The aminoacceptor stem of the yeast $tRNA^{Lys}$ contains determinants of mitochondrial import selectivity

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Abstract Yeast $tRNA^{Lys}_{CUU}$ is nucleus-encoded and is partially imported into the mitochondria. Another lysine isoacceptor, $tRNA^{Lys}_{SUU}$, is also nucleus-encoded but is not imported. These two tRNAs differ in 21 bases. We have previously localised import selectivity determinants in the anticodon arm. By in vitro import of mutant transcripts and by expression of mutant tRNA genes in vivo we show here that the first base pair (1:72) and the discriminator base 73 are also relevant to import selectivity. Replacement of bases 1:72 in $tRNA^{Lys}_{SUU}$ by those of $tRNA^{Lys}_{CUU}$ makes it importable with a transport efficiency similar to natural.

Key words: tRNA; Mitochondrial import; Aminoacceptor stem; *Saccharomyces cerevisiae*

1. Introduction

Mitochondrial import of tRNA is found in plants, protozoans and yeast [1–3]. The number of imported species of tRNA varies from one organism to another and ranges between one in yeast and the totality in trypanosomatids. The mechanism of tRNA import also seems to differ in different systems: in trypanosomes it was suggested that tRNAs are imported via interaction with an RNA-specific membrane protein [4], while in yeast co-import with the precursor of mitochondrial aminoacyl-tRNA synthetase via the pre-protein channel was proposed [5]. tRNA determinants of import selectivity were studied in trypanosomatids, where the D-loop region was found to be crucial [6], and in *Tetrahymena*, where the anticodon sequence determined the import [7]. No evident 'tRNA-import signature' was found in plant tRNAs [3]. In yeast a single species, $tRNA^{Lys}_{CUU}$ (further referred to as tRK1), is imported. The second cytoplasmic isoacceptor lysine-tRNA, $tRNA^{Lys}_{SUU}$ (tRK2), is localised exclusively in the cytoplasm, while the third one, $tRNA^{Lys}_{U*UU}$ (tRK3), is encoded by the mitochondrial DNA and is localised exclusively in the mitochondria [1]. We have previously shown that $tRNA^{Lys}_{CUU}$ and the majority of its in vitro synthesised mutant derivatives are imported after aminoacylation with the cognate cytoplasmic lysyl-tRNA synthetase, KRS. Interaction with the precursor of the mitochondrial lysyl-tRNA synthetase, pre-MSK, is also a prerequisite for import [5]. We suggest that the role of aminoacylation in import is to induce a conformational change in tRK1 to facilitate its binding to pre-MSK, which is likely to act as a carrier for mitochondrial translocation of the tRNA. One region important for determination of import selectivity is shown to be the anticodon arm, and especially position 34 (first position of the anticodon). Our previous results also indicate that the anticodon is not the only position affecting import efficiency [8]. Here we present a systematic study in vitro and in vivo of the role of the bases in the aminoacceptor helix of tRNA for mitochondrial import selectivity.

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Abbreviations: tRK1, cytoplasmic $tRNA^{Lys}_{CUU}$; tRK2, cytoplasmic $tRNA^{Lys}_{SUU}$; tRK3, mitochondrial $tRNA^{Lys}_{U*UU}$; KRS, cytoplasmic lysyl-tRNA synthetase; MSK, mitochondrial lysyl-tRNA synthetase; IDP, import directing proteins

2. Materials and methods

2.1. Strains, cloning and mutagenesis

Saccharomyces cerevisiae strain YPH499 [9] was used for isolation of mitochondria and in vivo expression of tRK2 mutant versions. In vitro import was directed by protein extracts (IDP, import direction proteins) from a pre-protein import deficient strain, $\Delta TOM20_{IRV}$ [10] carrying the plasmid pG11T6 with the MSK1 gene [11] to overexpress the precursor form of MSK. The procedure of IDP preparation was described elsewhere [5,8]. *Escherichia coli* DH5αF′ and CJ236 were used for cloning and mutagenesis. Cloning, isolation, analysis and sequencing of recombinant DNA were performed by standard methods [12]. The tRK1 gene was cloned by PCR from pY109 into M13mp18 under the control of a T7 promoter as described [8]. The tRK2 gene from plasmid pFD17 was subcloned in a similar way but the first residue of the tRNA coding sequence T1 was changed to G to allow efficient in vitro transcription and, accordingly, residue A72 was changed to C to restore base pairing at the end of the acceptor stem (see Fig. 1). In addition, the 23 bp intron of the tRK2 gene was removed by PCR deletion mutagenesis [8]. For in vivo expression of tRK2 mutant versions, the entire coding sequence with its intron and 50 bp of 5′ and 3′ flanking sequences was PCR-cloned into the pRS416 vector. Oligonucleotide-directed mutagenesis was performed by the method of Kunkel [13].

2.2. In vitro transcription and aminoacylation

*Bst*NI-linearised DNAs (to create the CCA 3′-terminal sequence) were transcribed with T7 RNA polymerase by a standard procedure [14]. Transcripts were gel-purified, dephosphorylated with calf intestine phosphatase and labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Gel-purified transcripts were refolded by a heat denaturation-renaturation cycle in the presence of 0.5 mM $MgCl_2$. Aminoacylation was done with purified yeast cytoplasmic lysyl-tRNA synthetase (KRS) as described [8].

Total yeast tRNA was prepared by phenol extraction. tRK1 and tRK2 were purified by reverse-phase chromatography followed by preparative gel electrophoresis and verified by partial sequencing and oligonucleotide hybridisation.

2.3. pre-MSK binding and tRNA import in vitro

The pre-MSK binding test was done mainly as described before [8]. Pre-MSK was overexpressed in the *Pichia pastoris* expressing system (Invitrogen) and purified from the culture medium by DEAE chromatography. Aliquots of 5′-^{32}P-labeled transcripts (or tRNAs) were mixed with 0.5 μg of pre-MSK or 20–50 μg of IDP in 50 μl of the standard import mixture (without mitochondria) and incubated for 10 min at 30°C. The mixture was centrifuged to remove insoluble aggre-

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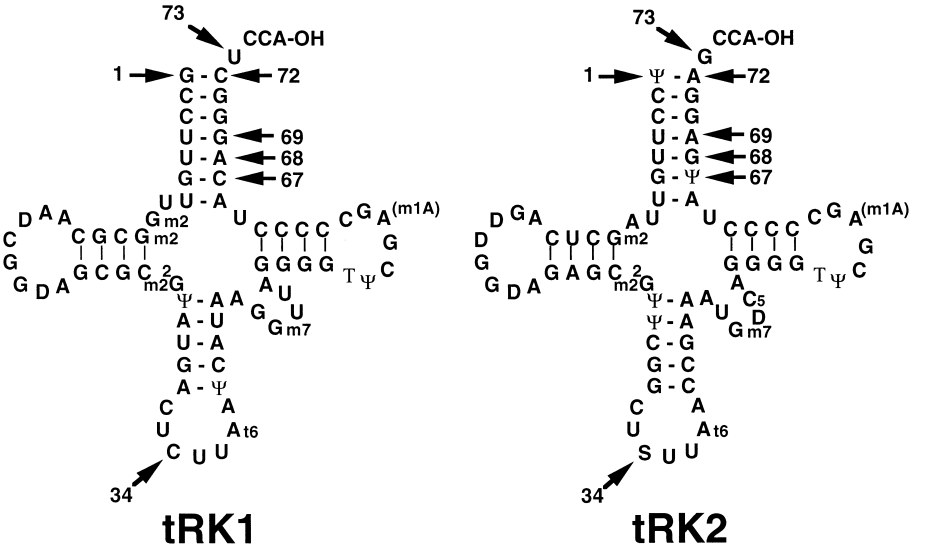

Fig. 1. Cloverleaf structures of tRK1 and tRK2 (based on [8]). Bases that differ between the two isoacceptors in the anticodon loop and aminoacceptor stem are indicated with arrows.

gates and 50 μl of lysis buffer (0.1% NP-40, 0.1 M Tris-HCl pH 7.5) and 1 μl of an appropriate dilution of anti-MSK antibodies were added to the supernatant. After incubation for 1 h on ice, 10% v/v of protein A-Sepharose (Pharmacia) was added and the mixture was shaken for 1 h at 4°C. Sepharose beads were collected by centrifugation, washed with lysis buffer and the radioactivity determined by Cerenkov counting. As a control, the same assay was done with pre-immune antibodies. The non-specific binding was on average $< 5\%$ of tRK1 binding.

The standard import reaction (100 μl) contained freshly isolated mitochondria (100 μg of mitochondrial protein), 3 pmol of 5′-^{32}P-labeled aminoacylated tRNA and 20–50 μg of IDP and 0.1–0.5 μg pre-MSK in 0.44 M sorbitol, 20 mM HEPES (pH 6.8), 20 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, 5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 0.1 mM diisopropyl fluorophosphate, 0.5 mM phosphoenolpyruvate and 4 units of pyruvate kinase. The import was carried out at 30°C for 20 min. Mitochondria were then treated with a mixture of RNases [15,16], mitoplasts were generated by hypotonic shock (10 mM HEPES, pH 6.8, 10 min at 0°C) and re-purified after addition of sorbitol to 0.44 M. Mitoplasts were lysed in 1% SDS, 0.1 M sodium acetate (pH 4.8) and 0.05% diethyl pyrocarbonate at 60°C and mitochondrial RNA was phenol-extracted at the same temperature. Imported tRNAs were analysed by denaturing 13% polyacrylamide gel electrophoresis and autoradiography. Quantitation of tRNA import was done by scanning autoradiographs onto a Phosphor Imager (Fuji, Bas-2000).

2.4. In vivo import quantitation

YPH499 cells were transformed by pRS416 carrying mutated versions of the tRK2 gene. Various amounts ranging from 0.01 to 1 μg of total and mitochondrial tRNA were dot-transferred onto nylon (Hybond-N) membrane. For this purpose isolation of mitochondria included an RNase treatment step and sucrose gradient centrifugation as described before [15,16]. Mutant tRNAs were detected by hybridisation with oligonucleotide probes ^{32}P-labeled with polynucleotide kinase. The following oligonucleotide probes were used: for detection of tRK1, 5′-AACCTTATGATTAAGAGT-3′; tRK2, GACATTTCGGTTAAAAG; tRK3, CAAGCATGGGTTGCTTAAAAG; tRK2 Ψ1:A72→G1:C72 mutation, TGGCGCCTCATAGGGGG; tRK2 S34→C mutation, GCGCTTAAGAGCCGAACGC; tRK2 G73→U73 mutation, TGGAGCCTCATAGGGGG. Hybridisation was done as described elsewhere [17]. Filter wash conditions were optimised for each probe. Radioactive signals were detected and quantitated onto Phosphor Imager. Expression and import were calculated taking into account signals obtained with probes which do not contain mismatches with mutated tRNAs. The signal corresponding to tRK1 was taken as 100% of expression. The amount of mutated tRNAs in the mitochondria was calculated in relation to the amount of tRK3. Obtained values were compared to the previously determined efficiency of expression (52% for tRK2 versus tRK1) and import (20% for tRK2-U34→C versus 100% of tRK1) [8].

3. Results

3.1. In vitro import of tRK1 and tRK2 mutant transcripts

The aminoacceptor stem of tRK1 differs from that of tRK2 in positions 1, 67–69, 72 and 73 (Fig. 1). We have constructed a panel of mutant transcripts which contained a backbone of tRK1 with bases corresponding to the sequence of tRK2 at these specific positions and vice versa (Table 1). One additional position, 34, was also mutated. This is the first position of the anticodon which was previously found to be one of the import determinants [8]. All the mutant transcripts were analysed for their ability to be imported into isolated yeast mitochondria, for aminoacylation with KRS, which was previously found to be crucial for the import of a majority of transcripts, and for pre-MSK binding, which was shown to be a pre-requisite for import [5].

The results are presented in Fig. 2 and Table 1. In agreement with our previous results, all imported transcripts retain pre-MSK binding capacities. Replacement of bases 67–69 in the context of tRK1 blocks pre-MSK binding and the import

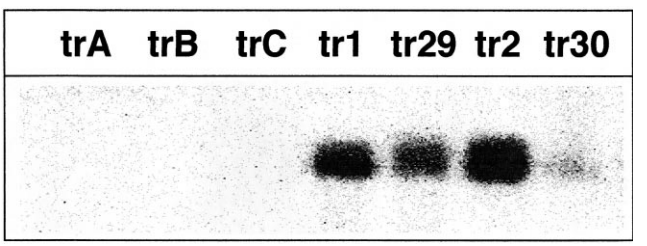

Fig. 2. Import of mutant tRK1 and tRK2 transcripts into isolated mitochondria. Autoradiographic detection of 5′-^{32}P-labeled transcripts after separation on a denaturing polyacrylamide gel. 'tr', in vitro synthesised transcripts. See Table 1 for description of mutants.

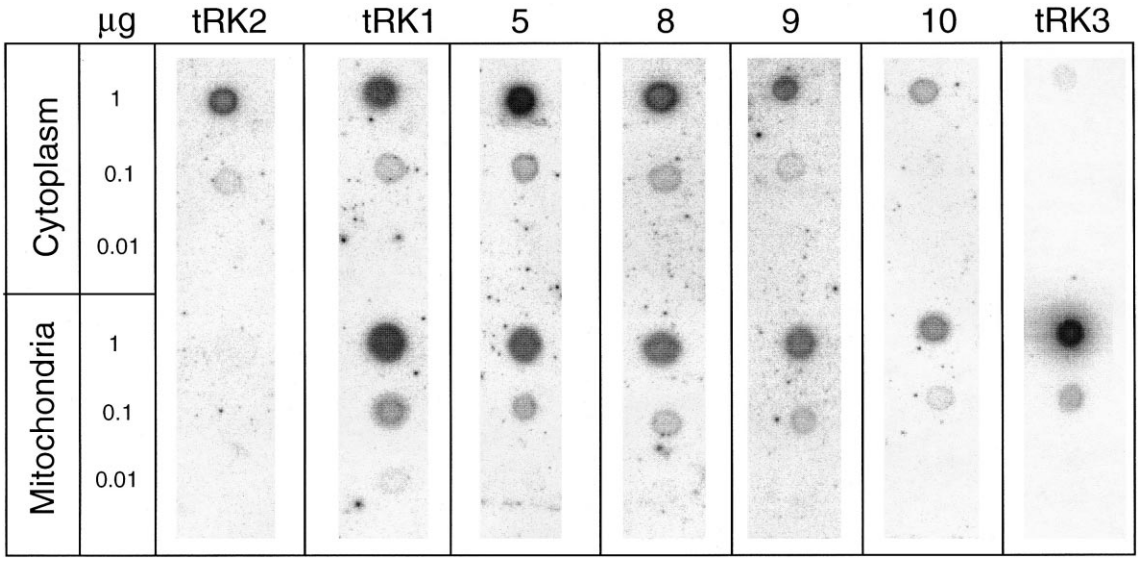

Fig. 3. In vivo import of tRK2 mutant versions. Dot hybridisation of total (Cytoplasm) and mitochondrial (Mitochondria) tRNA extracted from YPH499 cells harbouring corresponding mutant genes with mutation-specific oligonucleotide probes. 'µg', the amount of tRNA dotted; '5', '8', '9' and '10', numbers of tRK2 mutant versions (see Table 2 for their description); tRK1, 2 and 3, control hybridisations with normally imported (tRK1), non-imported (tRK2) and host mitochondrial (tRK3) tRNAs used for import efficiency evaluation (see Section 2 for details).

(transcripts C and 40). However, replacement of bases 67–69 in the context of tRK2 does not lead to its import (transcripts A and B), which suggests that in the tRK2 context these bases are not 'import determinants'. Changing of the discriminator base in tRK1 (transcript 29) does not significantly affect the import. On the other hand, replacement of the discriminator in tRK2 leads to the import of a mutant version with a similar efficiency as tRK1 (transcript 93). Transcript 93 is poorly aminoacylated but retains pre-MSK binding capacity in deacylated form. This transcript, similar to all other in vitro synthesised tRK1 and tRK2 derivatives, contains two additional mutations: to permit T7 transcription the first base pair Ψ:A was replaced by G:C. The natural first Ψ:A pair of the tRK2 aminoacceptor stem is a weakly interacting base pair. In order to verify if the stability of the first pair is in fact important, we tested versions with a weak G:U pair in the first position of the tRK1 and tRK2 aminoacceptor stem. In the context of tRK2 this replacement was done simultaneously with the replacement of the discriminator base, similar to the well imported transcript 93. The resulting transcripts A, B and C can be aminoacylated by KRS but lose pre-MSK binding and import capacities. Transcript 33 loses both aminoacylation ability and pre-MSK binding in deacylated form.

The results obtained clearly indicate that the stability of the first base pair in tRNA molecule can, indeed, influence the interaction with pre-MSK and, therefore, the mitochondrial import. It was not possible to verify the importance of the first nucleotide in tRK2 by experiments in vitro, since U in position 1 inhibits T7 transcription of the gene. For that purpose, we used an alternative in vivo approach.

Table 1
Aminoacylation, MSK binding and import of tRK1 and tRK2 mutant transcripts

tRNA	tRNA context of the transcript	Bases				MSK binding[a]		Aminoacylation			Import[c]	Ref.
		1:72	34	67–69	73	+KRS	−KRS	K_m (µM)	V_{max} (pmol/min)	V_{max}/K_m[b]		
tRK1	–	G:C	C	CAG	U	100	5	1.4	2600	16.20	100	[8]
tRK2	–	Ψ:A	S	ΨGA	G	<5	15	1.5	2500	14.50	0	[8]
tr1	tRK1	G:C	C	CAG	U	85	20	2.2	250	1.00	120	[8]
tr2	tRK2	G:C	C	UGA	G	100	40	1.5	450	2.60	150–200	[8]
tr29	tRK1	G:C	C	CAG	G	75	45	6.7	290	0.40	75–100	[8]
tr40	tRK1	G:C	C	UGA	U	10	5	1.8	5	0.03	<5	[8]
trC	tRK1	G:U	C	UGA	U	5	15	0.5	15	0.25	<5	this work
tr30	tRK2	G:C	U	UGA	G	20	20	1.2	15	0.10	5–10	[8]
tr33	tRK2	G:U	C	UGA	U	30	<5	4.2	10	0.02	<5	[8]
tr93	tRK2	G:C	U	UGA	U	45	100	5.9	10	0.02	75–100	[8]
trA	tRK2	G:U	U	CAG	U	<5	<5	0.7	70	0.85	<5	this work
trB	tRK2	G:U	U	CAG	–	10	<5	0.7	45	0.55	<5	this work

[a]MSK binding of the aminoacylated tRK1 is taken as 100%; +/−KRS, with or without aminoacylation prior to pre-MSK binding assay.
[b]Relative, V_{max}/K_m of transcript 1 is taken as 1.00.
[c]tRK1 import is taken as 100%.

Table 2
In vivo import of tRK2 mutant derivatives

	tRK1	tRK2	tRK3	Mutant versions of tRK2							
				3[d]	4	5	6	7	8	9	10
Anticodon	CUU	SUU	U*UU	U[e]UU	CUU	U[e]UU	U[e]UU	U[e]UU	CUU	U[e]UU	CUU
1:72 pair	G:C	Ψ:A	G:C	U:A	U:A	G:C	G×A	U:C	G:C	G:C	G:C
73 base	U	G	A	G	G	G	G	G	G	U	U
Cytoplasm[a]	100	30	1	52	55	110	1	70	45	25	20
Mitochondria[b]	45	–	100	–	5	27	–	–	12	10	10
Import efficiency[c]	100	–	–	–	20	55	–	–	60	90	110

[a]Total cellular pool, tRK1 signal is taken as 100%.
[b]Mitochondrial pool, tRK3 signal is taken as 100%.
[d]Efficiency of import was calculated as the ratio of mitochondrial to the total cellular pools and is expressed in percentage of the tRK1 import efficiency.
[d]tRK2 gene without mutations on the pRS416 vector.
[e]Presence of the base modification in the first anticodon position of mutant tRNAs was not tested.

3.2. *In vivo import of mutant tRK2 versions*

Yeast mitochondria normally import tRK1. That makes it difficult to quantitate the import of mutated tRK1 versions by hybridisation with oligonucleotide probes because of cross-hybridisation with natural tRK1. We therefore did all the in vivo analyses with mutant tRK2 versions. In fact, some mutations in tRK2, e.g. S34→C in the first position of the anticodon, confer on this normally non-imported tRNA a capacity for import [8]. Various versions of the tRK2 gene with replacements at positions 1:72 and 73 were constructed in a background of either U34 or C34 and introduced into yeast onto a centromeric vector. The mutant tRNAs were analysed for presence in the cell and in mitochondria, and efficiencies of their import were calculated (Table 2 and Fig. 3).

The normal tRK2 gene expressed on pRS416 (version 3) changes the steady-state concentration of tRK2 in the cell only slightly (Table 2), which may be due to a transcriptional control. The mutant versions of tRK2 are expressed ranging from 20 and 110% in comparison with tRK1. One version of tRK2 (No. 6) is not expressed. This tRNA contains a mispairing in the 1:72 positions (GxA) which might influence its processing and/or stability. Another version with UxC mispairing at the end of the aminoacceptor stem (No. 7) is well expressed, but not imported into mitochondria. This result is in agreement with our in vitro data, suggesting that the stability of the first base pair influences the import efficiency. Furthermore, introduction of a strong G:C pair in the tRK2 context leads to import of this tRNA, even with U34 in the anticodon sequence (version 5). This is clearly in favour of a determinant role of the first pair of bases for import. Version 5 with this replacement only is present in mitochondria in amounts comparable to those of tRK1. However, since its overall expression is higher, the efficiency of import was estimated to be 55%. Introduction of an additional mutation in the discriminator position 73 (version 9) decreases the expression, but increases the import efficiency, which becomes almost the same as for tRK1 (Table 2). Version 8, which differs from version 5 by C in position 34, has almost the same import efficiency. Finally, version 10 carrying all three changes, C34, G1:C72 and U73, has the lowest level of expression but a higher import efficiency. Since the efficiency of import of this version is similar to that of tRK1, we suggest that bases C34, G1:C72 and U73 act as an 'import signature' in the tRK2 context.

4. Discussion

We analysed here the role of the bases in the aminoacceptor stem of yeast lysine-tRNAs in modulation of their mitochondrial import. The first base pair is shown to be crucial for import selectivity, since the G:C pair specifies the import of tRK2, which is normally not imported, both in vitro and in vivo. The input of other bases of the aminoacceptor stem seems to refer only to the efficiency of import and is context-dependent. Position 73 at the end of the acceptor stem, which is referred to as the discriminator base, has been shown to contribute to the identity of virtually ever tRNA species and to influence the structure of the whole acceptor helix and particularly the stability of the first base pair onto which it is stacked [18,19]. G73 was assumed to favour dissociation of the first base pair [19]. Our in vitro and in vivo results also suggest that G73 reduces the efficiency of mitochondrial import, probably due to its influence on the interaction at positions 1–72. Positions 67–69 of tRK1 are involved in interaction with pre-MSK, since their replacement by those of tRK2 and vice versa resulted in a decrease of efficiency of pre-MSK binding and the total loss of mitochondrial import capacity independently of aminoacylation abilities.

It is interesting to note that the bases crucial or important for mitochondrial import are localised in the anticodon loop and the aminoacceptor stem. These are precisely the main regions of the tRNA molecule which normally interact with the cognate aminoacyl-tRNA synthetase [20]. However, if the domains normally important for interaction with aminoacyl-tRNA synthetase and for mitochondrial import are similar, the set of nucleotides involving in aminoacylation and import specificity is different. In fact, neither C34 nor G1:C72 bases are aminoacylation identity elements, since they are different among the two lysine isoacceptors, tRK1 and tRK2, but they are shown to be relevant to the import selectivity. This fact can be explained by the non-canonical character of the interaction between the carrier protein pre-MSK, precursor of the mitochondrial lysyl-tRNA synthetase, and tRK1. Although the cytoplasmic precursor of a mitochondrially imported protein is not a cognate aminoacyl-tRNA synthetase for tRK1 and tRK2 (the cognate enzyme being KRS), it is an aminoacyl-tRNA synthetase. We suggest that the specific character of interaction between pre-MSK and tRK1 might involve the same domains of tRNA as for the normal type of interaction

aminoacyl-tRNA synthetase tRNA, while a set of import-specific bases, C34, G1-G72 and U73, provides import selectivity.

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