Role of Nuclear Glycogen Synthase and Cytoplasmic UDP Glucose Pyrophosphorylase in the Biosynthesis of Nuclear Glycogen in HD33 Ehrlich-Lettré Ascites Tumor Cells

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ABSTRACT Biochemical and autoradiographic evidence show both glycogen synthesis and the presence of glycogen synthase (UDP glucose [UDPG]: glycogen 4-α-D-glucosyltransferase; EC 2.4.1.11) in isolated nuclei of Ehrlich-Lettré mouse ascites tumor cells of the mutant subline HD33.

5 d after tumor transplantation, glycogen (average 5–7 pg/cell) is stored mainly in the cell nuclei. The activity of glycogen synthase in isolated nuclei is 14.5 mU/mg protein. At least half of the total cellular glycogen synthase activity is present in the nuclei. The nuclear glycogen synthase activity exists almost exclusively in its b form. The K_m value for (a + b) glycogen synthase is 1×10^{-3} M UDPG, the activation constant is 5×10^{-3} M glucose-6-phosphate (Glc-6-P).

Light and electron microscopic autoradiographs of isolated nuclei incubated with UDP-[1-^{3}H]glucose show the highest activity of glycogen synthesis not only in the periphery of glycogen deposits but also in interchromatin regions unrelated to detectable glycogen particles. Together with earlier findings on nuclear glycogen synthesis in intact HD33 ascites tumor cells (Zimmermann, H.-P., V. Granzow, and C. Granzow. 1976. *J. Ultrastruct. Res.* 54:115–123), the results of tests on isolated nuclei suggest a predominantly appositional mode of nuclear glycogen deposition, without participation of the nuclear membrane system.

In intact cells, synthesis of UDPG for nuclear glycogen synthesis depends on the activity of the exclusively cytoplasmic UDPG pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridylyltransferase; EC 2.7.7.9). However, we conclude that glycogen synthesis is not exclusively a cytoplasmic function and that the mammalian cell nucleus is capable of synthesizing glycogen.

Intranuclear glycogen deposition has been observed in amphibian (19), avian (11), and a variety of mammalia cells, particularly in hepatocytes (5, 28, 30, 38), cells of renal collecting tubules (6), and cardiac muscle cells (8), partly under normal, partly under various pathological conditions. With respect to the origin of intranuclear glycogen, translocations of cytoplasmic glycogen particles into the nuclear compartment (30, 40) or the synthesis of the polysaccharide within the nucleus itself have been postulated. As for the occurrence of nuclear glycogen synthesis, only morphological evidence and no quantitative data emerged from in vitro experiments with intact Novikoff hepatoma (22) and Ehrlich ascites tumor (45) cells and with preparations of human gastric adenocarcinomas (31). So far, neither morphological nor conclusive biochemical data are available on glycogen synthesis in isolated mammalian nuclei.

From the experimental point of view, Ehrlich-Lettré ascites tumor cells of the HD33 subline established in our laboratory (15) proved to be especially suitable for investigating nuclear glycogen synthesis.[1] The cells descended from the glycogen-

[1] Granzow, C., V. Granzow, L. Edler, and W. Sauer. Establishment

free wild-type Ehrlich-Lettré ascites tumor, which was manipulated pharmacologically by means of N-methyl-colchicamide (27). A genome mutation of HD33 cells is associated with various phenotypical deviations from the wild type,[1] among which a pronounced nuclear and/or cytoplasmic glycogen storage is the most conspicuous (46). Characteristically, between days 4 and 6 after transplantation, glycogen deposits of increasing size are located chiefly within the tumor cell nuclei. Additional cytoplasmic glycogen storage is restricted to later stages of tumor development (46). On days 5 and 6, pronounced nuclear glycogen storage coincides with minimal cytoplasmic glycogen deposition in interphase cells. Thus, cells at this stage of tumor development provide a unique substrate for an analysis of their nuclear glycogen metabolism.

In previous communications (15, 45), nuclear glycogen synthesis from glucose in intact HD33 ascites tumor cells was demonstrated by cytochemical and autoradiographic techniques. We show here biochemical and autoradiographic evidence for the synthesis of glycogen from UDP glucose (UDPG) in nuclei previously isolated from HD33 ascites tumor cells harvested 5 d after transplantation. The responsible enzyme is intranuclear glycogen synthase. In intact cells, its substrate is provided by cytoplasmic UDPG pyrophosphorylase activity.[2]

MATERIALS AND METHODS

Chemicals and Reagents

NADP, UDPG, glucose-6-phosphate (Glc-6-P), bovine serum albumin, rabbit liver glycogen, phosphoglucomutase (EC 2.7.5.1) from rabbit muscle, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from yeast, hexokinase (EC 2.7.1.1) from yeast, UDPG pyrophosphorylase (EC 2.7.7.9) from beef liver, and amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* were obtained from Boehringer, Mannheim, W. Germany. In some tests, UDPG from Serva, Heidelberg, W. Germany, was used. UDP-D-[U-^{1};4C]glucose, 296 mCi/mmol sp act, was purchased from The Radiochemical Centre, Amersham, England. D-[6-^{3}H]glucose, 8.45 Ci/mmol sp act, and UDP-D-[1-^{3}H]glucose, 4.85 Ci/mmol sp act, were products of New England Nuclear, Boston, Mass. Dithiothreitol was obtained from Calbiochem-Behring, San Diego, Calif., anion exchange resin AG 1-X8, 100–200 mesh (acetate form), from BioRad Laboratories, Richmond, Calif., Szintigel-Roth from Roth, Karlsruhe, W. Germany, AR 10 autoradiographic stripping film from Kodak, London, England, Schiff's reagent, modified according to Graumann (16), from Chroma, Stuttgart, W. Germany, and vinylcyclohexene dioxide, nonenylsuccinic anhydride, diglycidyl ether of polypropylene glycol (D.E.R. 736), and 2-dimethylaminoethanol from Serva. All other chemicals (analytical grade) were products of E. Merck, Darmstadt, W. Germany.

Ascites Tumor Cells

Cells of the HD33 subline of the Ehrlich-Lettré mouse ascites tumor (15), were used.[1] The tumor was maintained by weekly intraperitoneal transfers of 10^7 ascites cells to male, specific pathogen-free NMRI mice, which were purchased from Sueddeutsche Versuchstieranstalt, Tuttlingen, W. Germany.

Preparation and Fractionation of HD33 Ascites Tumor Cells

5 d after transplantation, seven animals bearing typically developed ascites tumors were sacrificed by cervical dislocation. The tumors were aspirated aseptically, pooled, and processed further at 0°C. The tumor was washed three times with Earle's balanced salt solution (7), without glucose, pH 7.5, osmolality 290 ± 5 mosmol/kg (EBSS), and the pelleted (200 *g*, 5 min, 4°C) tumor cells were finally suspended to a density of 180 × 10^6/ml in EBSS. This suspension was used for the incubation of whole cells with [^{3}H]glucose, for the preparation of cell homogenates, and for the isolation of nuclei.

For preparing the cell homogenate, 1 vol of the cell suspension was mixed with 4 vol either of 5 mM EDTA, 100 mM NaF, 50 mM Tris-HCl, pH 7.5 (for the assay of glycogen and glycogen synthase), or of 1 mM dithiothreitol, 0.1 M Tris-acetate, pH 7.8 (for the assay of UDPG pyrophosphorylase). The mixture was equilibrated with N_2 under 100 bar for 30 min in a 4635 cell disruption bomb (Parr Instrument Co., Moline, Ill.). The cell homogenate obtained after decompression contained no identifiable cell organelles and could be used immediately for assaying glycogen and glycogen synthase or processed for the UDPG pyrophosphorylase assay.

For the isolation of ascites cell nuclei, the method of Mamaril et al. (29) was used with the following modifications. (*a*) According to phase-contrast microscopic observations to be discussed later, the duration of the swelling of the cells in 2 mM EDTA, pH 7.0, was reduced to 7 min before homogenization. (*b*) To avoid nuclear aggregation, 2 vol of the homogenate were poured into 1 vol of 0.44 M sucrose, 8 mM $CaCl_2$, and 1 vol of 25 mM K_2HPO_4, 18.8 mM KH_2PO_4, 20 mM $MgCl_2$, 4 mM EDTA, 0.6 mM $NaHCO_3$, pH 7.0, was added. The mixture was centrifuged (300 *g*, 10 min, 4°C). The postnuclear supernate was used for the assay of glycogen synthase and glycogen or processed for the assay of UDPG pyrophosphorylase. In some experiments, the postnuclear supernate was ultracentrifuged (94,000 g_{max}, 35 min, 0°C, SW 27 rotor, Beckman L5-65 ultracentrifuge; Beckman Instruments, Spinco Div., Palo Alto, Calif.), and glycogen synthase was assayed separately in the supernate and in the pellet which was resuspended in a small volume of 5 mM EDTA, 100 mM NaF, 50 mM Tris-HCl, pH 7.5. For the assay of glycogen, the postnuclear supernate was ultracentrifuged (131,000 g_{max}, 2 h, 0°C, SW 27 rotor), and the pellet was resuspended in the same buffer. (*c*) The second ultracentrifugation of the nuclear pellet was omitted. (*d*) 1 vol of the pellet after the first ultracentrifugation through 2.1 M sucrose in 2 mM EDTA, pH 7.0 (94,000 g_{max}, 35 min, 0°C, SW 27 rotor) was washed twice with 20–30 vol of 0.22 M sucrose solution containing 12.5 mM K_2HPO_4, 9.4 mM KH_2PO_4, 10 mM $MgCl_2$, 2 mM EDTA, 0.3 mM $NaHCO_3$, pH 7.0, and centrifuged (300 *g*, 10 min, 4°C).

The nuclear pellet obtained was either washed with 20–30 vol of EBSS, centrifuged as before, resuspended in 10 vol of EBSS, and used for light and electron microscopic investigations, or else it was washed twice with 20–30 vol of 100 mM NaF, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, centrifuged as before, resuspended in 10 vol of the same buffer, and homogenized with 40–50 strokes in a Potter-Elvehjem homogenizer with a glass pestle. The homogenate was used for the glycogen synthase assay.

For the assay of UDPG pyrophosphorylase, the nuclear pellet was washed with 20–30 vol of 1 mM dithiothreitol, 0.1 M Tris-acetate, pH 7.8, centrifuged as before, resuspended in 10 vol of the same buffer, and equilibrated with N_2 for 25 min under 130 bar in the cell disruption bomb. The homogenate obtained after decompression was processed for the assay of UDPG pyrophosphorylase activity.

Incubation of Cells and Isolated Nuclei

0.1 ml of the cell suspension was mixed with 0.8 ml of EBSS and preincubated in a water bath at 37°C for 10 min before adding [^{3}H]glucose (final concentration 5 mM, 30 μCi/ml) dissolved in EBSS.

In parallel tests, 0.1 ml of the suspension of isolated nuclei was mixed with 0.75 ml of EBSS and preincubated as the cells were before adding (*a*) [^{3}H]glucose (final concentration 5 mM, 20 μCi/ml or 20 mM, 120 μC/ml), or (*b*) UDP-[^{3}H]glucose (final concentration 4 mM, 5 μCi/ml), or (*c*) the same as (*b*), but with additional Glc-6-P (final concentration 10 mM); all of which were dissolved in EBSS. The incubation of cells and nuclei was continued for 30 min, stopped in an ice bath, and aliquots were processed for light and electron microscopic autoradiography.

Light and Electron Microscopy

For light microscopy, smears from native ascites tumors were fixed and stained according to Granzow and Granzow (14). Autoradiographs from test and control specimens of cells and isolated nuclei were prepared as described previously (15). For electron microscopy, cells and isolated nuclei were fixed and dehydrated according to Franke et al. (10). The specimens were embedded according to Spurr (41). Autoradiographs were prepared as described previously (45). For control purposes, the specimens were additionally treated after the prefixation either with 0.067 M of Sørensen phosphate buffer, pH 7.2, or with a solution of 14 U of amyloglucosidase/ml of this buffer for 6 h. All specimens were stained with lead citrate according to Reynolds (35) and viewed in a Siemens Elmiskop 102.

Assay of Glycogen Synthase

Glycogen synthase was determined according to Golden et al. (12), but with final concentrations of 4.5 mM UDP-[^{14}C]glucose (5–6 × 10^4 cpm/μmol) and 6.67 mM Glc-6-P. The eluate from the columns with resin in acetate form was

and growth kinetics of the mutant Ehrlich-Lettré ascites cell strain HD33 in permanent suspension culture. Submitted for publication.

[2] Some of the results were presented at the Eleventh Federation of European Biochemical Societies Meeting, Copenhagen, 14–19 August 1977 (Abstract A 1-9-118).

mixed with 7.5 vol of Szintigel-Roth and counted in a Beckman LS 9000 liquid scintillation counter. The activity was expressed in milliunits per milligram protein equal to nanomoles glucose incorporated into glycogen per minute per milligram protein at 30°C.

Assay of UDPG Pyrophosphorylase

Homogenates of cells and nuclei, as well as postnuclear supernates, were ultracentrifuged (27,000 g_{max}, 40 min, 0°C, SW 50.1 rotor). In the supernates, UDPG pyrophosphorylase was assayed according to Hansen et al. (17). The activity was expressed in milliunits per milligram protein equal to nanomole glucose-1-phosphate formed per minute per milligram protein at 30°C.

Protein Determination

Protein was determined according to Bensadoun and Weinstein (4), with bovine serum albumin as standard.

Glycogen Determination

Glycogen was isolated and enzymatically determined as described previously (13).

RESULTS

Glycogen Distribution in Intact HD33 Ascites Tumor Cells

5 d after transplantation, glycogen deposits of various sizes and numbers were cytochemically detected almost exclusively in the HD33 ascites tumor cell nuclei (Fig. 1). Cytoplasmic deposits were seen in the frequent mitotic figures and only occasionally in interphase cells. A minority of the tumor cells was cytochemically glycogen free. The frequency of host cells, predominantly macrophages and leukocytes, was <5%.

The morphological findings are confirmed by quantitative determination of the actual amount of glycogen in cells and cell fractions. The enzymatically determined average glycogen content of the tumor cells was 5–7 pg/cell. 9–14% of the total cellular glycogen was found in 131,000 *g* pellets of cytoplasmic fractions. Per milligram protein, 134.6 ± 27.6 μg glycogen (n = 4) was determined in isolated nuclei vs. 28.12 ± 2.35 μg (n = 3) in cell homogenates.

Morphology of Isolated HD33 Ascites Tumor Cell Nuclei

For the experiments described here, the evaluation of native preparations of isolated nuclei by phase-contrast microscopy was essential. Preparations were used consisting of regularly formed nuclei mixed with a few shrunken or fragmented nuclei. Fig. 2 shows the ultrastructural aspect of a typical preparation of isolated nuclei before incubation. The moderately shrunken nuclei were surrounded by the nuclear envelope. The external nuclear membrane frequently formed vesicular extensions. The general pattern of chromatin distribution typical for intact HD33 ascites cells (46) was preserved. Nuclear glycogen deposits remained mostly unaltered, but some of them appeared separated from the surrounding chromatin. They were never seen connected to membranous structures of the nucleus. Cy-

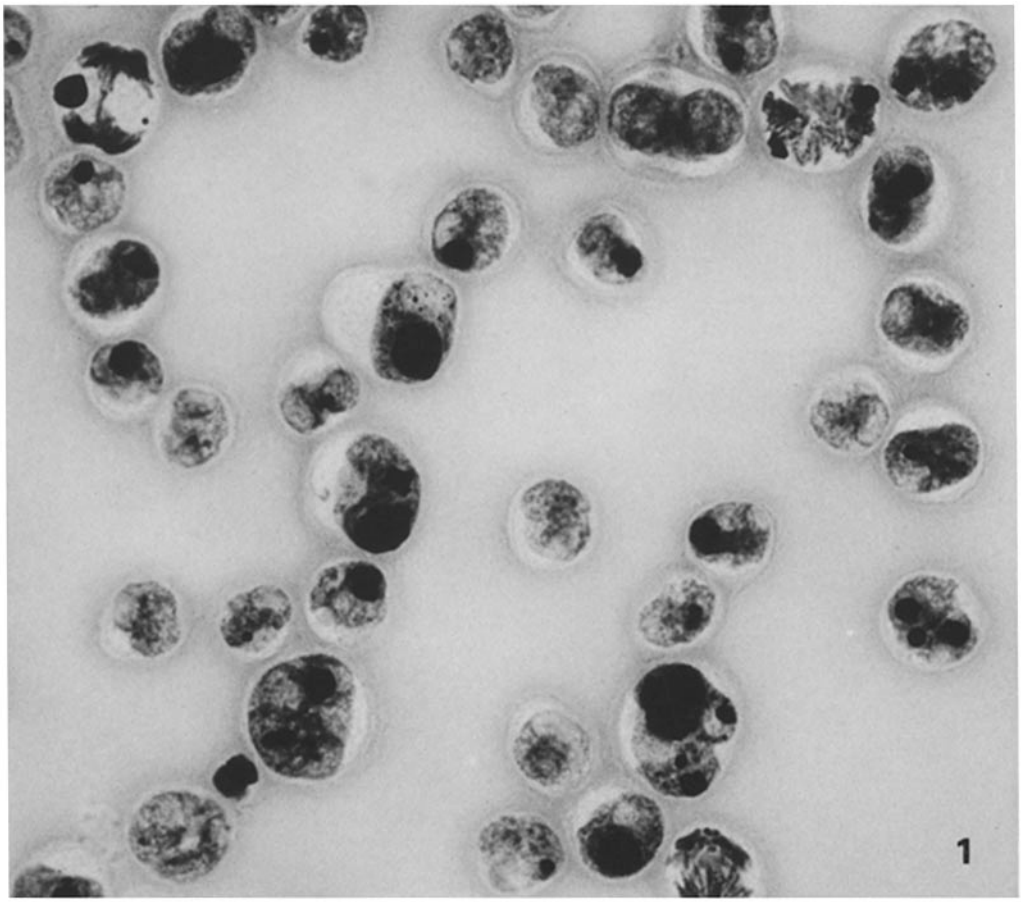

FIGURE 1 Population of HD33 ascites tumor cells 5 d after transplantation. Most of the nuclei contain single or multiple glycogen deposits of various sizes. Cytoplasmic glycogen deposits are restricted to mitotic figures. The tumor cells are coated by weakly periodic acid-Schiff(PAS)-positive material. Simultaneous reaction for Feulgen- and PAS-positive material (14). × 640.

toplasmic invaginations appeared enlarged and contained mainly vesicular structures. Except for some remnants of the endoplasmic reticulum, no further cytoplasmic structures could be identified.

Localization of Glycogen Synthesis in Isolated Nuclei

In autoradiographs of whole nuclei that had been incubated for 30 min with UDP-[^{3}H]glucose in the presence or absence of Glc-6-P, we observed single or multiple periodic acid-Schiff(PAS)-positive deposits similar to those in intact HD33 tumor cell nuclei. Accumulations of silver grains were seen over the deposits (Fig. 3 *a* and *b*), whereas PAS-negative areas of the nuclei and nuclei lacking such deposits showed only background labeling. Labeled, isolated PAS-positive deposits that were lost from the smeared nuclei could also be observed occasionally in such preparations. The labeling of the PAS-positive deposits was clearly more frequent and more intense in specimens incubated with 10 mM of Glc-6-P. Isolated nuclei incubated in the presence of Glc-6-P had a strong tendency to form aggregates.

Control specimens treated after fixation with amyloglucosidase dissolved in acetate buffer showed light areas resembling in form and size the nuclear deposits and background labeling only (Fig. 3 *c*); parallel specimens treated with the same buffer without amyloglucosidase were almost indistinguishable from the test preparations. Isolated nuclei incubated with either 5 or 20 mM [^{3}H]glucose showed background labeling only.

As shown in the electron microscopic autoradiographs (Figs. 4–6), the ultrastructure of isolated nuclei incubated for 30 min with UDP-[^{3}H]glucose was well preserved, in spite of a partial loss of the nuclear envelope. Most of the alterations seen in freshly isolated nuclei had been restored under the isotonic incubation conditions. Glycogen accumulations consisting predominantly of α-particles appeared less densely packed than those in nuclei immediately after isolation or *in situ*.

With respect to the autoradiographic reaction over ultrathin sections, three different labeling patterns were distinguishable. In the majority of nuclei with extended glycogen deposits, silver grains were seen predominantly related to the periphery of the glycogen accumulations (Fig. 4). In some nuclei, the label was evenly distributed over the deposit (Fig. 5). A third pattern of intense labeling unrelated to identifiable glycogen deposits was observed over sections with small glycogen accumulations (Fig. 6), as well as over single, apparently glycogen-free sections (not shown). In such instances, silver grains were found in serial sections over interchromatin areas and in the neighborhood of invaginations of the nuclear envelope. Nucleoli were always unlabeled. On the ultrastructural level, an

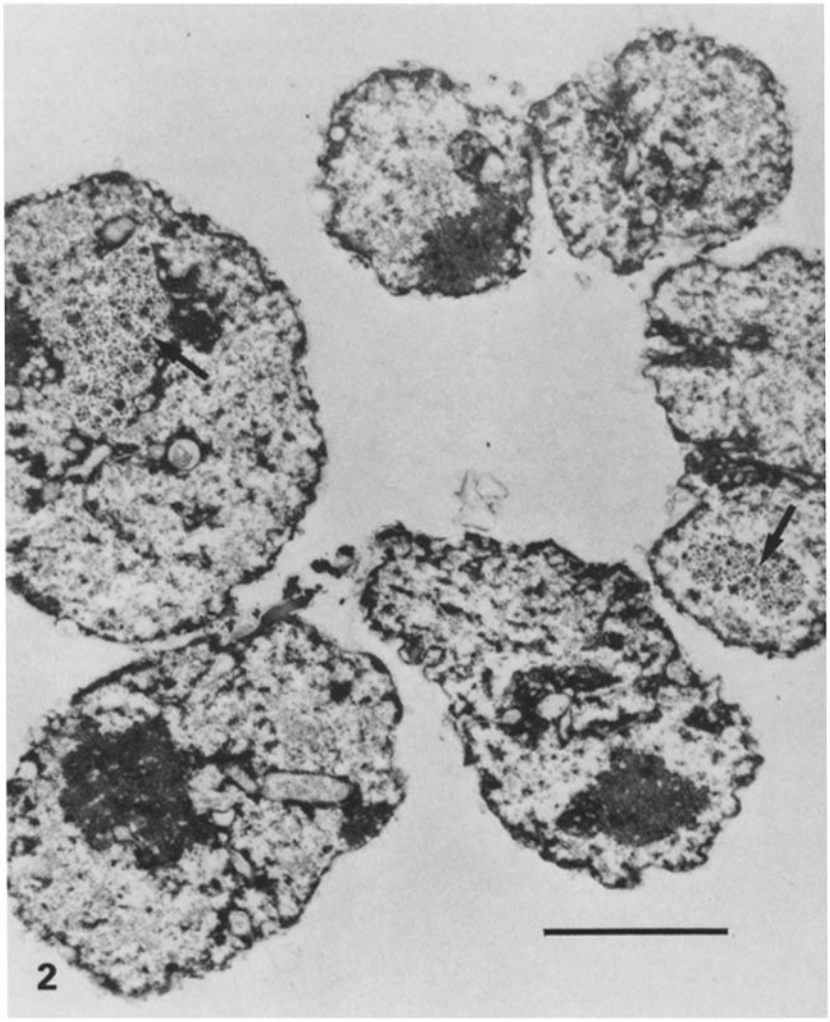

FIGURE 2. Electron micrograph of freshly isolated nuclei of HD33 ascites tumor cells harvested 5 d after transplantation. Intranuclear glycogen deposits (arrows) are well preserved. Bar, 1 μm. × 6,000.

increased labeling intensity was also obvious in specimens incubated with 10 mM Glc-6-P. Electron microscopic autoradiographs of isolated nuclei incubated with [^{3}H]glucose showed background labeling only. In contrast, the light and electron microscopic autoradiographs of intact tumor cells incubated as controls with [^{3}H]glucose revealed a labeling pattern over the nuclear glycogen deposits which was almost indistinguishable from that described above for isolated nuclei incubated with UDP-[^{3}H]glucose. In addition, the rare cytoplasmic glycogen deposits were specifically labeled, thus confirming earlier observations (45).

Control autoradiographs of sections treated with amyloglucosidase dissolved in phosphate buffer showed scarcely any background labeling. Many nuclei exhibited, after the enzyme digestion, large areas filled with a reticular network that corresponded in shape and extension to the deposits of glycogen particles seen in untreated sections. In control specimens treated with phosphate buffer alone, both label and glycogen deposits were preserved. The findings were in full agreement with earlier observations on whole HD33 cells (45), and imply a specific sensitivity of the label to the action of amyloglucosidase, as was also found in sections without identifiable glycogen deposits.

Due to a loss of sectioned, nuclear glycogen deposits during the preparation for electron microscopic autoradiography, some specimens of the test and control series contained nuclei with large empty areas which corresponded topographically to the glycogen deposits of intact nuclei.

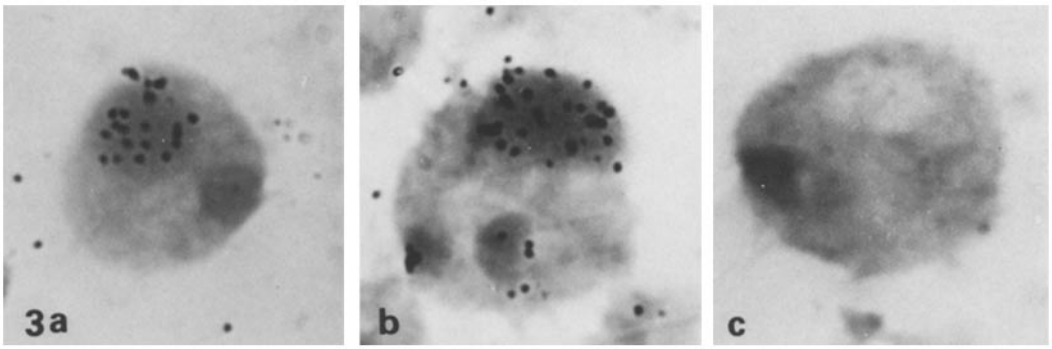

FIGURE 3 Autoradiographs of isolated HD33 ascites tumor cell nuclei incubated for 30 min at 37°C with UDP-[1-^{3}H]glucose. (*a*) Labeling over a nuclear glycogen deposit. (*b*) Intensified labeling over three nuclear glycogen deposits after incubation with additional Glc-6-P (10 mM). (*c*) Negative autoradiographic reaction and negative PAS reaction of a nucleus incubated as in Fig. 3 b and digested with amyloglucosidase. Reduced staining in an area corresponding in shape and size to the glycogen deposits seen in undigested specimens. Intense hemalum staining of the nucleolus. PAS reaction, Mayer's hemalum. × 2400.

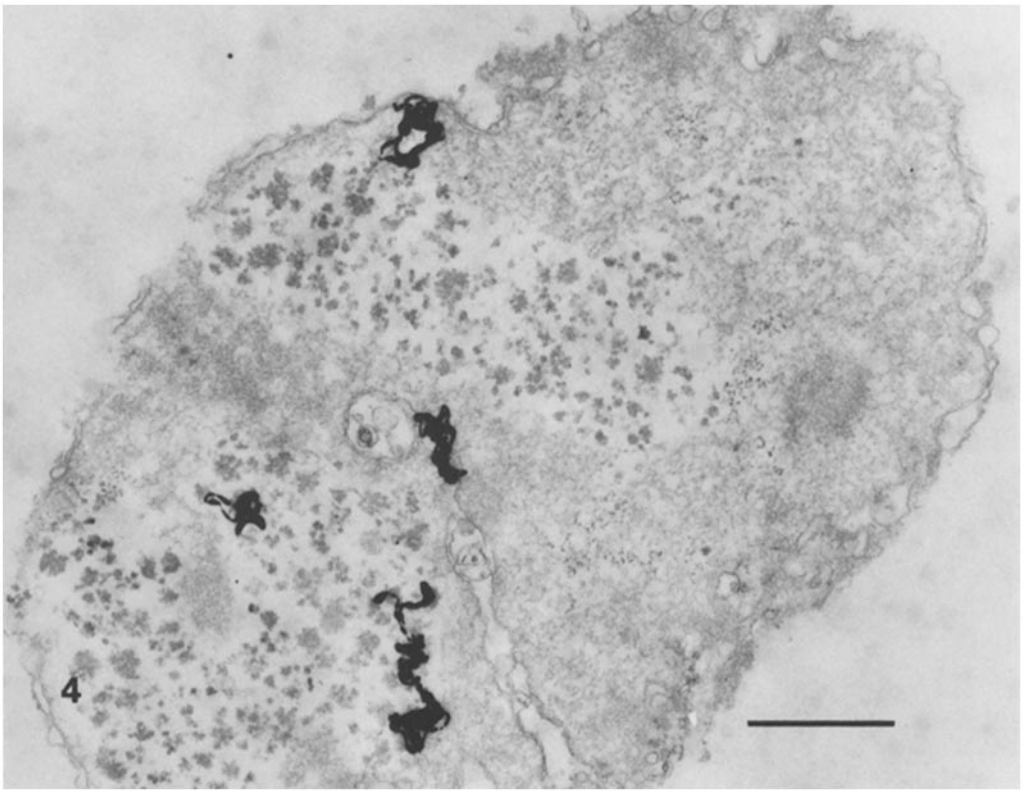

FIGURE 4 Autoradiograph of an isolated HD33 ascites tumor cell nucleus, incubated for 30 min at 37°C with UDP-[1-^{3}H]glucose and 10 mM Glc-6-P. Silver grains are located predominantly in the periphery of the glycogen deposits. Bar, 1 μm. × 18,000.

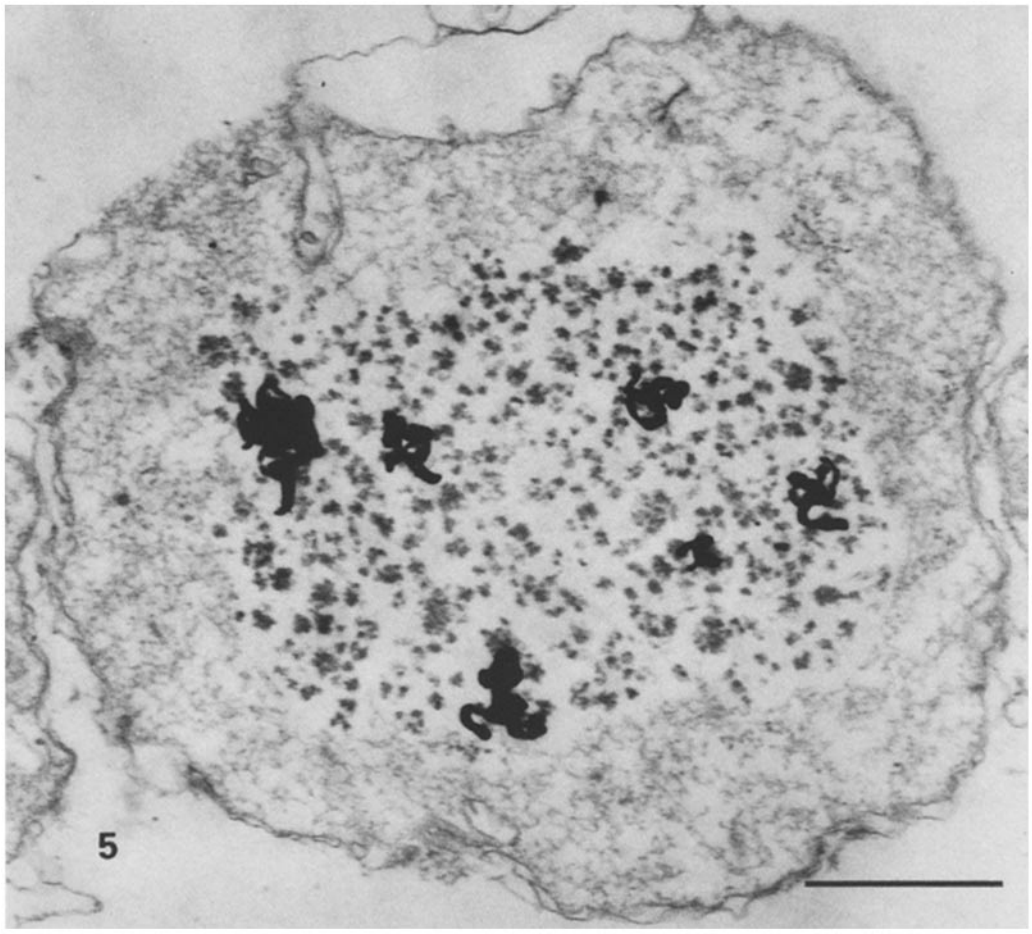

FIGURE 5 Autoradiograph of an isolated HD33 ascites tumor cell nucleus. Incubation conditions were as in Fig. 4. Silver grains are evenly distributed over a glycogen deposit. Bar, 1 μm. × 26,000.

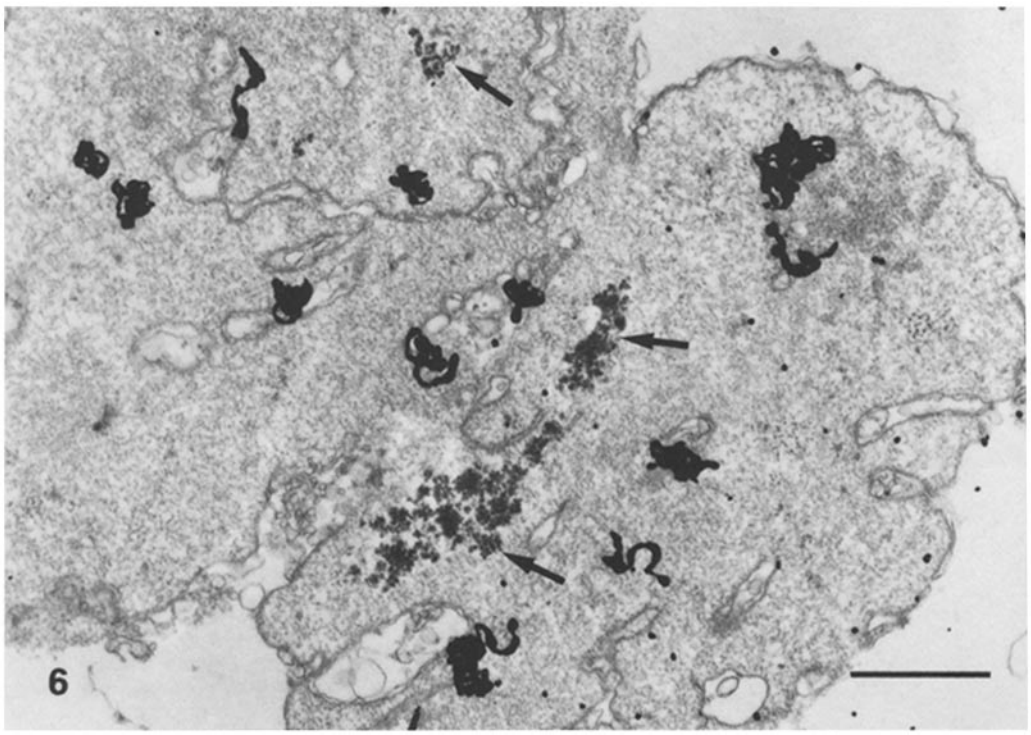

FIGURE 6 Autoradiograph of an isolated HD33 ascites tumor cell nucleus with numerous invaginations of the nuclear envelope. Incubation conditions were as in Fig. 4. Intense labeling, unrelated to glycogen particles (arrows), over interchromatin regions. Bar, 1 μm. × 20,000.

Glycogen Synthase Activity in HD33 Ascites Tumor Cells and Isolated Nuclei

The total glycogen synthase activity of HD33 ascites tumor cells harvested on day 5 of tumor development was 6.90 ± 1.08 U/10^8 cells/h (n = 5). Considerable differences in enzyme activity were observed between the various tested cell fractions (Table I). By far, the highest glycogen synthase activity was found in isolated nuclei. It was more than five times higher than that of postnuclear supernates. The glycogen synthase activity present in postnuclear supernates could be pelleted quantitatively, but could only be enriched threefold by ultracentrifugation. Intermediate values of glycogen synthase activity were found in homogenates of whole cells.

The total glycogen synthase activity in (a + b) form found in homogenates of 192 × 10^6 cells was 235 mU. The postnuclear supernate prepared from an identical number of cells contained 118 mU. Hence, the nuclear compartment contributed half of the total glycogen synthase activity in HD33 ascites tumor cells.

Invariably, only very low fractions of the total glycogen synthase activity were present in its a form (1.07, 2.7, and 1.3% in isolated nuclei, cell homogenates, and in 94,000 g pellets of postnuclear supernates, respectively). Hence, the glycogen synthase activity of isolated nuclei and cell homogenates was further investigated in the presence of 6.67 mM Glc-6-P. Under such conditions, the incorporation of UDP-[^{14}C]glucose into glycogen was linear with time for at least 20 min (Fig. 7). The effect of changing the UDPG concentration and the corresponding Lineweaver-Burk plots are shown in Fig. 8. In five independent tests, K_m values for glycogen synthase in (a + b) form ranging from 0.77 × 10^{-3} M to 1.17 × 10^{-3} M UDPG were obtained (mean value 1.03 × 10^{-3} M). The K_m values were identical in isolated nuclei and in cell homogenates. This is also true of the K_m values of glycogen synthase activity in a form that ranged from 0.66 to 1.00 × 10^{-3} M (not shown). The activation of glycogen synthase in isolated nuclei of HD33 ascites tumor cells with various concentrations of Glc-6-P is demonstrated in Fig. 9. The resulting K_a value is 5 × 10^{-3} M Glc-6-P.

Distribution of UDPG Pyrophosphorylase in HD33 Ascites Tumor Cells

As shown in Table II, the total UDPG pyrophosphorylase activity found in cell homogenates resided in the postnuclear supernate. If detectable at all, a contamination of the isolated nuclei by the enzyme was negligible.

TABLE I

Subcellular Distribution of Glycogen Synthase Activity in HD33 Ascites Tumor Cells

Fraction	Glycogen synthase	
	(a + b) form	a form
	mU/mg protein ± SE *	
Nuclei	14.53 ± 1.33 (n = 5)	0.156 ± 0.08 (n = 5)
Cell homogenates	5.75 ± 0.52 (n = 5)	0.155 ± 0.04 (n = 4)
Cytoplasm‡	2.55	0
94,000 g cytoplasmic pellet	8.13	0.106
94,000 g cytoplasmic supernate	0	0

* The number of independent experiments is given in parentheses, and the means ± SE are given when more than two experiments have been done. In each experiment, the results were obtained from at least triplicate determinations.

‡ The isolation of nuclei involves a low-speed centrifugation step after the cell homogenization. The resulting postnuclear supernate is designated cytoplasm.

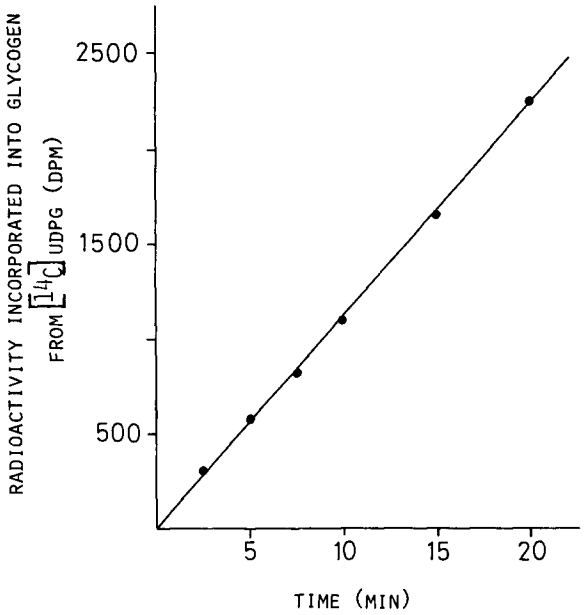

FIGURE 7 Time course of glycogen synthesis in homogenates of isolated nuclei of HD33 ascites tumor cells. The absolute radioactivity of glycogen formed was measured after incubation with 6.67 mM Glc-6-P under standard conditions and corresponds to 76 μg nuclear protein.

DISCUSSION

The mainly nuclear glycogen deposition in HD33 ascites tumor interphase cells 5 d after transplantation favors the assumption that glycogen synthesis takes place within the nuclei themselves. However, apart from a preliminary report (3), there is still a lack of cytochemical or biochemical evidence for the intranuclear occurrence of the responsible enzyme, glycogen synthase (43). Therefore, the possibility has to be excluded that glycogen synthase activity found in isolated nuclei might represent merely a contamination and, in fact, originate from the cytoplasm. The recent report (32) on the artificial presence of glycogen synthase in isolated rat liver nuclei may serve as an example for the importance of morphologically controlling nuclear preparations. The authors observed glycogen particles and other cytoplasmic structures within isolated liver nuclei whose membranes had been severely damaged and concluded that, in the course of the isolation procedure, such nuclei had trapped glycogen synthase activity associated with the cytoplasmic glycogen particles.

In striking contrast to liver cells, >85% of the total cellular glycogen of the HD33 ascites tumor cells investigated here is stored within the nuclei. The cytoplasm of most of the cells is cytochemically glycogen free (cf. Fig. 1). Therefore, a significant influence of cytoplasmic glycogen particles (or particle-associated glycogen synthase) on enzyme activities of isolated tumor cell nuclei appears unlikely. On regular, thorough electron microscopic examination of preparations of isolated nuclei, the only detectable cytoplasmic structures were remnants of the endoplasmic reticulum connected to the nuclear envelope and the vesicular contents of its characteristic invaginations. The glycogen deposits seen in undamaged, isolated nuclei were, as in intact cells (46), without a gap surrounded by chromatin. Hence, an adequate separation of the nuclei from cytoplasmic

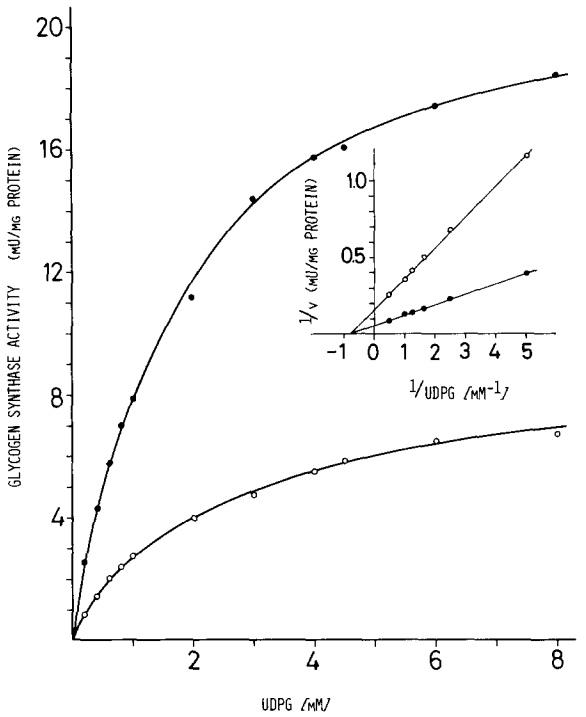

FIGURE 8 The effect of changing the UDPG concentration on the activity of glycogen synthase in (a + b) form. Homogenates of isolated nuclei (●) and of whole HD33 ascites tumor cells (○) were incubated for 15 min under standard conditions. *Inset:* Lineweaver-Burk diagram. (●) isolated nuclei, (○) cell homogenates.

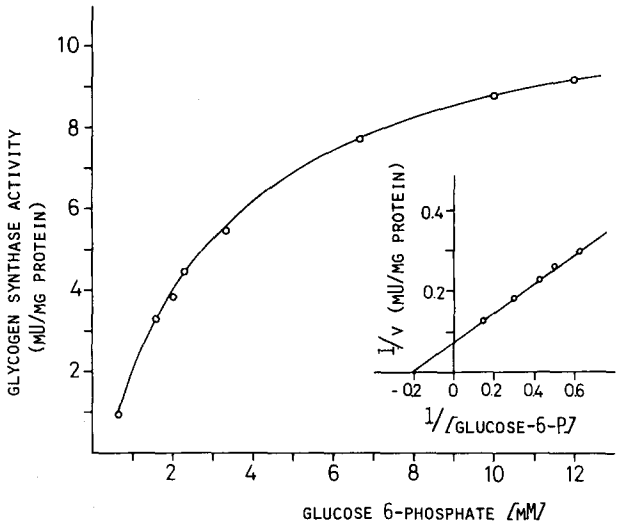

FIGURE 9 Activation of glycogen synthase with Glc-6-P. Homogenates of HD33 ascites tumor cells harvested 6 d after transplantation were incubated under standard conditions with the Glc-6-P concentrations indicated. *Inset:* Doubly reciprocal plot; the UDPG concentration was 4.5 mM.

structures was achieved. On the basis of these ultrastructural findings, an artificial contamination of the isolated tumor cell nuclei with glycogen synthase activity associated with trapped or adsorbed cytoplasmic glycogen particles can be excluded.

Nevertheless, a contamination of isolated nuclei with soluble matter of cytoplasmic origin could result from uptake during the hypotonic treatment of the tumor cells. When one follows the swelling of such cells in 2 mM EDTA by phase-contrast microscopy, the plasma membranes expand for several minutes and eventually burst. A swelling of the nuclei is not obvious up

TABLE II

Subcellular Distribution of UDPG Pyrophosphorylase Activity in HD33 Ascites Tumor Cells

Fraction	mU/mg protein*
Nuclei	0.11
Cell homogenates	37.70
Cytoplasm‡	39.60

* The values are means from two independent experiments. In each experiment, the results were obtained from at least triplicate determinations; in the case of nuclear fractions, samples containing up to 360 μg protein were assayed.

‡ Cytoplasm is defined as in Table I.

to this point, but develops gradually afterward. Prolonged swelling leads to the leaking of contrast-rich matter from the nuclei and, finally, to their rupture. Thus, the uptake of water by the nuclei and of solutes which may permeate the nuclear envelope is best prevented by homogenizing the swelling cells shortly before the bursting of the plasma membrane; careful phase-contrast observation of the individual samples proved, in fact, to be essential. An enzyme known for its complete solubility in isotonic or hypotonic media is UDPG pyrophosphorylase (42). Because it is abundant in the cytoplasm of HD33 ascites tumor cells, it can serve as an example of a protein expected to permeate the nuclear envelope artificially in the course of the aqueous isolation of nuclei. The absence of significant amounts of that enzyme from preparations of HD33 cell nuclei suggests that uptake and binding even of this highly soluble cytoplasmic enzyme by the nuclei was negligible.

The activity of glycogen synthase itself, when compared on a milligram protein basis, was found to be five times higher in isolated nuclei than in postnuclear supernates and almost twice as high as in the high-speed pellets of the latter. This result is incompatible with the assumption that glycogen synthase activity found in isolated nuclei could originate from cytoplasmic contamination. On the other hand, the reverse possibility, that enzyme activity released from mechanically or osmotically fragmented nuclei may contaminate the postnuclear supernate, cannot be excluded.

An estimate of the contamination by cytoplasmic enzyme activities actually to be expected in properly isolated nuclei can be made from the percentage of the UDPG pyrophosphorylase activity of cell homogenates which was traced in isolated nuclei. It was either absent or <1%, a figure in conformity with our ultrastructural findings.

The conclusion from these considerations, that HD33 ascites tumor cell nuclei contain, in addition to extended glycogen deposits, high autochthonous glycogen synthase activity, is confirmed by the autoradiographic findings on isolated nuclei. Whereas [^{3}H]glucose could not be utilized, UDP-[^{3}H]glucose was incorporated in structurally bound matter whose glycogen nature was evident from its specific sensitivity to amyloglucosidase digestion. Thus, the sites of autoradiographic reaction represent areas of the isolated nuclei in which glycogen was synthesized. They are located in close spatial relationship to glycogen deposits, predominantly in their periphery, sometimes also more evenly distributed over a deposit. That glycogen particles that can be visualized by electron microscopy are not a prerequisite for glycogen synthesis is indicated by the frequent observation of amyloglucosidase-sensitive labeling over glycogen-free interchromatin areas in the sections. Because in light microscopic autoradiographs of whole isolated nuclei, specific labeling was found exclusively over nuclei containing glycogen deposits, we assume that ultrathin sections showing

labeling without detectable glycogen particles have been cut from areas neighboring a nuclear glycogen deposit.

The topographic pattern of glycogen synthesis that emerges from the observations on autoradiographs of isolated HD33 nuclei centers on glycogen deposits of various sizes located in the interchromatin region, which are separated from the nuclear membrane system by chromatin. Glycogen is most actively synthesized in the periphery of glycogen deposits as well as in the surrounding interchromatin area, suggesting a predominantly appositional mode of growth of the deposits in interphase nuclei at the expense of the space available for the chromatin. This situation is in full agreement with earlier findings on nuclear glycogen synthesis in intact HD33 ascites tumor cells (45). The ultrastructure of a protein network associated with nuclear glycogen in HD33 ascites cells has been described in the paper cited.

The availability of the substrate for nuclear glycogen synthase, UDPG, depends on its flow through the nuclear envelope, for the activity of UDPG pyrophosphorylase is restricted exclusively to the cytoplasmic compartment. The nuclear envelope is to be looked at as a diffusion-restricting barrier with patent pore radii of ~45 Å (33). The space-filling model of the UDPG molecule in its stretched form (26) has a maximum molecular radius corresponding to 8 Å, allowing sufficient access of this diffusible cellular metabolite to the nuclear compartment. Thus, the availability of its substrate should be similar for glycogen synthase residing inside or outside the nuclear envelope. This is also valid for its activator, Glc-6-P, an even smaller molecule which is abundantly present in isolated liver nuclei (39).

The biochemical characteristics of glycogen synthase are identical in isolated nuclei, cell homogenates, and cytoplasmic fractions. Considering the permanent exchange between nucleoplasm and cytoplasm due to the high mitotic activity of the tumor cells, this result is not unexpected. It implies that the lack of extranuclear glycogen deposition in HD33 cells at the investigated stage of tumor development is caused by factors other than the absence of cytoplasmic glycogen synthase activity. A most striking phenomenon is that the enzyme exists to ~98% in its b form. A similar coincidence of pronounced glycogen storage with the presence of most of the glycogen synthase activity in its Glc-6-P-dependent form has been reported for human choriocarcinoma cells (21), human (34, 36, 44) and rat (37) polymorphonuclear leukocytes, human lymphocytes (18), and rat myogenic cells (20). Surprisingly, the activity of glycogen synthase of HD33 ascites tumor cells resembles closest that of glycogen synthase from the normal human white blood cells. Both the malignant and the normal type of single cells contain comparable amounts of glycogen, and <3% of the glycogen synthase activity occurs in its a form. The K_m values for UDPG in the presence of Glc-6-P and the activation constants for Glc-6-P are in the same range. It is, therefore, the more interesting that the activity of glycogen synthase found by others in glycogen-free Ehrlich ascites tumor cells is qualitatively and quantitatively distinguishable from that of the mutant cells described here. In homogenates of the glycogen-free Ehrlich ascites tumor cells, 10% of the glycogen synthase activity existed in its a form, the K_m value was 0.2×10^{-3} M UDPG, and the total enzyme activity was only 2.88 mU/mg protein (1). The fundamental difference between the cell types lies in the ability of the HD33 ascites cells to synthesize and to store glycogen. One might speculate that, in these mutant cells, the mechanisms are derepressed that otherwise inhibit these functions in Ehrlich ascites tumor cells.

The mode of initiation of glycogen synthesis within the nuclear matrix remains to be elucidated. Karasaki (22), who discovered nuclear glycogen synthesis in intact Novikoff hepatoma cells, suggested its initiation through glycogen particles erratically included in reconstructing nuclei after mitosis. Neither cytochemically nor by electron microscopy could an inclusion of glycogen into reconstructing daughter nuclei be detected, although glycogen is frequently present in mitotic HD33 ascites tumor cells (cf. Fig. 1). Taking into consideration the reports on the action of glycogen initiator synthase in rat liver cells (25), *Escherichia coli* (2), and *Neurospora crassa* (9), we assume that in nuclei of HD33 ascites tumor cells glycogen synthesis is also initiated by a similar enzyme activity.

A further important aspect is the regulation of nuclear glycogen synthesis. In glycogen-storing Ehrlich ascites tumor cells, glycogen synthesis could not be influenced by epinephrine, dibutyryl cAMP, and glucagon (23). However, in a preliminary report (24) it was shown that the activity of nuclear glycogen synthase in HD33 ascites tumor cells strictly correlates with the growth rate of the tumor. The mechanisms involved in this correlation, the mode of initiation of nuclear glycogen synthesis, and further biochemical characteristics of glycogen synthase in HD33 ascites tumor cells are currently being investigated.

To our knowledge, the reported findings on isolated nuclei represent the first complete evidence for the presence of autochthonous glycogen synthase activity in mammalian cell nuclei, as well as for the ability of isolated nuclei to synthesize glycogen *de novo*. This may contribute in particular to a better understanding of nuclear glycogen deposition and synthesis in the various types of cells mentioned in the Introduction. The more general implications are that glycogen synthesis in mammalian cells is not an exclusively cytoplasmic function and, further, that the mammalian cell nucleus is capable of synthesizing biopolymers other than nucleic acids.

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