**In silico motif analysis suggests an interplay of transcripational and translational control in mTOR response**

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Abbreviations: TSS, transcription start site(s); OP, oligopyrimidine tract(s) (DNA motif); TOP, terminal oligopyrimidine tract(s) (mRNA motif); mTOR, mammalian target of rapamycin; UTR, untranslated region

**The short 5'-terminal oligopyrimidine tract (TOP) of 5' UTRs is a well-known regulatory sequence motif of mRNAs that are subject to growth-dependent translation. Specifically, translation of TOP mRNAs is regulated by the mTOR signaling pathway that is involved in cell proliferation, cancer development and aging. High throughput data permit detailed study of specific features of the mRNA TOP motif and its DNA origins at transcription start sites (TSS). Recently, ribosome profiling was used to identify mRNA targets of the mTOR pathway in PC3 cells. A novel pyrimidine-rich translational element (PRTE) was reported to play a key role without positional preferences within the 5' UTRs, unlike 5' TOP, which are strictly located at the 5' ends. In this study, we couple recently reported ribosome profiling data on the mTOR mRNA targets with the annotation of TSS obtained by HeliscopeCAGE. We confirm the canonical TOP and strong positional preferences of respective oligopyrimidine tracts (OP) straddling the experimentally validated TSS regions at the DNA level. Such OP localization ensures that transcription from OP segments creates the 5'-terminal TOP in the corresponding mRNAs. We demonstrate that OP are not overrepresented in downstream regions of 5' UTRs of mTOR targets. Finally, we highlight several mTOR target genes with broad and multimodal TSS spanning dozens of nucleotides that are only partially covered with an OP. Therefore, in such cases only a fraction of all produced mRNAs carry a TOP regulatory motif and, thus, respond to mTOR via TOP mechanism. We hypothesize that the interplay between transcription and translation may play a crucial role in the regulation of the mTOR response.**

**Introduction**

The mammalian target of rapamycin (mTORC1) is one of major regulators of cell growth and proliferation, including protein biosynthesis, in higher eukaryotes.1,2 An important role of the mTOR pathway in cancer development pushes forward studies on mTOR translational regulation.3,4 Sequence features of 5' UTRs regulated by mTOR, have been studied extensively.5 Twenty years ago, it was discovered that the short 5'-terminal oligopyrimidine tract (TOP) of 5' UTRs plays an important role in translational regulation of ribosomal genes.6,9 Many of corresponding mRNAs carry evolutionary conserved TOP motif,10 characterized as cytidine followed by an uninterrupted oligopyrimidine sequence of 5–14 nt, which are located at the 5' ends of 5' UTRs.5

In parallel, the TCT-promoters were discovered as a specific class of promoter sequences. Normal transcription from a TCT-promoter depends on the TCT-motif localized at transcription start site (TSS).11,12 This suggests a possible interplay between transcription and translation through a double-purpose pyrimidine-rich motif. Furthermore, commonly used gene annotations do not always correctly localize TSS, which must be taken into account when studying terminal 5' UTR sequences.13 High-throughput ribosome profiling experiments challenged the assumption that the mTOR mRNAs carry terminal TOP motif suggesting that some mRNAs may contain nearly terminal TOP-like motifs14 or even downstream pyrimidine-rich translational elements (PRTE).15 Databases of experimentally determined TSS, such as DBTSS (http://dbtss.hgc.jp/),16 were
complicating the picture, having limited agreement with the annotated TSS while providing rather sparse data.

A new source of quantitative data on TSS appeared with HeliScopeCAGE, Cap Analysis of Gene Expression using Helicos single-molecule sequencing. HeliScopeCAGE allowed high-throughput quantitative analysis of TSS at a single-nucleotide resolution. Thus, it is tempting to couple high-throughput translational (ribosome profiling) and transcriptional (HeliScopeCAGE) data to study the sequence motifs involved in the mTOR translational response.

To further elucidate the role of oligopyrimidine motifs in mTOR regulation we, first, confirm the TOP motif by de novo motif discovery in 5' UTRs of mTOR target genes and highlight oligopyrimidine tracts, OP, straddling respective TSS. Second, we demonstrate that a commonly used TSS annotation is not precise enough to study features of terminal sequences in 5' UTRs. Third, we apply a motif finding approach to the 5' UTRs of mTOR targets and demonstrate significant OP enrichment in the vicinity of TSS and no significant enrichment in the downstream segments of the 5' UTRs. Fourth, we describe different OP motif subtypes for broad and narrow TSS. A possible functional role of broad and multimodal TSS only partially covered by OP is discussed.

**Results**

**Known TOP motif confirmed by de novo motif discovery**

By de novo motif discovery we have found a CT-rich motif similar to the mRNA TOP using 250 5' UTRs extended upstream by additional 100 nt to take into account possible TSS misannotation. The sequences corresponded to different 5'UTRs of UCSC annotated transcripts, OP, straddling respective TSS. Second, we demonstrate that a commonly used TSS annotation is not precise enough to study features of terminal sequences in 5' UTRs. Third, we apply a motif finding approach to the 5' UTRs of mTOR targets and demonstrate significant OP enrichment in the vicinity of TSS and no significant enrichment in the downstream segments of the 5' UTRs. Fourth, we describe different OP motif subtypes for broad and narrow TSS. A possible functional role of broad and multimodal TSS only partially covered by OP is discussed.

**OP positional preferences**

To study OP positional preferences we assessed the OP location relative to the verified TSS. We counted the number of 5' UTRs with OP hits at a given fixed position relative to the HeliScopeCAGE peak maximums (the precise TSS of major transcribed mRNA isoforms). We computed the statistical significance of the association between the mTOR targets and OP hits in 5' UTRs under the null hypothesis that the frequency and location of OP hits in the 5' UTRs of mTOR targets are the same as in the control data set of other protein-coding genes. The enrichment of OP upstream and in TSS-overlapping locations was highly significant (P << 0.05) independently of the OP detection threshold (Fig. 2, Supplementary File 2). Enrichment for nearby downstream OP had lower significance.
with some OP in the +1 through +4 positions showing \( P < 0.05 \) significance level (depending on the OP detection threshold). Also, we inspected the general OP positional preferences by looking for OP hits not farther than +19 nt downstream from the verified TSS (heads of the 5′ UTRs) and in the remainders of the 5′ UTRs, i.e., more than +19 nt downstream of the TSS (tails of the 5′ UTRs). Comparing to the control data set, OP hits were significantly overrepresented in heads (\( P < 0.05 \)) but not in tails of the 5′ UTRs of mTOR targets (\( P > 0.05 \), see Supplementary File 2). De novo motif discovery in 5′ UTR tails was also unable to detect any overrepresented pyrimidine-rich motifs.

The OP positional preference to TSS and the absence of other pyrimidine-rich motifs called into question the existence of the PRTE elements recently reported by Hsieh and colleagues,\(^{15}\) which were claimed to be located in downstream regions of 5′ UTRs.

**OP are different for narrow and broad promoters**

Translational genes are mostly transcribed from a special class of promoters, the TCT-promoters, which are characterized by the central TCT consensus and a TSS-straddling OP with a strict TSS positioning on the inner invariant C. The importance of the TCT consensus at the transcriptional level has been validated experimentally.\(^{12}\) This suggests an interplay between transcriptional and translational regulatory mechanisms, since the transcriptional TCT-motif significantly influences the presence of TOP in the transcribed mRNAs.

At the same time, not all mTOR-responsive mRNAs are transcribed from narrow TSS, such as TCT-promoters. We utilized the quantitative nature of HeliScopeCAGE data to estimate typical TSS width of mTOR targets. First, we tested the fraction of mRNAs transcribed from a particular genomic position having the maximal HeliScopeCAGE peak signal (Supplementary Figure 1). We found that more than half of mTOR targets have less than 50% of mRNAs transcribed from a single major TSS position. Thus, the mTOR targets, even taking into account the TCT-promoters, poorly fit the “single-position TSS” assumption.

Thus, for each UCSC annotated transcript we estimated the TSS width as the minimal width of the genomic region such that at least 2/3 of the mRNA pool would be transcribed from that region (Fig. 3, Supplementary File 3). Even with the best (minimal) values taken into account for each gene, nearly 30% of mTOR targets had a TSS width of over 10 nt.

We performed de novo motif discovery separately for 167 sequences of extended 5′ UTRs with narrow (width ≤ 10 nt) and 83 with broad (width > 10 nt) TSS. We found the motifs (Supplementary File 4) shown in Figure 4 (b,c) in 5′ UTRs of 138 and 63 transcripts with narrow and broad TSS, respectively. The motifs were similar, except for the major T in a motif found for broad TSS indirectly resembling the PRTE motif, suggesting that previous studies were possibly affected by these broad TSS.

OP and OP-free modes may exist in a single multimodal TSS

The OP motif straddles experimentally verified TSS regions whereby after transcription the corresponding mRNAs begin with the TOP sequence. The shape of the TSS peaks defines the fraction of the mRNA pool transcribed from each particular genomic position. For broad TSS, OP may cover only a fraction of the TSS region. An extreme example is given by multimodal TSS exhibiting several sharp peaks (modes) with not all of them carrying the start of the OP occurrence in permissive -9..+4 positions. We report several mTOR targets possibly having TSS with such properties (Supplementary File 5), supporting the hypothesis that mTOR translational response may be regulated on the transcriptional level. Figure 5 shows annotation of the classical TOP gene PABPC1, UBA52 with a multimodal TSS, and YBX1 as a special case with a broad TSS and a weak OP occurrence.
While our approach yielded several interesting observations on OP/TOP motifs, it is somewhat limited for several reasons. First of all, both the TSS width and the length of the OP region can vary. Thus, the gapless multiple local alignment and the positional weight matrix, typically used for motif modeling, may be not fully suitable to study the OP/TOP phenomena. Therefore, it may be worthwhile to look for a more flexible in silico model of the OP/TOP motifs. Another problem is connected with the TCT-promoter motif having its own structure linked with corresponding transcriptional mechanisms, making it is difficult to clearly separate the transcriptional and translational sequence features.

Also, HeliScopeCAGE data were produced for THP-1 and HeLa cells, while PC3 cells were used for ribosome profiling. Many mTOR targets are housekeeping genes with strictly defined TSS, with good agreement between HeliScopeCAGE peaks for THP-1 and HeLa (Supplementary Figure 2). However, it is possible that some TSS with cell type-specific expression were missed or shifted from PC3-specific locations and falsely detected as OP-overlapping or OP-free.

We detected dozens of target genes with OP-free TSS, TSS with weak OP occurrences, and broad TSS with fuzzy pyrimidine tracks. One such example is YBX1 with a very broad TSS leading to rare mRNA isoforms, one of which was previously used to validate functionality of putative PRTE. Recent experimental verification showed that translation of the truncated TOP-free YBX1 mRNA, that lacks any CT-rich segments in its 5’ UTR, is still regulated by mTOR (rabbit YBX1 5’ UTR having 100% identity with the truncated human sequence). Therefore, it is likely that mTOR translational response of YBX1 mRNA is controlled through an alternative OP-independent mechanism, possibly involving other regulatory elements.

We were unable to confirm the presence or significance of pyrimidine-rich motifs, such as PRTE, downstream of TSS. However, compared with the control genome-wide data set, the 5’ UTRs of mTOR targets have special features, e.g., generally shorter lengths and pyrimidine-rich nucleotide composition (Supplementary file 6). Therefore, the question of basic sequence-level features of 5’ UTRs and their correct statistical evaluation remains unresolved. The presence and significance of OP GC-tails as well as other downstream sequence features also require further investigation.

The discovered multimodal OP/OP-free TSS would benefit from experimental study. TSS modes may be simultaneously active, or may switch on and off in a particular tissue or growth conditions. It was shown, that growth-dependent translation of ribosomal mRNAs can be different in different cell types. Furthermore, in different tissues a particular gene can be transcribed from alternative TSS producing either TOP or TOP-free mRNAs with different base translational efficiency. We suppose that new high-throughput experimental data on tissue-specific TSS activity would provide additional insights into the transcription-translation regulatory interplay.

**Conclusion**

Transcription from a TSS inside the OP results in the TOP motif being present in the transcribed mRNA. Our observations on multimodal OP/OP-free TSS suggest that fine tuning of mTOR-driven regulation may occur at the transcription-translation interface. OP motifs associated with TOP mRNAs may contain purine substitutions and can be slightly shifted downstream of the major TSS.

We would like to emphasize the importance of precise transcription start annotation for sequence analysis of 5’ UTRs. Usage of improper annotation is unreliable leading to questionable discoveries, such as the downstream PRTE motif of mTOR mRNA targets, which we were unable to confirm neither by motif finding nor by de novo motif discovery.

Finally, we provide a comprehensive annotation of OP/TOP motifs in 5’ UTRs of mTOR mRNA targets along with the corresponding HeliScopeCAGE peak profiles as TSS annotation (Supplementary file 1).

**Materials and Methods**

**HeliScopeCAGE data preparation**

HeliScopeCAGE peak data of THP-1 and HeLa cells were taken from http://fantom.gsc.riken.jp/5/suppl/Kanamori-Katayama_et_al_2011/. For each of these two cell lines, values at each genomic position were averaged across replicates (rounding down to the nearest integer value) discarding the positive values if present only in a single replicate. The resulting data were then averaged across the two cell lines (rounding down to the nearest integer value as well). The HeliScopeCAGE signal

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**Figure 3.** The percentage of genes (X axis) having a given TSS width (Y axis). TSS width is defined as the minimal length of the region aggregating at least 2/3 of the total HeliScopeCAGE signal. For each gene, the best (minimal) value is taken among all corresponding transcripts of the UCSC genome annotation.
is proportional to the number of mRNAs transcribed from each particular genomic position.

**Sequence data sets preparation**

The set of protein-coding genes mapped to Entrez gene IDs was extracted from the HGNC database. Corresponding transcripts and basic TSS annotations were taken according to the UCSC hg18 human genome annotation. Intronic sequences were removed; 5' UTRs were additionally extended with +100 nt upstream segments to account for possibly misannotated TSS.

144 mTOR target genes identified in PC3 cells by ribosome profiling were taken from Hsieh et al. 142 genes had UCSC-to-Entrez mapping and at least one UCSC transcript annotation with non-empty HeliScopeCAGE peak profile. We refer to these 142 genes and respective extended 250 5' UTRs (Supplementary file 7) based on UCSC transcript annotations as the mTOR targets.

The set of all remaining protein-coding genes curated in HGNC with UCSC-to-Entrez mapping and non-empty HeliScopeCAGE peak profile for at least one UCSC annotated transcript was used as the control data set (17671 5' UTRs of 11027 genes in total).

**De novo motif discovery**

We used ChIPMunk motif discovery tool (http://autosome.ru/ChIPMunk/) for de novo motif discovery in 5' UTRs of mTOR targets. MEME software was additionally applied on the same data sets ensuring agreement with the ChIPMunk results. ChIPMunk was searching for the optimal gapless multiple local alignment accounting for local nucleotide composition. Motif discovery was performed in the extreme precision mode (with 100 times more random seeds than by default). The automatically determined motif length was 25 with 14 nt long OP sequence flanked by GC-rich tails. Thus, the fixed length of 14 nt was adopted to rediscover the OP motif. The presence and significance of GC-tails as well as GA-rich motifs downstream of TSS requires further investigation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Figure 5. HelScopeCAGE signal and OP motif occurrences in extended 5’ UTRs of three mTOR targets: PABPC1 (top panel), YBX1 (middle panel), UBA52 (bottom panel). The UCSC annotated TSS (hg18 genome annotation) are denoted by black triangles. Best OP motif occurrences in the region are highlighted. UBA52 has a multimodal TSS only partially covered by OP.

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