Uridine 5′-polyphosphates (p_4U and p_5U) and uridine(5′)polyphospho(5′)nucleosides (Up_nNs) can be synthesized by UTP:glucose-1-phosphate uridylyltransferase from *Saccharomyces cerevisiae*

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Abstract UTP:glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) from *Saccharomyces cerevisiae* can transfer the uridylyl moiety from UDP-glucose onto tripolyphosphate (P_3), tetrapolyphosphate (P_4), nucleoside triphosphates (p_3Ns) and nucleoside 5′-polyphosphates (p_4Ns) forming uridine 5′-tetraphosphate (p_4U), uridine 5′-pentaphosphate (p_5U) and dinucleotides, such as Ap_4U, Cp_4U, Gp_4U, Up_4U, Ap_5U and Gp_5U. Unlike UDP-glucose, UDP-galactose was not a UMP donor and ADP was not a UMP acceptor. This is the first example of an enzyme that may be responsible for accumulation of dinucleoside tetraphosphates containing two pyrimidine nucleosides in vivo. Occurrence of such dinucleotides in *S. cerevisiae* and *Escherichia coli* has been previously reported (Coste et al., J. Biol. Chem. 262 (1987) 12096–12103).

Key words: UTP:glucose-1-phosphate uridylyltransferase; UDP-glucose; Uridine polyphosphate; Uridine-oligophospho-nucleoside; Dinucleoside polyphosphate; *Saccharomyces cerevisiae*

1. Introduction

Dinucleoside polyphosphates (Np_nN's, where N and N′ are 5′-*O*-nucleosides and *n* represents the number of phosphate residues in the polyphosphate chain that links N with N′ esterifying the nucleosides in their 5′ positions) are naturally occurring compounds. Their biological roles are only partially understood. Particularly, diadenosine polyphosphates have been implicated in various intracellular processes [1,2] and extracellular purinergic signalling [3]. The level of Np_nN's in the cells can be regulated by enzymes involved in their synthesis [4] and by specific and non-specific enzymes that degrade these dinucleotides to mononucleotides [5,6]. Gp_4G [7] and Gp_3G [8] were the first Np_nN's described in biological material. These and other Gp_3Ns and Gp_4Ns can be produced by the GTP:GTP guanylyltransferase (EC 2.7.7.45) that is capable of transferring the GMP moiety to various NTPs and NDPs [9] via an enzyme:GMP complex [10]. However, the most investigated dinucleotides have been the adenosine-containing Np_nN's, Ap_nNs; mainly Ap_4A and Ap_3A [11]. The enzymes that have been demonstrated to synthesize Ap_nNs include various aminoacyl-tRNA synthetases [12–16], ADP:ATP adenylyltransferase [17,18], firefly luciferase [19,20], acyl:CoA ligase [21], non-ribosomal peptide synthetase [22], DNA [23,24] and RNA [25] ligases and the recently described plant enzyme, 4-coumarate:CoA ligase [26]. In reactions catalyzed by those enzymes the AMP moiety is transferred to an acceptor either from a mixed anhydride (acyl ~ pA) or from an enzyme ~ pA intermediate [4]. There exists, however, one report that the cells of the yeast *Saccharomyces cerevisiae* and bacterium *Escherichia coli* can accumulate, particularly during stress, all kinds of Np_4N's, including those which have exclusively pyrimidine nucleosides, such as Cp_4C, Cp_4U and Up_4U [27]. The authors suggested that these compounds may originate from the action of Ap_4A phosphorylase (EC 2.7.7.53). However, that enzyme is unable to use pyrimidine NTPs as potential acceptors of the NMP moiety from either UDP or CDP [17,18]. Some time ago we predicted that uridylyl- or cytidylyltransferases can catalyze the synthesis of the pyrimidine-containing Np_nN's [4,19]. Here, we present experimental evidence that one of those nucleotidyltransferases, the commercially available UTP:glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) from brewer's yeast (*S. cerevisiae*), has the catalytic capacity to produce uridylylated tri- and tetrapolyphosphates, p_4U and p_5U, and uridylylated nucleoside 5′-tri- and tetraphosphates, Ap_4U, Cp_4U, Gp_4U, Up_4U, Ap_5U and Gp_5U.

2. Materials and methods

2.1. Materials

All nucleotides including adenosine 5′-tetraphosphate (p_4A), guanosine 5′-tetraphosphate (p_4G), and UDP-sugars as well as tripolyphosphate (P_3), tetrapolyphosphate (P_4), glucose-1-P, firefly luciferase (used for the synthesis of standard Ap_4U [20]) and the yeast UTP:

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Abbreviations: Np_nN', nucleoside(5′)polyphospho(5′)nucleoside; P_2, pyrophosphate; P_3, tripolyphosphate; P_4, tetrapolyphosphate; p_4U, uridine 5′-tetraphosphate; Up_4N, uridine(5′)tetraphospho(5′)nucleoside

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glucose-1-phosphate uridylyltransferase were from Sigma, St. Louis, MO, USA. Stock solution of the transferase contained 250 U/ml (149 U/mg). One unit of the enzyme forms 1 μmol of glucose-1-P from UDP-glucose and PP_i per min at 30°C. Shrimp alkaline phosphatase (EC 3.1.3.1) was from Roche Molecular Biochemicals, phosphodiesterase I (EC 3.6.1.1) from snake venom and yeast inorganic pyrophosphatase (EC 3.6.1.1) were from Boehringer, Mannheim, Germany (now Roche Molecular Biochemicals). Recombinant exopolyphosphatase (EC 3.6.1.11) from *S. cerevisiae* [28] was supplied by Dr. S. Liu, Department of Biochemistry, Beckman Center, Stanford, CA, USA, recombinant (*asymmetrical*) Ap_4A hydrolase (EC 3.6.1.17) from humans [29] was obtained from Dr. A.G. McLennan, School of Biosciences, University of Liverpool, UK, and *symmetrical* Ap_4A hydrolase (EC 3.6.1.41) was prepared from *E. coli* as described [30]. [α-^{32}P]UTP (3000 Ci/mmol) was from Amersham. Thin layer chromatography (TLC) aluminum plates precoated with silica gel containing fluorescent indicator were from Merck and X-ray films from Konica. Radioactively labeled nucleotides were quantified by the use of an InstantImager (Packard Instruments). High performance liquid chromatography (HPLC) was carried out on a Hypersil ODS column (4.6×100 mm) from Hewlett Packard in a Hewlett Packard chromatograph (model 1090), with diode array detector commanded by an HPLC Chemstation as described [31].

2.2. Synthesis of [α-^{32}P]UDP-glucose

The reaction mixture (0.02 ml) contained 50 mM HEPES/KOH (pH 8.0), 1 mM α-glucose-1-P, 0.36 mM [α-^{32}P]UTP (2 μCi), 2 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 U/ml pyrophosphatase and 0.25 U of the uridylyltransferase. The mixture was incubated until consumption of UTP (usually for 10 min at 30°C) and then used directly as a source of [α-^{32}P]UDP-glucose. This preparation is normally contaminated with [^{32}P]UMP.

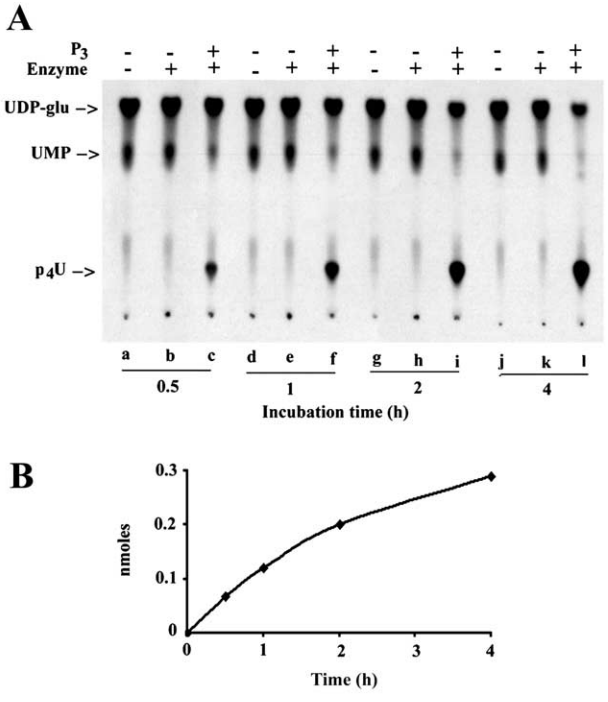

Fig. 1. Synthesis of [α-^{32}P]p_4U from [α-^{32}P]UDP-glucose and P_3 catalyzed by UTP:glucose-1-P uridylyltransferase from yeast *S. cerevisiae*. The reaction mixtures (0.02 ml) contained 0.03 mM [α-^{32}P]UDP-glucose (0.4 μCi), 10 mM $MgCl_2$, 4 mM P_3 and 0.25 U enzyme, where indicated. Other assay conditions were as described in Section 2. At the indicated times, aliquots were spotted on TLC plates, developed in dioxane:ammonium hydroxide:water (6:1:6) and subjected to autoradiography (A). Amount of [α-^{32}P]p_4U formed in the complete reaction mixture during incubation is shown in B.

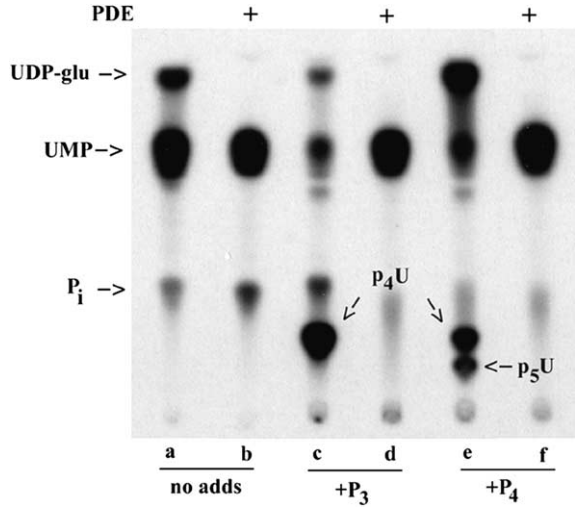

Fig. 2. Synthesis of [α-^{32}P]p_4U and [α-^{32}P]p_5U. The reaction mixtures (0.02 ml) contained 0.05 mM [α-^{32}P]UDP-glucose (0.2 μCi), 10 mM $MgCl_2$, 4 mM P_3 or P_4 where indicated, and 0.25 U uridylyltransferase. Other assay conditions were as described in Section 2. After incubation for 4 h at 30°C, the reaction mixtures were heated for 5 min at 90°C to inactivate transferase and treated further with phosphodiesterase (lanes b, d and f). Aliquots were spotted on TLC and analyzed as in Fig. 1.

2.3. Synthesis of p_nU and Np_nU

Standard reaction mixtures (0.02 ml) contained 50 mM HEPES/KOH (pH 8.0), 0.05 mM [α-^{32}P]UDP-glucose, 4 or 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 4 mM polyphosphate or nucleotide and 0.25 U of uridylyltransferase. After incubation at 30°C, aliquots (1.5 μl) were spotted onto silica gel plates and the chromatograms developed for 90 min in dioxane:ammonium hydroxide:water, 6:1:6 by volume (Figs. 1 and 2) or 6:1:5 (Fig. 3). The radioactivity was visualized by autoradiography and quantified by the use of an InstantImager. For characterization of the reaction products the mixtures were inactivated by heating for 5 min at 90°C and treated either with shrimp alkaline phosphatase (20 U/ml) or with phosphodiesterase (20 μg/ml) for 1 h at 37°C. In our TLC working conditions, the R_f value corresponding to P_i is concentration-dependent, i.e. at low concentration (50 μM) P_i migrates with $R_f \approx 0.17$, while at 4 mM it practically does not move from the origin. Therefore, in those samples that contained high concentrations of polyphosphates or NTPs, the P_i, formed during treatment with alkaline phosphatase, remained at the origin (see Fig. 3).

For the analysis by HPLC, the reaction mixtures (0.05 ml) contained 50 mM HEPES/KOH (pH 8.0), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 4 mM UDP-glucose, the indicated concentration of polyphosphate (P_3 or P_4) or one of the nucleotides (ATP, CTP, GTP, UTP or p_4A), 2 U of inorganic pyrophosphatase, and the yeast uridylyltransferase. After incubation, a 0.015 ml aliquot was transferred into 0.135 ml H_2O and heated for 1.5 min at 95°C. Precipitated protein was removed by centrifugation and 0.05 ml aliquots injected into a Hypersil ODS column and eluted as described [31].

3. Results

Our earlier prediction that transferases that catalyze the synthesis of NDP-sugars with concomitant release of PP_i should be able to produce Np_nN's [2,19] was confirmed using commercially available UTP:glucose-1-P uridylyltransferase from *S. cerevisiae*. When the enzyme was incubated in the presence of [α-^{32}P]UDP-glucose as an uridylyl donor and various potential uridylyl acceptors, the formation of new compounds could be observed. As shown in Fig. 1A, [α-^{32}P]p_4U was formed in the reaction mixture that contained P_3 (lanes c, f, i and l). The appearance of p_4U was concomitant with the

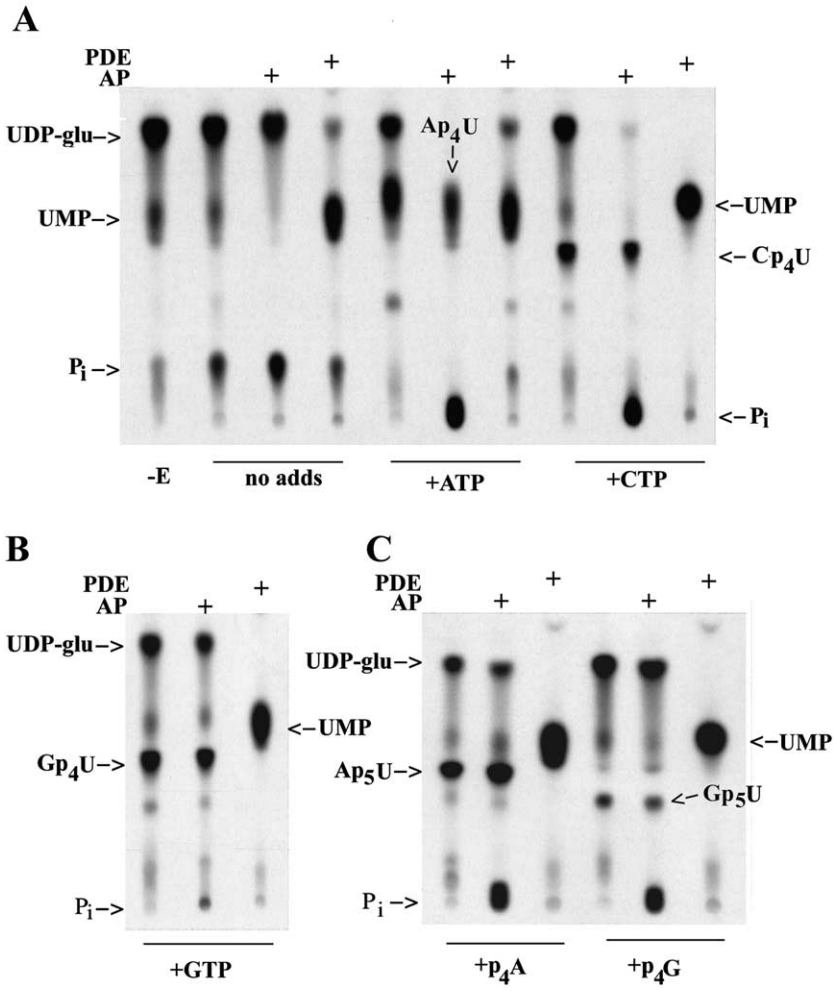

Fig. 3. Synthesis of uridine-[α-^{32}P]tetraphospho-nucleosides from [α-^{32}P]UDP-glucose and NTP. The reaction mixtures (0.02 ml) contained 0.05 mM [α-^{32}P]UDP-glucose (0.2 μCi), 4 mM $MgCl_2$, 4 mM of the indicated nucleotides and 0.25 U of enzyme. Other assay conditions were as described in Section 2. After 20 h incubation, aliquots (1.5 μl) of each mixture were spotted onto a TLC plate and the rest of the mixture was divided in two parts. One part was treated with alkaline phosphatase (AP) and the other with phosphodiesterase (PDE), as indicated in Section 2. After enzymatic treatment aliquots were spotted on TLC plates and developed in dioxane:ammonium hydroxide:water (6:1:5).

disappearance of UDP-glucose. (The radioactive spot that migrates below UDP-glucose corresponds to [^{32}P]UMP originating from the labeled UDP-glucose during heat inactivation of the transferase.) The time course of p_4U accumulation is shown in Fig. 1B. The result of a similar experiment where P_3 and P_4 were compared as acceptors of the UMP moiety is shown in Fig. 2. After 4 h incubation a new spot, probably p_5U, was observed in the reaction mixture containing P_4 (lane e). This spot was accompanied by a faster migrating one that corresponded to p_4U. Both p_4U and p_5U were susceptible to phosphodiesterase treatment since in both cases (lanes d and f) a main spot corresponding to UMP was observed. The presence of p_4U in the reaction mixture that contained P_4 was probably the result of spontaneous decomposition of p_5U in solution and/or synthesis of p_4U that could occur due to P_3 present in the P_4 sample as a contaminant.

Fig. 3 summarizes the result of incubating the transferase with [α-^{32}P]UDP-glucose in the presence of one of the nucleotides indicated: ATP or CTP (Fig. 3A), GTP (Fig. 3B), and p_4A or p_4G (Fig. 3C). After prolonged (20 h) incubation, there appeared new radioactive spots that could correspond to the uridylylated nucleotides: Ap_4U, Cp_4U (Fig. 3A), Gp_4U (Fig. 3B) and Ap_5U and Gp_5U (Fig. 3C). These compounds proved to be resistant to alkaline phosphatase, but susceptible to the phosphodiesterase treatment yielding, in all cases, [^{32}P]UMP as one of the reaction products. Notice that Ap_4U almost co-migrated with UMP (Fig. 3A).

The synthesis and characterization of uridylylated polyphosphates and uridylylated nucleotides were also analyzed by HPLC. Fig. 4 shows the result of incubating transferase with UDP-glucose and in the absence (a) or in the presence of a UMP acceptor: PP_i (b) or P_3 (c). In the mixture containing PP_i, UTP was formed as a result of the reverse reaction (reaction 2, see below) (Fig. 4b). In the mixture containing P_3 (Fig. 4c,d), a new product, presumably p_4U, was produced (see reaction 3). When the reaction mixture (shown in Fig. 4d) was supplemented either with exopolyphosphatase (a highly specific enzyme which hydrolyzes p_4Ns to appropriate NTPs and P_i [28]) (Fig. 4e) or with alkaline phosphatase (Fig. 4f), the presumed p_4U was degraded to UTP and uridine, respectively.

The HPLC profiles of the reaction mixtures that had been incubated for 7 h in the presence of UDP-glucose, one of the canonical nucleoside triphosphates or p_4A and yeast uridylyl-

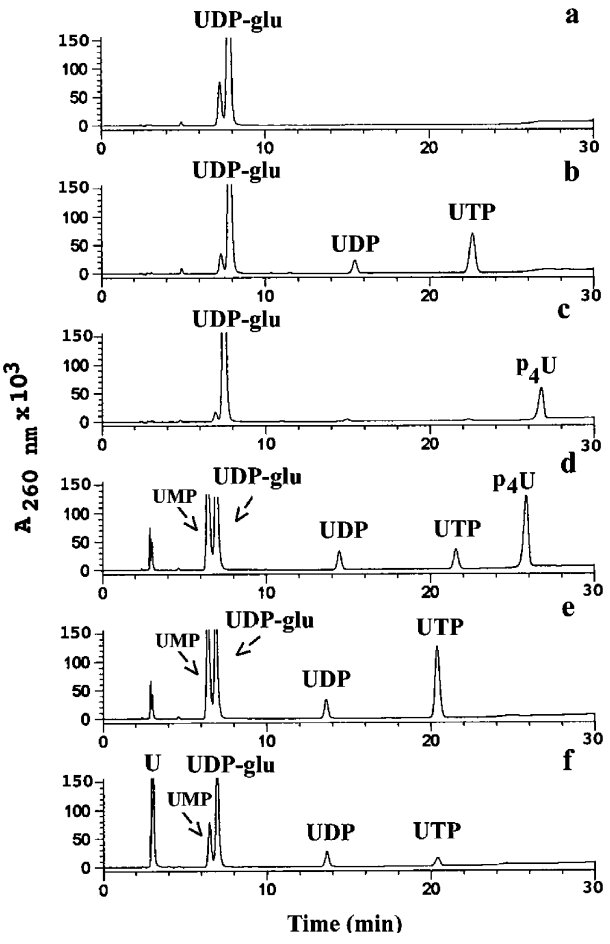

Fig. 4. Synthesis of UTP and p_4U. The reaction mixtures (0.05 ml) contained 4 mM UDP-glucose, 10 mM $MgCl_2$ (a), and either 4 mM P_2 (b) or 4 mM P_3 (c and d), and 1.5 U enzyme. Other conditions were as described in Section 2. After 7 h incubation aliquots were withdrawn, appropriately diluted, heat-inactivated and analyzed by HPLC. To characterize the reaction products, another sample that also contained P_3 was incubated for 20 h and an aliquot analyzed as above (d). The rest of the reaction mixture was divided into two parts, one part was treated with exopolyphosphatase (0.5 U, 1 h at 37°C) (e) and the other with alkaline phosphatase (2 U, 2 h at 37°C) (f).

transferase, showed (after alkaline phosphatase treatment) the presence of new compounds that could correspond to Ap_4U, Cp_4U, Gp_4U, Up_4U (Fig. 5) and Ap_5U (Fig. 6). Since commercially available NTPs always contain small contamination of the appropriate p_4Ns [32–35], the more slowly migrating peak that accompanied each Np_4U may correspond to Np_5U (Fig. 5). Formation of these new compounds was uridylyltransferase-dependent. In the parallel mixtures that were incubated for each NTP and for p_4A and in which the transferase was omitted none of those new compounds was synthesized. One example of such controls is shown in Fig. 5a. Each of the compounds (Figs. 5 and 6) was further characterized either by its UV absorption spectrum and/or by its susceptibility to specific Np_4N'-degrading enzymes. Ap_4U was additionally identified by comparison of its retention time with that of an Ap_4U standard. Ap_5U degradation by the (*asymmetrical*) Ap_4A hydrolase from humans is shown in Fig. 6. Four products: ATP, UDP, ADP and UTP were formed and judging from the area of the peaks it can be concluded that the cleavage that yielded ATP+UDP prevailed over the alternative one that gave UTP+ADP. Similarly, Gp_4U treated with the (*symmetrical*) Ap_4A hydrolase from *E. coli* split to GDP+UDP (profile not shown). The rate of synthesis of the uridylylated nucleotides with NTP and p_4A is approximately one order of magnitude lower than that of p_4U.

Finally, we have proved that the uridylylation reactions required Mg^{2+} as cofactor, that UDP-galactose did not function as an alternative donor of UMP moiety onto P_3 and that ADP was not a UMP acceptor since no Ap_3U was formed in the standard reaction mixture containing ADP (not shown). A K_m value of about 4.5 mM was estimated for P_3, in the synthesis of p_4U, using the conditions described in Section 2 and in the presence of a fixed (0.1 mM) concentration of labeled UDP-glucose and variable (1–4 mM) P_3 concentrations. In the same way, a K_m value of about 0.035 mM was determined for UDP-glucose, in the presence of fixed (4 mM) P_3 and variable (0.035–0.155 mM) UDP-glucose concentrations. From the V_{max} values obtained in these experiments and assuming a molecular mass of 400 kDa for the yeast transferase, a rate of the p_4U synthesis (k_{cat}) of 0.02 s^{-1} was calculated.

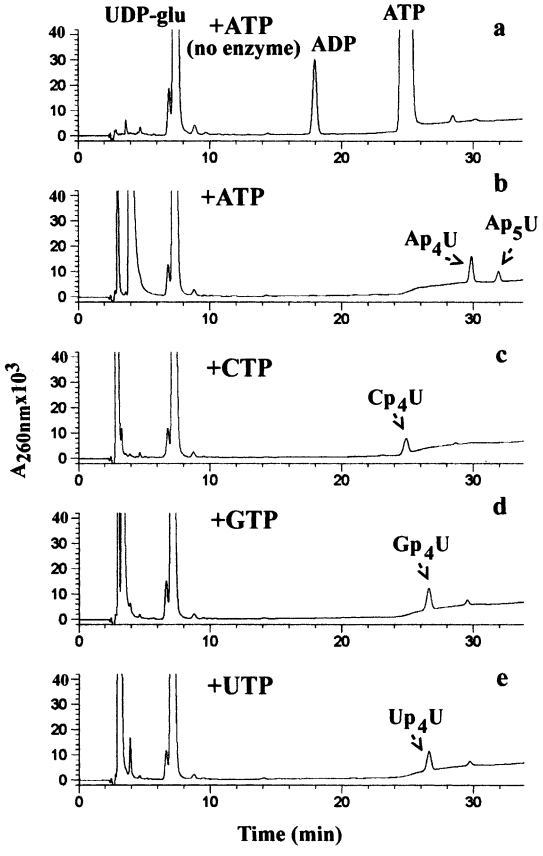

Fig. 5. Synthesis of dinucleotides from UDP-glucose and NTP. The reaction mixtures (0.03 ml) contained 4 mM UDP-glucose, 10 mM $MgCl_2$, 4 mM ATP, or CTP, or GTP, or UTP, as indicated, and 1 U enzyme (except for the control mixtures, one example of which is shown in profile a). Other assay conditions were as described in Section 2. After 7 h incubation the reaction mixtures were heated to inactivate the transferase, treated with 1 U alkaline phosphatase for 2 h at 37°C, and 10-fold diluted aliquots analyzed by HPLC. The peaks which eluted before 10 min correspond to nucleosides and UDP-glucose.

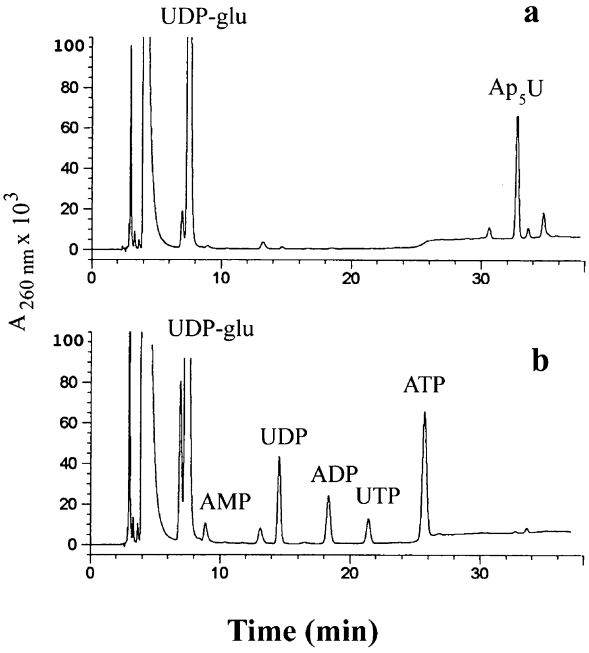

Fig. 6. Synthesis of Ap_5U and characterization of the compound by treatment with *asymmetrical* Ap_4A hydrolase. The reaction mixtures (0.05 ml) contained 4 mM UDP-glucose, 10 mM $MgCl_2$, 10 mM p_4A and 2 U uridylyltransferase. Other conditions were as described in Section 2. After 18 h incubation, the reaction mixture was heated to inactivate the transferase, treated with 1 U alkaline phosphatase for 2 h at 37°C, and a 10-fold diluted aliquot analyzed by HPLC (a). The rest of the reaction mixture was treated further for 10 min with 2 U of the human (*asymmetrical*) Ap_4A hydrolase and a 10-fold diluted aliquot analyzed by HPLC.

4. Discussion

Until now, the uridylyltransferase was known to catalyze the synthesis of UDP-glucose from UTP and glucose-1-phosphate (reaction 1) or degrade it in the presence of pyrophosphate in the reverse reaction 2

$$\text{UTP} + \text{glucose-1-P} \rightarrow \text{UDP-glucose} + \text{PP}_i \quad \text{(reaction 1)}$$

$$\text{UDP-glucose} + \text{PP}_i \rightarrow \text{UTP} + \text{glucose-1-P} \quad \text{(reaction 2)}$$

thus playing a key role in the metabolism of carbohydrates. Here, we show that the enzyme has also the catalytic capacity to transfer the UMP moiety from UDP-glucose onto P_3 or P_4 (reactions 3 and 4)

$$\text{UDP-glucose} + \text{P}_3 \rightarrow \text{p}_4\text{U} + \text{glucose-1-P} \quad \text{(reaction 3)}$$

$$\text{UDP-glucose} + \text{P}_4 \rightarrow \text{p}_5\text{U} + \text{glucose-1-P} \quad \text{(reaction 4)}$$

and onto NTP (p_3N) or p_4N (reactions 5 and 6, respectively):

$$\text{UDP-glucose} + \text{pppN} \rightarrow \text{Np}_4\text{U} + \text{glucose-1-P} \quad \text{(reaction 5)}$$

$$\text{UDP-glucose} + \text{ppppN} \rightarrow \text{Np}_5\text{U} + \text{glucose-1-P} \quad \text{(reaction 6)}$$

This observation widens the transferase substrate specificity and calls for further studies on its biological implications. The synthesis of Up_4Ns and p_4U would proceed only if there was enough UDP-glucose and UMP-acceptors (P_3, P_4 or NTPs) but no pyrophosphate; otherwise the synthesis of UTP would prevail (reaction 2). In fact UDP-sugars are among prominent nucleotides occurring in the yeast cells [36] and imbalance in their further utilization can facilitate uridylylation of polyphosphates and/or nucleoside polyphosphates. The occurrence of polyphosphates at millimolar concentration is well documented in the vacuoles of yeast cells [37]. However, the concentration of P_3 and P_4 is difficult to estimate in yeast cells mainly due to possible hydrolysis of polyphosphates by endopolyphosphatases, present in vacuoles, during protoplast isolation [37]. Moreover, levels of the short-chain (acid-soluble) polyphosphates depend upon different conditions and change during yeast culture growth. Their concentration was very low during exponential growth and rose rapidly and transiently at the time of transition between the exponential and stationary phases of growth. The production of acid-soluble polyphosphates correlated with the mitochondrial activities [38]. Earlier, Ludwig and co-workers showed that phosphorylated compounds, tentatively identified as P_3 and P_4, accumulated in *S. cerevisiae* when certain amino acids were added to the cultures [39]. The concentration of NTPs in yeast cells is high enough to support the synthesis of Np_4Us (ATP 1–2 mM, CTP 0.2 mM, GTP 0.2–0.3 mM and UTP 0.3–0.5 mM) [36].

The uridylyltransferase investigated here was not very effective in the uridylylation processes and in particular the accumulation of uridylylated nucleotides was observed only after prolonged incubations. However, it is plausible that other UMP-transferases might be more effective in these reactions.

Each enzyme that can potentially catalyze the synthesis of p_nNs and/or Np_nUs has to be experimentally verified. This postulate derives from earlier studies on aminoacyl-tRNA synthetases of which only some appeared to be effective 'Ap_4N synthases' and others were not active in that respect [14–16]. Also some mutants or molecular variants of the transferase may be more capable than others in uridylylation of polyphosphates or nucleoside polyphosphates. Here, the example can be the gain of function mutant M293P/K320L of coumarate:CoA ligase from *Arabidopsis thaliana* that proved to be more efficient in catalyzing p_4A and Ap_4A syntheses than its wild-type protein [26].

Finally, the fact that the UTP:glucose-1-phosphate uridylyltransferase catalyzes the synthesis of Np_4Us could be a potential enzymatic way in which different pyrimidine-containing Np_4N's might accumulate in the yeast and bacterial cells, a phenomenon observed by Coste and co-workers [27]. Our findings should provide a stimulus to look for other enzymes (transferases) involved in the synthesis of NDP-sugars or NDP-lipids as potential p_nN and Np_nN synthases; in particular those that could be capable of producing the pyrimidine mono- and dinucleoside polyphosphates.

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