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REVERSIBLE INHIBITION OF REPLICON INITIATION IN EHRLICH ASCITES CELLS BY ANAEROBIOSIS

Hans Probst and Volker Gekeler

Physiologisch-chemisches Institut der Universität Tübingen
Hoppe-Seyler-Straße 1, D 7400 Tübingen, Germany

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SUMMARY: The effect of anaerobiosis and oxygen recovery on the DNA synthesis of Ehrlich ascites cells was studied by alkaline sucrose gradient centrifugation after pulse labeling the daughter chain DNA of operating replication units by [^{3}H]-thymidine. Removal of oxygen from cells growing in culture caused a strong inhibition of initiation of new units. When oxygen was resupplied initiation reappeared subsequently. The kinetics of restoration of a normal length distribution of the daughter chains of operating units indicated that the initiation pattern in the reaerated cells was normal.

The nuclear genome of eucaryotic cells is organized for replication into multiple discrete units, replicons (for a review see (1)). Little is known about the molecular events leading to the initiation of DNA synthesis in a replicon. To study these events it would be of great advantage to have a reliable tool for suppressing initiation and permitting it again.

An inhibitory effect on replicon initiation has been described for several agents, e.g.: Actinomycin (2), cycloheximide (3), arabinofuranosylcytosine (4), novobiocin (5) and 2,4-dinitrophenol (6). The fastest method to demonstrate an inhibition of replicon initiation is the analysis (by alkaline sucrose gradient centrifugation) of the length distribution of the daughter strand DNA labeled by a [^{3}H]-thymidine pulse of a few minutes. The principle of this method was described by Huberman and Horwitz (7) and by Gautschi et al. (6). The latter authors applied it first on drug mediated inhibition of initiation.

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Using this method we searched for a suitable off/on switch of the kind mentioned above. So far, we had not been able to find conditions for any of the tested drugs under which a sufficient inhibition of replicon initiation occured and could be abolished to a satisfactory extent afterwards.

From published work is known so far: (i) 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, inhibits replicon initiation (6). (ii) The DNA synthesis of the Ehrlich ascites cells used in this laboratory ceases after 6 - 8 h of anaerobiosis (8). (iii) The cells reenter DNA synthesis and almost normal growth when aerobic conditions are reconstituted (8,9,10).

These facts stimulated us to examine the effects of anaerobiosis on replicon initiation.

Below, we present evidence that anaerobiosis suppresses replicon initiation in Ehrlich ascites cells. After oxygen recovery initiation too recovers quickly. An essentially normal length distribution of pulse labeled daughter DNA reappears when a period is elapsed which corresponds to about one complete replication round of the larger replicons occuring in these cells.

MATERIALS AND METHODS: The ascites cells and their growth in vivo and in vitro have been described (11). We used cells from the logarithmic growth phase of the in vivo tumor (4 days after inoculation). About 15 h before explantation to normal culture medium (6.5 x 10 cells/ml) the tumor mice were injected with 7.5 µC [2- ^{14}C]-thymidine (Amersham-Buchler, 60 mC/mMol) in 0.3 ml 0.9 % NaCl in order to label the mature DNA. 2 h after explantation anaerobiosis was initiated in one part of the cells (the other part served as aerobic control) by bubbling argon/ 5 % CO (deoxygenated and humidified according to ref. (10)) through the culture for 15 min. For the rest of the anaerobic period the gas was only flushed over the surface of the culture fluid. Anaerobiosis was abolished by bubbling air through the culture for 15 min. For pulse labeling of the nascent daughter DNA 1 ml cell suspension was incubated anaerobically or aerobically with 15 or 30 µC of [Me-^{3}H]-thymidine (NEN - Europe, 50 C/mMol) for 5 min. Radioactivity incorporation was stopped by pouring the cells into 10 ml of Hanks solution of 0 ^{o}C. The cells were washed twice with cold Hanks solution and then either lysed on the top of alkaline sucrose gradients (2 - 4

x 10^5 cells/gradient) or counted for their $^3H/^{14}C$-ratio after repeated washings with cold 5 % TCA and collection on membrane filters. The gradients (Rotor SW 27, Spinco) consisted of (12): 15 - 30 % sucrose, 0.25 M NaOH, 0.5 M NaCl, 1 mM EDTA, 0.1 % (w/v) sarcosyl. The lysis procedure of ref.(13) was used. After 6.5 h of lysis at 23°C in the dark the gradients were centrifuged at 23°C and 26 000 rpm for 6 h. 30 fractions of 1.2 ml were collected from the top (Isco gradient fractionator) and precipitated with 4 ml 15 % TCA after addition of 100 µg carrier DNA. The precipitates were collected on membrane filters which were counted after washing with 1 % TCA and drying in a toluene based scintillation fluid. The gradients were calibrated with ^{14}C-labeled lambda-, T5- and T4-phage. One fraction corresponds to 4.5 S

RESULTS: It is known from prior investigations with these cells (8,9,10) that the DNA synthesis rate drops progressively after establishment of anaerobic conditions and is practically zero after 7 - 8 h. In the experiment of fig. 1 the thymidine incorporation was 12 % of the aerobic control after 5 h of anaerobiosis. Reaeration caused a subsequent reincrease of the thymidine incorporation rate and, after 2 - 2.5 h, the initial level was reached again. (Anaerobiosis during more than 10 - 12 h may cause a lag of the reincrease under certain conditions (8,10)). Fig. 1 shows the alkaline sucrose gradient sedimentation patterns obtained in a 5 h anaerobiosis/2.5 h aerobiosis experiment. The peak of the [^{14}C]-prelabel (mature DNA) of all gradients was at about 120 S (depicted only for 2 of the 12 gradients contained in fig. 1). The length distributions of the [^3H]-pulse labeled daughter chains of the aerobic controls in fig. 1 are virtually indentical. Position and form of these distributions correspond largely to those described in the literature (4,6,12). This indicates an identical and essentially normal pattern of replicon initiation and operation in all control samples.

In contrast, the corresponding [^3H]-profiles obtained after 1.5, 3 and 5 h of anaerobiosis are progressively impoverished of radioactivity in the range of the shorter chain lengths. This

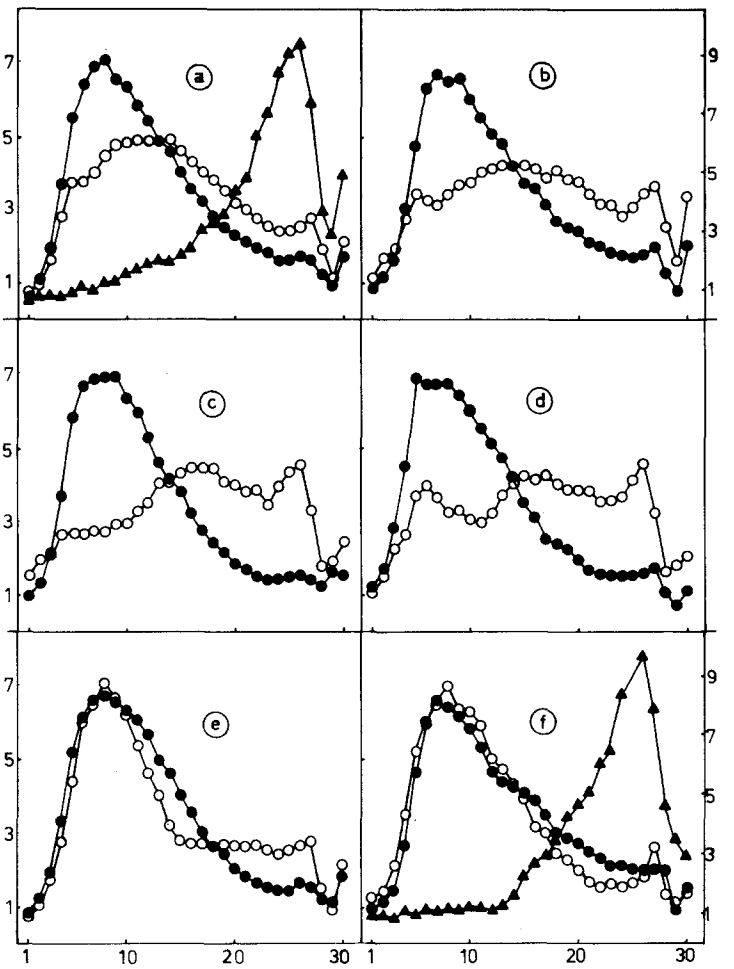

Fig. 1: Alkaline sucrose gradient centrifugation. Abscissa: Fraction number; ordinate: Percent of total counts. Direction of sedimentation is from left to right.
A freshly explanted culture of [^{14}C]-prelabeled cells was divided. One part was incubated aerobically (control), the other first anaerobically (5 h) and then again aerobically. At the times indicated below a sample of each culture was pulse labeled with [^{3}H]-thymidine, lysed on the top of alkaline gradients, centrifuged and counted as described in Methods. Each fig. in part contains the two [^{3}H]-profiles of each point of time (●—● = controls, ○—○ = anaerobic or anaerobic → aerobic; left ordinate). For better clarity, only two of the 12 (nearly identical) [^{14}C]-profiles are shown (▲—▲, right ordinate).
a) 1.5 h anaerobic, b) 3 h anaerobic, c) 5 h anaerobic, d) 5 h anaerobic → 20 min aerobic, e) 5 h anaerobic → 1 h aerobic, f) 5 h anaerobic → 3 h aerobic. Total [^{3}H]-cpm of the controls were between 16 733 and 43 561. The gradient of the most inhibited anaerobic sample contained totally 4 866 [^{3}H]-cpm.

indicates that the initiation of new replicons was largely suppressed (4,6,7,). Already 20 min after the begin of reaeration a distinct slowly sedimenting peak appeared. After 1 h the light flanks of the profiles of the control sample and of the reaerated sample essentially coincided again. After 2.5 h hardly any difference occured between the control and the reaerated sample, apart from a slight relative deficiency in the region of the very long chains of the latter. This indicates that a new and essentially normal steady state of replicon initiation, operation and termination had been established again.

DISCUSSION: The results show clearly that the initiation of new replicons is strongly inhibited when the cells are deprived of oxygen. This inhibition can be abolished easily and completely when oxygen is resupplied. The mean fork movement rate of our ascites cells in vitro is 0.6 - 0.7 um/min, the largest inter-initiation distances are 120 - 200 um (14). Replicons of this size would operate 90 - 150 min. Within this time an almost normal length distribution of the daughter DNA in the operating replicons reappeared. This indicates that the block had significantly altered neither the initiation frequency nor the replicon length distribution. A detailed kinetic analysis, including fiber autoradiographic studies, is in progress.

In a comparative study (to be published elsewhere) oxygen deprivation and resupply has been proved to be superior to a number of drugs (mentioned initially) as a reliable switch for replicon initiation. In praxi, this switch can be operated by simply turning a stopcock. Therefore, it may be useful in studying the molecular mechanism of replicon initiation. During the growth of an Ehrlich ascites tumor in the mouse a prolonged S-phase occurs (15) which is shortened again when the cells are

explanted (11,14). The supposition that this may be due to a reduced replicon initiation frequency, caused by the poor O_2 supply in the in vivo tumor (14), is supported by these results. The molecular basis of the O_2 dependence of the replicon initiation is not clear. The results presented in (8,9,10) indicate that glycolysis can supply sufficient ATP and that energy deficiency may not be the reason. To explain the analogous inhibitory effect of 2,4-dinitrophenol (which cannot be abolished as easy) Gautschi et al. (6) suspected that high energy intermediates of oxidative phosphorylation essential for both, ATP production and replicon initiation, might be involved. The present results support this hypothesis.

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