Expression of Poly(adenosine Diphosphate-Ribose) Polymerase Activity in Erythroleukemic Mouse Cells during Cell Cycle and Erythropoietic Differentiation*

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Accumulation of erythroleukemic mouse spleen cells (F4N-Ostertag) in the G-1 phase of the cell cycle was achieved in three ways: maintenance of cells 1) at high densities (3 to 5 x 10^6 cells ml^{-1}), 2) at low densities (1 x 10^6 cells ml^{-1}) in isoleucine-free medium, and 3) in the presence of 1.5 mM butyric acid or 3.8 mM hexamethylene bisacetamide, both potent inducers of erythropoietic differentiation, at these given concentrations in F4N cells.

These G-1 arrests were documented by microfluorometric determination of the DNA content of single cells. Poly(ADP-ribose) polymerase activity was followed during the process of G-1 accumulation. This enzyme catalyzes the synthesis of poly(ADP-ribose) from NAD. As poly(ADP-ribose) is covalently bound to chromosomal proteins, this system provides a possible means for connecting cellular NAD levels with regulative events in chromatin. Poly(ADP-ribose) polymerase had a fairly constant activity level in logarithmically growing cells and increased 3- to 4-fold when G-1 accumulation was achieved by either of the three methods. Although increased levels of poly(ADP-ribose) polymerase activity seemed to occur whenever F4N cells accumulated in G-1, it also appeared to be conditional for the expression of the erythroid differentiation program in these cells.

For determination of poly(ADP-ribose) polymerase activity we used an in vitro enzyme assay system. Under these conditions two variables which contribute to the enzyme activity could be excluded: fluctuations in nuclear NAD levels and poly(ADP-ribose) degradation. Enzyme concentration and number of polymer attachment sites on chromatin thus are the rate-determining factors.

The manifestation of the epigenetic induction event is observed at two levels: limitation of cell proliferative capacity and specific alterations in gene activity. With MeSO as inducer only four additional cell cycles occur after a stochastic commitment event (6) and initiation of S phase is transiently inhibited from 12 to 24 h after exposure to the inducer (7). A concerted shift in gene expression, representing a portion of the normal erythropoietic differentiaton program, is visualized in a decrease in total RNA synthesis (8) and a 10- to 100-fold increase in globin mRNA synthesis (3). Such alterations in the transcription program can be controlled by de novo synthesis or modification of chromatin-associated proteins. In Friend cells the de novo induction of a specific chromosomal protein occurs as an early event in the differentiation process (9) but no data are available so far on concomitant modifications of chromosomal proteins. Here we investigated poly(ADP-R) polymerase and related changes in the activity of this enzyme to initial events of the induction process. Poly(ADP-R) polymerase catalyzes the formation of a homopolymer of ADP-R units linked by 1'-2' glycosidic bonds (10, 11). The substrate for the reaction is NAD (11, 12). In the presence of DNA the enzyme successively adds ADP-R units onto an initial ADP-R residue (13), which had been reported to be covalently attached to various nuclear proteins, mainly histones (14, 15). As yet no firm evidence exists which indicates that poly(ADP-R)ribosylated chromosomal proteins are involved in the regulation of gene transcription, although a recent report suggests a role in differentiation (16). More evidence exists that poly(ADP-R) polymerase is correlated with some aspects of replication (13) and that synthesis of poly(ADP-R) causes perturbation of chromatin structure (17, 18). We showed that both increase in poly(ADP-R) polymerase activity and transition into G-1 phase occur concomitantly with induction of erythropoietic differentiation in Friend cells.

EXPERIMENTAL PROCEDURES

Erythroleukemic mouse spleen cells (Friend cells) undergo erythropoietic differentiation when cultured in the presence of inducers like Me_2SO (1-3), HMBB (4), or butyric acid (5).

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The abbreviations used are: MeSO, dimethylsulfoxide; HMBB, hexamethylene bisacetamide; poly(ADP-R), poly(adenosine diphosphate-ribose); DIPF, 4'-6-bis(2'-imidazolinyl)-4',5'-3'H-2-phenylindole.

J. Zlatanova and P. Swetly, manuscript in preparation.

Cell Culture—Dr. W. Ostertag kindly provided the erythroleukemic mouse spleen cell line F4N. Ostertag et al. (10) reported on its characterization and its properties in respect to hemoglobin induction and Friend virus synthesis. We cultured F4N cells in suspension using Eagle's flow minimum essential medium, supplemented with...
double amounts of amino acids, 4-fold amounts of glutamine, and 10% fetal bovine serum.

Materials - Mouse interferon had a specific activity of 8 × 10^8 units/mg of protein and was prepared according to Bodo et al. (20). Units are defined as reference research standards. We synthesized HMBA according to Reuben et al. (4) and obtained the detergent Kyro EOB as a gift from Dr. Hughes, Procter and Gamble Co. Imethy-3H-thymidine had a specific activity of 50 Ci/mmol and nicotinamide-14C (ADP-R) will be described in detail elsewhere. In short, we incubated rat thymus cell nuclei under standard assay conditions with 1 mM [14C]NAD (specific activity, 1 mCi/mmol) as a substrate. Purification included Hirt extraction (21) of the incubation mixture, treatment with nucleases, and chromatography on hydroxyapatite (22). For chain length determination of [γ-32P]poly(ADP-R) we digested the polymer by phosphodiesterase treatment and used subsequently paper chromatography in an isobutyric acid/ammonium hydroxide/water system for the quantitative determination of the products (23). Average chain length was 18 ADP-R units and an aliquot of 1500 cpm corresponded to 0.75 nmoI of poly(ADP-R).

Induction of Differentiation - For inducing differentiation we seeded F4N cells at a density of approximately 5 × 10^4 cells ml^-1 and added the inducer 12 h later. The concentration of butyric acid was 1 to 1.5 mM and that of HMBA was 3.8 mM. In daily intervals cells were collected by centrifugation at 20°C, 600 × g for 10 min and resuspended in fresh medium containing the respective inducer.

For determination of the percentage of hemoglobin synthesizing cells we centrifuged aliquots of the cell suspensions containing approximately 5 × 10^6 cells at 1000 × g for 5 min, resuspended the cell pellet in 0.1 ml of culture medium, and spread the suspension on slides. Benzidine staining was carried out by standard procedure (24) and the percentage of benzidine reactive cells was determined by counting at least 100 cells/slide.

For Friend virus determination we used cell supernatants filtered through 0.45-μm pore size Millipore filters and performed the XC plaque test (95) on SC1 cells (96). The measurements of the cellular NAD/NADH content were according to Nisselbaum and Green (27).

Cell Fractionation - For preparation of cell lysates we centrifuged 1 to 2 ml of F4N cell suspension (containing 1 to 2 × 10^7 cells) as determined by hemocytometer counting, from 1000 × g for 5 min, resuspended the cell pellet in 0.3 ml ice cold salt/P, (0.138 M NaCl, 2.7 mM KCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄), and lysed the cells by adding 0.5% Kyro EOB for 20 min at 0°C. For preparing cell nuclei we centrifuged the cell suspensions at 1000 × g for 5 min and resuspended the pellet in 0.5 ml of cold salt/P. The supernatant was recentrifuged at 2000 × g for 5 min to remove contaminating traces of nuclei and was used as cytoplasmic fraction.

Poly(ADP-R) Polymerase Activity Assay - We determined the incorporation of ADP-R into triphloroacetic acid-insoluble material according to the reaction: nNAD + (ADP-RXₙ + n-nicotinamide. We added either cell lysate, nuclei, or cytoplasm (volume 0.3 ml) to 0.2 ml of a reaction mixture containing, in a final volume of 0.5 ml: 0.1 M Tris-Cl (pH 8.3), 20 mM MgCl₂, 4 mM NaF, 60 mM KCl, 4 mM β-mercaptoethanol, and 1 mM [14C]NAD (specific activity, 0.5 mCi/ mmol). The reaction was allowed to proceed for 15 min at 25°C and was stopped by the addition of 5 ml of cold 7% trichloroacetic acid and the radioactivity was determined by liquid scintillation counting.

In the experiments where we determined the enzyme activity in nuclei we added 0.5% Kyro EOB to the nuclear suspension to attain identical incubation conditions like with cell lysates, although we found no influence of Kyro EOB on poly(ADP-R) polymerase activity. For determination of poly(ADP-R) degradation we incubated cell lysates or nuclei under the conditions of the poly(ADP-R) polymerase assay with the exception that we used unlabeled NAD instead of [14C]NAD and added 1500 cpm of [14C]poly(ADP-R).

Determination of DNA Synthesis - In order to determine the percentage of DNA synthesizing cells, we incubated 1 ml aliquots of cell suspension with 3 μCi of [3H]thymidine (specific activity, 50 mCi/mmol) for 10 min at 37°C, cooled them to 0°C, and washed the cells twice in cold salt/P. Cells were spread on slides, air-dried, and fixed in methanol. Then we stained the cells according to Schnedl et al. (28) for 15 min at 20°C with an aqueous solution of the fluorochrome DAPI at a concentration of 0.5 μg/ml. After washing with water and mounting in Sorensen buffer, pH 6.8, the fluorescence intensity of at least 200 single cells was measured using a Reichert Zetopan microscope connected with a Reichert microphotometer, which was equipped with an HBO 200 mercury burner.

RESULTS

Accumulation of Differentiating F4N Cells in G1-Phase of Cell Cycle - Butyric acid at a concentration of 1 to 1.5 mM induced erythropoietic differentiation in F4N cells. Successful stimulation with low cytotoxicity required that cells grew at low density (0.5 to 1 × 10^6 ml^-1) for at least 24 h before addition of butyric acid. Determination of benzidine-reactive cells at daily intervals provided a measurement for the percentage of hemoglobin-synthesizing cells. Butyric acid induction led to 63% benzidine positive cells after 2 days and a maximum level of more than 90% of hemoglobin-synthesizing cells after 3 days as shown in Table I.

Butyric acid-treated F4N cells doubled in number and subsequently reached a period of ultimate growth arrest (Fig. 1) despite transfer of cells to fresh butyric acid containing growth medium in daily intervals. Two different reasons might be responsible for this arrest: an increased mortality rate might compensate an unreduced rate of cell proliferation, or a prolongation of cell cycle duration might occur. Under the conditions applied, less than 3% of the cells in culture stained with trypan blue during butyric acid treatment. Prolongation of cell cycle seemed thus likely and we determined which phase of the cell cycle was prolonged. We harvested cells at different times after onset of butyric acid treatment and stained them with the fluorescent dye DAPI (28). In Fig. 2, A to C, the fluorescence intensities of at least 200 nuclei are presented in form of histograms. Untreated cells exhibited a broad distribution of DNA which is representative for the S phase of the cell cycle (Fig. 2A). The same pattern of DNA distribution was observed when mock-induced cells were harvested at 16 h or at 31 h after onset of treatment. Cells collected after 16 h of butyric acid treatment (Fig. 2B) showed a biphase distribution in their DNA content, with a minimum in the region of S phase cells (medium amounts of DNA) and a broad distribution of DNA which is representative for the S phase of the cell cycle (Fig. 2A).

<table>
<thead>
<tr>
<th>Time of Treatment (days)</th>
<th>Butyric acid</th>
<th>HMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>N. T.</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>92</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>65</td>
</tr>
</tbody>
</table>

* N. T., not tested.
peaks in the regions characteristic for G-1 phase and for G-2 phase. This type of distribution results when cells move out of S phase but cells in G-1 phase cease transgressing into S phase. Cells harvested after 31 h of butyric acid treatment no longer showed biphasic distribution but displayed a narrow spectrum with almost all the cells in the G-1 region (Fig. 2C). Thus by a time point clearly preceding phenotypic expression of erythropoietic differentiation the cells had moved through S and G-2 phases and accumulated in the G-1 phase of the next cell cycle. The transition probability from G-1 to S decreased more than 20-fold in the presence of butyric acid as the average duration time of G-1 phase shifted from 1 h in exponentially growing cells to more than 20 h. HMBA showed at a concentration of 3.8 \times 10^{-3} M no cytotoxicity in F4N cells and induced hemoglobin synthesis in 65% of the cells within 4 days of treatment (Table I). Cell division slowed down within 3 days but reached no state of arrest as with butyric acid. Fig. 2, D to F, displays the histograms of DNA content distribution after 24, 48, and 72 h of HMBA treatment. After 24 h (Fig. 2D), the distribution of DNA resembled closely that of untreated cells (Fig. 2A), whereas 48-h treatment led to a state with beginning G-1 accumulation and biphasic distribution. About 50% of the cells were in G-1 phase after 72 h (Fig. 2F) at which time 46% of the cells synthesized hemoglobin (Table I). These data suggest a temporal relationship between the number of differentiating and G-1 arrested cells.

**Induction of Erythroid Differentiation Led to Increase in Poly(ADP-R) Polymerase Activity**—We determined poly(ADP-R) polymerase activity in cell lysates of F4N cells harvested at various times after induction of erythroid differentiation by either butyric acid or HMBA. Fig. 3 shows that enzyme activity increased steeply between 8 and 20 h after exposure of cells to butyric acid and reached a level about 4 times above that of control cells. In HMBA-treated cells poly(ADP-R) polymerase activity increased 2-fold around 40 h after addition of the inducer. The lower level of induced enzyme activity correlated with a lower number of cells arrested in G-1, as Fig. 2 shows, and less cells in a benzidine-reactive state (Table I).

**Cell Lysates and Nuclei Had Similar Poly(ADP-R) Polymerase Activities**—Although poly(ADP-R) polymerase is a nuclear enzyme we used total cell lysates consisting of intact nuclei, cytoplasm, and membranes in our standard enzyme assay. This allowed us to assay a small number (about 10⁶) of cells and to avoid irreproducible loss of material which occurred during the preparation of nuclei. Before employing this

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**Fig. 1.** Arrest of F4N cell proliferation in the presence of butyric acid. F4N cells seeded at a density of 7.5 \times 10^{6} cells ml⁻¹ were exposed to 1 mM butyric acid. At daily intervals the cells were quantitatively transferred into fresh medium containing butyric acid. Control cells were cultured in the same way except that butyric acid was omitted. At the times indicated cell densities were determined by hemocytometer counting. Density of butyric acid-treated cells, O-O; that of control cells, - -

**Fig. 2.** Histograms of the DNA content of F4N cells at various stages of butyric acid- or HMBA-induced erythropoietic differentiation. F4N cells seeded at a density of 1 \times 10^{6} cells ml⁻¹ were treated either with 1 mM butyric acid or with 3.8 mM HMBA. In daily intervals the cells were transferred to fresh medium containing the respective inducer. Aliquots of 5 \times 10^{6} butyric acid-treated cells were removed from the cultures and the DNA content was determined by microfluorometry (28) after 16 h (B) and 31 h (C). From HMBA-treated cultures cells were removed after 24 h (D), 48 h (E), and 72 h (F). Panel A shows the DNA distribution of untreated F4N cells in logarithmic growth phase.
we tested the method with respect to the presence of cytoplasmic factors which might interfere. We assayed cell lysates and nuclei both of untreated and butyric acid-treated cells for enzyme activity and found similar activities in corresponding preparations (Table II). This indicated that no inhibitory or stimulating factors for the enzyme activity become apparent in total cell lysates which are not active in isolated cell nuclei. Further we compared the polymerase activity in total cell lysates with that after combining isolated nuclei and cytoplasmic fraction of butyric acid-treated cells and found similar levels of activity (Table II). We found, however, elevated levels of enzyme activity in a mixture of butyric acid-treated nuclei and cytoplasm from untreated cells.

These results made the presence of an interfering, inhibitory activity in the cytoplasm of F4N cells unlikely and established the assay of poly(ADP-R) polymerase activities in cell lysates as a reliable method. In all experiments we took care, however, that the exogenous NAD concentration in the assay mixture was higher than endogenous cellular NAD concentrations.

No Direct Influence of Butyric Acid or HMBA on Poly(ADP-R) Polymerase Activity in Vitro—We tested a possible direct influence of agents used for the induction of erythroid differentiation on poly(ADP-R) polymerase by adding either butyric acid or HMBA to the enzyme assay mixture. The concentration range of the inducers varied from 0.1- to 10-fold the concentration used for the stimulation of the cells. No alteration of enzyme activity occurred under these conditions which excluded a direct effect of the inducing substances on the enzyme reaction.

Increase in Poly(ADP-R) Polymerase Activity during Differentiation Was Not Correlated with Friend Virus Synthesis—F4N cells continuously produce Friend virus which can be assayed by the XC test (25, 29). It had been previously shown that induction of hemoglobin synthesis by Me2SO is at early stages accompanied by an increase in Friend virus production and that mouse interferon suppresses virus synthesis in F4N cells without interfering with hemoglobin synthesis (30). Here we investigated the effect of butyric acid and interferon either alone or combined on Friend virus synthesis and poly(ADP-R) polymerase activity. This should allow us to examine the possibility that increased poly(ADP-R) polymerase activity is a reflection of elevated virus synthesis. By assaying cell-free supernatants in the XC tests we found that Friend virus production had increased 6-fold within 18 h after butyric acid induction and decreased within 49 h to the level of uninduced cell supernatants. In this period the intracellular poly(ADP-R) polymerase activity increased 5-fold (Table III). Interferon at an activity of 500 units ml⁻¹ in the absence of butyric acid reduced the Friend virus concentration in the cell supernatants by more than 10-fold but did not affect the basic level of poly(ADP-R) polymerase within 49 h. In order to achieve an optimal antiviral effect already at early stages of differentiation, we pretreated cells for 38 h with 500 units ml⁻¹ of mouse interferon before inducing F4N cells with butyric acid in the continuous presence of interferon. Under these conditions the synthesis of hemoglobin (not shown) and the increase in poly(ADP-R) polymerase activity occurred simi-
larly in butyric acid-induced cells in the absence of interferon, whereas Friend virus synthesis was suppressed (Table III). Thus, interferon inhibited the chronic synthesis of Friend virus and its increased production at early stages of differentiation but did not interfere with elevation of poly(ADP-R) polymerase activity levels. This observation suggests that these two processes are not jointly controlled.

Factors Which Control Poly(ADP-R) Polymerase Activity—The observed increase in poly(ADP-R) polymerase activity could be a reflection of alterations in several parameters: 1) increase in the concentration of poly(ADP-R) polymerase; 2) increase in the intracellular NAD concentration; 3) decrease in the activity of poly(ADP-R) degrading enzymes like glycohydrolase or phosphodiesterase; 4) increase in the number of initiation sites for poly(ADP-R) synthesis in chromatin.

To test whether intracellular NAD present in the cell lysates contributed significantly to the in vitro assay and whether fluctuations in the cellular NAD pool might influence the total NAD concentration, we determined the NAD/NADH content of resting F4N cells. Cells harvested at a density of 4 x 10^6 cells ml^-1 contained 0.24 ± 0.06 nmol of NAD/NADH in 10^6 cells. We used for the in vitro assay cell lysates of up to 4 x 10^6 cells which contained roughly 1 nmol of endogenous NAD. As the assay mixture contained 500 nmol of exogenous NAD, even strong fluctuations in the cellular NAD pool should not contribute significantly to the substrate concentration. Poly(ADP-R)-degrading activity was determined under the conditions of the poly(ADP-R) polymerase test. As substrate for the polymer-degrading enzymes we used [14C]poly(ADP-R) with average chain length of 18 ADP-R units. We incubated the radioactive substrate together with unlabeled NAD at 30°C with either lysates or with nuclei of F4N cells, harvested at various stages of butyric acid-induced differentiation, and determined the acid-precipitable radioactivity. If the increase in poly(ADP-R) polymerase activity during differentiation is caused by a decrease in poly(ADP-R)-degrading activities, we should observe faster degradation of [14C]poly(ADP-R) with lysates of uninduced cells. From the data presented in Table IV we conclude that under the in vitro polymerase assay conditions cell lysates of neither uninduced nor induced F4N cells displayed degrading activities. This conclusion is restricted to degrading enzymes which act on free poly(ADP-R) chains and cannot be extended to poly(ADP-

### Table III

**Effect of interferon treatment on poly(ADP-R) polymerase and Friend virus production in untreated and butyric acid-treated cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation of [14C]poly(ADP-R) into acid-precipitable material</th>
<th>Virus titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cpm/10^6 cells)</td>
<td>(plaque-forming units/10^6 cells)</td>
</tr>
<tr>
<td>RA</td>
<td>RA + IF</td>
<td>IF</td>
</tr>
<tr>
<td>0</td>
<td>135 ± 40</td>
<td>166 ± 20</td>
</tr>
<tr>
<td>18</td>
<td>208 ± 30</td>
<td>350 ± 30</td>
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<tr>
<td>31</td>
<td>350 ± 30</td>
<td>166 ± 20</td>
</tr>
<tr>
<td>38</td>
<td>792 ± 20</td>
<td>641 ± 70</td>
</tr>
<tr>
<td>49</td>
<td>919 ± 20</td>
<td>641 ± 70</td>
</tr>
</tbody>
</table>

* N. T., not tested.

### Table IV

**Determination of [14C]poly(ADP-R) degrading activity in lysates and nuclei of F4N cells at different stages of butyric acid-induced differentiation**

Erythropoietic differentiation was induced in F4N cells by butyric acid as described under "Experimental Procedures." Aliquots of 2 to 4 x 10^6 cells were removed from the cultures for preparation of cell lysates and of nuclei. Determination of a [14C]poly(ADP-R)-degrading activity was carried out under the conditions of the poly(ADP-R) polymerase assay by using unlabeled NAD as substrate and 1500 cpm of [14C]poly(ADP-R)/assay mixture.

<table>
<thead>
<tr>
<th>Days of butyric acid treatment</th>
<th>Recovery of [14C]poly(ADP-R) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysate</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
</tr>
<tr>
<td>0</td>
<td>1452</td>
</tr>
<tr>
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<td>2</td>
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</tr>
<tr>
<td>3</td>
<td>1382</td>
</tr>
</tbody>
</table>

* N. T., not tested.

![Graph showing incorporation of ADP-R ribose in logarithmic and stationary growth phase of F4N cells](link to graph)
Poly(ADP-R) Polymerase in Friend Cells

R) bound to protein. However, all enzymes described so far which degrade poly(ADP-R) are exonucleases and should act on free polymer chains.

Thus, the polymerase activity which we measured reflected availability of initiation sites and polymerase concentration. At present these two possibilities are difficult to distinguish. Either of these or both increase during butyric acid-induced differentiation.

Cells in Resting State Had Increased Poly(ADP-R) Polymerase Activity—As shown in Fig. 2, accumulation of butyric acid-induced cells in the G-1 phase of the cell cycle either preceded or occurred concurrently with early events in the erythroid differentiation like the increase in poly(ADP-R) polymerase activity. We investigated whether this increase occurred also when cells accumulated in the G-1 phase without expressing the differentiated phenotype: F4N cells seeded at a low density (4.5 x 10⁶ cells ml⁻¹) ceased dividing after 3 days at 37°C without medium change at a density of 2.3 x 10⁶ cells ml⁻¹ and maintained this density for 3 days more. We assayed for poly(ADP-R) polymerase activity throughout this period and, as Fig. 4 demonstrates, observed a linear increase to a 4-fold activity synchronous with increase in cell density and accumulation in a resting state. We concluded from these data that the transition of F4N cells to a resting state with or without expression of a differentiated phenotype is accompanied by an increase in poly(ADP-R) polymerase concentration, in the number of initiation sites for ADP-ribosylation, or both.

Poly(ADP-R) Polymerase Activity Increased during Exposure of F4N Cells to Isoleucine-free Medium—The stationary growth phase of cultured cells is accompanied by a depletion of essential components from the culture medium. Among these factors is isoleucine which can induce cell proliferation when added to the culture medium of resting cells (31). The nutritional conditions of resting cells can be simulated by exposing cells to isoleucine-free medium and inducing their accumulation in a resting state. We proved the applicability of this method for F4N cells and showed by measuring fluorescence intensities of cell nuclei that within 20 h in isoleucine-free medium nearly all cells accumulated in the G-1 phase (Fig. 5). After 12 h in isoleucine-free medium a redistribution in the DNA content of the cells had occurred with an increase in the number of cells in the low DNA content region (Fig. 5B) when compared to untreated cells (Fig. 5A). Within 20 h almost all cells had shifted to the G-1 compartment (Fig. 5C). Incorporation of [³H]thymidine into DNA synthesis in F4N cells during arrest in isoleucine-free medium and during release by isoleucine addition F4N cells were washed three times in isoleucine-free medium and seeded in this medium at a density of 1.5 x 10⁶ cells ml⁻¹. After 40 h of incubation isoleucine was added to a final concentration of 0.8 mM and the culture was diluted with complete medium to give the double volume. At the times indicated aliquots of 3 to 6 x 10² cells were removed and assayed for DNA synthesis or lysed for determination of poly(ADP-R) polymerase activity (■). DNA synthesis was measured either by incorporation of [²H]thymidine into trichloroacetic acid-precipitable material (■–■) or autoradiographically by determining the percentage of DNA synthesizing cells (■–■).
trichloroacetic acid-precipitable material continually decreased throughout this period to nearly background levels after 16 h in isoleucine-free medium (Fig. 6), indicating that the majority of cells did not enter in a new S phase under these conditions. Poly(ADP-R) polymerase activity showed a 4-fold increase within 10 h of exposure to isoleucine-deficient medium. Poly(ADP-R) polymerase in F4N cells is thus likely to exist at two stable activity levels. The low level is expressed in exponentially growing cells, with more than 75% of the cells involved in DNA synthesis and few cells in a G-1 state. A high level state approximately 4 times above that of logarithmically growing cells is found in cells accumulating in G-1 because of either increased cell density, medium deficiency, or transition to a differentiated state.

Transgression of F4N Cells through S Phase Did Not Require Low Level Poly(ADP-R) Polymerase Activity State – F4N cells, arrested in the G-1 phase by isoleucine deficiency could be released into S phase when cells were transferred to isoleucine-containing growth medium. Cells resumed DNA synthesis within 2 to 3 h and cell number doubled after 18 h. In the right section of Fig. 6 we plotted the percentage of cells active in DNA synthesis after addition of isoleucine: about 70% of the cells synthesized DNA within 10 h. The distribution of F4N cells 11 h after transfer to isoleucine-containing medium according to their DNA content is shown in Fig. 5D. Most cells exhibited a medium DNA content characteristic for S phase cells. Poly(ADP-R) polymerase activity was at the high level state throughout this period to nearly background levels in fresh medium without butyric acid, showed at all time points a similar DNA distribution as cells in exponential growth phase. This G-1 arrest coincided with the period of transcriptional expression of the specialized proteins of the differentiated phenotype. HMBA induction occurred less rapidly in F4N cells under our culturing conditions. The pattern of DNA content showed similar characteristic shifts like in butyric acid-induced cells but with a delay. By 72 h both arrest in G-1 phase and number of benzidine-reactive cells represent a similar proportion of total cell number in culture. From these results we draw the following conclusions. In order to become successfully induced by butyric acid cells had to be in a replication active state, according to the cell cycle model of Smith and Martin (33). After butyric acid induction F4N cells had a significantly reduced G-1/S transition probability resulting in an altered generation time after commitment. The expression of hemoglobin synthesis can be correlated with G-1 arrest both in butyric acid- and HMBA-induced cells. The transgression to resting state induced by butyric acid occurred under nutritional requirements sufficient for keeping cells in the replicating state and differed thus from G-1 arrest in high density cultures which was caused by isoleucine depletion of the culture medium.

Poly(ADP-R) polymerase, the enzyme which in the presence of DNA catalyzes the formation of ADP-R homopolymers from NAD, may play a key role as a regulative intermediate between nuclear NAD levels and cell proliferative events. Several reports indicate a relationship between cell cycle events and poly(ADP-R) polymerase activity (34–39). We showed that poly(ADP-R) polymerase activity in erythroleukemic spleen cells increased by a factor of 4 when the cells were allowed to accumulate in a resting state, underwent erythropoietic differentiation induced by butyric acid, or accumulated in G-1 phase of the cell cycle by isoleucine depletion of the culture medium. HMBA-induced cells not only showed a lower degree of accumulation in G-1 phase together with a lower percentage of benzidine-reactive cells but also only a doubling of poly(ADP-R) polymerase activity. We concluded from these data that the resting state of the cell cycle is associated with a 4-fold elevated level of poly(ADP-R) polymerase activity as compared to the active replicating state. The increase in enzyme activity was an early event in the transition process. Similar results were obtained by Miwa et al. (40) who showed an increase in enzyme activity correlated with cell density.

As enzyme activity reflects several parameters, its increase could be related to increase in enzyme concentration due to a higher rate of synthesis or activation of an inactive precursor form, to an elevated number of acceptor sites for ADP-riboseylation on chromatin, to increased cellular NAD levels, or to
decreased activity of poly(ADP-R) degrading enzymes. We excluded the latter alternatives by demonstrating the absence of degrading enzyme activities under the enzyme assay conditions and by using saturating amounts of exogenous NAD.

A possible role of poly(ADP-R) polymerase during cellular differentiation was suggested by Caplan and Rosenberg (18), who showed that a strong correlation seemed to exist between the commitment of mesodermal cells of embryonic chick limbs for chondrogenic expression and the increase or maximum of poly(ADP-R) synthesis. The structural aspect of such a function is illustrated by the findings of Mullins et al. (41), who described a preferential association of the enzyme activity with template active regions of HeLa cell chromatin and by the fact that DNA is an essential factor for its activity and stability (42). More than 90% of poly(ADP-R) eluted from cell nuclei in accordance with histones, 85% thereof being associated with histone H1 (43). There exists also good evidence that major structural changes occur in chromatin during erythropoietic differentiation: globin structural genes were protected from DNAase digestion in avian fibroblastic cells and were sensitive to degradation in avian erythrocyes (44). During erythropoietic differentiation of erythroleukemic mouse spleen cells a chromosomal protein is induced which was extracted in accordance with histones (9). Two possible models could be put forward for how increased poly(ADP-R) polymerase activity could influence the expression of the differentiated phenotype. The (Ca\(^{2+}\), Mg\(^{2+}\))-dependent endonuclease present in chromatin can act as an acceptor protein for ADP ribosylation (45) and its ability to activate chromatin for DNA synthesis is impaired by this modification, resulting in an inhibition of DNA synthesis. Another possibility could be deduced from recent results by Ord and Stocken (46) who showed that ADP-ribosylated histones H1 and H3 were more easily released from nuclei than their unmodified forms. As the concentration of H1 is low in transcriptionally active chromatin, poly(ADP)-ribosylation of this histone and its subsequent release from chromatin could be a tool for activation of certain regions of chromatin during specialized transcription.

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Expression of poly(adenosine diphosphate-ribose) polymerase activity in erythroleukemic mouse cells during cell cycle and erythropoietic differentiation.
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