A Nuclear Membrane-Associated DNA Complex in Cultured Mammalian Cells Capable of Synthesizing DNA in Vitro†

Anthony A. Infante,‡* William Firshein, Peter Hobart, and Louann Murray

ABSTRACT: A DNA-nuclear membrane complex has been isolated by two different methods from the nuclei of cultured mouse fibroblast (3T3) cells. One method, utilizing the detergent sarkosyl (sodium lauroyl sarkosinate), yields a DNA-nuclear membrane complex (the M band), which contains virtually all of the DNA in the nuclei. However, treatment of the M band by sonication, vortexing, or freeze-thaw reduces the amount of DNA in the complex by approximately 50–80%, depending upon the phase of the cell cycle from which the complex was extracted. The remaining DNA is tightly bound to the nuclear membrane and resists further shearing procedures. Over 90% of the choline-labeled phospholipid present in nuclei is also found in these sheared M bands. The percentage of DNA associated with the nuclear membrane varies during the cell cycle and correlates well with the onset, continuation, and cessation of DNA synthesis. Thus, although DNA-membrane complexes can be detected throughout the cell cycle, the percentage of DNA bound to membrane increases during late G_1 and S and decreases during G_2. In addition, there are distinct qualitative differences in the type of DNA present in the membrane fraction, with a more highly d(A-T) rich DNA being present in confluent (G_0) cells than in cells during the S phase. This d(A-T) rich DNA may be related to the mouse satellite DNA identified by others. The M band can be separated into two DNA-nuclear membrane subfractions by centrifugation through a continuous sucrose gradient. The relative proportions of these two subfractions depend upon the percentage of sarkosyl present in the M band prior to centrifugation, with complete removal of sarkosyl resulting in a very large increase in the sedimentation velocity of the complex and in the formation of only one fraction. Evidence that this is a complex of DNA with membrane is given by the finding that DNA is dissociated from the complex with Pronase, deoxycholate, or high levels of sarkosyl. Removal of virtually all of the DNA with DNase from this rapidly sedimenting complex does not dissociate any of the phospholipid which still sediments rapidly as a single band. A second method, which yields a DNA-membrane fraction from nuclei, utilizes sedimentation of lysed nuclei to equilibrium in CsCl density gradients. This low-density CsCl fraction contains only 10–15% of the total DNA, but contains most of the nascent DNA, which may be chased into a membrane-free fraction. The DNA-membrane fraction from CsCl gradients possesses properties in common with the M-band fraction and can be converted into an M band. DNA membrane complexes from sucrose gradients, as well as the crude M-band preparation and a non-membrane-associated DNA fraction from nuclei can synthesize DNA in vitro without the addition of an external DNA template or DNA polymerase. In contrast to the activity in the non-membrane-associated DNA fraction, the membrane-associated polymerase activity is strongly stimulated by adenosine triphosphate and is unaffected by ethidium bromide. A separation of different DNA polymerases into the non-membrane- and membrane-associated DNA fractions is therefore indicated.

Considerable evidence has accumulated to suggest that DNA replication in procaryotes occurs in association with the cell membrane. For example, DNA-membrane complexes have been extracted from a variety of bacteria (Sueoka and Quinn, 1968; Tremblay et al., 1969; Firshein, 1972; Smith et al., 1970; Knippers and Stratling, 1970; Kornberg et al., 1974). These complexes contained all or most of the newly synthesized DNA (Tremblay et al., 1969; Firshein, 1972), as well as small DNA fragments involved in the initial stages of DNA replication (Firshein, 1972). In some cases, it has been shown that such DNA-membrane complexes are capable of DNA synthesis in vitro (Smith et al., 1970; Knippers and Stratling, 1970; Firshein, 1972) and in Pneumococcus it contains a complex of enzymes which also synthesizes DNA precursors (Firshein, 1972).

In eucaryotic systems, the evidence that DNA replication is associated with the nuclear membrane is much more controversial. Some biochemical and cell-fractionation studies have suggested the existence of a DNA-nuclear membrane complex (Freienstein et al., 1973; Barrieux et al, 1973; Yamada and Hanaoka, 1973; Hildebrand and Tobey, 1973; Infante et al., 1973; Cabradilla and Toliver, 1975) and that replicating cells contain a greater amount of membrane-bound DNA than quiescent cells (Hildebrand and Tobey, 1973). However, other studies suggest that such DNA-nuclear membrane complexes may be either the results of artifacts during preparation (Fakan et al., 1972; Huberman et al., 1973), true complexes but not the site of DNA replication in the nuclei (Comings and Okada, 1973; Franke et al., 1973), or only partly concerned with such replication (O'Brien et al., 1973).

Most of the cell-fractionation studies in eucaryotes used only one method of extraction and failed to determine whether a DNA-nuclear membrane complex could synthesize DNA in vitro. The use of separate methods of extraction, as well as a determination of the synthetic capabilities of the isolated DNA nuclear membrane complex, is extremely important in establishing the significance of the complex as a natural component of DNA replication. In this study, we have analyzed synchronized 3T3 fibroblast cells with the view of determining: (1) whether DNA-nuclear membrane complexes can be ex-

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tracted by two distinctly separate methods, (2) whether newly synthesized DNA is present in these complexes, (3) whether changes occur in such complexes during the cell cycle, and (4) whether DNA can be synthesized in vitro without addition of an external DNA template or enzymes.

Materials and Methods

(*a*) *Cell Lines and Media.* Embryonic Swiss mouse fibroblast 3T3 cells were purchased from the American Type Culture Collection. They were maintained in Falcon plastic tissue culture flasks in Dulbecco's modified Eagle's medium made from Gibco-powdered media, supplemented with 10% fetal calf serum (Gibco), 20 mM Hepes[1] buffer, 2.2 g/l. of sodium bicarbonate, 2.5 μg/ml of Fungizone, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (designated D-FC10). Cultures were grown in a humidified 5% CO_2 atmosphere, at 37 °C, using a New Brunswick CO_2 incubator. For experiments, approximately 5×10^5 cells were inoculated into 10 ml of D-FC10 in 100-mm Falcon plastic petri dishes. After 3 or 4 days, the medium was replaced with 10 ml of fresh D-FC10 and incubation was continued at 37 °C until the cells reached confluence.

(*b*) *Synchronization of 3T3 Cells.* After reaching confluency, the plates of 3T3 cells were medium changed with D-FC50 (50% serum). Cells underwent a round of DNA synthesis 18–20 h later, for a period of 6 h. Autoradiographic analysis of the cells pulse labeled at 22 h after the medium change showed that 50–60% of the nuclei were engaged in DNA synthesis. For M bands (see below), cells in S phase were collected 20–25 h after the medium change; cells in G_2 phase were collected 27–28 h after the medium change. G_0 cells were labeled during log phase and collected when confluent.

(*c*) *Labeling of Cells.* Continuously labeled DNA was obtained by adding 0.1 μCi/ml of [^{3}H]thymidine (New England Nuclear Corp., 20 Ci/mmol) to cells in log phase for 2–4 generations. For pulse-chase experiments, the plates were pulsed for 0.5–10 min with 1 or 10 μCi/ml of [^{3}H]thymidine. The labeled medium was removed, plates were rinsed twice with D-FC10 containing 3×10^{-5} M unlabeled thymidine, and D-FC50 plus 3×10^{-5} M thymidine were added for the chase. Membrane components were labeled with 0.02 μCi/ml of [*methyl*-^{14}C]choline chloride (New England Nuclear Corp., 30–50 mCi/mmol).

(*d*) *Isolation of Nuclei.* Enough cells were collected to yield approximately $1-2 \times 10^6$ nuclei. The medium was discarded and the culture surface was rinsed twice with ice-cold nuclear isolation buffer consisting of 0.01 M NaCl, 0.003 M $MgCl_2$, and 0.01 M tris(hydroxymethyl)aminomethane adjusted to pH 7.4 (Penman, 1966). All further operations were carried out at 4 °C. The cells were scraped off with a rubber policeman into isolation buffer, washed one more time by centrifugation, resuspended in 3 ml of isolation buffer, and allowed to swell for 15–25 min. The cells were then subjected to a vigorous homogenization in a close-fitting Dounce homogenizer. The nuclei were pelleted at 1000*g* and washed with 10 ml of TMK buffer (0.01 M Tris, 0.005 M $MgCl_2$, 0.1 M KCl, adjusted to pH 7.4) and then resuspended in 1.0 ml of TMK buffer for each gradient desired.

(*e*) *Detection of DNA-Nuclear Membrane Complexes.* Formation of M Bands. The first method involved the use of sodium lauroyl sarkosinate (sarkosyl; Geigy Chemical), which, in the presence of Mg^{2+}, coprecipitates with membrane-associated DNA at a density greater than that of "free" DNA producing the so-called M band and top fraction (the remaining nuclear extract) (Tremblay et al., 1969). To each ml of nuclear suspension, 3% sarkosyl was added rapidly to yield a 0.15% solution. Where indicated, samples were then immediately subjected to vortex shear in 10-s bursts or to sonication using a Branson sonifier, usually for 20 s at a setting of 2.5. The tubes were then maintained at 4 °C for 5 min to complete sarkosyl-Mg^{2+} crystal formation. One milliliter of the nuclear lysate was layered onto a discontinuous sucrose gradient consisting of 5.5 ml of 60% sucrose (w/v) and 5.5 ml of 15% sucrose (w/v) in TMK buffer. The gradients were centrifuged in a Beckman Spinco SW 41 rotor at 18 000 rpm for 18 min (4 °C). Ten fractions were usually collected from the top of each gradient with a syringe fitted with a no. 18 gauge canula. Radioactivity in each fraction was determined by Cl_3CCOOH precipitation and collection on glass-fiber filters, which were then washed with cold 5% Cl_3CCOOH, dried, and counted in toluene-based scintillation fluid. A typical distribution of DNA in the M band and top fractions is shown in the insert of Figure 1.

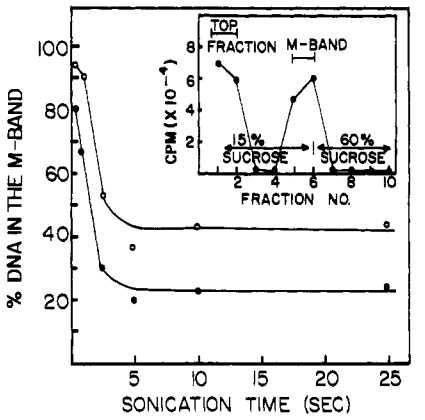

FIGURE 1: The effect of sonication on the isolation of sarkosyl DNA-membrane complexes from nuclei of 3T3 cells. Nuclei were prepared from density-inhibited (G_0) cells (●), and from synchronized cells in the S phase (24 h after medium change) of the cell cycle (○). Conditions for labeling the DNA, preparation of the sarkosyl M bands, and for sonication are given under Methods. The percentage of DNA present in the M band after the various times of sonication was determined from sucrose gradients. A typical "M-band gradient" showing the distribution of DNA from nuclei of 3T3 cells in the S phase of the cell cycle is given in the insert.

Buoyant Density Method. Nuclei prepared by the method described under part d were disrupted by three rapid freeze-thawing cycles in dry ice–acetone or sonicated for 20 s in the Branson sonifier at a setting of 2.5. Nuclear lysate (0.5 ml) was layered onto a partially preformed CsCl (4 M) gradient, which was prepared by layering 2.2 ml of 2 M CsCl in TMK on top of 2.2 ml of 6 M CsCl (Stone, 1974). The tubes were inverted twice slowly to form the gradient and then centrifuged in an SW 50.1 rotor at 35 000 rpm at 15 °C for 16 h. Fractions were collected and radioactivity in Cl_3CCOOH precipitates was determined as described above.

(*f*) *In Vitro Synthesis of DNA by the M Band and Its Subfraction.* Sonicated M bands and top fractions prepared by the method described under part e were dialyzed for 15 h against 500 volumes of TGA buffer (0.02 M Tris-HCl, pH 7.8, 20% (w/v) glycerol, 0.1% bovine serum albumin) and assayed for enzymatic activity or centrifuged in the 30–60% sucrose gradient prior to assaying for activity. For DNA polymerase

[1] Abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ATP, CTP, GTP, TTP, adenosine, cytidine, guanosine, and thymidine triphosphates.

assays, 0.2 ml of each M band (containing approximately 0.35 mg of protein) or top fraction (containing approximately 0.9–1 mg of protein) was incubated in a total volume of 0.3 ml containing: Tris 4 μmol, pH 8.0; $MgCl_2$, 5 μmol; KCl, 17 μmol; dithiothreitol, 0.5 μmol; ATP, 2 μmol; dCTP, dGTP, dATP, 0.06 μmol each; [^{3}H]dTTP (Schwarz/Mann) 0.50 μCi, 0.01 μmol. Controls were prepared in which the top or M-band fractions were either boiled (5 min) before addition of assay components or added after Cl_3CCOOH precipitation. The assay solutions were incubated for various periods of time up to 12 min. At each time period, an equal amount of cold 10% Cl_3CCOOH containing 1% sodium pyrophosphate was added to stop the reaction and the level of acid-insoluble radioactivity (indicating DNA synthesis) was determined.

Results

Isolation of DNA–Membrane Complexes from 3T3 Cells by Two Different Methods. Preparation of Sarkosyl M-band Fractions. Our approach to the isolation of DNA–membrane complexes from mammalian cells follows from our experience with another eucaryotic system, the developing sea urchin embryo (Infante et al., 1973). We have chosen to first isolate nuclei to avoid contamination of our preparation with mitochondrial DNA–membrane complexes (Yoshikawa-Fukada and Ebert, 1971) and to eliminate the bulk of the cellular protein and RNA. Generally, no special attempt was made to use highly purified nuclei but, in all cases, they were relatively free of cytoplasmic debris, as observed by phase microscopy.

When the sarkosyl M-band procedure used for sea urchin nuclei was applied directly to 3T3 cells, the resulting distribution of DNA was quite different in the two systems. In the dividing sea urchin embryo, only 10–30% of the DNA was present in the M band, whereas in 3T3 cells almost all of the DNA sedimented in the M-band fraction. We also obtained the latter result with L cells and WI38. This is in agreement with the work of others using mammalian cells (Freienstein et al., 1973; Hanaoka and Yamada, 1971). It has been shown that shearing procedures, such as vortexing, reduce the amount of DNA in the sarkosyl M-band preparations (Yamada and Hanaoka, 1973; Hildebrand and Tobey, 1973). We have confirmed this observation and have found that three procedures are suitable. These are: vortexing, sonication, and freeze-thaw. Figure 1 presents the effects of sonication on the percentage of DNA associated with the M-band fraction of cells in G_0 and in the S period of the cell cycle. Cells were grown for several generations in the presence of [^{3}H]thymidine to uniformly label the DNA. They were then allowed to become contact inhibited and one sample of cells was used directly in preparing an M band (G_0 cells). Another sample of cells was "medium changed" with 50% serum to synchronize the cells. Figure 1 shows that the percentage of DNA in the M band is very high for both the G_0- and S-phase cells when no shearing procedure was used. However, when the nuclei obtained from these cells were sonicated after addition of sarkosyl, more than half the DNA was removed from the final M band within 5 s of sonication. A significantly greater amount of DNA remains associated with the M band when the intensity of sonication is less than one-half that used in this study. However, if the intensity of sonication were doubled, or if the length of time of sonication were greatly increased (up to 25 s in Figure 1), little or no additional DNA could be dissociated from the complex. Thus, a limiting amount of DNA appears to be tightly bound to the complex. We have used 20 s for most of our studies when sonication was employed.

Figure 1 also shows that the limiting percentage of DNA in the M band is related to the cell cycle. There is approximately two times more DNA present in the M band from nuclei of S-phase cells compared to G_0 cells. This observation will be discussed in greater detail below. Finally, the insert of Figure 1 shows a typical distribution of DNA in an "M-band gradient" for the sonicated nuclei of S-phase cells. The two main fractions, M band and "top fraction" obtained with this technique are indicated.

In some experiments, nuclei were extensively cleaned by washing with Triton X-100 at either 0.1 or 0.5%. The "nuclear" structure that remains after this treatment did not yield an M-band fraction. Electron microscopy of nuclei treated in this way revealed that both the outer and inner membranes had been removed (cf. Barton et al., 1971). These observations support the conclusion that membrane is an integral component in the M-band fraction. It remains to be determined if this is contrary to the claim of Berezney and Coffey (1975) that DNA is attached to the non-membrane protein matrix of rat liver nuclei.

Preparation of CsCl Low-Density DNA–Membrane Fraction from Nuclei. We have reported that pulse-labeled DNA is preferentially associated with the DNA–nuclear membrane complex in the M band of sea urchin nuclei (Infante et al., 1973). To verify this in mammalian cells and to examine the utility of a different procedure for isolating DNA–nuclear membrane complexes, we examined the distribution of 3T3-cell DNA in CsCl density gradients. Under these conditions, the reduced buoyant density of a complex between DNA and membrane should permit such complexes to be readily separated from free DNA. In the experiments represented by Figure 2, 3T3 cells were grown to confluence, synchronized by medium change, and incubated for 22 h at 37 °C. At this time, two replicate cultures were pulsed for 5 min with [^{3}H]-thymidine followed in one replicate by a 30-min chase with nonlabeled thymidine. A third culture was pulsed continuously for 30 min with [^{3}H]thymidine. After the pulses or pulse and chase, nuclei were isolated, lysed by freeze-thaw, and centrifuged in CsCl gradients as described under Methods. Figure 2 shows the distribution of labeled DNA in CsCl gradients for the 5-min pulse and the 5-min pulse–30-min chase preparations. It can be seen that the DNA is clearly separated into two main fractions, one being very close to the bottom of the gradient and another fraction present near the top of the gradient. We have designated these fractions as the CsCl high (H)- and low (L)-density fractions, respectively. Since the density range in these gradients is from 1.40 to 1.55 g cm^{-3}, the DNA at the top of the gradient must be associated with material of density considerably less than 1.40 g cm^{-3}. Also, this complex, which may be tentatively identified as DNA with membrane, is clearly of sufficient strength to resist dissociation by the high-ionic conditions present in the gradient. The DNA in the CsCl high-density fraction sediments with purified DNA (added as a marker in several runs) and is largely free of membrane as determined by choline labeling (see below).

The distribution of labeled DNA between these two fractions is strongly dependent upon the length of labeling time (insert of Figure 2). Approximately one-half of the DNA synthesized during a 5-min pulse is present in the low-density fraction. However, when the 5-min pulse is followed by a 30-min chase, or if the incubation of the cells in the presence of labeled thymidine is continued for 30 min, the percentage of labeled DNA in the low-density fraction decreases to about 20%. We have always observed that only about 10% of the total bulk DNA in log growing cells is present in the low-density

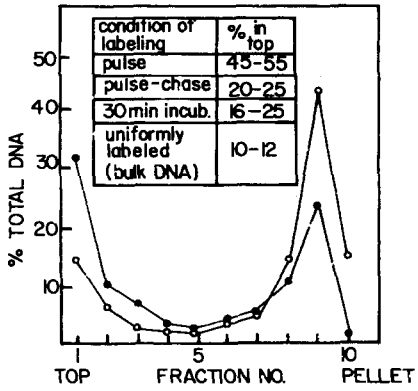

FIGURE 2: Association of pulse-labeled DNA with a low-density fraction in CsCl gradients. Synchronized 3T3 cells in the S phase were incubated with [^{3}H]thymidine, the nuclei were prepared and lysed by freeze-thaw, and the lysate was centrifuged to equilibrium in CsCl gradients as described under Methods. The distributions of DNA in two parallel gradients are shown. (●) 5-min pulse; (○) 5-min pulse followed by a 30-min chase in unlabeled thymidine. The insert gives the percentage of the total labeled DNA present in the low-density fraction for various labeling conditions.

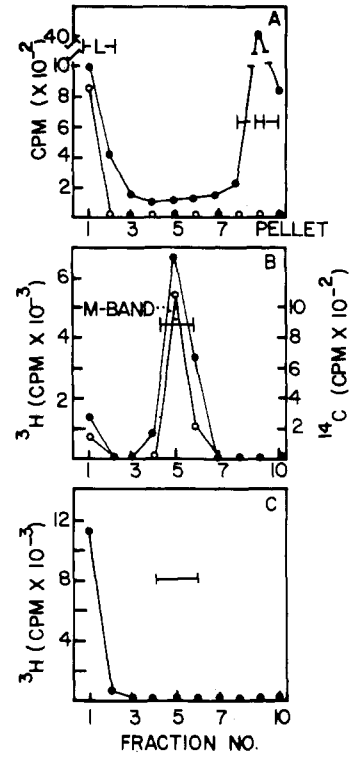

FIGURE 3: Relationship between the low-density component of CsCl gradients and the sarkosyl M-band fraction. (A) Distribution of 1-h labeled DNA and phospholipids on a CsCl gradient. (B) Fraction L, as indicated in A, was isolated from a CsCl gradient, dialyzed, made up to 0.15% sarkosyl in TMK buffer, and then centrifuged in an M-band sucrose gradient as described under Methods. (C) Fraction H from A treated with sarkosyl in same way as described for fraction L. (●) [^{3}H]DNA; (○) [^{14}C]phospholipids.

fraction. Thus, it appears that the CsCl low-density fraction is enriched with newly synthesized DNA, which may be chased into the free or bulk DNA fraction. These results are consistent with the possibility that the CsCl low-density fraction is analogous to the sarkosyl M-band fraction, where nascent DNA is preferentially located (Infante et al., 1973; Hildebrand and Tobey, 1973; Yamada and Hanaoka, 1973), and that the CsCl low-density fraction may consist of a DNA-nuclear membrane complex containing the sites for DNA replication. We will now present evidence indicating that this CsCl fraction is, in fact, operationally equivalent to the M band.

Synchronized 3T3 cells were incubated with [^{3}H]thymidine and [^{14}C]choline for 1 h after most of the cells had entered the S phase (22–23 h after medium change). Nuclei from these cells were disrupted by three freeze-thaw cycles and centrifuged to equilibrium in CsCl gradients. The distribution of labeled DNA and lipid material is given in Figure 3A. There is approximately 26% of the DNA and over 90% of the choline-labeled lipid located at the top of the CsCl gradient. In all of our experiments, radioactive choline was incorporated into lipids (presumably phospholipids), as tested by its complete extraction with chloroform–methanol (1:2). The low (L)- and high (H)-density CsCl fractions were isolated as indicated in Figure 3A, dialyzed for 24 h against TMK buffer, and then treated with sarkosyl and subjected to an M-band gradient centrifugation as described in Figure 1. In the case of the low density fraction, over 80% of the DNA and choline-labeled lipids sedimented as a sarkosyl M band (Figure 3B). In contrast, the high-density fraction from CsCl yielded no distinct M band when subjected to the same analysis (Figure 3C), with virtually all of the DNA remaining at the top of the M-band gradient. This behavior of the DNA from the CsCl high-density fraction is identical with that obtained when purified DNA is displayed on such M-band gradients. Therefore, it appears from these studies that we are dealing with an association of DNA with membranous material in both the low-density fraction of CsCl and in the M-band fraction. Further evidence for this conclusion is given in the next section.

Nature of the Sarkosyl M Band and CsCl Low-Density Fraction. In addition to the similarities given above, all of the procedures that reduce the amount of DNA in our M-band preparations (see Figure 6) also reduce the amount of DNA in the CsCl low-density fraction. These treatments include digestion with deoxycholate, Pronase, and DNase. For example, treatment of the nuclear lysate with Pronase prior to CsCl centrifugation results in all of the DNA sedimenting in the high-density (free DNA) fraction. Although precise membrane components were not determined in either the low-density CsCl fraction or the M band, all of the above results support the suggestion that these fractions contain a DNA–membrane complex.

To obtain further information concerning the nature of this DNA fraction and to initiate steps for its purification, the M-band complex was isolated and recentrifuged in linear sucrose gradients. These studies were patterned after those with a procaryote (pneumococci) in which similar experiments revealed the existence of two DNA–membrane subfractions (Firshein, 1976). Accordingly, sonicated M-band complexes were extracted from synchronized cells during the S phase after incubation with [^{3}H]thymidine and [^{14}C]choline. The complex was dialyzed for 15 h against the TGA buffer, described under Methods, and centrifuged for 2.5 h in a 30–60% sucrose gradient. Figure 4 shows that, similar to the results with pneumococci, two subfractions of DNA and phospholipid were obtained. In the course of these experiments, we found that a possible explanation for this result was that not all of the sarkosyl is removed during dialysis. The light fraction in Figure 4 could therefore be due to those sites with which sarkosyl remains bound while a sarkosyl-free DNA–membrane complex sediments to the 80% sucrose shelf (heavy fraction).

That this explanation is valid was seen in a series of reconstruction experiments in which the sonicated DNA–membrane

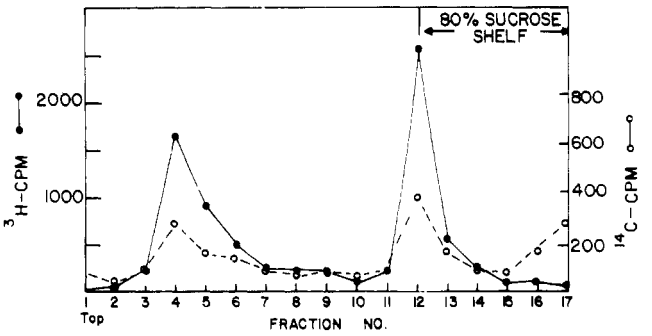

FIGURE 4: Sedimentation pattern of a dialyzed sarkosyl M-band fraction in a sucrose gradient. Synchronized 3T3 cells were labeled 60 min with [^{3}H]thymidine and [^{14}C]choline during the S phase of the cell cycle. The M-band fraction was prepared from nuclei, as described in Figure 1. The isolated M band was dialyzed for 15 h against TGA buffer (Methods, section f) and then 0.5 ml was layered on a 3.3-ml 30–60% (w/v) linear sucrose gradient containing 1.4 ml of 80% sucrose shelf in TMK buffer, as indicated. Centrifugation was for 2.5 h at 40 000 rpm in the Spinco SW 50.1 rotor. Radioactivity assayed by Cl_3CCOOH precipitation. (●) [^{3}H]DNA; (○) [^{14}C]phospholipids.

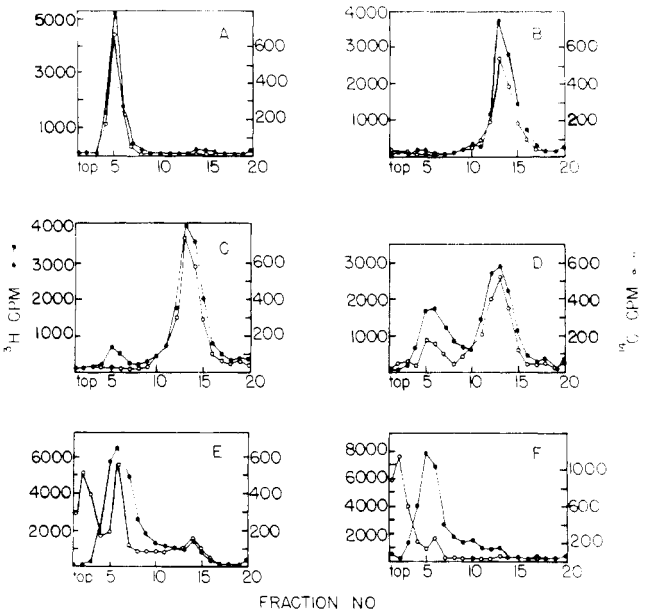

FIGURE 5: Effect of sarkosyl on DNA–nuclear membrane complexes of 3T3 cells. The sarkosyl M-band fraction was prepared as in Figure 4. This fraction, after various treatments, was resedimented in 30–60% sucrose gradients containing an 80% sucrose shelf as in Figure 4. (A) Sedimentation of an undialyzed M band. (B) Sedimentation of M-band fraction after extensive dialysis (24–36 h) against Tris–glycerol–albumin buffer (Methods) to remove sarkosyl. In C–F various concentrations of sarkosyl were added to the dialyzed material in B. (C) 0.01%; (D) 0.05%; (E) 0.1%; (F) 0.2%. Radioactivity is in Cl_3CCOOH-precipitable material; (●) [^{3}H]DNA; (○) [^{14}C]phospholipids.

fraction was dialyzed extensively against TGA buffer and then centrifuged in the sucrose gradient together with various concentrations of sarkosyl. First, Figure 5A shows the position of an undialyzed M-band preparation in this type of gradient. All of the DNA and labeled phospholipid sediment in the position of the light subfraction. After extensive dialysis of this fraction, a dramatic increase in the sedimentation velocity of the complex results, such that all of the radioactive DNA and phospholipid sediments to the 80% sucrose shelf (Figure 5B). Addition of increasing concentrations of sarkosyl (Figure 5C,D) resulted in a shift from this single fraction to two bands, each containing both DNA and phospholipid. At a concentration of 0.05% sarkosyl (Figure 5D), the distribution of DNA and lipid is similar to that obtained when the M-band is dialyzed for a shorter period of time (cf. Figure 4). At higher concentrations of sarkosyl, the heavy band is progressively lost and there is extensive dissociation of the DNA from the phospholipid (Figure 5E,F). The explanation we wish to offer for these results is that the removal of the detergent, sarkosyl, permits fragments of DNA–membrane complexes to coalesce into much larger aggregates. The driving force for this aggregation is presumably hydrophobic lipid–lipid interactions that are blocked when sarkosyl is present. Although electron microscopic examinations (unpublished observations) of the heavy fraction shown in Figure 5 demonstrate the presence of large membranous aggregates, there are currently no discernible discrete features of the complex that are readily evident. In the following section, biochemical evidence is presented that membrane–membrane interactions can explain the more rapid sedimentation of the DNA. DNA polymerase activity is detectable in both the light and heavy fractions in Figure 4.

Effect of Various Degradative Agents on the Sedimentation of the DNA–Membrane Fraction. The doubly labeled DNA–phospholipid fraction sedimenting near the 80% sucrose shelf after extensive dialysis was used to probe the composition and binding properties of the various macromolecules in the complex. Various degradative enzymes or deoxycholate were added to replicate samples of the complex and incubated from 2 to 20 min, and the complexes sedimented in the 30–60% sucrose gradient. Figure 6A,B shows the sedimentation profile of the heavy band after control incubations in the absence of degradative agents. In Figure 6C is shown the effect of deoxycholate on this complex. As in the experiments with high levels of sarkosyl (Figure 5F) and Triton X-100 (data not shown), this detergent also completely dissociates the rapidly sedimenting DNA complex and the phospholipid moiety no longer cosediments with the DNA. Digestion with Pronase (Figure 6D) produced the same extensive dissociation of lipid from DNA and destruction of the rapidly sedimenting complex. On the other hand, ribonuclease (Figure 6E) treatment had no discernible effect on the complex. An important result was obtained when the complex was subjected to DNase digestion (Figure 6F). Under our conditions, virtually all of the labeled DNA was degraded. Despite this lack of DNA, the labeled phospholipids still sedimented as a single band, although its sedimentation velocity was reduced. It is clear therefore that the DNA in our preparations is intimately associated with both lipid and protein components, and the sedimentation properties of this complex are largely determined by the integrity of the protein–lipid interaction. Since we are isolating our DNA fractions from purified nuclei, it is most likely that this interaction of protein and lipid is within the nuclear membrane, and that DNA is associated with this membrane. In this system, RNA does not appear to be involved in the DNA–membrane association. Hildebrand and Tobey (1973) arrived at the same conclusion with Chinese hamster cells.

Variation of DNA–Membrane Association During the Cell Cycle. We have reported that in sea urchin embryos a complex of DNA with the nuclear membrane is readily demonstrated when the embryos are synthesizing DNA, but such a complex is not observed when there is no DNA synthesis (Infante et al., 1973). Since this suggests that the association of DNA with membrane could be involved in the regulation of DNA synthesis, we sought to establish whether the DNA–membrane complex is present at all times in 3T3 cells or if it is formed only

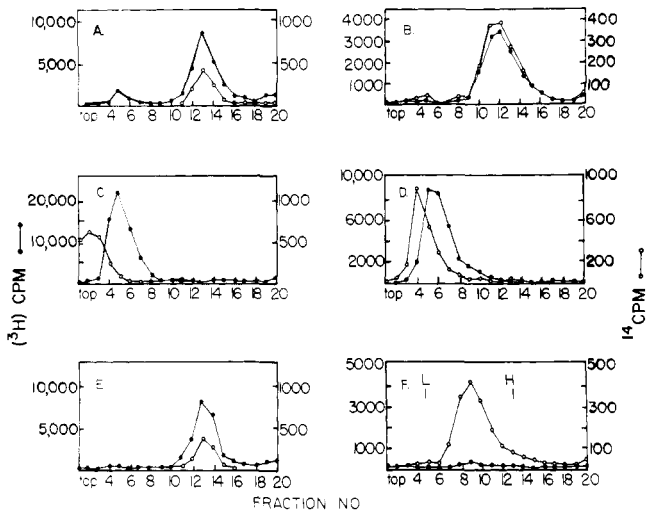

FIGURE 6: Effect of various agents on the sedimentation of the DNA-membrane complex. The same extensively dialyzed M-band fraction, as described in Figure 5B, was used. After the various treatments, the fraction was layered on sucrose gradients and sedimented as in Figure 5. A and B are controls for the treated samples: (A) sedimentation profile of complex after incubation at 20 °C for 20 min without any degradative agent; (B) incubation as in F in the absence of DNase; (C) DNA-membrane complex treated with 0.2% DOC at 20 °C for 2 min; (D) Pronase digestion, 100 μg/ml for 20 min at 20 °C; (E) RNase digestion, 10 μg/ml for 10 min at 20 °C; (F) DNase digestion, the DNA-membrane complex was made 0.005 M in $MgCl_2$ and incubated at 20 °C for 20 min with 20 μg/ml of DNase.

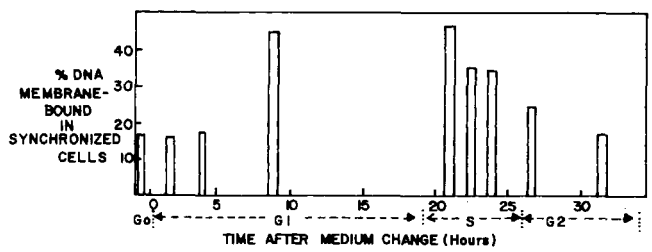

FIGURE 7: Cell-cycle dependent changes in the percentage of DNA associated with membrane. All values were determined as the limiting percentage of DNA associated with M bands after various lengths of sonication time as shown in Figure 1.

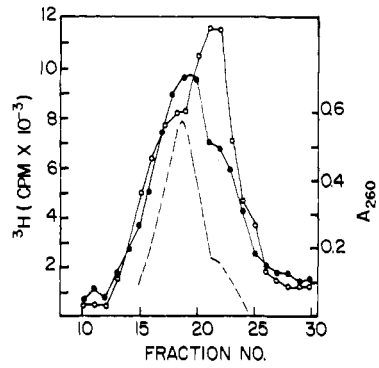

FIGURE 8: Buoyant density distributions of DNA from DNA-membrane complexes of synchronized 3T3 cells. The M-band fraction was isolated, as in Figure 1, from cells whose DNA had been uniformly labeled with [^{3}H]thymidine. The DNA from the M bands were extracted with the UP technique of Britten et al. (1974) using hydroxylapatite. Marker, mouse liver DNA was purified according to Flamm et al. (1972). Neutral CsCl gradients were prepared by adding solid CsCl to 100 μg of mouse liver DNA and 1.2-2.0 × 10^4 cpm of M-band DNA in 0.01 M Tris-HCl, pH 8.5. The density was adjusted to 1.705 g cm^{-3} in a final volume of 5 ml. Centrifugation was for 84 h at 33 000 rpm in the Spinco 50 Ti rotor. Approximately 40 fractions were collected and the position of the marker DNA was determined by measuring the absorbance of each fraction at 260 nm (dashed line). Radioactivity was determined by Cl_3CCOOH precipitation as given under Methods. The distributions of DNA on two parallel gradients are shown; the positions of the marker DNA in both gradients have been superimposed. (○) M-band DNA from cells in G_0; (●) M-band DNA from cells in the S phase.

at specific periods during the cell cycle. Accordingly, M bands were isolated from synchronized cells in which the DNA had been uniformly labeled, and the percentage of DNA bound to the membrane determined for different phases of the cell cycle. Figure 7 shows that, in contrast to the sea urchin system, DNA-membrane complexes are detected throughout the cell cycle, including contact-inhibited cells (G_0 phase), where about 15% of the DNA is isolated with the M band. However, the percentage of DNA in the M-band fraction approximately doubles by the mid-G_1 phase and remains at this elevated level through the S phase. There appears to be a reduction in the amount of complex after the S phase. Similar fluctuations have been observed with Chinese hamster cells (Hildebrand and Tobey, 1973; Yamada and Hanaoka, 1973).

Presence of d(A-T)-Rich DNA in the M Band. As shown above, some DNA appears to be associated with the nuclear membrane at all times during the cell cycle. It will be important to eventually determine if there are differences in the DNA-membrane complex isolated from various phases of the cell cycle. For example, do the physical properties and enzymatic composition of M bands in cells that are synthesizing DNA differ from the M bands of cells not synthesizing DNA? As part of this separate study, we report here that the DNA present in the M band of G_0 cells is readily distinguishable in terms of its base composition from the DNA in the M band of S-phase cells.

Figure 8 gives the buoyant density distributions of DNA present in the M bands of S-phase and G_0 cells. There is a skewing of both DNA preparations toward the light side of the main-band DNA. This is the region containing the mouse satellite DNA (Flamm et al., 1971), and has a higher d(A-T) content than the average DNA. In the case of membrane-bound DNA from G_0 cells, the displacement toward this low-density region is significantly more pronounced than the DNA from S-period cells. In the latter case, the buoyant density mode is almost the same as total mouse DNA, with the mean density mode for S-phase, M-band DNA being approximately 0.005 g cm^{-3} higher than G_0 phase M-band DNA. More extensive studies of the DNA present in M bands will be reported elsewhere. Of particular interest is the finding that mitotic cells also contain DNA-membrane complexes and this DNA is very d(A-T) rich (Margolis and Hobart, unpublished experiments). The importance of these results to the present study is that, in addition to the quantitative changes that occur in the composition of M bands during the cell cycle, there are apparently qualitative variations as well.

In Vitro Synthesis of DNA by M-Band and Various Subfractions. To determine whether DNA synthesis could be detected using the M-band and the top fraction derived from the nuclei of 3T3 cells, samples of each were incubated under various conditions with the appropriate precursors and cofactors. It can be seen first (Table I, section A) that both the M-band and top fraction were capable of synthesizing DNA, with most of the increase in activity in both fractions being detected during the first 4 min of incubation. After this time, the synthetic rate decreased, probably due to the presence of nucleases and other degradative enzymes in the crude preparation. The specific activity of DNA polymerase was consistently higher in the M-band fraction than in the top fraction.

TABLE I: DNA Polymerase Activity in Membrane and Non-Membrane Associated DNA Fractions Derived from Nuclei of 3T3 Cells.

A. Length of Incubation[a] (min)	Top Fraction (cpm/mg of protein)	M Band (cpm/mg of protein)
0	0	0
1	125	408
2	284	750
4	349	920
8	252	684
12	290	680

B. Conditions[b]	% Total Sp Act.	% Total Sp Act.
Complete	100	100
−dCTP, dATP, dGTP	20	15
−ATP	98	59
+Deoxyribonuclease (50 μg)	5	1
+Ethidium bromide (20 μm)	5	85
After sedimentation through sucrose gradient[c]		
Light fraction		230 (2100)
Heavy fraction		175 (1650)

[a] Kinetics of incorporation. The M-band and "top fraction" were prepared from 3T3 cells in the S phase as in Figure 1 insert. DNA polymerase activity was measured as described under Methods. Protein was measured by the method of Lowry et al. (1951). [b] Effect of various conditions or treatments on DNA synthesis in the M-band and top fraction. The percent total specific activity was determined by comparison to the activity obtained after the 4-min incubation period in section A. [c] The subfractions of the M band that were sedimented through sucrose gradient were prepared as in Figure 4. The heavy fraction is the one that sediments to the 80% sucrose shelf. Values in parentheses are the actual cpm/mg of protein incorporated in 4 min. All incorporation values were corrected by subtraction of radioactivity in zero-time incubation controls.

Table I, section B shows the effects of various conditions on the synthetic activity of the M-band and top fraction. It can be seen that the omission of three of the deoxyribonucleoside triphosphates inhibited the reaction in both fractions significantly, but not completely. This lack of complete inhibition has been observed by other investigators with relatively crude preparations from eucaryotic cells (Weissbach et al., 1971; Chiu and Baril, 1975). However, the addition of deoxyribonuclease completely inhibited the activity, showing the requirement for DNA in the reaction. The omission of ATP resulted in a decrease in activity in the M-band fraction but not the top fraction, whereas the addition of ethidium bromide inhibited DNA polymerase activity in the top fraction, but not the M band. These latter differential effects could suggest the presence of different polymerases in the nucleus of 3T3 cells, with one polymerase more firmly bound to the nuclear membrane than the other.

As shown in Figure 4, under certain conditions, the M band may be separated on 30–60% sucrose gradients into two fractions, whose relative proportions are dependent upon the amount of sarkosyl present in the preparation (Figure 5). Both of these fractions contain DNA polymerase activity (Table I, section B). Of importance here is the finding that the specific activities of both the slowly and rapidly sedimenting fractions are approximately two times greater than the original M band. Thus, we conclude that the DNA–membrane fraction is capable of DNA synthesis in vitro without the addition of an external template or enzymes and that this activity in the M band may be purified to some extent by sedimentation in the 30–60% sucrose gradients.

Discussion

Two methods have been used to isolate a fraction from nuclei of 3T3 cells which contains a complex of DNA with membrane. The rationale involved in using CsCl buoyant density gradients and the sarkosyl M-band procedure to isolate such complexes, and the evidence that we have isolated a DNA–membrane complex were discussed under Results. We will consider here the functional significance of this DNA–membrane fraction. Despite earlier compelling autoradiographic analysis of pulse-labeled cells showing a proximity of nascent DNA with the nuclear membrane (Comings and Kakefuda, 1968; O'Brien et al., 1972), more recent cytological studies have seriously questioned the role of the nuclear membrane in DNA synthesis (Comings and Okada, 1973; Huberman et al., 1973; O'Brien et al., 1973; Wise and Prescott, 1973). In general, the latter studies suggest that only some of the DNA (mostly heterochromatin which is synthesized late in the S period) is replicated near the membrane. These are in apparent contradiction to a number of biochemical studies with eucaryotes, which strongly implicate the nuclear membrane in DNA synthesis (e.g., Friedman and Muller, 1969; Hanaoka and Yamada, 1971; Infante et al., 1973; Mizuno et al., 1971; Cabradilla and Toliver, 1975). A major argument against these biochemical studies is that nascent DNA may be in a condition, such as possessing single-stranded regions, which causes it to become complexed with membrane upon disruption of the nucleus (Fakan et al., 1972; O'Brien et al., 1973; Wise and Prescott, 1973). The results presented here strongly argue against an artifactual association of DNA with membrane. First, there is a high percentage of DNA associated with membrane when no DNA synthesis is occurring, hence, replicating DNA is not the exclusive DNA in these complexes. This is substantiated by the finding that the percentage of DNA bound to membrane increases to the level present in the S-phase cells during the G_1 phase, well in advance of DNA synthesis (Figure 7). In addition to the increased percentage of DNA in the DNA–membrane complexes of S phase compared to G_0 cells, there is also a difference in the DNA contained in the two complexes. In G_0 cells the DNA associated with membrane is highly d(A–T) rich, whereas the corresponding DNA from S-phase cells more closely resembles total DNA. It should be pointed out that, since about 40% of our culture consists of cells that do not become induced (i.e., remain in G_0), the actual difference in the density of membrane-bound DNA between S phase and G_0 cells may be even greater than we observe. The reason for this qualitative change in membrane-associated DNA is not known but it may represent a greater number of binding points of DNA to the membrane when DNA synthesis takes place (Sueoka and Quinn, 1968). In all phases of the cell cycle examined, the DNA associated with the membrane contains more d(A–T)-rich regions than total DNA as determined by CsCl buoyant density analysis (Figure 8). Since thymidine was used in these studies as the radioactive precursor, it seemed possible that we were selecting for labeled d(A–T)-rich segments. This was not the case, since total labeled nuclear DNA displayed a density distribution that was identical to the marker mouse DNA used (unpublished determination). Thus, there appears to be a preference for d(A–T)-rich segments of DNA that bind to the membrane.

We cannot rule out the possibility that cell cycle dependent

alterations occur in the state of the DNA to account for our results on the basis of an artifactual binding during extraction. These hypothetical alterations in the DNA would then permit more DNA and specifically non-d(A-T)-rich DNA, to complex with the membrane at one phase compared to another. We are unable to postulate the alterations required for this to occur but it will be difficult to eliminate such a possibility. In the 3T3 system, as in others (e.g., Hildebrand and Tobey, 1973; Infante et al., 1973), negligible amounts of purified DNA (both double and single stranded) added to nuclei during extraction become complexed with membrane (unpublished observations). It would appear, therefore, that the evidence does not in any way support an artifactual basis for DNA-membrane complexes. However, the function of this complex is not clear. One possible role of these complexes is that they serve as sites for synthesis of DNA (Berezney and Coffey, 1975; Cabradilla and Toliver, 1975). Our previous results with sea urchin embryos strongly support this possibility. In this system, all of the pulse-labeled DNA is present in the M-band fraction, whereas only 10–30% of the total DNA is located therein. Also, virtually all of the DNA polymerase activity present in nuclei could be isolated with the M band. The present work indicates that in mammalian cells in culture the situation is quite different. First, in 3T3 cells the shortest pulse times used, although showing an enrichment of nascent DNA in the membrane fraction, always yielded significant amounts of pulse-labeled DNA in the nonmembrane fractions (Figure 2). This result might be due to the severe shearing conditions needed to disrupt the mammalian nuclei (Hatfield, 1972). Another interpretation is that, in this system, DNA synthesis occurs both associated with and not associated with nuclear membrane. This agrees with the conclusion of O'Brien et al. (1973) and is supported by our finding that a considerable percentage of total nuclear DNA polymerase is present in the non-DNA-membrane fraction. However, the two polymerase activities seem to be different and may serve different functions, such as repair and replication. The stimulation of the membrane-bound DNA polymerase by ATP suggests that this may be the replicative enzyme (Genta et al., 1976; Lynch et al., 1970).

Thus, we conclude that DNA-membrane complexes are present in mammalian cells. But, although such complexes are enriched with nascent DNA, they may not represent the only sites for DNA synthesis. Striking quantitative and qualitative changes occur in these complexes during the cell cycle, suggesting that they may perhaps be involved in some way with regulating DNA synthesis. Since several DNA polymerases have been isolated from eucaryotic cells (Weissbach, 1975), a more extensive analysis of the nature of the DNA polymerase present in this complex and the polymerase which is not associated with the complex should help to elucidate the role of DNA-membrane complexes.

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