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Localization of Adenovirus DNA Replication in KB Cells

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The localization of adenovirus type 5 DNA replication has been investigated by both fractionation of isolated nuclei and electron-microscope autoradiography. Nuclear fractionation by means of the M-band-technique of Tremblay *et al.* (Tremblay, G. Y., Daniels, M. J., and Schaechter, M. (1969). *J. Mol. Biol.* **40**, 65–76.) reveals that pulse-labeled adenovirus type 5 DNA cosediments with the nuclear membrane. On the basis of this finding it can be supposed that the site of viral DNA replication is at the nuclear membrane. However, electron-microscope autoradiography of infected cells shows that the pulse-labeled viral DNA is not located at the nuclear membrane but randomly distributed over the nucleus. It is concluded that the replication of adenovirus type 5 DNA occurs throughout the cell nucleus; the association of replicating viral DNA with a nuclear membrane complex should be considered as an artifact.

INTRODUCTION

In the replicon model of Jacob *et al.* (1963) it is postulated that a close association between DNA and the cell membrane is a prerequisite for DNA replication. In bacteria the association between DNA and cell membrane has been studied by a variety of techniques including biochemical analysis of rapidly sedimenting complexes (Ganesan and Lederberg, 1965; Smith and Hanawalt, 1967; Parker and Glaser, 1974) and autoradiography (Ryter and Jacob, 1963). In bacteriophage systems, rapidly sedimenting complexes containing replicating phage DNA and host-cell membrane have been observed (Knippers and Sinsheimer, 1968). From this it is assumed that bacteriophage DNA replicates in close association with the host-cell membrane. The intimate association of replicating DNA and cell membrane in prokaryotic cells may be required for the initiation event and the segregation of daughter chromosomes as proposed in the replicon model.

In eukaryotic cells the nuclear membrane may be involved in the replication of the cellular DNA, which contains multiple replication units. However, conflicting data have been reported in the literature. In 1969 Tremblay *et al.* introduced a technique for the isolation of DNA–cell membrane complexes from bacteria based upon the cosedimentation of membrane-bound nascent DNA and Mg^{2+}-Sarkosyl crystals (M-band). By analogy with bacterial systems, most cell fractionation experiments on the intracellular localization of DNA synthesis with the "M-band"-technique have been interpreted in favor of the attachment of replication sites to the nuclear membrane (Friedman and Mueller, 1969; Mizuno *et al.*, 1971; Hanaoka and Yamada, 1971; Yamada and Hanaoka, 1973; Infante *et al.*, 1973; Hildebrand and Tobey, 1973). However, most electron-microscope

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autoradiographic investigations reveal that even after very short pulses with [^{3}H]thymidine, DNA replication sites are randomly distributed throughout the nucleus (Williams and Ockey, 1970; Fakan *et al.*, 1972; Huberman *et al.*, 1973; Comings and Okada, 1973; Wise and Prescott, 1973).

The replication of adenovirus DNA occurs in the cell nucleus. From their experiments in which the M-band-technique was used, Pearson and Hanawalt (1971) concluded that the DNA replication of adenovirus type 2 (Ad2) may take place at the nuclear membrane. Shiroki *et al.* (1974) reported that replicating DNA of adenovirus type 12 (Ad12) is associated with the M-band. On the other hand Martines-Palomo and Granboulan (1967) and Shiroki *et al.* (1974) showed by electron-microscope autoradiography that, after labeling of Ad12-infected cells with [^{3}H]thymidine, the grains are randomly distributed throughout the nucleus.

In view of the contradictory data on the location of adenovirus DNA replication in eukaryotic cells we investigated the localization of Ad5-DNA replication in KB cells by both electron-microscope autoradiography and nuclear fractionation with special reference to the involvement of the nuclear envelope. From these experiments we conclude that the replication of Ad5 takes place randomly throughout the cell nucleus.

MATERIALS AND METHODS

Cells and virus. The growth of KB cells, the purification of Ad5 and its DNA were performed as before (van der Vliet and Sussenbach, 1972). Suspension cultures were obtained by trypsinization of monolayers and addition of the cells, at a concentration of 5×10^5 cells per ml, to Earle's balanced salt medium containing 0.5% lactalbumin, 2% calf serum, 100 units of penicillin G per ml and 100 μg of streptomycin sulphate per ml. Cells were infected with purified Ad5 at an m.o.i. of 2–3 $\times$ 10^3 physical virus particles per cell. Virus-particle concentration was determined from A_{260} measurements (Maizel *et al.*, 1968).

Conditions of labeling. Radiochemicals were obtained from the Radiochemical Centre, Amersham, England. Cellular DNA was labeled with either [^{14}C]T ([2-^{14}C]thymine, 60 Ci/mole, 1 μCi/ml) or [^{3}H]TdR ([6-^{3}H]thymidine, 22 Ci/mmole, 1 μCi/ml) during at least two generations of growth. The nuclear envelope was labeled with either [^{14}C]choline ([^{14}C]methylcholine chloride, 60 Ci/mole, 1 μCi/ml) or [^{3}H]choline ([^{3}H]methylcholine chloride, 16.5 Ci/mmole, 0.2 μCi/ml) during at least two generations of growth. For electron-microscope autoradiography cells were labeled with [^{3}H]choline at a concentration of 2 μCi/ml during two generations of growth. These labeling procedures were carried out on monolayer cultures of KB cells.

At 18 hr after infection, infected or mock-infected cells in suspension were concentrated to 5×10^6 cells per ml and subsequently exposed to [^{3}H]TdR (43 Ci/mmole) at concentrations ranging from 7–200 Ci/ml for different periods of time. The incorporation was stopped by dropping the reaction mixture into ice-cold phosphate-buffered saline containing 1 m*M* EDTA. The cells were washed twice with the same buffer, and samples were taken for electron-microscope autoradiography, preparation of nuclei or radioactivity measurement.

Formation of DNA–nuclear membrane complexes. Nuclei were isolated (Sussenbach *et al.*, 1972) and purified by discontinuous sucrose gradient centrifugation as described by Raskas (1971). The DNA–nuclear membrane complex was isolated by the M-band technique as described by Tremblay *et al.* (1969) with some modifications. Nuclei were suspended in 0.01 *M* Tris-HCl, 0.1 *M* KCl, pH 7.5, to a final concentration of 1–2 $\times$ 10^6 nuclei per ml. The suspension of nuclei (2 ml) was gently mixed with Sarkosyl NL 30 to a final concentration of 0.15% and kept on ice for 10 min. Subsequently $MgCl_2$ (final concentration 0.01 *M*) was added to the nuclear lysate. Where indicated, the nuclear lysate was sheared by means of Vortex mixing for a period of 40 sec (Hanaoka and Yamada, 1971). After the formation of the white

Mg^{2+}-Sarkosyl crystals (5 min in ice) the nuclear lysate was layered on top of a two-layer tube of sucrose (15 ml of 15% sucrose and 15 ml of 40% sucrose in 0.01 *M* Tris-HCl, 0.1 *M* KCl, 0.01 *M* $MgCl_2$, 0.001 *M* EDTA, pH 7.5). The M-band complexes were concentrated in a sharp, white band at the interface by centrifugation in an SW 27 rotor at 4° for 18 min at 18,000 rpm in a Spinco ultracentrifuge. The top and interface fractions from the gradient were collected and the acid-precipitable radioactivity was measured. From both fractions the viral DNA was extracted according to the Hirt procedure (van der Vliet and Sussenbach, 1972).

Isolation and analysis of DNA. Total DNA was extracted from isolated nuclei with phenol. Viral DNA could be extracted according to a modified Hirt procedure (van der Vliet and Sussenbach, 1972). The extracted DNA was analyzed by CsCl density gradient centrifugation. The nucleic acid solution in 0.15 *M* trisodium citrate, 0.01 *M* EDTA, pH 7.5, was mixed with solid CsCl to a density of 1.700 g/cm³ and centrifuged at 38,000 rpm for 72 hr in a Beckman Spinco 65 Ti angle rotor. Samples were collected from the bottom of the centrifuge tube. The density of the gradient fractions was determined from refractive indices measured in an Abbé refractometer at room temperature. Nucleic acid was precipitated with 5% trichlorocetic acid and collected on Whatman GFC filters for radioactivity measurements.

Lipid extraction. Isolated nuclei from cells labeled with [^{3}H]choline and [^{14}C]thymine for two generations were treated with 0.5 *M* perchloric acid at 0°. The precipitates were washed three times in order to remove unincorporated radioactivity. Lipids were extracted with a 2:1 mixture of chloroform and methanol as described by Folch *et al.* (1957).

Electron microscope autoradiography. Cells labeled with [^{3}H]TdR or nuclei isolated from cells labeled with [^{3}H]choline were fixed for 1 hr in a trial-dehyde-dimethyl sulfoxide (DMSO) fixation in 0.1 *M* NaH_2PO_4-Na_2HPO_4 buffer, pH 7.4 (3% glutaraldehyde-2% formaldehyde-1% acrolein-2.5% DMSO). The fixed cells were rinsed in the phosphate buffer (four changes over 1 hr) and postfixed in 1% OsO_4 solution containing the same buffer. Dehydration was performed in graded acetone. All procedures were carried out at room temperature. Embedding was done in Araldite. Thin sections (600-800 Å) were cut and picked up on copper grids covered with a thin film of Pioloform F. The preparations were then coated with a carbon layer (± 50 Å), stuck on microscope slides and covered afterwards with an Ilford L4 emulsion diluted to form a monolayer of silver halide crystals. After exposure for the desired time, the specimens were developed for 3 min at 19° in freshly prepared D 19. Fixation was done in 24% sodium thiosulfite. The preparations were then stained with PbOH according to Millonig (1961). Preparations were examined in a Philips EM 201.

Quantitative evaluation of the autoradiographs. For quantitative study of the distribution of grains within nuclei, photographs of sections of labeled specimens at final magnifications of 8,040 or 13,400× were used. Within each nuclear cross-section on the prints, lines were drawn that were at all points away from the nearest nuclear membrane for distances of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 μm, respectively. The maximal distance is 3.5 μm, because the average diameter of a nucleus in this cell type is about 7 μm. The lines divide the nuclear cross-sections in areas that have been denominated as *a*, *b*, *c*, *d*, *e*, *f* and *g*, respectively. Thus area *a* is located in close proximity to the nuclear membrane and area *g* is the most internal region of a nuclear section. The nucleolar regions were considered separately. The surface of each area was measured by integration, and the number of grains overlying these areas was counted. Grains lying outside the nucleus were ignored; the amount of grains derived from background (radioactivity) was negligible. The grain densities are expressed as grains per 100 μm². Calculation of the grain distribution in peripherally cut nuclear sections may give erroneous results, as some of the grains found in internal areas may actually belong to the nuclear periphery. Therefore, nu-

clear cross-sections smaller than 25 μm^2 were excluded.

RESULTS

Characterization of Pulse-Labeled DNA of Ad5-Infected KB Cells

The localization of adenovirus type 5 (Ad5)-DNA replication in KB cells by both electron-microscope autoradiography and nuclear fractionation can be established only if the radioactivity administered 18 hr after infection is incorporated into replicating viral DNA and not into cellular DNA. Biochemically, Ad5-DNA can be distinguished from host-cell DNA by its higher density in CsCl (Piña and Green, 1965). Figure 1 shows the density distribution of total DNA isolated from infected cells 18 hr after infection. Cellular DNA, prelabeled with [^{14}C]thymidine for two generations of growth, bands at a density of 1.700 g/cm^3 and is clearly distinguishable from DNA that has been pulse-labeled with [^{3}H]thymidine (TdR) for 1 min. The majority of the pulse-labeled DNA bands at the density of ^{32}P-labeled Ad5 marker DNA (1.716 g/cm^3) and at higher densities. Such a density distribution of pulse-labeled DNA molecules is characteristic for intermediates in the replication of Ad5-DNA (Sussenbach *et al.*, 1972; van der Eb, 1973). It can be concluded that at 18 hr after infection about 90% of the radioactivity is incorporated into replicating viral DNA; the synthesis of host-cell DNA is almost completely suppressed.

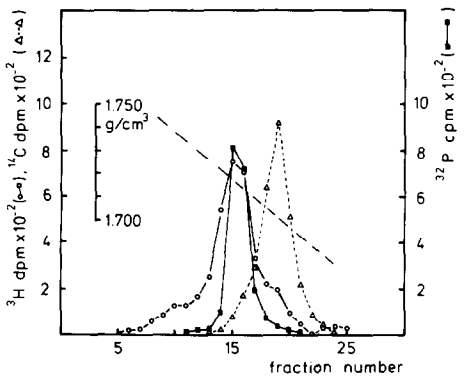

FIG. 1. CsCl equilibrium centrifugation of DNA from adenovirus infected cells. KB cells were prelabeled with [^{14}C]thymine for two generations of growth. At 18 hr after infection cells were labeled with [^{3}H]thymidine for 1 min at 37°. DNA from whole cells was extracted with phenol and subjected to CsCl gradient centrifugation in 0.15 *M* NaCl, 0.015 *M* trisodium citrate, 0.1% Sarkosyl, pH 7.5, at 38,000 rpm for 65 hr at 10° in a Spinco 50 Ti angle rotor. —○—, ^{3}H-labeled DNA; —■—, ^{32}P-labeled Ad5 marker DNA; - -Δ- -, ^{14}C-labeled KB-DNA.

Fractionation of Isolated Nuclei

Isolated nuclei, purified by centrifugation through a discontinuous sucrose gradient were fractionated using the M-band technique (Tremblay *et al.*, 1969), as described in Materials and Methods. After centrifugation of the nuclear lysate, virtually all of the cellular DNA, which has been prelabeled with [^{14}C]thymine is caught in the M-band complex (Table 1). Shearing of the lysate by agitation on a Vortimixer for 40 sec breaks the long cellular DNA filaments and results in the removal of a considerable portion of the cellular DNA from the M-band complex. Prolonged agitation does not remove more cellular DNA. The relative amount of cellular DNA removed by shear from the M-band complex is the same in infected and uninfected cells.

The location of the nuclear membrane after nuclear fractionation was determined using purified nuclei from cells prelabeled with [^{3}H]choline for two generations of growth. The [^{3}H]choline-containing fraction of the nucleus remains associated completely with the M-band complex even after shearing (Table 1). By means of a lipid extraction according to Folch *et al.* (1957), it could be demonstrated that labeled choline has been incorporated exclusively into the lipid moiety of the nuclei. More than 95% of the ^{3}H radioactivity was recovered in the lipid phase. The residual fraction consisting mainly of nucleic acids and proteins contained no radioactivity indicating that transmethylation or ^{3}H exchange does not occur extensively in these nuclei. Therefore, an M-band complex can be isolated from purified nuclei that contains a minor part of the cellular DNA in association with the nuclear lipids.

To investigate the location of Ad5-DNA replication inside the cell nucleus, cells were pulse-labeled with [^{3}H]TdR for differ-

TABLE 1

LOCALIZATION OF VIRAL AND CELLULAR DNA AFTER NUCLEAR FRACTIONATION[a]

Sample	Infection	Shear (40 sec)	Total cpm recovered	Top (%)	M-band (%)
Prelabeled Cellular DNA ([^{14}C]T)	−	−	1,775	9	91
	−	+	1,825	75	25
	+	−	4,705	12	88
	+	+	4,650	71	29
Nuclear membrane ([^{14}C]choline)	+	−	995	8	92
	+	+	770	5	95
1-Min pulse ([^{3}H]TdR)	+	−	976	3	97
	+	+	1,025	7	93
60-Min pulse ([^{3}H]TdR)	+	−	75,500	6	94
	+	+	76,420	76	24
1-Min pulse ([^{3}H]TdR), 60-min chase	+	−	2,916	4	96
	+	+	2,874	80	20

[a] KB cells were uniformly labeled with [^{3}H]TdR, [^{14}C]T or [^{14}C]choline. As indicated, the cells were pulse-labeled with [^{3}H]TdR for different periods of time, 18 hr after infection with Ad5. Purified nuclei were prepared from these cells and they were subjected to M-band analysis as described in Materials and Methods. When indicated, the nuclear lysate was sheared for 40 sec by means of Vortex mixing. The values are expressed as the relative amount of acid-precipitable radioactivity in the top and M-band fractions of the gradient compared with the total radioactivity recovered (average of three experiments). The recovery of radioactivity applied to the gradients was greater than 75%.

ent periods of time at 18 hr after infection. As mentioned before, at this time after infection the pulse-labeled DNA corresponds to viral DNA. Nuclei from pulse-labeled infected cells were fractionated according to the M-band procedure. After a 1-min pulse of [^{3}H]TdR almost all radioactivity is associated with the M-band complex, even if the nuclear lysate is sheared. In this respect, replicating Ad5-DNA behaves differently from the bulk of overall labeled cellular DNA (Table 1). However, after a 60-min pulse of [^{3}H]TdR or a 1-min pulse of [^{3}H]TdR followed by a 60-min chase, about 80% of the labeled viral DNA is removed from the M-band complex by shearing. The observed cosedimentation of pulse-labeled viral DNA and nuclear lipids, if a sheared nuclear lysate is used, strongly suggests that intermediates in Ad5-DNA replication are intimately associated with the lipid moiety of the nucleus, while mature viral DNA is not.

It is known that replicating Ad5-DNA molecules contain extensive regions of single-stranded DNA (Sussenbach and van der Vliet, 1972). The observed association of replicating Ad5 DNA with the M-band complex, if sheared nuclear lysates are used, may be the result of an artificial attachment due to the presence of such extensive single-stranded regions. To investigate this possibility, different forms of Ad5-DNA were added to isolated nuclei prior to nuclear fractionation (Table 2). It appears that native Ad5-DNA isolated from intact virus particles by phenol extraction is not associated with the M-band complex. When AdDNA is denatured by heat the single-stranded DNA molecules are to a certain extent attached to the M-band complex. However, isolated replicating Ad5-DNA molecules, although containing extensive regions of single-stranded DNA, are not associated with the M-band complex. Therefore it seems unlikely that the association of replicating Ad5-DNA with the M-band complex originates from the presence of single-stranded regions in these molecules.

Electron-Microscope Autoradiography

Since pulse-labeled Ad5-DNA and nuclear lipids cosediment in an M-band complex, it seems likely that the replication of Ad5-DNA takes place in proximity to the nuclear membrane. The location of the lipid moiety in the cell nucleus, which has

TABLE 2

ASSOCIATION OF DIFFERENT FORMS OF Ad5-DNA WITH THE M-BAND[a]

Sample	Shear (40 sec)	Total cpm recovered	Top (%)	M-band (%)
Native Ad5-DNA (^{32}P)	+	402	88	12
Denatured Ad5-DNA (^{32}P)	+	367	67	33
Replicating Ad5-DNA, 1-min pulse (^{3}H-TdR)	+	4830	99	1

[a] Nuclei purified from infected cells were mixed with either phenol-extracted, ^{32}P-labeled Ad5-DNA or its denatured form (10 min at 95° followed by rapid cooling to 0°) or ^{3}H-labeled intermediates in Ad5-DNA replication before nuclear lysis. This replicating Ad5-DNA was extracted from infected cells by a modified Hirt procedure, after a 1-min pulse with [^{3}H]TdR. After M-band analysis, as described in Materials and Methods, the acid-precipitable radioactivity was measured and the values are expressed as the relative amount of radioactivity in the top and M-band fractions of the gradients compared to the total radioactivity recovered (average of three experiments). The recovery of radioactivity applied to the gradients was almost 100%.

not yet been identified as the nuclear membrane, is investigated by means of electron-microscope autoradiography. With the same technique, the location of pulse-labeled Ad5-DNA in nuclei of infected cells is determined in order to investigate a possible structural relationship between replicating Ad5-DNA and the nuclear membrane.

Nuclei were isolated (Sussenbach *et al.*, 1972) from cells that were labeled with [^{3}H]choline for two generations of growth. Cytoplasmic contamination was removed by sedimentation of isolated nuclei through a discontinuous sucrose gradient (Raskas, 1971). An autoradiogram of these nuclei (Fig. 2) shows that most grains are located at or in close proximity to the nuclear membrane. The distribution of the grains over the nuclei is quantified as described in Materials and Methods and the results are listed in Fig. 3A. The majority of the grains is situated in area *a*, in which the grains are away from the nuclear envelope for a distance of 0.5 μm or less. This value represents twice the error limiting the autoradiographic resolution used (Bachmann and Salpeter, 1965). These results show that the [^{3}H]choline radioactivity, which represents the lipid moiety of the nucleus, is incorporated almost exclusively into the nuclear membrane. The origin of the grains in parts of the nucleus other than the region close to the nuclear envelope is not yet clear.

Electron-microscope autoradiography of infected cells pulse-labeled with [^{3}H]TdR for 1 min (Fig. 4) shows that the radioactivity is confined to the cell nucleus. In contrast to what has been observed in [^{3}H]choline-labeled nuclei, the grains are not restricted to the nuclear envelope or regions adjacent to it, but they appear to be randomly distributed over the nucleus. To quantify this observation the grain densities over the different areas (see Materials and Methods) have been determined. The results have been summarized in a histogram (Fig. 3B). It appears that the grain densities in most areas are similar. However, the grain density in the region close to the nuclear envelope (area *a*) is significantly lower than the densities in other areas. An essentially similar density distribution of grains is observed in nuclei from cells that were pulse-labeled with [^{3}H]TdR for 2 min (Fig. 3C) and 60 min (Fig. 3D). The data from the histograms further indicate that nucleoli are less involved in Ad5-DNA replication than the nucleoplasm. At 18 hr after Ad5 infection electron-dense, bandlike inclusion bodies are visible in the nuclei (Fig. 4). Their appearance is similar to that of the type II inclusion bodies described by Martinez-Palomo *et al.* (1967) in Ad12-infected cells. While others have reported a strict correlation between the grains and type II inclusion bodies (Martinez-Palomo and Grandboulan, 1967; Shiroki *et al.*, 1974), we find that the grains inside the nucleoplasm are only partially located over such inclusion bodies. This discrepancy may be due in part to the stage of infection.

The data from electron-microscope autoradiography reveal that the grain distribution in nuclei from infected cells pulse-

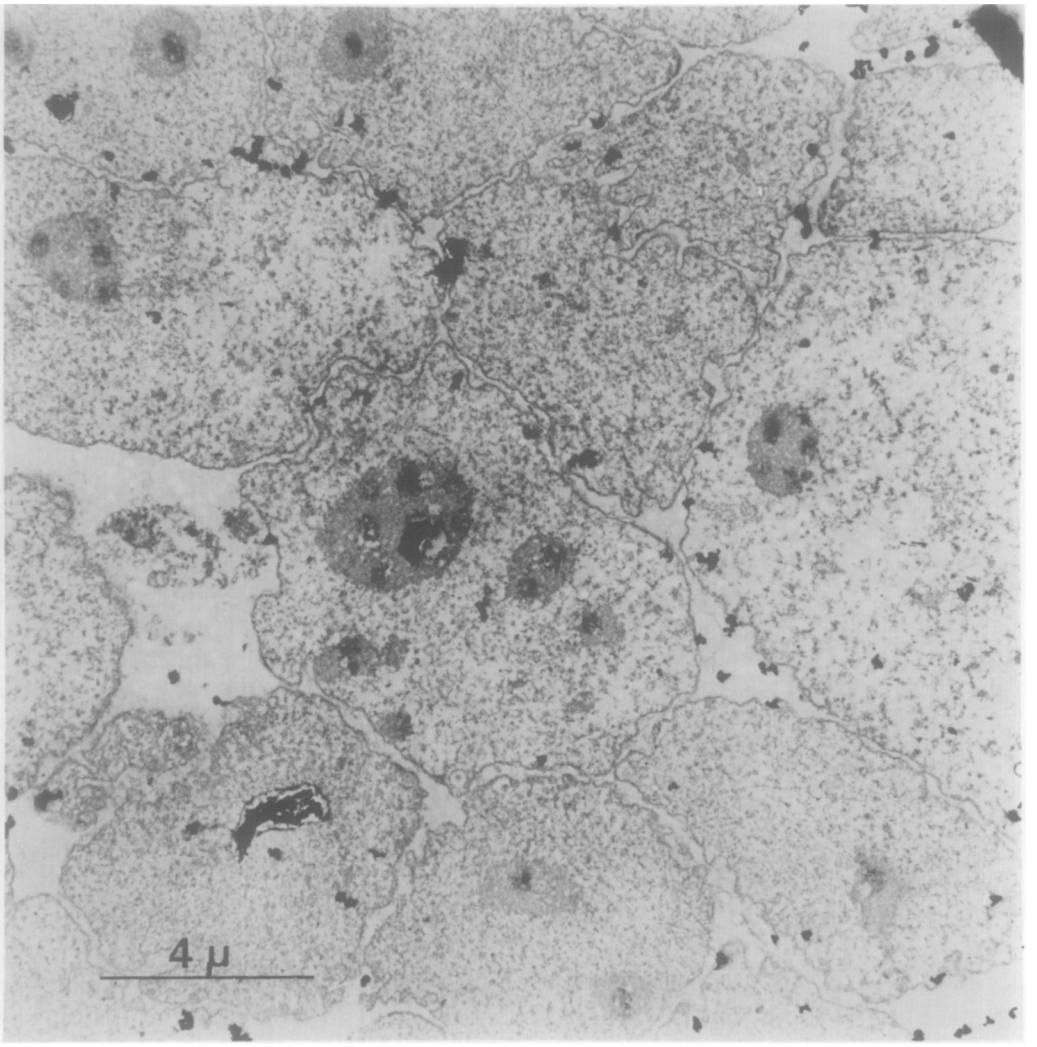

FIG. 2. Electron-microscope autoradiograph of nuclei from cells that had been grown for two generations in the presence of [^{3}H]choline (2 μCi/ml). Nuclei were purified as described in the text. The specimen was processed as described in Materials and Methods; the exposure time was 9 weeks. The bar represents 4 μm.

labeled with [^{3}H]TdR does not coincide with the grain distribution in nuclei labeled with [^{3}H]choline.

DISCUSSION

In the present study the localization of Ad5-DNA replication inside the cell nucleus has been investigated by both fractionation of isolated nuclei and electron-microscope autoradiography. For a correct interpretation of the results it has to be shown that, after pulse-labeling with [^{3}H]TdR 18 hr after infection, radioactivity is incorporated into viral DNA only. Several authors have shown that when the rate of viral DNA replication is at its maximum the synthesis of host-cell DNA is suppressed (Ginsberg *et al.*, 1967; Pearson and Hanawalt, 1971). On the other hand a stimulation of cellular DNA synthesis under these conditions is reported by Takahashi *et al.* (1969). We demonstrated that in KB cells the synthesis of cellular DNA is almost completely suppressed at 18 hr after infection; the newly synthesized DNA corresponds to viral DNA (Fig. 1). Therefore the radioactivity incorporated into DNA at 18 hr after infection can be

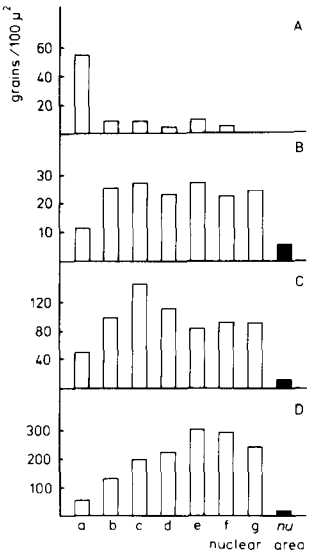

FIG. 3. Distribution of grains in nuclei from KB cells. The distribution of grains was measured in 30 nuclei isolated from cells labeled with [^{3}H]choline for two generations of growth (A) and in nuclei (40 for each pulse) from adenovirus-infected cells pulse-labeled with [^{3}H]TdR for 1 min (B), 2 min (C) and 60 min (D), as described in Materials and Methods. The grain density (expressed as grains/100 μm^2) in nuclear cross-sections was measured in different areas, which were at each point away from the nuclear membrane for distances of 0–0.5 (*a*); 0.5–1.0 (*b*); 1.0–1.5 (*c*); 1.5–2.0 (*d*); 2.0–2.5 μm (*e*); 2.5–3.0 (*f*) and 3.0–3.5 μm (*g*). The grain densities of the nucleoli (*nu*) were measured separately. See also Materials and Methods.

used to trace viral DNA in electron-microscope autoradiography and nuclear fractionation experiments.

To study the site of Ad5-DNA replication, nuclear preparations were fractionated according to the M-band technique of Tremblay *et al.* (1969) with some modifications. In our experiments the nuclear membrane was presumed to be monitored by the incorporation of [^{3}H]choline into the lipid moiety of the cell nucleus. We demonstrated by lipid extraction that this radioactivity is incorporated into the lipid moiety of the nucleus. This lipid moiety represents the nuclear membrane as concluded from electron-microscope autoradiography (Fig. 2). Upon fractionation of isolated nuclei the nuclear membrane is always found associated with the M-band complex as could be expected from the hydrophobic character of the nuclear envelope. After shearing of the nuclear lysate only a minor portion of the cellular DNA remains associated with the nuclear membrane in an M-band complex. This DNA fraction is reported to be enriched with replicating DNA (Pearson and Hanawalt, 1971; Yamada and Hanaoka, 1973; Hildebrand and Tobey, 1973; Infante *et al.*, 1973). Pulse-labeled Ad5-DNA cosediments almost completely with the nuclear membrane in an M-band complex unaffected by shear. When this pulse-labeled Ad5-DNA is chased into mature molecules the majority of the radioacitvity no longer cosediments with the nuclear membrane after shear. Our observations strongly suggest an intimate association of the nuclear membrane and the sites of Ad5-DNA replication. Similar results were obtained by Yamashita and Green (1974) and Pearson and Hanawalt (1971) for Ad2-DNA and by Shiroki *et al.* (1974) for Ad12-DNA.

The localization of Ad5-DNA replication in KB cells was also determined using electron-microscope autoradiography with special reference to the involvement of the nuclear membrane. As calculated from pulse-chase experiments (unpublished results) replication of Ad5-DNA proceeds at a maximal rate of about 1 μm/min, which is in agreement with rates for DNA replication in other mammalian systems. Therefore, a pulse of [^{3}H]TdR for 3 min or more could result in erroneous location of originally peripheral grains in a more central position in the nucleus. Thus, pulses of [^{3}H]TdR as short as 1 or 2 min are required to establish whether the nuclear membrane is actually involved in Ad5-DNA replication. Analysis of autoradiograms (Fig. 4) of cells pulse-labeled with [^{3}H]TdR for 1 min reveals that the grains are not restricted to the region adjacent to the nuclear envelope, although an intimate association between the nuclear membrane and the sites of viral DNA replication was suggested by nuclear fractionation experiments. The grains appear to be randomly distributed over the nucleus. Furthermore, we conclude (Fig. 3) that there are no major differences in grain distribution in nuclei of cells labeled with [^{3}H]TdR for different

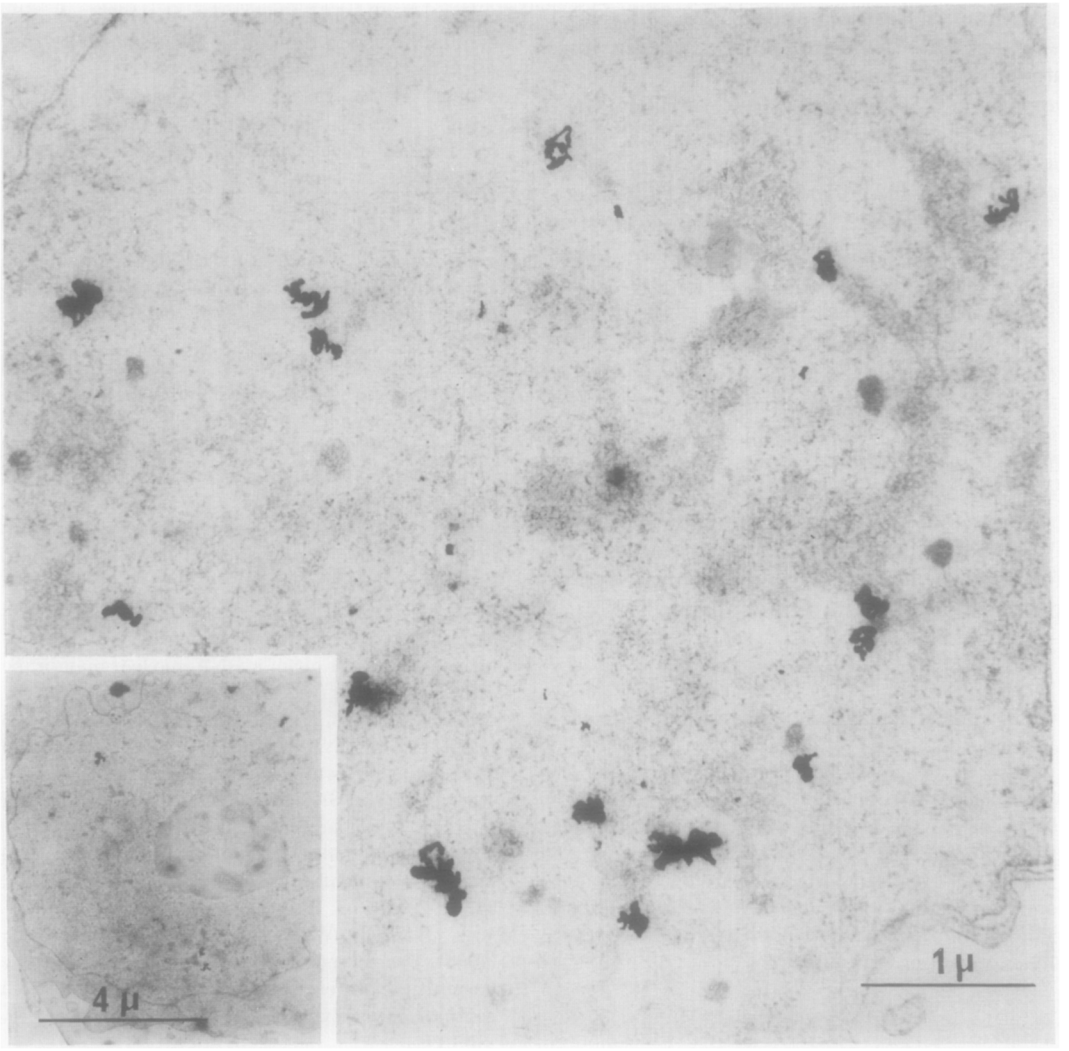

FIG. 4. Electron-microscope autoradiographs from Ad5-infected KB cells exposed to [^{3}H]TdR for 1 min. The specimens were processed as described in Materials and Methods; the exposure time was 16 weeks. The bar represents 1 μm. Inset: bar represents 4 μm.

periods of time. In all cases the region in close proximity to the nuclear membrane (area *a*) seems to be less involved in Ad5-DNA replication than other areas. One could speculate that the presence of peripheral heterochromatin concentrated at the nuclear membrane is prohibitive for the presence of viral DNA. These data indicate that the nuclear membrane is not involved in Ad5-DNA replication. On the contrary, the replication of Ad5-DNA seems to occur throughout the cell nucleus. In agreement with our data two recent electron-microscope autoradiographic studies showed that the replication of Ad12-DNA (Shiroki *et al.*, 1974) and Ad2-DNA (Simmons *et al.*, 1974) is not associated with the nuclear membrane.

The data from electron-microscope autoradiography and nuclear fractionation lead to opposite conclusions. It is most likely that the association of replicating viral DNA with the M-band complex has no functional implications. The temporal association between viral DNA and an M-band complex is highly dependent on the replicative state of the DNA, since mature Ad5-DNA is released from the complex

upon completion of replication. Since replicating Ad5-DNA molecules contain long stretches of single-stranded DNA, these regions may associate preferentially with the nuclear membrane or with the M-band complex, as has been suggested (Fakan *et al.*, 1972; Huberman *et al.*, 1973). Control experiments, however, indicate that the association of pulse-labeled Ad5-DNA with the M-band complex is probably not due to the single-stranded regions in replicating Ad5-DNA molecules. It is more likely that proteins present in a DNA replication complex are responsible for the association of replicating DNA and the nuclear membrane, as suggested by Yamada and Hanaoka (1973). Recently the existence of an Ad31 virus-coded protein involved in both DNA replication and the formation of the M-band complex was proposed by Suzuki and Shimojo (1974).

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