INDUCTION OF CELLULAR DNA SYNTHESIS IN CHICK EMBRYO FIBROBLASTS INFECTED WITH ROUS SARCOMA VIRUS IN CULTURE

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As has recently been found and confirmed by various research groups, the DNA-containing oncogenic viruses (polyoma virus, simian virus 40, adenovirus 12) induce the synthesis of cellular DNA in infected cultures of mammalian cells (Weil et al.,1965, Dulbecco et al.,1965, Winocour et al.,1965, Gershon et al.,1966, Henry et al.,1966, Ledinko,1967). In cells infected with these viruses the synthesis of a number of enzymes, which participate in the biosynthesis of pyrimidine deoxyribonucleotides and DNA, is also induced (Kit et al.,1966, Hartwell et al.,1966, Kára and Weil,1967). The ability to interfere with the genetic regulation of the synthesis of cellular enzymes and cellular DNA seems to be a characteristic property of the DNA-containing oncogenic viruses.

The question arose whether the RNA-containing oncogenic viruses carry the genetic information in their RNA for a similar function. It has been shown that the replication of Rous sarcoma virus (RSV) (RNA-containing oncogenic virus) in infected cells requires DNA synthesis and DNA-dependent RNA synthesis (Bader,1964, Temin,1964, Vigier and Goldé,1964). The synthesis of DNA in RSV-infected cells is necessary not only for the RSV replication but is also essen-

tial for the malignant transformation of the infected cells (Bader, 1965). Recently we have found (Kára,1968) that in the chick embryo fibroblast(CEF) cultures infected with RSV (strain Schmidt-Ruppin) the morphological and malignant transformation of cells is preceded by the induction of deoxycytidylate deaminase and uridine kinase and by the induction of cellular DNA synthesis. Induction of thymidine-^{3}H incorporation in chick embryo multinucleated myotubes by RSV has been described recently (Lee et al.,1968). Induction of the division cycle in resting stage human fibroblast after RSV infection was also reported (Macieira-Coelho and Pontén, 1967). Further experimental evidence of the induction of cellular DNA synthesis in CEF cultures infected with RSV (Schmidt-Ruppin strain) is presented in this communication.

Materials and Methods. The primocultures of CEF prepared by trypsinization of 9-day-old White Leghorn chick embryos (decapitated and deviscerated) were grown in a medium A described elsewhere (Kára,1968). The RSV preparation (strain Schmidt-Ruppin) was prepared from the tissue homogenate of Rous sarcoma (induced by this virus) according to Prince (Prince,1959). The cells were seeded in Petri dishes (60 mm in diameter,4 x 10^{6}cells per Petri dish) and cultivated at 39^{o}C in a thermostat in a humidified atmosphere with 5% CO_2. 24 hours after plating, the almost confluent cell-monolayer was infected with 0.5 ml RSV suspension (10^{6} focus forming units/ml) and incubated at 39^{o}C for 60 min. After virus adsorption the infected cultures were washed with 2 ml of medium A, 5 ml of the medium was then added and the cultures were further incubated at 39^{o}C. At this temperature the cell transformation process is accelerated in comparison with cultures incubated at 37^{o}C (Kára,1968).

Incorporation of thymidine-^{3}H into cellular DNA in RSV-infected CEF cultures as well as in uninfected ones was measured by the method of Mueller et al.(1962). Thymidine-6-^{3}H (specif.act. 25 C/mmole) was added to RSV-infected and to the uninfected CEF cultures in 3 ml medium A per Petri dish (4 μC/ml) at various time intervals after infection (p.i.) and the cultures were incubated for 60 minutes at 39°C. After this pulse labeling the cell monolayers were washed with isotonic saline, extracted with 0.6 M perchloric acid, dried with ethanol and ether and lysed in 0.4 ml 99% formic acid (Mueller et al.,1962). Aliquots of the lysate (0.1 ml) were dried on Whatman No.1 paper squares (25 x 25 mm) and radioactivity was measured in a Nuclear Chicago Mark I scintillation counter in toluene solution of 2,5-diphenyloxazole (PPO) and 2-p-phenylene-bis(5-phenyloxazole)(POPOP).

For autoradiography the RSV-infected CEF and uninfected CEF monolayers growing on microscopic slides in Petri dishes were labeled with thymidine-6-^{3}H (4 μC/ml) for 4 hours (24 - 28 hour p.i. and 68 - 72 hour p.i.). The cultures were then washed twice with 5 ml medium A and the free thymidine-6-^{3}H nucleotides were "chased" by incubation of labeled cells for additional 30 minutes in medium without thymidine-6-^{3}H. Autoradiograms of the fixed cells were prepared using Kodak AK 10 stripping film and 7 days exposure. The cells were stained with eosin and toluidine blue and the labeled nuclei were counted in total number of 4,000 cells per culture (two microscopic fields).

For isolation of labeled DNA the cultures were treated with thymidine-6-^{3}H in the same way as for autoradiography. Labeled DNA was extracted from RSV-infected and uninfected CEF cultures by the dodecylsulfate method (Watson and Littlefield,1960,Weil et

al.,1965, Hirt,1967). The pulse labelled cells were washed 3 times with 5 ml isotonic saline and than lysed by addition of 0.4 ml 0.6% sodium dodecylsulfate solution (pH 7.5) containing 0.01 M EDTA (ethylenediamine-tetraacetic acid). After 20 minutes at room temperature the viscous lysate was transfered with a Pasteur pipette into a glass tube, 0.1 ml 5M-NaCl was added and mixed with the lysate by gentle shaking for 1 minute. The mixture was stored at 4°C for 18 hours and then it was transfered with a Pasteur pipette into a plastic centrifuge tube and centrifuged at 12,000x g for 60 min. at 5°C to remove the major portion of SDS and protein. An aliquot (0.1 ml) was used for sedimentation velocity analysis (band centrifugation) according to the method described by Vinograd et al.(1965).The labeled DNA solution (0.1 ml) was layered over 3 ml of cesium chloride solution (ρ= 1.503 gm cm^{-3}, pH 7.0)in a 5 ml nitrocellulose tube and centrifuged in Spinco, model L centrifuge,in SW39 rotor at 35,000 r.p.m. and 4°C for 160 minutes. Then the bottom of the tube was pierced and 3 drops-fractions were collected. An aliquot (60 µl) of each fraction was used for the determination of radioactivity as described above.

Results and discussion. The incorporation of thymidine-6-^{3}H into DNA of RSV-infected CEF cultures and into DNA of uninfected cells was determined at 8, 24, 48, and 72 hours p.i. (Fig.1). It is evident that the thymidine-6-^{3}H incorporation in the RSV-infected cultures was about twice higher than in uninfected ones as early as 8 hours p.i. A similar relation was found at 24 hours p.i., when no morphologically transformed cells could be observed in the infected cultures. Thymidine-6-^{3}H incorporation in the infected cultures is higher at 48 hours p.i., when a small percentage of transformed cells appeared, and is maximal (about 8 times higher than in uninfected cultures) at 72 hours p.i., when about 50%

of cells in the culture is morphologically transformed.

The results of autoradiographic analysis of the RSV-infected and uninfected CEF cultures labeled with thymidine-6-^{3}H presented

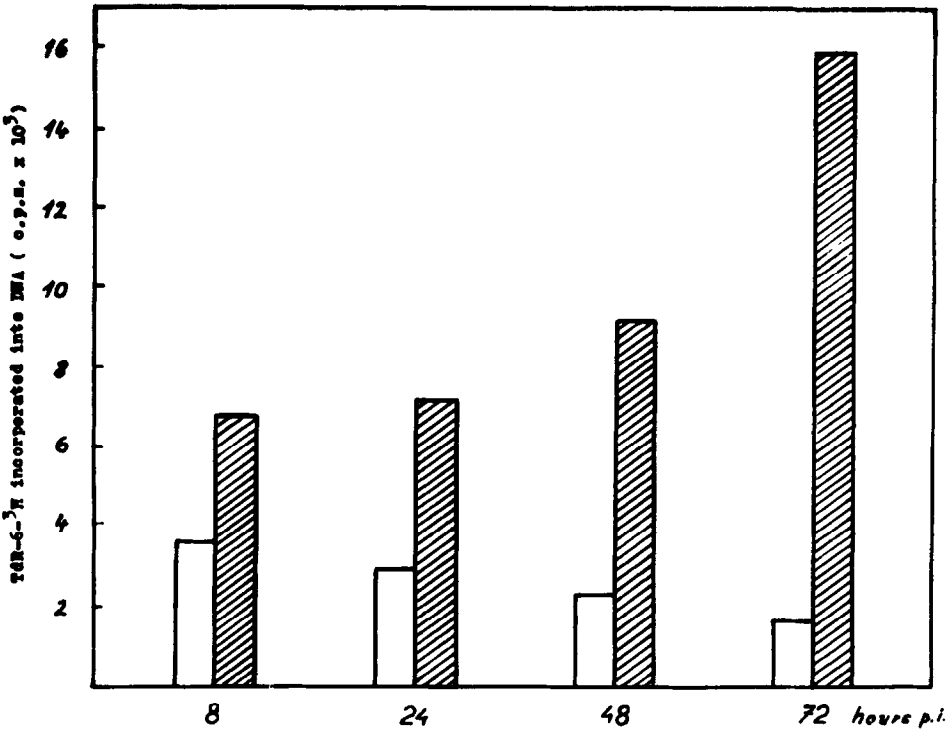

Fig.1. The incorporation of thymidine-6-^{3}H into cellular DNA of CEF cultures infected with RSV (shadowed columns) and in uninfected cultures (white columns). Each column represents the average radioactivity determined in two cultures.

Culture	hours p.i.	Number of ^{3}H-labeled nuclei per 4,000 cells	Percentage of ^{3}H-labeled cells
Uninfected	(24)	320	8
RSV-infected	24	750	18
Uninfected	(72)	248	6
RSV-infected	72	1,872	46

Tab.1. Autoradiographic analysis of the RSV-infected and uninfected CEF cultures labeled with thymidine-6-^{3}H.

in Tab.1 show that the percentage of thymidine-^{3}H labeled nuclei in the RSV-infected cell population at 24 hours p.i. is about twice higher in comparison with the uninfected CEF culture and about 8 times higher at 72 hours p.i. The grains of reduced silver were located only over the nuclei and the number of grains over a single nucleus did not differ significantly in the infected and uninfected cells.

The results presented in Fig.1 and Tab.1 indicate that the higher incorporation of thymidine-6-^{3}H into DNA in the RSV-infected cultures is due to an increased number of the DNA-synthesizing cells in the infected cultures. These results are in accord with the data published recently by Lee et al.(1968).

In order to prove that thymidine-6-^{3}H in the RSV-infected cultures is really incorporated into the cellular DNA molecules, the RSV-infected and uninfected CEF cultures were labeled with thymidine-6-^{3}H between 24 - 28 hours p.i., the labeled DNA was extracted and analysed by band centrifugation as described above. The results of the sedimentation velocity analysis (Fig.2) show that the sedimentation characteristics of the labeled DNA from RSV-infected and from uninfected CEF cultures are identical. The radioactivity is present mainly in the three "heavy fractions" of DNA (fractions number 8, 10 and 13 in Fig.2) and a low percentage is also in a "light fraction" (maximum in fraction number 26, Fig.2). Fraction 26 is a deoxyribooligonucleotide and is not split off from the "heavy" DNA molecules during the isolation. This fraction may be a subunit, which seems to be part of the cellular DNA (Thaler and Villee,1967). Higher radioactivity of the DNA extracted from the RSV-infected CEF culture (Fig.2) is in correlation with the higher number of DNA-synthesizing cells in this culture in comparison with uninfected CEF culture.

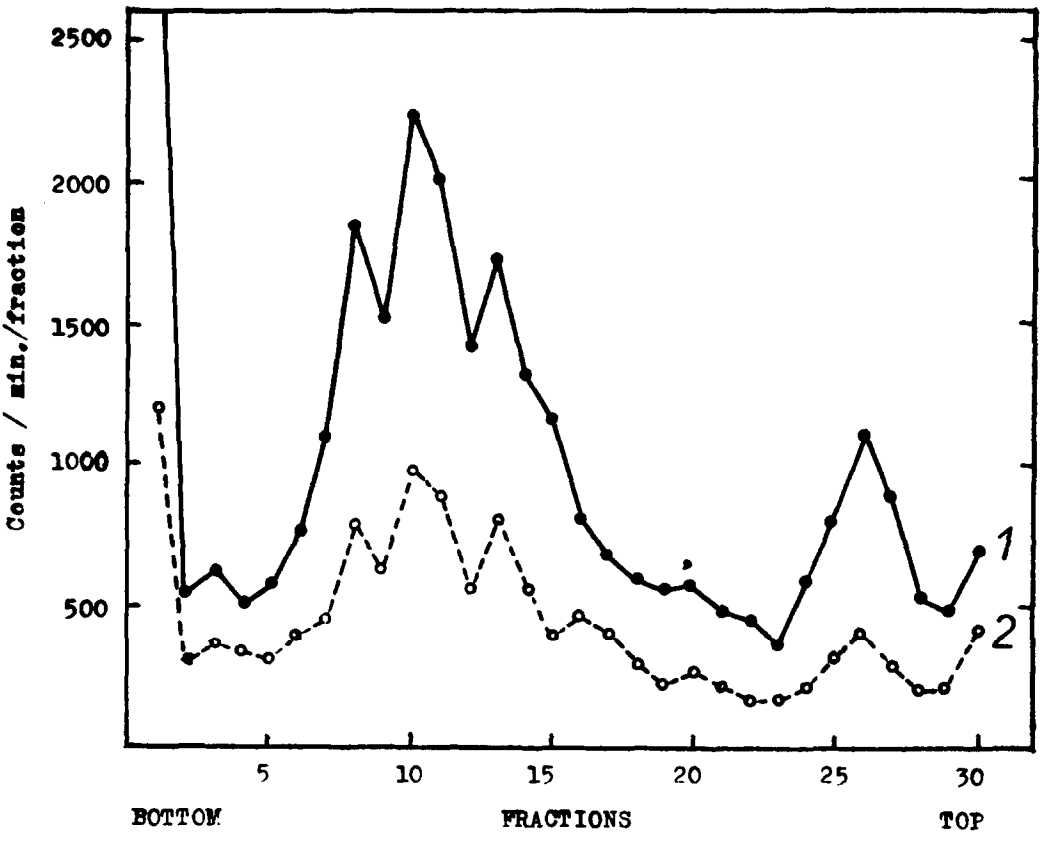

Fig.2. Sedimentation velocity analysis (band centrifugation) of thymidine-6-^{3}H labeled DNA isolated from CEF culture infected with RSV (1) and from uninfected CEF cultures (2).

The experimental results permit the conclusion that cellular DNA synthesis is induced in the chick embryo cells infected with RSV (Schmidt-Ruppin strain). This induction with the simultaneous induction of the cellular enzyme deoxycytidylate deaminase (Kára,1968) and probably with the induction of other enzymes of the pyrimidine deoxyribonucleotides biosynthetic pathway precedes the malignant transformation of the RSV-infected cells. It is interesting that one of the functions of RSV, the RNA-containing oncogenic virus, is the ability to induce cellular DNA synthesis, as has also been found with the oncogenic DNA-contai-

ning viruses. It is therefore probable that the induction of the cellular DNA synthesis is part of the mechanism of viral carcinogenesis.

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