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Presence of phosphorylated *O*-ribosyl-adenosine in T-Ψ-stem of yeast methionine initiator tRNA

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ABSTRACT

We report in this paper on isolation and characterization of two unknown nucleosides G* and [A*] located in the T-Ψ-stem of yeast methionine initiator tRNA, using the combined means of HPLC protocols, real time UV-absorption spectrum, and post-run mass spectrometry by electron impact or fast atom bombardment. The G* nucleoside in position 65 was identified as unmodified guanosine. The structure of the unknown [A*] in position 64 was characterized as an isomeric form of O-ribosyl-adenosine by comparison of its chromatographic, UV-spectral and mass spectrometric properties with those of authentic O-α-ribofuranosyl-(1"-->2')-adenosine isolated from biosynthetic poly(adenosine diphosphate ribose). Our studies also brought evidence for the presence of a phosphorylmonoester group located on this new modified nucleoside [A*], when isolated by ion exchange chromatography from enzymic hydrolysis of yeast initiator $tRNA^{Met}$ without phosphatase treatment.

INTRODUCTION

The nucleotide sequence of yeast methionine initiator tRNA (initiator $tRNA^{Met}$) was previously reported by Simsek and Rajbhandary (1). However, among the 75 nucleoside units of this tRNA, two nucleosides had not been characterized. These two nucleosides designated as A* and G* by the authors were located in the T-Ψ-stem at the positions 64 and 65, respectively. They were isolated as the dinucleotide A*pG*p after exhaustive T1-RNase digestion of yeast initiator $tRNA^{Met}$. This dinucleotide showed an UV-spectrum similar to reference dinucleotide ApGp. It behaved like a tetranucleotide on DEAE-cellulose columns, meaning additional anionic charges as compared to reference dinucleotide ApGp. The dinucleotide A*pG*p yielded adenine and guanine in equimolar amounts upon treatment with N hydrochloric acid at 100°C for 1 hour, but it was almost totally resistant to T2-RNase, N sodium hydroxyde or phosphodiesterase hydrolysis. From these last results, it was assumed by the authors that the ribose moiety attached to the unknown nucleoside A* could be a 2'-O-alkylated ribose.

In this paper, we report on the isolation of A*pG* dinucleotide by high

performance liquid chromatography (HPLC). The yeast initiator $tRNA^{Met}$ was hydrolyzed into its component nucleosides by nuclease P1 and bacterial alkaline phosphatase (BAP), and we found that A*pG* eluted at 44 minutes. This dinucleotide was then hydrolyzed into two nucleosides, [A*] and G*, using snake venom phosphodiesterase and BAP treatment. G* nucleoside was identified by HPLC retention time, UV-spectrum, and mass spectrometry as unmodified guanosine (Guo). The unknown nucleoside [A*] was characterized as an O-ribosyl-adenosine isomer by means of (i) real time UV-absorption spectrum, (ii) post-run mass spectrometric measurements, (iii) comparison of this modified nucleoside to authentic O-α-D-ribofuranosyl-(1"-->2')-adenosine (Ado-Rib) isolated from biosynthetic poly(adenosine diphosphate ribose), i.e. poly(ADP-Rib).

We also report our findings on one additional phosphorylation of this new modified nucleoside [A*] when isolated by ion exchange column chromatography from the pancreatic RNase and nuclease T2 hydrolysis of yeast initiator $tRNA^{Met}$ without phosphatase treatment.

Hall (2) described a general procedure for isolation of "minor" nucleosides from enzymic hydrolysates of RNAs, and observed the presence of O-ribosyl-adenosine. He later postulated that this modified nucleoside could come from poly(adenosine diphosphate ribose) which was a contaminant of RNA isolation and not a component of the RNA (3). In contrast, our studies show that A* is a modified phosphorylated nucleoside in yeast initiator $tRNA^{Met}$ with the following structure of 9[2'-O-ribosyl-β-D-ribofuranosyl]-adenine for the nucleoside moiety.

MATERIALS AND METHODS

Isolation and purification of yeast initiator $tRNA^{Met}$

Enriched or pure yeast initiator $tRNA^{Met}$ was isolated from unfractionated brewer's yeast tRNAs (Boehringer, Mannheim, GFR) by a three step procedure involving counter current distribution (4), followed by two successive column chromatography separations on BD-Cellulose and Sepharose-4B, conditions were as previously described (5,6).

Biosynthetic preparation of poly(ADP-Rib)

Poly(ADP-Rib) was synthesized *in vitro* by incubation of calf thymus homogenate in presence of NAD^+ 2 mM, Tris-HCl buffer 0.1 M pH 8 and $MgCl_2$ 0.03 M for 20 min at 37°C according to Miwa and Sugimura (7). Further purification was carried out after DNase, RNase and Pronase enzymic treatments, each one being followed by ethanol precipitation. Then, proteins

were removed by phenol-chloroform 0.5:0.5 (v/v) treatment. The final purification was performed on hydroxyapatite column chromatography (Biogel, HTP from Biorad). Poly(ADP-Rib) was adsorbed on the solid phase of the hydroxyapatite, whereas the deoxynucleotides and ribonucleotides resulting from the enzymic digestion by DNase and RNase flow through the column while charging. Poly(ADP-Rib) was finally desorbed using a linear gradient from 0.1 M to 0.5 M sodium phosphate at pH 6.8.

Isolation methods for A* and G* nucleosides from yeast initiator $tRNA^{Met}$

Method involving HPLC analysis - The pure yeast initiator $tRNA^{Met}$ sample (50 A_{260} units in 200 µl of distilled water) was hydrolyzed for 14 h at 37°C with 100 µg of nuclease P1 (Boehringer, Mannheim, GFR) in 100 µl of 30 mM acetate buffer pH 5.3 and 50 µl of 10 mM $ZnCl_2$. The solution of free 5'-phosphate nucleotides was then hydrolyzed for 2 hours at 37°C by addition of 200 µl of 0,5 M Tris buffer pH 8.3 containing 5 units of bacterial alkaline phosphatase (BAP) (Sigma, St Louis, MO, USA). The final hydrolysate containing free nucleosides had a total volume of 550 µl.

The hydrolysate (ca. 500 µl) was injected onto a Supelcosil LC 18S column, 250 mm x 4.6 mm (Supelco Inc, Bellefonte, PA). The liquid chromatography was carried out using the experimental conditions for the separation of ribonucleosides developed by Gehrke and Kuo (8-12) and described in Table I. The peak corresponding to the unknown phosphatase treated dinucleotide A*pG* (44 min retention time) was collected, and the collected fraction was evaporated to dryness under vacuum at room temperature in a Savant Speed Vac concentrator (Savant Instrument,Inc, Farmingdale, N.Y.). The dried residue was redissolved in 200 µl of distilled water. The total solution was finally injected onto the HPLC column and eluted with an elution gradient from 5 % to 70 % aqueous methanol solution (30 min run), for desalting. The peak of unknown phosphatase treated dinucleotide A*pG* was collected, and the desalted fraction was evaporated to dryness as described above.

To isolate the two nucleoside moieties of this pure dinucleotide, the following procedures were used :

(i) hydrolysis of the dinucleotide solution (about 0.25 A_{260}, ca. 10 µg in 100 µl of distilled water) for 4 hours at 37°C with 1.2 units of snake venom phosphodiesterase (Sigma, St Louis, MO, USA) in 60 µl of 5 mM magnesium acetate and 30 µl of 0.5 M Tris buffer pH 7.8,

(ii) BAP hydrolysis for 2 hours at 37°C as described above,

(iii) HPLC separation on 150 mm x 4.6 mm column of the two free

TABLE I : Standard elution gradient for ribonucleoside fractionation by reverse-phase high performance liquid chromatography.

Elution Buffers :			
A.	pH 5.30;	0.01 M $NH_4H_2PO_4$;	2.5 % CH_3OH
B.	pH 5.10;	" " " ;	20.0 % CH_3OH
C.	pH 4.90;	" " " ;	35.0 % CH_3CN

Elution Gradient Sequence Time (min)		Solvent (%)
1.	0.0 to 12.0	100% A Isocratic
2.	12.0 to 20.0	100% A to 90% A,10% B
3.	20.0 to 25.0	90% A,10% B to 75% A,25% B
4.	25.0 to 32.0	75% A,25% B to 40% A,60% B
5.	32.0 to 36.0	40% A,60% B to 38% A,62% B
6.	36.0 to 45.0	38% A,62% B to 100% B
7.	45.0 to 80.0	100% B to 100% C
8.	80.0 to 85.0	100% C Isocratic

Column : Supelcosil LC 18S 250 X 4.6 mm

Equilibrate for 20.0 min with buffer A at 1 ml/min

Flow Rate : 1 ml/min

Column Temperature : 26°C

nucleosides [A*] and G*, and collection of the two corresponding fractions,

(iv) desalting of each nucleoside fraction by HPLC as described above,

(v) after evaporation to dryness of the collected desalted fractions, the pure dry compounds [A^*] and G^* were further investigated for structural identification.

Method involving classic DEAE-cellulose column chromatography - Counter current fractions containing 3-6 % initiator $tRNA^{Met}$ were hydrolyzed by pancreatic RNase (Worthinghton, USA) (13). The resulting oligonucleotide mixture was chromatographed on fibrous DEAE-cellulose from Schleicher and Schüll (Dassel, GFR). Elution was performed with a linear gradient from 0 to 0.5 M using a volatile salt, triethylammonium bicarbonate (13,14). A hexanucleotidic fraction containing the dinucleotide A*pG* was collected, evaporated to dryness in a rotatory evaporator under vacuum in order to remove the triethylammonium carbonate salt.

Further hydrolysis of this hexanucleotidic fraction were carried out as

previously described (13-15) with T2-RNase and nuclease P1 to obtain the A*pG* dimer and mononucleotides, and with snake venom phosphodiesterase to give only the mononucleotides. The digests were chromatographed on a DEAE-cellulose column to determine the anionic charges extent of the resulting mono and dinucleotides.

Method involving thin layer chromatography - Thin layer chromatography (TLC) on cellulose plates (F1440 or G1440 from Schleicher and Schüll, Dassel, GFR) was used to separate our dinucleotides and mononucleotides resulting from digestions of yeast initiator $tRNA^{Met}$ samples with T2-RNase, nuclease P1, snake venom phosphodiesterase, or combinations of the three enzymes. The following solvents (15) were used for that purpose :

A - isobutyric acid - 25 % NH_4OH - H_2O (50:1.1:28.9, v/v/v)

B - 0.1 M sodium phosphate pH 6.8 - ammonium sulfate - n-propanol (100:60:2, v/w/v)

C - HCl - isopropanol - H_2O (15:70:15, v/v/v)

Isolation method for Ado-Rib from poly(ADP-Rib)

The poly(ADP-Rib) sample (5 A_{260} units in 150 µl of distilled water) was hydrolyzed for 3 hours at 37°C with 80 µg of snake venom phosphodiesterase (Sigma, St Louis, MO) in 90 µl of 0.5 M Tris buffer pH 8.3 and 20 µl of 10 mM magnesium acetate leading to O-α-D-ribofuranosyl-(1"-->2')-adenosine-5',5"-bis-(phosphate), i.e. Ado(P)-Rib(P). The solution was then submitted for 2 hours at 37°C to the action of 2 units of BAP in 40 µl of 0.5 M Tris-HCl buffer pH 8.3. The final hydrolysate will contain the free nucleoside (Ado-Rib) in a final volume of 300 µl.

After HPLC fractionation of this hydrolysate as described above, the peak corresponding to the Ado-Rib nucleoside was collected. The collected fraction was desalted and concentrated as described above. Finally, the pure dry compound was submitted to the same spectral investigations as [A^*] and G^* isolated from yeast initiator $tRNA^{Met}$.

Derivatization for gas chromatography and mass spectrometry

Sample aliquots of 0.1 A_{260} unit of the nucleosides (approximately 4 µg in 50 µl of distilled water) were used for conversion to volatile trimethylsilyl (TMS) derivatives (16-18). Each sample aliquot was transferred into a capillary tube for TMS derivatization. The capillary tubes were first heated in a furnace at 500°C for 5 hours prior to using, and kept in a clean dessicator. One end of the capillary tubes was sealed with a methane oxygen torch.

The solvent (H_2O) was evaporated to dryness under vacuum at room temperature in a Savant Speed Vac concentrator. Each sample was then dried three times with acetonitrile (ca. 15 µl) to remove traces of water. To the dry nucleoside, were added 7 µl of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (Pierce Chemical Company, Rockford, IL, USA) and 3 µl of pyridine, immediately followed by brief centrifugation for about 10 s to move all liquid to the bottom of the tube. The capillary tube was then sealed, ca. 1 cm from the capillary opening using a methane oxygen torch.

The sample was then converted to volatile TMS derivative by heating at 150°C for 20 min. Five to eight µl of sample solution were used for gas chromatography or direct-probe-MS analysis.

Gas Chromatography

Precise retention times of trimethylsilylated nucleosides were measured by gas chromatography (GC) using a 25 m Durabond DB-1 fused silica capillary column (J and W, Folsom, CA, USA) in a HP 5890 gas chromatograph (Hewlett Packard, Evry, France) equipped with split injector and flame ionization detector. Injector temperature was 270°C ; the column was programmed from 160°C to 300°C at 3°C/min.

Mass spectrometry analysis

Mass spectra of trimethylsilylated nucleosides by electron impact (EI-MS) were performed with a Kratos MS 25 mass spectrometer (Kratos Analytical, Ramsey, NJ, USA) equipped with a DS-55 data handling system. The samples were introduced by direct probe after removal of solvent-reagent in the probe vacuum lock : 70 eV ionizing energy, ion source temperature 200°C, probe temperature from 20°C to 450°C at 100°C/min.

Mass spectra of underivatized nucleosides by fast atom bombardment (FAB-MS) were acquired with a VG Analytical ZAB2-SEQ mass spectrometer equipped with a FAB gun using a neutral cesium beam of some 8 keV energy. High resolution at the multiplier detector was set using a multichannel acquisition system (MCA). The amount placed on the copper target ranged from 10 to 15 µg of nucleoside. The sample was mixed with thioglycerol and placed on the target surface.

RESULTS

Isolation of [A*] and G* nucleosides from yeast initiator $tRNA^{Met}$ by HPLC

Fig. 1a shows the HPLC nucleoside analysis of yeast initiator $tRNA^{Met}$ after exhaustive enzymic digestion by nuclease P1 followed by BAP hydrolysis. The peak eluted at 44 min retention time was designated as N44 unknown

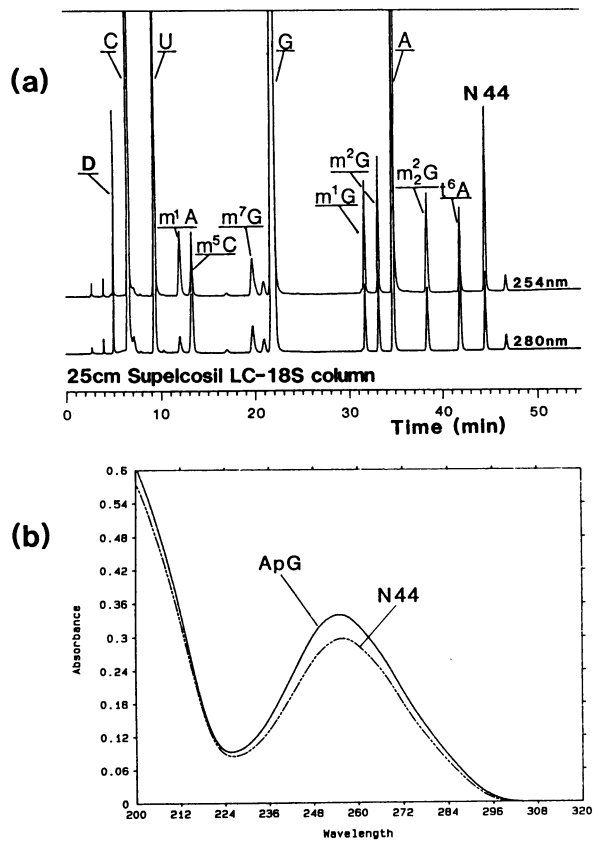


Fig. 1 -(a) : HPLC chromatogram of nucleosides resulting from nuclease P1 plus bacterial alkaline phosphatase digestion of yeast initiator $tRNA^{Met}$. The experimental conditions are described in Materials and Methods. Upper and lower plots correspond to UV-detections at 254 nm and 280 nm, respectively. The peak N44 corresponds to the unknown dinucleotide A*pG* eluted at 44 min retention time.

-(b) : UV-absorption spectrum at pH 7.0 of ApG reference dinucleotide (λmax 255.5 nm), as compared to that of unknown N44 dinucleotide, i.e. A*pG* (λmax 256 nm), isolated from nuclease P1 plus BAP digest of yeast initiator $tRNA^{Met}$.

compound. We examinated the TMS derivative of N44 by EI-MS, and it gave a high mass ion greater than for any mononucleoside. Thus, we concluded that this unknown compound was at least a dimer. Further studies showed that this isolated pure compound was a dinucleotide which was totally resistant to nuclease P1 and ribonuclease T2 hydrolysis. Since it was spectrally similar

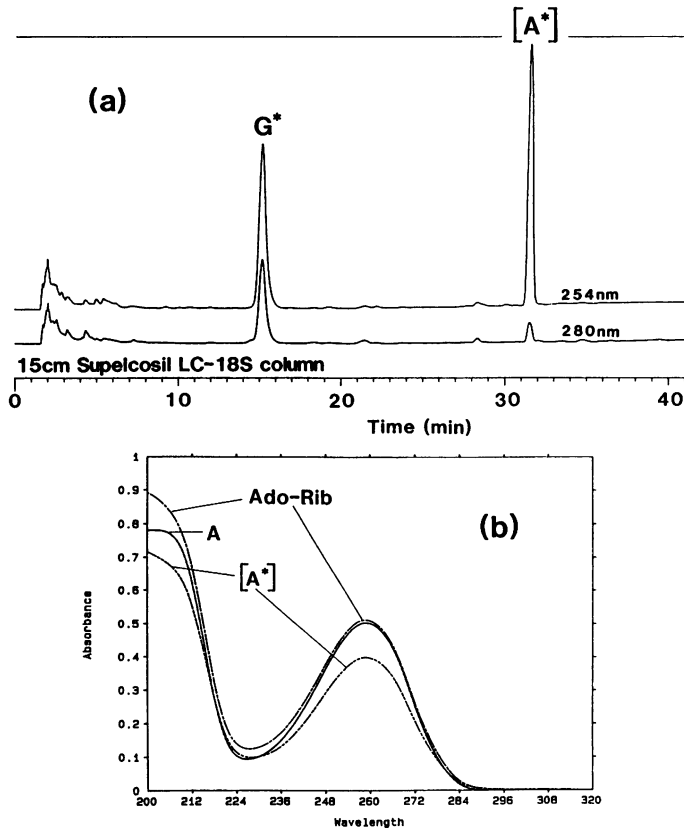
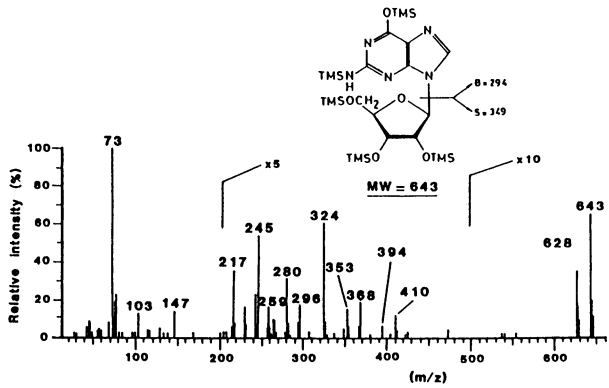


Fig. 2 -(a) : HPLC chromatogram of [A*] and G* nucleosides resulting from snake venom phosphodiesterase plus bacterial alkaline phosphatase hydrolysis of unknown N44 dinucleotide, i.e. A*pG*.
Use of 150 mm x 4.6 mm column. Upper and lower plots correspond to UV-detections at 254 nm and 280 nm, respectively.
-(b) : Comparative UV-absorption spectra at pH 7.0 of reference adenosine (λmax 259 nm), unknown [A*] nucleoside from yeast initiator $tRNA^{Met}$ (λ max 258.5 nm), and Ado-Rib nucleoside from poly(ADP-Rib) (λmax 258.5 nm).

to ApG reference dinucleotide (Fig. 1b), N44 was related to A^*pG^* dinucleotide described by Simsek and Rajbhandary (1)

As shown in Fig. 2a, A^*pG^* yielded two nucleosides in equimolar amount upon enzymic hydrolysis with snake venom phosphodiesterase followed by BAP treatment. These two nucleosides were designated as the unknowns G* and [A*] according to their UV-spectra which were very similar to those of guanosine and adenosine, respectively. G* and [A*] nucleosides were collected, desalted

(a) EI-MASS SPECTRUM OF (TMS) G*



(b) IONS-SERIES FROM THE EI-MS OF (TMS) G*

= ION-SERIES FROM THE EI-MS OF (TMS) G

Mass (m/z)	Positive Ion	Composition	Mass (m/z)	Positive Ion	Composition
643	M	Molecular Ion	410	B + 116	B + H + C_2H_2OTMS
628	M - 15	M - CH_3	394	B + 100	B + C_2H_2ODMS
			368	B + 74	B + H + TMS
259	S - 90	S - TMSOH	353	B + 59	B + DMS
245	S - 104	S - H - CH_2OTMS	324	B + 30	B + CH_2O
217	S - 132	C_3H_3(OTMS)$_2$	296	B + 2	B + H_2
103		CH_2OTMS	280	B - 14	B - CH_2

Fig. 3 : Electron impact mass spectrum (a), and fragment ion-series of G* nucleoside as TMS derivative (b). Fragmentation pathways of G* EI-mass spectrum are rigorously identical to those of reference guanosine.
S = sugar (ribose) moiety ; B = base (guanine) moiety.

and characterized by HPLC retention time, UV-spectrum absorption and MS analysis as described below.

Characterization of G* as guanosine

The identity of G* as unmodified guanosine (Guo) was easily substantiated by comparison of its HPLC retention time and UV spectrum with those of authentic Guo. The EI-MS fragmentation of silylated G* confirmed this structure (Fig. 3). Thus, the G* at position 65 in the T-Ψ-stem of yeast initiator $tRNA^{Met}$ is an unmodified guanosine. Therefore, we will name it G in the following pages.

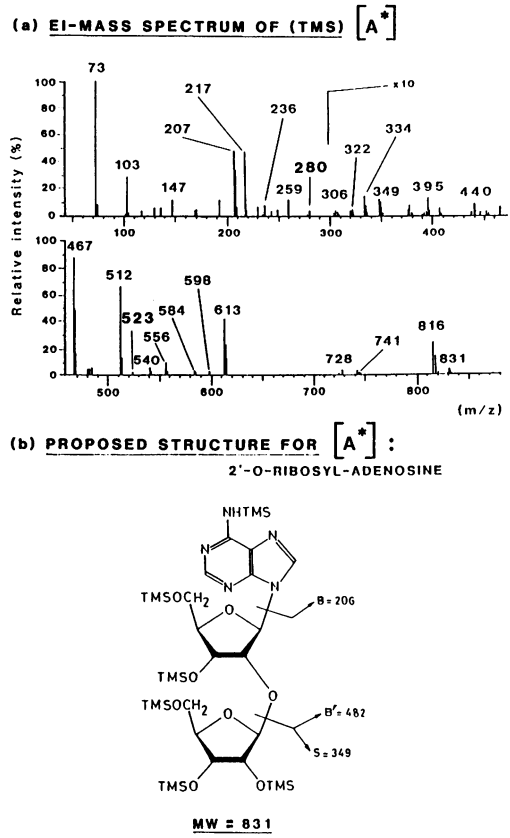


Fig. 4 : Electron impact mass spectrum of [A*] nucleoside as TMS derivative (a),.and proposed structure for [A*] as 2'-O-ribosyl-adenosine (b). This structure was deduced from the fragment ion-series described in Table II. S = sugar moiety ; B' = intact adenosine part ; B = adenine base.

Characterization of [A*] as O-ribosyl-adenosine

The UV-absorption spectrum of [A*] exhibited a profile which was a typical feature for substituted adenosine derivative (see the UV-spectrum of [A*] in Fig. 2b). However, the HPLC retention time of [A*] did not correspond to any of the modified adenosines so far identified in tRNAs. Thus, we have concluded that [A*] nucleoside is an unknown modified Ado.

Principal structural information was gained from the EI-mass spectrum of the silylated [A*], shown in Fig. 4a. Using the fragmentation processes described by Mc Closkey's group (18-21) for EI-MS of trimethylsilylated nucleosides, the chemical structure of [A*] was tentatively determined as O-

TABLE II : Fragment ion series from electron impact mass spectrum of A* nucleoside as trimethylsilylated derivative.

MASS (m/z)	POSITIVE ION	COMPOSITION	MASS (m/z)	POSITIVE ION	COMPOSITION
831	M	Molecular ion	334	B +128	B +C_3H_3OTMS
816	M -15	M -CH_3	322	B +116	B +C_2H_3OTMS
741	M -90	M -TMSOH	306	B +100	B +C_2H_2ODMS
728	M -103	M -CH_2OTMS	280	B +74	B +H +TMS
726	M -105	M -CH_3 -TMSOH	236	B +30	B +H +CHO
			207	B +1	B +H
613	B' +131	B' +$C_2H_2O_2$TMS			
598	B' +116	B' +C_2H_2OTMS	349	S	Ribose
584	B' +102	B' +CHO +TMS	259	S -90	S -TMSOH
556	B' +74	B' +H +TMS	230	S -119	$C_4H_4(OTMS)_2$
540	B' +58	B' +DMS	217	S -132	$CH_3(OTMS)_2$
523	B' +41	B' +C_2HO	103	-	CH_2OTMS
512	B' +30	B' +H +CHO			
467	B' -15	B' -CH_3			

M : intact molecular ion
B': intact adenosine part of the molecule
B : adenine base
S : sugar (ribose) moiety

ribosyl-adenosine (Fig. 4b) from four series of fragment ions observed in the EI-MS of [A*] (Table II). Resulting from decomposition of the molecular ion M^+ at m/z 831, the ion peaks at m/z 816, 741, 728 and 726 of the first series were assigned to the fragments M^+-CH_3, M^+-TMSOH, M^+-CH_2OTMS and M^+-CH_3-TMSOH, respectively. In the second series, eight ion peaks at m/z 613, 598, 584, 556, 540, 523, 512 and 467 were attributed to several cleavages in $ribose_2$ moiety. These ion peaks are specific for the O-ribosyl-adenosine structure by containing the intact adenosine part (B') plus portions of the $ribose_2$ moiety. The ion peaks at m/z 334, 322, 306, 280, 236 and 207 of the third ion series were identical to most of the base-ion peaks obtained by EI-MS of reference adenosine (Ado) as TMS derivative (19). Resulting from the cleavage of the N-C glycosidic bond, these fragment ions were assigned to contain the adenine base (B) plus portions of the $ribose_1$ moiety. Finally, numerous ions such as m/z 349, 259, 217, 103 were observed ; they are characteristic of ribonucleosides having an unmethylated sugar.

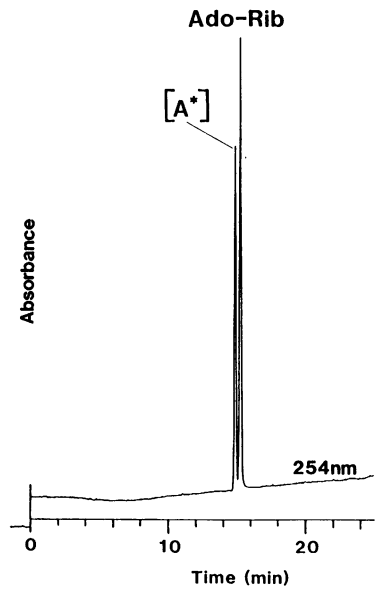


Fig. 5 : HPLC separation of [A*] and Ado-Rib isolated from yeast initiator $tRNA^{Met}$ and poly(ADP-Rib), respectively, by using of desalting HPLC gradient, i.e. elution gradient from 5 % to 70 % methanol aqueous solutions for 30 min run.

All these four ion series are in agreement with the proposed O-ribosyl-adenosine structure for [A*] as $(TMS)_6$ derivative (Fig. 4b).

Comparison of [A*] with authentic Ado-Rib isolated from poly(ADP-Rib)

The purified preparation of the biosynthetic poly(ADP-Rib) yielded authentic O-α-D-ribofuranosyl-(1"-->2')-adenosine (Ado-Rib) upon enzymic digestion with snake venom phosphodiesterase followed by BAP hydrolysis. This nucleoside was compared to [A*] by measurements of the exact molecular mass by FAB-MS, UV-spectral absorption, precise HPLC and GC retention times, and EI-MS fragmentation.

The exact masses of 399.1445 and 399.1424 were determined by high resolution FAB-MS of underivatized [A*] and Ado-Rib, respectively. These values support in both cases an atomic composition which can correspond to a nucleoside structure by including eight cycles and (or) double bonds in the molecule : C_{15} H_{21} N_5 O_8 (theoretical 399.1390). Since this composition corresponds to the known composition of Ado-Rib nucleoside (22), the chemical structure of [A*] is confirmed to be an O-ribosyl-adenosine structure,

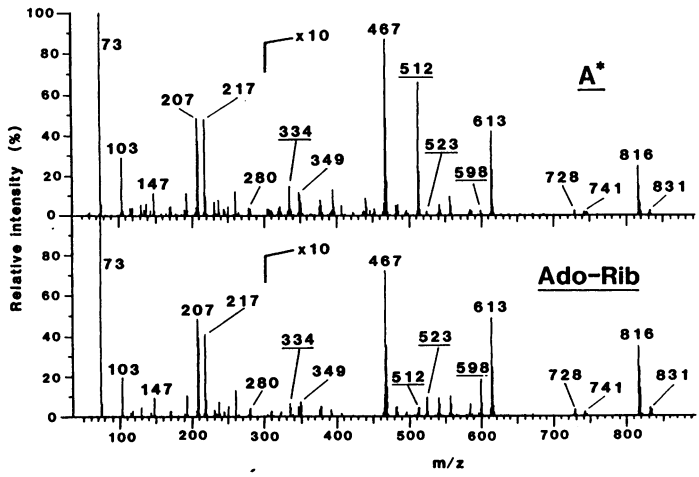


Fig. 6 : Electron-impact mass spectrum of TMS derivative of [A*] nucleoside, i.e. O-ribosyl-adenosine, isolated from yeast initiator $tRNA^{Met}$, as compared to that of TMS derivative of Ado-Rib isolated from poly(ADP-Rib).
The underlined m/z values correspond to the fragment ions having high relative intensity differences between the two EI-mass spectra.

because of already known conclusions from the EI-MS fragmentation of silylated [A*].

The UV-absorption spectra of [A*] and Ado-Rib were strongly similar, as shown in Fig. 2b. The HPLC retention times of [A^*] and Ado-Rib were very close in standard chromatographic conditions. However, the two compounds were separated (Fig. 5), when the chromatography was carried out using the desalting gradient, i.e. from 5 % to 20 % methanol aqueous solution (30 min run). Additional comparison was made by measurements of precise chromatographic retention times using capillary gas chromatography as described in Material and Methods. The elution positions of [A*] and Ado-Rib as TMS derivatives from DB-1 column were 44.26 min and 44.52 min, respectively. Thus, these HPLC and GC results indicated a structural difference between [A*] and Ado-Rib.

This structural difference between the nucleosides [A^*] and Ado-Rib was substantiated by comparing the EI-mass spectra of their silylated derivatives. As shown in Fig. 6, all the ion-series described above for EI-MS of silylated [A^*] are present in the EI-MS of silylated known reference Ado-Rib. However, significative differences are observed for the relative intensities of fragment ions m/z 598, 523, 512 and 334, especially.

From these comparative results, it is assumed that the unknown [A*] nucleoside is an O-ribosyl-adenosine having an isomeric structure partially different from the authentic Ado-Rib isolated from poly(ADP-Rib). This isomeric structure stays to be determined.

Study of A* nucleotide derivatives by DEAE-cellulose column chromatography : presence on A* of a phosphate group as the additional anionic charge.

The above structural studies used small amounts of completely dephosphorylated derivatives. Indeed, HPLC purification was performed on phosphatase treated nucleotides. In order to characterize the additional anionic charge described by Simsek and Rajbhandary (1) on A*pGp (we have proved that the second moiety is guanosine), large amounts of not phosphatase treated A* derivatives were isolated from counter current fractions containing 3-6 % initiator $tRNA^{Met}$. These fractions were hydrolyzed by pancreatic RNase and the resulting digest was chromatographed on a DEAE-cellulose column as described in Material and Methods.

The pancreatic RNase digestion led to a set of oligonucleotides containing GpA*pGpCp, a tetranucleotide which eluted on DEAE ion-exchange chromatography in the hexanucleotidic fraction. This tetranucleotide behaving like a hexanucleotide was further digested with RNase T2 or nuclease P1. The not phosphatase treated digests contained A*pGp or pA*pG, respectively, and these two dinucleotides behaved like tetranucleotides when chromatographed on a DEAE-cellulose column. They were well separated from mononucleotides and from alkali or RNase resistant dinucleotides carrying 2'-O-methyl groups. One further hydrolysis of A*pGp or pA*pG with snake venom phosphodiesterase led to A* or pA*, respectively. Without further phosphatase treatment, A* behaved like a mononucleotide and pA* like a trinucleotide on DEAE-cellulose column chromatography, while additional phosphatase treatment of A* or pA* led in both cases to a unique neutral nucleoside which did not stick to DEAE-cellulose, and behaved like adenosine. Further investigations showed that this nucleoside was identical to the phosphatase treated [A*] nucleoside found by HPLC, i.e. O-ribosyl-adenosine.

From these results, it is clear that the additional charge on not phosphatase treated A* belongs to a phosphorylmonoester group. The exact position of this phosphate group is still under investigation.

Separation of A* derivatives by thin layer chromatography

Using the TLC system on cellulose plates already described in Materials and Methods, we have characterized the positions of the A* derivatives that

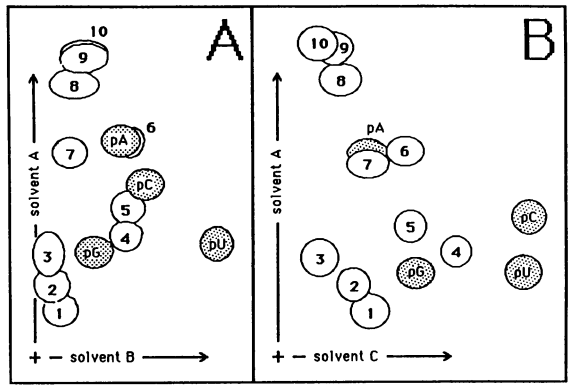


Fig. 7 : Two dimensional TLC separation of A* derivatives isolated from pure yeast initiator $tRNA^{Met}$, or from A* containing oligonucleotides.
A : Use of solvents A and B (see Materials and Methods).
B : Use of solvents A and C (see Materials and Methods).
The spots of the major nucleotides are dotted.
The numbered spots correspond to : 1:pA*pG, 2:A*pGp, 3:A*pG, 4:pA*, 5:Ado(P)-Rib(P), 6:A*, 7:Guanosine, 8:[A*], 9:Ado-Rib, 10:Adenosine (for details, see text).

we obtained enzymatically from pure initiator $tRNA^{Met}$ or from oligonucleotides prepared by DEAE-cellulose column chromatography.

These fragments are : (i) not phosphatase treated pA*pG, A*pGp, A*pG, pA* and A*, and (ii) phosphatase treated A*, i.e. [A*]. Their positions relative to the four major nucleotides are drawn on Fig. 7. In addition, the pattern shows also the exact position of Ado(P)-Rib(P), which is the major constituent of poly(ADP-Rib), as well as its phosphatase treated counterpart (Ado-Rib).

Spots 1, 2 and 3 located near the origin correspond to A*pG derivatives. They differ by the number and localisation (5' or 3') of the external phosphate(s). The removal of the guanylic acid from pA*pG by venom phosphodiesterase or from A*pGp by nuclease P1 followed by venom phosphodiesterase treatment leads to two completely distinct spots : pA* (spot 4) and A* (spot 6), respectively. In our conditions, pA* migrates in the Ado(P)-Rib(P) area (spot 5) whereas A* migrates like pA meaning that A* could carry one additional phosphate group. The phosphatase treatments of pA* (spot 4) and A* (spot 6) confirmed the presence of this phosphate group by leading to one unique nucleoside [A*] (spot 8). This nucleoside migrates in the adenosine (spot 10) and Ado-Rib (spot 9) area.

The slight difference of migration between [A*] and Ado-Rib is related to the close structural relation of the two compounds already established by the above HPLC, GC and EI-MS results.

DISCUSSION

The unknown A*pG* dinucleotide that we isolated from a very pure yeast methionine initiator tRNA after enzymic digestion with nuclease P1 and bacterial alkaline phosphatase is related to the not phosphatase treated dinucleotide A*pG*p reported by Simsek and Rajbhandary (1). This dinucleotide is located at positions 64 and 65 in T-Ψ-stem. The characterization of A* and G* was based on approximately 4 μg of material prepared from a snake venom phosphodiesterase hydrolysate of the A*pG* dinucleotide. We identified the unknown G* in position 65 as unmodified guanosine (G). The structure of the unknown A* was established in this paper as an isomeric form of O-ribosyl-adenosine which contains one phosphate group.

The dephosphorylated A* nucleoside, i.e. [A*], was compared chromatographically and spectrometrically with authentic Ado-Rib that we isolated from poly(ADP-Rib). We found that [A*] and Ado-Rib are not identical. According to our UV-spectral and EI-MS data, the difference of chemical structure between [A*] and Ado-Rib can be only a difference of isomerism. Poly(ADP-Rib) is a nucleic acid-like biopolymer of Ado-Rib residues linked through α(1"-->2')-ribose$_2$-ribose$_1$ glycosidic bonds (23,24). In the [A*] nucleoside, ribose$_2$ and ribose$_1$ could be linked through, either α(1"-->3'), or β(1"-->3'), or β(1"-->2') glycosidic bond. By comparing the EI-MS fragmentation pathways of [A*] to those of Ado-Rib, a β(1"-->2')-ribose$_2$-ribose$_1$ linkage in the chemical structure of [A*] seems more likely than an α- or a β-(1"-->3') glycosidic bond. Furthermore, an O-alkylation at the 2' position on ribose$_1$ is the nucleosidic modification which is most compatible with the usual linkage of nucleotides in tRNAs (3',5'-phosphodiester bonds). Investigations are in progress to confirm this β(1"-->2') spatial configuration for [A*] by using nuclear magnetic resonance spectroscopy.

On the other hand, the net charges of pA*pG and A*pGp dinucleotides isolated from successive enzymic hydrolysates of yeast initiator tRNA^{Met} without phosphatase treatment were identical to those of tetranucleotides. Additional anionic charges on the A* derivatives were confirmed by the retention time on DEAE-cellulose column chromatography of not phosphatase treated A* and pA* which behaved like a mononucleotide and a trinucleotide,

respectively. Further phosphatase treatment of A* and pA* brought evidence for a phosphorylmonoester group as providing the additional anionic charges. Investigations are in progress to determine the exact position of this phosphate group on the ribose moiety of O-ribosyl-adenosine.

The most significant result from this research is the identification of a phosphorylated O-ribosyl-adenosine as a minor nucleotide in yeast initiator $tRNA^{Met}$. From this result, it can be assumed that the O-ribosyl-adenosine found by Hall (2,3) in yeast RNA fractions was not a poly(ADP-Rib) contaminant of RNA isolation, but a nucleoside constituent of yeast initiator $tRNA^{Met}$, especially since poly(ADP-Rib) has not yet been found in yeast (25). The phosphorylated O-ribosyl-adenosine unit is located in the T-Ψ-stem of yeast initiator $tRNA^{Met}$, at position 64. It must be emphasized that such a modified nucleoside has not yet been identified in any tRNA sequence.

We do not know the real meaning of this modification in yeast initiator $tRNA^{Met}$ which is the only tRNA carrying this modification among 39 initiator tRNAs so far sequenced. Some eucaryote initiator tRNAs carry Gm at the same position 64, but this is not a general feature. Therefore, we wonder if the described modification has a discriminator role in initiation - elongation processes, or if a different function has to be found for such a modificatiion. Studies to elucidate the role of phosphorylated O-ribosyl-adenosine will be considered as soon as the rigorous chemical structures of A* and pA*pG have been defined.

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Abbreviations : initiator $tRNA^{Met}$ = methionine initiator tRNA; A* = phospho rylated O-ribosyl-adenosine; [A*] = phosphatase treated A* = O-ribosyl-adenosine; poly(ADP-Rib) = poly(adenosine diphosphate ribose); Ado-Rib = O-α-D-ribofuranosyl-(1"-->2')-adenosine; Ado(P)-Rib(P) = O-α-D-ribofuranosyl-(1"-->2')-adenosine-5',5"-bis(phosphate).

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