Cytosolic High K_m 5′-Nucleotidase and 5′(3′)-Deoxyribonucleotidase in Substrate Cycles Involved in Nucleotide Metabolism*

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5′-Nucleotidases are the catabolic members of the substrate cycles postulated to be involved in the regulation of intracellular deoxyribonucleoside triphosphate pools. Here, we attempt to identify the nature of the nucleotidases. Earlier, we constructed various mammalian cell lines that can be induced to overproduce the high K_m 5′-nucleotidase (hkm-NT) or the 5′(3′)-deoxynucleotidase (dNT-1). Now we labeled control and induced human 293 cells and hamster V79 cells with radioactive hypoxanthine or uridine and during a chase measured quantitatively the metabolism of ribo- and deoxyribonucleotides, DNA replication, and excretion of nucleosides into the medium. Overproduction of hkm-NT greatly increased excretion of inosine and guanosine but did not affect adenosine or deoxyribonucleosides. dNT-1 overproduction increased excretion of deoxycytidine, thymidine, and in particular deoxyuridine but also uridine and cytidine. We conclude that the hkm-NT is not involved in the regulation of deoxyribonucleotide pools but affects IMP and GTP pools. dNT-1, instead, appears to be the catabolic arm of substrate cycles regulating pyrimidine nucleotide pools.

DNA replication and repair requires a continuous and balanced supply of the four deoxyribonucleoside triphosphates (dNTPs).[1] Precursor pools are very small, suffice only for a few minutes of DNA replication in mammalian cells, and have to be continuously replenished during S phase (1). Imbalances in pool sizes are genotoxic and may in severe cases lead to cell death (reviewed in Ref. 2). A network of biosynthetic and catabolic enzymes regulates the size of each pool with the enzyme ribonucleotide reductase playing a pivotal role (1). This enzyme not only directs the total flow of metabolites into DNA but also, via an exquisite allosteric mechanism, divides the flow into separate channels to maintain the balance between the four pools.

A second level of control comes from the dynamic interplay between kinases, catalyzing the salvage of deoxyribonucleosides, and nucleotidases, involved in the catabolism of deoxyribonucleotides (1). The two groups of enzymes form substrate (or futile) cycles (3, 4) by catalyzing two opposite irreversible reactions whose net result is the hydrolysis of ATP to ADP and P_i (Fig. 1). This seemingly wasteful exercise provides the cell with a mechanism for the fine tuning of dNTP pools (1) that relies on the difference in cell permeability between nucleosides and nucleotides. Nucleosides move freely between the inside and outside of the cell, whereas nucleotides remain trapped inside (Fig. 1). When catabolism outweighs anabolism, deoxyribonucleosides accumulate and are excreted. In the opposite situation, they are imported and further metabolized to dNTPs. The excretion or the import of a nucleoside over the cell membrane can be used as a measure of the activity of substrate cycles.

"Auxiliary" catabolic enzymes (deaminases, phosphorylases, and hydrolases) remove some of the nucleosides and thereby shift the equilibrium in the direction of catabolism (Fig. 1). The importance of these enzymes for the regulation of dNTP pools is apparent from the finding that their genetic loss leads to severe dysfunctions that depend on the accumulation of specific dNTPs in special cell types (reviewed in Ref. 5). Thus, the loss of either adenosine deaminase or purine nucleoside phosphorylase leads to severe combined immune deficiency caused by the accumulation of dATP and dGTP, respectively, in T and B cells. These cells are low in nucleotidase activity (6), and the balance in their substrate cycles is normally shifted toward anabolism. They are therefore particularly sensitive to the excess of deoxyadenosine or deoxyguanosine in the blood stream caused by the absence of the deaminase or phosphorylase. The biological mechanism underlying the two immunodeficiency diseases provides considerable validation for the importance of substrate cycles. An additional similar example comes from the recent demonstration that the genetic loss of thymidine phosphorylase leads to severe mitochondrial dysfunction (7).

We have for some time used isotope flow experiments with cultured cells to study substrate cycles. Mouse 3T6 cells (8), hamster V79 (9, 10) and Chinese hamster ovary cells (11), and human CEM cells (12) were incubated with trace amounts of labeled precursors for purine or pyrimidine deoxyribonucleotides, and the flux of isotope through rNTP and dNTP pools into DNA and into deoxyribonucleosides released into the medium was determined. For these experiments we used both normal cells and cells carrying mutations in different enzymes involved in dNTP synthesis. The main results were as follows: (i) in all normal cells the bulk of newly synthesized purine and pyrimidine deoxyribonucleotides was incorporated into DNA, but a fraction was excreted into the medium as deoxyribonucleosides; (ii) inhibition of DNA synthesis greatly increased the excretion of deoxyribonucleosides, and inhibition of dNTP synthesis by hydroxyurea favored their import; and (iii) loss of thymidine kinase resulted in increased excretion of thymidine

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[1] The abbreviations used are: dNTP, deoxyribonucleoside triphosphate; hkm-NT, high K_m 5′-nucleotidase; dNT-1, cytosolic 5′(3′)-deoxyribonucleotidase; rNTP, ribonucleoside triphosphate; HPLC, high performance liquid chromatography.
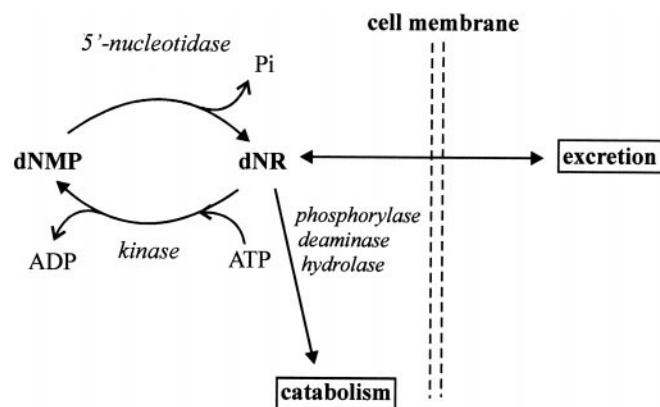
FIG. 1. **Schematic representation of a substrate cycle involving a deoxyribonucleotide (*dNMP*) and a deoxyribonucleoside (*dNR*).**

and deoxyuridine, and loss of deoxycytidine kinase resulted in increased excretion of deoxycytidine and deoxyadenosine.

Important for the concept of substrate cycles is the identification of the catabolic nucleotidase(s). Three ubiquitous classes of 5′-nucleotidases are known in higher organisms, differing in protein structure and substrate specificity: (i) the cytosolic high K_m nucleotidase (hkm-NT) (13); (ii) the cytosolic 5′(3′)-deoxynucleotidase (dNT-1) (14, 15) and a closely related mitochondrial enzyme (dNT-2) (16); and (iii) an ectonucleotidase attached to the cell membrane via a glycosyl-phosphoinositol anchor (17, 18). An additional cytosolic nucleotidase with a specificity for AMP and high activity in muscle and heart, was recently cloned (19).

Which nucleotidase participates in substrate cycles involving deoxyribonucleotides? Earlier isotope flow experiments from our laboratory appear to exclude the ectonucleotidase (20). Here we report experiments with human 293 and hamster V79 cell lines in which the cDNAs for the cytosolic nucleotidases were under the control of an inducible promoter. By isotope flow experiments with labeled hypoxanthine and uridine, we determined in induced and noninduced cells the incorporation of isotope into nucleotide pools and DNA and into the ribo- and deoxyribonucleosides excreted into the medium. Overproduction of the hkm-NT resulted in an increased excretion of inosine and guanosine but not adenosine or deoxyribonucleosides. Overproduction of dNT-1 gave an increased excretion of pyrimidine deoxyribonucleosides as well as uridine and cytidine. The results suggest that the hkm-NT participates in the regulation of the pools of some purine ribonucleotides and that dNT-1 is involved in substrate cycles of pyrimidine ribo- and deoxyribonucleotides.

EXPERIMENTAL PROCEDURES

Materials —[5,6-³H]uridine (40 Ci/mmol), [8-³H]hypoxanthine (30 Ci/mmol) for cell culture experiments, ³H-labeled thymidine, deoxycytidine for kinase assays, and ³²P-labeled dATP and dTTP for pool determinations were from Amersham Pharmacia Biotech. [¹⁴C]IMP and [³H]dUMP were from Moravek. For cell culture experiments, the precursors were used without further dilution; for enzyme assays the radioactivity was diluted to 300–400 cpm·nmol⁻¹. ³²P-Labeled nucleotides were diluted to ~2000 cpm·pmol⁻¹. Immucillin H (21) was a gift from Dr. Vern Schramm.

The human 293 and hamster V79 cell lines used for the isotope flow experiments had been stably transfected with constructs containing either the human hkm-NT (Hkig-2 cells; Ref. 22) or the murine dNT-l (15) under the control of an ecdyson- inducible promoter. They were routinely grown in a humidified incubator at 37 °C in Dulbecco's modified Eagle's medium and supplemented with 7% fetal calf serum, 1% glutamine and antibiotics. Cell cultures were tested periodically for absence of mycoplasma contamination.

Enzyme Assays—Cells were lysed in a solution of 20 mM Tris·HCl, pH

7.8, 15% glycerol, 2 mM EDTA, 1% Triton X, 1 mM dithiothreitol. After centrifugation for 10 min at 14,000 rpm in an Eppendorf centrifuge, dNT-1 and hkm-NT activities were determined in the supernatants with [¹⁴C]IMP (22) and [³H]dUMP (15), respectively, as substrates. Thymidine kinase and deoxycytidine kinase activities were determined from the phosphorylation of the corresponding deoxyribonucleosides (23, 24). All enzyme assays were made with two concentrations of crude protein to ascertain proportionality. Specific activities are defined as nmol of product formed during 1 min/mg of protein. Protein was quantitated as described (25).

Induction of 5′-Nucleotidases —Ponasterone A (4 μM for 293 cells and 8 μM for V79 cells) was added to the cultures during 48 h (induction of hkm-NT in 293 cells and dNT-1 in V79 cells) or 72 h (induction of dNT-1 in 293 cells) before addition of isotope. Extracts of noninduced cells showed a basal nucleotidase specific activity between 3 and 7. After induction, the extracts from 293 cells showed specific activities between 80 and 150, and extracts from V79 cells showed specific activities between 15 and 25.

Isotope Flow Experiments—The experiments were started by seeding 1.5–2 × 10⁵ cells on polylysine-coated dishes (diameter, 3.5 cm). Half of the cultures were induced with ponasterone, and the other half served as controls. Incubation was in 2.5 ml of medium at 37 °C. On the evening before addition of isotope, the medium was replaced with fresh medium containing dialyzed fetal calf serum to minimize the effect of the presence of nucleosides in the serum. After 2 or 3 days from seeding, when the cultures had reached a density of 5–7 × 10⁵ cells/dish, 1.5 ml of medium was withdrawn and retained in the incubator for the chase of radioactivity. The cultures were then incubated with either labeled hypoxanthine (final concentration, 0.3 μM) + 0.5 μM immucillin and 10 μM *erythro*-9-(2-hydroxy-3-nonyl)adenine or with labeled uridine (final concentration, 1.0 μM). After 2 h the radioactive medium was removed and replaced with the earlier withdrawn nonlabeled medium containing immucillin and *erythro*-9-(2-hydroxy-3-nonyl)adenine, where required. Dishes were removed for analyses after 1 and 2 h of further incubation at 37 °C. All handling of the cultures outside the incubator was in a climatized 37 °C room to avoid perturbations of cell metabolism.

After removal from the incubator, the dishes were washed twice with prewarmed saline solution. The dishes were placed on ice, and the cells were extracted with 1 ml of 60% methanol for 30 min (21). The methanolic solution was transferred to a centrifuge tube immersed in a boiling water bath and heated for 3 min. This served to kill all enzymes that may influence pool analyses. Methanol was removed by vacuum evaporation. The dry residue was dissolved in 0.2 ml of water and used for pool analyses.

Great care was taken during methanol extraction not to detach cells from the dish. After removal of methanol, 1 ml of 0.3 M NaOH was added, and the dish was incubated at 37 °C for 24 h to dissolve the cells and hydrolyze RNA. The absorbance at 260 nm of the clear solution provided a correction factor for unequal growth on different dishes. This correction that rarely amounted to more than 10% was made by dividing all the values for total radioactivity (Fig. 2, *shaded boxes*) by the absorbance at 260 nm of the corresponding NaOH solution. By multiplying this value by 2, we could then relate the values to 10⁶ cells because 10⁶ cells dissolved in 1 ml of 0.3 M NaOH showed an absorbance of 2 at 260 nm. A portion of the NaOH solution was precipitated with HClO₄ to measure isotope incorporation into DNA.

Determinations of Specific Activities of Nucleoside Triphosphates— These measurements were made on portions of the material extracted from the cells by 60% methanol. rNTPs were separated by HPLC chromatography on a Partisil SAX column (Whatman) with 0.4 M ammonium phosphate as the mobile phase with a Waters ML3255 machine. The total radioactivity present in each nucleotide peak was determined, and the amount of nucleotide was obtained by automatic integration of the peak area. From the two values we could then calculate the specific radioactivity (cpm/pmol) of each rNTP.

The specific activities of dNTPs were obtained by a modification of the method of Sherman and Fyfe (26). As originally described, this method measures the total amount of a given dNTP in a cell extract with a synthetic oligonucleotide and DNA polymerase. To permit also the determination of specific activity, we first constructed a standard curve for increasing amounts of the relevant dNTP, labeled with tritium, and an excess of ³²P-labeled dATP (or [³²P]dTTP for determination of [³H]dATP) under the conditions given by Sherman and Fyfe (26). With a determined large excess of polymerase the incorporation of ³²P into the oligonucleotide corresponded quantitatively to the increasing amounts of the relevant dNTP. Because of quenching on the DEAE paper, the ³H values only amounted to 50–70% of the ³²P values. In the
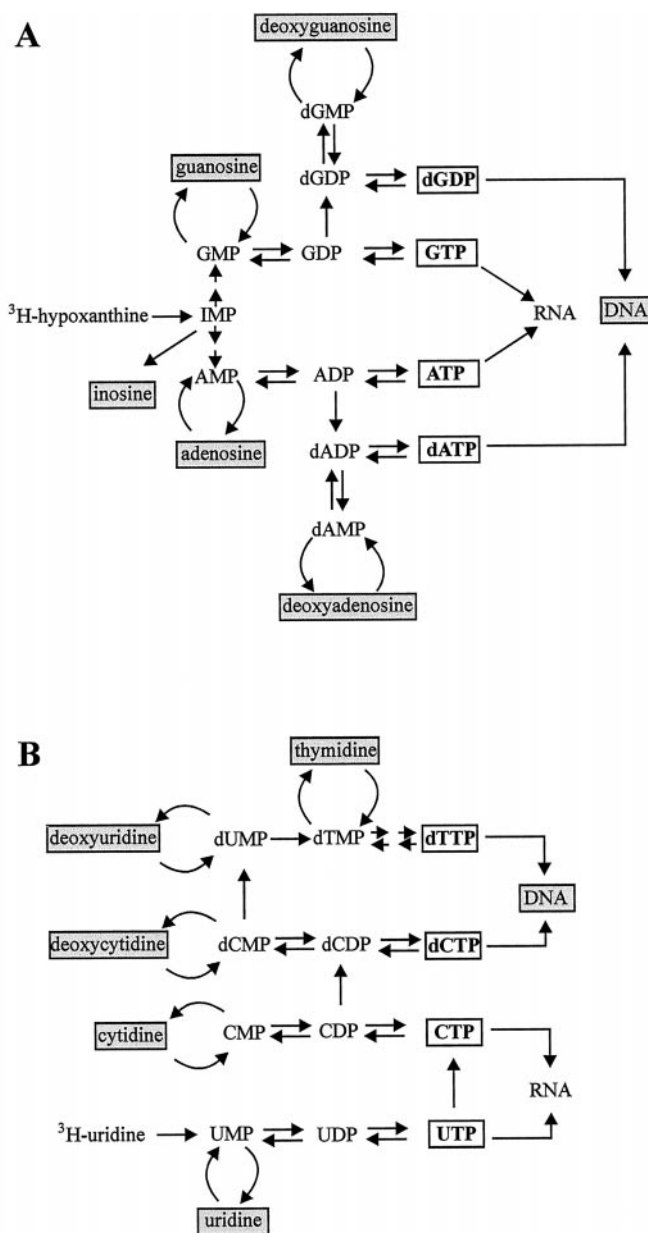
FIG. 2. **Sequential incorporation of radioactivity into cellular components from labeled hypoxanthine (*A*) or uridine (*B*).** The *shaded boxes* represent end products that accumulate isotope with time. Their amount can be calculated from the accumulation of isotope divided by the determined specific radioactivity of the nucleoside triphosphates (in *boxes*).

actual experiment, two different portions of cell extract were incubated with the ^{32}P-labeled dNTP under the conditions established for the standard curve. The amount of dNTP present in the extract was obtained directly from the incorporation of ^{32}P. From the quench-corrected incorporation of tritium we could then calculate the specific activity of the dNTP by dividing the ^{3}H value with the amount of the nucleotide. Once the required amount of DNA polymerase had been established, this method gave reproducible results for the specific activity of each of the four dNTPs in extracts from as little as 10^{4} cells.

Excretion of Nucleosides into the Medium—To remove all proteins 0.2 ml of ice-cold medium was precipitated with 0.005 ml of cold concentrated HClO$_4$ and centrifuged. The supernatant solution was neutralized immediately with 4 M KOH, and the precipitated KClO$_4$ was removed by centrifugation. It was important to work rapidly at low temperature to avoid hydrolysis of the purine *N*-glycosidic bonds of deoxyribonucleosides.

Nucleosides in 0.05 ml of the supernatant were separated by HPLC on a Nucleosil 5–100 C18 column (250 × 4.6 mm) at a flow rate of 1 ml/min. Purine nucleosides were separated by elution with 20 mM

ammonium acetate, pH 5.0, containing increasing amounts of methanol (5% during the first 20 min, followed by 10% during 25 min and finally 50% during 15 min). The following retention times were found: hypoxanthine, 8.5 min; guanine, 9.0 min; inosine, 14.5 min; guanosine, 17 min; deoxyinosine, 19 min; deoxyguanosine, 23 min; adenosine, 35.5 min; and deoxyadenosine, 43 min. Pyrimidine nucleosides were separated similarly with 40 mM ammonium acetate, pH 5.0, and increasing amounts of methanol (0% methanol during 20 min, followed by 50% methanol during 30 min). Following retention times were found: cytidine, 10 min; deoxycytidine, 13 min; uridine, 16 min; deoxyuridine, 21 min; and thymidine, 35 min.

The appropriate chromatographic fractions were used to determine the total radioactivity of each nucleoside. A correction was applied to this value as volume changes occurring during protein removal from the medium were difficult to control. An appropriate factor was obtained for each chromatogram from the absorption peak of an unknown component of the medium that had a retention time of 24 and 9 min, respectively, in the purine and pyrimidine chromatograms. The size of this peak was exclusively dependent on the amount of medium and was not affected by the amount of cells used in the experiment. Thus, it could be used safely for volume corrections. The total radioactivity of each nucleoside peak was normalized from the integrated absorption value of this peak.

RESULTS

Rationale of Experiments—The participation of a particular 5′-nucleotidase in substrate cycles was investigated by determining whether overproduction of the enzyme increased the excretion of nucleosides. The cDNAs for either hkm-NT or dNT-1 on plasmids under the control of an inducible promoter were transfected into human 293 or hamster V79 cells. Induction with ponasterone A increased the activity of the enzymes in cellular extracts 5–30-fold. Induced and control noninduced cells were first labeled from radioactive hypoxanthine as precursor of purine nucleotides (Fig. 2*A*) or uridine as precursor of pyrimidine nucleotides (Fig. 2*B*). During a chase we then measured the accumulation of radioactivity (as cpm) in various end products (nucleosides excreted into the medium and DNA, indicated in *shaded boxes* in Fig. 2) as well as the specific radioactivities (cpm/pmol) of NTPs (indicated in *boxes* in Fig. 2). From these values we transformed into pmol/min the cpm accumulated in each excreted nucleoside or incorporated into DNA.

Specific Activities of Precursor Pools and Incorporation into DNA—During the first 2 h of the experiments, increasing amounts of radioactivity were incorporated via "salvage" enzymes (hypoxanthine phosphoribosyl transferase for hypoxanthine, uridine kinase for uridine) into all purine or pyrimidine ribo- and deoxyribonucleotides, RNA, and DNA. After 2 h the labeled precursor was removed from the cultures by change of medium, and the specific radioactivities of the relevant rNTPs and dNTPs were determined immediately and after 1 and 2 h. As an example, Fig. 3 gives the results from two typical experiments with dNT-1 overproducing 293 cells: one labeled from radioactive hypoxanthine (Fig. 3*A*) and the other labeled from uridine (Fig. 3*B*). In these and all other experiments only small differences were observed between overproducing and control cells. The specific activities of the nucleoside triphosphates had not yet reached isotope equilibrium, but this does not affect our calculations that concern the chase period. After change of the medium, the values decreased with time by dilution from *de novo* synthesis. dNTPs were always more heavily labeled than rNTPs, suggesting that their synthesis in S phase cells occurred from a ribonucleotide pool with higher than average specific activity. The lower specific activity of dTTP, relative to dCTP, is due to loss of tritium from the 5 position of uridine during transformation of dUMP to dTMP. The specific activity of DNA increased continuously by incorporation of radioactivity from dNTPs.

We used such data to calculate rates of DNA synthesis in the different experiments. From the hypoxanthine experiment in Fig. 3*A* we calculate that the dATP and dGTP pools during the 2-h period had average specific activities of 340 and 307 cpm/pmol, respectively. During that period DNA accumulated radioactivity from the two dNTPs at a rate of 1075 cpm/min/10^{6}

FIG. 3. **Specific activities of deoxyribonucleoside- and ribonucleoside triphosphates and incorporation of radioactivity into DNA in control (■) and induced (□) 293 cells overproducing dNT-1, during the chase after labeling from [³H]hypoxanthine (*A*) or [³H]uridine (*B*).** *A*, the *continuous lines* represent dGTP or GTP, and the *dotted lines* represent dATP or ATP. *B*, the continuous lines are dCTP or CTP, and the *dotted lines* are dTTP or UTP.
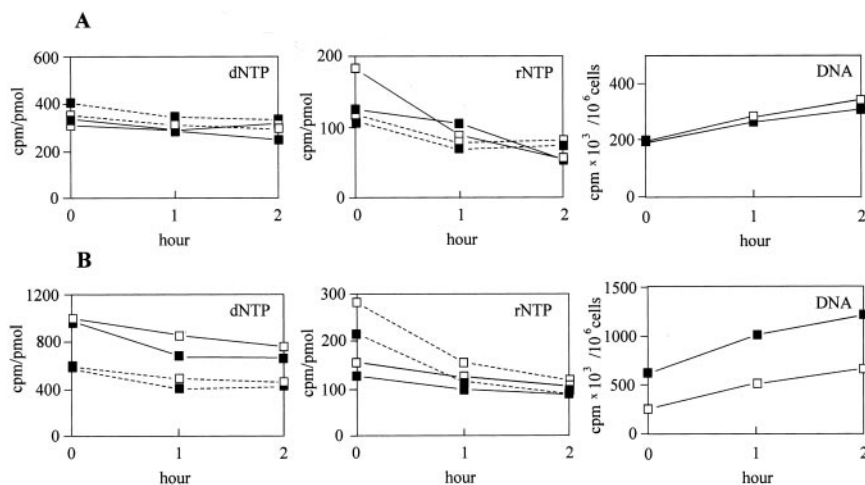
cells. Using an A/G ratio of 1.32 for mammalian DNA, we can transform this accumulation into the incorporation of 1.88 pmol/min of adenine and 1.42 pmol/min of guanine.

Excretion of Purine Nucleosides—A possible complication in experiments with labeled hypoxanthine was the degradation of purine nucleosides into free purine bases by adenosine deaminase and purine nucleoside phosphorylase. In a preliminary experiment we found only labeled inosine and traces of other labeled purine nucleosides in the medium. All amounts increased dramatically when we added *erythro*-9-(2-hydroxy-3-nonyl)adenine to inhibit adenosine deaminase and immucillin H (21) to inhibit purine nucleoside phosphorylase. Immucillin had to be used at 0.5 μM concentration to give full effect. Neither of the two compounds inhibited the incorporation of labeled hypoxanthine into RNA or DNA (data not shown), suggesting that cellular metabolism was not affected.

[³H]Hypoxanthine was used in three experiments to label purine nucleotides and DNA in 293 cells overproducing hkm-NT and in two experiments with 293 or V79 cells overproducing dNT-1. Figs. 4 and 5 illustrate the effects of enzyme overproduction on the excretion of ribo- and deoxyribonucleosides into the medium. Fig. 4 compares time curves for the excretion of purine ribonucleosides from control and induced 293 cells, with one line overproducing hkm-NT (Fig. 4*A*) and the other overproducing dNT-1 (Fig. 4*B*). For adenosine and guanosine the results are expressed as pmol/10⁶ cells, calculated from the amount of isotope that accumulated in the nucleosides and the specific activities of the relevant triphosphates. In the case of inosine, we present the data as cpm/10⁶ cells because the specific activity of IMP could not be determined. Induction of hkm-NT (Fig. 4*A*) lead to an ~10-fold increase in excretion of inosine and guanosine but had no effect on adenosine. In this experiment hkm-NT activity was 30-fold increased. In other experiments in which overproduction of hkm-NT was only 5-fold, similar results were obtained but with smaller increases in excretion of inosine and guanosine (not shown). In contrast, induction of dNT-1 (Fig. 4*B*) did not increase excretion of any ribonucleosides.

The excretion of deoxyguanosine and deoxyadenosine in the same experiments and in an additional experiment with V79 cells overproducing dNT-1 is illustrated in Fig. 5. Excretion of the deoxyribonucleosides is given as percentage of the total reduction of the corresponding ribonucleotide, taken as the sum of the incorporation of the relevant purine into DNA and the excretion of the deoxyribonucleoside.

Induction of either hkm-NT or dNT-1 decreased the excre-

tion of both deoxyribonucleosides. We found earlier (22)[2] that cells overproducing either nucleotidase contain smaller dNTP pools and therefore have lower substrate concentrations for a putative nucleotidase. This may explain the decreased excretion of deoxyribonucleosides after induction.

Excretion of Pyrimidine Nucleosides—[[5,6-³H]uridine was used to label pyrimidine nucleotides and DNA in induced and control 293 cells overproducing hkm-NT or dNT-1, and in V79 cells overproducing dNT-1. Time curves for the excretion of pyrimidine ribonucleosides are shown in Fig. 6*A* (induction of hkm-NT in 293 cells) and 6B (induction of dNT-1 in 293 cells). Enzyme induction increased the excretion of both uridine and cytidine.

Fig. 7 illustrates the excretion of pyrimidine deoxyribonucleosides in all three experiments. The values are again given as percentages of total ribonucleotide reduction. In this case, CDP reduction was the source of both dCTP and dTTP (Fig. 2*B*). Total reduction of CDP was taken as the sum of cytosine and thymine incorporation into DNA plus excretion of deoxcytidine, deoxyuridine, and thymidine. We neglect the small part of dTTP formed by reduction of UDP (9).

Overproduction of dNT-1 lead to an increased excretion of deoxycytidine (Fig. 7*A*) and deoxyuridine (Fig. 7*B*) in both cell lines. In 293 cells the effect on deoxyuridine is particulary striking. The data in Fig. 7 are the means from four parallel experiments with the cell lines overproducing dNT-1. The differences between induced and control cells were statistically significant with *p* values (Student's *t* test) of 0.05 or less. In 293 cells, the increase in thymidine excretion (Fig. 7*C*) was also statistically significant but not in V79 cells. This may be due to the smaller increase in nucleotidase activity which in these experiments was 6-fold in V79 cells and 20-fold in 293 cells. In a single experiment, induction of hkm-NT in 293 cells did not significantly affect the excretion of pyrimidine deoxyribonucleosides (Fig. 7).

Activity of Deoxyribonucleoside Kinases in 293 and V79 Cells—The anabolic components of the investigated substrate cycles are deoxycytidine kinase for the phosphorylation of deoxycytidine, deoxyadenosine and deoxyguanosine, and thymidine kinase for the phosphorylation of thymidine. It is evident from Figs. 5 and 7 that the inherent excretion of deoxyadenosine, deoxyguanosine, and deoxycytidine was much larger in 293 cells than in V79 cells. Assays in extracts from the two noninduced lines gave closely similar results for deoxyribo-

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[2] C. Gazziola, P. Ferraro, M. Moras, P. Reichard, and V. Bianchi, unpublished data.

FIG. 4. **Effect of the overproduction of hkm-NT (*A*) or dNT-1 (*B*) on the time course of the excretion of purine ribonucleosides by 293 cells labeled from [³H]hypoxanthine.** □, induced cells; ■, control cells.
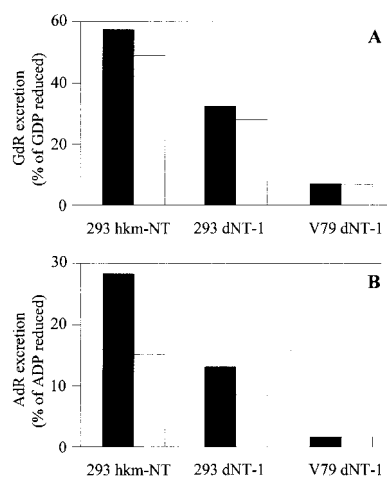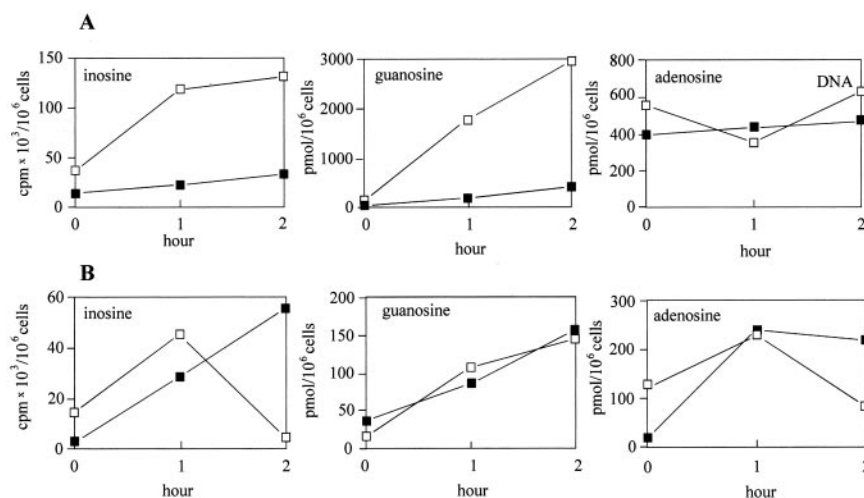

FIG. 5. **Effects of overproduction of hkm-NT or dNT-1 on the excretion of deoxyguanosine (*A*) and deoxyadenosine (*B*) during a 2-h period by different cell lines labeled from [³H]hypoxanthine.** The *black columns* represent the controls, and the *open columns* represent the induced cells.
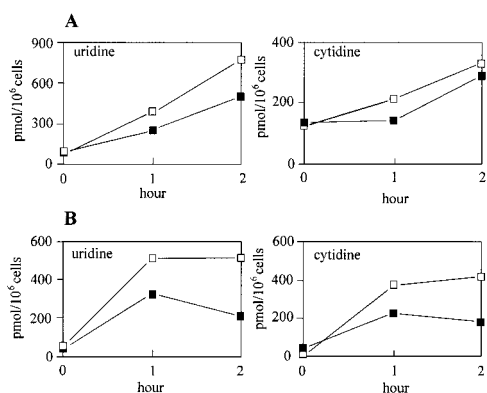

FIG. 6. **Time course of uridine and cytidine excretion by induced (□) and control (■) 293 cells labeled from [³H]uridine.** *A*, overproduction of hkm-NT. *B*, overproduction of dNT-1.

FIG. 7. **Effects of overproduction of hkm-NT or dNT-1 on the excretion of deoxycytidine (*A*), deoxyuridine (*B*), and thymidine (*C*) during a 2-h period by 293 and V79 cells labeled from [³H]uridine.** *Black columns*, controls; *open columns*, induced cells.

DISCUSSION

Genetic methodology provides a powerful tool to study substrate cycles. Thus, thymidine kinase was identified as the anabolic enzyme of the dTMP/thymidine substrate cycle from isotope flow experiments where the excretion of thymidine and deoxyuridine was increased stepwise from tk⁺/tk⁺ *via* tk⁺/tk⁻ to tk⁻/tk⁻ cell mutants (11). Deoxycytidine kinase was similarly identified for the dCMP/deoxycytidine and dAMP/deoxyadenosine cycles (11, 12). Cells devoid of 5′-nucleotidase activity are not known, and we could therefore not use the same technique to identify nucleotidases participating in substrate cycles. Instead, we investigated to what extent an increase in the activity of a particular nucleotidase affects nucleoside excretion. Increased excretion would suggest that this enzyme represents the catabolic arm of the substrate cycle.

We now show that overproduction of each of two cytosolic nucleotidases increases the excretion of specific groups of nucleosides. hkm-NT increases inosine and guanosine excretion (Fig. 4*A*), *i.e.* two ribonucleosides, but not that of deoxyribonucleosides or adenosine. In as single experiment (Fig. 6*A*)

nucleotidase activity, excluding differences in the breakdown of monophosphates. However, they differed widely with respect to the specific activity of deoxycytidine kinase which was 1.2 in 293 cells and 13 in V79 cells. The corresponding values for thymidine kinase were 34 and 42. The low deoxycytidine kinase activity of 293 cells may explain why these cells showed such a huge excretion of the deoxyribonucleosides whose phosphorylation is catalyzed by this enzyme.
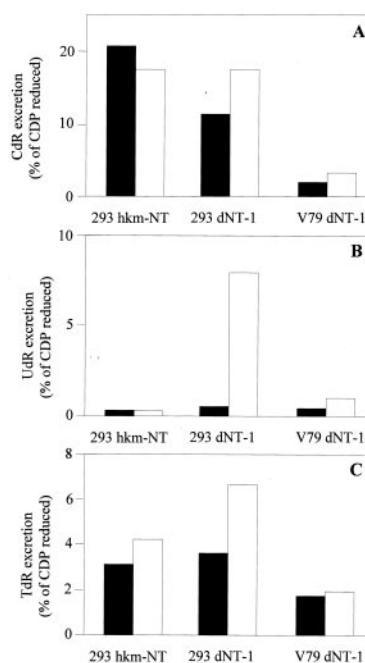
uridine and cytidine were also affected slightly, but this result requires further confirmation. The specificity for inosine and guanosine and lack of activity for adenosine agree well with the known substrate specificity of hkm-NT (13). Our results agree with recent conclusions by Sala-Newby *et al.* (27), who used a completely different technique to investigate the *in situ* specificity of the hkm-NT. They also demonstrated that the *in situ* dephosphorylation of AMP, but not that of IMP or GMP, was catalyzed by an AMP-specific 5′-nucleotidase (28).

The main objective of our work was to identify the 5′-nucleotidases that act on deoxyribonucleotides. That the hkm-NT did not increase excretion of deoxyadenosine was expected from the *in vitro* specificity of the enzyme (13) and from the finding that the resistance toward the toxicity of deoxyadenosine and 2-chloroadenosine of cells overproducing the enzyme was not increased (22). The lack of activity of the hkm-NT for dGMP was more surprising because the isolated enzyme dephosphorylates dGMP quite actively. A possible explanation is that the K_m for dGMP is high (0.1–0.3 mM) and that the dGTP pool of cells is the smallest of the four dNTP pools (5–10 pmol/10^6 cells). Assuming that the dGMP pool is less than 10% of the dGTP pool and that the volume of 10^6 cells is 0.2 μl, the intracellular dGMP concentration can be estimated to be ~1–4 μM.

The results with dNT-1 were more gratifying. Overproduction of the enzyme gave a clear increase in the excretion of deoxycytidine and in particular deoxyuridine in two separate cell lines (Fig. 7). The large effect on deoxyuridine agrees with the preference of the enzyme for dUMP as substrate (14, 15). In 293 cells induction of dNT-1 also increased thymidine excretion. The smaller increase in V79 cells was not statistically significant, which may be explained by the lower overproduction of the enzyme. These results are all the more significant because overproduction of the nucleotidases in several other cases resulted in a *decreased* excretion, probably caused by lowering dNTP pool sizes. Also the excretion of pyrimidine ribonucleosides was clearly increased in 293 cells (Fig. 6*B*). Our results thus indicate that dNT-1 overproduction increases the excretion of pyrimidine nucleosides and that the enzyme is involved in substrate cycles for pyrimidine ribo- and deoxyribonucleotides.

Our experiments were carried out with cells in different phases of the cell cycle, and the isotope data reflect the metabolism of cells both in and out of S phase. Ribonucleotides were labeled from hypoxanthine and uridine in all cells, but deoxyribonucleotides were labeled only in S phase cells, because ribonucleotide reductase is a cell cycle-regulated enzyme. As a consequence our data on ribonucleoside excretion come from a different cell population than the data concerning deoxyribo-

nucleoside excretion. The general conclusions given above should, however, not be affected by this consideration.

The large differences between 293 and V79 cells in the excretion of several deoxyribonucleosides deserve a final comment. We believe that they are explained by the large differences in deoxycytidine kinase activity between the two cell lines. This then is a further example of the function of substrate cycles in pool regulation.

The identification of dNT-1 as one of the nucleotidases involved in the turnover of deoxyribonucleotide pools has consequences for antiblastic and antiviral therapy. The sensitivity of the monophosphates of analogs to dephosphorylation by dNT-1 may become one important parameter in the evaluation of new drugs.

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