

Electron Microscopy of Adenovirus 12 Replication

II. High-Resolution Autoradiography of Infected KB Cells Labeled with Tritiated Thymidine

A. MARTINEZ-PALOMO¹ AND N. GRANBOULAN

Institut de Recherches sur le Cancer, 94-Villejuif, France

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Incorporation of tritiated thymidine by KB cells infected with oncogenic adenovirus 12 was studied by means of high-resolution electron microscopic autoradiography. After a 1-hr pulse with tritiated thymidine, infected and control cultures were fixed at 8, 16, 24, 30, and 36 hr. Infected cultures showed a higher percentage of labeled cells. During early stages, the frequency of silver grains in the nucleus and in the nucleolus was higher in infected material. From 24 hr on, there was an inhibition of nuclear and nucleolar deoxyribonucleic acid (DNA) synthesis. At late stages, one-third of the label was located over nuclear inclusions, type II and IV, previously shown to be composed of DNA and protein, while a large majority of the remaining grains were located over the nucleoplasm. The possibility is considered, that the early increase in nuclear and nucleolar DNA synthesis produced by adeno 12 replication could in part be due to newly synthesized cellular DNA, as has been reported by others with respect to other oncogenic DNA viruses.

The lytic cycle of adenovirus 12 induces in the host cell a sequence of complex alterations of the nuclear architecture. The fine structure and the sensitivity to enzymatic extractions of these lesions has been reported in a previous paper (22). Among such lesions, several types of nuclear inclusion bodies were found to be composed of protein and deoxyribonucleic acid (DNA). In addition, the nucleolus of infected cells showed early morphological changes which could reflect metabolic modifications similar to those described in SV40-infected cells (11). Therefore, the incorporation of tritiated thymidine was studied at the ultrastructural level in order to ascertain the alterations of nuclear and nucleolar DNA metabolism in cells replicating adenovirus 12. This technique facilitates the distinction between nuclear and nucleolar labeling and is particularly suited to study the incorporation rates of the different nuclear inclusions appearing late during the lytic cycle of adenovirus 12.

Two recent publications have demonstrated by means of light microscopic autoradiography that cultures infected with adenovirus 12 show a higher percentage of cells labeled with tritiated thymidine than do noninfected cultures (4, 25). A previous report on the ultrastructural localization of tritiated thymidine dealt mainly with the labeling of viral particles (17). In the present

study, advantage was taken, first, of the higher resolution obtained by electron microscopic autoradiography which was further increased by the use of a fine-grain emulsion (13); and, second, of a technique, allowing quantitative autoradiographic studies (14), that has been shown by statistical analysis to provide reliable data (7, 12). Thus, differentiation between nuclear and nucleolar labeling and that of nuclear inclusions becomes feasible.

Our results show that during the eclipse period the incorporation of tritiated thymidine per nuclear and nucleolar surface is increased in infected cells. During late stages of viral replication, tritiated thymidine is preferentially incorporated by two types of nuclear inclusions.

MATERIALS AND METHODS

A continuous line of human KB cells was grown at 37 C in Eagle's medium containing fourfold concentrations of vitamins and amino acids, and 10% calf serum. Monolayers of 10^6 to 2×10^6 cells were grown in 125-ml prescription bottles. On the second day of culture, they were infected with adenovirus 12 at a multiplicity of 2 ID_{50} per cell, yielding 80 to 100% of cells with early antigens detectable at 24 hr by the immunofluorescence technique (21). Techniques for electron microscopy were similar to those reported previously (22).

For electron microscopic autoradiography, tritiated thymidine (Radiochemical Center, Amersham, England; specific activity 20.60 c/mmole) diluted to 50

¹ Fellow of the National University of Mexico.

$\mu\text{C}/\text{ml}$ in medium containing 4% calf serum, was added to the cultures at 7, 15, 23, 29, and 35 hr after infection. The cultures were then incubated at 37 C for 1 hr and subsequently fixed. Uninfected cultures were similarly incubated with the precursor and fixed at the same intervals. Thin sections of Epon or glycolmethacrylate-embedded cells were cut with an LKB ultratome and laid with a plastic ring onto glass slides covered with a thick layer of collodion (14). The slides were then dipped into Gevaert 307 emulsion, allowed to dry, and stored at 4 C. The sections were exposed during 6, 8, or 11 days and were developed in Kodak D-19 at 18 C for 5 min. After fixation, the slides were dried and the membranes were floated onto distilled water; the sections were then picked up with 200-mesh grids and stained with uranyl acetate and lead citrate after the gelatin layer was digested with a $n/40$ acetic acid solution for 30 min. The background of the emulsion batches employed was always lower than 0.30 grains per $100 \mu^2$.

For quantitative analysis of grain distribution Epon sections approximately 600 A thick were used. Care was taken to use only sections with the same interference color to minimize thickness variation. Sections of infected cells and their corresponding controls were put on the same glass slides. All sections were developed under similar conditions 11 days after exposure. Micrographs of 50 cells of each group showing more than five reduced silver grains per nucleus were taken at a nominal magnification of $6,000\times$. The number of silver grains over the nucleus and the nucleolus was counted directly over the photographic plates with the aid of a dissecting microscope. A total of 500 cells was counted. The total surface of nuclear and nucleolar areas was estimated by drawing the borders of each nucleus and each nucleolus on a white paper; the regions were then cut out and the paper was weighed. Weights were converted to μ^2 by comparison with the weight of a known surface area of the same paper. The location of grains over different nuclear regions in infected cells was determined on photographic enlargements (total magnification $18,000\times$) of 20 cells possessing the full range of nuclear alterations. The surface areas of the different regions were cut out, weighed, and converted to square microns.

RESULTS

Intranuclear location of label. At all times examined, a majority of the reduced silver grains were diffusely distributed over the nucleoplasm of noninfected cells (Fig. 1). Occasionally, grains were preferentially located over the chromatin clumps disposed along the nuclear membrane and over the nucleolus-associated chromatin. Eight hr after infection the distribution of grains was similar, but in some cells a large proportion of silver grains was located over the nucleolus-associated chromatin and over the intranucleolar chromatin (Fig. 2). At this time, an important increase in nucleolar chromatin bands was found

in some infected cells. This was in sharp contrast to the sparsity of nucleolar chromatin observed in noninfected KB cells (Fig. 3 and 4).

After 24 hr, inclusion types I, II, and III as described in our previous paper, became visible (22). Type I inclusions are formed by large, round, homogeneous accumulations of dense material readily attacked by proteolytic enzymes. Type II inclusions are made up of irregular accumulations of moderately dense fibrils digestible by proteolytic treatment followed by prolonged deoxyribonuclease digestion. Type III inclusions are formed by thin fibrils of low density that are also susceptible to combined proteolytic and deoxyribonuclease extraction. At this time, reduced silver grains were in general associated with type II inclusions (Fig. 5).

At 30 hr, silver grains were still predominantly located over type II inclusions, and the remainder over the nucleoplasm (Fig. 6). At 36 hr the label was again localized over type II inclusions and also over type IV inclusions, which are considered to be formed by a condensation of the second type of inclusions (22), shown in Fig. 7. As expected, viral particles were not labeled due to the shortness of the pulse.

Labeling rate. At all times examined, the percentage of labeled cells was higher in the infected population. In control cultures, 25 to 30% of cells were labeled; 8 hr after viral inoculation, 40% of cells were labeled; and the percentage of labeled cells increased progressively until late stages, when 95% of the cells showing the nuclear lesions induced by viral replication were labeled.

Electron microscopic autoradiography was found to be particularly suitable for the differentiation of nuclear and nucleolar labeling. At 8 hr after viral infection, the mean number of silver grains per nuclear surface was nearly twice that seen in uninfected material. This situation was reversed from 16 hr on, as control cells showed uniformly more grains per nuclear surface than infected cells did (Table 1). Similarly, analysis of the distribution of silver grains per nucleolar surface revealed that at 8 hr the average labeling rate was nearly twice as intense as that seen in controls (Table 2). From 24 hr on, nucleolar labeling dropped rapidly to very low levels.

The percentage of grains and the grain frequencies over the different nuclear regions 36 hr after infection is shown in Table 3. The highest concentration of grains was located over the nucleoplasm and over type II and type IV inclusions. The frequency of grains per $10 \mu^2$ was highest in these two types of inclusions.

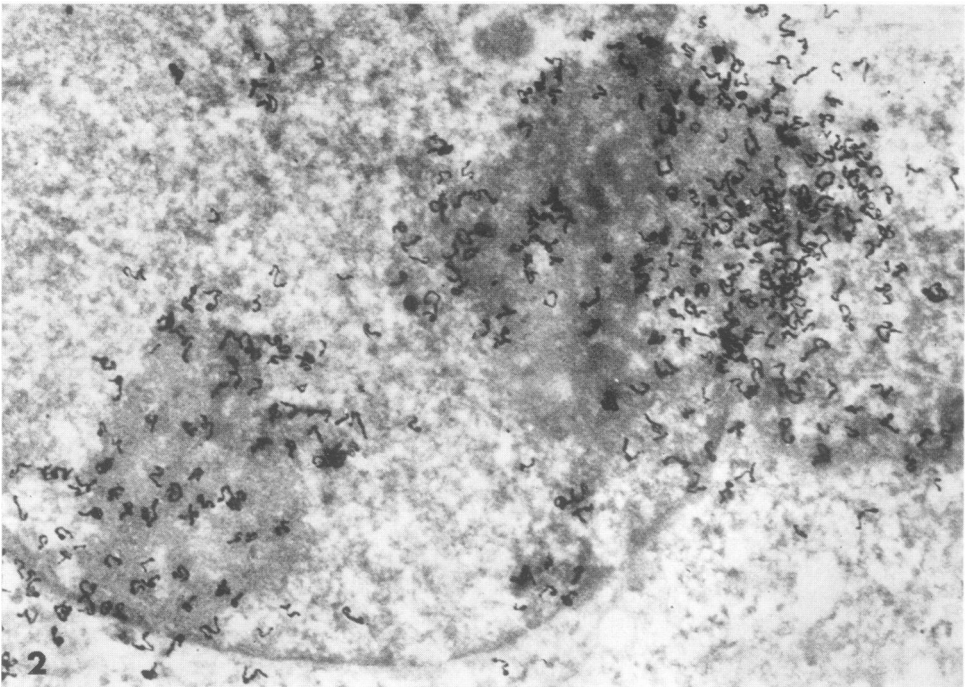
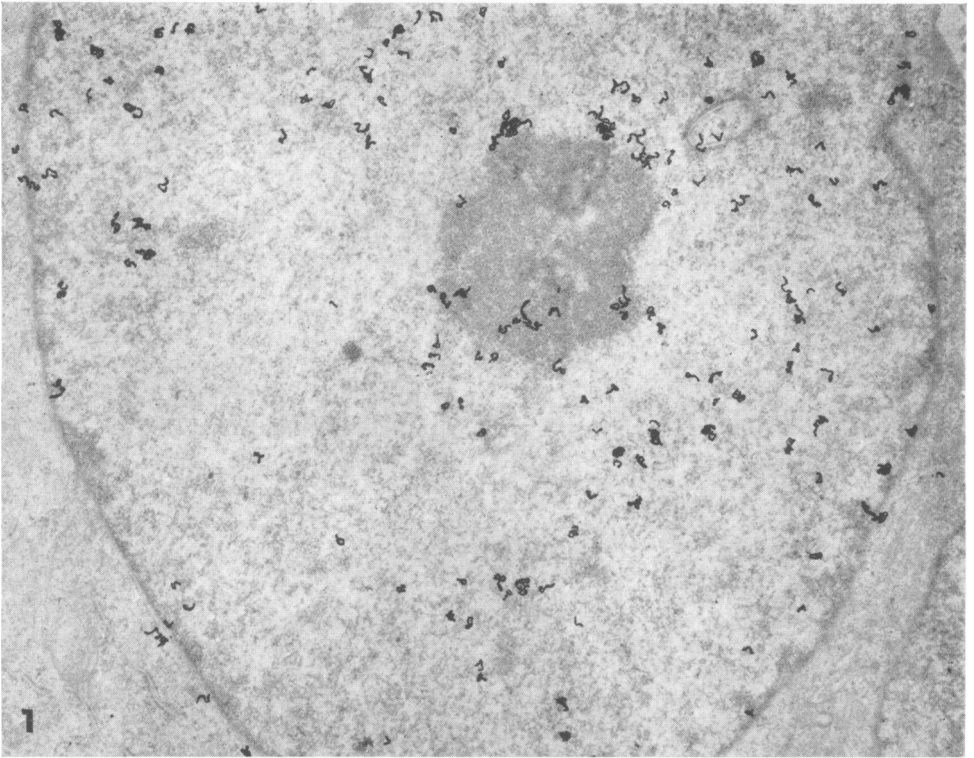


FIG. 1. *Electron microscopic autoradiograph of a noninfected KB cell labeled 1 hr before fixation with tritiated thymidine (control, 8 hr). Silver grains are located mainly over the nucleoplasm; a few grains are seen over the peripheral chromatin and over the nucleolus. Epon embedding. $\times 13,000$.*

FIG. 2. *Electron microscopic autoradiograph of a KB cell 8 hr after inoculation with adenovirus 12. One-hr pulse with tritiated thymidine. Intense labeling of two nucleoli. Glycolmethacrylate embedding. $\times 20,000$.*

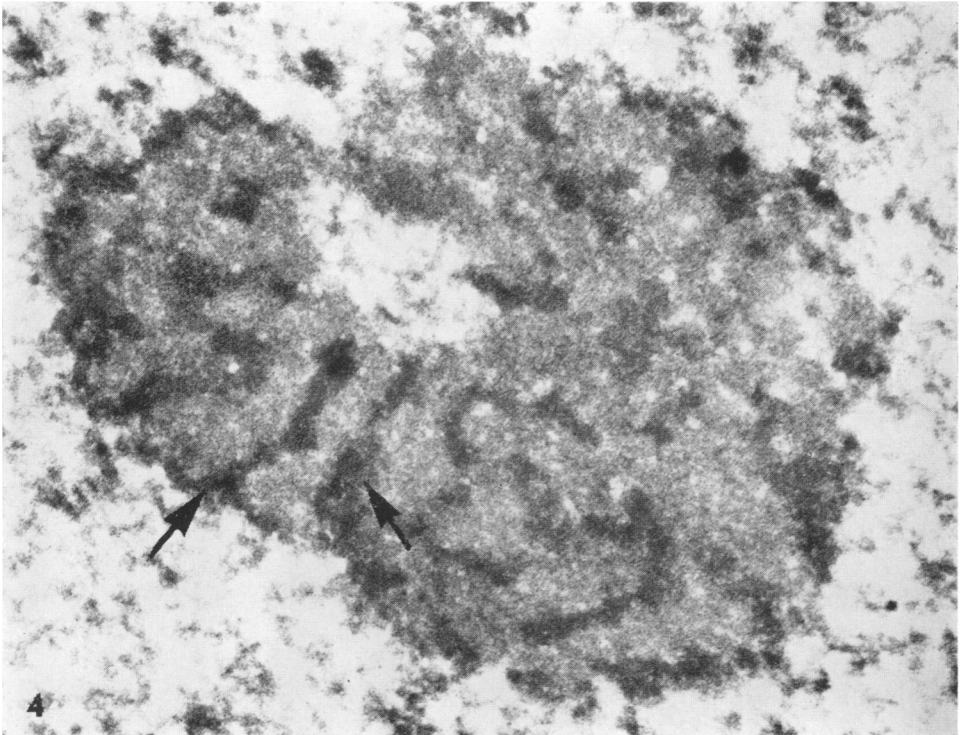
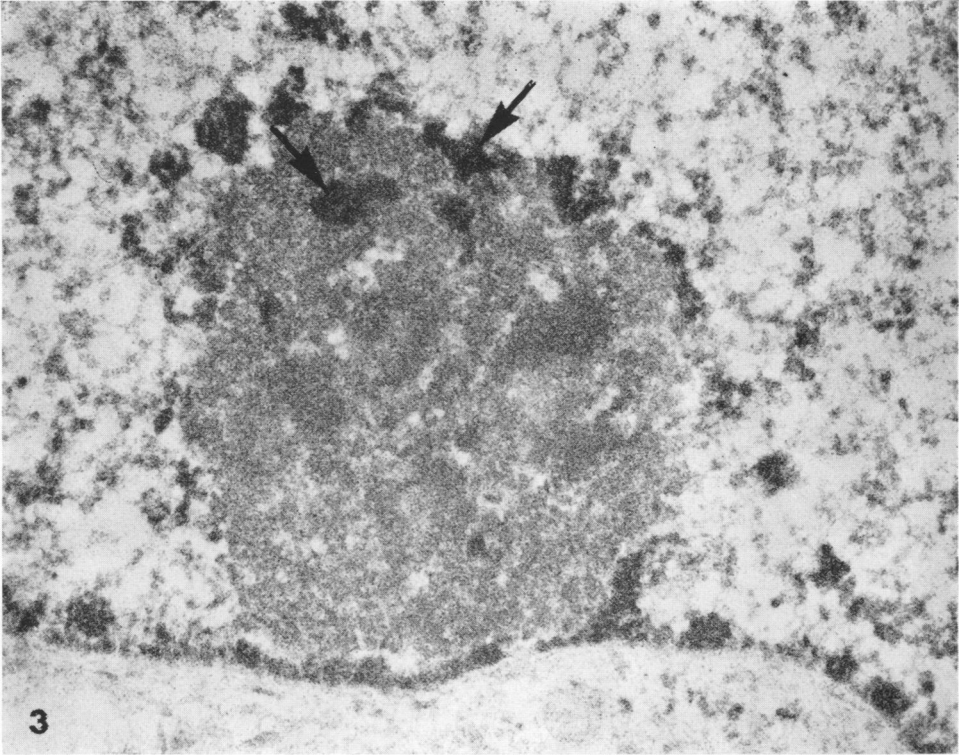


FIG. 3. Noninfected KB cell, 8 hr. The section was floated for 1 hr over a 0.1% ribonuclease solution to increase the contrast of chromatin, which appears as dense, irregular masses distributed within and around the nucleolus (arrows). Glycolmethacrylate embedding. $\times 40,000$.

FIG. 4. KB cell 8 hr after inoculation with adenovirus 12. The section was treated 1 hr with a 0.1% ribonuclease solution. Prominent chromatin bands are seen around and inside the nucleolus (arrows) in contrast with the sparse nucleolar chromatin clumps observed in Fig. 3. Glycolmethacrylate embedding. $\times 40,000$.

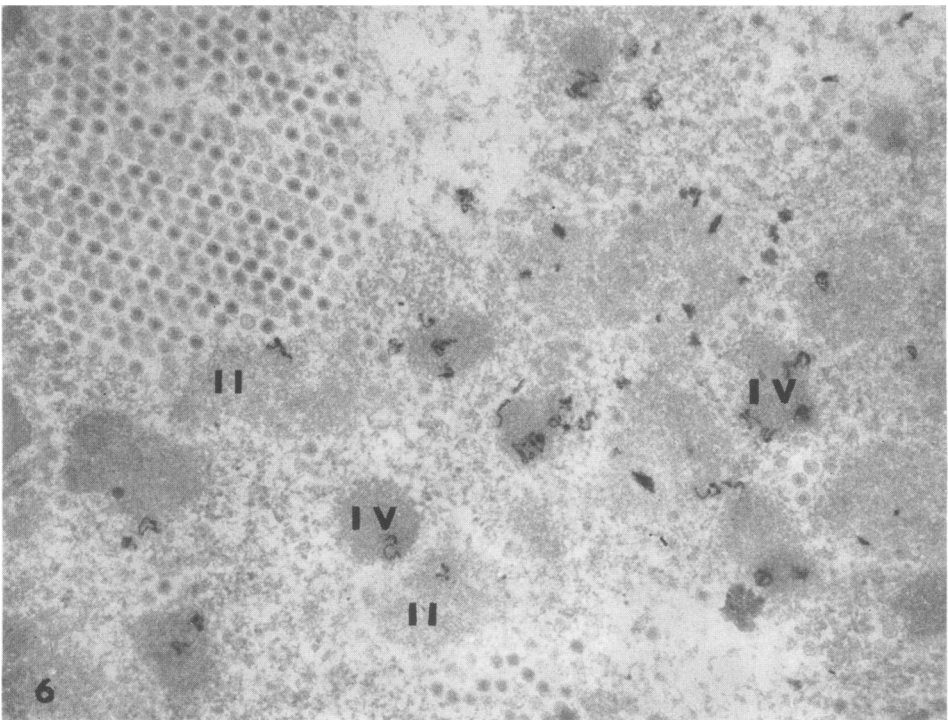
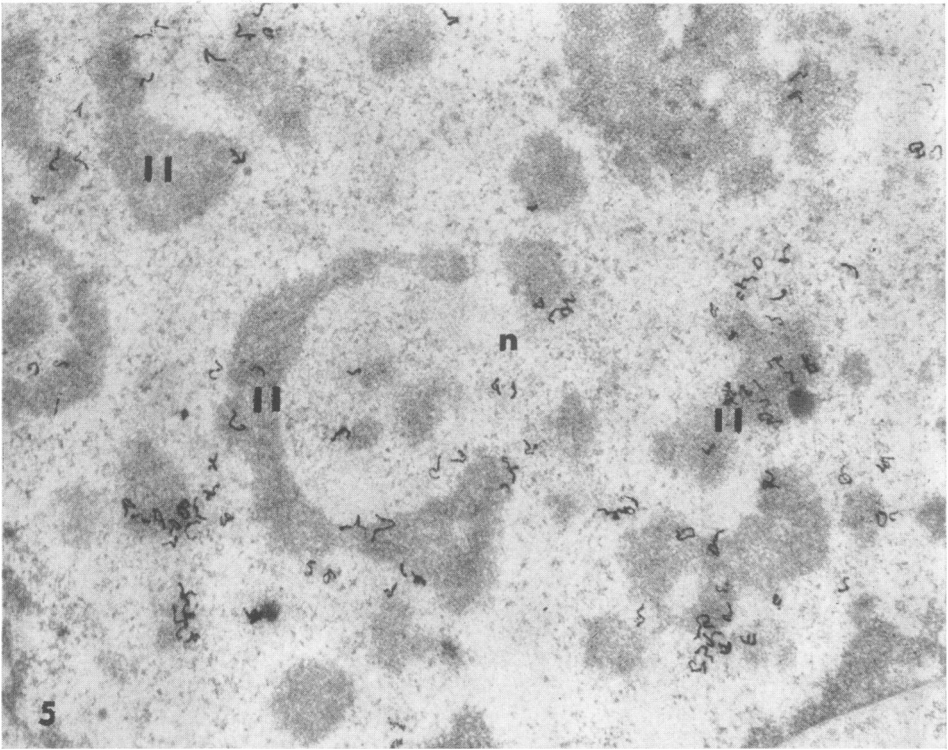


FIG. 5. Electron microscopic autoradiography of a KB cell 24 hr after infection with adenovirus 12. One-hr pulse with tritiated thymidine. Silver grains are located over the nucleoplasm (n) and over type II (II) inclusion. Epon embedding. $\times 18,000$.

FIG. 6. Electron microscopic autoradiography of a KB cell 30 hr after infection with adenovirus 12. One-hr pulse with tritiated thymidine. Inclusion type II (II) and type IV (IV) have been labeled. No silver grains are found over the viral crystal. Epon embedding. $\times 18,000$.

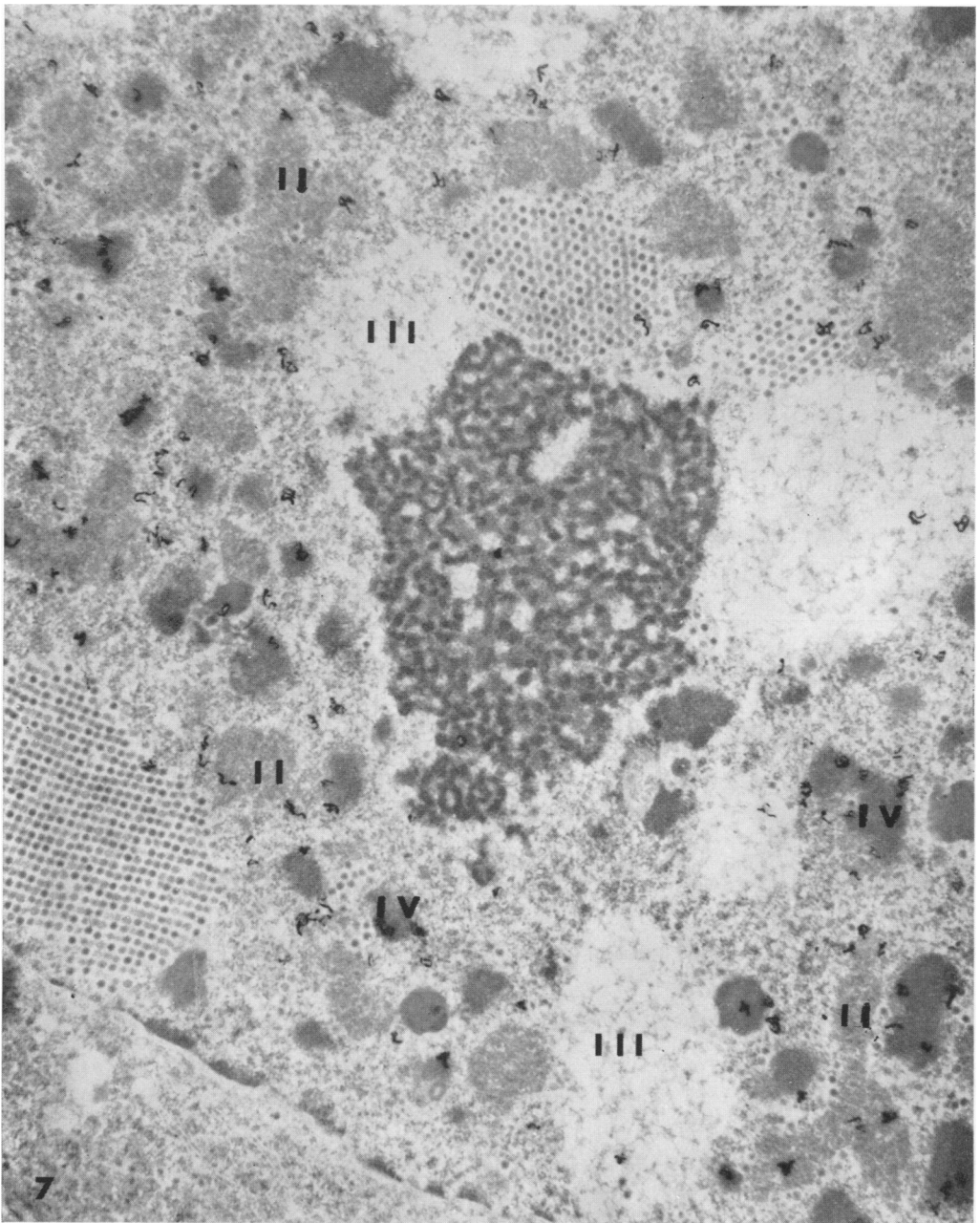


FIG. 7. Electron microscopic autoradiography of a KB cell 36 hr after infection with adenovirus 12. One-hr pulse with tritiated thymidine. Preferential labeling of inclusion type II (II) and type IV (IV). Poor labeling of type III inclusion. $\times 12,000$.

DISCUSSION

The present work demonstrates that adenovirus 12 replicating in KB cells induces an increase in the percentage of cells labeled with tritiated thymidine, and that during early stages nuclear and nucleolar labeling rates increase in infected

cells. Thus, our results are in agreement with previous light microscopic autoradiographic data indicating that cells exposed to adenovirus 12 have a higher percentage of labeling with tritiated thymidine than do uninfected cells (4, 25). With respect to tritiated thymidine uptake per in-

TABLE 1. Nuclear incorporation of tritiated thymidine (nucleolus excluded)

Time (hr)	No. of grains per $10 \mu^2$	
	Noninfected	Infected
8	29.1	57.4
16	25.1	24.2
24	17.2	11.4
30	19.8	9.7
36	21.8	10.7

TABLE 2. Nucleolar incorporation of tritiated thymidine

Time (hr)	No. of grains per $10 \mu^2$	
	Noninfected	Infected
8	33.5	63.3
16	29.0	30.2
24	24.0	3.0
30	17.5	2.4
36	24.8	2.6

TABLE 3. Distribution of silver grains over different regions in KB cells 36 hr after inoculation with adenovirus 12

Region	No. of grains	Per cent of total	Area (μ^2)	Grains/ $10 \mu^2$
Inclusion I.	3	0.1	7.5	4.0
Inclusion II.	511	32.3	97.3	52.5
Inclusion III.	111	7.0	205.0	5.3
Inclusion IV.	183	11.6	17.4	105.1
Virus crystals.	13	0.8	10.1	12.6
Nucleoplasm.	737	46.5	687.4	10.7
Nucleolus.	11	0.6	41.7	2.6
Cytoplasm.	13	0.8	547.1	0.2

dividual cell, Coto et al. (4) found no difference between infected and control HeLa cells, while Takayashi et al. (25) observed a higher degree of labeling in control than in adenovirus-12 infected cells 14 and 24 hr after infection. Our results confirm that infected KB cells show fewer silver grains per nuclear surface after 16-hr infection, but indicate that at 8 hr the number of grains per nuclear surface is higher in infected cells.

In addition to the early increase in nuclear DNA, we have found an augmentation of nucleolar DNA demonstrated by the higher incorporation of tritiated thymidine and by the increase of intranucleolar chromatin observed during early phases of viral replication. This result may be likened to the increased synthesis of nucleolar DNA found in SV40-infected cells in early stages (11). From the foregoing, it seems that the

nucleolus has an active participation in the biochemical events leading to the replication of small DNA viruses.

The location of a considerable proportion of silver grains over inclusion bodies, types II and IV, provides additional evidence of the DNA content of such bodies previously shown to be susceptible to deoxyribonuclease digestion after proteolytic treatment (22). This DNA may account, in part, for the 1.5- to 2.0-fold increase in DNA content of adeno-12 infected cells recently reported by Green (16), and may correspond to the excess viral DNA found by him in adeno-2 (15) and by Ginsberg and Dingle (10) in adeno-4 infected cells. It is very likely that the DNA present in such inclusions is viral DNA, since the associated protein has been found to be antigenic to adenovirus 12 (21).

Whether the increase in DNA synthesis induced by adeno-12 virus refers solely to an increase of viral DNA or whether adeno-12 stimulates the synthesis of both cellular and viral DNA, remains to be determined. This problem is particularly interesting in view of recent reports on the stimulation of host DNA synthesis by other oncogenic DNA viruses, polyoma and SV40. Productive infection of cell cultures with polyoma virus may alter the normal pattern of host DNA in two ways, depending on the physiological stage of the cell. On the one hand, in stationary cells with a low level of DNA synthesis, polyoma virus induces the synthesis of cellular DNA, as found independently by Dulbecco et al. (6), Weil et al. (27), and Winocour et al. (28). It has been subsequently demonstrated that the induction of cellular DNA occurs specifically in productively infected cells (26), and that this induction depends on a function of the viral genome (1, 8). On the other hand, polyoma virus inhibits host DNA synthesis in growing cultures with higher levels of DNA synthesis (3, 5, 23, 24). It has been found that the induced DNA is an unstable, rapidly degrading form of DNA (2). Induction of cellular DNA is also observed in abortive polyoma infection (8, 24), as well as in SV40 replicative (19) and transforming systems (9, 20).

With respect to DNA modifications induced by oncogenic adenovirus, very little biochemical evidence is available at present. From their autoradiographic study, Takayashi et al. (25) consider that the increased incorporation of tritiated thymidine may be the result of viral DNA synthesis, in view of the fact that adeno-12 infection decreases the mitotic index and that the label is located over viral inclusions. Furthermore, suppression of cellular DNA synthesis by X-irradiation does not alter the synthesis of viral DNA in productive or in abortive adeno-12 infections

(18). From our results it is evident that more than one-third of the label is located over inclusion bodies containing both DNA and protein (22) antigenic to adenovirus 12 (21). However, the nature of the DNA responsible for the early high levels of thymidine incorporation, and that of the DNA which accounts for more than 50% of the total labeling at late stages of viral replication, is uncertain. It is tempting to speculate that adenovirus 12 may induce the synthesis of host DNA and that at least part of the label which is not associated with viral inclusion bodies may be related to newly synthesized cellular DNA.

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