O-Ribosyl-phosphate purine as a constant modified nucleotide located at position 64 in cytoplasmic initiator tRNA\textsuperscript{Met} of yeasts

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ABSTRACT

The unknown modified nucleotide G*, isolated from both \textit{Schizosaccharomyces pombe} and \textit{Torulopsis utilis} initiator tRNA\textsuperscript{Met}, has been identified as an O-ribosyl-(1"—2")-guanosine-5"-phosphate, called Gr(p), by means of HPLC, UV-absorption, mass spectrometry and periodate oxidation procedures. By comparison with the previously published structure of Ar(p) isolated from \textit{Saccharomyces cerevisiae} initiator tRNA\textsuperscript{Met}, the (1"—2")-glycosidic bond in Gr(p) has been postulated to have a /3-spatial conformation. The modified nucleotide Gr(p) is located at position 64 in the tRNA\textsuperscript{Met} molecules, i.e. at the same position as Ar(p). Since we have also characterized Gr(p) in \textit{Candida albicans} initiator tRNA\textsuperscript{Met}, the phosphoribosylation of purine 64 can be considered as a constant nucleotide modification in the cytoplasmic initiator tRNA\textsuperscript{Met} of all yeast species so far sequenced. Precise evidence for the presence of Gr(p) in initiator tRNA\textsuperscript{Met} of several plants is also reported.

INTRODUCTION

In eukaryotic cells, the cytoplasmic methionine initiator tRNAs (initiator tRNA\textsuperscript{Met}) so far sequenced have several structural characteristics which differentiate them from their methionine elongator tRNA counterpart as well as from all other cytoplasmic elongator tRNAs. Such characteristics have been observed in their primary structure (1). One of them is located in the acceptor stem where the first A1-U72 base pair has never been found at that position in any elongator tRNA. Another characteristic concerns the first (position 54) and the last (position 60) nucleotides of the T-loop. There are adenosines in cytoplasmic initiator tRNA\textsuperscript{Met}, whereas elongator tRNAs carry always pyrimidines at these positions, including the widespread T\textsubscript{54}. Finally, several cytoplasmic initiator tRNA\textsuperscript{Met} from yeasts and plants carry, at position 64, an unknown modified purine which could also differentiate them from those of the other eukaryotic species.

Recently, we determined the chemical structure of the unknown nucleotide located at this position (64) in the \textit{Saccharomyces cerevisiae} (S.cerevisiae) initiator tRNA\textsuperscript{Met} (2,3). It was found to be O-β-ribosyl-(1"—2")-adenosine-5"-phosphate, i.e. Ar(p). This result prompted us to check whether other yeast initiator tRNA\textsuperscript{Met}, especially those of \textit{Schizosaccharomyces pombe} (S.pombe) and \textit{Torulopsis utilis} (T.utilis), did or did not contain the same nucleotide modification on their unknown purine at the position 64. The authors who sequenced the \textit{T. utilis} initiator tRNA\textsuperscript{Met} described in position 64 a modified G* with structural characteristics similar but not identical to Gm (4). In addition, \textit{S. pombe} has dG in position 64 of its gene (5).

Starting with enriched \textit{S. pombe} and \textit{T. utilis} tRNA fractions containing the initiator tRNA\textsuperscript{Met}, we prepared, therefore, about one mg of G\textsubscript{64}-G\textsubscript{65} dinucleotide. This dinucleotide was then hydrolyzed into two nucleosides [G*] and G using snake venom phosphodiesterase (SV-PDE) and bacterial alkaline phosphatase treatments.

We report in this paper our investigations on the structure determination of the unknown nucleotide G\textsubscript{64} in yeasts, by using the combined means, previously used for Ar(p) identification (2,3), of reverse phase-high performance liquid chromatography (RP-HPLC) protocols, UV-absorption spectrum, mass spectrometric measurements and periodate oxidation on several mono- and dinucleotides containing G*.

MATERIALS AND METHODS

Purification of Initiator tRNA\textsuperscript{Met} from \textit{T. utilis} and \textit{S. pombe}

Pure or enriched initiator tRNA\textsuperscript{Met} from both \textit{S. pombe} and \textit{T. utilis} were prepared by BD-cellulose and Sepharose 4B column chromatographies performed on total tRNAs from the above yeasts, followed by two dimensional polyacrylamide gel electrophoresis, as previously reported (6—9).
Purification of dephosphorylated [G*] nucleoside

The dephosphorylated [G*] nucleoside was isolated by HPLC from nuclease PI + bacterial alkaline phosphatase (BAP) digests of S.pombe and T.utilis initiator tRNAs^Met, using the analytical procedure previously described for the isolation of Ar nucleoside from yeast initiator tRNA^Met (2). Ribonucleoside analyses by HPLC were performed on a Spectra-Physics liquid chromatograph equipped with a Spectra Focus detector (Spectra-Physics France, Les Ulis, France) and a Supelcosil LC 18S column, 250 mm x 4.6 mm (Supelco France, St Germain-en-Laye, France). The liquid chromatography was carried out using the experimental conditions for the separation of ribonucleosides developed by Gehrke's group (10-12).

The [G*] nucleoside can also be obtained from BAP treatment of G* or pG* mononucleotides isolated and purified as described below.

Purification of di- and mononucleotides containing G*

Partially purified initiator tRNA^Met from S.pombe or T.utilis was hydrolyzed by pancreatic RNase (Worthington, Freehold, USA). The resulting oligonucleotide mixture was separated on fibrous DEAE-cellulose column as previously described (13). The nonanucleotidic fraction containing the heptanucleotide ApG*pGpApGpApCp was collected and desalted.

Further hydrolysis of the above fraction was carried out with RNase-T2 or nuclease PI. In addition to mononucleosides and nucleosides, the corresponding digests contained G*pGp or pG*pG, respectively. These dinucleotides eluted like tetranucleotides when chromatographed on DEAE-cellulose column. They were further purified from contaminating tetranucleotides by thin layer chromatography (TLC) on cellulose plates (F1440 or G1440 from Schleicher and Schüll) (9).

Finally, the G* and pG* mononucleotides were purified and desalted by HPLC from SV-PDE digests of G*pGp and pG*pG dinucleotides, respectively.

Mass spectrometry analysis

A sample aliquot of 0.1 A_260 unit of the phosphatase treated [G*] mononucleoside was used for conversion to volatile trimethyl-silyl (TMS) derivative as previously described (2).

Mass spectra of the trimethylsilylated [G*] nucleoside by electron impact (EI-MS) or by chemical ionization (CI-MS) were performed in the 'Centre Européen de Spectrométrie de Masse' (Spiral, Couternon, France) with a Nermag R10-10C mass spectrometer (Delfi-Nermag, Argenteuil, France) equipped with...
a Digital Micro PDP-11/23 data system. The samples were injected by direct probe introduction after removal of solvent-reagent in the vacuum lock. For EI-MS analysis, ionizing energy was 70 eV, ion source temperature 150°C, probe temperature from 50°C to 280°C at 2°C/s. For CI-MS analysis, ammoniac was used as chemical ionization gas; source pressure was 10⁻¹ bar, and ionization current 100 mA.

Periodate oxidation–β-elimination procedure

The periodate oxidation–β-elimination procedure (P.O.–βE. procedure) described by Keith and Gilham (14) for the stepwise degradation of polyribonucleotides was adapted to the chemical degradation of G* containing di- and mononucleotides isolated from S. pombe or T. utilis initiator tRNAsMet.

The dinucleotides or mononucleotides (ca. 100 nmol) in 150 µl of distilled water were treated at 0°C for 60 min with 20 µl of 0.2 M sodium periodate (Sigma, St Louis, USA), followed by 20 µl of 0.4 M rhamnose (Sigma, St Louis, USA) at 0°C for 30 min in order to neutralize the remaining sodium periodate. To the mixture, were then added 60 µl of 2 M cyclohexylamine hydrochloride, and the solution was incubated at 45°C for 90 min. The products present in the final solution were analyzed and identified by HPLC.

RESULTS

Isolation of [G*] nucleoside from S. pombe initiator tRNAMet

Fig. 1 shows the HPLC nucleoside analysis of S. pombe initiator tRNA after exhaustive nuclease P1 digestion followed by BAP hydrolysis. The peak eluted at 43.7 min retention time, between m/z 2G and t6A, was collected and designated as N44 unknown compound. Further studies showed that this compound was a dinucleotide which was totally resistant to nuclease P1 and RNase-T2.

As shown in Fig. 2, the dinucleotide N44 yielded two mononucleotides in equimolar amount upon enzymatic hydrolysis with SV-PDE followed by BAP treatment.

One of these mononucleotides was easily identified as unmodified guanosine by comparison of its HPLC retention time and UV-absorption spectrum with those of authentic guanosine.

The second mononucleotide exhibited a UV-absorption spectrum whose profile was typical for substituted guanosine derivatives. However, its HPLC retention time did not correspond to any of the modified guanosines so far identified.

An identical enzymatic and HPLC procedure was performed on T. utilis initiator tRNA leading also to the isolation of the same derivative. We concluded that this modified nucleoside was an unknown guanosine derivative designated [G*] nucleoside.

Characterization of [G*] nucleoside as O-ribosyl-guanosine (Gr)

Identification by electron impact-mass spectrometry (EI-MS)

Most structural information was gained from the electron-impact mass spectrum (EI-MS) of the silylated [G*] obtained by direct probe introduction (Fig. 3).

Trimethylsilyl derivatization of [G*] yielded two TMS derivatives: a major (TMS)7 derivative leading to the molecular ion M+ at m/z 919, and a very minor (TMS)6 derivative leading to the molecular ion M'+ at m/z 847. According to the fragmentation process previously described for EI-MS of trimethyl-

Fig. 3. Electron impact–mass spectrum and structure of Gr nucleoside as trimethylsilyl (TMS) derivative. S = sugar (ribose) moiety; B' = intact guanosine part; B = guanine base; (TMS) = trimethylsilyl group = Si(CH3)3.
silylated O-ribosyl-adenosine (Ar) from yeast initiator tRNA^Met (2), the chemical structure of [G*] nucleoside was determined as O-ribosyl-guanosine (Gr) from the EI-MS fragmentation process of the molecular ion M^+ at m/z 919. This fragmentation process presented in Table I can be interpreted as follows:

- the ion peaks at m/z 904, 829, 816, ... of a first ion-series correspond to the removal of CH_3, (TMS)OH or CH_2O(TMS) from M^+,

- the ion peaks at m/z 701, 686, 672, ... of a second ion-series are attributed to several cleavages in ribose moiety; they are specific for the O-ribosyl-guanosine structure by containing the intact guanosine part (B') plus portions of the ribose_2 moiety,

- in a third ion-series, numerous other fragment ions at m/z 483, 425, 410, ... contain the guanine base (B) plus portions of the ribose_1 moiety,

- finally, several ions at the low mass regions such as m/z 349, 259, 243, 217, 169 and 103 are characteristic of silylated ribonucleosides carrying an unmethylated sugar.

All these four ion-series are in agreement with the O-ribosyl-guanosine structure for [G*] as (TMS)_7 derivative. However, the carbons involved in the glycosidic bond between the two ribose moieties stayed to be determined.

**Confirmation of O-ribosyl-guanosine (Gr) structure by chemical ionization-mass spectrometry (CI-MS).** The ammoniacal chemical ionization-mass spectrum of [G*] nucleoside as TMS derivative (results not shown) confirmed the O-ribosyl-guanosine structure for [G*] by leading to the protonated molecular ion MH^+ (m/z 920) of the (TMS)_7 derivative as base peak of the CI-mass spectrum, as well as to the minor protonated molecular ion M' + H^+ (m/z 848) of the (TMS)_6 derivative. In addition, several ion peaks of high relative intensity were observed (m/z 600, 556, 382, 368, 324, 296, ...), resulting from some cleavages in ribose_2 or ribose_1 moiety.

**Study of non phosphatase treated G* derivatives**

The above structural studies were performed on [G*], i.e. phosphatase treated G*.

groups on carbons 2' and 3' of ribose2 moiety in pGr(p) are free. Thus, the additional phosphorylmonooester group is located on the carbon 5'.

**Structure of the ribose-ribose glycosidic bond in G*.** The nuclease P1 digestion of the dinucleotide G*pGp, i.e. Gr(p)pGp, led to 3'-dephosphorylated Gr(p)Gp. Upon P.O. −βE. treatment, this latter yielded a mixture of two oxidative compounds which were identified as guanine and guanosine-3'-phosphate by their HPLC retention times and their UV-absorption spectra. Thus, the chemical degradation of Gr(p)pG dinucleotide by P.O. −βE. was efficient for the cleavage of the G nucleoside moiety with its subsequent conversion to guanine. In the same time, it was also efficient for the removal of the phosphor-ribose; part from Gr(p) moiety, leading to the release of guanosine-3'-phosphate (Fig. 4b).

These results provide the evidence for the 3',5'-phosphodiester structure of the bond between the two nucleoside moieties in Gr(p)pG dinucleotide. Consequently, the linkage between ribose2 and ribose1 in Gr(p) nucleotide can be only a (1'--2')-glycosidic bond. These results also confirmed the location of the additional monophosphate group at the position 5' of ribose2 moiety.

**DISCUSSION**

The above results demonstrate that the unknown modified nucleotide G* isolated from both initiator tRNAsMet of *S.pombe* and *T.utilis* is an isomeric form of phosphorylated O-ribo-syl-guanosine. Since the linkage between the two ribose moieties of G* was determined as a (1'--2')-glycosidic bond, this new modified G has been called by the abbreviation Gr(p). The additional phosphate group carried by Gr(p) is located on the carbon 5' of the ribose2 moiety. Because of the close structural relationship between Gr(p) from *S.pombe* and *T.utilis* initiator tRNAsMet and Ar(p) from *Saccharomyces cerevisiae* initiator tRNA^Met^ (2,3), the (1'--2')-glycosidic bond in Gr(p) can be postulated to have the same spatial conformation as Ar(p), i.e. a β-spacial conformation. Thus, the final structure for Gr(p) nucleotide in *S.pombe* and *T.utilis* initiator tRNAsMet was deduced as O-β-ribo-yl(1'--2')-guanosine-5'-phosphate (Fig. 5).

According to the sequence of *T.utilis* initiator tRNA^Met^ previously published (4), the location of Gr(p) nucleotide was found to be in the T-stem at the position 64, i.e. at the same position as Ar(p) in *S.cerevisiae* initiator tRNA^Met^. This might also be the case in *S.pombe* initiator tRNAs^Met^ whose gene has dG at the position 64 (5). Structural investigations on this tRNA should confirm this location for Gr(p). Preliminary studies recently conducted in our laboratories have shown that Gr(p) is also present in a partially purified sample of *Candida albicans* initiator tRNA^Met^ provided by M.Santos.

From these results, we assume that a phosphorysylated purine 64 is a constant modified nucleotide in the initiator tRNAs^Met^ of all yeast species so far studied. Such a modification could play a special discriminatory role in the initiation process of translation in the cytosol of these euukaryotic cells, as already described in the case of Ar(p) in *S.cerevisiae* initiator tRNA^Met^ by Sprinzl and coworkers (15). In the same paper, these authors attributed the Gr(p) structure to the unknown nucleotide G* located at the position 64 in wheat germ initiator tRNA^Met^ (16). This identification was postulated by means of enzymatic and periodate reactions on the whole tRNA molecule, and on the basis of our earlier results on Ar(p) (2,3). Using our analytical procedure, we recently obtained precise evidence for the presence of Gr(p) in a commercial preparation (Sigma, St. Louis, USA) of unfractionated wheat germ tRNAs. We have also characterized Gr(p) in pure initiator tRNAs^Met^ from *Lupinus luteus* seeds provided by J.Barciszewski, and from soybean provided by P.Guillemaut.

In addition to the typical features of euukaryotic initiator tRNAsMet described above (base pair A1-U72, adenosines 54 and 60 in T-loop), the phosphorysylation of purine 64 (adenosine or guanosine) could be a specific modification not only in the cytoplasmic initiator tRNAs^Met^ of yeast species, but also in those of different plants. Further studies on tRNAs of more yeast and plant species are needed to confirm or not this general feature. Other euukaryotic initiator tRNAs^Met^ like those from mammals, fishes, frogs, insects, ... have an unmodified pyrimidine nucleotide at position 64. This could mean that phosphorysylation might be necessary for the initiation process only when the nucleotide 64 is a purine, whereas a pyrimidine at that position together with other initiator tRNA^Met^ discriminants would be discriminatory enough to restrict its use for initiation.

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