Nucleic Acids Research, Vol. 19, No. 19 5199–5203

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

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Received July 23, 1991; Revised and Accepted September 6, 1991

ABSTRACT

The unknown modified nucleotide G*, isolated from both *Schizosaccharomyces pombe* and *Torulopsis utilis* initiator tRNAs^{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 *Saccharomyces cerevisiae* initiator tRNA^{Met}, the (1″→2′)-glycosidic bond in Gr(p) has been postulated to have a β-spatial conformation. The modified nucleotide Gr(p) is located at position 64 in the tRNA^{Met} molecules, i.e. at the same position as Ar(p). Since we have also characterized Gr(p) in *Candida albicans* initiator tRNA^{Met}, the phosphoribosylation of purine 64 can be considered as a constant nucleotide modification in the cytoplasmic initiator tRNAs^{Met} of all yeast species so far sequenced. Precise evidence for the presence of Gr(p) in initiator tRNAs^{Met} of several plants is also reported.

INTRODUCTION

In eukaryotic cells, the cytoplasmic methionine initiator tRNAs (initiator tRNAs^{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 A_1-U_{72} 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 tRNAs^{Met}, whereas elongator tRNAs carry always pyrimidines at these positions, including the widespread T_{54}. Finally, several cytoplasmic initiator tRNAs^{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 *Saccharomyces cerevisiae* (*S.cerevisiae*) initiator tRNA^{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 tRNAs^{Met}, especially those of *Schizosaccharomyces pombe* (*S.pombe*) and *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 *T.utilis* initiator tRNA^{Met} described in position 64 a modified G* with structural characteristics similar but not identical to Gm (4). In addition, *S.pombe* has dG in position 64 of its gene (5).

Starting with enriched *S.pombe* and *T.utilis* tRNA fractions containing the initiator tRNAs^{Met}, we prepared, therefore, about one mg of G^*_{64}-G_{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^*_{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 tRNAs^{Met} from *T.utilis* and *S.pombe*

Pure or enriched initiator tRNAs^{Met} from both *S.pombe* and *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).

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Purification of dephosphorylated [G*] nucleoside

The dephosphorylated [G*] nucleoside was isolated by HPLC from nuclease P1 + 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×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 developped 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 (Worthinghton, 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 P1. 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

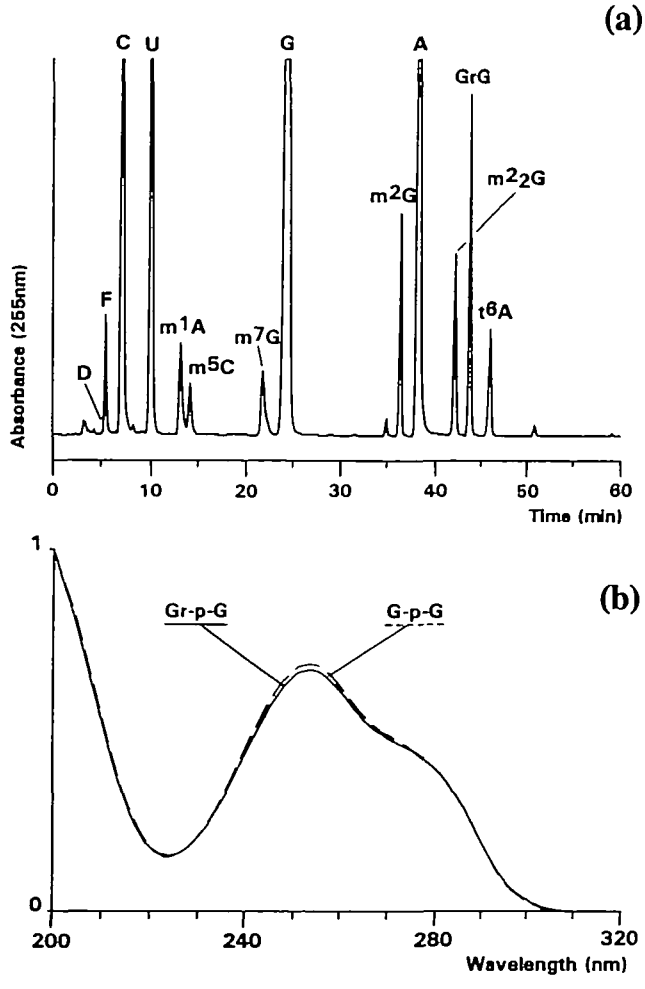

Fig. 1. (a): HPLC chromatogram of nucleosides resulting from nuclease P1 plus bacterial alkaline phosphatase (BAP) digestion of *S.pombe* initiator $tRNA^{Met}$. The peak GrG corresponds to the unknown dinucleotide G*pG eluted at 43.7 min retention time. (b): UV-absorption spectrum at pH 7.0 of GpG reference dinucleotide (λmax 253 nm), as compared to that of the unknown G*pG isolated from *S.pombe* initiator $tRNA^{Met}$.

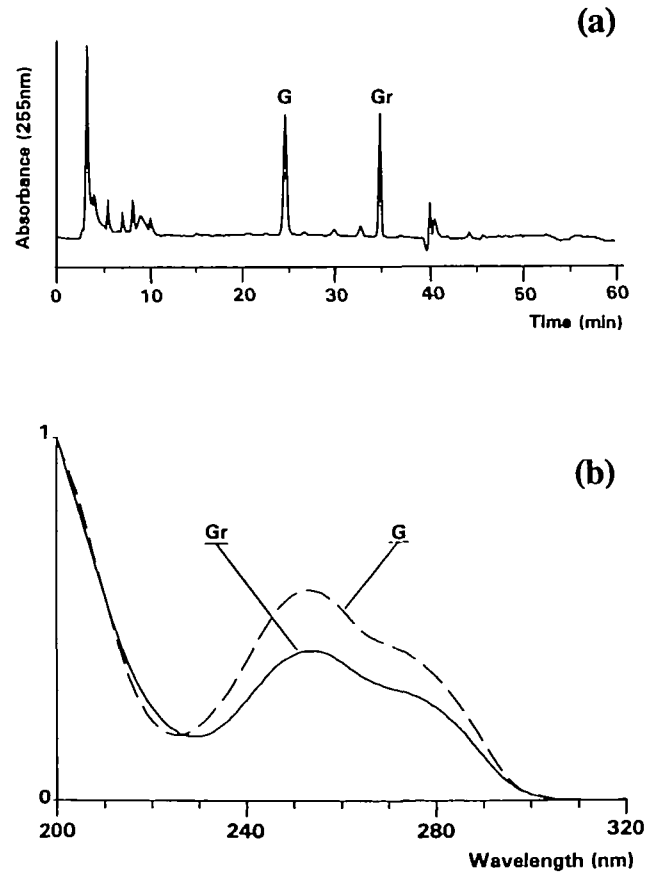

Fig. 2. (a): HPLC chromatogram of Gr and G nucleosides resulting from snake venom phosphodiesterase plus BAP hydrolysis of the unknown G*pG dinucleotide, i.e. Gr-p-G. (b): UV-absorption spectrum at pH 7.0 of reference guanosine (λmax 254 nm), as compared to that of the new modified nucleoside Gr.

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 $tRNAs^{Met}$.

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 $tRNA^{Met}$

Fig. 1 shows the HPLC nucleoside analysis of *S.pombe* initiator $tRNA^{Met}$ after exhaustive nuclease P1 digestion followed by BAP hydrolysis. The peak eluted at 43.7 min retention time, between $m^2$2G and t^6A, was collected and designated as N_{44} 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 N_{44} yielded two mononucleosides in equimolar amount upon enzymatic hydrolysis with SV-PDE followed by BAP treatment.

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

The second mononucleoside 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 $tRNA^{Met}$ 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-

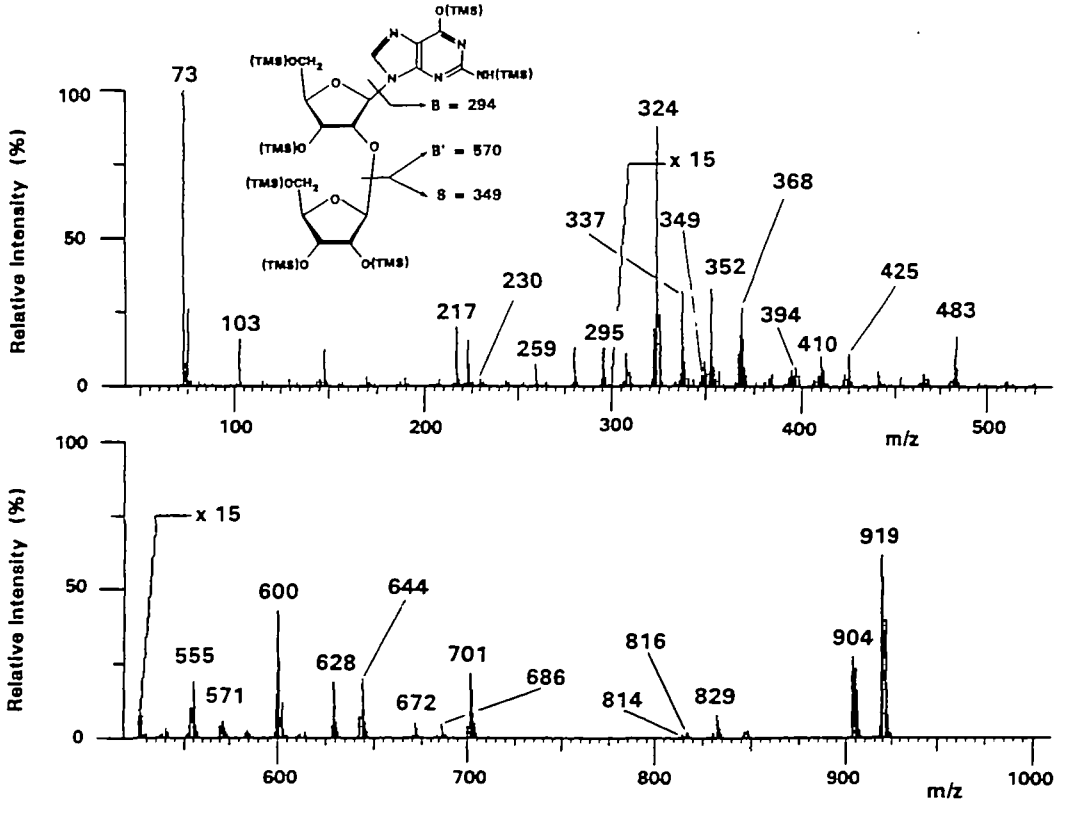

Fig. 3. Electron impact–mass spectrum and structure of Gr nucleoside as trimethylsilyl (TMS) derivative. S = sugar (ribose) moiety; B' = intact guanosine part; B = guanine base; (TMS) = trimethylsilyl group = $Si(CH_3)_3$.

Table I. Fragment-ion series from electron impact−mass spectrum of G* nucleoside isolated from *S.pombe* initiator $tRNA^{Met}$, as trimethylsilylated derivative. M: intact molecular ion, B: guanine base, B′: intact guanosine part, S: sugar (ribose) moiety.

MASS (m/z)	POSITIVE ION	COMPOSITION	MASS (m/z)	POSITIVE ION	COMPOSITION
919	M	Molecular ion	483	B +188 +1	B +H +$C_2H_2O_2$(TMS)$_2$
904	M -15	M -CH_3	425	B +131	B +$C_2H_2O_2$(TMS)
829	M -90	M -(TMS)OH	410	B +116	B +C_2H_3O(TMS)
816	M -103	M -CH_2O(TMS)	394	B +100	B +$C_2H_2OSi(CH_3)_2$
814	M -105	M -CH_3 -(TMS)OH	368	B +74	B +H +(TMS)
			352	B +58	B +$Si(CH_3)_2$
701	B′ +131	B′ +$C_2H_2O_2$(TMS)	337	B +58 -15	B +$Si(CH_3)_2$ -CH_3
686	B′ +116	B′ +C_2H_2O(TMS)	324	B +30	B +H + CHO
672	B′ +102	B′ +CHO +(TMS)	295	B +1	B +H
644	B′ +74	B′ +H +(TMS)			
628	B′ +58	B′ +$Si(CH_3)_2$	349	S	Ribose
600	B′ +30	B′ +H +CHO	259	S -90	S -(TMS)OH
571	B′ +1	B′ +H	230	S -119	C_4H_4(OTMS)$_2$
555	B′ -15	B′ -CH_3	217	S -132	CH_3(OTMS)$_2$
			103		CH_2O(TMS)

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 $ribose_2$ 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*.

As already mentioned in 'Materials and Methods', enzymatic hydrolysis of ApG*pGpApGpApCp heptanucleotide without phosphatase treatment leads to G*pGp and pG*pG which behaved like tetranucleotides on DEAE-cellulose column chromatography.

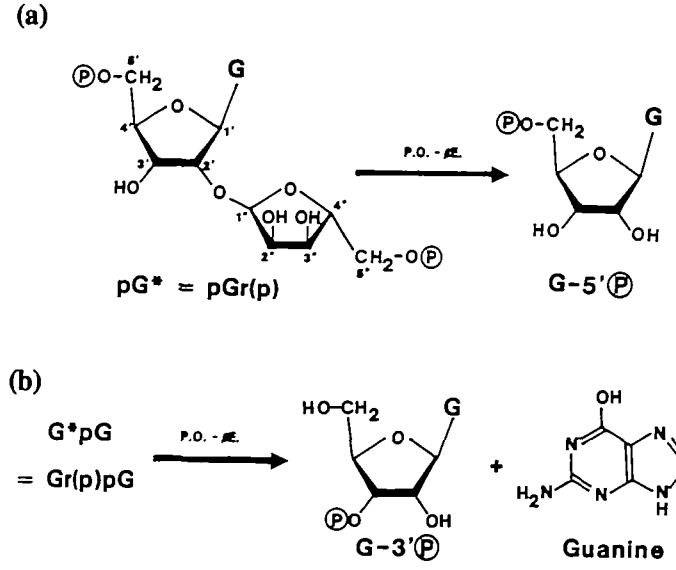

Fig. 4. Summarized results of periodate oxidation followed by β-elimination (P.O.−βE.) procedure applied: (a) to pGr(p) mononucleotide: identification of guanosine-5′-phosphate (G-5′P) as oxidative compound, (b) to Gr(p)pG dinucleotide: identification of guanosine-3′-phosphate (G-3′P) and guanine as oxidative products.

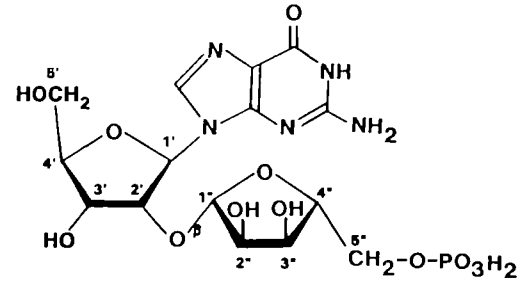

Fig. 5. Chemical structure of O-β-ribosyl(1″→2′)-guanosine-5″-phosphate, i.e. Gr(p), located at the position 64 in *S.pombe* and *T.utilis* initiator $tRNAs^{Met}$.

Further hydrolysis of G*pGp or pG*pG with SV-PDE led to G* or pG*, respectively. G* behaved like a mononucleotide and pG* like a trinucleotide when chromatographed on a DEAE-cellulose column, while additional phosphatase treatment of G* or pG* led to a unique neutral nucleoside. This latter was identical to the phosphatase treated [G*] nucleoside, i.e. Gr.

From these results, it was clear that the additional anionic charge carried by G* comes from a phosphorylmonoester group. The exact location of this phosphate group, as well as the carbons involved in the glycosidic bond between the two ribose moieties were determined by using the chemical procedure of periodate oxidation and subsequent β-elimination with cyclohexylamine (P.O.−βE.) on pG* mononucleotide and G*pG dinucleotide.

*Location of monophosphate group on G**. When applied to the 5′-mononucleotide pG*, i.e. pGr(p), the P.O.−βE. procedure led to guanosine-5′-phosphate by removing the phosphoryl-$ribose_2$ moiety from the original molecule. From this result summarized in *Fig. 4a*, one can conclude that the hydroxyl

groups on carbons 2″ and 3″ of ribose$_2$ moiety in pGr(p) are free. Thus, the additional phosphorylmonoester group is located on the carbon 5″.

*Structure of the ribose$_2$-ribose$_1$ glycosidic bond in G**. The nuclease P1 digestion of the dinucleotide G*pGp, i.e. Gr(p)pGp, led to 3′-dephosphorylated Gr(p)pG. 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 phosphoryl-ribose$_2$ 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 ribose$_2$ and ribose$_1$ 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 ribose$_2$ moiety.

DISCUSSION

The above results demonstrate that the unknown modified nucleotide G* isolated from both initiator tRNAs^{Met} of *S.pombe* and *T.utilis* is an isomeric form of phosphorylated O-ribosyl-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 ribose$_2$ moiety. Because of the close structural relationship between Gr(p) from *S.pombe* and *T.utilis* initiator tRNAs^{Met} 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-spatial conformation. Thus, the final structure for Gr(p) nucleotide in *S.pombe* and *T.utilis* initiator tRNAs^{Met} was deduced as *O*-β-ribosyl-(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 tRNA^{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 phosphoribosylated 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 eukaryotic 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 eukaryotic initiator tRNAs^{Met} described above (base pair A_1-U_{72}, adenosines 54 and 60 in T-loop), the phosphoribosylation 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 eukaryotic initiator tRNAs^{Met} like those from mammals, fishes, frogs, insects, ... have an unmodified pyrimidine nucleotide at position 64. This could mean that phosphoribosylation 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.

ACKNOWLEDGEMENTS

We are indebted to Dr. J.Barciszewski, Dr. P.Guillemaut and Mr. M.Santos for providing us *Lupinus luteus* seeds, soybean and *Candida albicans* initiator tRNA^{Met} samples, respectively. We acknowledge the expert technical assistance of Mrs. C.Fix. Part of the work was carried out in the Laboratory of Prof. G.Dirheimer who is gratefully acknowledged for valuable comments and constant support. We are also grateful to Mr. J.-L.Guyot who conducted the MS studies in the 'Centre Européen de Spectrométrie de Masse' (Spiral Co., Couternon, France) managed by Dr. M.Prost. This research had financial support from 'Ministère des Universités', 'Association pour la Recherche sur le Cancer', 'Ligue Bourguignonne contre le Cancer' et 'Centre National de la Recherche Scientifique'.

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