Oxygen Dependent Regulation of DNA Synthesis and Growth of Ehrlich Ascites Tumor Cells in Vitro and in Vivo

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ABSTRACT

Ehrlich ascites cells were cultured under different O₂ partial pressures from <0.1 ppm to 2 × 10⁻⁶ ppm. During the artificial hypoxia and following reoxygenation the DNA synthesis rate was measured and the relative frequency of replicon initiations was examined by analyzing the length distributions of replicative daughter strand DNA. These studies were complemented by evaluation of growth and cycling of the cells and by biochemical analyses. It was demonstrated that the reversible shutdown of clusters of replication units already described before (Probst, H., Gekeler, V., and Helftenbein, E. Exp. Cell Res., 156:327-341, 1984) occurred between 0.25 and about 2.5 μM dissolved O₂. Above 2.5 μM, a transition range to aerobiosis extended to about 16 μM O₂. Below 0.25 μM O₂ the cells suffered damage impairing the reversibility of the shutdown. The observed changes of growth and cycling correlated well with the respective changes of replication. Analogous oxygen dependent regulatory events in replication were also observed during growth of the cells as an in vivo ascites tumor. Obviously, the particular oxygenation conditions in the peritoneal cavity strongly influence tumor growth via the oxygen dependent regulation of replication.

INTRODUCTION

It is generally accepted that the natural microenvironment of malignant tumor cells is frequently hypoxic to varying extents. Especially microelectrode measurements on multicellular spheroids as in vitro tumor models (1) furnished exact and reliable data. The oxygenation state is a crucial contributory factor of the proliferative behavior of tumor cells (2-4) and greatly modifies their susceptibility to different drugs (5) and radiation (6, 7). Probably, certain interdependences exist between the cell proliferation and the susceptibilities mentioned. The underlying molecular mechanisms, however, are still not understood.

In connection with work on the molecular mechanisms of DNA replication in animal cells we found that deprivation and recovery of oxygen was a suitable means for specifically and reversibly suppressing the initiation of replication units ("replicons") in the DNA of cultured Ehrlich ascites cells (8). Obviously, the suppression of initiation was not caused by any impairment of the cellular sources of energy or DNA building blocks or cofactors of DNA synthesis; DNA chain elongation and maturation were essentially not affected (9). The suppression and reactivation of replicon initiation occurred strictly orderly in groups ("clusters") of replicons (9). Hypoxic cells in G₁ of the cell cycle did not initiate the first replicon clusters of the S phase; in S-cells the initiation of further clusters progressively failed to appear while the replication of already active clusters was completed normally. By reoxygenation, initiations could be reactivated within less than 10 min.

As these events obviously occurred independently of intracellular shortages and were coordinated at the hierarchical level of replicon clusters, which is the level of control for the run of replication in "committed" cells (10-12), they were regarded as a manifestation of an oxygen dependent regulation. By this regulation the cells probably can enter a special kind of resting state which is quite different from resting in the G₂-G₁ or G₂ of the cell cycle and rather exhibits analogies to the resting observed with MCA-11 mouse mammary tumor cells under metabolic constraints (13). The existence of a regulation of this kind has important implications for modeling tumor growth and for the design of therapeutic regimes in tumor treatment. The present communication deals with systematic studies on the conditions of the release of the O₂-dependent regulation of the replication machinery of Ehrlich ascites cells growing in culture or as in vivo tumors. In addition, we summarize the results of control studies on growth, cycling, etc. of the cells, performed parallel to analysis of the DNA replication under different O₂ conditions.

MATERIALS AND METHODS

Cells. The growth and the cytokinetic properties of the Ehrlich ascites cells used (designated ELT Bonn according to Nielsen and Granzow (14) have been described elsewhere (15). In vivo ascites tumors were maintained by transplanting about 25 × 10⁶ cells from mouse to mouse every 7 days. The in vitro experiments were performed with suspension cultures in the second passage explanted from 4-day-old in vivo tumors. Hypoxic Standard Cell Culture. High purity argon/5% CO₂ was purchased from Linde, Karlruhe, Federal Republic of Germany. The supplier guaranteed an O₂ content <5 ppm. This gas was mixed with the desired portions of O₂ (medical grade) in preevacuated gas cylinders under manometric control. For low O₂ concentrations two dilution steps were used. For elimination of O₂ to <0.1 ppm the argon/CO₂ mixture was passed through a heated column (150°C) filled with oxygen absorbing catalyst (R3-11/M3610; BASF, Ludwigshafen, Federal Republic of Germany) directly before use. For humidification, gases were bubbled through 0.9% NaCl solution (boiled before use) at 37°C. All tubing consisted of stainless steel or glass connected by flexible joints of thick-walled butyl tubing made as short as possible.

Gassed standard cell cultures were grown in Fernbach-type culture flasks (20 cm diameter) equipped with an NS19 ground joint side neck accommodating the O₂ electrode. The main neck was closed by a large rubber stopper, or for experiments using gas mixtures containing <0.1 ppm O₂, also by a ground joint. From the main neck two capillary glass tubes, serving as a gas inlet or resp. as combined gas/culture-sample outlet, reached to opposite bottom edges of the flask 6-8 mm above the surface of the culture fluid. For experiments, usually large volume (200-500 ml) starting cultures were prepared (4-5 × 10⁶ cells/ml). After about 2 h aerobic growth, 100 ml culture fluid were transferred to each gassing flask and to a normal aerated culture flask (control). Initially the gassing flask was tilted by about 50° in order to immerse the gas inlet tube into the culture fluid and to bring the end of the outlet tube in the uppermost region of the flask. About 100 ml gas/min were bubbled for 30 min through the culture fluid. Then, the flask was reverted to normal position and the gas was henceforth merely conducted over the surface of the culture. Culture samples for analyses or radioactive incubations could be extruded without contamination by air

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into appropriate vessels equipped with an inlet and outlet, respectively. These vessels were connected to the outlet line of the Fernbach flask and were flushed with the emanating gas usually 1–2 h before the sample was extruded by transiently tilting the flask and thereby immersing the end of the outlet capillary tube into the culture fluid. Cell culture samples to be reoxygogenated were extruded into 0.25 volume of prewarmed medium saturated with argon (gas mixture 99% Ar/1% CO₂).

Determination of O₂. For polarographic O₂ measurements an Orbisphere Oxygen Indicator Model 2607 with sensor 2112 (Orbisphere Laboratories, Geneva, Switzerland) was used. The sensor was routinely placed in the side neck of the culture flask.

For the chemical determination of dissolved O₂ the method of Altman (16) was specially adapted for analysis of cell culture samples. The reaction was performed in 12-ml “multidose vials” sealed up with a 4-mm thick rubber septum under an argon atmosphere and then stored under argon. About 10 ml of culture fluid were extruded into preweighed multidose flasks through injection needles pierced through the septum. Subsequently, 25 ml of 40% MnCl₂·4H₂O and 100 ml of 20% NaOH were injected and 25 s later, 200 ml of a mixture of 7 volumes acetic acid and 1 volume 4% Leukoberbellin Blue 1 (Boehringer-Mannheim) were added. Before reading A₄₂₂ at d = 1 or 5 cm against a blank with 50 μl of MnCl₂·4H₂O, precipitated material was separated by centrifugation. The Leukoberbellin Blue 1 [bis-(4-dimethylaminophenyl)-phenyl-o-sulfonic acid methanol] was synthesized according to the method of Altman (16). All solutions were prepared and stored under strict exclusion of air. The all-glass syringes for injection of the reagents were flushed with argon to correct for A₄₂₂ not representing physically dissolved O₂, we gassed cell cultures with <0.1 ppm O₂ for 30 h. Then, after adding 100 ml 20% NaOH/10 ml, the cultures were boiled for 15 min while continuing gassing. After cooling, 250 μl of 4% MnCl₂·4H₂O/10 ml were added under vigorous shaking followed by addition of acetic acid/Leukoberbellin Blue 1 and photometry as above.

Analysis of DNA Replication. The [³H]cpm measured in [³H]dTdTh incorporation studies performed according to the method of Probst et al. (17) were related to a ¹³C prelabel obtained by preincubating the cells with [¹³C]thymidine (16–18 h, 10⁶ cells in 200 ml medium containing 0.5 μCl [¹³C]thymidine, 50–60 μCl/mmol, obtained from Amersham-Buchler, Braunschweig, Federal Republic of Germany). Roughly 80% of the radioactivity was incorporated into DNA under these conditions. The autoradiographic procedures for evaluating thymidine pulse labeling index, continuous labeling curves, and labeled mitoses curves are described in (15).

For analysis of the length distributions of replicative daughter strand DNA by alkaline sedimentation 2-ml culture samples were labeled for 8 min with [³H]dTdTh and processed as described in (8). For labeling the tumor cells in vivo, 200 μCi [methyl-³H]thymidine in isotonic saline were i.p. injected in case of tumors not older than 5 days. From the sixth day on 300 μCi [¹³C]thymidine (16–18 h, 10⁶ cells in 200 ml medium containing 0.5 μCi [¹³C]thymidine, 50–60 μCl/mmol, obtained from Amersham-Buchler, Braunschweig, Federal Republic of Germany). Roughly 80% of the radioactivity was incorporated into DNA under these conditions. The autoradiographic procedures for evaluating thymidine pulse labeling index, continuous labeling curves, and labeled mitoses curves are described in (15).

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is shown in the *inset* is practically identical with that measured with the $O_2$ electrode in all experiments of this study using gas mixtures with $<1000$ ppm $O_2$. At later times of the experiments, the relative distance between such curves became more significant, because of the quasi-steady state mentioned. This is illustrated by the *broken lines* in Fig. 1A indicating the course for gassing with 150 and 300 ppm $O_2$, respectively.

Fig. 1B summarizes the relationship between the dissolved $O_2$ and the $O_2$ content of the respective gassing mixtures for all $O_2$ portions between $<0.1$ ppm (i.e., the most extreme hypoxic mixture that can be feasibly used by us) and $2 \times 10^4$ ppm after a 10–12 h gassing period. The situation of four “hypoxic stringency ranges” designated (a)–(d) is also shown in Fig. 1B. As outlined below, these ranges reflect the biological responses of the cells in standard experiments using gassing mixtures containing different portions of $O_2$: (a) $2 \times 10^3$ (=atmospheric)–$2 \times 10^4$ ppm, range of no response; (b) $2 \times 10^4$ to $2 \times 5 \times 10^3$ ppm, range of incomplete response; (c) $2 \times 5 \times 10^3$ to $150$–$200$ ppm, range of reversible transition to hypoxic resting state; (d) $150$–$200$ ppm to the most extreme hypoxic gassing mixture that can be feasibly used by us ($<0.1$ ppm $O_2$), range of cell damage.

Note that the relationship of these ranges to the dissolved $O_2$ scale is not a true one during the initial decline of dissolved $O_2$. DNA Synthesis in Cultured Cells during Hypoxia and after Reoxygenation. Following is a summary of the results of a series of 13 identically scheduled experiments in which standard cell cultures were gassed with mixtures containing different portions of $O_2$. Parts were reoxygenated 5 and 10 h after the start of gassing. During gassing and up to 100 min after reoxygenation we monitored the $[^3H]$dThd incorporation rate. In addition, we examined the relative frequency of freshly initiated replication units by analyzing the length distributions of pulse labeled daughter strand DNA at 0, 2, 5, and 10 h under gassed conditions and 20, 40, and 60 min after reoxygenation. The same analyses were performed with the aerated control cultures derived from the same starting cell populations.

Earlier work (9) had shown that hypoxia increased the cellular pool for exogenous thymidine equivalents. We reexamined this effect systematically at different $O_2$ conditions by measuring the extent of $[^3H]$bromodeoxyuridine substitution in the newly formed DNA (data not presented). This enabled us to correct the $[^3H]$dThd incorporation data for the hypoxic pool changes.

Fig. 2 shows 3 of the altogether 13 curves of thymidine incorporation rates. All 13 curves qualitatively displayed the same characteristic course already observed earlier (9), i.e., a decay-like decrease during the hypoxic gassing and a recovery exhibiting an artificial swerve after reoxygenation. It was therefore possible to compile the relevant quantitative features of all 13 curves in a single graph (Fig. 3) which shows the relative levels of the DNA synthesis rates 5 and 10 h after gassing start together with the respective recoveries following the reoxygenations. The 13 experiments of this series comprised more than 100 alkaline sucrose gradients run with $[^3H]$dThd pulse labeled cells. Fig. 4 shows some examples of the $^3H$ sedimentation patterns obtained. These patterns represent length distributions of replicative daughter chains which occurred at the end of the three experiments of Fig. 2 preceding and following reoxygenation.

With respect to the different hypoxic stringency ranges (a)–(d) (Fig. 1B) the entirety of the results of the present series revealed the following.

In range (a) the DNA synthesis rate in gassed cultures was...
indistinguishable throughout from that of the aerated controls. Equally, the chain lengths of replicative daughter DNA exhibited no detectable changes, neither during hypoxia nor after reoxygenation. As hypoxia was only moderate in range (a) the "reoxygenation" by addition of 0.25 vol medium saturated with pure O<sub>2</sub> elevated the dissolved O<sub>2</sub> in the cultures partly noticeably above the aerated level. It was not determined to what extent the slight excess of [<sup>3</sup>H]dThd incorporation following addition of O<sub>2</sub> medium to the culture gassed with 2 × 10<sup>4</sup> and 2 × 10<sup>5</sup> ppm O<sub>2</sub> represented triggering of a few additional replicon initiations not detectable in the sedimentation profiles.

The effects observed in range (c) were compatible throughout with our earlier findings (8, 9, 19), when we simply evaluated results between hypoxic and aerobic conditions, respectively. The alkaline sedimentation patterns obtained from cells labeled during hypoxic gassing consistently revealed that the relative frequency of replicon initiations was considerably decreased (see e.g., Fig. 4C). DNA fiber autoradiography had demonstrated that the suppression of initiations, also measurable by alkaline sedimentation, was the essential cause of the hypoxic drop of the DNA synthesis rate while DNA chain growth remained essentially unaffected (9). Fig. 2C and the data concerning range (c) in Fig. 3 indicate that missing initiations were accumulated during the hypoxic period in a state ready to be activated immediately after reoxygenation: (i) the replication activity of the reoxygenated cells exceeded that of the controls at the end of the 100 min observation period partly by >100% and further increased during the subsequent 30–60 min (not shown); (ii) the response to reoxygenation was distinctly more pronounced after 10 h hypoxia than after 5 h. Strengthening the hypoxia within the limits of range (c) also led to an enhanced response. The latter may reflect an earlier drop of dissolved O<sub>2</sub> below the critical level when gassing mixtures contain less O<sub>2</sub>. Burst-like initiations of new replicons triggered by the reoxygenation are clearly visible in Fig. 4C.

In range (b) the above described effects successively emerged. However, further experiments indicated only poor correlation between stringency of hypoxia and the extent of the hypoxic responses of replication within the limits of range (b). The transition between ranges (b) and (c) was rather poorly defined. Conceivably, the total extension of range (b) only reflects local and temporal changes of dissolved O<sub>2</sub> in the diffusion depleted zone surrounding the cells (24) brought about by intermittent agitation of the gassed cultures during withdrawal of samples. Apart from this, the cells can be heterogenous with respect to the individual O<sub>2</sub> threshold eliciting the hypoxic responses (25). Finally, the breadth of transition of a hypothetical redox system serving as O<sub>2</sub> sensor (26) may be of importance.

In range (d) the hypoxic decay of replication became very pronounced. Extreme hypoxic gassing (<0.1 ppm O<sub>2</sub>) let the DNA synthesis rate drop to near zero within 7 h (Fig. 2A). However, at the same time, the cells progressively lost the ability of fast recovery following reoxygenation. Already after gassing with 100 ppm O<sub>2</sub>, the DNA synthesis rate did not reattain the level of the control culture during the 100 min observation period following readmission of oxygen. In marked contrast to range (c), prolonging the hypoxic gassing period and/or further strengthening the hypoxia caused additional impairment of recovery (Fig. 2A). After 12 h gassing with <0.1 ppm O<sub>2</sub>, reoxygenation produced no significant recovery within the first 2 h; after 6 h only 30% of the control was attained (data not shown). The impaired recovery of the DNA synthesis rate correlated with the absence of new replicon initiations upon reoxyenation (Fig. 4, A and B). It seems reasonable to suppose that the cells suffered progressive damage when exposed to extremely hypoxic conditions for longer periods. This damage manifested itself, perhaps besides failures of other cellular functions, in an inability to restart effectively the replication machinery which was switched off before when the cells passed through ranges (b) and (c).

Cell Growth and Related Parameters. A separate series of experiments was designed to correlate the above observations with growth, cycling, and the supply of ATP energy of the cells. Furthermore, accumulation of cellular protein was measured and glucose, lactate, and pH of medium were controlled. For each experiment three standard cultures were established from the same large starting culture and gassed with the same gassing mixture. Each of the cultures in part served for examination of 2–3 of the parameters mentioned, usually at 4-h intervals. The gassing mixtures used had the following O<sub>2</sub> contents: 2 × 10<sup>-4</sup>; 1.3 × 10<sup>-3</sup>; 7 × 10<sup>-3</sup>; 3.6 × 10<sup>-3</sup>; 200; and <5 ppm.

Each of the experiments of the series additionally included examination of the length distribution of replicative daughter strand DNA by alkaline sedimentation 10 and 20 h after gassing start. The results confirmed those described in the preceding paragraph.

The cell growth was analogous (data not shown) to the already described activity of DNA replication under the same O<sub>2</sub> conditions. At any O<sub>2</sub> concentration within range (a) completely normal aerobic growth occurred. Within range (b), cell multiplication was progressively retarded and finally adopted the behavior which was typical for ranges (c) and (d). There, the cells grew by less than 20% during the first 5–8 h of gassing and were then completely arrested. After 20–22 h of gassing,
all cultures were reaerated and cell number was reexamined 16–20 h later. In cultures having been gassed before with mixtures corresponding to ranges (a)–(d) the cells caught up with the aerobic control cultures by about 80%. Range (d) cultures grew only insignificantly within this period.

Analyses using the labeled mitoses technique indicated that even in range (b) a significant fraction (27) of the cells cycled as if growing in a normal aerated culture (15). Continuous [3H]dTTh labeling during the hypoxic period demonstrated that the entry of new cells into DNA synthesis was strongly inhibited when gassing mixtures contained <3.6 × 10⁻⁵ ppm O₂.

DNA histograms, produced by flow cytofluorometry routinely recorded during all experiments of this series at 4-h intervals, indicated in accordance with the above growth data and autoradiographic data, virtually no differences to the aerated control cultures within range (a). In the lower half of range (b), we found a slight and transient accumulation of cells exhibiting G₁ DNA content. In range (c), this accumulation was distinct and persistent, at least during the 20-h period observed, and occurred preferably at the expense of G₂ cells. The example shown in Fig. 5, A and B demonstrates this effect with the aid of an asynchronous culture gassed for 12 h with 200 ppm O₂. In a separate experiment, we gassed also relatively pure G₁ cells [selected by a zonal centrifugation procedure developed in our laboratory (15, 28)] for 12 h with 200 ppm O₂ and demonstrated that the accumulation was brought about by preventing G₁ cells from entering S phase and not by shifting the steady state of cycling cells in favor of G₁ (Fig. 5, C and D). This type of G₁ arrest, however, turned out to be distinctly different from that produced by starvation of serum or growth factors where the initiation of DNA synthesis first occurs 6–10 hours after resupplementation (29): G₁ cells arrested by hypoxia under conditions like that of the experiment of Fig. 6 normally enter DNA synthesis within <10 min after reoxygenation. Flow cytofluorometry fails to demonstrate this quick response of the cells on resupplementation with O₂; it is the subject of a separate communication presenting evidence that the initiation of early S-phase replication units triggered by reoxygenation of hypoxic cells occurs with excellent synchrony. Our zonal centrifugation procedure also permits the selection of fully viable cells with DNA content intermediate between G₁ and G₂ (28). Such cells equally did not increase their DNA content during 12-h gassing with 200 ppm O₂ and could also be stimulated to continue cycling by reaeration. Thus, the reversible arrest at the G₁-S-phase border as well as the arrest in different stages of the S phase is consistent with the results of numerous studies using DNA flow cytometry, as reported e.g., in Refs. 30–32. Only cells of the very late S phase were reported to attain G₂ under hypoxic conditions (31). In range (d), the DNA histograms exhibited the same accumulation of G₁ cells as in range (c). However, the entry of the accumulated cells into S phase after reoxygenation appeared to be progressively delayed and retarded in dependence on the extent of the O₂ deficiency and its duration. This observation corresponds to the impairment of recovery of replication under the same conditions.

The adenylate energy charge (33) measured in the course of all experiments of this series was without exception between 0.8 and 0.9. Independent cultures held under the same O₂ conditions usually exhibited larger differences than two parallel cultures derived from the same starting population but exposed to gas mixtures differing largely in their O₂ content. Even reoxygenation of severely hypoxic cultures produced no significant changes of cellular ATP/ADP/AMP. This confirms the belief that tumor cells are not dependent on respiratory ATP (34).

The determinations of glucose and lactate in the medium indicated that the cells, already in range (b), enhanced their glycolysis which was relatively high in the aerated state anyhow. The ratio lactate produced:glucose consumed rose from 1.6 to 1.9. The pH remained above 6.8–6.9 during the 20-h gassing period in all cases. The glucose concentration never fell below 5 mM (initial value, 11 mM).

Coincidentally with the increase of glycolysis, the net increase of total cellular protein dropped to about 10% of the normal aerated rate and remained at this level also in range (c). According to Pettersen et al. (35) the hypoxic drop of protein accumulation relies on enhanced (energy consuming) protein degradation rather than on diminished synthesis and probably is part of a regulatory answer of the cells to decreased O₂ tension itself. It is perhaps no pure chance that the changes of glycolysis and protein metabolism occurred together with the switching of DNA synthesis at O₂ tensions about two orders of magnitude above the level affecting the respiratory activity of Ehrlich ascites cells (36) and at an obviously completely normal supply of ATP energy.

In Vivo Ascites Tumors. Relatively long ago, Harris et al. (37) suggested that the microenvironment of the cells of in vivo ascites tumors is generally hypoxic. The cells were thought to become only transiently oxygenated when fluctuating into the vicinity of blood vessels of the peritoneum. Clearly, the frequency of such transient oxygenations will diminish as tumor volume increases. We expected that a situation of this kind would intermittently suppress and retrigger replicon initiations, thereby causing temporal dissociation of S-phase replication. Consequently, the S-phase duration should be prolonged in vivo in comparison to permanently aerated cell culture. This was exactly what we observed earlier with our cells (15) despite enhanced DNA chain growth under in vivo conditions (28). Furthermore, the S-phase duration should increase in the course of in vivo tumor growth. This is also a published fact (38). Continuing this reasoning, we expected to see corresponding differences in the length distributions of replicative daughter

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Fig. 5. Influence of gassing with 200 ppm O₂ on the DNA histograms of asynchronous cells and of G₁ cells selected by zonal centrifugation. The cultures A (aerated control) and B (gassed) were parts of the same starting culture and analyzed after 12 h. C, G₁ cells directly after selection; D, after 12 h hypoxic gassing. The analyses were performed by a Bio-Physics Model 4801 Cytofluorograph.

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strand DNA because these reflect the mean relative replicon initiation frequency of cell populations (8, 9).

Fig. 6 demonstrates that the relative frequency of replicon initiations was indeed distinctly decreasing in the course of in vivo ascites tumor growth. Fig. 7A shows the drastic difference between growth as in vivo tumor and as aerated cell culture. The observed changes correspond well with the labeled mitoses curves recorded earlier under analogous conditions (15). Fig. 7, B and C show the results of attempts to manipulate the replicon initiation frequency of the cells during growth in the peritoneal cavity of the mice by allowing the animals to breathe elevated (Fig. 7B) or diminished (Fig. 7C) O₂ concentrations, respectively. As expected on the basis of the oxygen dissociation curve of mouse blood (39), the effect of increased O₂ was only marginal (but reproducible) whereas breathing of decreased O₂ concentrations caused distinct diminution of short labeled DNA chains originating from recently initiated replicons. Thus, the results were completely compatible with the predictions deduced by applying the model of intermittent oxygenation (37) to oxygen-dependent suppression and triggering of replicon initiations. Our estimations of ATP/ADP/AMP in in vivo tumor cells consistently revealed adenylate energy charges between 0.8 and 0.9 in all tumor stages tested (2–12 days). Therefore, the old proposal of an involvement of ATP/ADP/AMP (37) must be replaced by the assumption of a direct regulatory influence of O₂ tension on DNA replication. Moreover, the possible points of impact must be expanded from the G₁-S-phase transition to additional stages within the S phase. It is expected that relatively short shutdown periods do not interrupt S-phase replication but cause execution in a more staggered manner, thus prolonging its duration. Thereby the fraction of S-phase cells should increase rather than decrease. This was, indeed, demonstrated by the thymidine pulse labeling index which was higher in 4-day-old in vivo tumors than in cell cultures prepared from the same, although cell growth was markedly enhanced in the continually aerated cultures (15). More detailed analyses of pulse labeling index and of DNA histograms obtained by cytofluorometry supported the observation that the oxygen dependent regulation of replicon initiation is crucially involved in the control of ascites tumor growth in vivo (40).

Concluding Remarks. It is obvious that DNA replication switches to an economical state when the concentration of dissolved O₂ in the environment of the cells decreases below 3–6 µM, despite a normal cell respiration (36) and at a virtually normal supply of ATP energy. The economical state is characterized by reversible suppression of replicon initiations while the other partial processes of replication remain essentially unaffected. DNA fiber autoradiography (9) demonstrated that the switching was strictly coordinated at the level of the regulatory units of eucaryotic DNA replication, the replicon clusters (10–12). It seemed that the cells omitted initiating clusters which were forthcoming to be activated according to the cellular program (10, 41) organizing sequential genome replication. By normally completing the sections of the program already started before, the cells can achieve ordered escape from the expensive task of genome duplication without leaving genetic information in the replicative state and thus inaccessible when required for maintaining basic vital functions. According to our prior proposal (9), the cells which are generally "in cycle" and committed to DNA synthesis thereby change into a special kind of resting state ("S₀ phase") basically different from resting in G₁-G₀ or G₂ of the cell cycle. The cells can reach this state coming from several stages of the S phase located before the start of duplication of distinct sections of the genome. The exact location is not known so far with one exception: the stage immediately before the initiation of the very first clusters of the S phase. There, significant accumulation occurs when cells are transferred from aerated to hypoxic conditions. The synthesis of the very first replicons can be synchronized by exploiting this. It is not clear whether the resting in S phase can lead to a complete deadlock or only to a strong retardation of S-phase replication (9, 13, 31). It is conceivable that the same regulation of replication is also subject to other environmental signals than O₂ decrease as it was proposed in the study by Allison et al. (13). Vice versa, it seems equally possible that other metabolic sequences or cellular processes, e.g., glycolysis or mitosis, are also subject to the same O₂-dependent regulation.

However, extended exposition of the cells to O₂ concentrations below about 0.2 µM [range (d)] caused cell damage affecting the reversibility of the hypoxic shutdown of replication and cell growth. We experienced the damage range first on account of an, at first glance, mysterious failure: when we "improved" our equipment used earlier (8, 9) by replacing several meters of silicone tubing (providing a poor diffusion barrier for O₂) by...
stainless steel tubing, we abolished reversibility after gassing periods >3–4 h. Catching up now for the omitted O2 measurements, we found that the silicone tubing, although fed with a severely hypoxic gas mixture, delivered about 200 ppm O2 into the culture flask, by chance, an almost optimal value for eliciting the regulatory responses.

The O2 tension at the beginning of the damage range corresponded almost exactly to the respiratory constant of Ehrlich ascites cells (36) i.e., the value of O2 concentration at which cell respiration occurs at 0.5 of aerated rate. We therefore believe that the perturbations occurring in range (d) are caused by direct deficiency of O2 for metabolic reactions which depend, for example, on the respiratory chain by using flavins as reductants (e.g., dihydorotate dehydrogenase of uridine monophosphate synthesis). Normal adenylate energy charges indicated that in our experiments deficiency of respiratory ATP played no role even in range (d).

A signal function of oxygen tension for various physiological and metabolic regulations is generally accepted (26). However, in no case could a redox system serving as O2 sensor be identified. Our own search for a sensor directing the replication regulation was equally unsuccessful so far. According to Reithard (42), Löfler et al. (43), and Löfler (44) and to results of our laboratory the deoxytubidine triphosphate level may have a function in the intracellular transmission of the oxygen signal. The hypoxia-like effect of low doses of cycloheximide points to an involvement of a short lived protein (45). Influences of hypoxia on protein degradation (35) and protein synthesis (46) have been described.

The described regulation of the expensive process of genome replication may represent a surviving principle of mammalian embryonic cells. Before connection to the maternal circulation by nidation in the endometrium early embryos can undergo, although engaged in intensive DNA replication, severe metabolic constraints probably announced first by decreasing O2 tension. Ongoing studies on different cell lines revealed that BHK cells also possess this O2 dependent regulation of replication and we expect to find them in additional nontransformed (and transformed) mammalian cell lines.

Similar to early embryonic cells, tumor cells are generally “in cycle.” The benefits of the embryonic strategy may improve their adaptability and thus enhance their malignant potential. Because actively replicating cells are preferential targets for many drugs and for radiation, the regulation has also implications for tumor therapy.

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OXYGEN DEPENDENT REGULATION OF DNA REPLICATION

Oxygen Dependent Regulation of DNA Synthesis and Growth of Ehrlich Ascites Tumor Cells \textit{in Vitro} and \textit{in Vivo}

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