied by velocity sedimentation (see Fig. 2).

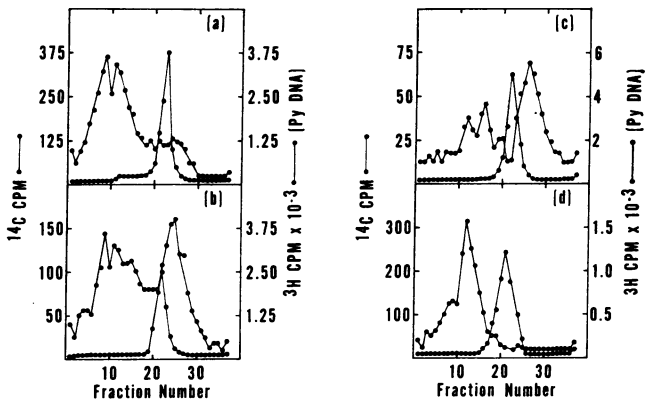

FIG. 2. Velocity sedimentation of DNA from cell fractions. Fractions 19–21 (*a*), 23–25 (*b*), and 26–29 (*c*) from the 13,000 G ↓ DNA, and fractions 18–24 (*d*) from nuclear DNA were collected separately from the EthBr gradients shown in Fig. 1 and dialyzed for 24 hr against 0.001 EDTA–0.02 M Tris·HCl, pH 8.0. A 0.2-ml sample of the DNA in each pool was sedimented through a CsCl–0.01 M EDTA (pH 7.5) solution (ρ = 1.50 g cm^{-3}) for 2.5 hr at 35,000 rpm at 25°C in the SW50.1 rotor. O——O, ^{14}C; ●——●, ^3H-labeled polyoma (Py) DNA marker (20S).

untreated DNA sedimented to the lower third of the gradients, between densities of 1.198 and 1.135 g cm^{-3}, whereas treatment with deoxycholate released 90% of the rapidly sedimenting DNA (Fig. 3). Treatment with phospholipase C caused the DNA to sediment slightly faster and sharpened the band (Fig. 3). The position of sedimentation of the DNA–membrane complex varied depending on the particular lot, or the exact concentration, of NP-40 used, relative to the number of cells. The optimum concentration of NP-40 for preservation of the rapidly-sedimenting DNA–membrane complex varied between 0.1 and 0.5% for 5.0 × 10^8 cells suspended in 2.0 ml of RSB buffer (see Fig. 1).

The fact that each cell has IgG associated with its plasma membrane (5) was used to locate the position of sedimentation of plasma-membrane fragments in sucrose gradients. Cells were incubated for 60 min at 4°C with 1 μg of immunospecifically purified, sheep anti-human IgG labeled with ^{125}I. After the sheep antibody had bound to the IgG on the plasma membrane, cells were lysed and the cytoplasmic fraction was sedimented in sucrose gradients. The ^{125}I-labeled sheep antibody bound to plasma membrane fragments sedimented to the same position in the gradients as the DNA (Fig. 3).

The above findings suggested that DNA was bound to the plasma membrane. To confirm this, we labeled DNA uniformly with [2-^{14}C]thymidine and prepared the plasma membranes by both the fluorescein mercuric acetate and zinc chloride methods (18). These preparations contained mainly intact plasma membranes, without morphologic evidence for contamination with other subcellular structures. Plasma membranes prepared by both methods were associated with CCl_3-COOH-precipitable radioactivity. The nature of the DNA–membrane association was investigated as shown in Table 1. DNA can be quantitatively released by deoxycholate and pronase, suggesting that the DNA is on the surface of the membrane, since it is completely susceptible to DNase. The

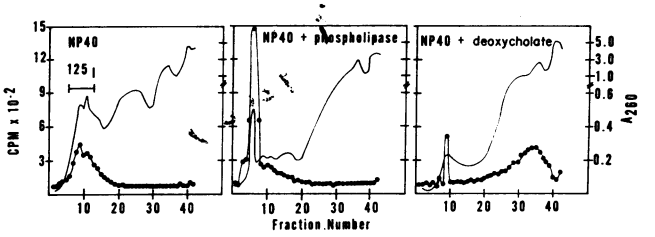

Fig. 3. Velocity sedimentation of the DNA–membrane complex. The DNA from 6.0 × 10^8 cells was uniformly labeled with ^{14}C for two cell generations as in Table 2. Cells were collected and lysed by NP-40, and nuclei were removed as described in Fig. 1. Aliquots of the supernatant fluid were either not treated further, made 1% with deoxycholate, or treated with phospholipase C (200 μg/ml) at 37°C for 30 min. In a parallel experiment, 6.0 × 10^8 cells were washed three times in Earle's salts at 4°C and resuspended in 50 ml of Earle's salts containing 2.0 × 10^{-6} g of immunospecifically purified, sheep anti-human IgG labeled with ^{125}I (1.0 × 10^6 cpm/1.0 × 10^{-6} g protein). Cells and antibody were incubated at 4°C for 60 min, sedimented at 800 × *g* for 2 min, and washed three times in cold (4°C) Earle's salts. Cells were lysed and nuclei removed as described above. Lysates were sedimented at 95,400 × *g* for 45 min through a 36-ml 7.5–45% (w/w) linear sucrose gradient in the SW27 rotor at 4°C. Fractions were collected through a Gilford recording spectrophotometer (0.5-cm light path) and radioactivity was determined. —A_{260}; ●—● cpm ^{14}C; |—| position of sedimentation of ^{125}I.

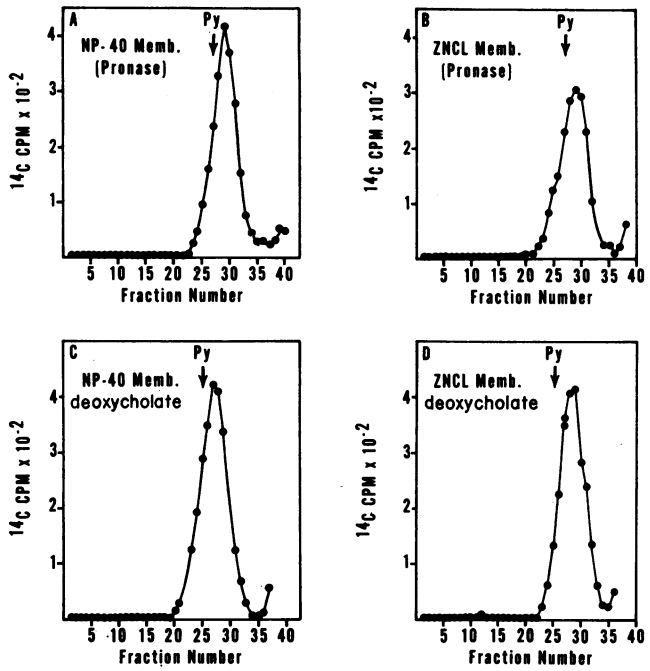

FIG. 4. Sedimentation velocity of membrane-associated DNA. Purified plasma membranes ($ZnCl_2$) or membrane fragments from NP-40 lysates were prepared. Aliquots were treated with either deoxycholate (1%) or pronase (500 μg/ml) for 60 min at 37°C; 0.2 ml was layered onto 3.0 ml CsCl (1.50 g cm^{-3}) and centrifuged for 95 min at 114,000 × *g* at 25°C. Fractions were collected and the acid-precipitable radioactivity was determined. Purified polyoma (Py) DNA (20S) was used as a marker.

fact that the DNA can be released from the membranes by pronase, but not by phospholipase C (Fig. 3), suggests a protein–DNA linkage in the membrane.

The DNA released from purified plasma membranes ($ZnCl_2$ method) or from membrane fragments obtained after lysis of cells with NP-40 was compared and characterized with respect to sedimentation equilibrium in EthBr–CsCl, neutral and alkaline CsCl, and sedimentation velocity. No supercoiled DNA was detected. DNA released from membrane fragments had a buoyant density in CsCl equal to that of nuclear DNA (1.699 g cm^{-3}). The density for membrane-associated DNA corresponded to a mole fraction of guanine plus cytosine (G + C) of 40% in good agreement with the results found in human-spleen-cell DNA (19). When nuclear and membrane-associated DNA were centrifuged to equilibrium in CsCl (pH 12.4), they both banded as a single species, with a buoyant density of 1.756. DNA released by either deoxycholate or pronase from purified membranes or membrane fragments sedimented with a mean sedimentation coefficient of 16 (Fig. 4). This would correspond to a molecular weight for a linear DNA molecule of about 3.0 × 10^6. By contrast, nuclear DNA deliberately sheared by passage 10 times through a 22-gauge needle sedimented as a band at 23 S.

TABLE 1. *Release of plasma-membrane-associated DNA*

Treatment	Per cent released from membrane: Total released	Per cent released from membrane: Acid-precipitable released
Deoxycholate	96	100
Pronase	99	100
DNase	99	2
RNase	<2	...

The DNA from 5.0 × 10^8 cells was uniformly labeled for three cell generations with [2-^{14}C]thymidine, and purified plasma membranes were prepared by the $ZnCl_2$ method. Equal aliquots of membrane fragments were resuspended in RSB buffer (see Fig. 1) and were treated for 45 min at 37°C with either deoxycholate (1%), pronase (600 μg/ml), DNase (200 μg/ml), or RNase (80 μg/ml). After treatment, membrane fragments were sedimented at 6000 × *g* for 15 min, washed once in buffer, and resedimented. The radioactivity remaining with membranes and in the supernatant fluid was measured before and after precipitation with 5% Cl_3CCOOH.

Synthesis of membrane-associated DNA in synchronized cells

To compare the synthesis of membrane-associated DNA to nuclear DNA in different phases of the cell cycle, we labeled cells uniformly with [2-^{14}C]thymidine and synchronized them in the G_1 phase (15). After release from G_1 arrest, cells were pulse-labeled with [^{3}H]thymidine during the G_1 and S phases, and membrane fragments and nuclei were prepared after lysis of cells with NP-40. The membrane fragments and nuclear DNA were selectively extracted and the DNA was sedimented to equilibrium in EthBr–CsCl gradients in order to remove any possible contamination from supercoiled mitochondrial DNA. The ratio of ^{3}H to ^{14}C incorporated into nuclear and membrane DNA in the G_1 and S phases of the cell cycle was calculated (Table 2). These results indicate that the membrane-associated DNA replicates in both G_1 and S, and that the ^{3}H/^{14}C ratio for membrane-associated DNA is different from that of nuclear DNA in both phases. If a linear rate of incorporation of [^{3}H]thymidine is assumed, and if the incorporation during only 1 hr is considered, then the rate of nuclear DNA synthesis during G_1 is 3% of that in S, whereas

TABLE 2. *Specific activity of plasma-membrane-associated DNA during the cell cycle*

Source of DNA	Phase of cell cycle: G_1 ^{3}H/^{14}C	Phase of cell cycle: S ^{3}H/^{14}C
Nuclei	5	40
Membranes	16	25

500 ml of media was seeded with cells at a concentration of 2.0 × 10^5/ml. The cells were grown in the presence of [2-^{14}C]-thymidine (0.1 μCi/ml, 50 Ci/mmol) to a cell concentration of 2.0 × 10^6/ml. At this point (stationary phase) cells are arrested in the G_1 phase and the rate of total cellular DNA synthesis is only 2–3% of that in S phase (15). To release cells from G_1 arrest, they were resuspended in fresh, warmed (37°C) medium and pulse-labeled with [*methyl*-^{3}H]thymidine (2 μCi/ml, 15 Ci/mmol) for either the first 4 hr of G_1, or for 1 hr at the peak of S (20 hr after release). Nuclei and membrane fragments were prepared after lysis of cells with NP-40; the DNA was selectively extracted (16) and sedimented to equilibrium in EthBr–CsCl gradients for 56 hr at 31,000 rpm in the SW65 rotor. Fractions were collected, and the ratio of ^{3}H/^{14}C counts in the peak was determined after precipitation with Cl_3CCOOH.

cytoplasmic DNA synthesis in G_1 is 16% that of S. The fact that nuclear DNA synthesis in G_1 was only 3% of that in S demonstrates the high degree of cell synchrony obtained. These studies seem to exclude the possibility that the membrane-associated DNA originates from the bulk of nuclear DNA. Similar differences between the replication pattern of membrane-associated DNA and nuclear DNA are obtained if the DNA from plasma membranes ($ZnCl_2$) is compared to nuclear DNA, or if DNA is not selectively extracted prior to study.

Electron microscopy of plasma-membrane-associated DNA

To visualize membrane-associated DNA, samples were prepared by a modification of the spreading procedure of Kleinschmidt and Zahn (20) and were examined in the electron microscope. DNA molecules are clearly associated with membranes (Fig. 5,*A–D*). Although some DNA molecules appeared to dissociate from membranes suspended in 1.0 M ammonium acetate, little or no dissociation occurred with membranes suspended in NaCl–Mg–Tris (see Fig. 1). Every membrane fragment of the about 1000 examined was associated with DNA. When purified plasma membranes were treated for 30 min with deoxyribonuclease (50 μg/ml), no DNA was seen (Fig. 5*E*). DNA was not usually visible unless membranes were treated with formamide. This suggests that the DNA is part of the membrane and not simply "stuck" in a random fashion to the membrane surface.

To determine whether membrane-associated DNA was predominantly linear or circular, DNA released by treatment of membranes ($ZnCl_2$) with sodium dodecyl sulfate was examined. More than 99% of the molecules seen were linear. Occasionally, "mini" circles similar to those described by Radloff *et al.* (17) were observed.

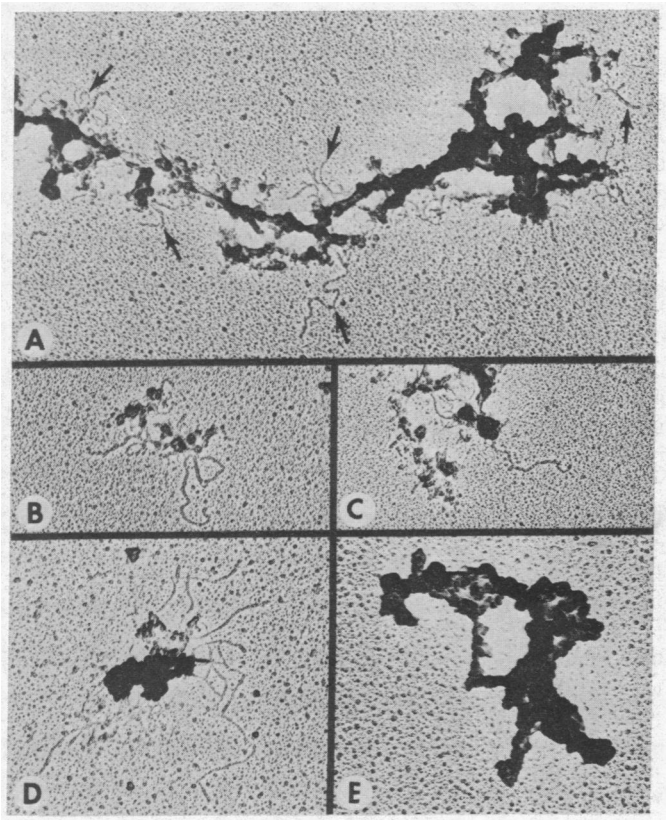

FIG. 5. Electron micrographs of plasma membranes. Samples for electron microscopy were suspended in either RSB (see Fig. 1) (*A, B, C, E*) or 1.0 M ammonium acetate, pH 5.5 (*D*) and made 42% with formamide and 0.01% with cytochrome *c*. The solutions were spread on a hypophase of 0.3 M ammonium acetate (pH 5.5) and the films were allowed to age for 10–15 min. The films were transferred to carbon-coated collodion support films on 200-mesh copper grids and rotary shadowed (120 rpm) with platinum. DNA molecules are seen associated with membrane fragments (*arrows, A; B–D*) (×14,800). No DNA molecules are seen when membranes were treated with deoxyribonuclease (*E*).

DISCUSSION

Evidence for the association of DNA with the plasma membrane of diploid human lymphocytes has been presented. Since this DNA represented about 0.5% of total cellular DNA, a major problem was to demonstrate that contamination of plasma-membrane preparations with nuclear, mitochondrial, mycoplasmal, or viral DNA did not account for its presence.

First, the cell line studied is in many ways suitable for analysis of plasma-membrane-associated DNA. These cells have very little endoplasmic reticulum and few lysosomal structures (21, 22). Thus, contamination with extraneous membraneous structures or lysosomal enzymes (e.g., nucleases) is minimized.

Contamination from nuclear DNA, while not ruled out, is unlikely. The use of NP-40 to lyse cells, sucrose gradient centrifugation to remove any nuclei not sedimented at 1000 × *g*, and selective extraction to remove any high molecular weight DNA is a combination of methods that efficiently removes free nuclear DNA. This fact is illustrated by our failure to detect any DNA in the 100,000 × *g* supernatant. Furthermore, neither deliberate shear nor selective extraction of nuclear DNA from these cells gave DNA as small as the species associated with plasma membranes. The specific activities of membrane-bound and nuclear DNA and the different time of synthesis during the cell cycle would exclude any random "leak" of nuclear DNA.

The probability that we have isolated a DNA replication complex that is membrane-associated from nuclei is unlikely; relative to nuclear DNA, more membrane-associated DNA was synthesized in G_1 than in S. Also, in S, the specific activity of pulse-labeled membrane-associated DNA was less than total nuclear DNA; this is the opposite of what would be expected of DNA from a replication complex. The possibility that a unique species of DNA is synthesized in the nucleus and exits to associate with the plasma membrane cannot be excluded; this would be of considerable interest.

Contamination from mitochondrial DNA seems unlikely, since few circular and no supercoiled molecules are detected in DNA released from membranes. Furthermore, the buoyant density of our membrane-associated DNA in neutral and alkaline CsCl is different than mitochondrial DNA. The density of human mitochondrial DNA in neutral CsCl differs from nuclear DNA by 0.007 g cm^{-3}, whereas in alkaline CsCl the complementary strands of mitochondrial DNA separate into "heavy" and "light" components, with respective densities of 1.766 and 1.727 g cm^{-3} (23). By contrast, the membrane-associated DNA described here has a density in neutral CsCl equal to that of nuclear DNA (1.699 g cm^{-3}), and in alkaline CsCl only a single component is observed.

Contamination from mycoplasmal DNA can be excluded. These cells were shown repeatedly by electron microscopy to be free from structures that resemble mycoplasma (21). Mycoplasma could not be cultured from these cells in attempts by Dr. Mary Pollock of this institution. Furthermore, the G + C content and size of this DNA are quite different from almost all mycoplasma (24–26), particularly those known to contaminate cell cultures.

The possibility that these cells carry a latent viral genome is more difficult to exclude. The cells were repeatedly studied by electron microscopy and serologic methods, and no evidence for morphological structures resembling virus or production of viral antigens was obtained (21, 22). Also any viral genome carried would have to have the same G + C content as host nuclear DNA and to be distributed throughout the plasma membrane.

Finally, the fact that 100% of the membrane fragments obtained from purified plasma membrane preparations can be shown to be associated with DNA makes it unlikely that all the DNA studied originates from other contaminating DNA–membrane complexes. For example, if we argue that our preparations contain 10% mitochondrial DNA complexes, then 90% of membranes should have been free from DNA. A similar argument can be made against DNA–membrane complexes of nuclear or mycoplasmal origin.

Although the function or precise origin of this plasma-membrane-associated DNA are unknown, several speculations in relation to immunogen recognition can be considered. If an analogy to the replicon model of Jacob (27, 28) is made, then union between the IgG receptor in the plasma membrane and the immunogen might cause a conformational change in the membrane that allows the DNA to either replicate or be transcribed. The result of replication could be gene amplification, which may accompany lymphocyte differentiation (29), whereas initiation of transcription might result ultimately in the synthesis of a regulatory polypeptide. Another possibility is that plasma-membrane-associated DNA is involved in the transfer of genetic information from cell to cell (e.g., thymus to bone marrow); in this sense, it would be similar to colicinogenic and F factors in bacteria (30).

An interesting relationship to consider is the ratio of membrane-associated DNA to IgG in the plasma membrane. The cell line studied has 1.8×10^4 membrane-associated molecules of IgG per cell (5). If one assumes 1.0×10^{-11} g of DNA per diploid cell, 0.5% of which is associated with the plasma membrane, then there is about 1 molecule of DNA (3.0×10^6 daltons) for each molecule of membrane-associated IgG. DNA of 3.0×10^6 daltons is sufficient to code for the synthesis of about 1700 amino acids. This is larger than is necessary for the synthesis of the IgG molecule.

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