Simian virus 40 (SV40)-infected CV1 cells exposed to hypoxia show an inhibition of viral replication. Reoxygenation after several hours of hypoxia results in new initiations followed by a nearly synchronous round of SV40 replication. In this communication, we examined the effect of glucose on inhibition of viral DNA replication under hypoxia. We found that glucose stimulated SV40 DNA replication under hypoxia in two different ways. First, the rate of DNA synthesis, i.e. the fork propagation rate, increased. This effect seemed to be mediated by inhibition of mitochondrial respiration by glucose (Crabtree effect). Inhibition of mitochondrial respiration probably resulted in a higher intracellular oxygen concentration and an activation of oxygen-dependent ribonucleotide reductase, which provides the precursors for DNA synthesis. This glucose effect was consequently strongly dependent on the strength of hypoxia and the extent of intracellular respiration; hypoxic gassing with 10 ppm instead of 200–400 ppm O2 or treatment of hypoxic cells with a mitochondrial uncoupler (carbonyl cyanide m-chlorophenylhydrazone) reduced the glucose effect on replication, whereas antimycin A, an inhibitor of respiration, increased it. The second effect of glucose concerned initiation, i.e. stimulation of unwinding of the viral origin. This effect was not influenced by the strength of hypoxia or the extent of cellular respiration and seemed, therefore, not to be mediated through a Crabtree effect. No evidence for a direct correlation between the cellular ATP concentration and the extent of SV40 replication under hypoxia was found. The effect of glucose on replication under hypoxia was not restricted to SV40-infected CV1 cells but was also detectable in HeLa cells. This suggests it to be a mechanism of more general validity.

DNA replication in mammalian cells is subject to a fast acting regulation that depends on the O2 tension in the cellular environment. This regulation results in inhibition of cellular replication when the concentration of O2 falls below 0.1%. Regulation of cellular replication by O2 has first been demonstrated for Ehrlich ascites cells (1–4). Further studies revealed that it is also valid for HeLa and CCRF cells (5), suggesting it to be a mechanism of general importance that adapts the cellular DNA replication to the supply of O2 and other nutrients. This seems to be of particular significance during embryonic growth, wound healing, or tumor cell propagation. Inhibition of replication under hypoxia primarily affects replicon initiation. Additionally, the rate of DNA chain growth is frequently reduced. Readmission of O2 after several hours of hypoxia reverses the suppression of DNA replication within a few minutes. This remarkably fast response suggests that the O2-dependent replication control acts very directly on the replication apparatus.

The molecular mechanism of the oxygen-dependent replication control is largely obscure. A reduction of the intracellular concentrations of deoxynucleoside triphosphates, especially of dCTP, has been observed under hypoxia (6). This may be explained by the inhibition of the O2-dependent action of ribonucleotide reductase (3, 7). As inhibition of mammalian replicon initiation and elongation under hypoxia is relieved by addition of deoxycytidine even without reoxygenation, it is possible that ribonucleotide reductase acts as a sensor of O2-dependent replication control transmitting its signal through alterations in the deoxynucleoside triphosphate pool.

O2-dependent regulation of replicon initiation was also demonstrated for viral DNA replication in simian virus 40 (SV40)-infected CV1 cells (8, 9). Like in HeLa or CCRF cells, replicon initiation as well as elongation of SV40 replication are inhibited under hypoxia. Upon reoxygenation, elongation of DNA strands resumes a normal rate within 2 min. New initiations, indicated by the appearance of a highly negatively supercoiled topoisomer of SV40 DNA (form U), are detectable 3 min after reoxyge- nation. SV40 form U has been shown to be the product of unwinding of the viral origin (9, 10). The new initiations are followed by a nearly synchronous round of SV40 replication.

Besides inhibition of replication, hypoxia induces numerous other molecular responses in the cell, some of which are also inducible by hypoglycemia. This was demonstrated, for example, for induction of glucose/oxygen-related proteins (11), up-regulation of vascular endothelial growth factor (12–14) and glucose transporter Glut 1-mRNA (14, 15), accumulation of proteasomes in the nucleus (16), and increase in metastatic capacity of tumor cells (17, 18).

On the other hand, glucose is able to prevent some hypoxia-induced cellular effects. This was shown for inhibition of hypoxia-induced apoptosis and necrosis of tumor cells (19) and reversion of hypoxia-mediated reduction of radiosensitivity in Ehrlich ascites cells (20).

In this study, we examined the influence of glucose on hypoxia-mediated regulation of simian virus 40 replication.
Glucose Stimulates SV40 Replication under Hypoxia

Glucose-induced inhibition of SV40 replication in CV1 cells. We show that glucose partially prevents the effects of hypoxia on replication. First, addition of glucose to hypoxic and hypoglycemic SV40-infected cells stimulates new viral initiations. Second, glucose increases the rate of viral DNA chain growth under hypoxia. The second effect may be explained by a better intracellular availability of oxygen, due to partial inhibition of mitochondrial respiration by glucose (Crabtree effect). The better availability of oxygen in the cell in turn may lead to a higher activity of oxygen-dependent ribonucleotide reductase, which provides the precursors for DNA synthesis. We further show that the effect of glucose on replication is not restricted to SV40-infected cells but is also valid for HeLa cells.

Experimental Procedures

Transient Hypoxia, Reoxygenation, and Radioactive Labeling—Monkey CV1 cells (ATCC CCL 70) were grown and infected with SV40 as described previously (21). Transient hypoxia experiments were performed under different conditions. When formation of SV40 form U was examined, experiments were done 24 h post-infection (p.i.) for 10 h at 0.02% (200 ppm) O2, 5% CO2, argon to 100%. All other experiments were performed 36 h p.i. for 6 h at 400 ppm O2, 5% CO2, argon to 100% (8), if not stated. Glucose was added to a defined concentration before start of incubation. The respective concentration is indicated in the figure legends. For reoxygenation, 0.25 volumes of medium equilibrated with 95% O2, 5% CO2 was added to hypoxic HeLa cells, and gassing was continued with artificial air (8).

Glucose, bromodeoxyuridine, and [methyl-3H]deoxythymidine were either added directly to the cells or, under hypoxic culture conditions, by plunging a spatula carrying the appropriate quantity in dried form into the culture medium.

Aphidicolin (Roche Molecular Biochemicals), antimycin A (Sigma), and carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) (Sigma), dissolved in MeSO, were applied to hypoxic cell cultures on a spatula after gassing in a hypoxic chamber for 15 min.

To stop incubations, the culture medium was removed by aspiration, and the cells were washed once with ice-cold phosphate-buffered saline (PBS: 150 mM NaCl, 10 mM NaHPO4, pH 7.0). Determination of acid-insoluble radioactive activity was described previously (22). For determination of specific [methyl-3H]deoxythymidine incorporation, cell cultures were prelabelled with 2.5 nCi of [3H]deoxythymidine/ml 1.5 h p.i. Cultivation of HeLa cells was described previously (5).

Analysis of SV40 DNA Isolation from Cell Cultures, Chloroquine Gel Electrophoresis, Southern Blotting, and Hybridization—SV40 DNA from whole cells was isolated as described (9). Washed cells were lysed and digested in 0.25 M EDTA (pH 8.0), 1% sodium lauryl sarcosinate, 100 μg of proteinase K/ml at 55 °C for 3 h. The lysate was then extracted twice with phenol/CHCl3 and dialyzed against 1 mM Tris/HCl, 0.1 mM EDTA (pH 8.0) at 4 °C overnight. After digestion with RNase A (100 μg/ml at 37 °C for 1 h), 100 ng of isolated DNA per slot was loaded onto a 20%–40% linear sucrose gradient in 10 mM Tris, 1 mM EDTA. Electrophoresis was carried out at 2.5 V/cm and 4°C for 20 h. Southern blotting was performed under alkaline conditions (23). SV40 DNA was detected by hybridization against a 32P-labeled full-length SV40 probe (24).

Determination of Glucose and ATP—Glucose concentration in the cell culture supernatant was determined using an enzymatic glucose assay (Sigma). ATP was determined luminometrically after boiling of the stopped SV40-infected cells in 1 ml of 50 mM Tris/HC1, 4 mM EDTA (pH 7.8) for 5 min using the ATP Bioluminescence CLC assay (Roche Molecular Biochemicals).

Bromodeoxyuridine (BrdUrd) Density Labeling and Isopycnic CsCl Centrifugation—SV40-infected cell cultures were simultaneously labeled with BrdUrd (5 or 20 μg) and [methyl-3H]deoxythymidine (10 μCi/ml). After incubation, the labeled cells were processed essentially as described (25). Cells were washed once with ice-cold PBS and lysed in 2 ml of lysis buffer (20 mM Tris/HC1, 10 mM EDTA, 0.5% sodium lauryl sarcosinate, 100 μg/ml proteinase K (Merck) (pH 8.0)) for 1 h at 37 °C. Thereafter, a lysate of reference cells, prepared in the same way from about 60,000 SV40-infected CV1 cells labeled under normoxic conditions with [3H]deoxythymidine (200 nCi/ml) for 6 h, was added; the combined lysates were sheared by 10 passages through a 25-gauge injection needle followed by 5 passages through a 27-gauge injection needle. The lysates were then diluted with 10 mM Tris/HC1, 1 mM EDTA (pH 8.0) to 6 ml, mixed with 8.0 or 8.5 g of solid CsCl (resulting density ~1.72 and 1.74 g/ml), and then, after being overlaid with paraffin oil, centrifuged in the Beckman 70 Ti rotor at 20 °C and 37,000 rpm for 50–60 h. The gradients were fractionated from below into 40 fractions of 200 μl which were analyzed for acid-precipitable 14C and 3H radioactivity according to Ref. 22.

Determination of Cellular Oxygen Consumption—For determination of cellular O2 consumption, SV40-infected cells were trypsinized for 3 min at 37 °C and resuspended in glucose-free Dulbecco’s modified Eagle’s medium. 1 ml of the cell suspension was put in the electrode chamber of a Clark-type oxygen electrode, and respiration was recorded before and after addition of glucose.

Results

High Glucose Permits SV40 DNA Replication Despite Hypoxia—Viral replication in SV40-infected cells is inhibited under hypoxic culture conditions. This is demonstrated by a decrease of [methyl-3H]deoxythymidine incorporation into viral DNA (8). After reoxygenation, incorporation increases again and exceeds incorporation levels of normoxically cultivated cell cultures within a few minutes (8).

As glucose can mimic or modulate diverse cellular responses induced by hypoxia (11, 13, 14, 19, 20), we asked whether SV40 DNA replication is also influenced by variations of the glucose concentration in the cell culture medium of virus-infected CV1 cells. To answer this question, we compared the effects of two different glucose concentrations on SV40 DNA replication as follows: 0.5 mg/ml, which is about the concentration we have used in previously published communications (8, 9), and 1.5 mg/ml. We further term these two concentrations as “low glucose” or “high glucose.” The glucose concentrations were adjusted at the beginning of each experiment (24 or 36 h p.i.) and fell to below 0.2 mg/ml (low glucose, see Fig. 8) or to about 0.9 mg/ml (high glucose, data not shown) at the end of a 6-h lasting hypoxic or normoxic incubation.

In first experiments, we examined the incorporation of [methyl-3H]deoxythymidine into SV40 DNA at the two glucose concentrations by pulse labeling of SV40-infected cells at different times after start of hypoxic or normoxic incubation. After reoxygenation, the cells were processed for determination of radioactive incorporation into viral DNA (Fig. 1).

Under normoxic culture conditions incorporation rates remained more or less constant, regardless of whether the glucose concentration in the cell culture supernatant was low or high. In hypoxically cultivated cell cultures, the course of [methyl-3H]deoxythymidine incorporation depended on the glucose concentration in the cell culture medium. At low glucose, the incorporation decreased to about 5% within 4 h and then remained constant. Upon reoxygenation, incorporation rapidly increased and exceeded the rates at the start of hypoxia.
Glucose Stimulates SV40 Replication under Hypoxia

Usage of Externally Supplied DNA Precursors Is Increased under Hypoxia and High Glucose—The oxygen dependence of ribonucleotide reductase allows only residual activity under the hypoxic culture conditions used in the experiments described here (6, 7). The results shown in Figs. 1 and 2, on the other hand, suggest that SV40 DNA replication is not significantly obstructed under hypoxia and high glucose. To clarify this apparent contradiction, we tested whether SV40-infected cells grown under hypoxia/glucose use more of the externally supplied thymidine analogue 5’-bromodeoxyuridine (BrdUrd) for DNA synthesis than normoxically cultivated control cells. As BrdUrd (–triphosphate) is incorporated instead of dTTP into DNA, a higher degree of substitution would indicate a reduced supply of intracellularly generated dTTP.

As Fig. 2 shows, high glucose concentrations under hypoxia resulted in almost identical sedimentation profiles, regardless of whether the cell cultures were pulse-labeled before or after reoxygenation. This indicates that no accumulation of initiation-competent SV40 replicons takes place under these conditions. Moreover, the sedimentation profiles shown in Fig. 2 closely resembled those of normoxically incubated cells (data not shown), suggesting that SV40 DNA replication proceeds asynchronously under hypoxia and high glucose.

FIG. 1. Temporal course of the incorporation of [methyl-\(^{3}\text{H}\)]deoxythymidine into SV40 DNA at different glucose concentrations. SV40-infected cells were grown under hypoxic (400 ppm) or normoxic conditions, labeled with [methyl-\(^{3}\text{H}\)]deoxythymidine for 10 min at the indicated times, and processed for determination of incorporated radioactivity. Part of the hypoxic cultures were reoxygenated after 6 h of hypoxia and labeled at different times thereafter. ■, hypoxic gassing, 0.5 mg of glucose/ml at the start of incubation; □, normoxic gassing, 0.5 mg of glucose/ml at the start of incubation; ●, hypoxic gassing, 1.5 mg of glucose/ml at the start of incubation; ○, normoxic gassing, 1.5 mg of glucose/ml at the start of incubation.

FIG. 2. Alkaline sedimentation profiles of hypoxic and reoxygenated SV40-infected cells cultivated under high glucose conditions. Glucose concentration was adjusted to 1.5 mg/ml, and cells were grown hypoxically (400 ppm) for 6 h and labeled with [methyl-\(^{3}\text{H}\)]deoxythymidine before (□), at (●), or 10 min after (△) reoxygenation for 6 min. Sedimentation was from left to right.

BrdUrd (5 μM) and [methyl-\(^{3}\text{H}\)]deoxythymidine (20 μCi) were simultaneously added to the cells, and incubation was continued for a further 30 min under hypoxic or normoxic conditions. The cell cultures were then processed for CsCl density gradient centrifugation. As a control, each gradient contained a \(^{14}\text{C}\)-labeled DNA, which was not substituted with BrdUrd.

As Fig. 3 shows, DNA isolated from virus-infected CV1 cultures, which were cultivated under hypoxic conditions in presence of high glucose, appeared at higher densities than DNA from normoxic cell cultures in the CsCl gradients. This indicates an increased usage of extracellularly supplied BrdUrd into DNA, most probably as a consequence of the reduced activity of ribonucleotide reductase under hypoxia.

Incorporation of [methyl-\(^{3}\text{H}\)]deoxythymidine into SV40 DNA was shown to be the same under normoxic culture conditions as well as under hypoxia/glucose (Fig. 1). As the usage of externally supplied thymidine analogues is, however, increased under hypoxia/glucose (Fig. 3), it follows that the rate of DNA synthesis must have been reduced in comparison to normoxic culture conditions.

In a further experiment, we compared the times necessary to obtain new semiconservatively synthesized SV40 DNA under hypoxia and glucose and under normoxia. We simultaneously labeled SV40-infected CV1 cells with [methyl-\(^{3}\text{H}\)]deoxythymidine (10 μCi/ml) and BrdUrd (20 μM) for increasing times under both culture conditions. This successively led to SV40 DNA labeled with BrdUrd in one strand (hemi-substituted DNA) and, after a second round of replication, to DNA labeled in both strands (double-substituted DNA). After separation in a CsCl density gradient, we compared the times, which were necessary to get the same ratios of radioactivity of hemi- and double-substituted DNA for hypoxic and normoxic culture conditions.

Fig. 4 shows that about 7 h were necessary to get the same amount of radioactivity in hemi- and double-substituted DNA under normoxia, but more than 8 h were taken to reach the same ratio under hypoxic conditions. This indicates that the rate of SV40 DNA replication is reduced under hypoxia/glucose compared with the rate under normoxia. The effect is, however, not very pronounced.

Glucose Triggers New Initiations in Hypoxic SV40-infected CV1 Cells—The results presented above show that high glucose partially prevents the hypoxia-induced inhibition of SV40 rep-
Glucose Stimulates SV40 Replication under Hypoxia

Fig. 3. Incorporation of bromodeoxyuridine into normoxically and hypoxically cultivated SV40-infected cells. Cells were grown under normoxic (○) or hypoxic (400 ppm, □) culture conditions. Glucose concentration at the start of incubation was 1.5 mg/ml. 6 h later, cells were simultaneously labeled with bromodeoxyuridine and [methyl-3H]deoxythymidine for 30 min. DNA was isolated and separated by CsCl density gradient centrifugation. Sedimentation was from right to left. □, 14C-labeled control DNA, not density labeled.

In principle, addition of glucose to hypoxic, virus-infected cells containing little glucose should therefore allow SV40 replication even without reoxygenation.

We tested this hypothesis by addition of 1 mg of glucose/ml to SV40-infected CV1 cells, which were cultivated hypoxically at low glucose for 6 h. After glucose addition, hypoxic gassing was continued, and cells were labeled with [methyl-3H]deoxythymidine (20 μCi/ml) for 6 min at different times. After labeling, the cells were lysed, and DNA was analyzed by alkaline sucrose gradient centrifugation.

Fig. 5 shows that glucose triggered a new round of viral replication under hypoxia. This was indicated by the increasing shift of labeled DNA molecules to higher S values with increasing incubation times. The course of the viral replication round after glucose addition proved to be nearly identical to that observed after reoxygenation (9). Comparing the sizes of the growing SV40 daughter strands at identical time points after glucose addition or reoxygenation, respectively, revealed, however, that the propagation rate of replication forks was diminished, when initiation of SV40 replication was triggered with glucose instead of oxygen (see also below).

We also tested the effect of glucose addition to hypoxic cells concerning the formation of SV40 form U, which was shown to be a product of the unwinding of the viral origin region (9, 10). SV40-infected cells (24 h p.i.) were subjected to 10 h of hypoxia at 200 ppm O2 at low glucose. Then 1 mg of glucose/ml was added, and cells were further incubated hypoxically for various times and then stopped. Form U was detected after chloroquine gel electrophoresis of whole cell DNA and blotting by hybridization against a SV40-specific probe.

Form U was detectable between 3 and 10 min after glucose addition (Fig. 6). Again, this result closely resembled those obtained in earlier experiments, when hypoxic SV40-infected cells were reoxygenated (9). Thus, elevation of the glucose concentration under hypoxia relieves the hypoxia-induced block of SV40 replication and leads to new initiations and a synchronous round of viral replication.

In a further experiment, we determined the glucose concentration necessary to induce formation of form U in hypoxic cells by adding different amounts of glucose to SV40-infected cells incubated for 10 h under hypoxia. 6 min after glucose addition, hypoxic incubation was stopped.

The results obtained show that form U was induced when the concentration of glucose in the cell culture supernatant exceeded 0.1–0.2 mg/ml (Fig. 7). Densitometric evaluation revealed that half-maximal stimulation of form U was obtained between 0.2 and 0.4 mg of glucose/ml (data not shown).

Changes in Cellular ATP Concentration Are Not Responsible for Hypoxia-induced Inhibition of Replication—Oxygen and glucose are main substrates of cellular ATP generation through oxidative phosphorylation and glycolysis. Therefore, it may be speculated that loss of cellular ATP under hypoxia causes inhibition of SV40 replication. In a previous communication, we have shown that ATP is not in short supply in Ehrlich ascites cells grown under hypoxic culture conditions (26). To examine whether ATP is significantly diminished under hypoxia in SV40-infected CV1 cells, we concomitantly determined incorporation of [methyl-3H]deoxythymidine, glucose concentration in the cell culture supernatant, and cellular ATP concentrations at different times after the start of hypoxic incubation (Fig. 8A). As a control, the same parameters of normoxically cultivated cells were measured (Fig. 8B). Moreover, we determined the extent of form U formation, i.e. SV40 initiations, during the course of hypoxia and normoxia (Fig. 8C). As SV40 form U is barely detectable without synchronization of SV40 replication, we treated the cell cultures with aphidicolin (2 μg/ml) for 15 min immediately before the end of hypoxic or normoxic incubation. This leads to an inhibition of DNA elongation and accumulation of form U (9).

Fig. 8A shows that [methyl-3H]deoxythymidine incorporation decreased between 2 and 3 h after the start of hypoxic incubation. After 5 h, incorporation was almost totally inhibited. Initiations showed a similar decrease of [methyl-3H]deoxythymidine incorporation during the course of hypoxic gassing and were undetectable after 5 h of hypoxia (Fig. 8C). The glucose concentration in the cell culture supernatant also decreased until 5 h after the start of hypoxic incubation and then remained constant. Correlation of form U generation and the respective glucose concentration showed that above about 0.2–0.3 mg of glucose/ml near maximal initiation rates were achieved. Below 0.1 mg of glucose/ml, initiations ceased completely. The relation of glucose concentration and extent of initiations found in this experiment agrees well with the results shown in Fig. 7.

Intracellular ATP concentration remained more or less constant until 4 h after the start of hypoxic gassing. Between 4 and 6 h of hypoxia, ATP slightly decreased. At this time, however, [methyl-3H]deoxythymidine incorporation into viral DNA and initiations have nearly reached their minimum. Thus, ATP concentration seems not to be decisive for the onset of inhibition of SV40 DNA replication under hypoxia. But as Fig. 8 and further experiments (data not shown) suggest, there is an inverse correlation between inhibition of SV40 DNA replication and the glucose concentration under hypoxia.

Under normoxic culture conditions, [methyl-3H]deoxythymidine incorporation and the extent of initiations remained more or less unchanged (Fig. 8, B and C). ATP concentration remained constant for the first 4 h of incubation and then increased by about 60% between 4 and 5 h. Glucose consumption was approximately at the same rate as under hypoxia, although the absolute glucose concentration was always about 0.1 mg/ml higher than under hypoxia at the same time after the start of incubation. This suggests that SV40-infected CV1 cells meet a significant amount of their cellular ATP demand through glycolysis, regardless of whether they are incubated normoxically or hypoxically.

A Crabtree Effect Probably Contributes to Stimulation of SV40 DNA Replication under Hypoxia/Glucose—High rates of
glycolysis under normoxic culture conditions are frequently observed in fast proliferating cells if enough glucose is available (27, 28). Concomitantly, cellular respiration is inhibited (Crabtree effect).

We tested whether a Crabtree effect, i.e. inhibition of cellular respiration through glucose, is also detectable in SV40-infected cells. To that purpose, the cells were incubated 36 h p.i. in glucose-free medium, and cellular respiration was measured with a Clark electrode before and after addition of various amounts of glucose.

Fig. 9A shows that glucose inhibited respiration in SV40-infected CV1 cells in a concentration-dependent manner. Half-maximal inhibition was observed at 0.2 mg of glucose/ml. Notably, about the same concentration of glucose stimulated half-maximal generation of form U under hypoxic conditions (Figs. 7 and 8). This points to the possibility that the effect of glucose on SV40 DNA replication under hypoxia is mediated via inhibition of cellular respiration; at glucose concentrations above about 0.2 mg/ml, cellular respiration is partially inhibited.

When the concentration falls, glycolysis ceases while at the same time respiration increases. Under normoxia, the higher energy yield of oxidative phosphorylation may give rise to an increase in ATP concentration as observed in Fig. 8B. Under hypoxia, however, beginning of respiration leads to a further shortage of intracellular oxygen and to the formation of an oxygen gradient between inside and outside the cell. Such gradients have been demonstrated in respirating cells (29). The intracellular oxygen shortage could lead to a further reduction of the activity of ribonucleotide reductase followed by inhibition of replication.

To test this hypothesis, we compared the effect of glucose on hypoxic SV40-infected cells in the presence or absence of CCCP, a mitochondrial uncoupler, or antimycin A, an inhibitor of respiration. As Fig. 9B shows, CCCP (10 μM) enhanced cellular O2 consumption about 2-fold, irrespective whether glu-
cose was present or not. Antimycin A (0.2 μM), on the other hand, completely blocked respiration. If the effects of glucose on initiation and replication are mediated by the availability of oxygen in the cell, CCCP-treated cells should not reinitiate upon glucose addition. In antimycin A-treated cells, the effect of glucose should be more pronounced than in untreated cells.

We first examined the effect of CCCP and antimycin A on the generation of form U after addition of glucose to hypoxic cells. CCCP (10 μM) and antimycin A (0.2 μM) were added 5 min before addition of glucose; 6 min after glucose addition, the cells were stopped and processed for detection of form U.

Fig. 10 shows that form U appeared regardless of whether the cells were treated with the effectors or not. This indicates that the Crabtree effect is not responsible for the induction of new initiations after glucose addition to hypoxic cells, i.e. glucose does not induce new initiations through an increase of the intracellular oxygen availability.

In a second experiment, we examined the effect of CCCP and antimycin A on the fork propagation rate during the synchronous viral replication round, which succeeds glucose addition to hypoxic cells. This was done by analyzing the shift of growing pulse-labeled viral DNA strands to higher S values by alkaline sucrose gradient sedimentation at various times after glucose addition. As a control, reoxygenated cells were examined.

Fig. 11 shows that reoxygenated cells exhibited the highest fork propagation rate (especially evident 16 min after release from hypoxia). 26 min after reoxygenation, they had largely completed their viral replication round, as indicated by the high portion of supercoiled SV40 DNA appearing around fraction 17.

When the suppression of SV40 DNA replication under hypoxia was released by glucose instead of oxygen, DNA fork propagation and completion of the initiated synchronous replication round were retarded. This retardation was most pronounced in the CCCP-treated cells and least in the antimycin A-treated cells (Fig. 11), indicating that the intracellular oxygen availability is decisive for the fork propagation rate during the synchronous SV40 replication round. This suggests that a Crabtree effect is involved in the glucose-mediated stimulation of SV40 DNA replication under hypoxia.

To confirm these results further, we repeated some of the transient hypoxia experiments described above at 10 ppm oxygen where ribonucleotide reductase is essentially inactive (6, 7). As a consequence, DNA synthesis should be completely inhibited regardless of whether mitochondrial oxygen consumption is diminished by a Crabtree effect or not. The glucose concentration in the medium should therefore have no influence on the SV40 DNA replication rate. In a first experiment, we examined the course of [methyl-3H]deoxythymidine incorporation into viral DNA during 6 h of hypoxia under low and high glucose.

Fig. 12 shows that, in contrast to the results obtained at 400 ppm (Fig. 1), incorporation was inhibited by hypoxia of 10 ppm O₂, regardless of the given glucose concentration. Stimulation of replication by reoxygenation was still possible after 6 h of...
hypoxia, indicating that the cells were not irreversibly damaged at this oxygen concentration (Fig. 12). Addition of glucose to SV40-infected CV1 cells cultivated for 10 h at 10 ppm oxygen under low glucose led to generation of form U (data not shown). The initiated replicons were, however, virtually not elongated during a further 26 min of hypoxic incubation at 10 ppm O₂ following glucose addition, as determined by alkaline sedimentation analysis (Fig. 13, compare also with Fig. 5 and Fig. 11).

Glucose Stimulates Initiations in Hypoxic HeLa Cells—To show that stimulation of replication by glucose is not restricted to SV40-infected CV1 cells, we also investigated the effect of glucose addition (2 mg/ml) to HeLa cells, which were incubated hypoxically (200 ppm) for 8 h. After glucose addition, the cells were further incubated hypoxically for 20 min and then processed for alkaline sedimentation analysis. Cells reoxygenated for 20 min were taken as control.

As Fig. 14 shows, glucose triggered new initiations in hypoxic cells, although the effect of glucose was not as strong as that of reoxygenation. This again may be explained by the fact that ribonucleotide reductase shows reduced activity at 200 ppm oxygen. The experiment, however, unambiguously demonstrates that HeLa cells react in a similar way as SV40-infected CV1 cells. This in turn suggests that stimulation of replicon initiations by...
Glucose Stimulates SV40 Replication under Hypoxia

In previous communications we have shown that SV40-infected CV1 cells and other eucaryotic cells are subjected to O₂-dependent regulation of replicon initiation (1–5, 8, 9). With respect to SV40 replication in vivo, it was shown that reduction of the pO₂ to 0.02–0.2% results in reversible suppression of initiation of viral DNA replication. Initiation was blocked before unwinding of the viral origin. Reoxygenation after several hours of hypoxia triggered the resumption of SV40 replication, beginning with the unwinding of the SV40 origin region. In the present study, we show that the reversible shutdown of SV40 DNA replication can be partially prevented by glucose concentrations exceeding about 0.2 mg/ml in the cell culture supernatant. Addition of glucose to SV40-infected cells cultivated hypoxically at low glucose concentration led to a similar burst of new replicon initiations as reoxygenation.

The results presented in this communication suggest that glucose exerts two separable effects on SV40 DNA replication. First, glucose addition to hypoxic cells increases the rate of viral DNA chain growth. This effect seems to depend on the intracellular oxygen availability (Fig. 11 and Fig. 12) and may be most easily explained by a partial inhibition of mitochondrial respiration by glucose (Crabtree effect). As cellular respiration may cause oxygen gradients between inside and outside the cell (29), partial inhibition of respiration by glucose possibly results in an increase of intracellular availability of oxygen, especially under hypoxic culture conditions. The improved supply of oxygen may increase the (oxygen-dependent) activity of ribonucleotide reductase (6, 7), which provides the precursors of DNA synthesis. The above assumption is supported by the fact that the effect of glucose depended on the oxygen concentration under hypoxia. At 400 ppm and high glucose concentrations, almost normal SV40 DNA replication was possible (Figs. 1, 2, and 4). At 10 ppm oxygen, on the other hand, DNA replication and replication fork propagation (Figs. 12 and 13), was nearly undetectable, regardless of whether glucose was present or not. This can be explained by the fact that intracellular ribonucleotide reductase retains some activity at an oxygen concentration of 400 ppm but is essentially inactive at 10 ppm.

The second effect of glucose concerns initiation of SV40 DNA replication. Glucose addition to hypoxic cells led to generation of form U, an SV40 topoisomerase that was demonstrated to be indicative for unwinding of the viral origin region (9, 10). The effect of glucose on generation of form U was always at least as strong as reoxygenation. At very low oxygen concentrations (10 ppm) or in cases, where cellular respiration was stimulated (e.g. in CCCP-treated cells), the form U signal after glucose addition was even stronger than that after reoxygenation (Fig. 10 and data not shown). This may be explained by accumulation of form U due to inhibition of DNA elongation if O₂ is under short supply in the cell. A similar accumulation of form U was observed, when elongation is inhibited by aphidicolin in hypogenated cells (9).

For two reasons, the release of new initiations in hypoxic cells after glucose addition seems not to be mediated by a
Glucose Stimulates SV40 Replication under Hypoxia

Crabtree effect and the resulting better availability of oxygen in the cell. First, CCCP, which prevents the Crabtree effect of glucose on respiration (Fig. 9B), did not prevent the generation of form U after glucose addition (Fig. 10). Second, glucose induced form U in hypoxically cultivated SV40-infected cells, regardless of whether the oxygen concentration was 10 or 400 ppm (data not shown).

Under normoxic conditions, initiations were independent of the glucose concentration in the cell culture medium. This indicates that glucose and oxygen are equally able to stimulate SV40 initiation. Moreover, neither addition of glucose to normoxic cells cultivated under low glucose conditions (100 μg/ml) (data not shown) nor reoxygenation of hypoxic cells under high glucose conditions (Fig. 2) stimulated further initiations (form U) in SV40-infected cells. This means that either of the stimuli is sufficient to induce the maximal degree of initiations under the respective culture conditions.

The signal transduction pathway leading from glucose addition to initiation remains to be elucidated. Despite the fact that oxygen and glucose are main substrates of cellular ATP generation, it seems unlikely that the intracellular ATP concentration regulates initiation of SV40 replication. First, inhibition of SV40 replication preceded the decrease of intracellular ATP under hypoxia (Fig. 8). Moreover, glucose addition to SV40-infected cells that were cultivated hypoxically for 10 h increased the overall cellular ATP concentration only by about 20%, resulting in an overall cellular ATP level, which was only one-third the level at the start of inhibition of SV40 replication (2–3 h after the start of hypoxic gassing) (data not shown). Thus, there is no correlation between the intracellular ATP concentration and the onset of inhibition of SV40 DNA replication as we have demonstrated previously, no hints were found that ATP was under short supply in hypoxic Ehrlich ascites cells (26).

As most of the glucose is consumed through glycolysis, an intermediate of this pathway may be involved in the stimulation of initiation under hypoxia. Interestingly, some glycolyisis enzymes are not restricted to the cytoplasm but have also been demonstrated in the nucleus, they might act as signal transducers, connecting the availability of glucose to DNA replication.

The stimulation of replication by glucose may also have some influence on the growth of solid tumors. Depending on the distance to the vascular system, these tumors contain regions of different supply of nutrients and oxygen (39). In poorly supplied regions of the tumor, it seems to be advantageous to save oxygen by inhibition of mitochondrial respiration via the Crabtree effect, as this enables a higher activity of ribonucleotide reductase and thereby a better DNA synthesis. Moreover, it may be speculated that a high level of aerobic glycolysis in the well supplied upper cell layers may reduce the oxygen consumption in favor of cells more distant from the vascular system. Such a mechanism has been discussed for the articular cartilage of pigs (40).

The replication-promoting effects of glucose are also a possible explanation for an observation concerning the radiosensitivity of Ehrlich ascites cells (20). Under hypoxia, these and other cells show reduced sensitivity to radiation, which may be explained by the fact that DNA replication ceases under these conditions. Addition of glucose to hypoxic Ehrlich ascites cells, however, restored radiosensitivity, possibly by restoration of cellular replication.

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